Sturctural studies of lipoxygenases: cloning, expression and purification of lipoxygenases from Anabaena and Fusarium

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STRUCTURAL STUDIES OF LIPIDOXGENASES: 
CLONING, EXPRESSION AND PURIFICATION OF LIPIDOXGENASES FROM 
*ANABAENA* AND *FUSARIUM*

A Thesis

Submitted to the Graduate Faculty of the 
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requirements for the degree of 
Master of Science

In 
The Department of Biological Sciences

By 
Pratik Dhar 
M.S., Indian Institute of Technology, May 2002 
B.S., Indian Institute of Technology, May 2000 
August, 2007
DEDICATION

I would like to dedicate this thesis to my parents, Mr. Gora Chand Dhar and Mrs. Kumkum Dhar, whose constant inspiration and support have helped me attain the academic position which I am presently at. I would also like to dedicate this thesis to my loving wife, Sanchita Dhar, who has stood beside me in thick and thin, and is the woman behind my success.
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PREFACE

This thesis is composed of an introduction on lipoxygenases followed by five chapters on the work done with the lipoxygenase proteins from the cyanobacterium *Anabaena* and the fungus *Fusarium*. While the long range goal of this research is to understand the mechanism of action of this family of proteins by crystallizing these proteins and solving their structures by X-ray crystallography, the thesis describes the cloning, expression and purification work done with these proteins.
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ABSTRACT

Lipoxygenases (LOX) are non-heme, non-sulfur, iron containing enzymes that catalyze the dioxygenation of unsaturated fatty acids. LOX enzyme is found in higher plants, some multicellular animals and some bacteria. In plants, the substrates for lipoxygenase enzymes are 18 carbon unsaturated fatty acids (linoleic and linolenic acids) while in animals, the substrate for this family of enzymes is the 20 carbon unsaturated fatty acid (arachidonic acid).

The LOX enzyme from the cyanobacterium Anabaena sp. strain PCC7120, which produces the smallest known LOX, and the fungus Fusarium verticillioides, which produces a LOX with dual specificity and remarkably high activity, were overexpressed using Escherichia coli (E. coli) bacterial expression systems. Both enzymes were also purified using conventional protein purification techniques such as affinity and size exclusion chromatography. Assays performed using linoleic acid as the substrate confirmed the purified enzymes to be in their active states and size exclusion chromatography confirmed sufficient purity for crystallization. The fusion protein from Anabaena, containing both LOX and AOS (allene oxide synthase), which is the naturally occurring form of the protein, has also been expressed using bacterial expression system.

New constructs encoding Fusarium LOX were engineered and cloned in pET-28b. The new constructs were designed to express protein lacking the T7 tag at the N-terminus, in an attempt to overcome crystallization problems. Both the new constructs have been successfully utilized to express the proteins, and protein obtained from one of the constructs has been overexpressed and purified.
CHAPTER 1 - INTRODUCTION

Lipoxygenases (LOXs) are non-heme, non-sulfur, iron-containing enzymes which catalyze the stereo- and regio-specific dioxygenation of polyunsaturated fatty acids (Nelson et al. 1994; Burger et al. 2000; Radmark 2002; Liavonchanka et al. 2006).

\[
\text{Fatty acid} + \text{O}_2 \xrightarrow{\text{LOX}} \text{Fatty Acid Hydroperoxide}
\]

Lipoxygenases are found in both plants and animals (fig. 1) (Brash 1999). The substrates for the plant enzymes are 18 carbon fatty acids, linoleic (18:2) and linolenic acids (18:3) (Hamberg et al. 1967), while the animal enzymes catalyze the peroxidation of arachidonic acid (20:4), a 20 carbon unsaturated fatty acid (Khanapure et al. 2007). Products of human lipoxygenases are part of the leukotriene pathway or are involved in other diverse cell functions.

![Diagram](image)

**Fig 1.** Distribution of lipoxygenases, its substrates and final products (Hydroxy fatty acids in plants, eicosanoids in animals (Schneider 2007))
While animal 5-LOXs require Ca\(^{2+}\), ATP and a lipoxygenase activator protein for activity, other LOXs have the capability of carrying out their functions on their own (Feussner et al. 2002). Mammalian LOXs have been found to be associated with the pathogenesis of several inflammatory conditions, such as arthritis, psoriasis, bronchial asthma and cancer (Di Venere et al. 2003; Ding et al. 2003; Lutteke et al. 2003). They are also thought to have a role in atherosclerosis, brain aging, HIV infection and kidney diseases (Di Venere et al. 2003; Ding et al. 2003; Lutteke et al. 2003). The plant LOXs convert the 18 carbon substrates into a variety of bioactive mediators involved in plant defense, senescence, seed germination, plant growth and development (Porta et al. 2002). The LOXs found in other organisms have been found to be functionally similar to human LOX (Musser et al. 1992), catalyzing the dioxygenation of unsaturated fatty acids. LOX converts the poly-unsaturated fatty acid substrate to hydroperoxy eicosatetraenoic acid (HPETE). LOX catalysis includes the following steps (Nelson et al. 1994; Schneider 2007) (fig. 2):

i) the stereospecific hydrogen removal from a pentadiene group

ii) delocalization of the electrons.

iii) stereospecific insertion of molecular oxygen.

Crystal structures of LOXs from

![Fig. 2 The LOX reaction mechanism](image)
soybean (Minor et al. 1996; Youn et al. 2006) (fig. 3), coral (Oldham et al. 2005) and rabbit (Gillmor et al. 1997; Borngraber et al. 1999)) have been solved, and striking similarities have been observed in the structures of plant and animal LOXs (Gillmor et al. 1997). The enzymes are composed of an amino terminal domain with an anti-parallel β-barrel, and a carboxyl terminal domain with α-helices, π-helices and anti-parallel β-sheets.

![Image](image.png)

**Fig 3. Crystal structure of soybean lipoxygenase (Minor et al. 1996)**

The C-terminal domain has two π helices (fig. 4), which is one of the rare forms of helical structures found in proteins (Gaffney 1996; Minor et al. 1996; Borngraber et al. 1999; Weaver 2000). One unique feature noticed in the known LOX structures is that the carboxyl terminal isoleucine residue loops around and forms part of the active site (Minor et al. 1996; Youn et al. 2006) where it coordinates with the non-heme iron. Isoleucine has a hydrophobic side chain, and so it is actually the carboxyl oxygen which coordinates with the ferrous iron. In plants, the Fe^{2+} coordinates with three histidine residues, one isoleucine residue and
one asparagine residue while the sixth position is taken up by a water molecule (Minor et al. 1996) (fig. 5), while in animals, the Fe$^{2+}$ shows interactions with either the same residues as in plants or with four histidine residues, one isoleucine residue and a water molecule (Kuban et al. 1998). Interaction with the isoleucine residue has been observed in all known LOXs, and the notable point is that the isoleucine is the last residue in the carboxyl terminus of the protein, which folds back into the protein to become part of the active site.

The focus of this thesis is the expression and purification of LOX from the cyanobacterium *Anabaena*, and the fungus *Fusarium*. The reason for choosing LOX enzymes from *Anabaena* and *Fusarium* is that both of these enzymes are exceptional examples from the LOX family of proteins. In *Anabaena* the LOX protein, which exists as a part of a fusion protein with allene oxide synthase (AOS), is significantly smaller than other known LOXs, and *Fusarium* LOX has dual specificity (Nelson et al. 1994) and an exceptionally high activity. The
study of the structures of these two LOXs might reveal significant answers involving the dual specificity and mechanism of action of this family of enzymes.
2.1. Introduction

*Anabaena* is a heterocyst-forming photosynthetic cyanobacterium (Rajaniemi *et al.* 2005; Prasanna *et al.* 2006). It carries out aerobic photosynthesis as well as nitrogen fixation (Rajaniemi *et al.* 2005; Prasanna *et al.* 2006). *Anabaena* (fig. 6) belongs to:

- Phylum: *Cyanophyta*
- Order: *Nostocales*
- Family: *Nostocaceae*
- Genus: *Anabaena*

Its cellular structure is comprised of long barrel-shaped vegetative cells, arranged one after the other forming long chains or filaments called *trichomes*. *Anabaena* is commonly referred to as “beads on a string” because of its characteristic appearance. *Anabaena* is one of the main nitrogen fixing organisms in the environment. *Anabaena* is normally found in a symbiotic relationship with the plant *Azolla* (Peters 1977).

Structurally, *Anabaena* is made up of 3 kinds of cells (fig. 7) (Rajaniemi *et al.* 2005).
1. *Anabaena* is made up mostly of barrel-shaped or spherical vegetative cells of uniform size (Rajaniemi *et al.* 2005). They form the filaments or the characteristic trichomes. These cells carry out oxidative photosynthesis. They contain the typical cyanobacterial pigments such as phycobilins and chlorophyll, and carry out photosynthesis with the help of both photosystem 1 (PS1) and photosystem 2 (PS2) (Ying *et al.* 2002).

2. Nitrogen fixation is carried out by large, thick-walled cells known as heterocysts (Rajaniemi *et al.* 2005). The most important requirement for nitrogen fixation (conversion of N$_2$ to NH$_3$) in cyanobacteria is the enzyme nitrogenase which is localized in these specialized cells. Since nitrogenase cannot function in the presence of oxygen, a heterocyst has 3 additional cell wall layers outside its primary cell wall to prevent the entry of oxygen. The rate of oxygen diffusion into the heterocyst is 100 times lower than into the vegetative cells. During the time of low environmental nitrogen, about 1 cell out of every 10 differentiates into a heterocyst. The presence of photosystem 2 is eliminated and the rate of respiration is stepped up to use up the excess oxygen. These heterocysts supply neighboring cells with fixed nitrogen in return for the products of photosynthesis. Heterocysts can still carry out photosystem 1 mediated reactions as this process does not generate oxygen. PS1 provides the necessary energy and reducing power.
for the nitrogenase enzyme (Neilson et al. 1971; Huang et al. 2005). The location of heterocysts varies in Anabaena. They can be found in between the vegetative cells ("intcalary heterocysts") or at the ends of the trichomes or filaments ("terminal heterocysts") (Huang et al. 2005).

3. The third kind of cells found in Anabaena are called akinetes. In Anabaena, they are present on either side of the heterocyst. They are also known as “resting spores”. Akinetes are ellipsoidal in shape and are slightly larger than the vegetative cells. They remain dormant until activation of the defense mechanism is required. In case of an emergency, such as the presence of some fungus in the environment, they produce chemical deterrents for the protection of the organism (Agrawal et al. 2000; Rajaniemi et al. 2005).

The fatty acids which have been shown to be present in Anabaena are 16:0 (30.55%), 18:0 (0.77%), 14:1 cis-7 (2.50%), 14:1 cis-9 (3.1%), 16:4 cis-4 (0.95%), 16:3 cis- 6 (2.16%), 16:1 cis-7 (1.44%), 16:1 cis-9 (6.53%), 16:1 trans-9 (4.02%), 16:1 cis-11 (1.62%), 18:2 cis-9 (10.16%), 18:3 cis-9 (18.19%), 18:1 cis-9 (4.01%), 18:1 trans-9 (0.92%); and the branched-chain fatty acids iso-16:0 (2.50%) and iso-15:1 (0.34%) (Caudales et al. 1992). Since Anabaena is a photosynthetic bacterium, showing many similarities with plants, Anabaena LOX probably uses the 18 carbon unsaturated fatty acids, linoleic acid (18:2) and linolenic acid (18:3) (fig. 8), as substrates. Also, arachidonic acid (20:4), which is a substrate for mammalian LOXs and not
for plant LOX, cannot be the normal substrate for this enzyme since arachidonic acid is not found in *Anabaena* (Caudales *et al.* 1992; Li *et al.* 2004).

Since *Anabaena* behaves very much like plants (oxidative photosynthesis), and *Anabaena* LOX can catalyze the dioxygenation of 18 carbon fatty acids *in vitro*, it is predicted that the LOX protein from *Anabaena* also behaves like the LOX found in plants. In plants, the fatty acids are converted to the hydroperoxides and passed on to AOS, to form allene oxide. Allene oxide is further modified to form defense molecules (Spiteller 1993). The defense cells for *Anabaena* are the akinetes. As LOX is part of the defense pathway, one possibility is that this protein resides in the akinetes. Another possibility which cannot be disregarded is that LOX uses oxygen for the dioxygenation reaction (Neilson *et al.* 1971). So, LOX might also be present in the heterocysts, as an oxygen scavenger.

The lipoxygenase structures solved to date (soybean, coral, and rabbit) show remarkable similarity to one another based on both their primary and secondary structures. Also, a BLAST search performed with the sequence of any of these known protein sequences used as the query returns results with high similarity to other LOXs whose primary sequences are known. This sequence homology of lipoxygenases is seen to be conserved in most eukaryotes. On the other hand, *Anabaena* LOX is very different. Though it exists as a fusion protein with AOS (allene oxide synthase), just like some other known LOXs such as coral LOX, and also performs the same function of dioxygenation of unsaturated fatty acids, the primary amino acid sequence shows very modest similarity with other known LOXs. In addition, *Anabaena* LOX is much smaller in size (~52kDa) than the other known LOXs. Once the crystal structure for this protein is obtained, it might be possible to understand the reason for the difference between *Anabaena*
LOX and other LOXs. Until then, only different hypotheses can be put forward, some of which are as follows:

**Conserved Active Site – Convergent or Divergent Evolution**

A sequence comparison of *Anabaena* LOX with other LOXs shows that the iron binding residues (3 histidines, 1 asparagine and 1 isoleucine) are conserved along with some other residues in lipoxygenases. This might be an indication that the active site, the main region for the activity of the enzyme, is conserved, and thus, the enzyme retains its function. This might be a sign of either convergent or divergent evolution. Evidence for both convergent and divergent evolution have been proposed for various proteins, leading to different primary structure and similar functions or similar structure and different functions, respectively. For example, regulatory mechanisms for phosphorylases from various eukaryotic organisms have diverged, but the phosphorylases are clearly from the same family of proteins (Hwang *et al.* 1986).

One very strong point in support of the divergent evolution theory will be the absence of the amino terminal β-barrel domain in the *Anabaena* lipoxygenase protein. While all the other known LOXs have the amino terminal domain which is used to direct the protein to the membrane in some cases, *Anabaena* lacks this part of the protein. This explains the smaller size compared to other proteins in the LOX family. In higher organisms the β-barrel domain may have an advantage by mediating the association of LOXs with their substrates. Perhaps the low level of similarity between the LOX from *Anabaena* and LOX from higher organisms results from changes in the catalytic domain as a result of association with the β-barrel in the eukaryotic LOXs.

This protein can also be thought to be in some point of the convergent evolution process. The present LOX protein from *Anabaena* might have had some other functions, and is gradually
evolving to take up lipoxygenase functions. Since the evolution is in the process of happening, though the protein performs the catalytic activity of dioxygenation of linoleic and linolenic acids, it is not fully developed yet, and still has relics of what this protein used to be, i.e., the amino acid residues from its previous form. The active site is completely or almost completely developed, as the enzyme can perform its catalytic activity of dioxygenation of linoleic acid with ease. It might be that over the course of many years, the *Anabaena* LOX protein will become similar in sequence to the other LOXs, and also develop the amino terminal β-barrel domain, which will be directing the protein to the membrane.

**Different Environment Means Difference in Structure**

The environment of a protein largely determines its structure (Petsko 2004). There might be a major difference in the environment in which *Anabaena* LOX performs its function when compared to LOXs from other organisms. The difference in environment might be a major deciding factor in the amino acid sequence of this protein. Some LOXs (e.g. 5-LOX) use the amino terminal β-barrel domain for membrane binding, while *Anabaena* LOX does not have the amino terminal domain. 5-LOX is requires Ca$^{2+}$, ATP and FLAP (5-lipoxygenase activator protein) for membrane binding and activity (Feussner et al. 2002), while *Anabaena* LOX does not. The β-barrel with its membrane-targeting function may have evolved to bring to bring LOX in contact with its substrate in the larger eukaryotic cell.

The substrates are also different for the different LOXs. Mammalian LOXs turn over 20 carbon fatty acids (arachidonic acid), while plant LOXs are active with 18 carbon fatty acids (linoleic and linolenic acids). *Anabaena* LOX is used in the catalysis of the conversion of linoleic and linolenic acids to hydroperoxides. However, it is likely that having a substrate two carbons smaller than animal LOXs would result only a few differences in amino acids. Furthermore,
soybean LOX also has a 18-C substrate and its sequence, too, is very different from that of \textit{Anabaena} LOX.

\textbf{Structure is Conserved While Sequence is Not}

Secondary structure prediction was performed for the \textit{Anabaena} LOX protein using the Advanced Protein Secondary Structure Prediction Server (http://imtech.res.in/raghava/apssp/) (Raghava 2000). The software predicts the secondary structure of proteins from their primary sequence information using a nearest neighbor and neural network approach. A close analysis of the results disclosed fascinating information which might be the answer that is being sought from the crystal structure. Though an alignment of the primary amino acid sequences of \textit{Anabaena} LOX and soybean LOX shows less than 20\% similarity, predicted secondary structures for the two proteins are highly similar. Since the secondary structure prediction was done independently, without any reference to the soybean LOX crystal structure, the similarity in the predicted secondary structure around the residues which coordinate with the Fe atom (3 histidines, 1 asparagine and 1 isoleucine) were free from any bias. The prototypic crystal structure of soybean LOX was modified based on the information obtained from the predicted secondary structure of \textit{Anabaena} LOX. The amino terminal domain was removed as it is not present in \textit{Anabaena} LOX. If the prediction has any relevance, the tertiary structure of \textit{Anabaena} LOX should look like the modeled molecule. Though the primary sequence of \textit{Anabaena} LOX is different from the other known eukaryotic LOXs, the shape of the active site should be conserved if the secondary structures are the same. Thus, the LOX from \textit{Anabaena} and other eukaryotes likely have similar structures even though the primary sequences are quite dissimilar. So, it can be hypothesized that the protein has conserved the secondary structure information even though it has significantly changed the primary sequence information.
2.2. Uniqueness of Lipoxygenase from *Anabaena*

The LOX protein from the cyanobacterium *Anabaena* naturally exists as part of a fusion protein with allene oxide synthase (AOS). The uniqueness of the LOX domain in this fusion protein is that its size is smaller than any other known LOXs, but it still shows high activity.

2.3. Expression of *Anabaena* LOX in Bacterial Expression System

*Anabaena* LOX was successfully over-expressed using an *E.coli* bacterial expression system. The gene for the protein in pET-17b was obtained from Dr. Alan Brash at Vanderbilt University, Tennessee.

2.3.1 Starter Culture

The bacterial plasmid containing the gene for *Anabaena* LOX was transformed into BL21(DE3) cells (Novagen) and plated on LB + ampicillin plates. A 100 ml volume of TB (Terrific Broth) media was autoclaved in a 500 ml conical flask and 100 µl of the antibiotic ampicillin (100 mg/ml) was added to the media. A single bacterial colony was picked from the overnight plate and placed in the media. The conical flask was shaken overnight at 250 rpm at 37°C.

2.3.2 Bulk Culture

Two liters of TB media were autoclaved in four 2.8L conical flasks and 500 µl of ampicillin (100 mg/ml) was added to each flask. The overnight starter culture was added equally to each flask. The conical flasks were shaken at 37°C at 250 rpm for 21 hours. The cultures were
then cold shocked at 4°C for 30 mins, before being put back in the shaker. The flasks were then shaken at 20°C at 250 rpm for 21 hours.

### 2.3.3 Lysing the Cells

The cells were spun down at 7000 rpm for 45 mins in centrifuge tubes. The supernatant was discarded and the pellets were frozen at -80°C. The pellets were then thawed and transferred to a 150 ml beaker. Bugbuster™ (Novagen) was added at 5 ml per 1 gm of pellet to lyse the cells. The protease inhibitor pepstatin and DNase were also added with Bugbuster™. The pellets were suspended in the lysis buffer, and let stand at room temperature for 30 mins. The cells were then sonicated on ice. The lysate was transferred to centrifuge tubes and centrifuged at 46000g for 30 mins. The supernatant was transferred to a 150 ml beaker using a 25 ml pipette.

### 2.4. Purification of *Anabaena* LOX

#### 2.4.1. Ni-NTA Chromatography

The protein was purified using an Amersham Biosciences AKTA FPLC Chromatography system. The first step of purification was performed using Ni-NTA chromatography. The 5 ml Ni^{2+} column was equilibrated with five column volumes of Buffer N (50 mM sodium phosphate pH 8.0, 500 mM NaCl, and 10% glycerol). The supernatant from the cell lysis step was loaded onto the column at 1.0 ml/min. The flowthrough was collected, and the column was successively washed with Buffer N, Buffer N1 (50 mM sodium phosphate pH 8.0, 1 M NaCl, 70 mM glycine, 10% glycerol), Buffer N and Buffer N2 (50 mM sodium phosphate pH 8.0, 500 mM NaCl, 20 mM imidazole, 10% glycerol), until the UV Absorption was below 0.05 AU in each wash. The protein bound to the column was eluted using an imidazole gradient from 20 mM to 200 mM in
Buffer N. The 5 ml fractions were collected using a fraction collector, and separated on a 10% SDS-PAGE gel by electrophoresis (fig. 9) to check purity.

![Fig 9. 10% SDS-PAGE gel showing purified *Anabaena* LOX after Ni-NTA chromatography](image)

2.4.2. Size Exclusion Chromatography

After Ni-NTA chromatography, size exclusion chromatography was used to further purify the protein and to ensure that all the protein obtained was of the same oligomeric form, as this is one of the primary criteria for obtaining diffraction quality protein crystals. First, the fractions from the Ni-NTA run containing the LOX protein were pooled and concentrated by centrifugation in a centricon tube.

The 120 ml Superdex 200 column was equilibrated with one column volume of 20 mM Tris HCl pH 8.0, 150
mM NaCl and 10% glycerol. The protein was loaded on to the column using a 2 ml loop, and eluted with one column volume of the buffer. Fractions of 1.3 ml were collected. A comparison of the elution profile of the protein (fig. 10) with the standard curve for the column showed that while some amount of the protein eluted as an aggregate, most of the pure protein eluted in the dimer form. The fractions containing the protein in the dimer form were pooled, concentrated to 10 mg/ml, and stored at -80°C.

2.5. Activity Assay

The concentrated dimer protein was assayed with linoleic acid, the natural substrate for this enzyme, using an Agilent 8453 spectrophotometer. 550 nM Anabaena LOX was assayed with 70 µM linoleic acid to check for activity. The dimer was found to be highly active in converting the linoleic acid into the hydroperoxy fatty acid, the formation of which was monitored by absorption of the hydroperoxide group.

**Fig 11.** Activity assay for Anabaena LOX with linoleic acid. UV absorption was measured at 235nm, the wavelength of absorption of the hydroperoxidized fatty acid. The initial product concentration is zero, and the initial amount of product measured in the assay is due to a delay in the collection of the first data point.
in the product at 235 nm (fig. 11). The turnover number of the protein was calculated to be approximately 9 per second.
CHAPTER 3 - THEORETICAL STUDIES ON *ANABAENA* LIPOXYGENASE

3.1. Primary Sequence Comparison with Soybean Lipoygenase

Primary sequence comparison of *Anabaena* LOX with the sequences of LOXs from coral, rabbit and soybean (fig. 12) was performed using CLUSTALW (Thompson *et al.* 1994). Low sequence similarities of less than 25% were observed between the primary sequence of *Anabaena* LOX and the primary sequences of coral, rabbit and soybean LOXs, even though they are all bona fide LOXs. A closer look at the sequences showed that the five important residues (shown in red in the figure) which form the coordinating sphere with the Fe$^{2+}$ atom are conserved in the LOXs being compared. In rabbit LOX, unlike the other LOXs, one histidine residue coordinates with the Fe$^{2+}$ atom instead of the asparagine, and the histidine is shown in blue in fig. 12.

![Fig 12. Primary sequence alignment of *Anabaena* LOX with coral, rabbit and soybean LOX using CLUSTALW (Thompson *et al.* 1994). The conserved residues that coordinate with Fe$^{2+}$ are shown in red and blue.](image-url)
3.2. Prediction of Structure of *Anabaena* LOX

Secondary structure prediction of *Anabaena* LOX was performed using the Advanced Protein Secondary Structure Prediction (APSSP) server (Raghava 2000). The program predicts the secondary structure of a protein from its primary sequence by using data from structures obtained from the PDB database. The results showed striking secondary structure similarity when compared with the structure of prototypic soybean LOX (fig. 13). Though the amino acid sequence is poorly conserved in the *Anabaena* LOX sequence, the tertiary structure and the structure of the active site may well be conserved. So, based on this similarity, an attempt was

Fig 13. Primary sequence alignment of *Anabaena* LOX and soybean LOX with secondary structure elements from the soybean LOX crystal structure and the *Anabaena* LOX predicted secondary structure.
made to predict the structure of the *Anabaena* LOX protein using the crystal structure of prototypic soybean LOX (fig. 14A) as a template. First, the amino terminal membrane binding domain was deleted from soybean LOX since no sequence was found in *Anabaena* LOX that could represent a similar structure. After that, the three regions of soybean LOX catalytic domain that do not align with any part of *Anabaena* LOX were deleted (fig. 14B). As the other predicted secondary structure elements from *Anabaena* LOX align relatively well with the secondary structure elements from the crystal structure of soybean LOX, it was assumed that the structure of *Anabaena* LOX might be like what is shown in fig. 14C. Two different views of the predicted structure are shown in fig. 14D & 14E.

 ![Fig 14. A. Soybean LOX crystal structure B. Soybean LOX Crystal structure with amino terminal domain C. Predicted *Anabaena* structure based on soybean LOX structure and predicted secondary structure elements from *Anabaena* LOX D and E. D and E. Predicted *Anabaena* LOX structure from different angles](image-url)
CHAPTER 4 - EXPRESSION AND PURIFICATION OF LIPOXYGENASE ENZYME FROM *FUSARIUM*

4.1. Introduction

*Fusarium* is a filamentous fungus (Matsuda *et al.* 1978). *Fusarium* is found abundantly in soil and in association with plants. *Fusarium* (fig. 15) belongs to:

Kingdom: *Fungi*
Phylum: *Ascomycota*
Order: *Sordariomycetes*
Family: *Hypocreales*
Genus: *Fusarium*

Though most species of *Fusarium* are known to be harmless (Seaver 1913), and are found abundantly in the environment, some species have been found to produce mycotoxins which can cause diseases in plants and animals by entering the food chain. The main toxins produced by these species are fumonisins and trichothecenes (Papsta *et al.* 2005), and fumonisin is a fatty acid derivative (Apsimon 2001). Since lipoxygenases are known to catalyze the conversion of unsaturated fatty acids to dioxygenated products which are subsequently used to produce chemical deterrents, it can be speculated that lipoxygenase in *Fusarium* is involved with the production of these toxins. Lipoxygenase from *Fusarium oxysporum* has been purified and
crystallized, but the structure has not been solved (Matsuda et al. 1978). The research work done for this thesis was with the LOX enzyme from *Fusarium verticillioides*.

### 4.2. Uniqueness of Lipoxygenase from *Fusarium*

There are two reasons why the LOX protein from the fungus *Fusarium* makes interesting subject for research. Firstly, this is one of the known LOX proteins which exhibits dual specificity. *Fusarium* LOX is a 9/13-LOX (Matsuda et al. 1978). This means that the enzyme can catalyze the dioxygenation of linoleic or linolenic acid at the 9 and the 13 positions (fig. 2). Also, this enzyme shows remarkably high activity when assayed with its natural substrates, linoleic and linolenic acids (Matsuda et al. 1978).

### 4.3. Expression of *Fusarium* LOX in Bacterial Expression System

*Fusarium* LOX was successfully over-expressed using an *E.coli* bacterial expression system. The gene for the protein in pET-17b was obtained from Dr. Alan Brash at Vanderbilt University, Tennessee. The same protocol as used for the expression of *Anabaena* LOX was used for the expression of *Fusarium* LOX.

### 4.4. Purification of *Fusarium* LOX

#### 4.4.1. Ni-NTA Chromatography

The protein was purified using an Amersham Biosciences AKTA FPLC Chromatography system. Since the expressed protein had a histidine tag near the amino terminus, the first step of purification was performed using Ni-NTA chromatography. The 5 ml Ni$^{2+}$ column was
equilibrated with five column volumes of Buffer N (50 mM sodium phosphate pH 8.0, 500 mM NaCl, and 10% glycerol). The supernatant from the cell lysis step was loaded onto the column at 1.0 ml/min. The flow through was collected, and the column was successively washed with Buffer N, Buffer N1 (50 mM sodium phosphate pH 8.0, 1M NaCl, 70 mM glycine, 10% glycerol), Buffer N and Buffer N2 (50 mM sodium phosphate pH 8.0, 500 mM NaCl, 20 mM imidazole, 10% glycerol), until the UV Absorption was below 0.05 AU in each wash. The protein bound to the column was eluted using an imidazole gradient from 20 mM to 200 mM in Buffer N. The 5 ml fractions were collected using a fraction collector, and separated on a 10% SDS-PAGE gel (fig. 16) by electrophoresis to check purity.

Fig 16. 10% SDS-PAGE gel showing purified *Fusarium* LOX after Ni-NTA chromatography
4.4.2. Size Exclusion Chromatography

After Ni-NTA chromatography, the fractions with minimum impurities were pooled, and size exclusion chromatography was used to ensure that all the protein obtained was in the same oligomeric form, as this is one of the primary criteria for obtaining diffraction quality protein crystals. First, the fractions from the Ni-NTA run containing the LOX protein from *Fusarium* were pooled and concentrated by centrifugation in a Centricon tube. The 120 ml Superdex 200 column was equilibrated with one column volume of 20 mM Tris HCl pH 8.0, 150 mM NaCl and 10% glycerol. The protein was loaded on to the column using a 2 ml loop, and eluted with one column volume of the buffer. Fractions of 1.3 ml were collected. A comparison of the elution profile of the protein (fig. 17) with the standard curve for the column showed that the pure protein eluted in the monomeric form. The fractions containing the protein were pooled, concentrated to 10 mg/ml, and stored at -80°C.

![Size exclusion chromatography profile for Fusarium LOX](image)

**Fig 17. Size exclusion chromatography profile for **Fusarium** LOX**
4.5. Activity Assay

The concentrated protein was assayed with linoleic acid, the natural substrate for this enzyme, using an Agilent 8453 spectrophotometer. Seventeen nM Fusarium LOX was assayed with 140 µM linoleic acid to check for activity. The protein was found to be highly active in converting the linoleic acid into the hydroperoxy fatty acid. The formation of the hydroperoxidized product was monitored by UV absorption at 235 nm wavelength (fig. 18), the wavelength at which the hydroperoxide group absorbs. The turnover rate of the protein was calculated to be approximately 73 per second.

![Activity Assay for Fusarium LOX (235nm)](image)

Fig 18. Activity assay for *Fusarium* LOX with linoleic acid. UV Absorption was measured at 235nm, the wavelength of absorption of the hydroperoxide group of the product. The initial product concentration is zero, and the initial amount of product measured in the assay is due to a delay in the collection of the first data point.

4.6. X-Ray Absorption Near the Edge Spectroscopy (XANES)

X-Ray Absorption near the edge Spectroscopy (XANES) experiments were performed at Center for Advanced Microstructures and Devices (CAMD), Baton Rouge, LA, to confirm the presence
of iron in the ferrous state in the protein. The absorption peak observed (fig. 19) confirmed the presence of iron in the *Fusarium* LOX expressed in the lab.

![Energy vs eV graph](image)

**Fig 19.** XANES data collected using *Fusarium* LOX

### 4.7. Theoretical Studies on *Fusarium* LOX

Primary sequence comparison of *Fusarium* LOX and soybean LOX (PDB ID: 1YGE) was performed (fig. 20) using CLUSTALW (Thompson *et al.* 1994). Even though both proteins are LOXs, very little sequence similarity (~20%) was observed between them. A closer look at the conserved residues showed that the five important residues which form the coordinating sphere with the Fe$^{2+}$ atom are conserved in both the enzymes.
Fig 20. Primary sequence alignment of Fusarium LOX and soybean LOX using CLUSTALW (Thompson et al. 1994). The conserved residues that coordinate with Fe$^{2+}$ are shown in red boxes.
5.1. Introduction

The gene for *Fusarium* LOX obtained from Dr. Alan Brash was in the vector pET-17b. pET-17b introduces a T7 tag near the amino terminus of the expressed protein. As the T7 tag might cause a problem in the crystallization of the protein, it was decided to sub-clone the gene sequence in pET-28b, which can be used to retain the histidine tag but lacks the T7 tag. Two new constructs were successfully created in pET-28b, one with the histidine tag, and one without.

5.2. Cloning of *Fusarium* LOX in pET-28b

Two 5' primers were designed. In one the start codon was in an NdeI site, and in the other it was in an NcoI site. The one with the NdeI restriction site was for a construct with histidine tag while the NcoI primer would yield a construct without histidine tag. The 3' primer contained a HindIII site immediately after the stop codon. The primers were ordered from Operon.

FusLOXNde: 5’-CTTCATATGTCTGCAGTTGCCAGTCAC –3’
FusLOXNco: 5’-CTTCCATGGCTGCAGTTGCCAGTCAC –3’
FusLOXHind: 3’-CTTAAGCTTCTAGATTTAAATTGAATGGCAGTG -5’

Each of the primers was made up to 1 μg /μl with TE (10 mM Tris /1 mM EDTA pH 8.0) buffer, and stored at -20°C. The primers were diluted 1:10 in water prior to use. Polymerase chain reaction (PCR) was performed using *Pfu UltraII™* DNA polymerase (Stratagene) according to the manufacturer’s protocol (Table 1).
The PCR products were separated by electrophoresis on a 1% agarose gel, which confirmed the success of the PCR reaction. Next, the DNA was tagged with a poly-adenine tail by 20 mins incubation with dATP and GoTaq® polymerase (Promega), so that the gene could be inserted in the pGEM®T-Easy vector, which has poly-thymine overhangs.

The gene was then inserted into pGEM®T-Easy vector by overnight ligation at 4°C using T4 DNA Ligase. The ligation products were transformed into α-Select competent cells (Bioline), and plated on LB+X-Gal+ampicillin. Six separate white colonies were picked from each plate (FusLOXNde and FusLOXNco) and grown overnight on LB+ampicillin at 37°C. Plasmid DNA was extracted and stored in TE buffer at -20°C. A restriction digestion was performed using EcoRI to confirm the presence of the insert in the pGEM®T-Easy vector, and it was confirmed by the two separate bands seen on the agarose gel, one for the vector and one for the insert. The plasmids were digested with NcoI and HindIII in the case of FusLOXNco and NdeI and HindIII in the case of FusLOXNde, and subjected to electrophoresis through 1.2% agarose. The inserts were excised from the gel, purified and ligated into pET-28b vector digested with the same enzymes. Ligations were transformed into E.coli BL21(DE3) cells and were plated on LB +

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Table 1. Manufacturer’s protocol for PCR using Pfu Ultra II
Kanamycin (30 mg/ml). Individual colonies were picked and grown overnight in LB + kanamycin. Plasmid DNA was prepared and digestion with NdeI or NcoI and HindIII confirmed presence of inserts in all.

5.3. Expression of *Fusarium* LOX (from new constructs) in Bacterial Expression System

*Fusarium* LOX was successfully over-expressed using an *E.coli* bacterial expression system (fig. 21A and 21B). The plasmids, FusLOXNco/pET-28b and FusLOXNde/pET-28b, were prepared under the guidance of Dr. Sue Bartlett. The plasmids were transformed into BL21(DE3) cells (Novagen), and the protein was over-expressed using the same expression protocol that was used to express *Anabaena* LOX. The only difference was that since the gene for this protein was in pET-28b, the antibiotic used was kanamycin instead of ampicillin.

![Fig 21. 10% SDS-PAGE gel showing expression of A> FusLOXNde and B> FusLOXNco](image-url)
5.4. Purification of *Fusarium* LOX

5.4.1. Ni-NTA Chromatography

FusLOXNde protein was purified using an Amersham Biosciences AKTA FPLC Chromatography system. The first step of purification was performed using Ni-NTA chromatography. The 5 ml Ni\(^{2+}\) column was equilibrated with five column volumes of Buffer N (50 mM sodium phosphate pH 8.0, 500 mM NaCl). The supernatant from the cell lysis step was loaded onto the column at 1.0 ml/min. The flow through was collected, and the column was successively washed with Buffer N, Buffer N1 (50 mM sodium phosphate pH 8.0, 1 M NaCl, 70 mM glycine), Buffer N and Buffer N2 (50 mM sodium phosphate pH 8.0, 500 mM NaCl, 20 mM imidazole), until the UV Absorption was below 0.05 AU in each wash. The protein bound to the column was eluted using an imidazole gradient from 20 mM to 200 mM in Buffer N. The 5 ml fractions were collected using a fraction collector, and separated by electrophoresis on a 10% SDS-PAGE gel (fig. 22) to check purity.

**Fig 22.** 10% SDS-PAGE gel showing purified FusLOXNde after Ni-NTA chromatography
5.4.2. Size Exclusion Chromatography

After Ni-NTA chromatography, size exclusion chromatography was used to ensure that all the protein obtained was in the same oligomeric form, as this is one of the primary criteria for obtaining diffraction quality protein crystals. First, the fractions from the Ni-NTA run containing the LOX protein with minimum impurities were pooled and concentrated by centrifugation in a centricon tube. The 120 ml Superdex 200 column was equilibrated with one column volume of 20 mM Tris HCl pH 8.0 and 150 mM NaCl. The protein was loaded on to the column using a 2 ml loop, and eluted with one column volume of the buffer. Fractions of 1.3 ml were collected. A comparison of the elution profile of the protein (fig. 24) with

![Fig 23. 10% SDS-PAGE gel showing purified FusLOXNde after size exclusion](image)

![Fig 24. Size exclusion elution profile of FusLOXNde (fraction numbers in red)](image)
the standard curve for the column showed that while some amount of the protein eluted as an aggregate, most of the pure protein eluted in the monomeric form. The protein in fractions 52 to 55 was pooled, concentrated to 8 mg/ml, and stored at -80°C.

5.5. Activity Assay

The concentrated protein was assayed with linoleic acid, the natural substrate for this enzyme, using an Agilent 8453 spectrophotometer. Seventeen nM protein was assayed with 140 µM linoleic acid in 700 µl of buffer to check for activity. The protein was found to be highly active in converting the linoleic acid into the dioxygenated product, the formation of which was monitored by UV absorption at 235 nm wavelength (fig. 25). The turnover number of the protein was calculated to be around 125 per second.

![Activity Assay for Fusarium LOX Nde (235nm)](image)

**Fig 25.** Activity assay for *Fusarium* LOX Nde protein with linoleic acid. UV Absorption was measured at 235nm, the wavelength of absorption of the hydroperoxide group in the product. The initial product concentration is zero, and the initial amount of product measured in the assay is due to a delay in the collection of the first data point.
6.1. Introduction

The lipoxygenase protein from *Anabaena* exists as part of a naturally occurring fusion protein with allene oxide synthase (AOS), a heme containing enzyme which uses the hydroperoxide product of LOX as a substrate (Tijet et al. 2002). Lipoxygenase hydroperoxidizes the unsaturated fatty acid, and passes the product on to AOS, which converts it to allene oxide.

6.2. Expression of Anabaena Fusion Protein in Bacterial Expression System

*Anabaena* fusion protein was successfully over-expressed using an *E.coli* bacterial expression system. The gene for the protein in pET-17b was obtained from Dr. Alan Brash at Vanderbilt University, Tennessee. The plasmid was transformed into Rosetta(DE3) cells (Novagen). Rosetta(DE3) was used because of the presence of rare codons in the gene sequence for this protein.

6.2.1 Cultures

Overexpression of this protein was obtained by using a step-up procedure (fig. 26). The culture was started with 1 ml LB (Luria Broth) in 15 ml Falcon tubes with one bacterial colony from the transformation plate at 37°C for 4 hours. A volume of 200 µl
of the starter culture was then transferred to 10 ml of TB in 50 ml conical flasks at 37°C. After 3 hours, the 10 ml cultures were transferred to 100 ml TB in 500 ml flasks at 28°C. Overexpression was achieved in 18 hours with shaking at 200 rpm.

**6.2.2 Lysing the Cells**

The cells were spun down at 7000 rpm for 45 minutes. The supernatant was discarded and the pellets were frozen at -80°C. The pellets were then thawed and transferred to a 150 ml beaker. Bugbuster™ (Novagen) was added at 5 ml per 1 gm of pellet to lyse the cells. The protease inhibitor pepstatin and DNAse were also added with Bugbuster™ (Novagen). The pellets were dissolved in the lysis buffer, and let stand at room temperature for 30 mins. The cells were then sonicated on ice. The lysate was transferred to centrifuge tubes and centrifuged at 46000g for 30 mins. The supernatant was transferred to a 150 ml beaker.

**6.3. Purification of Anabaena Fusion Protein Using Ni-NTA Chromatography**

The protein was purified using an Amersham Biosciences AKTA FPLC Chromatography system. The first step of purification was performed using Ni-NTA chromatography. The 5 ml Ni²⁺ column was equilibrated with five column volumes of Buffer N (50 mM sodium phosphate pH 8.0, 500 mM NaCl, and 10% glycerol). The supernatant from the cell lysis step was loaded onto the column at 1.0 ml/min. The flow through was collected, and the column was successively washed with Buffer N, Buffer N1 (50 mM sodium phosphate pH 8.0, 1 M NaCl, 70 mM glycine, 10% glycerol), Buffer N and Buffer N2 (50 mM sodium phosphate pH 8.0, 500 mM NaCl, 20 mM imidazole, 10% glycerol), until the UV Absorption was below 0.05 AU in each wash. The protein bound to the column was eluted using an imidazole gradient from 20 mM to 200 mM in
Buffer N. The 5 ml fractions (F1, F2 and F3) were collected using a fraction collector, and separated by gel electrophoresis on a 10% SDS-PAGE gel (fig. 27) to check purity.

Fig 27. 10% SDS-PAGE gel showing purified *Anabaena* fusion protein after Ni-NTA chromatography

6.4. Assay for Presence of Heme

As the AOS in the fusion protein contains heme, UV Absorption was measured at 406 nm, the wavelength at which heme absorbs (McCoubrey *et al.* 1997), and the absorption peak at 406 nm (fig. 28) confirmed the presence of heme in the expressed protein.

Fig 28. UV-Absorption to check the presence of heme. The reading was taken at 70 times dilution of the protein.
CONCLUSIONS

Lipoxygenase (LOX) is a regio-stereo specific, non-heme, non-sulfur, iron containing enzyme found both in plants and in animals. It has been found to be associated with many diseases, both in plants in animals. It might be possible to prevent these diseases if the mechanism of action of these proteins is better understood. This makes lipoxygenases a good target protein for biochemical studies.

Anabaena LOX, the smallest known LOX, was over-expressed using an E. coli expression system. The enzyme was successfully purified using Ni-NTA chromatography and size exclusion chromatography. The over-expressed protein migrated like a dimer in the latter. The enzyme was assayed with linoleic acid, the natural substrate for the enzyme, and was shown to be active. Theoretical studies were performed to predict a plausible structure for this protein based on information from the crystal structure of soybean LOX. Future work with this protein should be setting up crystallization experiments with the purified protein to obtain diffraction quality crystals. Once the structure is obtained, it might be helpful in understanding how Anabaena LOX, despite its small size and minimal similarity to other known LOXs, still shows robust activity.

Fusarium LOX, a LOX protein which demonstrates dual specificity and high activity, was successfully over-expressed using an E.coli expression system. The enzyme containing the histidine tag and the T7 tag, was successfully purified using Ni-NTA chromatography and size exclusion chromatography. The over-expressed protein was monomeric. The enzyme was assayed with linoleic acid, the natural substrate for the enzyme, and was active. Two new constructs for this protein were designed using the pET-28b expression vector, one with and one
without the histidine tag. Both of the constructs were used to over-express the protein. The over-expressed protein from the construct containing the histidine tag was purified using Ni-NTA chromatography and size exclusion chromatography. Theoretical studies were performed to check the similarity of this protein with soybean LOX. Future work with this protein should be setting up crystallization experiments with the purified protein, both with and without the T7 tag, to obtain diffraction quality crystals. Once the structure is obtained, it might be helpful in improving our understanding of the mechanism of action and dual specificity of lipoxygenases.

The *Anabaena* fusion protein, the naturally occurring LOX and AOS fusion protein, was expressed in Rosetta(DE3) cells. Purification was not performed due to low expression of this protein. Future work with this protein should be tweaking the expression conditions (temperature, media etc.) to obtain better yield of the protein. After the protein is purified, crystallization trials should be performed to obtain diffraction quality crystals. The crystal structure should be useful in improving our understanding the mechanism of action of the smallest known LOX, as well as that of LOX enzymes which naturally exist in the fusion form with AOS.

Though structural information is available for lipoxygenase proteins from a few organisms, a complete understanding of the mechanism of action of this family of enzymes has not still been attained. The crystal structures of LOX protein from *Anabaena* and *Fusarium* might assist in improving the understanding of the reaction mechanism of lipoxygenases.
REFERENCES


APPENDIX A - ANABAENA LOX

(computed using ProtParam)(Gasteiger E. 2005)

Sequence
GVSGALVHYF GSI VRAERTQ YLYGSKDDLPLGKPVYFPLPV TEIPSKRFLF LLEKYNFLTD
NSYPESDGEHD KI EALVSAMP TTALDLAVGT TDPTDIPDSY FLERRLNGYNTLDPGAI RESSQ
EGWTHELTHN LAKYDIKGPL HIFPFDVQCRLFVDKQNGVKLHSI KIDDIHEITPCQEQMVYA
KRTYLPQAEFL SQELKLHLAR CHFNI EIVYM AIP KRRRLAPTH PVRAFNPHLEGFI NASSA
VPKI I GSTGF I PI ASMLTVSISVDMKNELSKLSYMNPISPIDPRDPGDIFTPAATAYW
ELLNNYVEQG LLQPFEDELR TEVNAIVQDE LFAMKERSLYSDQPPKYYDSLKSSLMY
1 YHSFLHS VANIFQYDDA GNPNVHSMDGRSQDYIQTQDKIRFSQRSLTELSSYRNYV
VAVYGSDLKK QLI REKSSIL EPIGLPLEDLMMISNI

Number of amino acids: 455
Molecular weight: 51916.9
Theoretical pI: 5.41

Amino acid composition:

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Pro (P)  28   6.2%
Ser (S)  38   8.4%
Thr (T)  20   4.4%
Trp (W)  6   1.3%
Tyr (Y)  24   5.3%
Val (V)  22   4.8%

Total number of negatively charged residues (Asp + Glu): 57
Total number of positively charged residues (Arg + Lys): 42
Total number of atoms: 7278

**Extinction coefficients:**

Extinction coefficients are in units of M⁻¹ cm⁻¹, at 280 nm measured in water.

Ext. coefficient  68885
Abs 0.1% (=1 g/l)  1.327, assuming ALL Cys residues appear as half cystines

Ext. coefficient  68760
Abs 0.1% (=1 g/l)  1.324, assuming NO Cys residues appear as half cystines

**Instability index:**

The instability index (II) is computed to be 38.53
This classifies the protein as stable.
**APPENDIX B - *FUSARIUM LOX***

*(computed using ProtParam)(Gasteiger E. 2005)*

Sequence

```
M5AVASHVP  PRSKLDSI LS  SGLEHNI DHD  PLEVWDKGVF  LNELLKQGI A  LSTNENGTLD
GELVADEGLK  KG5YKGTLRA  LTEI YSI LED  AAVSHFDKRG  YEP1 FPVKRE  LDLKKR1 YQW
SDGTGDYPHP  LKVDGNDEAN  LPADERQSKP  GSARSEGVGQ  I FDMQETAFV  SKI AQAVSF1
I PKDI DHENT  PYKGPTLADV  EKFNQAFPKP  TDGQASNQDN  LNKAA1 DIKMG  RNI GEYDVMVY
SDARFAQQHF  SGVNPS1ET  ASQDKI KEYI  SEAOKQGLDK  VKAI LEDKD1  IL I QDYSYFR
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ATNRI I PEGE  LLYEI LSPHW  FRTLSSLNAAA  RKLLVPGVI A  RI AGFQTPSP  SLDKFGNN1AF
KLI DM6YKNF  NFQOKYI PND  LKKRGFDI KG  DKSQ1KYKNYP  YANDM11LVG  I I RNFKVTVI
ESQYTSDHV  QKDPYI GGMNC  KEI QTNGQI P  TFPTI TTVEQ  LI DAVTMCI H  TASPQHTAVN
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LLSYKVQQDY  NLI TYAKSLY  NVKNNRT1 TE  NTKFN1CT1 K  KAAADFYSHL  KSAGVEFENY
SKGQTAGTVE  YPVLQPETTA  I SI LI
```

Number of amino acids: 745

Molecular weight: 83635.2

Theoretical pI: 5.46

**Amino acid composition:**

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Count</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala (A)</td>
<td>61</td>
<td>8.2%</td>
</tr>
<tr>
<td>Arg (R)</td>
<td>23</td>
<td>3.1%</td>
</tr>
<tr>
<td>Asn (N)</td>
<td>36</td>
<td>4.8%</td>
</tr>
<tr>
<td>Asp (D)</td>
<td>56</td>
<td>7.5%</td>
</tr>
</tbody>
</table>
Cys (C)  5     0.7%
Gln (Q)  33   4.4%
Glu (E)  44   5.9%
Gly (G)  44   5.9%
His (H)  17   2.3%
Ile (I)  53   7.1%
Leu (L)  61   8.2%
Lys (K)  58   7.8%
Met (M)  7    0.9%
Phe (F)  27   3.6%
Pro (P)  38   5.1%
Ser (S)  45   6.0%
Thr (T)  52   7.0%
Trp (W)  12   1.6%
Tyr (Y)  36   4.8%
Val (V)  37   5.0%

Total number of negatively charged residues (Asp + Glu): 100
Total number of positively charged residues (Arg + Lys): 81
Total number of atoms: 11725

**Extinction coefficients:**

Extinction coefficients are in units of M$^{-1}$ cm$^{-1}$, at 280 nm measured in water.

Ext. coefficient  119890
Abs 0.1% (=1 g/l)  1.433, assuming ALL Cys residues appear as half cystines

Ext. coefficient  119640
Abs 0.1% (=1 g/l)  1.430, assuming NO Cys residues appear as half cystines

**Instability index:**

The instability index (II) is computed to be 32.72

This classifies the protein as stable.
APPENDIX C - ANABAENA FUSION

(computed using ProtParam)(Gasteiger E. 2005)

Sequence

```
MDLNTYLKL 
SHSLFTPGK 
VFGPTARLY 
SKKSRYARF 
NIVVEYLQA 
HYDLKLPNSY 
I PSKRFLL 
ERRLINGYNPG 
I K I DDHEITP 
RAF INPHLEG 
LPRDI PGDLF 
GDPQPKYDSS 
RFSQRSRTW 
```

```
NLLDSESQKI 
VGGAKDIALI 
KSDLASQL 
RLLNPQNSTE 
FRSI EDVAVD 
SVDDFASLGV 
EKYNFLTDNS 
AI RESSQEQG 
COEQMQYAKR 
LI FI NSSAVP 
TPAATAYWEL 
ELKSLLMYII 
LSSI RYNSSVA 
MLELQAMFSA 
NGRGSARI G 
KR YAVEALR 
GGLLDSSVEI 
CSNI WDPNTY 
SGALVHYFGS 
YPSDGEHDKI 
WITHELTHNL 
TABQAEFLSQ 
LI I GSTGFI P 
LNNYVEQGLL 
YHSSFLHSWA 
VYGSDLLKQL 
AGLALRGRGT 
NRGRGSAIRI G 
RRYAVEALR 
GPRLVLPRKR 
P WILDIAAI VL 
I VIRAERTQYL 
YGSKDDLPGBK 
EALVSAMPTT 
ELKHLHLARCH 
F  I VIRAERTQYL 
LALAVGGITTD 
PDFVQCRLFV 
ALDLAVGTTD 
DKQNGVKLHS 
P TDI PSYFL 
PTDI PSYFL 
QDESENDYY 
GKDSMLASGSL 
RLEAVTAPPTT 
GDTREKNYLR 
ALDLAVGTTD 
NEFRQRLTDDG 
Q DESENDYY 
LLDLVLINTGE 
NIGDDLSKPR 
HTQLCYEWWD 
LTVLHSSDVP 
HTDGIIVKGN 
LTVLHSSDVP 
```

```
Number of amino acids: 773
Molecular weight: 87860.3
Theoretical pI: 5.50

Amino acid composition:

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Count</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala (A)</td>
<td>45</td>
<td>5.8%</td>
</tr>
<tr>
<td>Arg (R)</td>
<td>40</td>
<td>5.2%</td>
</tr>
<tr>
<td>Asn (N)</td>
<td>39</td>
<td>5.0%</td>
</tr>
<tr>
<td>Asp (D)</td>
<td>56</td>
<td>7.2%</td>
</tr>
<tr>
<td>Cys (C)</td>
<td>5</td>
<td>0.6%</td>
</tr>
<tr>
<td>Gln (Q)</td>
<td>33</td>
<td>4.3%</td>
</tr>
<tr>
<td>Glu (E)</td>
<td>43</td>
<td>5.6%</td>
</tr>
<tr>
<td>Gly (G)</td>
<td>46</td>
<td>6.0%</td>
</tr>
</tbody>
</table>
```
His (H)  22   2.8%
Ile (I)  46   6.0%
Leu (L)  92  11.9%
Lys (K)  37   4.8%
Met (M)  13   1.7%
Phe (F)  32   4.1%
Pro (P)  40  5.2%
Ser (S)  62   8.0%
Thr (T)  34   4.4%
Trp (W)  9   1.2%
Tyr (Y)  40  5.2%
Val (V)  39  5.0%

Total number of negatively charged residues (Asp + Glu): 99
Total number of positively charged residues (Arg + Lys): 77
Total number of atoms: 12305

**Extinction coefficients:**

Extinction coefficients are in units of $\text{M}\cdot\text{cm}^{-1}$, at 280 nm measured in water.

<table>
<thead>
<tr>
<th>Ext. coefficient</th>
<th>Abs 0.1% (=1 g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>109350</td>
<td>1.245, assuming ALL Cys residues appear as half cystines</td>
</tr>
<tr>
<td>109100</td>
<td>1.242, assuming NO Cys residues appear as half cystines</td>
</tr>
</tbody>
</table>

The amino terminal of the sequence considered is M (Met).
The instability index (II) is computed to be 37.10

This classifies the protein as stable.
Pratik Dhar was born in Kolkata, West Bengal, India, on December 14, 1978. He received his Master of Science (MSc.) and Bachelor of Science (BSc.) degrees from Indian Institute of Technology, Kharagpur, India. After spending two years working for a pharmaceutical company, Aurigene Discovery Technologies in Bangalore, India, Pratik joined Louisiana State University to work on X-ray crystallography under the guidance of Dr. Marcia Newcomer. Pratik wishes to pursue higher studies in the field of management after his graduation.