STRUCTURAL CHARACTERIZATION OF CHITINASE

A THESIS

submitted in partial fulfilment of the
requirements for the award of the degree
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by

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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled "**Structural Characterization of Chitinase**" in partial fulfilment of the requirements for the award of the Degree of Doctor of Philosophy and submitted in the Department of Biotechnology of Indian Institute of Technology, Roorkee. Roorkee. This is an authentic record of my own work carried out during a period from Jan 2006 to Feb 2009 under the supervision of Dr. Pravindra Kumar and Dr. A. K Sharma, Assistant Professors, Department of Biotechnology, Indian Institute of Technology, Roorkee, Roorkee.

The matter presented in this thesis has not been submitted by me for the award of any other degree of this or any other institute.

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This is to certify that the above statement made by the candidate is correct to the best of our knowledge.

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(MANALI DATTA)

DEDICATED TO

MOM

I OWE YOU EVERYTHING

LIST OF PUBLICATIONS

1) Dipak N Patil, **Manali Datta**, Anshul Chaudhary, Shailly Tomar, Ashwani K.Sharma & Pravindra Kumar. Isolation, purification, crystallization and preliminary crystallographic studies of chitinase from Tamarind seeds. *Communicated*

2) **Manali Datta**, Dipak N Patil, Anshul Chaudhary, Shailly Tomar, Ashwani K. Sharma & Pravindra Kumar. Structural studies of chitinase from Tamarind seeds. *(Under preparation).*

LIST OF SYMBOLS AND ABBREVIATIONS

Å – (Angstrom) 10-10 meters

a, b, c – crystal unit cell dimension

α, β, γ – crystal unit cell angles

$\sigma(\tau)$ – standard deviation of torsion angle

ANS -8-anilino-1-naphthalene sulfonate)

CCP4 – Collaborative Computing Project Number 4

CM- Carboxy methyl

CTAB- Cetyl ammonium trimethyl bromide

DEAE-Diethyl amino ethane

DTT-Dithreiotol

EBI- European bioinformatics institute

EDTA- Ethyl diamine tetra acetate

EtBr- Ethidium bromide

GlcNAc- N-acetyl glucosamine

kDa- Kilodalton

MES- 2-(N-morpholino) ethanesulphonic acid

MPD- Methane pentane diol

OD- Optical density

ORF- Open reading frame

PAGE- Polyacrylamide gel electrophoresis

PCR-Polymerase chain reaction

PDA-Potato dextrose agar

PDB – Protein Data Bank

R – Crystallographic R factor

RF – Rotation Function

RMSD – root mean square deviation

SDS– Sodium dodecyl sulphate

SEC-Size exclusion chromatography

TE- Tris EDTA

Abstract

A protein (CHT) with chitinase activity was purified from the seeds of *Tamarindus indica* (Tamarind) by three step column chromatography involving an affinity and two ion exchange matrices. CHT was found to be an abundant protein of tamarind seed kernel. The molecular mass of the protein was determined to be 34 kDa by SDS-PAGE and SEC analysis. The protein was also showed to be a glycoprotein. The protein could be concentrated to 15 mg/ml. It is the characteristic of class III chitinases to have an antifungal and lysozyme activity but CHT lacked both antifungal and lysozyme activity. The enzyme was shown to be activated in presence of EDTA and monovalent ions. The activity was inhibited in the presence of divalent cations and reducing agents. N-terminal sequencing showed significant homology to class III chitinase (class III chitinase from *V. vinifera*).

The partial CHT cDNA and gene was amplified using PCR. The gene was demonstrated to have introns. The PCR product from genomic DNA and mRNA gave a product of size ~ 730bp in length. The product was sequenced. Comparison of the retrieved sequence showed 61% identity to class III chitinase from *Glycine max* (from nr database) and 57% identity to hevamine from *Hevea brasielinsis* (from pdb repository).

Fluorescence study for CHT was done using denaturing conditions of temperature, βME, urea and guanidium chloride. In presence of urea and GndCl, there was considerable denaturation of the CHT although formation of hydrophobic pockets was not observed when urea and GndCl was used in presence of ANS. HCl tend to denature the protein completely above 1.2 M HCl. Higher temperature tend to cause a

red shift in the tryptophan fluorescence intensity curve indicating in total unfolding of the protein and exposure of buried tryptophan residues.

CHT was crystallized in the presence of PEG 4000 at pH 6. The crystal belonged to tetragonal $P4_1$ space group and diffracted to 2.6 A□. The unit-cell parameters a = b = 67, c = 173.09 Å. The crystals contain two molecules in asymmetric unit which corresponds to a crystal volume per unit molecular weight (V_M) of 2.50 A□^{3} Da^{-1}. The structure was solved by molecular replacement method using the structure of hevamine from *H. brasienlinsis* as a search model. After series of refinement cycles, the model was fitted well in electron density and was refined to R factor of 28.6% with overall correlation coefficient of 84.13%. Structural analysis showed an overall conservation of the topology belonging to class III chitinases. CHT has an $\alpha\beta_8$ barrel structure with a substrate binding groove between the two chains. Each chain has three disulphide bridges and hydrogen bonding network which stabilizes the conserved residues present in the active site of the protein. Docking studies using HEX indicated that Tetra-NAG was fitting well in the substrate binding groove of CHT with an E-value of -273.71

CONTENTS

Chapter 1

LITERATURE REVIEW

1.1. Chitin.

Chitin is an unbranched polymer of N-acetyl β-D glucosamine, which are covalently linked as β 1,4 – linkages and is present ubiquitously except in mammals.(Fig 1.1)

The polymer is formed by enzymatic transfer of N-acetyl D glucosamine from uridine diphosphate *N*-acetyl-D-glucosamine to a preformed acceptor, forming a long chain polysaccharide which is insoluble in nature.

It is the major component of the cell wall of fungi (Kuranda *et al.*, 1991), protective exoskeletons and lining of digestive tract of many insects (Cohen .,1993), nematodes (Wu Y *et al.*, 2001) and crustaceans.

The deacetylated form of chitin is known as chitosan. Naturally it occurs in the cell wall of certain fungi and bacteria (Kolodziejska *et al.*, 1995; Tsigos *et al.*, 2000) but the percentage is less. Chitosan is produced commercially from chitin obtained from exoskeletons of crustaceans mostly shrimps and crabs. Chitosan as a polymer has more beneficial properties as it is biodegradable, biocompatible and has got increased permeability properties. Thus it has found its applicability in industries for purifying wastewater, as plant growth enhancers and plant defense boosters (Stevens, W.F., 1996) and as a wound-healing agent (Pusateri .,*et al* 2003) and an ion-exchange resin (Takuo *et al.*,2004).

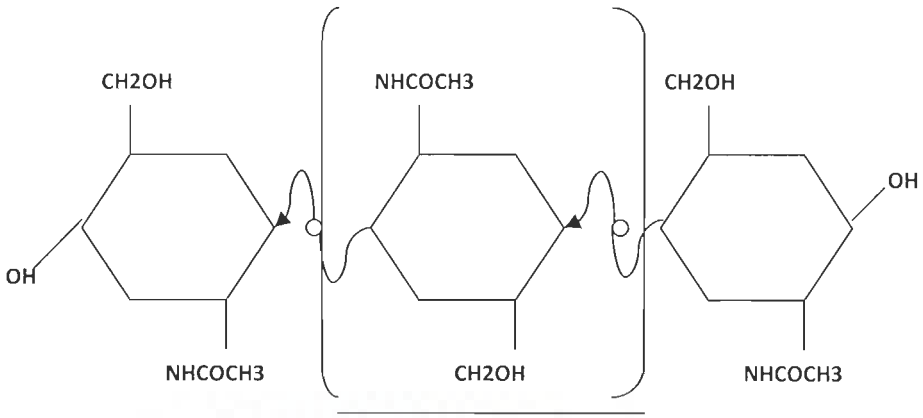


Fig1.1.Chitin -polymer of N-acetyl glucosamine

1.2. Chitinases: A preview

There are primarily three enzymes involved in metabolism of chitin namely chitinase, N-acetyl glusosaminidase and N- acetyl glucosamine synthase. In co-ordination they are involved in biosynthesis and degradation of chitin. Of all the three enzymes playing a fundamental role, chitinase is the most prominent one.

Enzymes that catalyze the breakdown of chitin by hydrolysis of the β 1,4 – glycosidic linkage are known as chitinases (EC.3.2.1.14).(Fig 1.2). Chitinases can have either exo or endo chitinolytic activity. The endochitinases are predominantly found in plants and fungi whereas exochitinases are found in bacteria. In case of endochitinases, chitin is cleaved randomly resulting in variation of the product length with a degree of polymerization ranging from 1-8 examples being chitotetraose, chitotriose, and diacetylchitobiose. On the other hand exochitinases can be subdivided into two classes: chitobiosidases (EC 3.2.1.29), which catalyzes the release of diacetylchitobiose starting with the nonreducing end of chitin

polymer, and β-(1,4) N-acetyl glucosaminidases (EC 3.2.1.30), which cleave the by-products of endochitinases and chitobiosidases, generating monomers of GlcNAc (Sahai and Manoch,1993).

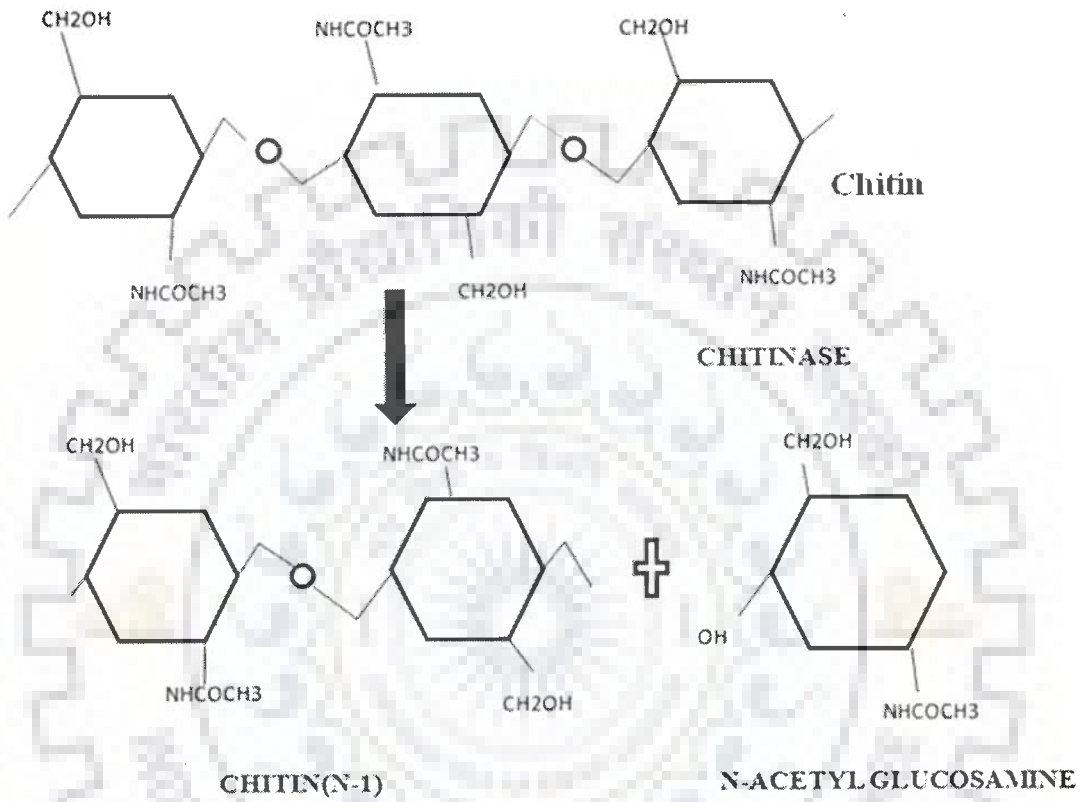


Fig 1.2. Chitinase action on chitin polymer giving N-acetyl glucosamine and chitin (n-1) as product.

Another pathway in which chitin is degraded to GlcNAc is utilizing the concerted action of chitin deacetylating enzyme and chitosanase. (Fig 1.3)

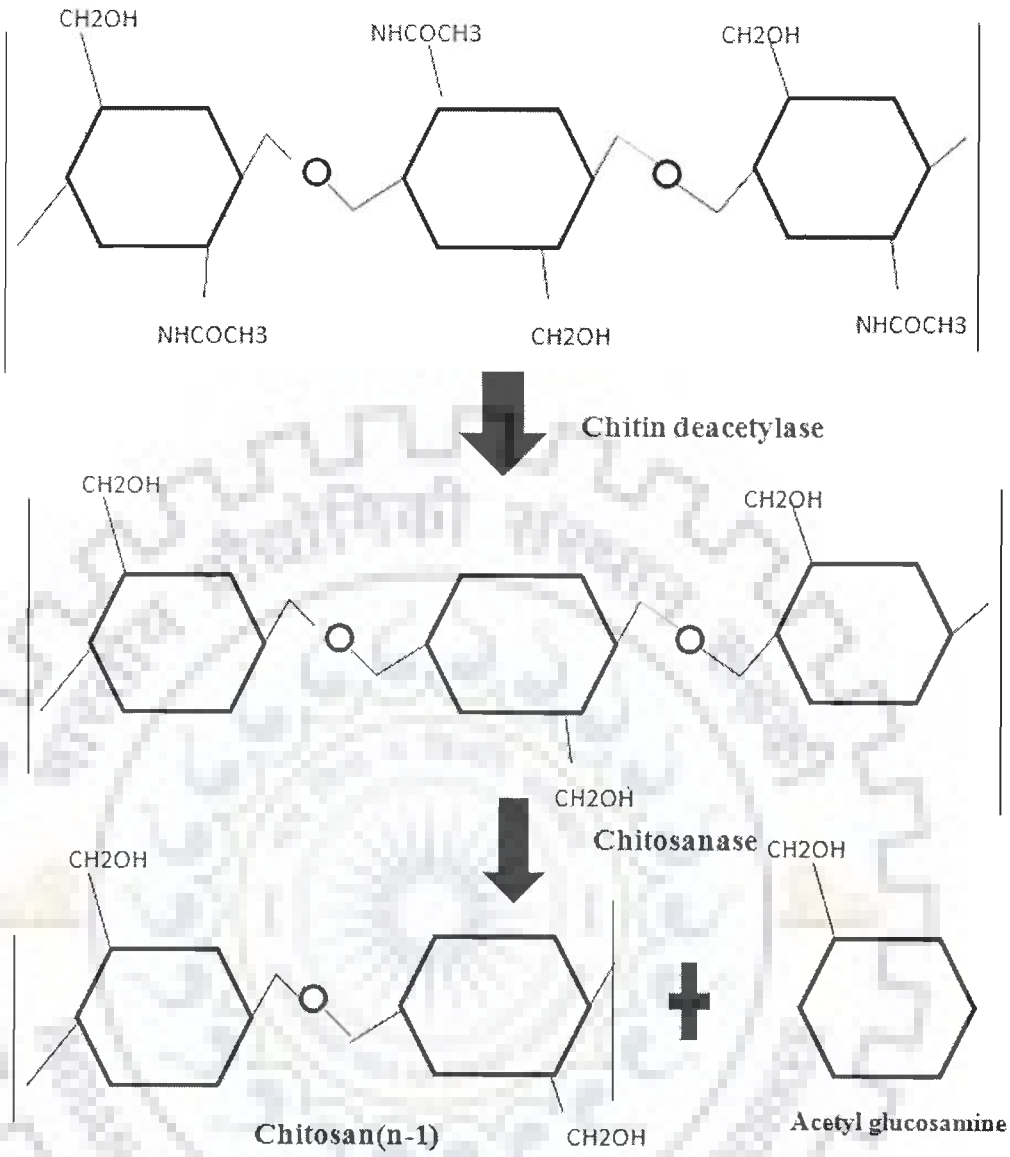


Fig 1.3. Degradation of chitin via chitosanase pathway giving acetyl glucosamine and chitosan (n-1) as product.

Some chitinases generally from a plant source also tend to show a dual lysozyme activity (Scheltinga *et al.*, 1994). Lysozyme hydrolyses the peptidoglycan moieties present in bacterial cell walls.

There are many ways in which the environment can affect the higher plants. Environmental factors can be abiotic or biotic in nature and they interact with different organisms sometimes resulting in stress conditions. Abiotic factors causing stress are mainly due to presence of heavy metals in the soil like mercury, presence of high amount of salt, drought and low or high temperatures. Biotic factors resulting in stress are mainly infection as well as symbiosis and mechanical damage during herbivory by animals. Plants defend themselves against these assaults by mainly three mechanisms:- by strengthening their cell walls by lignifications and suberization, by producing low molecular weight toxic compounds and by production of variety of proteins aptly named pathogenesis related proteins(PRP). Pathogenesis related protein encompasses a large variety of proteins like chitinases, glucanases and thaumatin like proteins. While the mode of action of many PR-proteins in plant-defense remains unclear, chitinase, a hydrolytic PRP, appears to exert its effect through degradation of the chitin-containing fungal cell wall and release of elicitor molecules. There is a growing body of evidence supporting the notion that higher chitinase activity helps plants resist fungal infection (Grison *et al.,* 1996; Terakawa *et al.*, 1997).

Chitinases occur in a wide range of organisms including viruses, bacteria, fungi, insects, higher plants, and animals. The functions of chitinase in these organisms are very diverse. Several nomenclatures tend to exist for chitinases, on the basis of their glycosyl hydrolase activity, as pathogenesis related proteins or simply as chitinases.

1.3 Classification of Chitinase: Based on primary structure

Based on their primary structures, plant chitinases have been classified into two major

families of glycosyl hydrolases, family 18 and family 19 which are in turn are segregated into seven classes, class I through VII of chitinases (Henrissat, 1997). Glycosyl hydrolases are the enzymes which are the majorly involved in carbohydrate metabolism. All glycosyl hydrolases act by generally by acid catalysis mechanism which involves two conserved amino acid residues catalyzing a single/double displacement reaction. The two different families of chitinases display no sequence similarities with each other and have different three-dimensional structures with different mechanisms of substrate catalysis.

1.3.1 Family 19 chitinase

Chitinases of the family 19, comprises of chitinases belonging largely to the plant family although reports have been made from bacteria (Tsuyoshi *et al.*, 1996; Kong., 2001; Mitsuhiro *et al.*, 2003). It has been proposed that transfer of this class of chitinase gene has occurred from plants to bacteria through horizontal gene transfer. The enzymes have an α-helical domain and a globular domain which incorporates the active site residues which are generally 2 glutamates. Family 19 chitinases operate by an inverting mechanism which involves a direct attack of a nucleophilic water molecule on the sugar anomeric carbon and producing an α-anomeric product (Fukamizo *et al.*, 1995). This mechanism does not involve the acetamido group at C-2 and, thus allows the hydrolysis of β-linked GlcNAc as well as GlcN. This class of glycosyl hydrolases encompasses the chitinases of class I, II and IV (Davies *et al.*, 1995).

1.3.1.1 Class I and Class II chitinases

Class I and II chitinases belong to the PR-3 family of pathogenesis-related

proteins.Class I chitinases have a N-terminal chitin-binding domain (CBD) which is rich in cysteine moieties and bears homology to hevein, a chitin-binding lectin from the rubber tree. For a tobacco class I chitinase (Fig 1.4), the deletion of CBD and the spacer region singly or in combination reduces the hydrolytic activity by 50% and antifungal activity is reduced by 80% (Suarez *et al.*, 2001). Class I chitinases are synthesized as propeptides, directed to the secretory pathway and eventually directed to the vacuole by a short C-terminal signal sequence known as the vacuolar targeting peptide (VTP). Deletion of this C-terminal targeting peptide redirects a class I chitinase to the apoplast while retaining the enzymatic activity (Grover *et al.*, 2001).

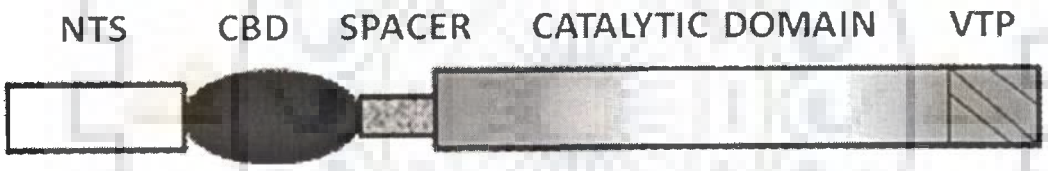


Fig 1.4: Model of class I chitinase from *Nicotiana tobacum* showing N-terminal sequence (NTS), chitin binding domain(CBD), spacer, catalytic domain and vacuolar targeting peptide(VTP) (Iseli *et al.*,1993).

Class II chitinases are similar to class I but lack the N-terminal CBD and the vacuole targeting C-terminal sequence which results their presence maximally in the extracellular spaces. In the catalytic domain they tend to have a deletion as compared to class I chitinases. Class II chitinases lacking the Cys-rich C terminal domain have been found to lack antifungal activity by themselves and sometimes limited activity in combination with 1, 3-glucanases

(Sela-Buurlage *et al.*, 1993).

1.3.1.2 Class IV chitinases

This class corresponds to a group of extracellular chitinases have a cysteine-rich domain in their N-terminal region. This cysteine-rich domain has been shown to be essential for substrate binding, but not for catalytic or antifungal activity (D.B. Collinge *et al.,* 1993).

The class IV is characterized by presence of many important deletions which are present in all other family 19 chitinases. A characteristic loop in the CBD is missing which does not affect its chitin binding affinity. There are three characteristic deletions in the main catalytic domain namely loop 1, loop 3 and loop 4. The loss of these three loops reduces the amount of protein present on the outer surface at a distance from catalytic loop the significance being that it requires its proximity to the substrate to efficiently catalyze it. The analysis suggests that the class IV enzyme recognizes an even shorter segment of the substrate than class I or II enzymes. This observation might help to explain why class IV enzymes are better suited to attack against pathogen cell walls (Takuji et al.,1992).

1.3.2 Family 18 chitinase

Family 18 chitinases are omnipresent throughout the living kingdom and are evolutionary diverse. Chitinases belonging to the Family 18 includes the class III, class V, class VI and class VII chitinases. All chitinases belonging to the family 18 of glycosyl hydrolases (Henrissat.,1995) possess a common fold known as the $(\beta/\alpha)_8$ barrel carboxylic end catalytic domain which has six sugar binding subsites (Van alten *et al.*,2000, Perrakis A *et al.*,1994). Since chitin hydrolysis by family 18 chitinases needs the N-acetyl group of the

sugar in the -1 subsite, productive substrate binding requires an N-acetylglucosamine to be bound in this subsite. Other subsites show less stringency in this respect, and may productively bind to, e.g. GlcN (Aalten *et al.,* 2001; Tews., 1997; Honda., 2000). The hydrolysis product of the family 18 catalyzed reaction is a β- anomeric in nature (retaining mechanism).

It also encompasses chitinases like lectins which lack enzymatic activity due to amino acid substitutions. These are addressed as "chitolectins", and are involved in tissue remodeling and have undergone this evolution of the catalytic acid/base (Fusetti *et al.*, 2003; Houston *et al.*, 2003; Mohanty *et al.,* 2003; Chang *et al.*, 2001; Varela *et al.*, 2002).

1.3.2.1 Class III chitinases

Class III chitinases are unique in having a structure and sequence unrelated to any other class of plant chitinases. These chitinases belong to the PR-8 family and family 18 of glycosyl hydrolases.

Members of family 18 glycosyl-hydrolases catalyze sugar hydrolysis with the retention of configuration at the anomeric carbon. Class III chitinases generally have been reported to have an additional lysozyme activity and appear to be more closely related to the bacterial chitinases. Class III chitinase have been purified from the seeds of *Benincasa hispida* (white gourd/wintermelon), a Chinese medicinal plant (Shih *et al.*, 2001). The enzyme is a 29 kDa protein with 27 amino acid N-terminal signal peptide (as deduced from N-terminal amino acid and genomic DNA sequences). The length of signal peptides and molecular weights are similar in other class III chitinases such as a pumpkin chitinase of 29

kDa with a 27 amino acid signal peptide (Kim *et al.*, 1999) and a sugar beet chitinase of 29 kDa with a 25-amino acid signal peptide (Nielsen *et al.*, 1993). This is unusual for a class III chitinase since they do not have a chitin-binding domain.

Class III chitinases show a wide range of isoelectric points, activity over a wide range of pH, and temperature stability at 60-70°C. The *B. hispida* chitinase has a pH optimum of 2 and retains approximately 50% activity at pH 8 (Shih *et al.*, 2001). Some class III chitinases, such as a yam enzyme, show two pH optima and heat stability at 80°C (Tsukomoto *et al.*, 1984).

The three-dimensional structure of hevamine, a chitinase/lysozyme from rubber tree, and its complex with the inhibitor allosamodin has been determined (Scheltinga *et al.*, 1994, 1995, and 1996). The structure is an $(\alpha/\beta)_8$ barrel similar to the bacterial family 18 glycosyl-hydrolases without significant sequence identity. These enzymes contain a substrate-binding cleft located at the C-terminal end of the β-strand in the barrel structure. The active site residue Glu127 of hevamine is required for activity whereas Asp125 allows a wider pH range for catalysis. The catalysis occurs by retention of configuration at the anomeric carbon, and is substrate assisted.

Generally class III chitinases also act as lysozymes. However, Bokma *et al.* (1997) showed that hevamine hydrolyzes the glycosidic bond of the peptidoglycan between C-1 of N-acetylglucosamine and C-4 of N-acetylmuramic acid as opposed to lysozyme which catalyzes the hydrolysis of peptidoglycan by cleavage of C-1 of N-acetylmuramic acid and C-4 of N-acetylglucosamine. Therefore, heveamine and possibly other class III plant chitinases are not strictly lysozymes. Some class III chitinases such as a sugar beet enzyme do not

exhibit lysozyme activity (Nielsen *et al.*, 1993). A recent study shows kinetic constants of hevamine by an improved assay method (Bokma *et al.*, 2000). Class III chitinase genes in *Sesbania rostrata* (Goormachig *et al.*, 2001), *Beta vulgaris* (Nielsen *et al.*, 1993), *Lupinus albus* (Regalado *et al.*, 2000), and *Cucurbita sp.* (Kim *et al.*, 1999) exist as single copies. In contrast, heveamine from *H. brasiliensis* is encoded by a small multigene family (Bokma *et al.*, 2001). Also, class III chitinase genes from *B. Hispida, Vitis vinifera* and *H. brasiliensis* (Shih *et al.*, 2001; Akihiko *et al.*, 2003 and Bokma *et al.*, 2001, respectively) lack introns.

Various class III chitinase genes showed distinct regulation upon stress treatment. For example, a *Lupinus albus* gene was shown to be induced by infection with *Colletotrichum gloesporioides*, by treatments with UV light, and by wounding (Regalado *et al.*, 2001). Both acidic and basic isoforms of tobacco class III chitinases were induced upon infection of plants with TMV (Lawton *et al.*, 1992). A grape class III chitinase was also shown to be induced in infected and non-infected leaves upon fungal infection (Busam *et al.*, 1997). A pumpkin class III gene was also responsive to the fungal elicitor and glycol chitin (Kim *et al.*, 1999).

1.3.2.2 Class V, VI, and VII Chitinases

Class V, VI, and VII chitinases belong to the PR-3 family of pathogenesis related proteins. These PR proteins are not expressed constitutively in the organism but are generated on induction. Class V chitinases from plants do not possess a genuine chitin binding domain corresponding to a hevein domain. In sugar beet the CBD binding domain is highly truncated and additional aromatic amino acids are missing which further reduces the possibility of binding chitin. Class VII chitinase is characterized by a single example from rice in which it

lacks a CBD but contains a catalytic domain homologus to class IV chitinases. Thus it can be said class V, VI, and VII chitinases each tend to have unique structures and are represented by few example each. (Table 1)

Table 1.1. Examples of less prevalent class of chitinases

CHITINASE CLASS	EXAMPLES
CLASS V	*Momordica charantia* (Xiao *et al.*,1992) *Nicotiana tobacum*(Melchers *et al.*, 1994)
CLASS VI	*Beta vulgaris(* Berglund., *1995)*
CLASS VII	*Oryza sativa* (Neuhaus., 1999)

1.4. Chitinases: Physiological roles in nature and potential applications

1.4.1. Role in insects

Insect chitinases belonging to family 18 glycosyl hydrolases, have been found to be present in molting fluid and gut tissues. These enzymes have a size range of 40–85 kDa, which is typically larger than the masses of plant (~25–40 kDa) and bacterial (~20–60 kDa) chitinases. They can be active in the pH range of ~4–8 and their isoelectric points range from pH ~5–7. (Gopalakrishnan et al., 1995; Wang et al., 1996) The insect chitinases catalyze the digestion of chitin present in the exoskeleton and peritrophic membrane (PM) in the gut to small oligosaccharides known as chitoologosaccharides. Chitinases have been shown to have

a role in during the molting of filariae and in the hatching of larvae from the eggshell (Adam. *et al.*, 1996, Arakane., 2003).

Manduca sexta has been used as a test model for characterizing chitinase in insects. (Kramer *et al.,* 1993) It has been demonstrated in *M. sexta* that level of chitinase expression increased before each molting process thus indicating direct involvement (Zheng *et al.*, 2002). In the *M. sexta* chitinases, two highly conserved regions of amino acids were found to be present in separate β-sheets. These conserved regions were involved in substrate binding and/or catalysis (Watanabe *et al.*, 1993). W145 was found to be present in these β-sheets. Protein-substrate docking studies revealed that this tryptophan has close proximity to the cleavage site of the bound substrate, $(GlcNAc)_6$. Site-directed mutagenesis was employed to check the importance of this particular tryptophan in the catalytic mechanism substitution of tryptophan with glycine at this particular position led to total loss of enzymatic activity but retaining the chitin binding ability (Xin Huang *et al.*, 1999). Role of tryptophan residue was highlighted in rye chitinase, W131, which is buried partially in the catalytic cleft, was also directly involved in catalysis.

1.4.2 Role as allergens

Chitinases constitute an important class of panallergens. The main reason for chitinases of plants acting as a panallergen is due to presence of hevein-like domain which is present at the N-terminus of class I chitinases. Hevein is one of the most important latex allergens. Chitinases present in fruits like avocado, banana and chestnut are the panallergens responsible for latex fruit allergy (Diaz-Perales *et al.*, 1998). In *Dermatophagoides pteronyssinus,* two chitinases, namely Der p 18 and Der p 15 which cause allergy in both man

and dogs are found (O'Neil., 2006). A class IV chitinase with molecular weight of 34 Kd from *Cryptomeria japonica* was also shown be a major cause of pollinosis (Fujimura *et al.*, 2005).

1.4.3 Role as storage protein

Chitinase have been found to act as storage proteins in banana (Peumans *et al.*, 2002) and alfalafa (Mignery *et al.*, 2003). In *Medicago sativa* the 32 kDa vegetative storage protein bears high homology with class III chitinases. Although no activity has been observed for the enzyme it was observed that the expression of protein increased during abiotic stress indicating that the protein may have an adaptive role during stress conditions (Avice *et al.*, 2003). Another member bearing homology to class II chitinase from *Vicia narbonensis* has also been observed to be a seed storage protein (Sumner *et al.*, 1919).

1.4.4. Chitinase and symbiosis

Chitinases represent a marker for enzymes induced in later stages of the symbiotic interactions. A class III chitinase gene from *Medicago truncatula* was shown to be specifically expressed in root cortical cells containing developing or mature arbuscules (Bonanomi *et al.*, 2001). This gene expression was limited to cells harboring the arbuscules. The proof as to expression of chitinase was not a result of fungal infection was displayed by the absence of fungal elicitors and absence of hydrogen peroxide production, a hallmark of defense response, (Salzer *et al.*, 1999) and that the other defence related proteins such as β-

1,3-glucanases were present at low levels in these cells (Blee and Anderson, 2000).

Another class III chitinase from *Sesbania rostrata* lacking the active site glutamic acid residue and devoid of chitinase activity was induced during nodule development (Goormachtig *et al.*, 2001). The active site glutamate is mutated to a lysine residue in this protein and therefore renders this protein a chitin binding lectin. This gene was induced within 4 h of inoculation with nodulation bacteria, and the protein was localized to the outer cell layer of nodules. Investigators suggest a role for this protein in Nod factor binding which would protect, concentrate, or facilitate its interaction with a receptor protein. Increased chitinase activity and induction of new isoforms have been observed in other plants, such as soybean, in symbiosis with nodulation bacteria (Xie *et al.*, 1999).

1.4.5. Chitinase in stress response

Overexpression of chit33 from *Trichoderma* in tobacco plants significantly enhances their tolerance to saline stress and high concentrations of heavy metals in *in vitro* conditions. (Dana *et al.*, 2006) In *Bromus inermis* suspension cells it was shown that mRNA expression corresponding to a chitinase increases on exposed to ice cold conditions although it did not prevent the formation of ice crystals. (Toshihide Nakamura *et al.*, 2008) It has been shown for *Nicotiana tobacum* cultivars that the exposure of a single pulse of ozone markedly increased the production of chitinase and β-1, 3 glucanase(Schraudner *et al.*,1992).

In *Secale cereale* (winter rye), during winter acclimatization two chitinases which act as antifreeze proteins are expressed. These proteins accumulates at cold temperature in the

apoplast of winter rye leaves and shown to exhibit both endochitinase and antifreeze activities, thus proving they are dual function enzyme. Expression of the chitinases, CHT9 and CHT46 occurs during developmental process that occurs at a later phase of cold acclimation, possibly at a time when plants in the field are more prone to be exposed to subzero temperatures and/or low temperature pathogens for long periods (Yeh *et al.*, 2000).

Evidence for other physiological functions of chitinases in flowering, reproduction, germination, and plant growth are also beginning to emerge. It has been implicated in embryogenesis. A mutant cell line of carrot was unable to form embryos and on exposure to the supernatant from wild type plant forms viable embryos. The rescuing factor was identified as class IV chitinase (Jong *et al.*, 1992).

1.5. Applications in biotechnology and pharmaceuticals.

1.5.1. Role as biopesticide

It was known that chitinase has an inhibitory effect on the growth and development of insects. It has been proved in vivo that chitinase tends to cause perforation in the gut lining of the insects as was observed when *Streptomyces griseus* chitinase was fed to anopheles (Shahabuddin *et al.*, 1993). Perforations were also observed in the case of spodopteran larva midgut peritrophic lining when *Serratia marcesens* chitinase was applied *in vitro*.

Fungi such as *Metarhizium anisopliae*, *Beauveria bassiana*, *Nomuraea rileyi*, and *Aspergillus flavus* have the chitinolytic ability to hydrolyze the chitinous exoskeleton of insects (St Leger *et al.*, 1991b; El-Sayed *et al.*, 1989). Chitinase from a nematode *B. malayi*

can break the chitinous extracellular sheath of the host mosquito to gain entry. It was also observed that first and fourth instar larvae of mosquito *A. aegypti* was killed within 48 h with the help of the crude preparation from *M. verrucaria* (Mendonsa *et al.*,1996). Though 100% mortality was observed within 48 h, purified endochitinase lethal times (LT_{50}) were 48 and 120 h for first and fourth instar larvae, respectively. Due to the proof of direct involvement of chitinases in insect molting they have been used to produce transgenic virus for insect control. Gopalakrishnan *et al* produced a recombinant baculo virus which on infection produces a 85 kDa protein which hydrolyzes chitin. Another polyhedron virus *Autographa californica* expressing a recombinant chitinase was able to kill the larvae of *S. frugiperda.*

The recombinant chitinase from tobacco hookworm was introduced in tobacco plant via agrobacterium mediated transformation. The chitinase expressed although truncated was enzymatically active (Wang *et al.,* 1996). When leaves from transgenic tobacco plant were fed to larvae of the tobacco budworm, *Heliothis virescens* there was a sixfold decrease in mass of larvae (Ding *et al.,* 1997).

1.5.2. Role as fungicide.

Plant chitinase is known to be one of proteins induced in plants through infection by pathogens. They have the capability to digest fungal cell wall, lysing growing tips and inhibiting mycelia growth (Shapira *et al.*, 1989, Woloshuk *et al.*, 1991, Schlumbaum *et al.*, 1986). The chitinase obtained from *Dioscorea batatas* was sprayed onto strawberry infected by powdery mildew. As an after effect, white-colored mildew prominent visible on the fruit and leaf of strawberry disappeared on the day following the spray and was in a controlled state for two weeks. This happened as the chitinase led to degradation of the mycelia (Koga

et al., 2004).

1.5.3. Role as antimicrobials.

Chito-oligosaccharide (COS) is generally known to possess many specific biological functions, especially antibacterial activity, depending on its size.A specific size range of antibacterially active COS by adjusting the degree of deacetylation (DD) of β-chitosan in a *Trichoderma harzianum* chitinase-hydrolysing process was developed. COS produced at 90% DD showed superior activity against most Gram-negative bacteria tested, with a minimum inhibition concentration (MIC) ranging from 55 ± 27 to 200 ± 122 μ g mL^{-1}(Lin *et al.*, 2009). *Monascus purpureus* CCRC31499 produced an antimicrobial chitinase when it was grown in a medium containing shrimp and crab shell powder (SCSP) of marine wastes. The unique characteristics of the purified chitinase include high molecular weight, nearly neutral optimum pH, protease activity, and antimicrobial activity with bacteria and fungal phytopathogens (Wang *et al.*, 2002).

The protozoan *Phytomonas* is responsible diseases in different crop plant species. The finding that chitin is an exposed cell surface polysaccharide in *Phytomonas françai* made this organism a susceptible target for action of chitinases. The recombinant plant chitinases *Urtica dioica* agglutinin (UDA) and *Arabidopsis thaliana Chia4* (ATCHIT4) proteins were shown to have an interaction with *P. françai* surface (Rocha *et al.*, 2003).

1.5.4. Chitinase in medicine

The main application of chitinase in medicine is in production of chito-

oligosaccharides which have immense pharmaceutical importance. Specific combinations of chitinolytic enzymes are required to obtain the desired chain length of the oligomer. For example, the production of chitooligosaccharides requires high levels of endochitinase and low levels of N-acetylglucosaminidase and exochitinase, whereas the production of GlcNAc requires higher proportion of exochitinase and N-acetylglucosaminidase (Aloise *et al.*, 1996). COS like Chitohexaose and chitoheptaose have shown potential anticarcinogenic activity.

Increase in serum levels of chitinase are indicators of T_H2 type inflammation and asthma. Studies against these enzymes may help in designing drugs which would help in reducing the symptoms Chitinases can be employed in human health care, such as making ophthalmic preparations with chitinases and microbiocides. A direct medical use has been suggested for chitinases in the therapy for fungal diseases in potentiating the activity of antifungal drugs (Pope and Davis., 1979; Orunsi and Trinci., 1985). They are also be used as additives in antifungal creams and lotions due to their topical applications.

1.5.5 Chitinase in transgenic plant development

The production of enzymes capable of degrading the cell walls of invading phytopathogenic fungi is an important component of the defence response of plants. This natural host defense mechanism has been utilized to produce fungal-resistant plants. Transgenic tobacco seedlings constitutively expressing a bean chitinase gene under control of the cauliflower mosaic virus 35S promoter demonstrated an increased survival and delayed susceptibility in soil infested with the fungal pathogen *Rhizoctonia solani* symptoms. (Brogue K.*et al*, 1991). Transgenic tobacco (*Nicotiana tabacum*) lines that overexpress the

endochitinases CHIT33 and CHIT42 from the mycoparasitic fungus *Trichoderma harzianum* have been produced and an evaluation has been done for their tolerance against biotic and abiotic stress. Both CHIT33 and CHIT42, individually, conferred broad resistance to fungal and bacterial pathogens, salinity, and heavy metals. Such broad-range protective effects came off with no obvious detrimental effect on the growth of tobacco plants (Dana M *et al.*, 2006). When a chitinase gene from the mycoparasitic fungus *Trichoderma harzianum* was transformed into tobacco and potato plants, transgenic plants showed enhanced resistance to various fungal pathogens including *Alternaria alternata*, *A. solani*, *Botrytis cinerea* and *Rhizoctonia solani* (Lorito *et al.*, 1998).

1.5.6. Chitinase in waste management

Large quantities of waste and by products are generated from seafood processing industries. Chitinolytic bacteria produce multiple chitinases which act synergistically to degrade native crystalline chitin (Romaguera *et al.*, 1992). Many microorganisms, e.g., *Serratia marcescens*, *Streptomyces* spp., are known to produce a multi-chitinolytic-enzyme complex (chitinases and chitobiases), which has shown numerous applications in waste treatment. In Chi40, a 40 kDa chitinase from *Streptomyces thermoviolaceus,* site directed mutagenesis was performed at positions W149, W263, F286, Y311 and W335 to study the whether these changes could bring about hydrolysis of highly packed chitin as present in shellfish waste. It was observed in case W149A the activity increased sixfold than the native enzyme. This study implied huge possibility of protein engineering for the establishment of advanced bioconversion methods of chitinous biomass (Muzzarelli., 1996; Miyashita *et al.* 1991).

1.6. Molecular characterization of chitinase

1.6.1. Biochemical characterization.

The chitin degrading enzyme from various sources has been assayed by using different methods. These methods can be grouped as follows:-

1.6.1.1. Colorimetric assay

The colorimetric based chitinase assay is done according to the protocol given by Imoto and Yagishita (1971). It is most commonly used for chitinase assay. Basis for this method lays in the fact that chitinase degrades chitin resulting in formation of more reducing ends. Presence of a reducing colour agent like potassium ferricyanide is thus used to monitor the amount of reducing ends produced by measuring absorbance at 420 nm spectrophotometrically.

Activity staining methods based on polyacrylamide gel electrophoresis has been done using dyes which are visible in daylight. In this method PAGE is performed for chitinases followed by overlaying of the gel on chitin agar plates containing dyes like congo red and ruthenium red at 0.001% (w/v). Dark activity bands may be observed in the presence of chitinases in daylight (Gohel *et al.*, 2004).

1.6.1.2. Fluorescence based assay

The fluorescence based chitinase assay is done according to the protocol given by Somashekhar *et al* (1997). The principle underlying this assay is that chitin binds to fluorescent dye ranipal and calcofluor white giving a characteristic peak around 406 nm. Decrease in amount of chitin can be easily monitored spectroscopically after addition of chitinases.

In situ activity staining method is also done by incorporating 0.01% (w/v) soluble glycol chitin in PAGE followed by staining of the gel in freshly prepared calcofluor white (0.01%). Dark activity bands against a blue background are observed against a blue background under UV (Trudel and Asselin., 1989).

1.6.1.3. Viscometric assay

Viscometric assay is based on the turgidity changes brought about by action of chitinases on chitin (Ohtakara *et al.*, 1988). It requires a soluble form of chitin (glycol chitin) which is sensitive to endochitinases. Chitinases activity is monitored by decrease in viscosity of the substrate solution by monitoring it with help of viscosimeter. This method is not widely used as it is too cumbersome.

1.6.1.4. Radiochemical assay

This method is sensitive and applicable for both endochitinases and exochitinases. This method is based on tritium labeled chitin which releases soluble oligosaccharides on hydrolysis. Released oligosaccharides are monitored radiochemically using scintillation counter (Molano *et al.*, 1977).

1.6.2 Biophysical characterization

1.6.2.1 Circular dichroism

Circular dichroism (CD) spectroscopy is a well-established method to understand the

structure function relationship of proteins. The sensitivity of far-UV protein CD spectra to protein secondary structure is used in one of the most successful applications of CD to determine the secondary structure composition of a protein (Yang *et al.*, 1986; Greenfield, 1996; Venyaminov and Yang, 1996; Sreerama and Woody, 2000, 2004). CD spectroscopic method depends upon the differential absorption of left- and right-circularly polarized light by optically active molecules. Alpha-helix, beta-sheet, and random coil structures each give rise to a characteristic shape and magnitude of CD spectrum. The far UV CD spectra of alpha-helical proteins are characterized by three peaks, a negative peak at ~222 nm, at ~208 nm and a stronger positive peak at ~192 nm. However, CD at 222 nm displays the strong concentration dependence characteristic of dissociative unfolding and usually the negative value at 222 nm is considered to measure the helix content. (Holtzer *et al.*, 2000) In general, spectra arising from β-sheets are characterized by a small negative peak near 217 nm and a positive peak near 195 nm that has approximately half the intensity of the α-helix peak in this region. β-Sheets give rise to considerably less intense signals than helices and show far more variation in spectral characteristics; the latter is partly attributable to the fact that β-sheets are much more structurally diverse than α -helices, with strands which may run parallel or antiparallel to each other, and with sheets displaying differing degrees of twisting. The weak β-sheet signal makes it more susceptible to distortions caused by the CD signals of aromatic side chains, which can produce peaks or dips between 225 nm and 235 nm. CD spectra are usually presented in molar ellipticity [θ] units (degrees-cm^2 dmol^{-1}). For proteins, the mean residue ellipticity (*MRE*) can be calculated as follows: $MRE = (MRW)\ \theta/10cl$, where *MRW* is the mean residue (amino acid) weight, *c* is the concentration of protein and *l* is the pathlength in cm. The α-helical content for each protein was calculated using the standard equation for [0] at 222 nm (28): $[0]_{222} = -30.300f_H - 2.340$, where f_H is the fraction of α -helical content (f_H

x 100, expressed in %). Spectra are presented as the mean residue ellipticity. Thermal stability is assessed using CD by following changes in the spectrum with increasing temperature.

In case of *Bacillus licheniformis* , CD melting unfolding study was able to distinguish between the full-length and truncated mutant molecules indicated by two phases transition temperatures in the mutants. The results indicated that 145 amino acid residues, including the putative C-terminal chitin-binding region and the fibronectin (III) motif of *B. licheniformis* chitinase, were not vital for maintaining the structure and hydrolysis of insoluble chitin (Chuang *et al.*, 2008).

The effects of cations and freeze-thaw cycles on the secondary structure of chitinases from winter rye (*Secale cereale*) were examined by CD in the far-UV range. A loss in α-helical and a loss of β-structure was observed when the protein was given freeze thaw cycles in the absence of cations. Moreover, increased negative mean residue ellipticity observed at 198 nm indicated an increase in random coil. The addition of $CaCl_2$ to the chitinases resulted in the change in protein secondary structure that was accompanied by loss of α-helical structure to β structure rather than formation of random coils. After three freeze-thaw cycles in the presence of $CaCl_2$, the proteins showed a small increase in random coil (Stressmann *et al.*, 2004).

1.6.2.2. Fluorescence spectroscopy

Fluorescence spectroscopy is a very powerful technique to monitor conformational changes in proteins. The fluorophores can be either intrinsic (tryptophan) or extrinsic (ANS: 8-anilino-1-naphthalene sulfonate) probes. The fluorescence property of tryptophan has long

been utilized to understand the folding/unfolding, substrate binding and conformational heterogeneity in different physicochemical conditions. The tryptophan fluorescence usually varies in somewhat unpredictable manner in various proteins. Some proteins exhibit large increases in their intensities and lifetimes and others, decreases, on unfolding of the chain. The ANS anion (8-anilino-1-naphthalene sulfonate) is a much utilized 'hydrophobic probe' for proteins (Slavik, 1982). ANS in water is nonfluorescent but it fluoresces upon binding to a nonpolar (hydrophobic) site of proteins. In fact, ANS binding to proteins depends on protein cationic charge and solution pH, and occurs largely through the ANS sulfonate group (Matulis and Lovrien, 1998). The dependence of ANS binding on electrostatic interaction between the sulfonate group and protein cationic groups indicate that ANS binding does not require pre-existing hydrophobic site on or in protein molecules to start the binding reaction. With increasing conformational unfolding state in different physicochemical conditions, the ANS fluorescence intensity increases.Thus,the increase in fluorescence intensity is a characteristic of relaxed or molten state where more access of ANS to hydrophobic core of a protein is allowed (Semisotnov *et al.*, 1991; Price, 2000).

The intermolecular interactions involved in oligosaccharide-protein recognition, binding and cleavage are complex. Study of complex interactions is important to understand the biochemical role these proteins play. Fluorescence spectroscopy is one of the methods used to study the binding between a substrate and enzyme. Chitinase-oligosaccharide binding affinity studies were performed in case of an inactive mutant form of *S. marcesens* ChiA. Results indicated that the binding constants (K_a) for the oligosaccharides increased as their number of GlcNAc units or length increases. This phenomenon was limited when the saccharide unit extended to 5. On the basis of fluorescence spectroscopy it was proposed that

$GlcNAc_6$ binds in the –4 to +2 subsites and there exists different binding modes for chitinases and chitolectins (Dalal *et al*., 2007).

Similarly in case of *Vibrio harveyi* chitinases, fluorescence studies were employed to deduce the binding conformation for $(NAG)_5$ and $(NAG)_6$ to the enzyme (Songsiriritthigul *et al*., 2008).

1.7. Three dimensional structure of chitinase

In cases where function of protein is known the three dimensional structure of protein can provide valuable insights subunit and quaternary interactions, modes of substrate binding, allosteric regulation and the identification of putative active site residues.

The two families of glycosyl hydrolases namely family 18 and family 19 consisting of chitinases do not bear any structural similarity. Family 19 Chitinases tend to bear similarity with chitosanases and lysozymes (Hart *et al*., 1995; Monzingo *et al*., 1996). Detailed three dimensional structural analysis of the family 19 chitinases revealed that they tend to have a catalytic site comprised of two lobes rich in alpha helical content. The non polar residues tend to constitute the hydrophobic core of the enzyme where they play a major role in constituting the active site geometry. The conserved polar residues invariantly lie in the cleft meant for substrate binding and catalysis. Three invariant residues Gln118, Tyr123 and Asn124 bind the substrate in barley chitinase. The structure of bacterial chitinases of this family varies from plants due to deletion of several loop regions that extend into the substrate binding groove. In accordance with these structural features, detailed analysis of the degradation of chitooligosaccharides by chitinase G, a single-domain family 19 chitinase from the Gram-positive bacterium *Streptomyces coelicolor* showed that the enzyme has only four subsites (-

four subsites (- 2 to +2), as opposed to six (-3 to +3) for plant enzymes. The most prominent structural difference leading to reduced size of the substrate-binding groove is the deletion of a 13-residue loop between the two putatively catalytic glutamates. The key difference between the class IV chitinase, represented by corn, and the class I and II molecules is two large deletions. The deletion in class IV chitinase represented by corn chitinase occurs in the loop region between barley residues 71 and 83 downstream from the catalytic Glu67 at the end of the C helix. (Hoell *et al.*,2006)

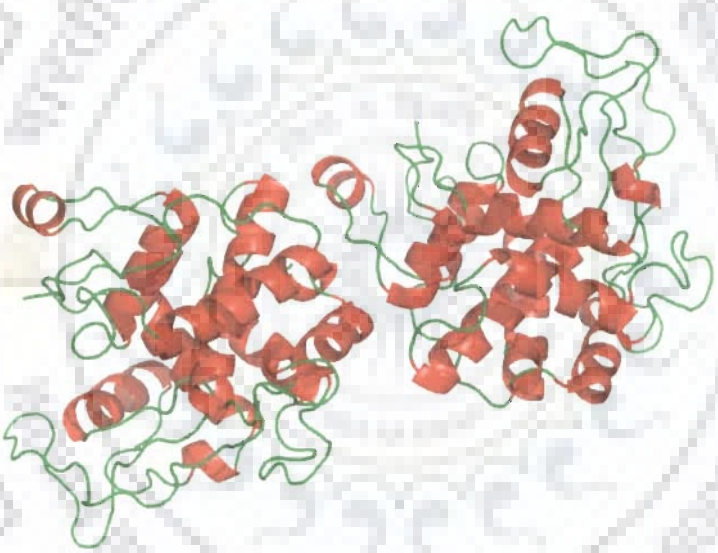


Fig 8. Structure of Chitinase I belonging to family 19 glycosyl hydrolases from barley at 2.0 angstrom.(PDB accession code-1 CNS) showing α-helical region(red) and loop region(green)

Chitinases belonging to the family 18 have a α/β_8 fold in their structure. A canonical α/β_8 fold consists of eight parallel β strands wrapped around by a series of eight α helices. This fold is present in 26 enzyme families according to SCOP database and 28 families

according to CATH database. According to the common architecture of the α/β_8 barrels active site is located on the C terminal of the central barrel. (Vega *et al.*, 2003).

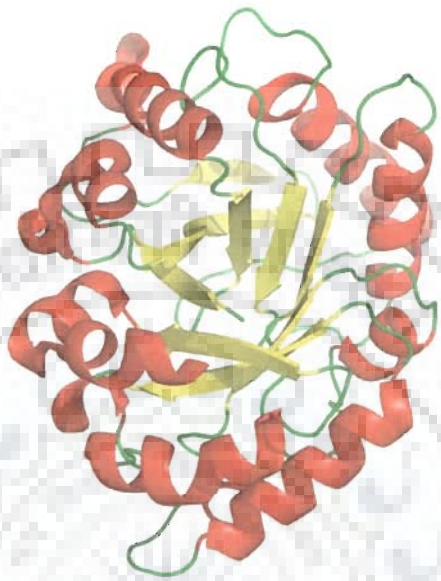


Fig 8. Structure of Chitinase III from rubber tree at 2.2 angstrom.(PDB accession code -1 HVQ) showing the $\alpha\beta_8$ barrel structure with α helices in red, β regions in yellow and connecting loops in green

Hevamine from *Hevea brasiliensis* was the first chitinases crystallized from the family 18 class of glycosyl hydrolases. The presence of a conserved glutamate residue (Glu127) is essential for substrate catalysis. Three disulphide bridges are present and they help in maintaining the integrity of the enzyme. (Scheltinga *et al.*, 1994) According to the model of created for bacterial type of enzymes, chitin binds to the active by stacking the N-acetyl glucosamine moieties on the conserved aromatic amino acids present on the lining of

the groove forming the active site. The major sugar binding site are formed by Trp384,Trp137 and Phe157 termed as the -1,+1 and +2 subsites with cleavage site occurring between -1 and +1 subsite. Plant type family 18 chitinases contain less identifiable subsites when compared to bacterial chitinases of this family. The MXYD motif seems to be conserved in family 18 chitinases as a part of the active site. Another motif conserved is the DXDXE (Asp173-Glu177) which is essential for activity of chitinases. *Aspergillus fumigatus* chitinase B1 (AfChiB1) possesses most of the conserved residues characteristic of the family 18 chitinases. (Aalten *et al.*, 2001; Bokma *et al.*, 2002; Watanabe *et al.*, 1994)

The mechanism of degradation of chitin has been elucidated on the basis of either structure or site directed mutagenesis. Entry of chitin in the substrate binding cleft is facilitated by the presence of surface loops which enable the chitin to enter unidirectionally. The residues facilitating entry of substrate in case of *S. marcesens* are Trp33, Trp69, Phe 232 and Trp245, whereas in case of *Aeromonas caviae* it was found to be ser33, Trp70 , Trp232 and Trp245. (Hult *et al.*,2005;Imai *et al.*,2002)

Three dimensional structures of many family 18 chitinases have been determined with or without substrates. The catalytic domain was found to be formed of β/α_8 barrel with the loops forming the substrate binding cleft. The C-terminus is composed of 8 parallel β strands with one of them containing the proton donor of the hydrolysis reaction. The crystal structures of complexes have helped in determining the various binding positions in the enzyme's active site. The solvent exposed tryptophan enables the binding of GlcNac of chitin in characteristic boat formation. Binding of chitin results in reorientation in which the hydrogen bond is formed between the Glu177 and -1 acetamido group of substrate replacing

the hydrogen bond that is originally present between Asp175 and Asp173. Nucleophilic attack of the carbonyl oxygen of same pyranose occurs with protonation of the glycosidic oxygen by the catalytic acid this result in formation of the leaving group from reducing end of chitin as well as an oxazolinium intermediate. Subsequent hydrolysis of this oxazolinium intermediate result to reversion to original stereochemistry as well as protonation of Glu177 and reformation of hydrogen bonding between Asp175 and Asp173. (Scheltinga *et al.*, 1997; Aalten *et al.,* 2001; Brameld *et al.*,1998)

A wide range of leguminous plant seeds contain chitinase which functions as a storage protein and also play a role in plant-pathogenesis. The seeds of leguminous plants are rich stores of proteins and therefore are used as valuable food sources. Current studies have implicated the involvement of chitinases in food allergies and responsible for causing the latex-fruit cross-sensitivity syndrome.

The tamarind belongs to the pea family, Leguminosae or Fabaceae, scientific name being *Tamarindus indica*. Tamarind is a large evergreen tree that grows in the tropics. It is native to Asian countries. Tamarind is popular in India as a condiment. Technology has now been utilized to manufacture pectin, tartarates and alcohol from its pulp. The pulp of tamarind has medicinal virtues and is used in treating loss of appetite, as a laxative, as an anti-helminthic and for healing wounds and cuts. The seed has found its applicability as textile thickener and in textile sizing for sizing jute and sometimes cotton. It has also been used in creaming rubber latex. The industries using the fruit pulp as the source have a large amount of tamarind kernel as the by product. Thus it becomes quite an economic source for isolation of any protein of interest.

Chapter 2

PURIFICATION AND BIOCHEMICAL CHARACTERIZATION OF CHT

2.1. Introduction

The modes of purification of proteins from various sources include chromatographic techniques of which ion exchange and gel permeation chromatography mainly comes into focus. The principle of ion exchange chromatography (IEC) relies on charge-charge interactions between the proteins in applied sample and the charges immobilized on the resin of choice. Ion exchange chromatography can be categorized into cation exchange chromatography, in which positively charged ions bind to a negatively charged resin; and anion exchange chromatography, in which the binding ions are negative, and the immobilized functional group is positive. Commonly used cation exchange resins are S-resin (sulphate derivatives) and Carboxy-Methyl resins (carboxylate derived ions). Commonly used anion exchange resins are Q-resin (Quaternary amine) and DEAE resin (Di Ethyl Amino Ethane). The principle of gel permeation / size exclusion chromatography (SEC) is that proteins of different sizes elute through a stationary phase at different rates. This results in the separation of a solution containing proteins based on their hydrodynamic size. Columns are often calibrated using 4-5 standard samples (e.g., folded proteins of known molecular weight), and a sample of the very large molecule blue dextran to determine the void volume. The elution volumes of the standards are divided by the elution volume of the blue dextran (Ve/Vo) and

plotted against the log of the standard's molecular weights. The plotted graph is then used to determine the molecular weight of an unknown protein. Molecular weight of proteins can also be determined by running them of SDS polyacrylamide gels. It is known that the electrophoretic mobility of the proteins when plotted with the logarithmic value of the molecular weight of the standard molecular weight markers gives a standard curve (Weber K *et al.*,1969). This standard curve is then utilized to determine the molecular weight of unknown protein.

Seeds are an important reserve for variety of proteins required by plant during germination. The proteins present can be classified as storage, structural and functionally active proteins. It also covers other widely distributed groups of proteins including lectins, enzyme inhibitors, antifungal proteins, thionins and oleosins.

Most plant chitinases have been purified from different seeds, especially *H. vulgare* (Leah *et al.*,1991), *V unguiculata* (Gomes *et al.*, 1996), *G. max*(Yeboah *et al.*, 1997), *S. bicolor* (Krishnaveni *et al.*,1999), *P. glaucum* (Radhajeyalakshmi *et al.*, 2000), *O. sativa* (Baek *et al.*, 2001)], *A. pavonina* (Santos *et al.*,2004) and *S. cereale* (Taira *et al.*, 2000). Soybean chitinase has an apparent molecular mass of 28 kDa as determined by SDS-PAGE, and has chitinase specific activity of 133U/mg protein at pH 5.2 and an pI of 5.7, was purified from mature dry seeds. Chitinases in seeds have been implicated in roles like defence during germination (Hirano et al., 1990) and as seed storage proteins.

The tamarind belongs to the pea family, Leguminosae or Fabaceae. Its scientific name is *Tamarindus indica*. Tamarind is a large evergreen tree that grows in the tropics. It is native to Asian countries. Tamarind is popular in India as a condiment. Technology has now been

virtues and is used in treating loss of appetite, as a laxative, as an anti-helminthic and for healing wounds and cuts. The seed has found its applicability as textile thickener and in textile sizing for sizing jute and sometimes cotton. It has also been used in creaming rubber latex. The industries using the fruit pulp as the source have a large amount of tamarind kernel as the by product. Thus it becomes quite an economic source for isolation of any protein of interest. A class III chitinases from tamarind has been characterized previously (Rao *et al.*, 2008). This enzyme has a pH optima of 5 and temperature optima of 40-50°C.

We have isolated and structurally characterized a class III chitinase from the kernel of *T. indica*. The importance of my work lies in the fact that family 18 chitinases have been isolated from diverse sources with equally diverse substrate preference. The basis for this difference lies in the three dimensional arrangement of the amino acid moieties on the surface and active site of the enzyme which effects the functioning of the enzyme. Thus it becomes important to study the 3-D structure of chitinase to perform an interpretation of the structure function relationship.

2.2. Materials and Methods

2.2.1. Materials

Fruits of tamarind were obtained locally. Centricon and Centriprep were purchased from Amicon Millipore (Beverly, MA). Molecular weight standards, protein assay kit (concentration estimation), Affi-Gel Blue matrix, MacroPrep DEAE and MacroPrep CM (carboxymethyl) support were obtained from Biorad (Bio-Rad Laboratories, Hercules, CA, USA). Dialysis membrane was obtained from Pierce (USA). Gel filteration column Superdex -200 was obtained from GE Healthcare. All other chemicals were purchased from Himedia chemicals. Fluorescent brightener 28 and Glycol chitin were obtained from Sigma-Aldrich Pvt. Ltd.

2.2.2. Methods

2.2.2.1. Purification of protein

Fruits from a single tree were collected and the pulp was removed manually. The seeds removed were soaked in buffer. The coat was then removed manually using forceps to obtain the kernel of tamarind seed. The seed kernel (10 g) thus obtained were crushed with mortar and pestle and soaked overnight at 4°C in 60 ml of 100 mM Tris-HCl buffer pH 7.4. The homogenate was filtered using a muslin cloth followed by centrifugation at 12,000 x g for 40 min, 4 °C to obtain a clear supernatant. The supernatant was loaded onto affi-gel blue affinity column equilibrated with 100 mM Tris-HCl buffer, pH 7.4. The column was washed extensively to remove unbound molecules and this was crosschecked simultaneously by measuring absorbance at 280 nm. Bound molecules were eluted with a NaCl step wise elution from 50 to 1000 mM in the same buffer. Fraction containing proteins was monitored by

OD_{280}. The purity of the eluted fractions was analyzed on 15% SDS –PAGE (Laemli, 1970). Fractions containing the protein of interest was pooled (500 mM NaCl) and concentrated by using Centriprep followed by extensive dialysis against the native buffer (100 mM Tris-HCl buffer, pH 7.4) to remove traces of salt. The protein was further purified by using a series of ion exchange column.

The dialyzed sample obtained was loaded directly on a pre-equilibrated CM cation exchange column. The equilibration of the column was done by using 100 mM Tris-HCl buffer, pH 7.4. The flow through of the column was collected and molar elution for the bound fraction was done using a gradient from 100 mM to 1M NaCl in 100 mM Tris-HCl buffer, pH 7.4. The elution profile was again checked for the protein of interest on 15% SDS -PAGE. The protein was detected to be present in the unbound fraction. The flow through was subsequently concentrated and loaded on DEAE weak anion exchange column pre-equilibrated with 100 mM Tris-HCl buffer, pH 7.4. The bound fractions were eluted using a NaCl gradient. The purity of the protein was checked on 15% SDS polyacrlyamide gel. The pure sample obtained was concentrated using centriprep and centricon and dialyzed extensively to remove any trace of salt present in the sample. The integrity of the sample was determined by gel permeation chromatography. The protein obtained was subsequently analyzed on both reducing and non-reducing SDS-PAGE.

2.2.2.2. Protein estimation

Protein concentration in crude extract and fractionated protein samples were estimated by standard dye-binding method using bovine serum albumin (Sigma) as standard. For crude extract, mature seeds (10 g) were crushed with mortar and pestle and soaked overnight at 4°C

in 40 mL of 50 mM Tris-HCl buffer, pH 7.4. The homogenate was filtered using a muslin cloth and cleared by centrifugation at 12,000 x g for 40 min and the supernatant was used to determine the soluble protein concentration. The purified protein sample was concentrated and concentration checked by Biorad (Bradford) assay.

2.2.2.3. SDS-PAGE analysis

Sodium dodecyl sulfate-polyacrylamide gel (15%) electrophoresis (SDS-PAGE) under both reducing and non reducing conditions was done as described by Laemli (Laemli, 1970). Relative molecular weight was determined by performing SDS-PAGE of protein with molecular weight standards under reducing condition calibrated on Tris-HCl gel. The molecular weight standards used were myosin (209 kDa), β-galactosidase (124 kDa), bovine serum albumin (80 kDa), ovalbumin (49 kDa), carbonic anhydrase (34.0 kDa), trypsin inhibitor (28 kDa), lysozyme (20.6 kDa) and aprotinin (7.1 kDa). The proteins were detected by staining the gel with 0.1% Coomassie brilliant blue R-250. Molecular weight of CHT was determined by comparing the distance travelled by protein compared to the molecular weight markers.

2.2.2.4. Characterization of protein as chitinase.

2.2.2.4.1 Colorimetric assay

The enzyme purified in 0.1 M Tris-HCl pH 7.4 was extensively dialyzed and subsequently buffer exchanged into sodium acetate 0.05 M pH 5. This was reported to be pH optima for most of the chitinases. The chitinase assay was done according to the protocol given by Imoto and Yagishita (1971). Briefly a .05% solution of glycol chitin was prepared in 0.1 M sodium acetate buffer pH 5 and incubated with increasing range (0-80μg) of the

enzyme at 40°C for 30 min. This was followed by incubation with 0.2 ml of potassium ferricyanide (potassium ferricyanide prepared in 0.5 M sodium carbonate) and the mixture was immediately heated to 95°C continuously for 15 min to terminate the reaction. Potassium ferricyanide binds to reducing ends produced by the action of chitinases and is converted to colourless potassium ferrocyanide. After boiling the mixture was cooled and absorbance was measured at 420 nm. A control reaction containing no enzyme had been prepared.

2.2.2.4.2 Fluorescent assay

The fluorescent dye based assay for chitinase was done using fluorescent whitener 28 [calcofluor white] (Somashekhar 1996). For the fluorescent dye based assays, 0.4 ml of 0.05% glycol chitosan was incubated with 0.5 ml of protein solution and 0.6 ml of the sodium acetate buffer pH 5 for 1 hour at 40°c. To the control tube, all the constituents were added sequentially except the enzyme. The control and the experimental tubes were boiled simultaneously for 15 min and placed in cold water. To these tubes 0.01% calcofluor white freshly prepared in distilled water was added and incubated for 1 hour at room temperature. The calcofluor white binds to unhydrolysed chitin and gives a characteristic peak at 406 nm. The absorbance was monitored at 406 nm.

Chitinase plate assay was also done according to the protocol of Gohel *et al.*, 2004. Briefly agarose plate containing 0.05% glycol chitin and 0.01% Fluorescent brightener 28 was prepared. After running the SDS-PAGE electrophoresis, gel was incubated in 0.1 M sodium acetate buffer, pH 5 containing 1% triton X-100. This was followed by extensive washing of the gel in distilled water for approximately 30 min. The washed gel was then placed carefully on the chitin-agarose and layered with 1 ml of sodium acetate buffer pH 5 to

allow efficient diffusion of the enzyme in the agarose plate. The gel was allowed to incubate on plate for 5 hrs at 37°C followed by extensive washing in distilled water. The plate was then observed under UV transillumination. Dark bands against the fluorescent background are an indication of chitinases action.

2.2.2.4.3. Effect of additives on activity

It has been observed that presence of metal ions tend to affect the activity of chitinases (Frankowski *et al.*, 2001, Wang *et al.*, 1997, Giambatitista *et al.*, 2001). Activity was assayed by reaction mixtures consisting of K^+(K_2SO_4), Na^+ (NaCl), Ca^{2+}($CaCl_2$), Li^+ (LiCl), Mg^{2+}($MgCl_2$), Cd^{2+}($Cdcl_2$) and Zn^{2+}($ZnCl_2$) metal ions at final concentration of 1 mM. Enzyme activity was observed at optimal pH 5 and temperature (40°C) after pre-incubation with the additive at 37 °C for 1 h. Effect of EDTA (5mM) was also monitored using the same protocol.

The enzyme was also incubated with reducing agents like 0.1 mM DTT and 10 mM β-mercaptoethanol followed by enzymatic assay according to Somashekhar *et al.*, 1996 with glycol chitosan as the substrate. All the experiments were performed in duplet and the results was averaged and plotted.

2.2.2.5. Lysozyme activity assay for CHT

Lysozyme activity for enzyme is measured at OD_{450} by tracking the decrease in optical density as the substrate's (*Micrococcus luteus*) cell wall is broken by the enzyme. For monitoring the activity lyophilized culture of *M. luteus* was regenerated using 0.9 M saline solution and plated on Luria agar plate followed by incubation at 37°C overnight. The

regenerated cells were subcultured for 12 hrs and subsequently pelleted by centrifugation at 5000 rpm at 4° C. Suitable amount of culture was then suspended in 0.5 M acetate buffer pH 5 so as to obtain OD_{450} ~0.800. This constituted the standard substrate solution for the enzyme CHT. Similarly substrate solution for hen egg white lysozyme (+ve control) was prepared in 0.1 M phosphate buffer pH 6.6. The reaction for the experiment and the control was set up by adding serial dilutions of CHT and lysozyme to 5 ml of standard substrate solution containing *M. luteus*. The reaction sets were then incubated at 37°C for 15 min with gentle agitation. The absorbance was observed at 450 nm against a buffer blank for each set of reaction.

2.2.2.6. Assay for antifungal activity of CHT

For checking the potential antifungal activity of CHT, potato dextrose agar plate assay was performed for *Aspergillus fumigatus*, *Aspergillus flavis*, *Alternaria brassica* and *Candida albicans*. The protein was dissolved in 0.1 M acetate buffer pH 5 to a concentration of 6 mg/ml and filtered using 0.22 micron syringe filter. PDA plates containing ampicillin at the concentration of 80µg/ml were prepared and actively growing spores were placed in the centre of the PDA plates for *Aspergillus fumigatus*, *Aspergillus flavis* and *Alternaria brassica*. Similarly for *Candida albicans* actively growing liquid culture was spread uniformly on the plate. Six holes were punched in each of the plates and increasing concentration of protein corresponding to 100-500 µg of CHT was placed in each well. The control well was kept with autoclaved 0.1 M acetate buffer pH 5. The plates were subsequently kept at 27°C for 72 hrs.

2.2.2.7. The N-terminal sequencing

Pure protein was subjected to SDS-PAGE (15%) and electroblotted onto a PVDF membrane (Immobilone-P ^{SQ} Millipore) in 100 mM Caps-buffer, pH 11 (Matsudaira., 1987). The N-terminal amino acid sequence analysis was performed by Edman degradation on a Shimadzu Automated Protein Sequencer (PPSQ-20) at the Biophysics Department, All India Institute of Medical Sciences, New Delhi, India.

2.3. RESULTS

A protein with a chitinase property was found to be a major constituent of a crude extract obtained from the kernel of tamarind. The protein was purified to homogeneity by a three step chromatographic procedure. Initial requirement was procuring a clear supernatant after centrifugation of the crude extract at 12000 x g which was loaded on to a affi-gel blue matrix packed column equilibrated with 100 mM Tris-HCl buffer, pH 7.4. Two major protein bands were obtained in 0.5 M NaCl elute. The elute thus obtained was dialyzed extensively using a 10 kDa cutoff membrane to remove any trace of salt present in the fraction. The elute when loaded on cation exchange matrix was freed from other minor impurities leaving two major bands both of which were obtained in the flow through of CM. The protein (CHT) was successfully purified using the anion exchange matrix(DEAE) at a molar elution with 0.1 M NaCl in 100 mM Tris-HCl buffer, pH 7.4.(Fig 2.1) The purity of the protein was confirmed by SDS-PAGE analysis. Analysis with both reducing and non-reducing PAGE confirmed that CHT consisted of a single polypeptide chain.

The purified protein was loaded on Superdex-200 gel permeation column and was eluted with 100 mM Tris-HCl buffer, pH 7.4 to give a single distinct peak.(Fig 2.2) The

protein mass was calculated to be ~34 kDa. A single peak indicated that protein is a homogenous mixture which is an essential prerequisite for crystallization.

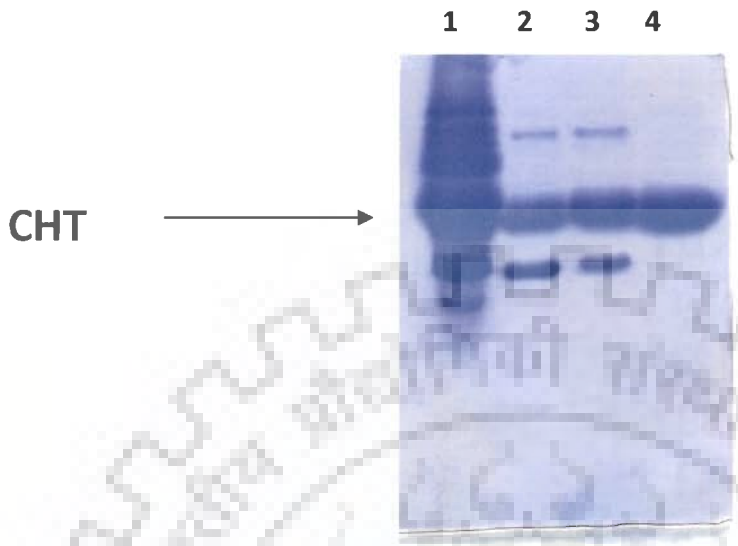


Fig 2.1: Elution profile of CHT on SDS-PAGE indicating 1: crude extract; 2: Affi-gel blue (0.5 M elute) ; 3: CM flow through; 4: DEAE (0.1 M elute)

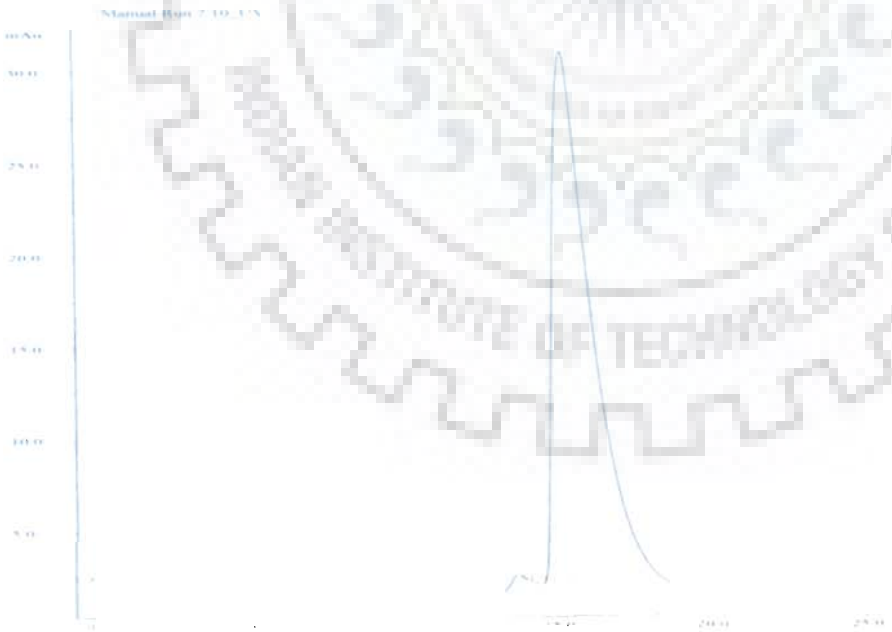


Fig 2.2: Elution profile of CHT from *Tamarindus indica* from gel permeation column Superdex -200.

The SDS-PAGE analysis of CHT along side protein molecular weight marker indicated the molecular weight of the protein to be approximately 34 kDa.(Fig 2.3)

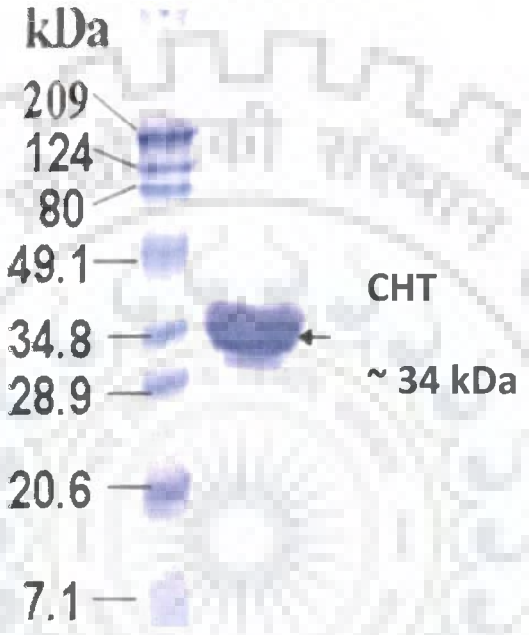


Fig 2.3: SDS-PAGE analysis of molecular weight of CHT from *Tamarindus indica* L1- molecular weight marker; L2- pure protein CHT

The protein was concentrated to 15mg/ml and the yield was about 0.5 mg/gm of protein. Purified concentrated samples of CHT were utilized to perform the activity assay with potassium ferricyanide (Fig 2.4) and calcofluor white (Fig 2.5) as indicators. The total reaction mixture was 3 ml containing increasing concentration of CHT.

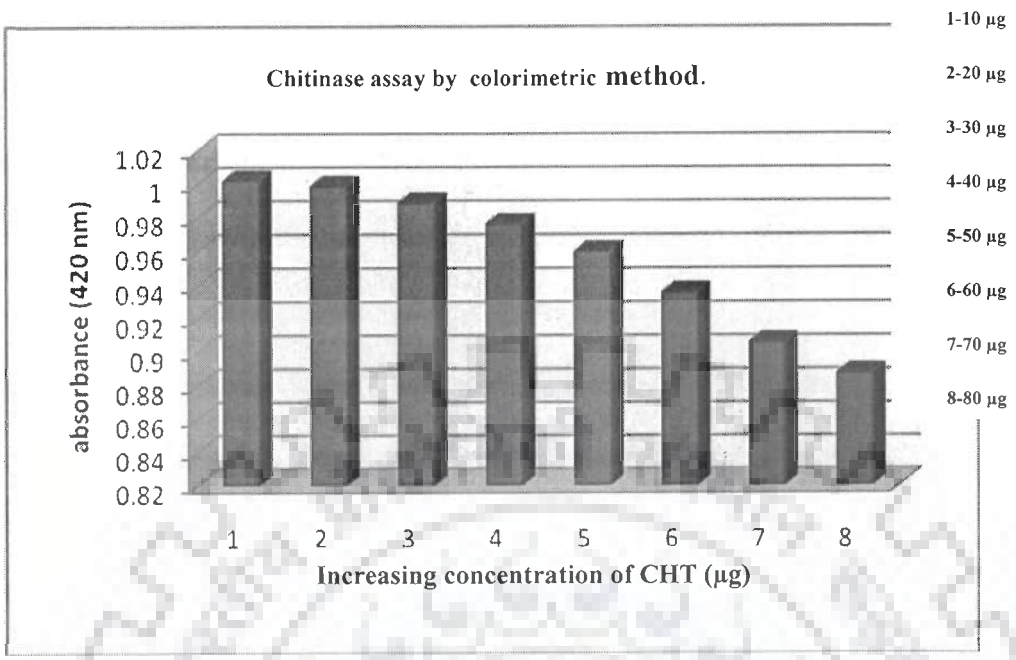


Fig 2.4: Confirmation of chitinase activity of CHT by colorimetric based activity assay using potassium ferricyanide.

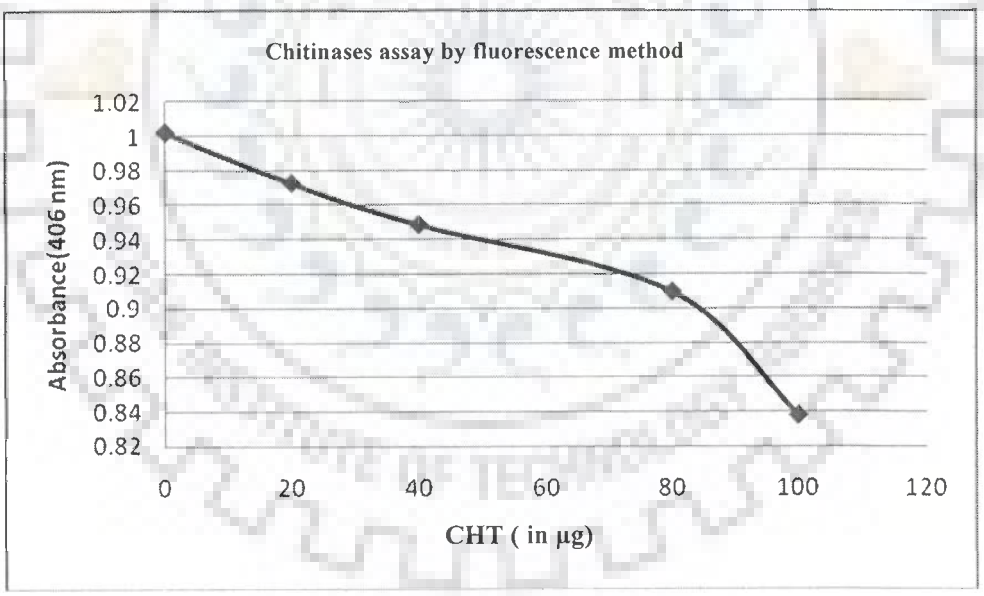


Fig 2.5: Confirmation of chitinase activity of purified CHT by fluorescence based activity assay (calcofluor white).

The enzymatic activity of CHT was examined after addition of metal ions and other additives like EDTA, DTT and βME.(Fig 2.6) The results were compared with the activity of the enzyme without any additives Zn^{2+} inhibited enzyme activity by 15% whereas in case of Mg^{2+} ions inhibited enzyme activity by 10%. EDTA at a molar concentration of 5 mM inhibited the CHT activity by 9% (Table 2.1).

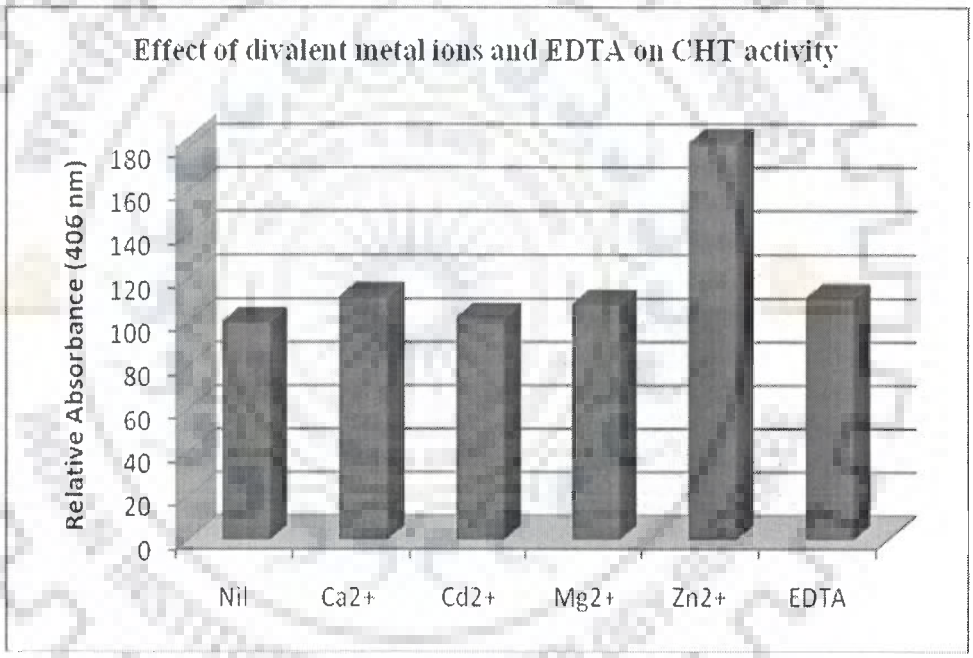


Fig 2.6 : Effect of divalent cations like Ca^{2+}, Cd^{2+}, Mg^{2+}, Zn^{2+} and EDTA on the activity of CHT from *Tamarindus indica*

Table 2.1. Influence of presence of divalent metal ions on chitinase activity

S .no	Additive	Absorbance	Relative absorbance	Change in activity(%)
1	Nil	0.8712	100	-
2	Ca^{2+}	0.9725	111.6	10
3	Cd^{2+}	0.8898	102.1	2
4	Mg^{2+}	0.9424	108.1	7
5	Zn^{2+}	1.29	182	15
6	EDTA	0.9665	110.9	9

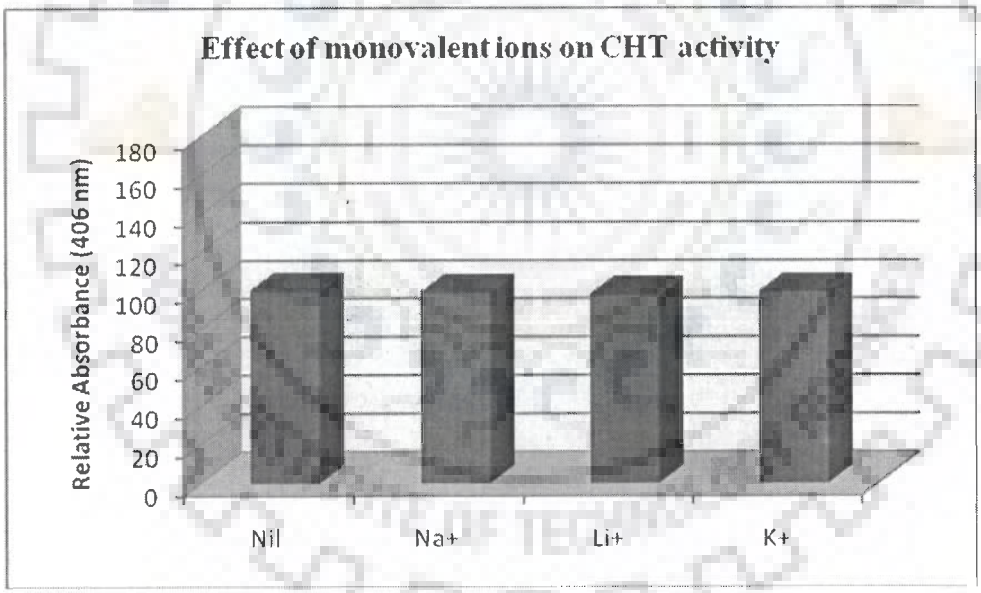


Fig 2.7: Effect of monovalent cations like Na^+, Li^+ and K^+ on the activity of CHT from *Tamarindus indica*

For monovalent metal ions (Fig 2.7) there was marginal enhancement of the enzyme activity. Li^+ at a concentration of 1mM, 2% increase in activity was observed (Table 2.2)

Table 2.2. Influence of presence of monovalent metal ions on chitinase activity

S .no	Additive	Absorbance	Relative absorbance	Change in activity(%)
1	Nil	0.8712	100	-
2	Na^+	0.8632	99.08	0.8
3	Li^+	0.8532	97.9	2
4	K^+	0.8673	99.5	0.4

In case of DTT at a concentration of 0.1mM and βME at a concentration of 10 mM (Fig 2.8) , both reducing agent, fall in the activity of enzyme was noticed the change being 12% and 8.7% respectively. (Table 2.3)

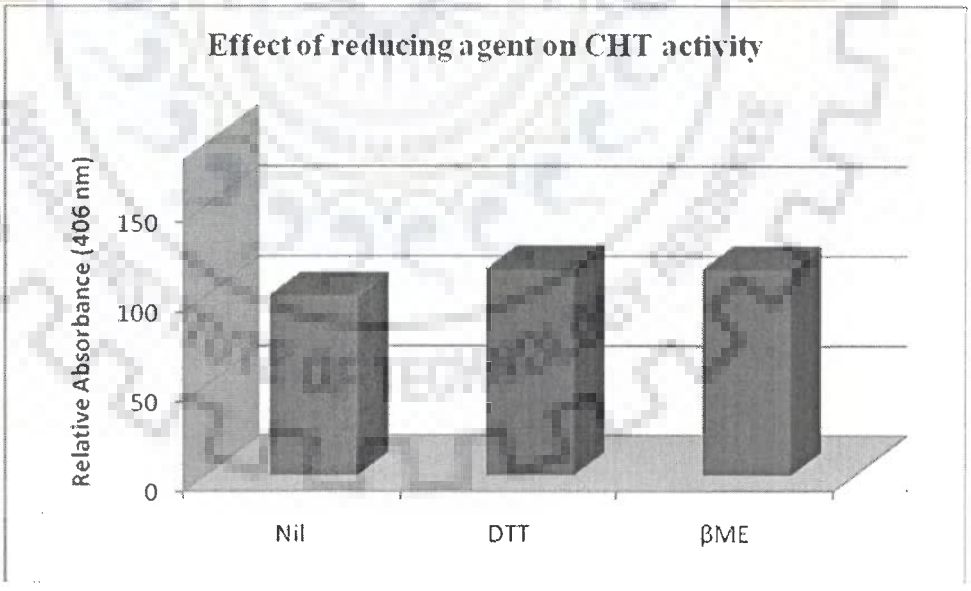


Fig 2.8: Effect of DTT and βME on the activity of CHT from *Tamarindus indica*

Table 2.3. Influence of presence of additives on chitinase activity

S .no	Additive	Absorbance	Relative absorbance	Change in activity(%)
1	Nil	0.8712	100	-
2	DTT	0.9987	114.6	12
3	βME	0.9581	114.5	8.7

Activity of CHT was examined as a lysozyme with the cells of *Micrococcus luteus* as a substrate for the enzyme. The results were obtained by observing OD_{450}. There was no decrease in OD_{450} even at a concentration of 1mg/ml of CHT. This indicated that the enzyme CHT has no lysozyme activity.

Antifungal activity was tested against three fungal plant pathogens namely *Aspergillus fumigatus*, *Aspergillus flavis*, *Alternaria brassica* and a human pathogen *Candida albicans*. CHT was found to be inactive against all four fungal pathogens.

Cycles of sequence data were obtained from the analysis of the protein sample. The sequence of the first 20 N-terminal amino acids was Trp-Asp-Asp-Ala-Ala-Tyr-Ala-Gly-Val-Ile-Ser-Val-Tyr-Trp-Gly-Gln-Asn-Gly-Ser-Asp. The obtained amino acid sequence was searched against the NCBI BLAST short sequence search protein data base. The 20 amino acid residue did not show any conserved domain in this stretch of amino acid. The sequence gave 78% identity with adzuki bean chitinase [Vigna angularis] followed by substantial identity with class III chitinases from *V. Vinifera* (Fig 2.11).

CHT	WDDAAYAGVISVYWGQNGSD	
Name of protein	**Sequence**	**Identity**
Acidic chitinase (*Vigna angularis*)	7 AGVISVYWGQNGSD 20 AG ISVYWGQNG++ 25 AGGISVYWGQNGNE 38	78%
Class III chitinase (*Vitis vinifera*)	6 YAGVISVYWGQNGSD 20 YAG I++YWGQNG++ 24 YAGGIAIYWGQNGNE 38	66%
Chitinase (*Mesembryanthemum crystallinum*)	3 DAAYAGVISVYWGQ 16 DA+ Y G I+ VYWGQ 21 DASYGGGIAVYWGQ 34	71%
Acidic chitinase (*Gossypium hirsutum*)	3 DAAYAGVISVYWGQNGSD 20 + +YAG I++ YWGQNG++ 22ETSYAGDIAIYWGQNGNE 39	55%

Figure 2.11: Sequence homology study by NCBI BLAST showing the N-terminal amino acid sequence of CHT (residues 1-20) having sequence identity with chitinases of different species.

2.4. DISCUSSION

Tamarindus indica is a well known plant belonging to the leguminaceae family with many medicinal and industrial applications. It is widely used in India as a condiment. In this chapter, the isolation, purification and characterization of a chitinase from the seed kernel of *Tamarindus indica* has been described. The chitinase (CHT) was purified by a three step procedure. The first step involved usage of an affinity matrix affi-gel blue gel followed by anion CM and cation DEAE column matrix at pH 7.4. The approximate molecular mass of the protein was determined to be 34 kDa from SDS-PAGE analysis and SEC. The protein yield was 0.5 mg/gm of seed and consisted of a homogenous mixture. It was found out to be the most abundant protein in tamarind seed kernel, thus implying a possible role as storage protein.

Effect of metal ions on the activity of chitinases has been widely studied. The effect of each metal ion on chitinases may differ from species to species. For CHT it was observed that divalent ions like Ca^{2+}, Cd^{2+} Mg^{2+} and Zn^{2+} tend to inhibit the activity of enzyme whereas the monovalent ions like Na^{+}, Li^{+} and K^{+} had a very low activating effect. The divalent ions like Ca^{2+} and Mg^{2+} did not exert any inhibitory activity in case of chitinase from *Alcaligenes xylosoxydans* even at an concentration upto 5mM. Similarly in case of *Serratia plymuthica* Ca^{2+} activated chitinases by 20%. The difference in the effect of metal ions on chitinases may depend on the mechanism of interaction of metals with the amino acid residues and the modulation which occurs due to their presence. It is a known fact that heavy metal ions tend to bind strongly to the sulfhydryl groups of amino acids (Viarengo, 1985).

Sulfhydryl binding changes the structure and enzymatic activities of proteins and causes toxic effects evident at the whole organism level (Hodson, 1988). The presence of 5 mM EDTA tends to inhibit the activity of CHT enzyme by 9% which signifies that requirement of metal ion for optimal activity of this enzyme. Reducing agents were also used to study the effect on the activity of CHT. DTT and βME inhibited the enzyme CHT by 12% and 9% respectively bringing into focus the importance of the disulphide bridges which are well known to play key roles in stability, folding and functions of proteins.

It has been observed in some cases of class III chitinases that there is an additional lysozyme activity (Scheltinga *et al.*,1994), but no such activity was found to be prevalent in CHT. This may be due to absence of some critical amino acid residues which enables the activity of chitinases as a lysozyme. The CHT was also found not to be a potent antifungal agent as it was unable to inhibit the growth of four fungal pathogens namely *Aspergillus fumigatus*, *Aspergillus flavis*, *Alternaria brassica* and *Candida albicans.* This characteristic has been found to be a common observation for class III chitinases. Chitinases mainly have been reported to act as antifungal agents synergistically in the presence of β,1,3- glucanases (Volgensang and Barz,1993).

The amino acid sequence analysis revealed the isolated protein was indeed a chitinase. The N-terminal of the protein showed 78% homology with acidic chitinases from *Vigna ungulata* and 66% with a class III chitinases from *Vitis vinifera* confirming that the isolated protein belongs to family of class III chitinases.

To sum up CHT is a single polypeptide 34 kDa protein belonging to class III chitinases isolated from the kernel of tamarind seed. This enzyme lacks antifungal and lysozyme activity. CHT may have the requirement of some metal ion for its optimal activity as presence of EDTA inhibited activity of the enzyme. Inhibition of enzyme activity in presence of reducing agents indicated the importance of disulphide bonds in maintaining the integrity and functioning of the enzyme. The purified protein CHT can be utilized for various industrial applications and waste management as the source is economical and easily available.

Chapter 3

MOLECULAR CLONING AND BIOINFORMATIC ANALYSIS OF CHT

3.1. Introduction

Members of the class III chitinases are unique in having no sequence similarity with any other classes of chitinases. The structural features of class III chitinases also vary quite vastly. Many chitinases, like other plant defense proteins, are acidic extractable, resistant to proteases, are secreted extracellularly and can either be acidic or basic in nature. Usually, the acidic forms are secreted into the apoplast or extracellular environment, whilst the basic forms accumulate intracellularly in the vacuole. Deduced amino acid sequences for putatively vacuolar, basic chitinases differ from the homologous extracellular, acidic isoforms by the presence of a C-terminal extension (Neuhaus *et al.,* 1991). Hydrolytic enzymes present in vacuoles of higher plant defend against pathogens and therefore, the higher plant vacuoles are believed to be analogous to animal lysosomes (Sahai *et al.*,1993 and Punja *et al.,* 1993). Basic endochitinases in bean leaves, for example, were localised in the vacuoles and contained most of the intracellular chitinase activity. In pineapple (*Ananas comosus*) a class chitinases named PL Chi-A was purified from the leaves but the chitinases did not have any antifungal activity. Besides leaves, seeds are a good source for chitinases as has been observed in the cases of barley (Leah *et al.*,1991), cowpea (Gomes *et al.*,1991), soybean (Yeboah *et al.*,1998), sorghum (Krishnaveni *et al.*,1999), pearl millet (Radhajeyalakshmi *et*

al., 2000), rice (Baek *et al.*, 2001) and rye (Taira *et al.*, 2001)

Chitinases belonging to the class III have been cloned from numerous sources like rice (Nagasaki *et al.*,1997), chickpea (Vogelsang *et al.*,1993), grapes (Ano *et al.*,2003), jelly fig (Li *et al.*,2005), muskmelon (Balde′ *et* al.,2004) and cucumber (Bovie *et al.*,2004) to name a few. A peculiar feature of this class of chitinases is that some of the members tend to lack introns in their gene sequence.

In this chapter, we have described the molecular cloning of CHT partial cDNA and its gene from *T. indica* The nucleotide sequence that was obtained from sequencing of cDNA was translated to obtain the amino acid sequence of CHT. Further, with the help of bioinformatic tools such as BLAST, Clustal W, EsPript, the obtained protein sequence of CHT was used for doing sequence and structural analysis.

3.2. Materials and methods

3.2.1. Materials

RNase A, plant RNA isolation kit, MMLV (Molony murine leukemia virus) reverse transcriptase, Taq polymerase and DNA gel extraction kit were obtained from Bangalore GeNei (Bangalore). All other chemicals were obtained from Himedia. Sequencing of cDNA and gene was done at Ocimum Biosolutions, Hyderabad.

3.2.2. Methods

3.2.2.1. Genomic DNA isolation

Leaves were harvested from the tree and frozen in liquid nitrogen. They were frozen at –80°C until required. 3.0 g of leaf sample was ground to a fine powder in liquid nitrogen using a pre-chilled mortar and pestle. Some liquid nitrogen was poured in just before adding

the leaves for crushing. The finely crushed leaves were quickly transferred to a pre-chilled 50-mL falcon tube. 15 ml of CTAB buffer [2% (w/v) CTAB containing 1.4M NaCl, 20 mM EDTA, 100 mM Tris HCI (pH 8.0)] was quickly added to the tube and was mixed thoroughly. The tube was incubated at 65°C for 1 hour with frequent swirling. This was followed by addition of an equal volume of chloroform: isoamyl alcohol (IAA) (24:1) and the total sample was mixed thoroughly for 20 min at room temperature. Subsequently the sample was centrifuged 5000 rpm for 10 min at room temperature to separate the phases. The supernatant was carefully transferred to a new tube using a wide mouth pipette tip. To the obtained supernatant 2/3 volume of ice cold isopropanal was added and the supernatant was mixed well by inverting the tube gently 4-5 times followed by incubation at 4°C for 10 min. Mixture was centrifuged to precipitate the suspended DNA and washed with 200 µl of ice cold 70% ethanol twice and the final precipitated DNA was dried and suspended in 1X TE buffer (Tris-EDTA pH 8).

RNA was removed from genomic DNA preparation by RNase A treatment. RNase A (DNase free) was used at a final concentration of 0.1 mg/ml and the reaction was carried out at 37°C in dry bath for 2 hrs. The DNA was extracted once with phenol:chloroform:IAA and once with chloroform:IAA followed by precipitation by isopropanol and dissolved in 1X TE buffer pH 8 and analyzed on a 0.8% agarose gel.

3.2.2.2 cDNA cloning

3.2.2.2.1. RNA extraction

RNA from seeds of tamarind was extracted using RNA extraction kit (Bangalore Genei). Polypropylene centrifuge tubes were pretreated with 0.1% diethyl pyrocarbonate (DEPC) for one hour at room temperature and the tubes were then autoclaved for 30 minutes

to destroy residual DEPC. Frozen freshly harvested 1 gm tamarind seeds were placed in liquid nitrogen and grounded to fine powder under liquid nitrogen using pre-chilled mortar and pestle. The liquid nitrogen was allowed to evaporate and the ground tissues was transferred to a sterile 15 ml centrifuge tube. 4 ml of prewarmed buffer A (from kit) + phenol mixture (80° C) was added and mixed thoroughly by inverting the tube for 5 minutes. Addition of equal volume of chloroform:isoamyl alcohol(24:1) was done to the tube and carefully mixed by inverting 3-5 times followed by incubation on ice for 30 minutes. The tube was centrifuged at 5000 x g for 20 minutes at 4°C and the top aqueous phase, containing RNA, was carefully removed.

3.2.2.2.2. RNA precipitation

To the obtained supernatant, precipitation buffer B (from kit) was added and incubated for 4hrs at 4°C. This was followed by centrifugation at 7000g for 20 minutes at 4°C to get the pellet. The pellet was resuspended in 1-2 ml of buffer C (from kit) and incubated on ice till the pellet is solubilizes totally and again pellet was obtained following centrifugation at 7000g for 20 minutes at 4°C. This step was repeated twice to remove any trace of impurity from the RNA pellet.

3.2.2.2.3. RNA washing

The pellet obtained was finally washed by adding a minimum of 5 ml of ice-cold 75% ethanol and centrifuged at 10,000 x g for 10 minutes at 4°C. The step was performed twice, the pellet was air dried in RNase-free environment for 20 minutes and obtained RNA was dissolved in nuclease-free water. The RNA pellet obtained was run on a 1% denaturing formaldehyde agarose gel to check the integrity of RNA.

3.2.2.2.4. Reverse transcription

The reverse transcription reaction was performed with the enzyme MMLV (Molony murine leukemia virus) reverse transcriptase. The first strand ssDNA synthesis reaction was performed by taking oligo-$(dT)_{26}$ with T_m value 48°C as a down stream primer. The RNA extracted was denatured prior to addition to the reverse transcription mix by heating to 75°C for 5 minutes in a dry bath. 20 µl of the reverse transcription reaction mix was prepared containing 3.5 µl nuclease-free water, 4.0 µl 5 x reaction buffer, 1.0 µl of 20 mM dNTP mix, 0.5 µl ribonuclease inhibitor, 5.0 µl MMLV reverse transcriptase. In 0.2 ml PCR tube, to this reaction mix 5 µl of denatured RNA and 1µl primer mix was added, giving the final reaction volume of 20 µl. The reaction was placed in a controlled temperature heat block equilibrated at 37°C and incubated for 60 minutes. This was followed by incubation at 95°C for 15 minutes to inactivate the reverse transcriptase.

3.2.2.2.5. Polymerase chain reaction

Polymerase chain reaction (PCR) is a common method of amplifying specific fragments of DNA. Immediately after completion of reverse transcription reaction, the synthesized first stand was used to amplify our gene of interest through polymerase chain reaction using gene specific primer. The primer had been designed on the basis of the N-terminal sequence of purified protein and the conserved C-terminal region in class III chitinases which was based on the results obtained after multiple sequence alignment. The gene specific primers were designed based on the percentage of GC content, least susceptibility to form hairpin structures and melting temperature (Table 3.1).

Table 3.1: Primer sequence

S.No	Primer sequence	%GC	Length	Tm(°C)
F1	**TGGGATGATGCTGCTTATGCTGGT**	50	24	62.71
F2	**TATTGGGGCCAAAACGGYAAYGA**	47.82	23	60.64
R1	**Oligo-$(dT)_{26}$**	-	26	48
R2	**CCAAAGAACCATAACACCACCATA**	41.66	24	59.3

50 µl of reaction mix was prepared containing 37.5 µl of nuclease-free water, 5 µl 10X reaction buffer, 2.0 µl 20 mM dNTP, 1 µl of 5 mM gene specific upstream primer, 1 µl of 5 mM downstream primer, 0.5 µl Taq polymerase and 3.0 µl RT reaction product. The PCR reaction mix was placed in a thermal cycler that has been pre-warmed to 94°C and following PCR program was used: an initial denaturation at 94°C for 5 minutes and then 94°C for 60 seconds; 45-58°C for 60 seconds and 72°C for 60 secs over 30 cycles, the run ended with an extension at 72°C for 10 minutes followed by cooling to 4°C.

For the post-PCR analysis 5 µl from the PCR-reaction was mixed with 1 µl gel loading dye (6X) and loaded on 1% agarose gel containing ethidium bromide.

3.2.2.2.6. Cloning of CHT gene

The genomic DNA isolated from leaves was used as a template for PCR amplification reaction using R2 and F2 as the reverse and forward primers. Same conditions were also used while doing PCR for amplifying the gene using genomic DNA as the template. Amplified product of PCR was analyzed on 1% agarose gel.

3.2.2.2.7. DNA Gel extraction

The amplified product obtained was extracted from gel using the gel extraction kit (Bangalore Genei). The amplified product was run on low melting agarose gel and after resolution of the full product, the amplified PCR product was manually sliced from the agarose gel on an UV-transilluminator. Weight of the excised pieces of gel were kept in 1.5 microfuge tubes was measured. Weight of the excised band was around 0.7 mg. Precautions were taken not to include excess volumes of agarose gel during the manual slicing. Subsequently 3 times volumes of gel solubilization buffer was added to one volume of gel containing PCR product. The mixture was incubated at 50°C for 10 min with proper vortexing every 2-3 minutes. 100 µl of 3 M sodium acetate pH 5 was added to the mix followed by addition of 700 µl of isopropanol. This was followed by addition of this mix to a DNA spin column(from kit) and the DNA was eluted from the column by washing, using wash buffer and eluted with 20 µl of elution buffer as provided in the kit.

The obtained DNA was quantified using spectrophotometer at OD_{260} and on 1% agarose gel containing ethidium bromide. The DNA obtained was subsequently sequenced by gold standard 96-capillary 3730xl DNA Analyzer at Ocimum Biosolutions, Hyderabad.

3.2.2.3. Bioinformatic analysis

The nucleotide sequence obtained was searched using the NCBI blast suite in nucleotide search database to find the identity of the amplified cDNA. The nucleotide sequence was subsequently translated to corresponding protein sequence using the Expasy Reverse Translate tool. The protein sequence containing one ORF was selected and subsequently matched in the NCBI protein non redundant database.

The amino acid sequences showing maximum similarity to chitinase CHT were retrieved in the FASTA format and alignment was performed using the CLUSTAL W tool (Thompson, J.D., 1994) from EBI site. The obtained alignment obtained was further analyzed.

Based on molisch test it was found that carbohydrate moieties are attached to the protein,hence CHT is a glycoprotein. Thus the protein glycosylation sites were searched and analyzed using the NetNGlyc 1.0 server. Protein analysis tools present in expasy server were used further to characterize the protein (Gasteiger *et al.*, 2005).

3.3. Results

3.3.1. Genomic DNA isolation

Fresh leaves were used to isolate genomic DNA from *Tamarindus indica*. The leaves were crushed in liquid nitrogen to effectively pulverize the tissue. Following pulverization the tissue was treated with 2% CTAB, incubated at 65˚C for 1 hour (which lyses the cells releasing its contents in the solution). Presence of EDTA chelates metal ions which might result in cleavage of DNA. Addition of chloroform and isoamyl alcohol results in separation of proteins and fat in the organic phase leaving the DNA in the aqueous phase. Addition of isopropanol results in effective precipitation of DNA from the aqueous phase which is washed with 70% ethanol and precipitated DNA suspended in 1X TE buffer pH 8. The genomic DNA run on 0.8% agarose gel containing EtBr gave a single distinct band confirming successful isolation. (Fig 3.1)

L1

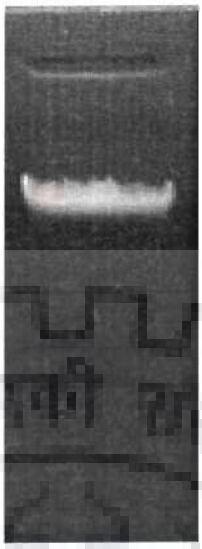


Fig 3.1: Genomic DNA of *T.indica*; Lane L1:- 10 µl of DNA

3.3.2 Isolation of RNA and reverse transcription

Seeds at various stages of maturation had been collected and monitored for the expression of CHT. Fresh seeds from the tree were directly collected after 40 days from flowering and immediately stored in liquid nitrogen. RNA was extracted from the seeds using the plant RNA extraction kit (Bangalore Genei).

Two distinct bands corresponding to 28S and 18S rRNA bands were seen on agarose in the isolated RNA sample. The 28S rRNA band was more intense as the 18S rRNA band. Thus, total RNA was successfully isolated from the seeds of *Tamarindus indica* in intact form. (Fig 3.2)

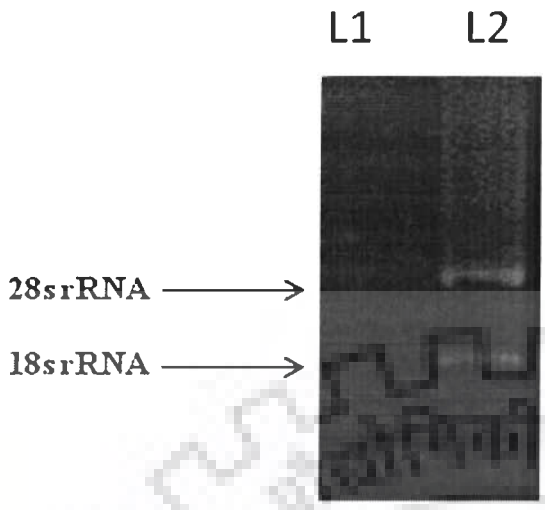


Fig 3.2: RNA denaturing formaldehyde gel of total RNA from the seeds of *Tamarindus indica* showing 28S and 18S rRNA bands. Lane L1: showing 1µl of RNA sample; lane L2: showing 5µl of RNA sample.

RNA extracted on storage tends to attain secondary structures hence denaturation of RNA by heating to 95°C prior to RT-PCR was done. For reverse transcription Oligo-dT was used for priming cDNA synthesis. The ssDNA product of RT-PCR was subsequently used as a template for PCR reaction.

For PCR two sets of forward primer and two reverse primers had been synthesized. The reaction in which oligo-d(T)[R1] was used as a reverse primer failed to give PCR product. The primer 5'-CCAAAGAACCATAACACCACCATA-3' [**R2**] with 5'-TATTGGGGCCAAAACGGYAAYGA-3' [**F2**] gave an amplified PCR product of ~ 700bp in length at an annealing temperature of 57.8°C (Fig 3.3). Same result were also obtained when primers R2 and F2 were used in a PCR reaction with same conditions as above and using genomic DNA as a template.

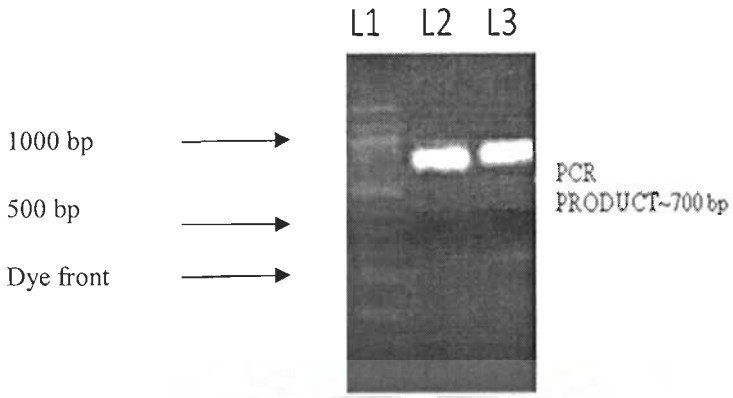


Fig 3.3: cDNA of CHT from Tamarindus indica. 1% DNA agarose gel showing amplifie PCR product with lane L1 showing the 100 bp DNA ladder (Bangalore Genei) and lane L and L3 showing amplified products for CHT.

The PCR product obtained was gel extracted and was sequenced (Fig 3.4)

The DNA sequence obtained for the cDNA of CHT by sequencing the amplified PC product is shown below in fig 3.5. The primer sequence was placed at the 5' and 3' end of th obtained DNA sequence. This DNA sequence was translated into the amino acid sequence using the translate tool of Expasy server to obtain the primary sequence of CHT protein fro *T. indica*. The amino acid sequences obtained on translation contained six different ORF' The deduced amino acid sequence of each ORF was further used for BLAST homolog searching against nonredundant (nr) protein databases of NCBI. An ORF was identified tha showed high homology with chitinase enzymes. This ORF (shown below in fig. 3.5) wa selected and used for further sequence analysis.

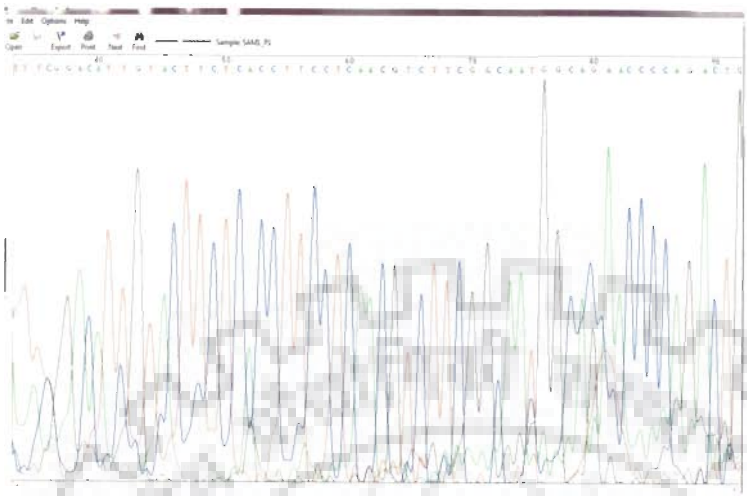


Fig 3.4: DNA sequence chromatogram of CHT (showing a portion of obtained CHT nucleiotide sequence, 32 nt to 92 nt)

equence. The protein sequences obtained in the translate tool were present in six different ORF's and thus they were matched to obtain the best match for the CHT sequence in the NCBI BLAST suite. Thus the protein sequence for CHT from Tamarindus indica was obtained.

'-TATTGGGGTCAAAATGGTTCTGATAATTCTGAAGGTTCTCTTTCTCAAGCT
Tyr Trp Gly Gln Asn Gly Ser Asp Asn Ser Glu Gly Ser Leu Ser Gln Ala

'GTGAAACTGGTAATTATGATTTTGTTCTTCTTCATTTTCTTAATACTTATCT
ys Glu Thr Gly Asn Tyr Asp Phe Val Leu Leu His Phe Leu Asn Thr Tyr Leu

ATGGTGGTGAACCTGATCTTAATCTTGCTGGTCATTGTGGTGGTGGTGGTGGT
sn Gly Gly Glu Pro Asp Leu Asn Leu Ala Gly His Cys Gly Gly Gly Gly Gly

TGTACTAAACTTCAATCTGAAATTAAATATTGTCAATCTCAAAATATTAAAGTT
Cys Thr Lys Leu Gln Ser Glu Ile Lys Tyr Cys Gln Ser Gln Asn Ile Lys Val

CTTCTTTCTCTTGGTGGTACTACTGGTAATGGTTCTCTTAATTCTACTGAAGAT
Leu Leu Ser Leu Gly Gly Thr Thr Gly Asn Gly Ser Leu Asn Ser Thr Glu Asp

GCTCAAGAACTTGCTAATTATCTTCCTAATAATTTTCTTAATGGTAATGCTGGT
Ala Gln Glu Leu Ala Asn Tyr Leu Pro Asn Asn Phe Leu Asn Gly Asn Ala Gly

CTCTTGGTGATGTTGAACTTGATGATATTGATTTTGATATTGAATCTGATGGT
Pro Leu Gly Asp Val Glu Leu Asp Asp Ile Asp Phe Asp Ile Glu Ser Asp Gly

TCTACTCTTTATTATGATGATCTTGCTCGTGCTATTAATCTTGATACTACTGGT
Ser Thr Leu Tyr Tyr Asp Asp Leu Ala Arg Ala Ile Asn Leu Asp Thr Thr Gly

CGTAAAATTTATCTTTCTGCTGCTCCTGAATGTCCTTATCCTGATAATTATCTT
Arg Lys Ile Tyr Leu Ser Ala Ala Pro Glu Cys Pro Tyr Pro Asp Asn Tyr Leu

AATAAAGCTGTTCAAACTGGTCTTGTTGATTATATTTTTGTTCAATTTTATAAT
Asn Lys Ala Val Gln Thr Gly Leu Val Asp Tyr Ile Phe Val Gln Phe Tyr Asn

AATCCTCCTTGTTCTTATTCTTCTGGTAATACTGGTGGTCTTTTTTCTGCTTGG
Asn Pro Pro Cys Ser Tyr Ser Ser Gly Asn Thr Gly Gly Leu Phe Ser Ala Trp

AATACTTGGACTTCTAATGTTCCTAATTCTCTTGTTTTTATGGGTCTTCCTGCT
Asn Thr Trp Thr Ser Asn Val Pro Asn Ser Leu Val Phe Met Gly Leu Pro Ala

TCTCCTGAAGCTGCTGGTTCTGGTGGTTATGTTCCTCCTGATGTTCTTAATTCT
Ser Pro Glu Ala Ala Gly Ser Gly Gly Tyr Val Pro Pro Asp Val Leu Asn Ser

CAAATTCTTCCTGCTATTAAAGGTTCTTCTAATTATGGTGGTGTTATGCTTTGG
Gln Ile Leu Pro Ala Ile Lys Gly Ser Ser Asn Tyr Gly Gly Val Met Leu Trp

AATAAATAT

Asn Lys Tyr

Fig 3.5. CHT nucleotide sequence and deduced amino acid sequences are shown below the respective nucleotide sequences.

GVISVYWGQNGSDNSEGSLSQACETGNYDFVLLHFLNTYLNGGEPDLNLAGHCGGGG
GCTKLQSEIKYCQSQNIKVLLSLGGTTGNGSLNSTEDAQELANYLPNNFLNGNAGPLG
DVELDDIDFDIESDGSTLYYDDLARAINLDTTGRKIYLSAAPECPYPDNYLNKAVQTGL
VDYIFVQFYNNPPCSYSSGNTGGLFSAWNTWTSNVPNSLVFMGLPASPEAAGSGGYVP
PDVLNSQILPAIKGSSNYGGVMLWN

Fig 3.6 Protein sequence of CHT from *T. indica*

3.3.3. Bioinformatic analysis

The protein sequences in the nr database matching with our query CHT sequence were retrieved in fasta format and sequentially aligned to find the conserved regions present in CHT protein using Clustal W from EBI server.

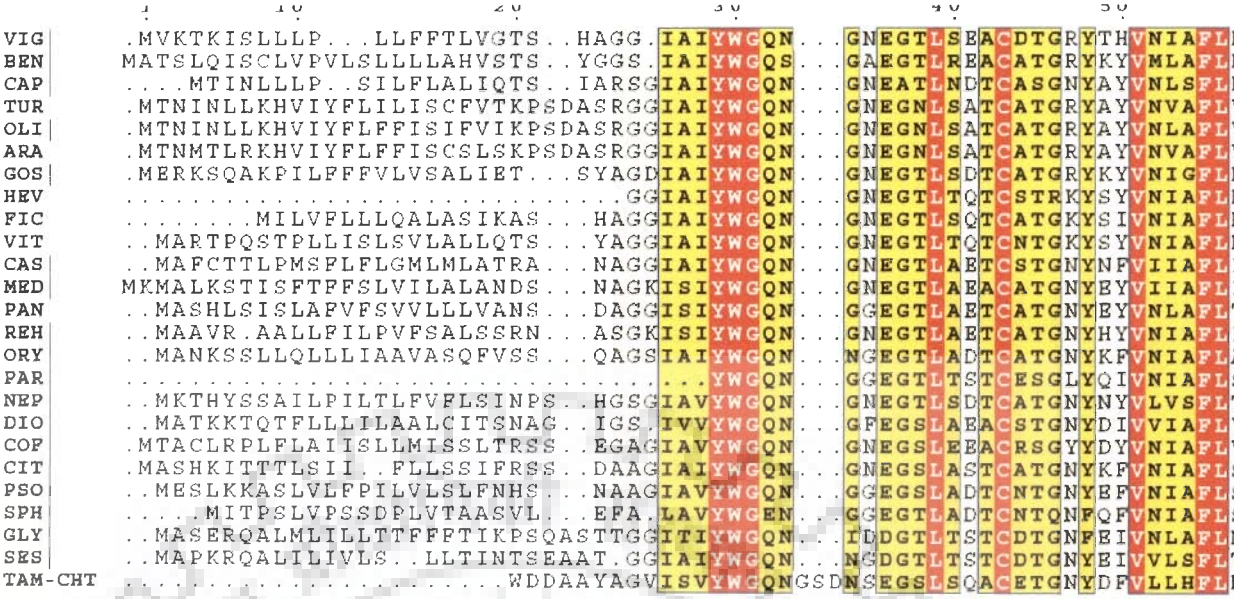
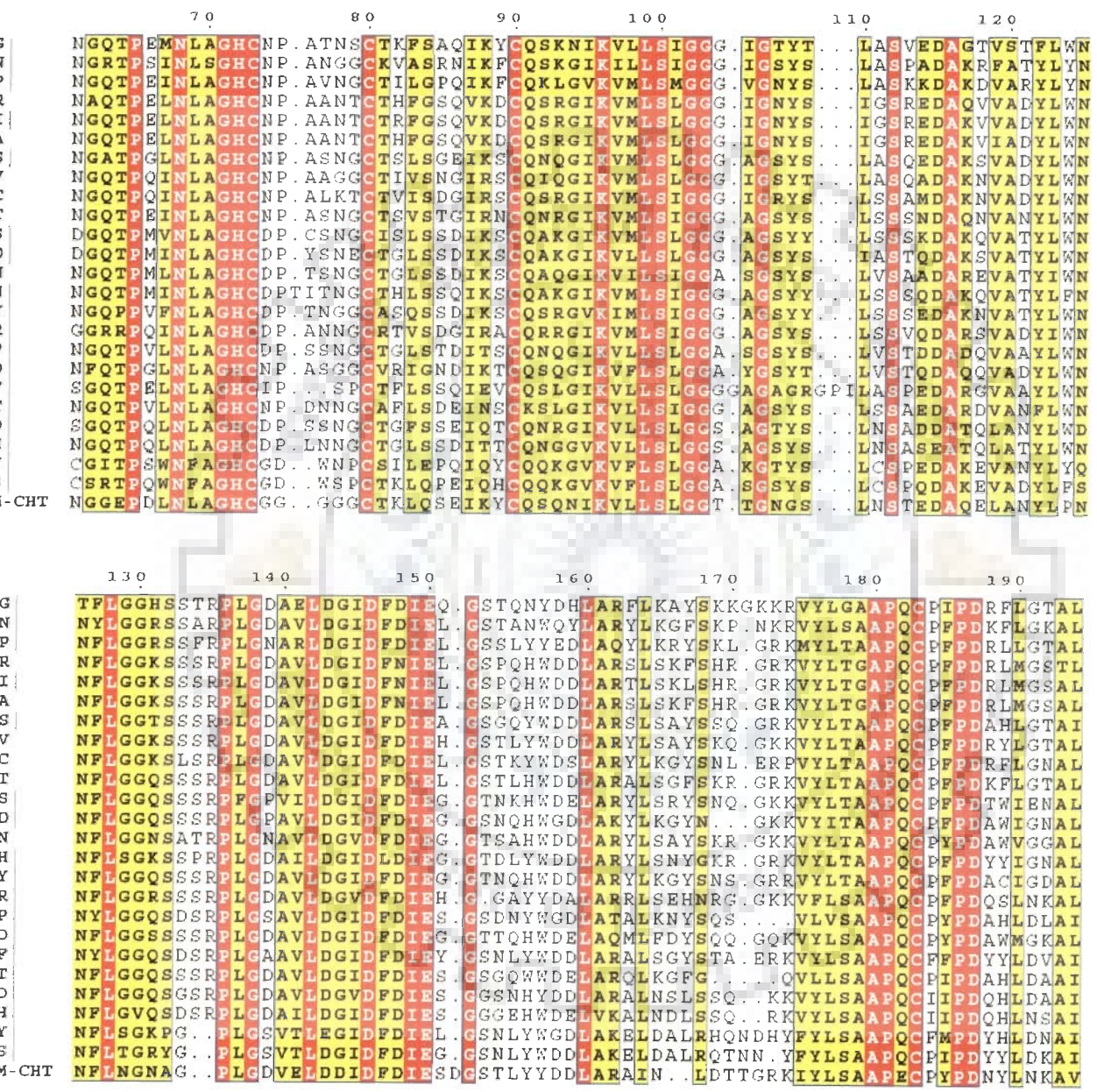
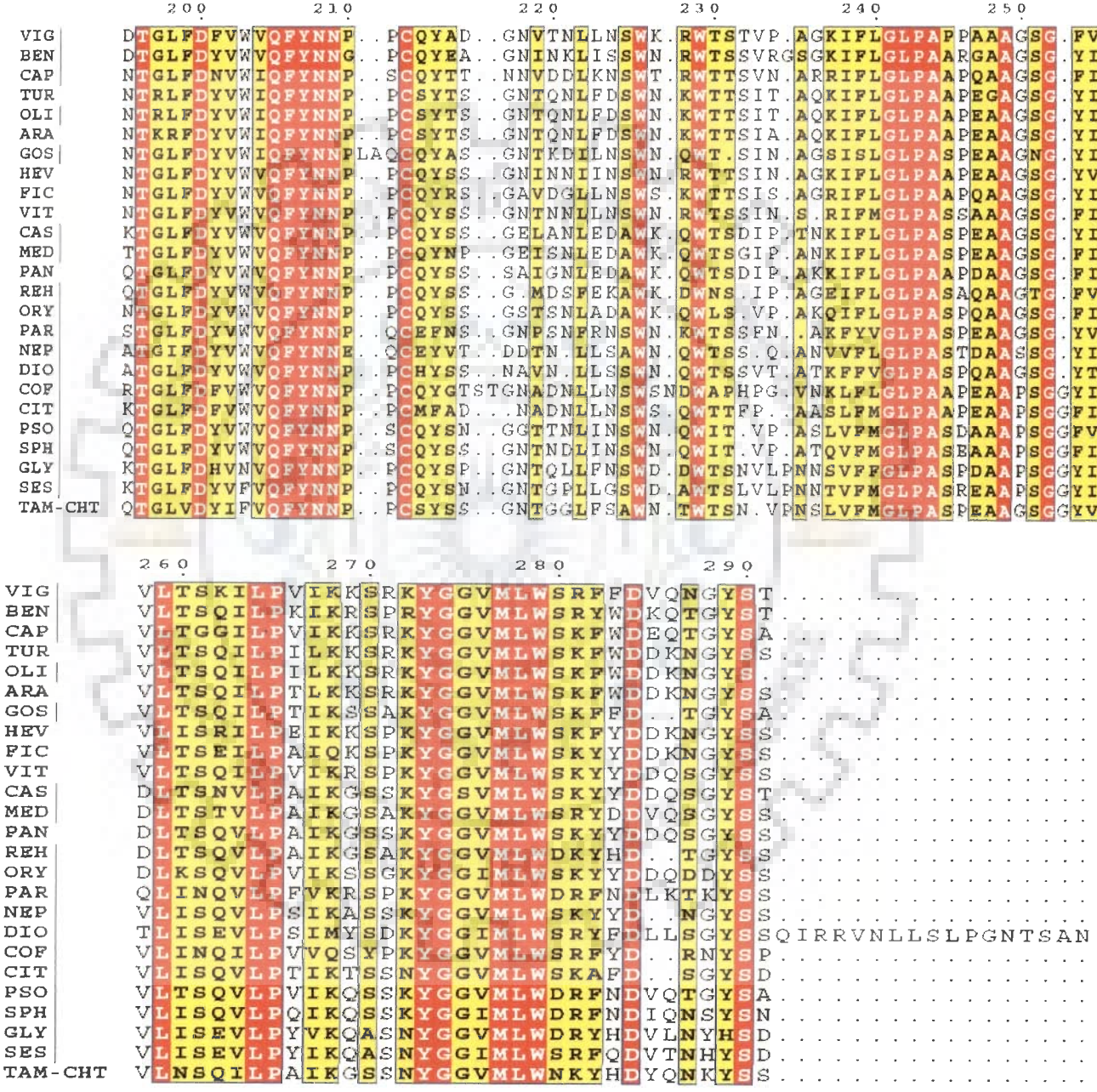


Fig 3.7: Multiple sequence alignment of **TAM-CHT** with other class III chitinases. VIG: *V angularis;* BEN: *B.hispida*; CAP: *C. Annuum*; TUR: *T. glabra;* OLI: *O. pumila;* ARA: *A. thaliana*; GOS: *G. Hirsutum*; HEV: *H. brasienlisis*; FIC: *F.awkeotsang;* VIT: *V. vinifera* CAS: *C. glauca;* MED: *M. sativa;* PAN: *P. ginseng;* REH: *R. glutinosa;* ORY: *O. sativa;* PAR: *P. platycephala;* NEP: *N. rafflesiana;* DIO COF: *C. arabica;* CIT : *C. lanatus;* PSO: *Psocarphus;* SPH *S. stenocarpa;* GLY: *G. Max;* SES: *S. rostrata.*




200
210
220
230
240
250
VIG
BEN
CAP
TUR
OLI
ARA
GOS
HEV
FIC
VIT
CAS
MED
PAN
REH
ORY
PAR
NEP
DIO
COF
CIT
PSO
SPH
GLY
SES
TAM-CHT
260
270
280
290

Amino acid	% composition
Ala	6.5
Arg	0.7
Asn	9.8
Asp	6.9
Cys	2.2
Gln	3.6
Glu	3.6
Gly	12
His	1.1
Ile	3.6
Leu	10.5
Lys	2.9
Met	0.7
Phe	2.9
Pro	5.1
Ser	9.5
Thr	4.7
Trp	1.8
Tyr	4.8
Val	4.7

Table 3.2: Amino acid composition for CHT (Expasy Protparam) (Gasteiger E et al., 2005)

The sequence identity given by CHT with respect to chitinases III from different species of plant is given in Table 3.3. The sequence identity of CHT was found to be highest with class III chitinase from glycine max and an acidic chitinases belonging to family 18 glycosylhydrolase from Psophocarpus at 61% and both along with tamarind belong to leguminosae family.

S .no	Species	Identity(%)
1	*Glycine max*(Acidic chitinases III)	61
2	*Psophocarpus*	61
3	*Sesbania rostrata* (Chitinases homologue)	60
4	*Vitis vinifera* (Class III chitinase)	59
5	*Medicago sativa* (Vegetative root storage protein)	58
6	*Ananas comosus*	58
7	*Vigna angularis* (Class III chitinases)	57
8	*Rehmannia glutinosa* (Class III chitinases)	56
9	*Arabidopsis thaliana* (Class III chitinases)	54
10	*Benicasa hispida* (Class III chitinases)	53
11	*Casuarinas glauca* (Class III chitinases)	53

Table 3.3. Sequence identity of CHT with class III chitinases from different plant species.

Since CHT was found to be a glycoprotein, potential N-glycosylation sites wer determined using NetNGlyc 1.0 server. Three regions containing the NGS glycosylatio motifs were identified at position 10[NGSD], 86[NGSL] and 90[NSTE] in CHT amino aci sequence with glycosylation potential of 0.7262, 0.6314 and 0.7516 (Fig 3.7).

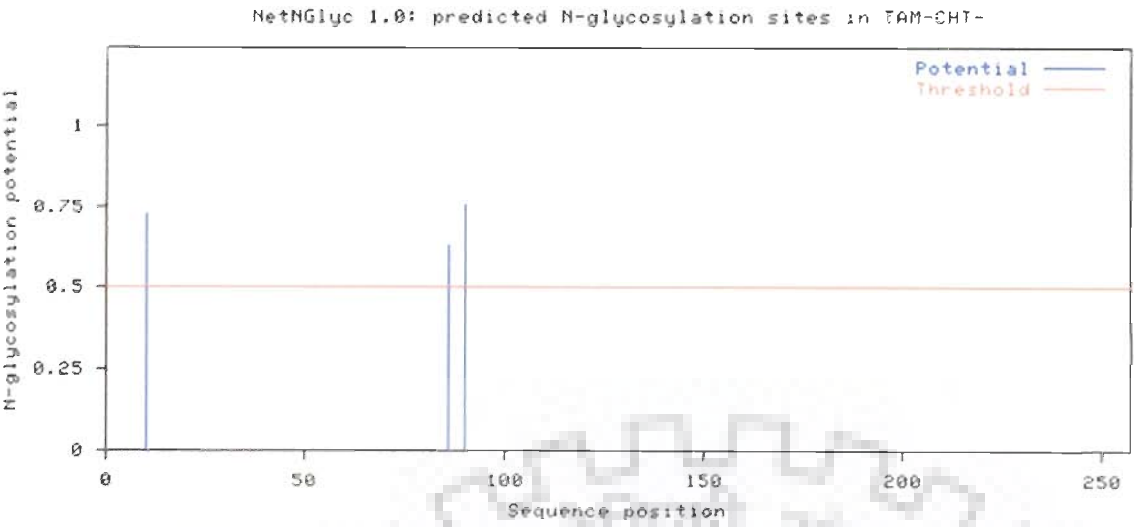


Fig 3.8: Predicted potential N–glycosylation sites in the sequence of CHT (NetNGlyC 1.0)

3.4. Discussion

The amino acid sequence for *T. indica* CHT protein was unknown. Structural, biochemical and biophysical analysis of a given protein is incomplete without its primary sequence. Therefore, amino acid sequence was required for characterization of CHT from *T. indica* using various biochemical and biophysical techniques including X-ray crystallography. For this purpose molecular cloning of CHT gene and cDNA was done by isolating the genomic DNA and total RNA which was followed by PCR and RTPCR respectively. The source for isolating genomic DNA was tamarind leaves and the DNA was isolated using the CTAB procedure. (Wagner *et al.*,1987). Freshly harvested seeds were used to get viable total RNA indicated by two nucleic acid bands in denaturing formaldehyde gel corresponding to 28s rRNA and 18s rRNA. The total RNA was then used to amplify the

chitinases cDNA by RTPCR method and the obtained amplified product was ~730 bp in length. The genomic DNA was used as the template for amplification of the CHT gene using PCR method. The amplification product of PCR was of the same size as obtained by RTPCR indicating that introns are absent in the open reading frame of CHT from *T. indica*. The absence of introns in CHT from T. indica was confirmed by sequencing the amplified gene. It has been found that introns are absent in case of class III chitinases genes from *V. vinifera*, *B. hispida* and *H. brasiliensis*. The DNA sequence obtained was reverse translated using the reverse translate tool from expasy and a single open reading frame was selected. This ORF encoded 241 amino acids to which the primer sequence encoding amino acids were added to obtain 257 amino acid residues. The theoretical pI of the protein is predicted to be 4.10 and the extinction coefficient of ~ 55810 M^{-1} cm^{-1}. The amino acid sequence comparison of CHT with other citinases showed that CHT has three intra-disulphide bonds.

On comparing the sequence of CHT, highest homology was found with class III chitinases from *Glycine max* at 78%. The sequence is showing highest sequence similarity to class III chitinases which also acts as seed storage proteins from leguminous plant. This indicates that CHT can also act as a storage protein in *T .indica*. On the whole the sequence had consensus region throughout indicating conservation of the overall primary structure of chitinases.

Chapter 4

BIOPHYSICAL CHARACTERIZATION AND STRUCTURE DETERMINATION OF CHT

4.1. Introduction

To understand structure-activity relationship, it is important to study the changes brought about by the external factors like denaturant on the stability of the protein. The stability of protein can be studied as a function of temperatures, pH, salts and detergents using various biophysical methods. Circular dichroism and fluorescence spectroscopy are very important tools to evaluate the secondary structure and conformational stability under various physichochemical conditions. In fluorescence studies, both ANS (8-anilino-1-naphthalene sulfonate) and tryptophan fluorescence were performed at different temperature, pH, salts, and detergents to understand the folding/unfolding nature of this protein.

Based on sequence and structural information, the chitinases are classified into two evolutionarily unrelated groups, designated as families 18 and 19 of the glycosyl hydrolases. Family 18 includes a number of evolved variants: endoglycosidases function being hydrolysis of chitobiose core of N-linked glycoproteins, and lectins, which are catalytically inactive but become active during tissue remodelling/differentiation. Three-dimensional structures of many Family 18 proteins from bacterial, fungal and plant sources have been determined by X-ray crystallography including several structures of inactive mutants with bound chitin oligosaccharides (Watanabe *et al.*, 2001, Papanikolau *et al.*, 2001 & Aalten *et al.*, 2001) as

well as with transition-state analogues (Bortone *et al.*, 2002, Houston *et al.*, 2002, Scheltinga, *et al.*, 1994, Matsumoto *et al.*, 1999). The catalytic domain of Family 18 chitinases consists of a $(\beta/\alpha)_8$-barrel with a deep substrate-binding cleft formed by the loops following the C-termini of the eight parallel β-strands. A conserved glutamate residue at the C-terminus of β_4 has been identified as the proton donor in the hydrolysis reaction (Brameld *et al.*, 1998, Tews *et al.*, 1997, Watanabe *et al.*, 1993). The crystal structures of complexes indicate that bound chitin oligosaccharide is the β-anomer and occupies subsites +1 and +2.(Fig 4.1) GlcNAc at -1 is the glycosyl unit protonated by the catalytic glutamate residue with disaccharide unit being the most common product released from chitin by family 18 chitinases. Double displacement at the glycosyl C-1 is accomplished by a substrate-assisted mechanism in which the carbonyl oxygen of the N-acetyl group at C-2 of the -1 GlcNAc, resulting in protonation of O-1 of the -1 GlcNAc and an oxazolinium intermediate formation. This positively charged intermediate is stabilized through interaction with a conserved aspartate residue located two amino acids upstream from the glutamate residue (Scheltinga *et al.*, 1995, Waddling *et al.*, 2000, Prag *et al.*, 2000).

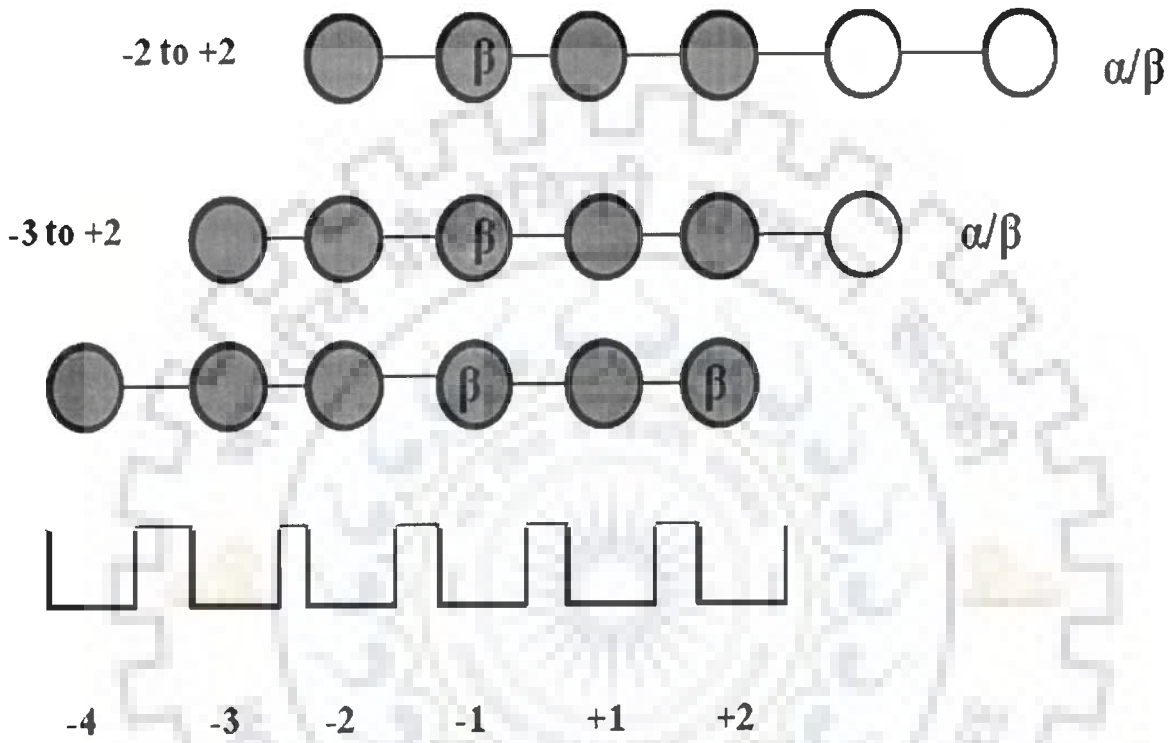


Fig 4.1. Predicted products from different binding modes of chitin hexasaccharide in the substrate cleft of Family 18 chitinase. (Adapted from Aronson *et al.*, 2003)

4.1.1. Protein crystallization and X-ray diffraction.

Nucleation and crystal growth of protein is affected by many physical and chemical parameters, method of crystallization being one of them. Protein crystallization is done mainly by three methods, dialysis, vapor diffusion and batch method. The principle of vapor

diffusion is with equilibriation, concentration of the constituents will increase in the droplet containing protein. The protein concentration in the droplet starts increasing from undersaturated state to supersaturated stage. At optima, the protein tends to forms crystals.

Exposure of protein crystals to X-ray radiation often results in disruption of chemical bonds, reduction of metal centres, decarboxylation of acidic amino acids (Asp and Glu), oxidation of hydroxyl group of serine, etc., (Ravelli and Garman, 2006) thus making a technique called cryo-mounting preferable. This technique holds the protein crystal in a cryo-protectant solution maintaining the protein crystal in a glassy surrounding and preventing ice formation. Exposure to gaseous N2 is done at 100K with with the protein held in cryo-loop. The composition of cryo-protectant solutions varies depending on the protein crystal and the crystallization condition. The diffraction pattern obtained should not correspond to ice giving well shaped spots. Small organic molecules such as glycerol, ethylene glycol, MPD, etc., are commonly added as cryo-protectants.

4.1.2. Phase Determination by Molecular Replacement

Molecular replacement is a versatile method for determination of unknown protein structures using a known structure exhibiting similar primary and tertiary structures (Rossmann, 1990). It is useful to consider the method in terms of operations involving Patterson function. In this framework, the method involves placing the known structure into the unit cell of the unknown structure by superposing Patterson functions. The known and unknown structures of the proteins are represented by separate Patterson functions. The Patterson function is calculated as follows: where, *u, v* and *w* are coordinates of positions in the Patterson unit cell; *P (u, v, w)* is the value of the Patterson function at the position (*u, v,*

w); *Vc* is the volume of the Patterson unit cell; *h, k* and *l* are indices of a particular reflection; *hkl F* is the amplitude of the structure factor of the reflection with indices *hkl*. The Patterson function of the known structure is calculated using the structure factor amplitudes determined from the model, whereas, the Patterson function of the unknown structure is determined from the intensities measured during the X-ray diffraction experiments. Placement of the "search model" (known structure) onto the unit cell of the target (unknown) structure with correct orientation takes place in two steps: Proper orientation of the search model followed by placement of the search model at the correct site within the unit cell. The software commonly used for performing molecular replacement method is MOLREP (Vagin, and Teplyakov, 1997), available within the *CCP4* program suite (Bailey, 1994).

4.1.3. Structure Refinement and Model Building

Refinement is a crucial step in any structure determination and it involves adjusting some parameters of the atomic model so as to improve the agreement between observed and calculated structure factor amplitudes. During refinement, for each atom four parameters (*x*, *y*, *z* and *B*) are refined against the observed data while restraining stereochemical parameters such as bond lengths, bond angles and torsion angles to values obtained from high resolution structures of small molecules or peptides which improves model with the observed data without deviating from normal stereochemical parameters. After each model building state, refinement is done repeatedly Model building involves construction and fitting the components of the refined model into the electron density maps calculated during the refinement procedure. Validity of the refinement process is monitored by *R* and *Rfree*, both scoring the difference between amplitudes of the observed and calculated structure factors (Bruger., 1992).

The value of *Rfree* is usually higher than *R* and for a successful refinement the values of both terms should decrease, which indicates improved agreement of the model with the experimental data. During refinement the difference between *R* and *Rfree* should not be too large otherwise it indicates bias or over-fitting the data. Refinement is continued until the values of both *R* and *Rfree* do not fall after an additional round of refinement. The calculated electron density is visualized using the molecular graphics COOT (Emsley and Cowtan., 2004). Residues were manually built into the electron density using these graphics programs. Finally water molecules are added both by manual inspection of the electron density and by using the program ARP/wARP (Perraksis *et al.*,1997) present within the REFMAC (Murshudov *et al.*, 1997) program.

4.1.4. Structure Validation

The stereochemical properties of the refined models were analyzed by the program PROCHECK (Laskowski *et al*, 1993). Stereochemistry of polypeptide backbone is analyzed by using the Ramachandran plot (Ramakrishnan and Ramachandran, 1965), which is a plot of all observations of the backbone the dihedral φ and Ψ angles (generated by PROCHECK).

4.2. Materials and methods

4.2.1 Materials

Protein was purified as described earlier. ANS (8-anilino-1-naphthalene sulfonate), guanidium hydrochloride, MES, PEG 8000 were purchased from Sigma-Aldrich Pvt. Ltd. All other chemicals were purchased from Himedia chemicals, India. Crystallization trays were obtained from Hampton research, Germany.

4.2.2 Methods

For fluorescence studies, protein was taken at a concentration of 3.5 μM. The ANS fluorescence measurements were performed in different physicochemical conditions, like a gradient of HCl concentration ranging from 0 – 2 M and denaturants like urea (0 – 7 M), guanidine hydrochloride (0 – 8 M) and β-mercaptoethanol (0 - 2 M). Overnight incubation of the protein at different denaturing conditions was done. For ANS binding experiments, the molar ratio of protein and ANS was taken to be 1:50. The excitation wavelength was set at 360 nm and the emission spectra were measured in the range 400 – 700 nm using an excitation and emission slit widths of 5.0.

ANS fluorescence studies as a function of different temperature was also performed. ANS (8-anilino-1-naphthalene sulfonate) dye was dissolved in 100 mM Tris-HCl, pH 8.7, to a concentration of 50 mM. The final concentration of protein in the reaction mixture was 3.5 μM and ANS concentration was 175 μM. The fluorescence was read immediately at after incubating the samples at different temperatures ranging from 30 to 90°C at an interval of 10°C using an excitation wavelength of 360 nm and emission spectra were measured in the range 420-700 nm using an excitation and emission slit width of 5.0 nm.

The tryptophan fluorescence measurements were also performed in presence of SDS (0 – 9%) at a protein concentration of 3.5 μM. The excitation wavelength was set at 280 nm and the emission spectra were measured in the range 290 – 400 nm using an excitation and emission slit widths of 5.0 nm.

Crystallization experiments were performed using the sitting-drop and hanging drop vapour diffusion method at 293 K. Crystals of CHT were grown in 2 μl drops, containing

equal volumes of protein (15 mg ml^{-1} in 100 mM Tris-HCl, pH 7.4) and reservoir solution. Various combinations of precipitants, salts and buffer conditions were used. The crystallization trays were incubated in vibration free crystallization chamber at 298K. Crystals were obtained in a range of PEG 4000 concentrations (10%-25%) at 0.1M MES pH 6.

A single diffraction quality crystal was mounted in cryoloops (Hampton Research) using reservoir buffer and 10 % glycerol as the cryoprotectant. Crystals were mounted and flash-cooled by direct immersion in liquid nitrogen prior to X-ray diffraction analysis. Data were collected with a MAR 345 dtb imaging-plate system using Cu *Kα* radiation generated by a Bruker Microstar H rotating-anode generator operated at 45 kV and 60 mA and equipped with Helios optics. Data were collected as 90 images with a crystal-to-detector distance of 200 mm with 1° oscillation per image. The time of exposure was 5 min and the diffraction data for the protein was obtained. The data generated was integrated using AUTOMAR (Bartels and Klein., 2003) and merged using SCALEPACK (Otwinowski & Minor., 1997). The sequence obtained from N-terminal and PCR –amplification based sequencing was fitted into the electron density. Some of the residues were modelled according to the electron density and based on based on multiple sequence alignment of the chitinase III family proteins.

Molecular replacement method (MOLREP) (Vagin and Teplyakov., 1997) was performed for determining structure. The rigid body followed by restrained body refinement was carried out using program REFMAC available within the CCP4 interface. Model building was performed using program COOT. Docking experiments were performed for CHT using the program HEX. (D.W. Ritchie., 2003)

4.4. Results

4.4.1. Fluorescence studies-Results

ANS fluorescence experiments were performed in different physicochemical conditions to monitor the extent of changes in native structure of CHT and relate them to activity. Intrinsic fluorescence intensity for CHT during thermal denaturation study was performed at temperature from 30 to 90°C is shown in Figure 3.2. There is sudden increase in fluorescence up to 40°C indicating change in the structure of the native protein. However, a fluorescence intensity then tends to fall down when temperature was at 50°C with characteristic red shift at 90°C indicating opening of the protein three dimensional conformation.(Fig 3.2) In the presence of extrinsic fluor ANS , the fluorescence intensity tend to increase systematically with increasing indicating progressive formation of hydrophobic pockets and thus denaturation.(Fig 3.3)

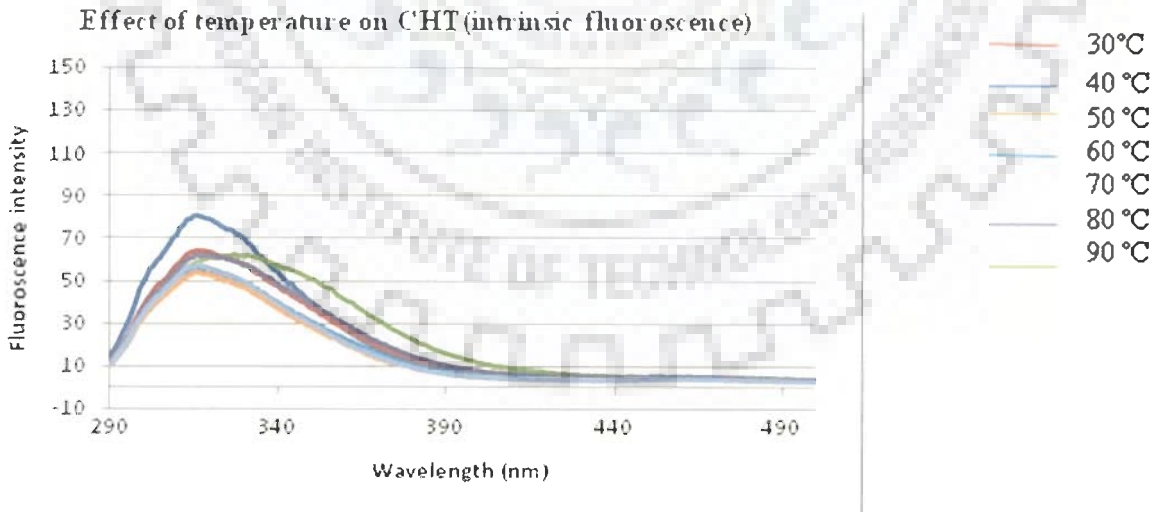


Fig 3.2: Intrinsic fluorescence of CHT with increasing temperature.

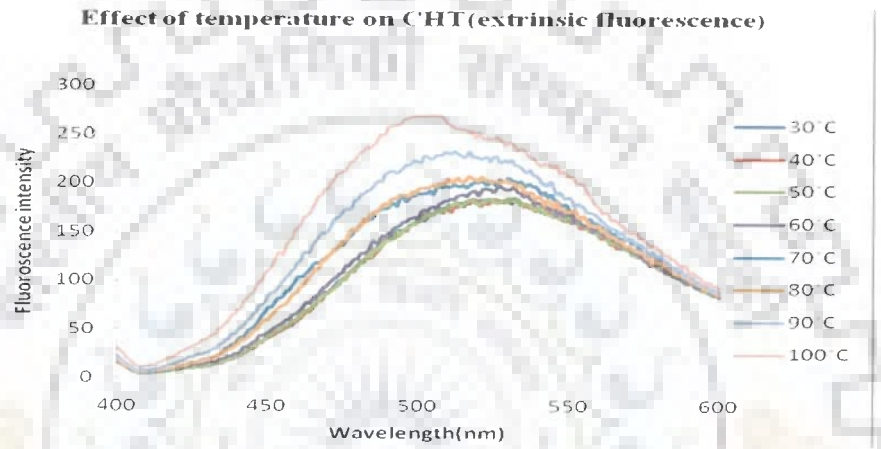


Fig 3.3: Extrinsic fluorescence of CHT in presence of ANS with increasing temperature

Fluorescence intensity measured for βME in presence of ANS showed increase in intensity with increase in concentration from 0 to 90 mM thereafter which there was decrease in fluorescence intensity indicating aggregation of the protein sample. **(Fig 3.4)** There was no visible aggregation.

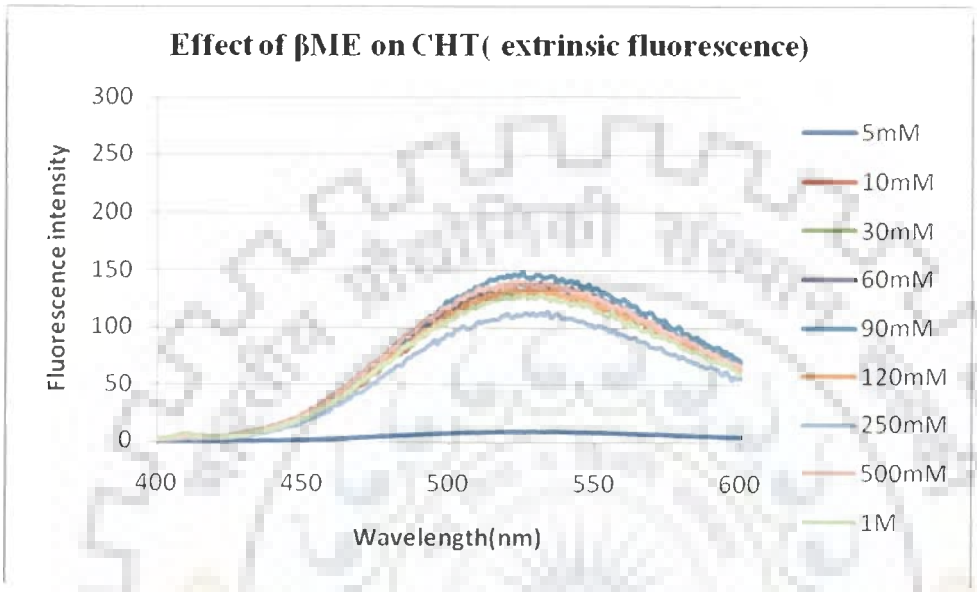


Fig 3.4: Extrinsic fluorescence of CHT in presence of ANS with increasing concentration of βME

There was an observed blue shift from ~530 nm to ~520 nm in case of increasing concentration of HCL in presence of ANS. The ANS fluorescence measured at various concentration of HCl showed increase in ANS fluorescence with increasing concentration of HCl up to 1.2 M indicating a gradual unfolding of the native structure. Above 1.2 M HCl, the intensity decreased significantly which finally touched the baseline at 2M indicating the

complete denaturation of protein (Fig 3.5)

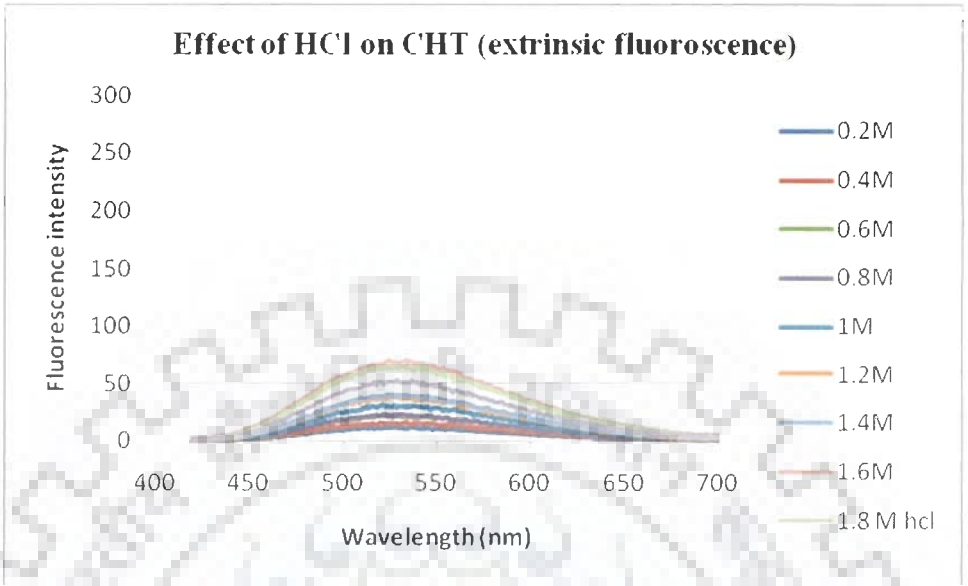


Fig 3.5: Extrinsic fluorescence of CHT in presence of ANS with increasing concentration of HCl.

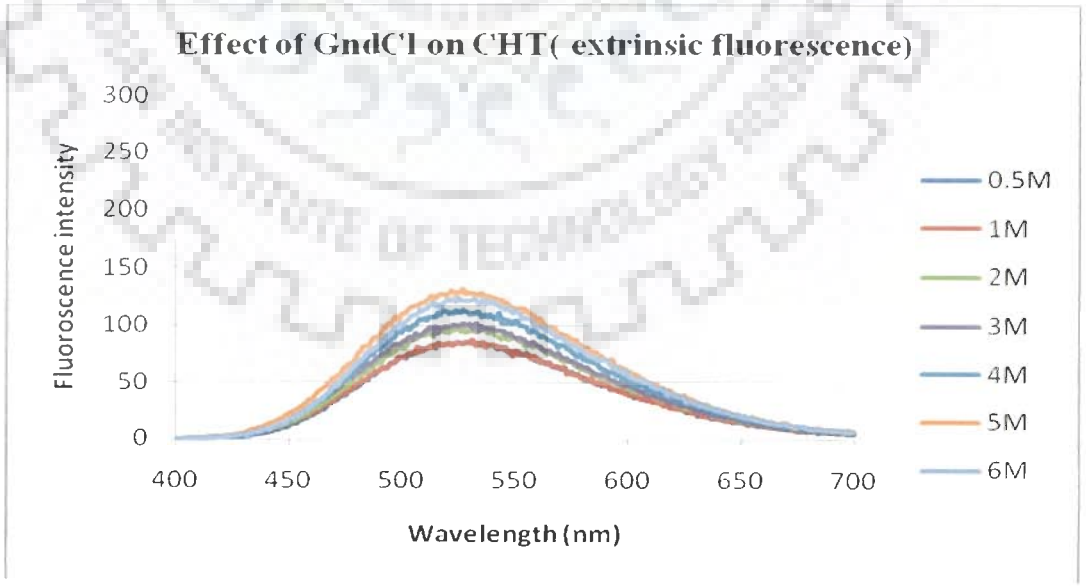


Fig 3.6: Extrinsic fluorescence of CHT in presence of ANS with increasing concentration of

GndCl.

At different concentrations of urea (0 to 7 M) and guanidine hydrochloride (0 to 6 M), a minor increase in fluorescence intensity was observed with increasing concentration of urea and guanidine hydrochloride (Figure 3.6, 3.7). A slight peak shift was observed in case of ANS fluorescence in urea mediated denaturation.

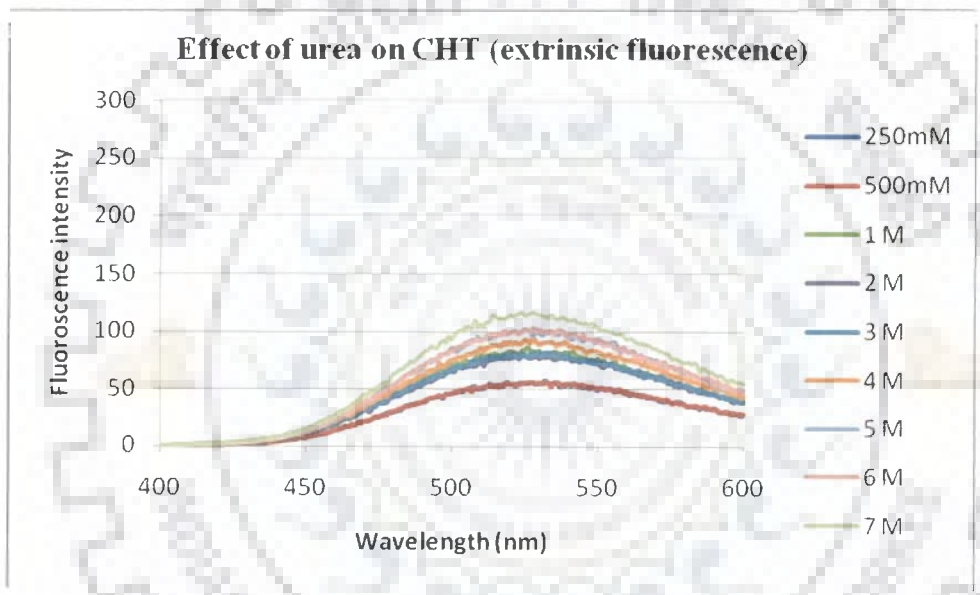


Fig 3.7: Extrinsic fluorescence of CHT in presence of ANS with increasing concentration of urea.

4.4.2. Structure determination: Results

The diffraction quality crystals of CHT were obtained in fifteen days in the reservoir

solution containing 100 mM 2-(N-morpholino) ethanesulphonic acid (MES), pH 6.0 and polyethylene glycol (PEG) 4000 as precipitant.(Fig 3.8)

Fig 3.8: Crystals of CHT from tamarind seeds

Crystal belong to the tetragonal space group $P4_1$ and diffracted to 2.6 Å resolution in-house (Fig. 3.9).

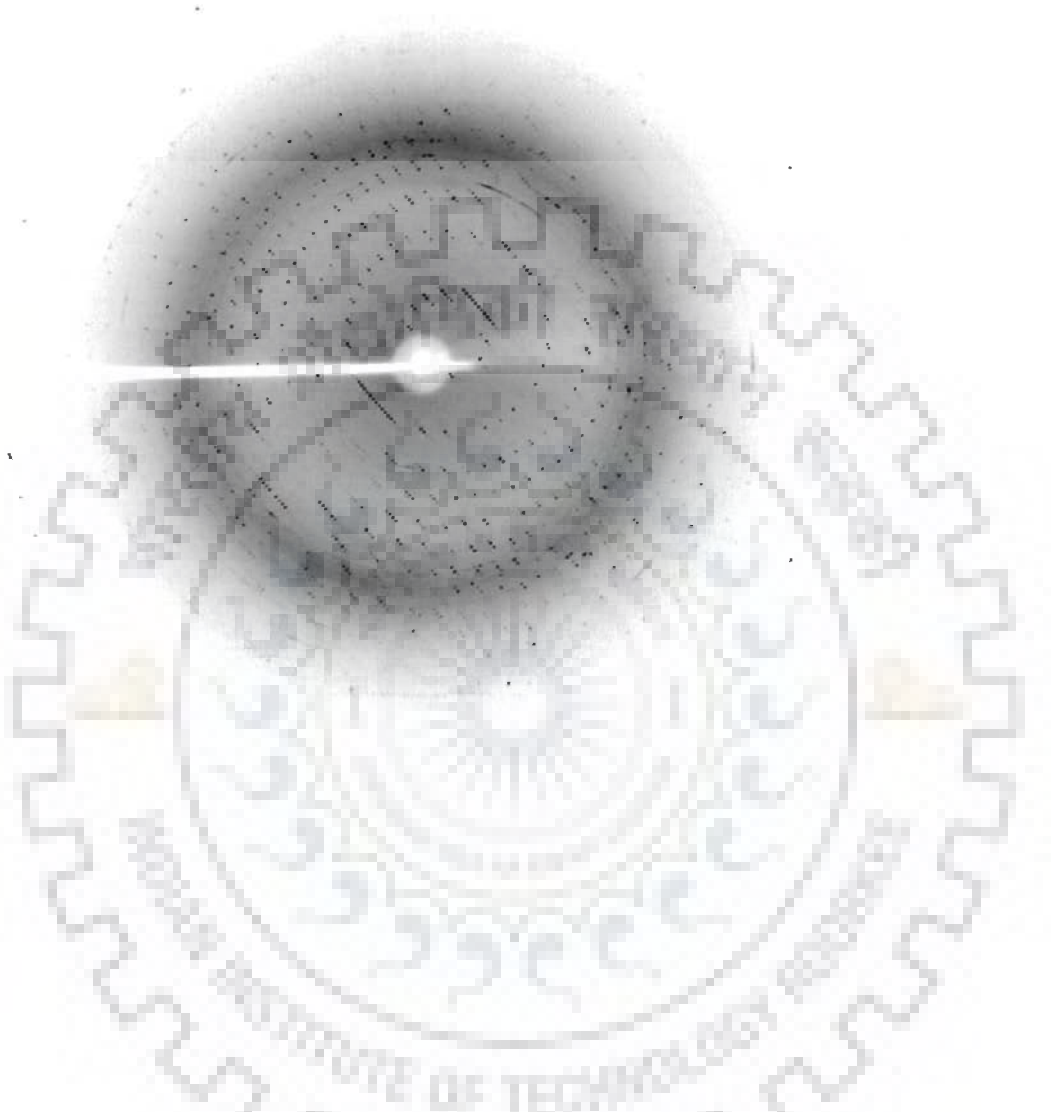


Figure 3.9: Diffraction pattern obtained for CHT crystals using in-house radiation at the MCU, IIC. The resolution at the edge of the plate is 2.6 Å.

The unit-cell parameters were a = b = 67.00 Å, c = 173.09 Å with two molecules per asymmetric unit this corresponds to a crystal volume per unit molecular weight (V_M) of 2.50 $Å^3$ Da^{-1}, given the molecular weight of 34 kDa for the protein. The data corresponds to a solvent content of 51%. R_{sym} for the 34343 unique reflections to 2.6 Å resolution was 4.6% (16% for the highest resolution shell 2.7-2.6 Å). The multiplicity for all the data was 7.9 $I/\sigma(I)$ in the last resolution shell was 2.1. The overall completeness of the data is 98% (99% in the highest resolution shell 2.7-2.6 Å). The data-collection statistics are summarized in Table 4.1.

Table 4.1: Data-collection statistics for Tamarind seed CHT

Values in parentheses are for the highest resolution shell.

Resolution (Å)	50–2.6 (2.69–2.60)
Completeness (%)	98.8 (99.0)
R_{sym}[†] (%)	4.65 (16.34)
Mean $I/\sigma(I)$	7.9 (2.1)
Space group	$P4_1$
Unit-cell parameters (Å)	a=b=67.00 Å, c=173.09 Å
No. of observed reflections	102311
No. of unique reflections	34343
Molecules per ASU	2
Mathews coefficient	2.50
Solvent content (%)	51

[†] $R_{sym} = \sum_h[\sum_i |I(h)_i - \langle I(h)\rangle|/\sum I(h)_i]$, where $I(h)_i$ is the ith observation of reflection h and $\langle I(h)\rangle$ is the mean intensity of all observations of h.

The structure was solved by molecular replacement with the MOLREP program (Vagin and Teplyakov, 1997) from the CCP4 software suite (Collaborative Computational Project, Number 4, 1994; Dodson et al., 1997) using the *Hevea brasiliensis* hevamine protein (PDB code 1HVQ) as a search model (Onesti et al., 1991). The rotation function was calculated with diffraction data from 20 to 3 Å resolution within a sphere radius of 20 Å. The first peak in the output of rotation function gave distinct solution. This solution was used for translation function calculations. It gave correlation coefficient of 31.2% and an R factor of 51.1%. This structure was used for refinement with twenty cycles of rigid-body refinement and subsequent iterative cycles of maximum-likelihood refinement using *REFMAC*5 (Murshudov *et al.*, 1997). After the first 20 cycles of maximum-likelihood refinement, the *R* and R_{free} values were 39.6% and 46.6%, respectively. Rigid-body refinement and restrained refinement were carried out with REFMAC (Collaborative Computational Project, Number 4, 1994) and model building with the COOT. Appropriate amino acid changes were carried to convert the molecular model of hevamine into CHT. Several steps of model building interspersed with restrained refinement using REFMAC yielded the *R* and R_{free} values were 26.6% and 31.2%, respectively. After manual checking of the chain A and B residues, it was confirmed there was no unambiguous electron density. Furthermore water molecules were placed in F_0-F_C map with ARP/wARP and inspected manually. Finally several cycles of refinement were carried out and it gave the final R and Rfree value of 22.6% and 27.6% respectively.

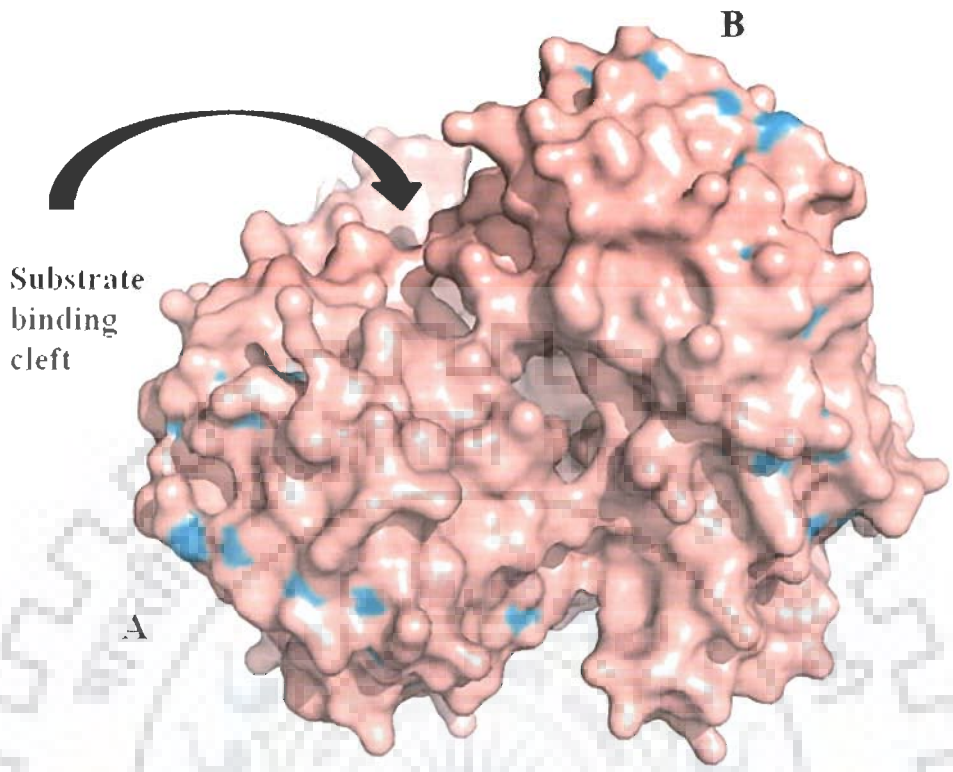


Fig 3.10: Overall surface diagram of CHT with chain A and B and substrate binding cleft. The pink regions indicate the loops and cyan regions indicate helical portions.

Topology of the CHT (Fig 3.10) was conserved in having a $(\alpha\beta)_8$ barrel domain(Fig 3.11) structure with three disulphide bonds in between cysteine residues at positions 20-67, 50-57 and 158-187 in each of the subunit.

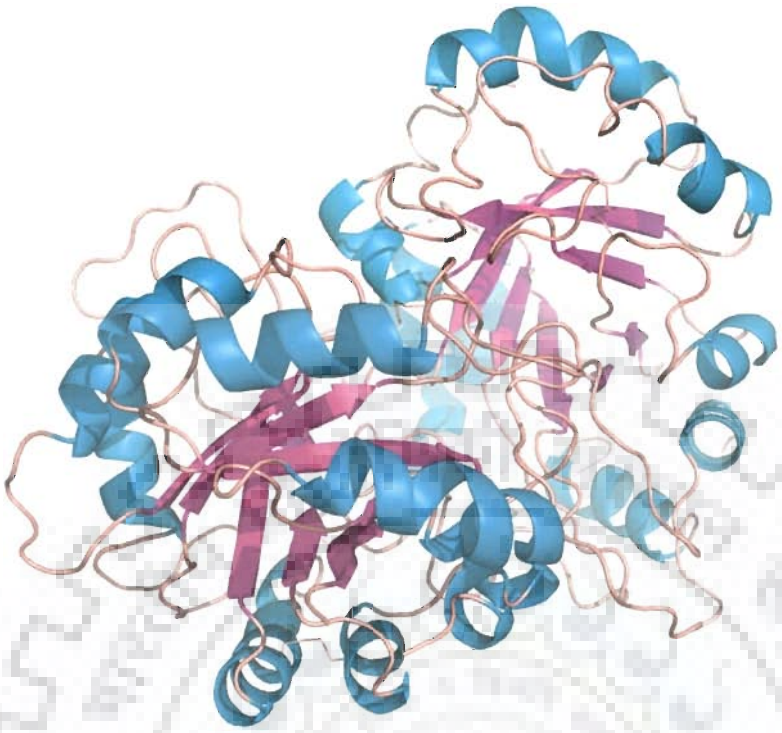


Fig 3.11. Overall structure of CHT with chain A and B and substrate binding cleft. The pink regions indicate the loops and cyan regions indicate helical portions.

The fraction of solvent content came to be about 51%. The conserved glutamate present in the C –terminus of the β4 helix is placed at position 134 in CHT. The aspartate residue which has a role in stabilizing the oxazolinium intermediate of the chitin oligosaccharide is placed optimally at position 132. These two residues tend to form an important part of the active site residues. Glu134 is fixed in this position by a hydrogen bonding to Asp132 (Fig 3.12). The other potential aspartates which can contribute to stabilization are

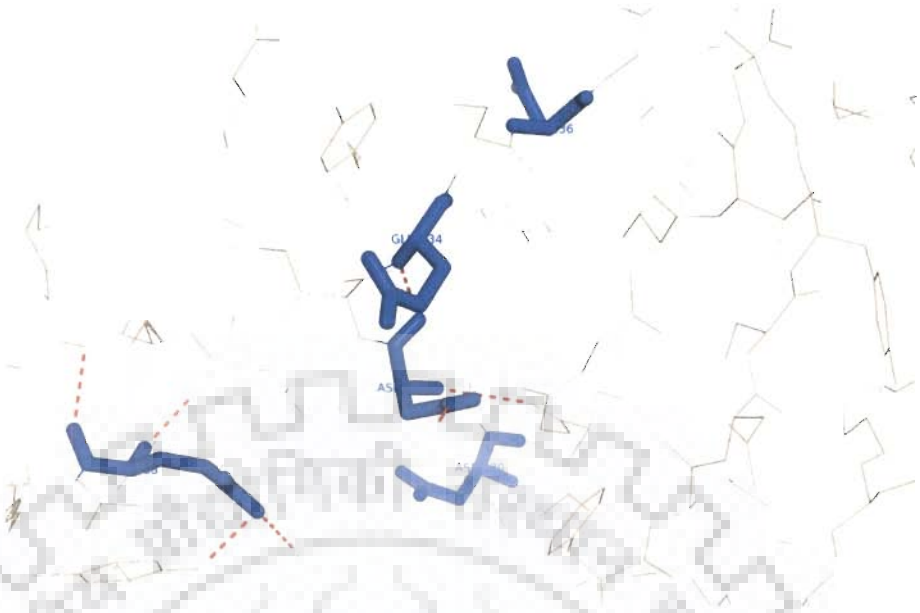


Fig 3.12. Geometry indicating proposed active site with Glu134 and Asp132 residues conserved near the active site.

placed far away from the active site and hence may not play important role in stabilization of the substrate.

The baverage value for the A and B chains of CHT was calculated using the baverage programme present in the CCP4 suite. The values given are indicated in Table 4.2.

Procheck programme from CCP4 suite was used to find the positioning of the peptides in three dimensional confirmations. The results indicated 90.6% residues are present in the most favoured regions and 9.4% in the generously allowed regions.

Table 4.2. Baverage results obtained from CCP4 suite

		Chain A	**Chain B**
Baverage	Main chain	11.805	12.46
	Side chains	11.58	12.06
	All atoms	11.7	12.273
RMSD	Main chain	0.424	0.426
	Side chain	1.026	1.011

The CHT protein shares 57%, 52%, 42% and 31% sequence similarity with hevamine from *H. brasielienisis*, concanavalin B from *Canavalia ensiformis*, PPL2 from *Parkia platycephala and* XIP-1 from *Oryza sativa*. The three dimensional structure of CHT was superimposed onto those of hevamine, concanavalin B and PPL2 with root mean square deviation (r.m.s.d) for Cα atoms to be 1.161A□, 1.262 A□ and 1.296 A□ respectively.(Fig 3.13)

The loops connecting the carboxy terminus of helix and amino terminus of the strand are generally 4-7 amino acids long with only one exception (Leu114-Asp127). The connections made between carboxy terminus of strand to amino terminus of helix vary in length from7-12 amino acids. Apart from the characteristic 8 β strands there exists 2 extra strands in CHT structure after β2 which among themselves form an anti-parallel β-sheeted structure.

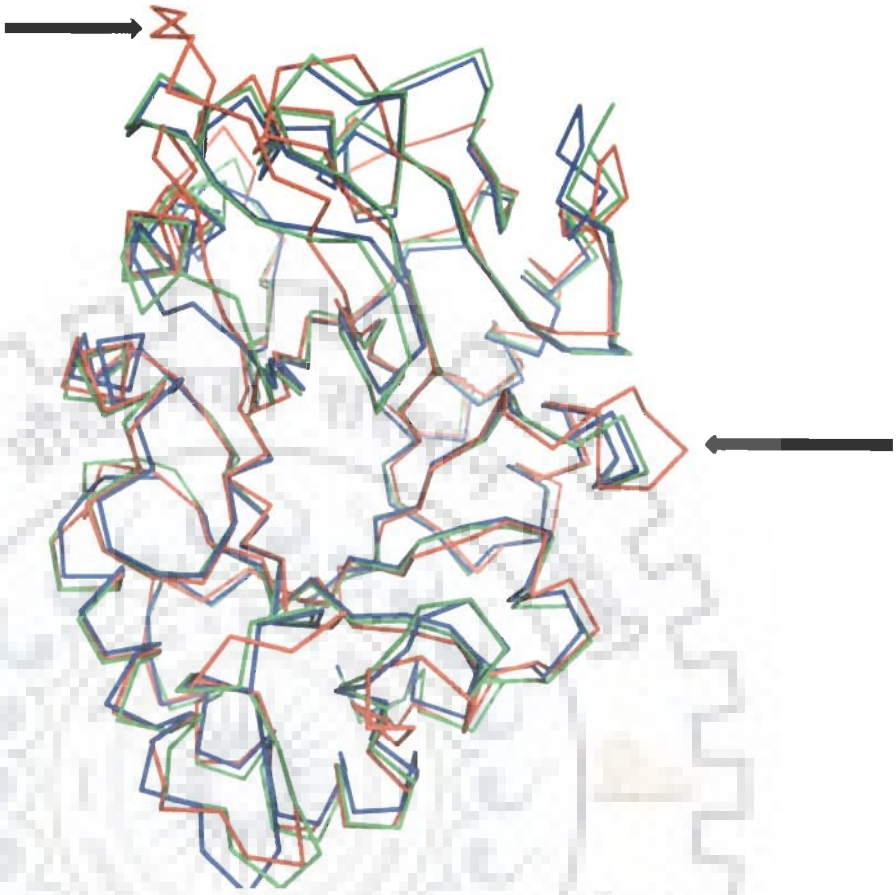


Fig 3.13. Overall superimposition of class III chitinases from 1HVQ(green), 2GSJ(blue) and CHT(red). Different orientation in loop regions indicated by arrows

The sequence corresponding to the loop region at positions 91-100 is TGNGSLNSTED, which when fed in the blast suite gave 88% similarity with proteins having zinc finger RNA binding domain.

Fig 3.14 Hydrogen bonding network between Asp126, Asp 127 and Val 82.

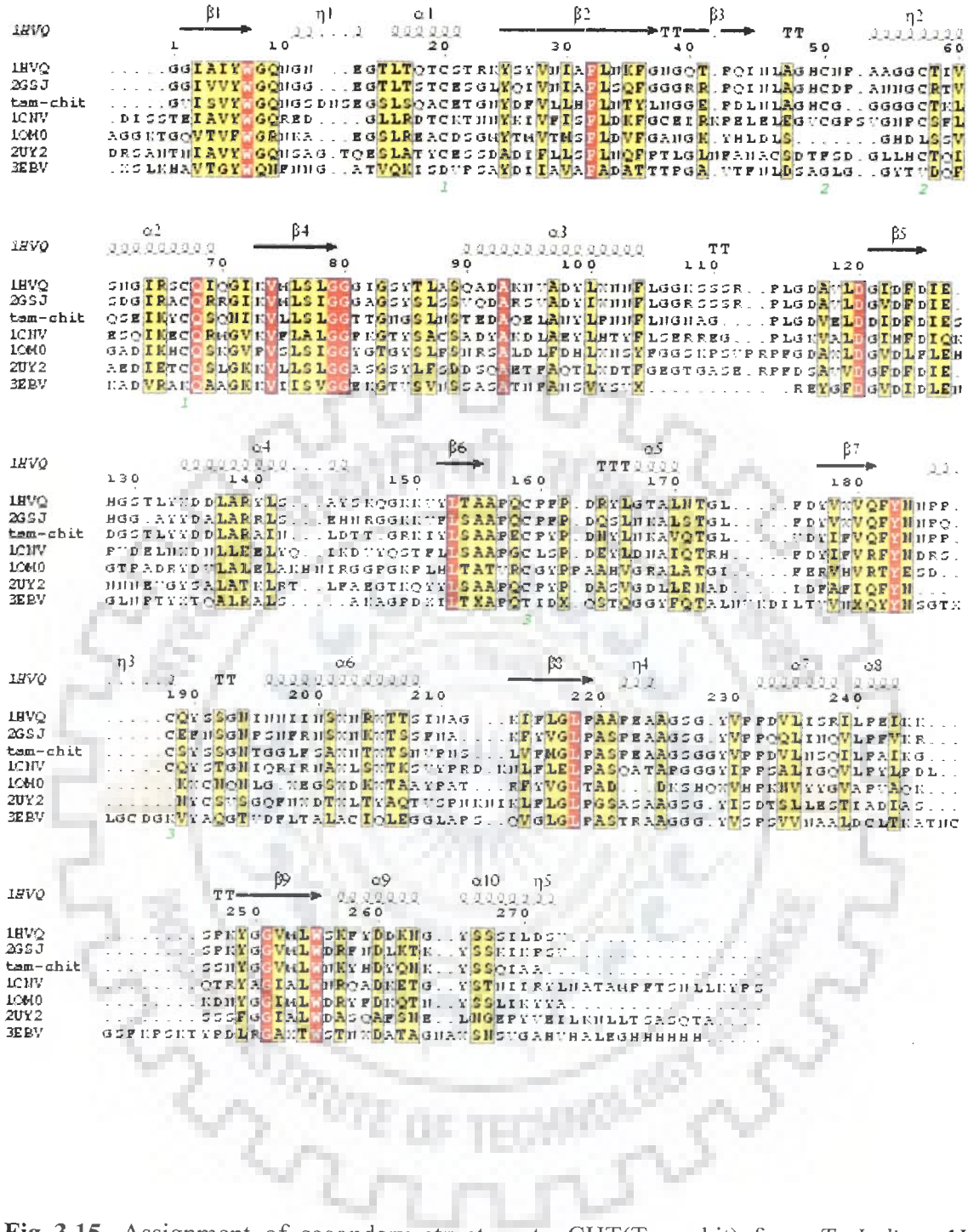


Fig 3.15. Assignment of secondary structure to CHT(Tam-chit) from *T. Indica;* 1HVQ: Hevamine from *H. brasielienisis,* 2GSJ: PPL2 from *Parkia platycephala,* 1CNV:concanavalin B from *Canavalia ensiformis* 1OM0: xylanase inhibitor protein from *Oryza sativa,* 2UY2: chitinase from *S. Cerevisae,* 3EBV: chitinases from *Streptomyces coelicolor.*

The consensus motifs characteristic of family 18 glycosyl hydrolases is conserved β3 and β4 strands. Although the β4 strand is totally conserved with respect to 1HVQ and 2GSJ, the β3 strand has some amino acid substitution in case of CHT (when compared to 1HVQ and 2GSJ).(Fig 3.15) Another motif characteristic of this family is the concerted hydrogen bonded network formed between Asp170,Gly121 and Val74 in case of 1HVQ. In CHT the residue at 121 position is substituted by Asp although a hydrogen bonded network is still very much present (Fig 3.14).

The docking analysis of $(NAG)_n$ with CHT was carried by HEX docking software. Docking is the process of fitting together of two molecules in three dimensional space. The molecules binding to the protein could be considered as a suitable substrate /substrate analogue. The collection of Tri-NAG (Fig 3.16), Tetra-NAG (Fig 3.17), Penta-NAG (Fig 3.19) and allosamidin (Fig 3.20) complexes with CHT were identified via docking and their relative stabilities were evaluated using molecular dynamics. Docking results for the substrate and inhibitor are shown in Table 3

Table 4.3.Docking results of CHT with substrates and inhibitor

SUBSTRATE/INHIBITOR DOCKED	E-VALUES
Tri-NAG	-216.23
Tetra-NAG	-273.71
Penta-NAG	-253.29
Allosamidin	-174.23

There was no stearic hindrance observed during binding of the substrates to CHT as inferred with the absence of any bumps. The lead molecule is the one having maximum interaction with lowest negative e-value. Thus on the basis of docking studies it can be said that Tetra –NAG is the best fitting substrate for CHT and allosamidin may not prove to be a very potent inhibitor for CHT.

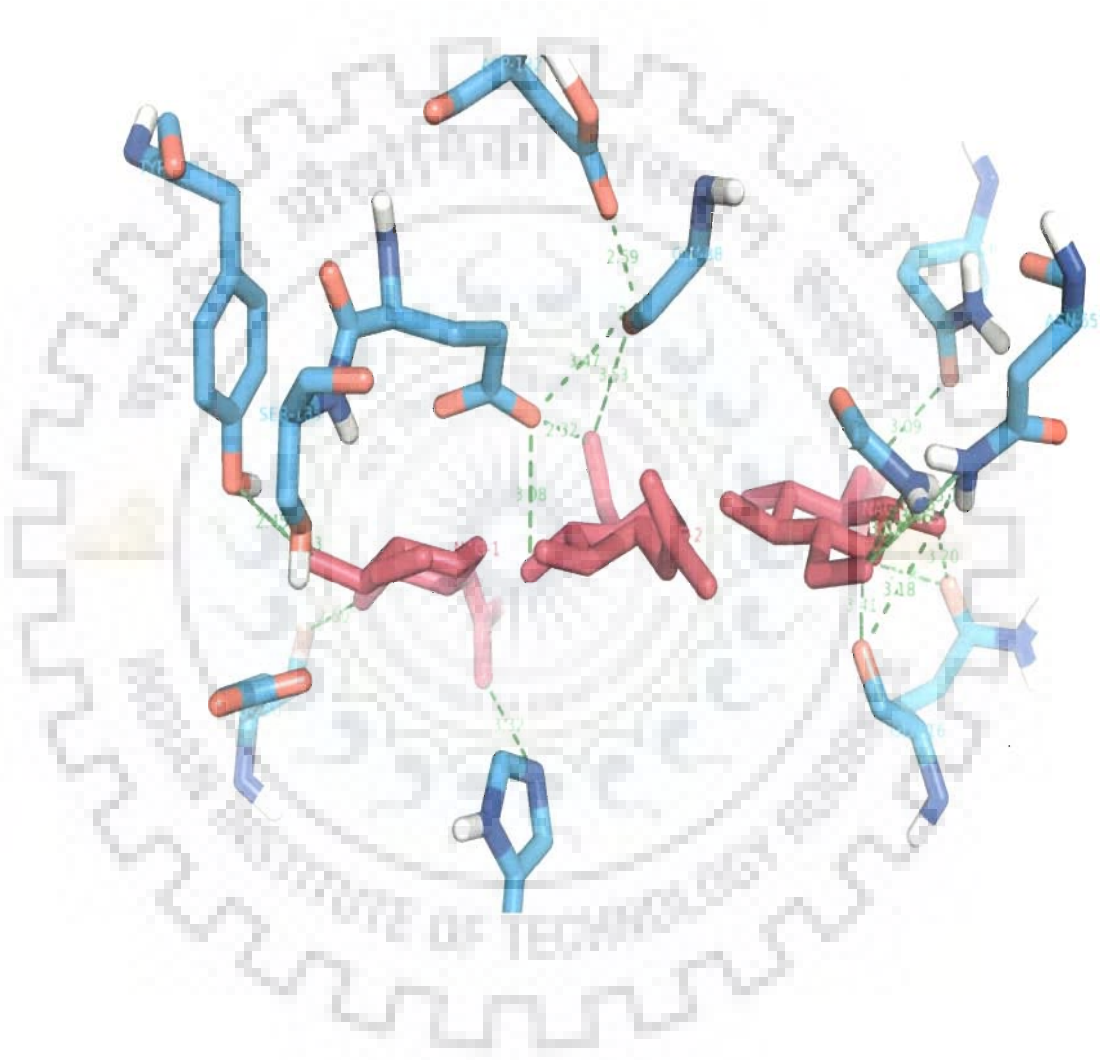


Fig 3.16: Polar interactions between tri-NAG and amino acid residues of CHT namely His 267, Glu 230, Tyr 172, Ser 135, Glu 134, Gln 16,Asn 55 and Asn 44

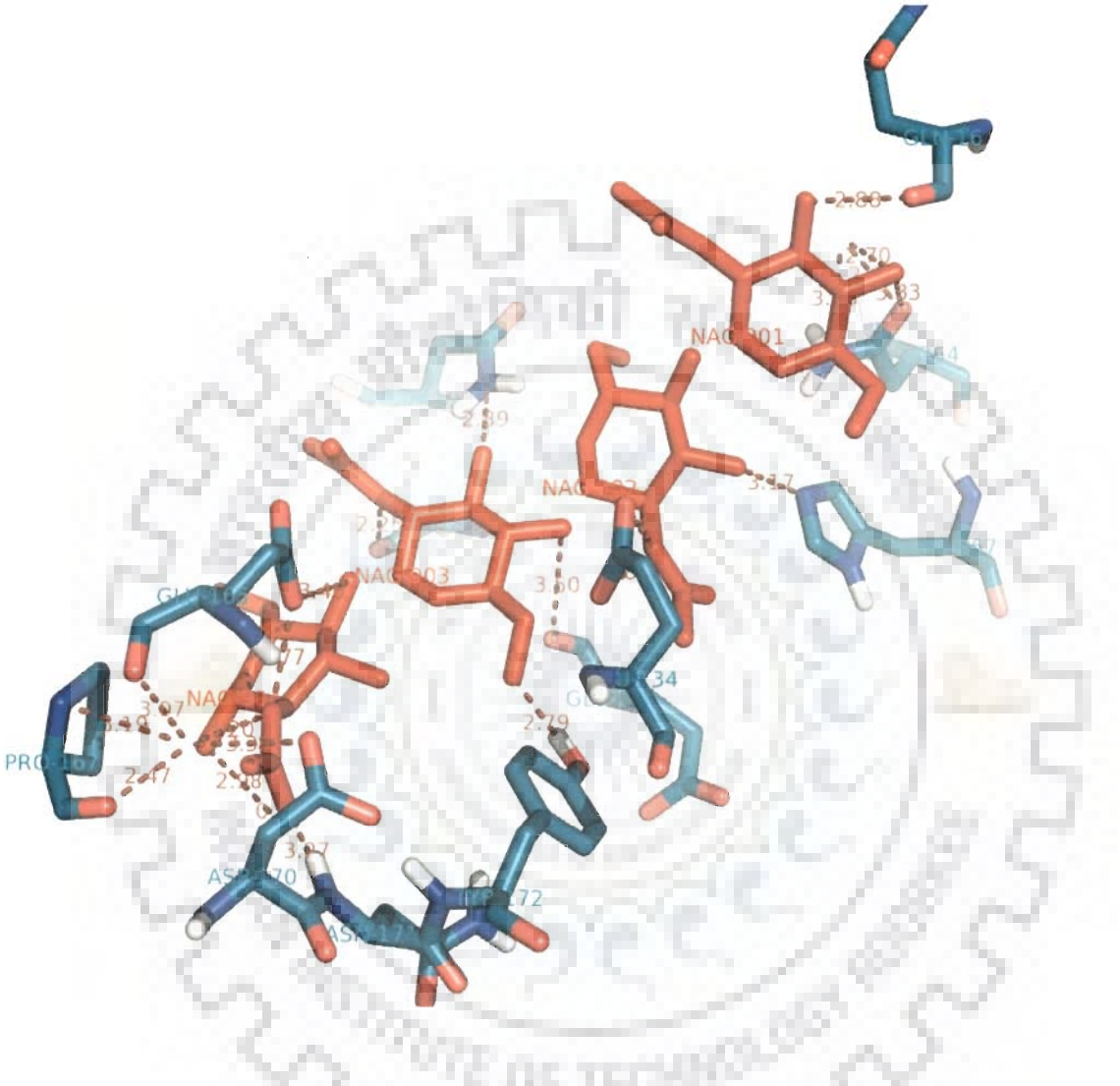


Fig 3.17 : Polar interactions between Tetra-NAG and amino acids residues of CHT namely His 267, Asn 264, Glu 230, Tyr 172, , Glu 134, Gln 16 Asn 191 and Ala 232.

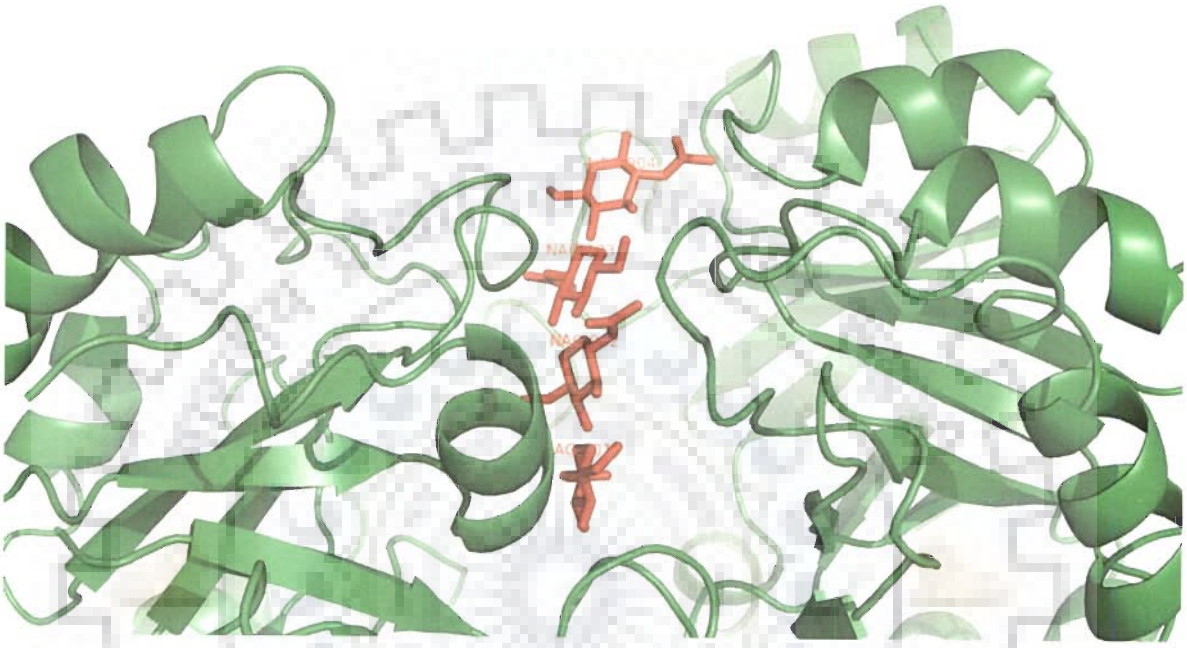


Fig 3.18: Substrate entry site for tetra-nag between two lobes of the protein constituted by chain A and chain B

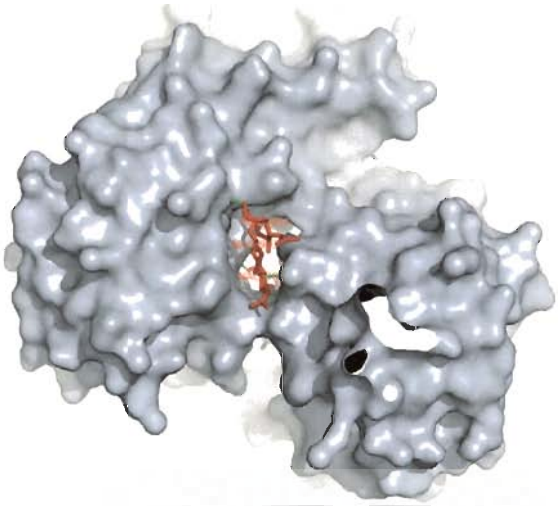


Fig 3.19: Surface diagram of CHT indicating Penta –NAG in the binding pocket

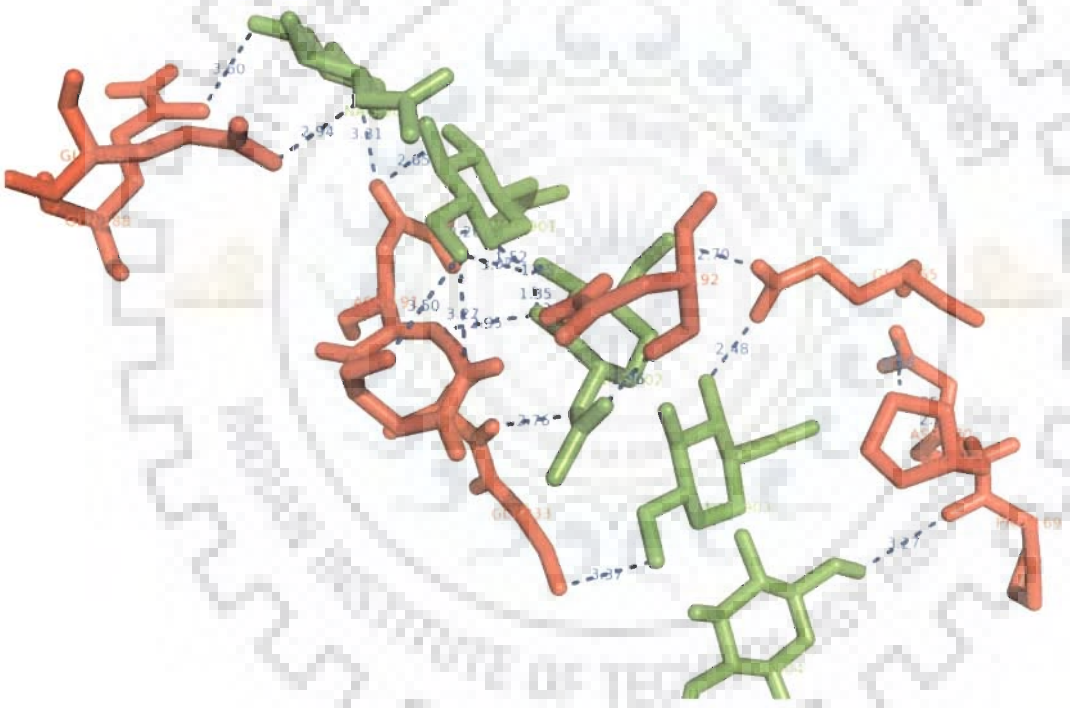


Fig 3.20: Polar interactions between Tetra-NAG and amino acids residues of CHT namely Asn 192, Glu 165, Gly233, , Glu 165, Gln 16, Gln 188, Asn 191 and Asp170.

4.5. Discussion

Fluorescence studies revealed the conformational stability of CHT at different physicochemical conditions. The results indicate that the conformational changes occurring in the structure as a function of guanidine hydrochloride and urea does not significantly expose the hydrophobic pockets for ANS binding. However, the significant unfolding of the CHT structure was observed with increasing temperature as determined by ANS and tryptophan fluorescence respectively. In presence of HCl gradual unfolding of CHT structure was observed up to 1.2 M HCl concentration with complete unfolding above this concentration. With increasing concentration of βME the protein folding occurs but at concentration above 90mM there is aggregation of protein.

X-ray studies have shown that enzymes of GH18 family showing chitinase activity have a conserved Asp125, Glu127 and Tyr183 amino acids (hevamine numbering) in the active site. The exact mechanism is although not known but Glu127 is considered as the proton donor to the glycosidic bond and Asp125 and Tyr183 stabilize the intermediate. Corresponding amino acids were found to be present in CHTas Glu134 and Asp 132. The residues required for catalysis are well placed near the catalytic and form stabilizing hydrogen bonding network between them. Conserved functional and structural features present in CHT indicate the mechanism of catalysis is same as compared to 1HVQ. The consensus motifs characteristic of family 18 glycosyl hydrolases is conserved β3 and β4 strands. Structural variance is associated with active site cleft loops where some loops tend to match with 2GSJ and some with 1 HVQ. The crystal structure for CHT confirmed an overall topology identical to the class III chitinases with major differences in the loop region which might explain it inability to hydrolyze bacterial cell wall as a substrate. The docking studies

indicated that Tetra-NAG was the ideal substrate for CHT. Further confirmation can be done on the basis of CHT-complex crystal formation.

Conclusions

- The *Tamarindus indica* chitinase was purified to homogeneity by three step purification protocol. In first step, the protein was partially purified by affinity chromatography followed by purification on a cation and anion exchange column. CHT was found to be the most abundant protein in the seeds of *Tamarindus indica* plant
- The SDS-PAGE(reducing and non reducing conditions) and SEC analysis confirmed that CHT is composed of a single polypeptide chain with molecular mass of 34 kDa..
- Chitinase activity was confirmed by ferri-ferrocyanide method and calcofluor dye based method. The activity of the enzyme was activated by 2 % in the presence of Li^+ and inhibited upto 15% in the presence of Zn^{2+}. Presence of reducing agent also inhibited the activity of enzyme(12% by DTT and 8.7% by βME). EDTA also inhibited the enzyme by 9%.
- CHT has no antifungal and antibacterial activity in 0.1M acetate pH 5
- N-terminal sequence analysis, with a NCBI BLAST short sequence search, showed homology to a class III chitinase from *V.vinifera.*
- CHT gene and cDNA was amplified by PCR giving ~730 bp product. The product obtained from mRNA and genomic DNA was same size indicating absence of introns in the CHT gene.
- Sequence analysis of CHT gave 78% identity with *G.max* class III chitinase. The sequence had consensus region throughout indicating conservation of the overall primary structure of chitinases.

- The theoretical pI of the protein was found to be 4.10 and the extinction coefficient was found of 55810 M^{-1} cm^{-1} .The sequence comparison showed that CHT has three disulphide bonds.
- CHT is progressively denatured by increasing concentrations of urea, HCl and GndCl. In case of HCl, complete opening of the protein 3 –D structure occurred. In presence of reducing agent βME aggregation of protein occurred at concentration above 90mM. At 90°C there was red shift in fluorescence intensity indicating complete unfolding of the protein polypeptide chain.
- Crystals of CHT was obtained at 20% PEG 4000 at 0.1 M MES pH 6.
- The unit-cell parameters were a = b = 67.00 Å, c = 173.09 Å with two molecules per asymmetric unit this corresponds to a crystal volume per unit molecular weight (V_M) of 2.50 $Å^3$ Da^{-1}, with a solvent content of 51%.
- Cycles of refinement was carried out and it gave the final R and R_{free} value of 22.6% and 27.6% respectively.
- Topology of the CHT was conserved in having a $(\alpha\beta)_8$ barrel domain (Fig 3.11) structure with three disulphide bonds in between cysteine residues at positions 20-67, 50-57 and 158-187 in each of the subunit.
- The docking studies indicated that Tetra-NAG was the ideal substrate for CHT. Further confirmation can be done on the basis of CHT-complex crystal formation.

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