5.1. Introduction

*Aspergillus flavus* is a pathogenic fungi of several plants and animals species, including humans and domestic animals. *A. flavus* generally infects seeds of corn, cotton, peanuts and nut trees. The fungus can be seen sporulating on injured seeds such as maize kernels (Payne, 1998). Growth of the fungus on a food source often leads to contamination by the production of potent mycotoxins, secondary metabolites called aflatoxins which are both immunosuppressive and carcinogenic (Bokhari, 2002; Hedayati et al., 2007). In humans, a broad spectrum of diseases ranging from hypersensitivity reactions to invasive infections associated with angioinvasions is caused by *A. flavus*. The second leading cause of invasive and non-invasive aspergillosis is *A. flavus*. Patients infected with *A. flavus* often have shortened immune systems. The primary target of aflatoxin is the liver and the functioning may fail depending on the duration of feeding on contaminated grain or food products and the amount of aflatoxin ingested. In developing countries, many individuals are not only malnourished but are also chronically exposed to high levels of mycotoxins in their diet (Akpan et al., 1999). One of the serious cause of food safety and quality problem of worldwide is by the aflatoxin contamination to various agricultural crops (Woloshuk et al., 1997).

*A. flavus* produces α-amylases which convert starch into simple sugars and these are used as the energy source by the organisms. α-amy late is an enzyme which aids in the breakdown of starch to maltose by the organism as its energy source. α-amylase inhibitors have been reported to inhibit the growth of fungi. A few reports exist on different proteins that act as fungal α-amylase inhibitors. Chen et al (1999) have reported that a corn trypsin inhibitor with antifungal activity can inhibit *A. flavus* α-amylase. Reducing the availability of simple sugars has been found to limit the ability of the fungus to produce aflatoxins. Fakhoury and Woloshuk (2001) have reported that the α-amylase activity can be inhibited by a lectin like protein from *Lablab purpureus* and was also found that that lectin can control the growth of *A. flavus*. Lectins, the widely distributed sugar-binding proteins which are found in plants, animals and microorganisms, are found to be highly specific for their sugar moieties. Among the total lectins studied, plant lectins are in majority and that have been isolated and characterized with respect to their molecular structures and carbohydrate-binding specificities (Abdullaev and Gonzalez, 1997; Rubinstein et al., 2004). Lectins have become a topic of interest to researchers due to their biological properties especially the sugar binding properties and the other features
including antitumor (Abdullaev and Gonzalez, 1997), immunomodulatory (Rubinstein et al., 2004), antifungal (Barrientos and Gronenborn, 2005), antibacterial (Pusztai et al., 1993), and mitogenic (Wimer, 1990) activities.

Analysis of the defense mechanism of lectins revealed that it is mainly by the inhibitory properties of lectins against some phytopathogenic organisms (Ciopraga et al., 1999; Freire et al., 2002) and yeasts (Klaflke et al., 2013). Pinheiro et al reported the growth of the filamentous fungus *Microsporum canis* was completely inhibited by *Talisia esculenta* lectin at a concentration of 2mg/ml (Pinheiro et al., 2009). There are also reports about antimicrobial lectins from plants such as *Canavalia brasiliensis, Mucuna pruriens, Clitoria fairchidiana, Dioclea virgate* and *Bauhinia variegata* (Pinheiro et al., 2009). Crystal structure of lectins from *Canavalia brasiliensis* (PDB code: 4h55) and from *Dioclea virgate* (PDB code: 3rs6) are available.

*Butea monosperma* lectin (BML) belongs to the legume lectin family. Crystal Structure of BML is an octamer consisting of quaternary tetramers consisting with two heterodimeric, α and β chains (Abhilash et al., 2011). The chain consists of 256 and β chain consists of 242 amino acids. The 18 β strands of BML form a jelly roll as in the other legume lectins. The jelly roll model consists of 6 anti-parallel β-sheets, curved 7 stranded front β-sheet and a short 5 membered β-sheet at the top of the molecule. The sheets are connected by many loops (Van Damme et al., 1998; Mitra et al., 2002). In this work an attempt was made to check the possibility of fungal α-amylase inhibition using the BML and the analysis of the inhibition was carried out by means of enzyme kinetics and Isothermal Titration Calorimetric analyses and molecular docking studies.

5.2. Materials and methods

5.2.1. Purification of fungal α-amylase from *A. flavus* and lectin from *Butea monosperma*

*A. flavus* strain which was obtained from MTCC, Chandigarh, India was grown and sub cultured repeatedly in Potato Dextrose Agar (PDA). α-Amylase from *A. flavus* was purified based on the protocol reported by El-Safey and Ammar (2004) with minor modifications. It was purified from cultures grown in 50 ml of the starch medium (1.4 g of KH₂PO₄, 10 g of NH₄NO₃, 0.5 g of KCl, 0.1 g of MgSO₄·7H₂O, 0.01 g of FeSO₄·7H₂O, 20 g of soluble starch dissolved in 1000 ml
of distilled water with pH 6.5) at 28°C, with shaking at 150 rpm. After 8 days of incubation, cultures were centrifuged at 5,000 x g for 10 min, supernatants were filtered through Miracloth (Calbiochem-Behring, San Diego, CA, U.S.A.) and the precipitant of 30% ammonium sulphate was discarded initially and the precipitate of 85% saturation of ammonium sulphate was collected and dialyzed for 20 hours against phosphate buffer (pH 6.9). This was used for the enzyme assay. Minimum inhibitory concentration of BML (MIC) was determined in a 96-well microtitre plate. Spore suspensions were prepared in potato dextrose broth and adjusted to final inoculums of 0.4 - 5 x 10^4 spores/ml. BML at five different concentrations ranging from 0.3 mg/ml to 1.5 mg/ml in the phosphate buffer were added to each well along with the organism. The plates were incubated at 25 -28°C for 48 h. The growth in each well was monitored.

Purification of lectin from Butea monosperma seeds was carried out using the protocol described in Chapter No.2 (Abhilash et al., 2011).

5.2.2. α-Amylase activity assay

![Figure 5.1](a) Plot of optical density (OD) against different concentration of maltose
(b) Plot of optical density (OD) against different concentrations of α-amylase in the reaction mixture

α-Amylase activity was assayed as described by Andrew et al, (1969). The assay mixture contained 1 ml of crude enzyme, 1 ml of 1% (w/v) soluble starch solution incubated for three minutes at 25°C. Reaction was terminated by adding 2 mL of DNS (3, 5-Dinitrosalicylic acid) in the reaction tube and then immersing the tube in boiling water bath (100°C) for 5 minutes. The absorbance was measured at 540 nm and the amount of maltose liberated was calculated.
using a standard graph (Figure 5.1). Aqueous maltose solution with concentration 0.25 to 2.0 mg/ml mixed with 2 ml of DNS was used to plot the standard graph.

Enzyme concentration in units/ml was calculated using the formula.

Enzyme concentration (units/ml) = \( \frac{\text{Maltose in mg (released)} \times \text{df}}{V} \)

\( \text{df} = \) dilution factor.
\( V = \) Volume (in milliliter) of enzyme used.

Similarly, the purification of lectin from *Butea monosperma* seeds was carried out by the reported protocol of Abhilash et al, 2011 (Chapter 2). The purity of the protein was confirmed by the SDS-PAGE analysis and haemagglutination test.

### 5.2.3. Enzyme Kinetics Studies

In order to obtain the enzyme kinetics parameters, one ml of enzyme solution (0.01 mM) was prepared and incubated with different concentrations (0.25, 0.5, 0.75, 1.00, 1.25, 1.50, 1.75 and 2.00 mg/ml) of substrate (starch) for three minutes at 20°C. The whole experiment was repeated with the enzyme pre-incubated with equal volume of 0.03 mM of BML. To ascertain the type of inhibition, Lineweaver-Burk plot (LB Plot) was drawn for the native and inhibited enzyme. The Michaelis constant \( (K_m) \) and maximal velocity \( (V_{max}) \) were determined from the LB plot. (Palmer and Bonner, 2007).

The inhibition constant, \( K_i \) was determined using the equation

\[
V'_{max} = \frac{V_{max}}{1 + (\frac{I_0}{K_i})}
\]

### 5.2.4. Isothermal Titration Calorimetric assay

Isothermal titration calorimetric (ITC) analysis was performed to identify the binding affinity of BML with \( \alpha \)-amylase. The concentration of \( \alpha \)-amylase was 0.01 mM and that of lectin was 0.1 mM in water. 1.8 ml of the enzyme solution was taken in the sample cell and 290 μl of lectin solution was taken in the syringe and injected to the sample cell in steps of 5 μl. The calorimetric titrations were performed at the temperature 298.15 K using VP-ITC isothermal titration...
calorimeter from Microcal (Northampton, MA, USA), as described in the manufacturer’s instruction manual. In each step, 5 μl of BML solutions was injected and the time given for each injection was 5 seconds. A time interval of 120 seconds was also set between each injection to allow the peak resulting from the reaction to return to the baseline. Total 57 injections were made. The reference power was set as 10 μcal and the stirring speed was adjusted to 307 rpm. The volume of the 1st injection was set 2 μl to avoid inaccuracy. The data at the end of the injections was fitted by a nonlinear least squares method using ORIGIN software from Microcal. Once $\Delta H$ and $\Delta S$ are obtained from graph $\Delta G$ can be calculated ($\Delta G = \Delta H - T\Delta S$).

**5.2.5. Molecular Modeling of α- amylose and missing residues of lectin**

The 3D structure of α- amylose of *A. flavus* has not been reported so far. Hence the structure modeled using Swiss model web server (Tintu et al., 2012) with α- amylose of *A. oryzae* (PDB code: 7taa) (Brzozowski and Davis, 1997) as the template structure (98% similarity). The modeled structure was optimized by extensive energy minimization and subjected to 150 ps molecular dynamics (MD) simulation in the presence of TIP3P water molecule using AMBER 9 software (Case et al., 2005).

**5.2.6. Molecular docking using shape complementarities by Fourier correlation.**

The docking study was performed based on the geometric surface recognition and electrostatic attraction methods. All these were carried out using Fast Fourier Transform (FFT) and Fourier Correlation Theory (FCT) implemented in HEX 6.3 (Ritchie, 2003). The α-amylase and BML was loaded on the workspace and shape based complementarities was searched by rotating the receptor and ligand about their centroids at an intermolecular distances of 40 Å. Initially, both the proteins were assigned in to three euler rotation angels and rotation of each had been done on their intermolecular axis. The program was searched an initial steric scan at steps N=16, followed by a final search at steps N=25 by default. From the total orientations generated, the best model was selected based on the appropriate electrostatic contribution.
5.2.7. Molecular Dynamics

Prior to molecular dynamics (MD), the docked complex was subjected to energy minimization about 2000 steps using conjugate gradient (CG) algorithm implemented in SPDBV programme (Guex and Peitsch, 1996). All MD simulations were performed by Schrodinger 9.1 with AMBER 94 force field (Cornell et al., 1995). A box with the dimensions of 121 Å³ was selected and filled with TIP3P water molecules. The molecular dynamics method was done at a constant temperature of 300 K with verlet algorithm. A non bonded cut off distance was assigned as 12.0 Å. The Steepest descent minimization method was adopted. There were 1,00,000 iterations with convergence threshold of 0.0500. The total simulation lasted for 2 ns time and the equilibration time was set as 0.1 ns.

5.3. Result and Discussion

From the assay result (Figure 5.1), the presence of α-amylase in the sample was confirmed. The OD was found to increase with respect to time. The haemagglutination test and SDS PAGE analysis also confirmed the presence of pure BML in the sample. The mode of inhibition of α-amylase with BML was identified by the LB plot (Figure 5.2). The Michaelis constant ($K_m$) and maximal velocity ($V_{max}$) for α-amylase are 0.625 mg/ml and 0.192 mg/ml/min respectively. In the presence of BML, the $V_{max}$ value was found to decrease to 0.057 mg/ml/min and the $K_m$ value was remains to be the same. The result indicated a non competitive binding of BML towards α-amylase. The ITC assay result of binding of lectin to the α-amylase at 303 K is shown in Figure 5.3. Total of 57 injections were made and a non linear least squares fitting method was applied to interpret the data. The binding constant ($K$), change in enthalpy ($\Delta H$), change in entropy ($\Delta S$) and binding free energy ($\Delta G$) were calculated and shown in Table 5.1. The isothermal curve was best-fitted with the stoichiometry $n =1$. The negative $\Delta G$ value indicated that the binding was thermodynamically favorable.
Figure 5.2. Lineweaver-Burk plots for the α-amylase inhibition by BML. [V] is the velocity of reaction and [S] is the substrate concentration in mg/ml. The values are averages of three independent measurements ± sd. $K_m$ value is 0.625 mg/ml and $V_{max}$ is 0.192 mg/ml/min for α-amylase. In the presence of lectin, the $K_m$ value was the same and a decrease in $V_{max}$ to 0.057 mg/ml/min was observed.

Figure 5.3. Isothermal titration calorimetric analysis of α-amylase with lectin. The curve represents the non-linear least-squares fit of the energy released as a function of the amount of lectin added during the titration.
Table 5.1. ITC results of the Titration of BML and α-amylase

<table>
<thead>
<tr>
<th>Name</th>
<th>N</th>
<th>K in M⁻¹</th>
<th>T in K</th>
<th>H cal/mol</th>
<th>S cal/mol</th>
<th>G Kcal/mol</th>
</tr>
</thead>
<tbody>
<tr>
<td>BML- α-amylase</td>
<td>0.804</td>
<td>9.70 x 10⁴</td>
<td>298.15</td>
<td>-9.22 x 10⁵</td>
<td>-3.07 x 10³</td>
<td>-7.179</td>
</tr>
</tbody>
</table>

The target structure (α-amylase of *A. flavus*) was modeled using homology modeling. The sequence identity between the template and target sequences was 98%. After 150 ps MD simulation, an average structure was calculated. The RMS deviation between the Cα atoms of the target and template structures was 0.53 Å and that between all backbone atoms was 0.66 Å. The modeled structure consisted of 14 alpha-helices, 21 beta strands and 31 turns. The conformational quality was tested using Ramachandran map, and found that all the residues were lying in the allowed region.

In order to identify the molecular mechanism of the inhibition, a rigid surface docking has been carried out with α, β chains and whole lectin (in octameric from). It was found that both chains are binding to α-amylase more or less in the same manner. Since α, β chains consist of similar sequences, the binding of all the chains was found to be unique. The binding mode of α chain is discussed herewith. It was found that α chain of BML binds at the opening of the active site of α-amylase (Figure 5.4).

Structural changes have been observed for the protein complex as a result of 2 ns MD simulation. The total number of hydrogen bonds, van der Waals contacts and salt bridges were calculated after MD. It was seen that loops are interacting with the α-amylase. Even though the loops are bound at the opening of the active site of α-amylase, no residues of lectin was found to interact directly with the catalytic residues of amylase. The ‘C-loop’ (residues 61-67) is arranged in a ‘U’ shaped conformation on the α-amylase surface (Figure 5.5).
From the docked pose it was observed that five loops, 60-67, 116-121, 145-152, and 196-205 of chain D and 185-189 of chain C of BML made contacts with α-amylase. There were two hydrogen bonds between BML and α-amylase. Hydrogen bonds from α-amylase Ser266 and...
Tyr256 were with the Phe240 and Lys202 respectively of BML chain D. Among the 61 van der Waals contacts, 20 were with chain C and 41 were with chain D of the BML. Apart from these, two salt bridges were also seen between amylase and chain C of the BML. The residues involved were Glu156 of amylase and Lys190 of chain C of the BML. The NZ atom of the Lys190 of Chain C had two salt bridges with the OD1 and OD2 atoms of Glu156.

The α-amylase of *A. flavus* has three domains, in which the catalytic domain is responsible for the cleavage of substrate (Swift et al., 1991; Rodenburg et al., 1994; Juge et al., 1995). The substrate of amylase (amylose) is recognized by the substrate binding groove which has a V-shaped depression (Davies and Henrissat, 1995). The substrate binding region extends up to the surface from the catalytic centre. The amylose enters the substrate binding groove through one side, getting hydrolyzed and releasing the product through the other side.

The overall structural changes occurring to α-amylase-BML complex were analyzed. The conformational changes occurring at the binding, interfacial region of two proteins due to the protein-protein interactions were also identified by super imposition analysis by least squares fitting method. 1.18 and 1.89 Å deviations were found in the Cα atoms of lectin and amylase respectively of the binding, interfacial region of two proteins. Similarly, 1.67 and 3.08 Å deviations were found in the side chain atoms of lectin and amylase respectively of the binding, interfacial region of two proteins. The structural changes observed after MD simulation were found to enhance the favorable binding of BML to α-amylase and close the entry of the substrate into the active site of the enzyme.

### 5.4. Conclusion

From the thermodynamic studies it is observed that the binding free energy ($\Delta G$) for the interaction of α-amylase and lectin was -7.179 kcal/mol. The negative $\Delta G$ value indicates the binding is thermodynamically stable.

By the molecular docking studies it has been revealed that the binding of lectin on α-amylase is in such a manner that it can mask one part of the substrate binding cleft. The bound lectin does not block the substrate from entering to the active site but may hinder the release of the product after catalysis.

The present study reveals that BML can inhibit fungal α-amylase and thereby can be used against *A. flavus*. 