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Biophysical study of the SH2 domain of human tensin

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BIOPHYSICAL STUDY OF THE SH2 DOMAIN OF HUMAN TENSIN

by

Yogesh Mohan Kulkarni, BE

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We hereby recommend that the dissertation prepared under our supervision by Kulkarni Yogesh Mohan

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ABSTRACT

Cell migration is a key characteristic of embryogenesis, inflammation, wound healing, tumor cell metastasis and a broad range of other normal and pathological processes. Accumulating evidence suggests that the protein tensin provides a physical link between transmembrane receptors, proteins commonly associated with signal transduction, and the actin cytoskeleton. Adhesion involves three broad classes of macromolecules: ExtraCellular Matrix (ECM) molecules, transmembrane adhesion receptors, and intracellular adhesion plaque proteins. Tensin is particularly enriched in Fibrillar Adhesions (FA's), though it is also present to a modest extent in Focal Contacts (FC's). Src Homology 2 (SH2) domains function in the transmission of molecular signals that start at the cell surface, pass through the plasma membrane, and engage the inner workings of the cell. SH2 domains carry out their function by binding with high affinity to phosphotyrosine-containing protein targets in a sequence-specific and largely phosphorylation-dependent manner. In this work, SH2 domain of protein tensin, a component of cell-substrate contacts with close connections to cancer is used as an investigative tool, to view the scientific problem from the perspective of a biophysicist.

Recombinant DNA technology was used to clone the SH2 gene and overexpress the recombinant SH2 domain. Molecular biology techniques like Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE), immunblot analysis were used to identify and characterize the protein molecular mass. Circular Dichroism (CD)

spectroscopy was used to determine the secondary structure of the protein and to demonstrate that the SH2 domain folds into a compact, stable molecule. Chemical denaturation and heat denaturation studies were done using Differential Scanning Calorimetry (DSC) to determine the thermostability of the SH2 domain. These studies reveal that the tensin-SH2 domain is highly stable as compared with some other known SH2 domains. Detailed knowledge of the structure and function of tensin will accelerate acquisition of more detailed knowledge of other focal adhesion components, advancing the development of molecular models of cell attachment and migration. Such knowledge is of interest to basic science as well as medicine. Moreover, it could also provide a model for nanotechnology development, providing inspiration for the design of novel types of molecular recognition and functionality, and materials design and fabrication.

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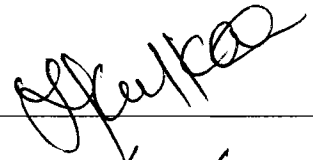
Author 

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DEDICATION

Dedicated to my wife, Jhelum, who offered me unconditional love and support throughout the course of this work, and to the fulfillment of our dreams.

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CHAPTER 1

INTRODUCTION

1.1. Biology

1.1.1. Cell Migration

Cell migration is a key characteristic of embryogenesis, inflammation, wound healing, tumor cell metastasis and a broad range of other normal and pathological processes (Douglas et al., 1996; Alberts et al., 1994). Distinctive features of cell migration are polarization of cell shape, extension of the membrane, formation of cell-substratum attachments, generation of contractile force and traction, and release from attachment (Douglas et al., 1996). Migration is influenced, regulated, or directly switched on or off by changes in growth factor concentration, ExtraCellular Matrix (ECM) receptor binding avidity, and receptor-cytoskeleton interactions (Huttenlocher et al., 1995; Duband et al., 1991; Kassner et al., 1995). Accumulating evidence suggests that the protein tensin provides a physical link between transmembrane receptors, proteins commonly associated with signal transduction, and the actin cytoskeleton. Tensin was first identified as a barbed-end actin capping protein immunolocalized at various adherens junctions and Z-lines of muscle (Wilkins et al., 1986).

1.1.2. Cell Adhesion

Adhesion involves three broad classes of macromolecules: extracellular matrix (ECM) molecules, transmembrane adhesion receptors, and intracellular adhesion plaque proteins. Cell adhesion plays a crucial part in regulating embryonic development, tissue architecture, tissue function, and transmembrane signaling (Abercrombie et al., 1975; Burridge et al., 1988; Walton et al., 1993; Jockush et al., 1995; Craig et al., 1996). Adhesion of any type of adherent cell is characterized by three common features: mediation by transmembrane ECM receptors called integrin, involvement of the actin cytoskeleton, *i.e.* microfilaments, and involvement of a complex assembly of anchoring proteins (Critchley, 2000; Jockusch et al., 1995; Yamada and Geiger, 1997). Integrins enable the cell to form close contacts with the ECM. Adhesion Complexes (AC's) are structurally and functionally diverse (Zamir et al., 1999, 2001). Two types of AC's seen in cultured fibroblasts are Focal Contacts (FC's) and Fibrillar Adhesions (FA's). FC's are found mainly at the cell periphery, and FA's more in the center of the cell. The detailed molecular compositions of these structures are quite different, as are their functions and dynamics. Tensin is particularly enriched in FA's, though it is also present to a modest extent in FC's (Zamir et al., 2000). Figure 1.1 shows a schematic diagram of an adhesion complex.

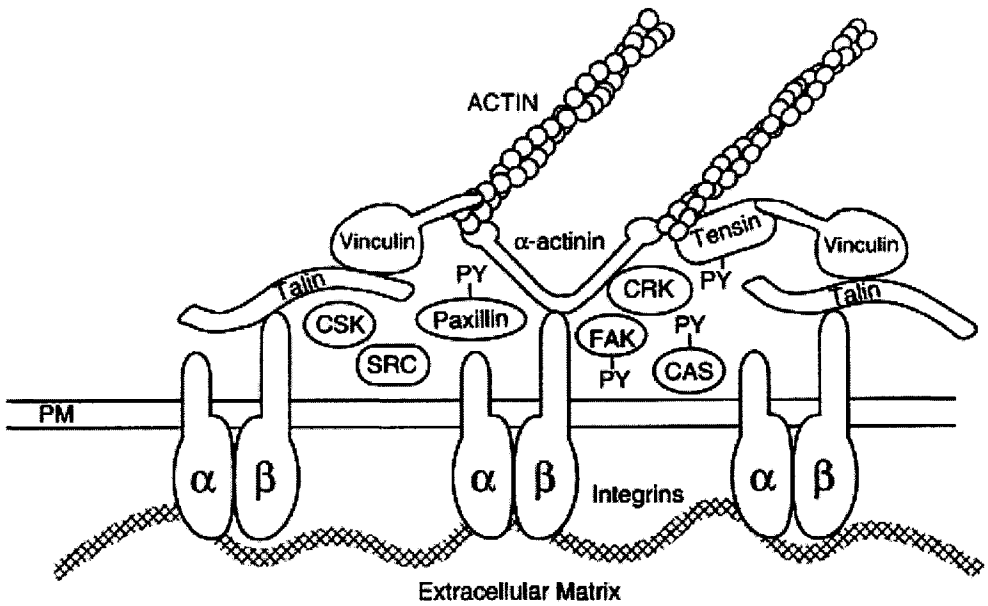

Figure 1.1. Interactions Between the Cytoplasmic Tails of Integrin and Cytoskeletal Proteins Provide a Physical Link Between the Actin Cytoskeleton and the ECM. Vuori, K. (1998). Journal of Membrane Biology **165:** 191-199.

AC's have at least two significant cellular functions: to transmit force or tension at adhesion sites and to serve as signaling centers from which numerous intracellular pathways emanate. The former helps a cell maintain strong attachments to the underlying ECM, and the latter plays a role in the regulation of cell growth and gene expression (Jockush et al., 1995; Abercrombie et al., 1975; Burridge et al., 1988). AC's have been identified in various cell types of cellular structures: living and fixed fibroblasts cultured *in vitro* on glass or plastic, myotendinous Z-junctions of skeletal muscle, adherent epithelial and endothelial cells, activated blood platelets, and structures called podosomes (Jockush et al., 1995; Abercrombie et al., 1975; Burridge et al., 1988).

AC's are dynamic structures that can be assembled, dispersed, and recycled as cells migrate or enter into mitosis. Two stages in AC formation and maturation have

been identified: formation of initial or immature focal adhesions (Bershadsky et al., 1985; Izzard, 1998; Izzard and Lochner, 1980; Izzard and Izzard, 1987), also called focal complexes, and maturation of focal adhesions (Bershadsky et al., 1985). Alteration of the structure and composition of AC's during cell migration is thought to play a role in cell attachment and detachment. Large-scale reorganization of AC's during the cell cycle or neoplastic transformation correlates with loss of adhesion and concomitant morphological changes. Aberrant changes in the activities of protein kinases and phosphatases at AC's may lead to a number of diseases, including cancer (Ishida et al., 1995). AC's thus may be important for tumor suppression.

There are two general patterns of ECM reorganization in cultured fibroblasts. In one, tensin-containing FC's move slowly, along the cell margin. In the other, dynamic tensin-rich FA's are found closer to the cell center. FA's can also translocate laterally, but they contain little phosphotyrosine (pTyr or pY). FA's are described as elongated or beaded, mirroring association with fibronectin fibrils. They are enriched in $\alpha_5\beta_1$ integrin and tensin (Zamir et al., 2000). They are found $\leq$100 nm from the substrate; interact with fibronectin fibrils, migrate slowly, and play a role in assembly of the fibronectin matrix. Geiger et al. (1995) have proposed that FA formation depends on ECM reorganization and segregation of cellular components from FC's. Studies of the ECM have shown that in primary fibroblasts, $\alpha_5\beta_1$ integrin associates with fibronectin fibrils, forming FA sites containing an enriched quantity of tensin but a low level of other common FC elements, e.g. paxillin, vinculin, and tyrosine-phosphorylated proteins (Zamir et al., 2000). Fibronectin promotes formation of FC's when covalently linked to the substrate, indicating that cytoskeletal organization and phosphorylation at FC's

depends on physical properties of the ECM (Zamir et al., 2000). Other details of the complex formed among integrins and their extracellular ligands and appropriate cytoplasmic anchor proteins may regulate the local tension of a FC, activate tyrosine phosphorylation, and promote formation of various types of matrix adhesion in fibroblasts (Zamir et al., 2000).

The transmembrane receptor molecules called integrins have a short cytoplasmic tail region apparently devoid of enzymatic activity. The interaction of integrins with their ligands enables cell adhesion. This interaction may trigger signaling responses but does not generally induce the formation of focal complexes. Integrin clustering may induce association with intracellular scaffolding proteins that play a key role in signaling. A focal complex is apparently a nascent adhesion site, the transformation depending on appropriate external stimuli or intracellular signaling. The assembly of focal adhesions is regulated by the GTP-binding protein Rho. Rho stimulates contractility which, in cells tightly adherent to the substrate, generates isometric tension. This tension leads to the bundling of actin filaments and the aggregation of integrins in the plane of the membrane. The aggregation of integrins activates the focal adhesion kinase (FAK) and leads to the assembly of a multicomponent signaling complex (Burridge and Chrzanowska-Wodnicka, 1996).The structural dynamics of a focal adhesions are governed by heat exchange with the local environment and changes in rate constants regulated by phosphorylation and other types of adhesion promoting stimuli.

FA assembly is triggered by ligation of integrins by the ECM. This process includes activation of a cascade of intracellular signaling events involving tyrosine kinases and GTPase Rho–A, and phosphorylation of cytoskeletal substrates which results

in cell migration, a highly-integrated macroscopic biological process. FA's appear to develop from one pole of FC's. The FA translocation process depends on an intact microfilament system and active myosin II.

FC formation requires integrins and other membrane-associated proteins being directed rapidly toward the cell periphery, including the leading edge and integrin clustering. These proteins tend to remain in place after this clustering. Proteins involving multiple binding domains enable molecular interconnections to be enhanced, modified, or linked to signaling pathways. The physical properties of these interconnections, such as their response to and ability to transmit mechanical forces, are almost certainly governed by signal-induced chemical modifications and are linked to be a key aspect of regulation of migration. Tensin is recruited early in the process of FC assembly (Miyamoto et al., 1995).

Tensin-containing fibrils emerge from the medial ends of FC's and translocate towards the cell center. Such short tensin structures can merge with each other to form FA's, containing little or no paxillin, vinculin, and FAK or pTyr.

1.1.3. Protein Phosphorylation in Cell Adhesion

Reversible phosphorylation of proteins is a ubiquitous mechanism of control of intracellular signal transduction pathways. Phosphorylation can be triggered by extracellular effectors such as hormones, mitogens, and cytokines, and by nerve impulses. Such processes as metabolism, gene expression, cell division, differentiation, development, transport, and locomotion are regulated at least in part by the phosphorylation and resultant activation or inactivation of proteins in critical biochemical pathways. Protein phosphorylation thus constitutes the molecular basis of control of

numerous complex physiological events within cells. In some cases control is achieved by phosphorylation on a specific amino acid; the assembly of multi-protein complexes can be directly regulated by the recruitment of phospho-specific binding modules. The level of tyrosine phosphorylation of a given protein *in vivo* is regulated by the opposing actions of Protein Tyrosine Kinases (PTK's), Protein Tyrosine Phosphatases (PTP's), and Dual-Specificity Phosphatases (DSP's). PTP's and in some cases DSP's are hydrolytic enzymes that catalyze removal of the phosphoryl group of a specific phosphotyrosine, phosphoserine (pSer) or phosphothreonine (pThr) from a specific substrate macromolecule.

Formation of adhesive structures in cells is strongly correlated with covalent modification of proteins by tyrosine phosphorylation. Various cytoskeleton-associated proteins are phosphorylated on tyrosine on cell adhesion to a substratum or extension of a filopodium. The most prominent and best characterized of these phosphoproteins are FAK, paxillin, and tensin (Otey et al., 1993; Pavalko et al., 1993, Schaller et al., 1995, Guan et al., 1991; Kornberg et al., 1991; Burridge et al., 1992; Bockholt and Burridge, 1993). These proteins are key players in the formation of FC's, and they are the earliest adhesion-related proteins to undergo phosphorylation (Pfaff et al., 1998). v-Src induced tyrosine phosphorylation on tensin and other FC proteins is reduced when cell-substratum adhesion is lost (Sabe et al., 1997).

FAK, a particularly interesting and well-studied component of FC's, binds talin and vinculin via its Carboxyl-terminus (C-terminus), and the β subunit of integrin by its Amino-terminus (N-terminus). Once bound to other focal adhesion components, FAK autophosphorylates on Tyr 397 and binds the SH2 domain of Src. FAK phosphorylates

numerous proteins on different tyrosine residues. In addition to an SH2 site, FAK also has binding sites for structural proteins such as paxillin, talin, and integrin (Pavalko et al., 1993, Calderwood et al., 1999, Hannigan et al., 1996). Tensin, known to become phosphorylated during FA assembly, may be a substrate of FAK. Tensin could also be a binding partner of Src. Recruitment of Src to the developing FA may be responsible for recruitment of tensin; phosphorylation of tensin may be involved in the regulation of a FA. Another key point of FAK is self-regulation through independent expression of a portion of the FAK gene (Richardson et al., 1996).

Inhibitors of tyrosine phosphorylation inhibit phosphorylation of FC components and correspondingly diminish the ability of a cell to migrate or spread (Loo et al., 1998). Phosphorylated tyrosine residues in FAK, paxillin, tensin and other FC proteins, along with other binding sites in these proteins, are likely play key roles in the assembly of FC's.

This discussion on protein phosphorylation of FA proteins is particularly relevant to a complete description of tensin structure and function, because amino acid sequence comparisons have revealed that tensin has several phosphotyrosinyl recognition modules. Fibroblast tensin is phosphorylated on tyrosine, serine, and threonine, with tyrosine phosphorylation increasing on oncogenic transformation and growth factor stimulation (Davis et al., 1991).

1.1.4. Tensin

1.1.4.1. Introduction

The large 220 kDa, cytoskeleton-associated docking protein tensin was first found in adhesive junctions of animal cells (Wilkins et al., 1986), including Z lines in skeletal

muscle. Tensin derived its name from its putative role in anchoring actin filaments to focal adhesion sites. Thus it maintains tension in the focal adhesion sites and enables cell migration. It is recruited early in the development of cell-substrate contacts (Miyamoto et al., 1995), where it binds the cytoplasmic domain of integrin β_1 (Calderwood et al., 2003) and caps the barbed ends of filamentous actin (Chuang et al., 1995). The N terminus of tensin is a close homolog of a protein with phosphatase homology and tensin homology (PTEN) (Figure 1.3), known to be an established tumor suppressor (Li et al., 1997). A model of tensin in focal adhesions is shown in Figure 1.2.

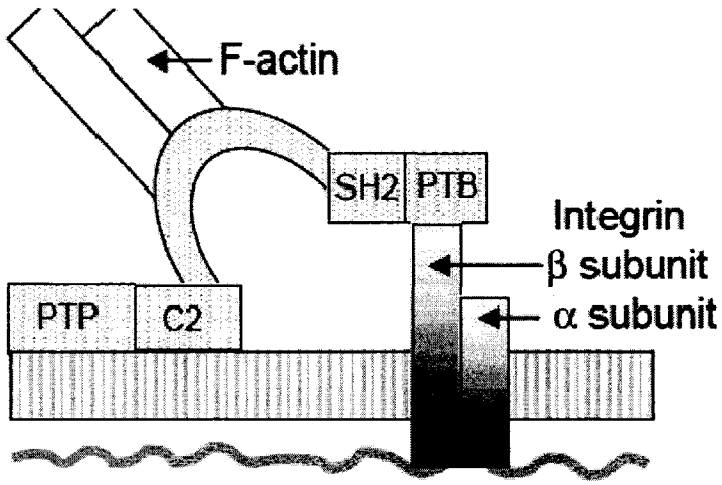

Figure 1.2. Molecular Model of Interaction of Tensin with Other Focal Adhesion Components, Including the Plasma Membrane.

Tensin is found in numerous eukaryotic organisms, including humans and nematode worms. The latter have but *c.* 1,000 cells at adulthood. In *H. sapiens* tensin has been mapped to chromosome 2 (locus *q*35-36). A second transcript of this gene has been described, but its full-length nature has not been determined (Lo et al., 1994a). Tensin is a substrate of calpain II, an FA protease involved in the assembly and disassembly of FC's. Some biochemical properties of tensin are given in Table 1.1.

Table 1.1. Synopses of Properties of Tensin

Property	References
Localized at:	
Focal adhesion sites in cultured fibroblasts	Wilkins and Lin (1986)
Dense plaques in smooth muscle	North et al., (1993)
Neuromuscular junctions, myotendinous junctions sarcolemma of skeletal muscle	Bockholt et al., (1992), North et al., (1993)
Present in low abundance in cultured fibroblasts and other cell types	Wilkins and Lin (1986)
Highly sensitive to proteolysis	Wilkins and Lin (1986)
Difficult to purify	Wilkins and Lin (1986)
Recruited to focal adhesion sites by $\alpha_5\beta_1$ integrin aggregation	Miyamoto et al., (1995), Pankov et al., (2000)
Newly expressed (along with FAK) in granulation tissue myofibroblasts in a type of focal adhesion involving extracellular fibronectin and stress fibers that contain α-smooth muscle α-actin	Dugina *et al.*, (2001)
Normal muscle development in tensin -/- mouse	Lo et al., (1997)
Not necessary for embryogenesis in mice, but possibly required for normal renal function in adults	Lo et al., (1997)
Phosphorylated on serine and threonine	Davis et al., (1991)
Increased phosphorylation on tyrosine from:	
Spreading of fibroblasts following attachment to ECM	Bockholt and Burridge (1993)
Treatment of certain cell types with platelet-derived growth factor, thrombin, or angiotensin	Jiang et al., (1996), Ishida et al., (1999)
Transformation of certain cell types with *v-src* or *bcr/abl*	Davis et al., (1991), Salgia et al., (1995)
Phosphorylated by protein kinase C, cdc2 kinase, FAK, epidermal growth factor receptor, and Src *in vitro*	Lo et al., (1994b)
Middle region of polypeptide inhibits actin polymerization by capping with the barbed end of filaments	Chuang et al., (1995), Lo et al., (1994c)
May also cross-link actin filaments	Chuang et al., (1995), Lo et al., (1994c)
Substrate of calpain II, a calcium-regulated focal adhesion protease	Chen et al., (2000)
Contains large number of PEST regions	Chuang et al., (1995)
SH2 domain identified near C-terminus	Davis et al., (1991)

SH2 domain can interact with certain tyrosine-phosphorylated proteins, notably PI3K and p130Cas	Salgia et al., (1995), Auger et al., (1996)
PTP domain that is non-catalytic identified at N-terminus	Haynie and Ponting (1996)
Identified as close homolog of PTEN	Steck et al., (1997), Li et al., (1997)
PTB domain identified at C-terminus	Haynie and Ponting (personal communication, 1997)
C2 domain identified near N-terminus	Lee et al., (1999)
PTB domain binds integrin $\beta 1$	Haynie and M. Humphries (unpublished data, 1998), Margolis (1999)
Both N- and C-terminal domains required to promote cell migration	Chen and Lo (2003)

There are three regions in the full-length polypeptide: N-terminus, central region, C-terminus. Three signal transduction-related domains have been identified in tensin: a Protein Tyrosine Phosphatase (PTP) domain, a Src Homology 2 (SH2) domain and a PhosphoTyrosine Binding (PTB) domain. Protein tyrosine phosphorylation and dephosphorylation is crucial for cell growth, tissue differentiation, inter-cellular communication, and the immune response. Tensin also contains a C2 domain, the function of which is not known. The domain architecture of human tensin is shown in Figure.1.3. The actin-binding region was determined *in vitro* by actin-binding assays.

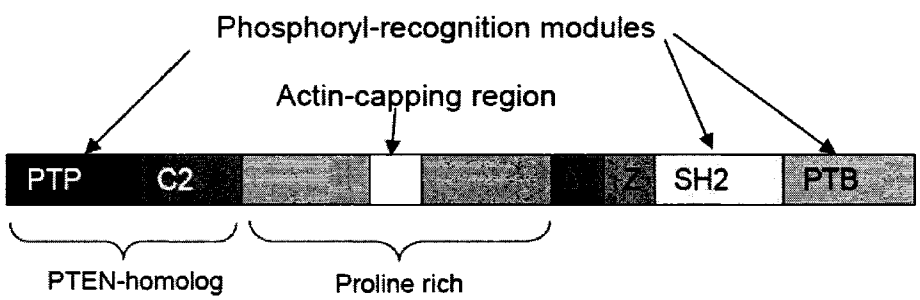

Figure 1.3. Putative Domain Architecture of Tensin. "Y" and "Z" are Putative Domains (D.T.Haynie, Unpublished Results, Permitted by D.T.Haynie).

SH2, PTP, and PTB domains recognize phosphotyrosine (or some other phosphorylated chemical group). It might also be true of the C2 domain in tensin (Lee et al., 1999). These four domains were identified in tensin by amino acid sequence comparisons. They are likely to be independently thermostable. Each of the known domains individually and corporately implicates tensin in intracellular signal transduction.

PTP-C2 is the molecular architecture of PTEN/MMAC1 (phosphatase and tensin homolog deleted on human chromosome 10 or mutated in multiple advanced cancers), a protein of considerable current interest (Li et al., 1997; Steck et al., 1997). PTPs and DSP's have the same fold. This similarity suggests that the tensin PTP domain will recognize phosphotyrosine, phosphoserine, phosphothreonine or, in view of the activity of PTEN, some form of phosphatidylinositol. SH2 domains can bind phosphotyrosines of activated tyrosine kinase receptors, their substrates, and other proteins (Walton et al., 1993). The PTB domain will recognize phosphorylated tyrosine in its target. Similarity to the PTB domain of Src homologous and collagen (Shc) suggests that tensin may bind specifically to a transmembrane receptor. Basic structural properties of the actin-binding region of tensin, identified by biochemical assays, have not yet been determined. Research on the molecular physiology of tensin involves analysis not only of interactions between a modular binding domain of tensin and its targets in other proteins, but also intramolecular interactions in tensin. Domain alignments of some tensin homologs are shown in Figure 1.4.

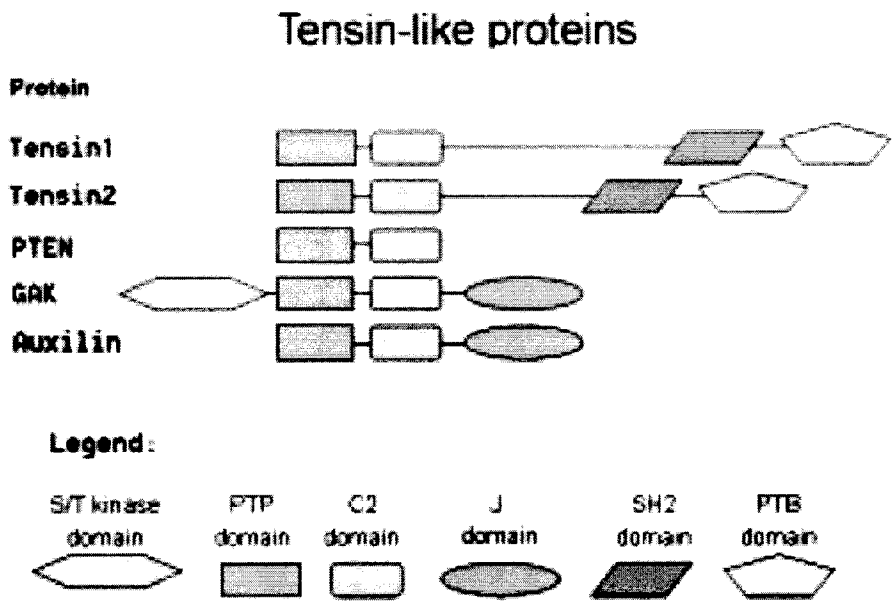

Figure 1.4. Domain Architecture of Tensin-like Proteins (D.T. Haynie, Unpublished, Permitted by D.T. Haynie).

Human tensin2 cDNA encodes a 1,285 amino acid sequence that shares extensive homology with tensin1 at its amino- and carboxyl-terminal ends, which include the actin-binding domain, the Src homology 2 (SH2) domain, and the phosphotyrosine binding (PTB) domain. Analysis of the genomic structures of tensin1 and tensin2 further confirmed that they represent a single gene family. Examination of tensin2 mRNA distribution revealed that heart, kidney, skeletal muscle, and liver were tissues of high expression. The endogenous and recombinant tensin2 were expressed as a 170-kDa protein in NIH 3T3 cells. The subcellular localization of tensin2 was determined by transfection of Green Fluorescence Protein (GFP)–tensin2 fusion construct. The results indicated that tensin2 is also localized to focal adhesions. Finally, functional analysis of tensin genes has demonstrated that expression of tensin genes can promote cell migration on fibronectin indicating that the tensin family plays a role in regulating cell motility (Chen et al., 2002).

1.1.4.2 Carboxyl terminus of tensin

Tensin1 is an actin-binding protein localized to focal adhesions (Lo et al., 1994), which play a crucial role in many biological events such as cell adhesion, migration, proliferation, differentiation, and apoptosis. The N-terminus of tensin1 binds to actin filaments (Lo et al., 1994), whereas the center region retards the G-actin polymerization (Lo et al., 1994). The C-terminus contains the SH2 (Davis et al., 1991) and the PTB domain. In addition, tyrosine phosphorylation of tensin1 is enhanced by extracellular matrix, growth factors, or oncogenes (Bockholt et al., 1993, Jiang et al., 1996, Salgia et al., 1995). Tyr phosphorylation of tensin1 suggests that tensin1 plays important roles in organizing actin cytoskeleton and mediating signal transduction (Lo et al., 1994a). Chen et al. (2002) have shown that expression of tensin1 promotes cell migration. Furthermore, analysis of tensin1 knockout mice has demonstrated a critical role of tensin1 in renal function and wound healing (Lo et al., 1997, Ishii et al., 2001). Because the tissue-restricted phenotypes found in tensin1 knockout mice were unexpected because of tensin's broad expression pattern, Chen et al. (2002) decided to search for other family member(s) and reported the identification of tensin2 (Chen et al., 2002). Human tensin2 contains 1285 amino acid residues and shares extensive homology with tensin1 at its N- and C-terminal ends, although the center portions of the molecules are divergent. The homologous regions span the Albumin-Binding Domain (ABD), the SH2 domain, and the PTB domain. Functional analysis also indicates a conserved role of tensin2 in cell migration. A recent study reported the cDNA sequence and the genomic structure of a more distant member of the tensin family, Carboxyl terminal tensin-like (cten) protein. In contrast to tensin1 and tensin2, Cten is a shorter polypeptide and is preferentially

expressed in prostate and placenta. To date, Cten is the first and only focal adhesion molecule that is specifically expressed in prostate gland. Interestingly, expression of Cten is down-regulated in prostate cancer, suggesting that Cten may be involved in prostatic cell transformation and may have a potential use in disease diagnosis and prognosis during treatment (Chen et al., 2002).

1.1.4.3. SH2 domain

1.1.4.3.1. Evolution of SH2 domain

The evolution of multicellular animals appears to be coincident with the development of the unique signaling motif called the SH2 domain. SH2 domains have thus far been found only in animals and viruses and apparently do not occur in fungi or plants (Branden et al., 1999). The name comes from its being discovered in the genomes of Sarcoma (Src) viruses. Similar sequences were later found in many other intracellular signal-transducing proteins. SH2 domains function in the transmission of molecular signals that start at the cell surface, pass through the plasma membrane, and engage the inner workings of the cell. SH2 domains can fold independently into a compact, stable structure like most other domains. The SH2 domain in human tensin corresponds to residues 1463 to 1557; it is located near the C-terminus.

1.1.4.3.2. Function of SH2 domain

SH2 domain was first identified as a conserved sequence domain between the oncoproteins Src and Fps. Similarly conserved sequences were subsequently found in many other intracellular signal transducing proteins. More specifically, SH2 domains are regulatory modules of intracellular signaling cascades. SH2 domains play a role in the intracellular response to growth factor stimulation by binding to phosphotyrosine

containing proteins (Charifson et al., 1997). SH2 domains carry out their function by binding with high affinity to phosphotyrosine-containing protein targets in a sequence-specific and largely phosphorylation-dependent manner (Branden et al., 1999, Wandless 1996, Furga 2000). The SH2 domain of one protein binds to a second protein that contains a phosphorylated tyrosine side chain in a specific amino-acid sequence context (Furga 2000). In some cases, however, an SH2 domain can bind to the target protein or ligand in a non-pTyr dependent manner (Forman-Kay et al., 1999). For instance, the SLAM-Associated Protein (SAP) SH2 domain binds Signaling Lymphocytic Activation Molecule (SLAM) (Li et al., 1999), and the Growth factor receptor bound protein 10 (Grb10) SH2 binds the C2 domain of Nedd4 (Vecchione et al., 2003) in the absence of phosphorylation. Such non-pTyr dependent binding could also occur in tensin. The SH2 domain could possibly interact with the C2 domain of the same tensin molecular or another tensin molecule. Alternatively, the SH2 domain of tensin could possibly interact with a pTyr residue in the same tensin molecule (intramolecular association, as in Src) or in another tensin molecule (intermolecular association, in this case dimerization).

A cross-sectional view of an animal cell is shown in Figure 1.5, with the cell membrane depicted in yellow. A hormone is bound by its specific transmembrane receptor (both in red). Activation of the receptor causes phosphorylation of the tyrosine (phosphotyrosine in green), which enables a signaling protein containing an SH2 domain (purple) to recognize the receptor. The signaling protein contains a catalytic domain (white), which transmits a signal when the SH2 domain is engaged. The SH2 phosphotyrosine interaction allows specific molecular pairings to be established in the crowded environment of the cell.

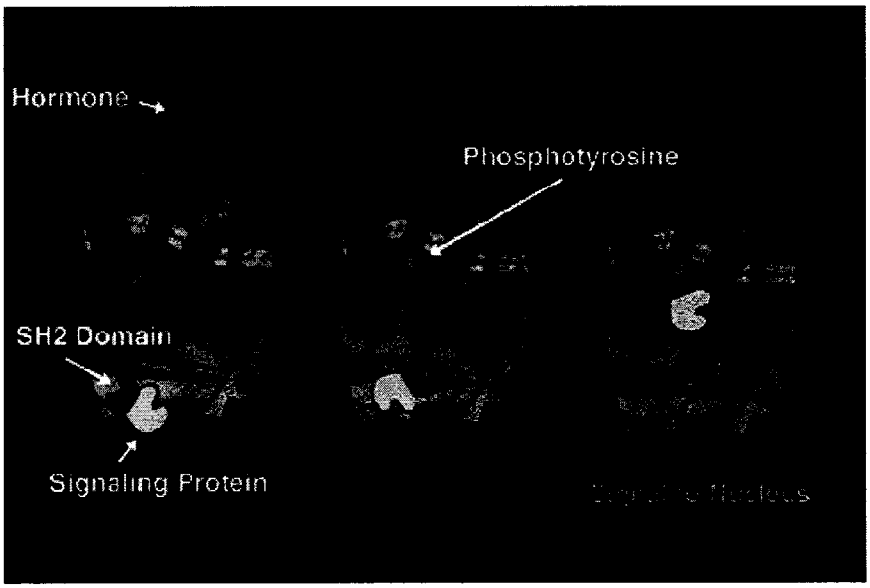

Figure 1.5. Cross Sectional View of an Animal Cell Depicting the Function of SH2 Domain (Kuriyan et al., 1999).

The SH2 domain is a molecule that functions in protein–protein interactions. Its binds the region containing it to a second regions that contains a phosphorylated tyrosine side chain in a specific amino acid sequence context (Branden et al., 1999, Furga et al., 2000). The SH2 domain fold includes a central beta stranded sheet and two alpha helices on either side of it. In Src family SH2 domains, a hydrophobic pocket recognizes a leucine or isoleucine residue at position pY+3 in high affinity peptides (Alberts et al., 1994).

Proteins bind to other proteins through several types of interface. Proteins can bind to another protein in at least three ways: a portion of the surface of one protein contacts an extended loop of polypeptide chain on a second protein. Such an interaction allows the SH2 domain of a protein to recognize the phosphorylated polypeptide as a loop on second protein. It also enables a protein kinase to recognize the proteins that it will phosphorylate (Alberts et al., 1994). Two α helices, one from each protein, pair together

to form a coil. This type of interaction is found in several gene regulatory proteins. The precise matching of one rigid surface with that of another occurs; this is the common type of the three. Such interaction can be tight because a large number of weak bonds can form between two surfaces that match well. This interaction is specific, enabling a protein to select just one partner from thousands of different proteins found in a cell (Alberts et al., 1994).

1.1.4.3.3. Binding partners for SH2 domain

The SH2 domain is a compact plug-in molecule that can be inserted almost anywhere in a polypeptide chain without disturbing other aspects of protein folding or function. Each domain has distinct sites for recognizing phosphotyrosine and for recognizing a particular amino acid side chain; different SH2 domains recognize phosphotyrosine in the context of different flanking amino acid sequences. SH2 motifs in certain cytoplasmic protein are crucial in the signaling pathways of the tyrosine kinase growth factor responses. Short, conserved motifs, primarily three to four amino acids on the C-terminal side of a phosphotyrosine residue, may carry the sequence-specific information for SH2 recognition (Alberts et al., 1994, Wandless 1996).

Phosphopeptide libraries have been used to determine the sequence specificity of the peptide binding sites of SH2 domains. One group of SH2 domains (Src, Fyn, Lck, Fgr, Abl, Crk, and Nck) prefer sequences with the general motif pTyr-hydrophilic-hydrophilic-Ile/Pro while another group (SH2 domains of C-γ and SHPTP2) bind to the general motif pTyr-hydrophobic-X-hydrophobic. Individual members of this group bind to unique sequences, except the Src sub-family (Src, Fyn, Lck and Fgr), which all bind to pTyr-Glu-Glu-Ile. The variability in SH2 domain sequences in likely sites of contact

provides a structural basis for the phosphopeptide selectivity of these families (Charifson et al., 1997). Subgroups of SH2 domain and their known ligands are given in Table 1.2.

Table 1.2. SH2 Family and Their Ligand Binding Partners

Subgroups of SH2 domain	Amino acid sequence of binding partner
Src, Fyn, Lck, Fgr, Abl, Crk, Nck	pY-hydrophilic-hydrophilic-I/P
p8, phospholipaseC-gamma, and SHPTP2	pY-hydrophobic-X-hydrophobic
Src sub family (Src, Fyn, Lck, Fgr)	pY-E-E-I

1.1.4.3.4. Structure determination of tensin-SH2 domain and comparison with other known SH2 domains

The function of a protein is determined by its structure, which in turn is determined by the sequence of amino acids in the polypeptide. Several approaches could be used to determine the structure of tensin-SH2 domain. One of them was to determine the secondary structure of tensin-SH2 domain and compare it with the secondary structures of other known SH2 domains. The other was to determine if the tensin-SH2 domain is stable on its own and if it folds independently of the upstream or downstream part of the protein. Also, the tensin-SH2 domain could be modeled using softwares like CharmM, based upon the available knowledge of the crystal structures of other SH2 domains.

Protein structures have been divided into four classes on the basis of their secondary structures: (α), containing only α-helices; (β), containing primarily β-sheet structure; ($\alpha + \beta$), containing helices and sheets in different parts of the structure; and (α/β), in which helices and sheets interact and often alternate along the polypeptide chain.

In (α) proteins, about 60% of the residues are in α-helices, and the helices are usually in contact with each other. In (β) proteins, there are always two β-sheets, both usually anti-parallel that pack against each other. In the ($\alpha + \beta$) proteins, there may be a single β sheet, usually antiparallel; the helices often cluster together at one or both ends of the β sheet. SH2 domains are known to be a part of ($\alpha + \beta$) structure class. The (α/β) proteins have one major β-sheet of primarily parallel strands; a helix usually occurs in each of the segments of polypeptide chain connecting the β-strands, probably owing to the necessarily long lengths of these connections. The helices pack on both sides of the sheet unless the sheet is closed into a barrel, in which case the α-helices pack around outside of the barrel.

1.2. Methodology

1.2.1. Recombinant DNA Technology

Recombinant DNA (rDNA) has various definitions, ranging from simple to strangely complex. The following are examples of how recombinant DNA is defined:

1. A DNA molecule containing DNA originating from two or more sources.
2. DNA that has been artificially created. It is DNA from two or more sources that is incorporated into a single recombinant molecule.
3. According to the NIH guidelines, recombinant DNA are molecules constructed outside of living cells by joining natural or synthetic DNA segments to DNA molecules that can replicate in a living cell, or molecules that result from their replication.

Recombinant DNA, also known as *in vitro* recombination, is a technique involved in creating and purifying desired genes. Molecular cloning (i.e., gene cloning) involves

creating recombinant DNA and introducing it into a host cell to be replicated. One of the basic strategies of molecular cloning is to move desired genes from a large, complex genome to a small, simple one. The process of *in vitro* recombination makes it possible to cut different strands of DNA, *in vitro*, with a restriction enzyme and join the DNA molecules together via complementary base pairing.

1.2.2. Steps Involved in Making rDNA

The following is a summary of the process of making rDNA:

1. Treat the DNA taken from both sources with the same restriction endonuclease.
2. The restriction enzyme cuts both molecules at the same site.
3. The ends of the cut have an overhanging piece of single-stranded DNA called "blunt ends."
4. These blunt ends are able to base pair with any DNA molecule that contains the complementary blunt end.
5. Complementary blunt ends can pair with each other when mixed.
6. DNA ligase is used to covalently link the two strands into a molecule of recombinant DNA.
7. To be useful, the recombinant DNA needs to be replicated many times (i.e. cloned). Cloning can be done *in vitro*, via the Polymerase Chain Reaction (PCR), or *in vivo* using unicellular prokaryotes (e.g. *E. coli*), unicellular eukaryotes (e.g. yeast), or mammalian tissue culture cells.

1.2.3. Choice of Host Organism

Among the many systems available for heterologous protein production, the Gram-negative bacterium *Escherichia coli* remains one of the most attractive because of its ability to grow rapidly and at high density on inexpensive substrates, its well-characterized genetics and the availability of an increasingly large number of cloning vectors and mutant host strains. Although there is no guarantee that a recombinant gene product will accumulate in *E. coli* at high levels in a full-length and biologically active form, a considerable amount of effort has been directed at improving the performance and versatility of this workhorse microorganism. Different types of organisms and the advantages and disadvantages of each are listed in Table 1.3. Many additional details and references can be found in Makrides' (1996) review.

Table 1.3. Choice of Host Organisms, With the Advantages and Disadvantages of Each.

Host	Advantages	Disadvantages
Bacteria e.g. *Escherichia coli*	Many references and much experience available	No post translational modification
	Wide choice of cloning vectors	Biological activity and immunogenicity may differ from natural protein
	Gene expression easily controlled	High endotoxin content in gram negative bacteria
	Easy to grow with high yields (target protein can form upto 50% of total cell protein)	
	Product can be designed for secretion into the growth media	
Bacteria e.g. *Staphylococcus aureus*	Secretes target protein into the growth media	Does not express at high levels as compared with *E. coli*
Mammalian cells	Same biological activity as native proteins	Cells can be difficult and expensive to grow
	Mammalian expression vectors are available	Cells grow slowly
	Can be grown in large scale	Manipulated cells can be

	cultures	genetically unstable and low productivity as compared with microorganisms.
Yeasts	Lacks detectable endotoxins	Gene expression less easily controlled
	Fermentation less expensive and facilitates glycosylation and formation of disulfide bonds	Glycosylation not identical to mammalian systems
Cultured insect cells *Baculovirus vector*	Many processing systems similar to eukaryotic cells	Lack of information on glycosylation mechanisms
	Baculovirus vector FDA approved for clinical trials	Product not always fully functional
	Virus stops host protein amplification; high level expression of target protein	Few differences between functional and antigenic properties between target and native proteins
Fungi e.g. *Aspergillus sp.*	Well established systems for fermentation for filamentous fungi	High levels of expression not yet achieved
	Growth inexpensive	Genetics not well characterized
	Can secrete large quantities of product into growth media, source of many industrial enzymes	
Plants		Low transformation efficiency and long generation time

1.2.4. Plasmid

1.2.4.1. Introduction

Plasmids are small, circular, extrachromosomal DNA molecules found in bacteria, which can replicate on their own, outside of a host cell. They have a cloning limit of 100 to 10,000 base pairs or 0.1-10 kilobases (kb). A plasmid vector is made from natural plasmids by removing unnecessary segments and adding essential sequences. Plasmids make excellent cloning vectors for various laboratory techniques, including rDNA.

Transformation is the modification of the genotype of a cell (usually prokaryotic) by introducing DNA from another source. During transformation, genetic information is

transferred via the uptake of free DNA. Often these sources of DNA come from plasmids that are deliberately introduced into a cell, transforming the cell with its genes. Transformation occurs naturally, and the resulting uptake of foreign DNA by the cell is not typically considered rDNA. If the plasmid being introduced to the cell has not been genetically altered, *in vitro*, the plasmid is not considered to contain rDNA.

Plasmids that have been genetically altered, such as by the insertion of an antibiotic resistance gene, do contain rDNA because the original genetic composition of the plasmid has been artificially altered. These plasmids can be used to incorporate bacterial cells with the antibiotic resistance gene via transformation.

1.2.4.2. Choice of plasmid

To clone the gene of interest, all engineered vectors have a selection of unique restriction sites downstream of a transcription promoter sequence. The choice of vector family is governed by the host. Once the host has been selected, many different vectors are available for consideration, from simple expression vectors to those that secrete fusion proteins. However, as for the selection of a suitable host system, the final choice of vector should take into consideration the specific requirements of the application and will, of course, be influenced by the behavior of the target protein. One key factor that has led to the increased use of fusion protein vectors is that amplification of a fusion protein containing a tag of known size and biological function can greatly simplify subsequent isolation, purification and detection. In some cases the protein yield can also be increased. Table 1.4 reviews some of the features of fusion protein amplification that may influence the final choice of vector.

Table 1.4. Advantages and Disadvantages of Fusion Proteins Over Non-fusion Proteins

Advantages	Disadvantages
Fusion proteins	
Provide a marker for expression	Tag may interfere with protein structure and affect folding or biological activity
Simple purification using affinity chromatography under native or denaturing conditions	Cleavage is not always 100% specific if tag needs to be removed
Easy detection	
Refolding achievable on chromatography column	
Ideal for secreted proteins as the product is easily isolated from growth media	
Non-fusion proteins	
No cleavage steps necessary	Purification and detection not as simple
	Problems with solubility may be difficult to overcome, reducing potential yield

1.3. Objectives

The short-term objectives of this work are as follows: (1) to express and purify the SH2 domain utilizing standard tools in molecular biology, (2) to assess the purity of the recombinant protein obtained using Capillary electrophoresis as a tool, (3) to calculate the exact mass of the SH2 domain using Mass spectrophotometer, and (4) to determine the biophysical and biochemical properties of this protein using circular dichroism spectrophotometer and differential scanning calorimetry as investigative tools.

The long term objective of this work is to use tensin as a tool to investigate to elucidate the physiology of cell substrate contacts at the molecular scale, and determine the molecular basis of focal adhesion and cell migration.

1.4. Organization

Chapter One is introduction and literature review followed by the specific objectives of this work in Section 1.3. Chapter Two is materials and methods which covers in some detail the various molecular biology and biophysical techniques used as tools in this work. Results are presented in Chapter Three and Chapter Four is a discussion which explains the significance and implications of the results presented in the preceding chapter. Chapter Five summarizes the work done with conclusions and recommendations for further work to acquire more detailed knowledge about the SH2 domain of tensin.

CHAPTER 2

MATERIALS AND METHODS

2.1. Molecular Biology Techniques

2.1.1. Bacterial Transformation

2.1.1.1. Materials

2.1.1.1.1. Plasmid

The pET-14b plasmid vector (Novagen, USA) was used for gene cloning. A map of the plasmid is shown in Figure 2.1. The size of PET-14b vector is 4671 bp with G-C content of 53.6%. Proteins expressed using a pET-14b vector carry a N-terminal His·tag coding sequence, that aids in purification of the recombinant protein using affinity chromatography, followed by a thrombin cleavage site and three cloning sites. Unique sites are shown on the vector map. The sequence is numbered by the pBR322 convention, so the T7 expression region is reversed on the circular map. The cloning/expression region of the coding strand transcribed by T7 RNA polymerase is also shown in the map. SH2 gene and plasmid vector (pET-14b) were enzymatically digested using *Nde I* and *Xho I* enzymes at 37 °C for 4 h. The vector and insert were ligated in ratio 1:10 (Velicharla, 2004) and used for transformation using BL21 DE3 (pLysS) cells.

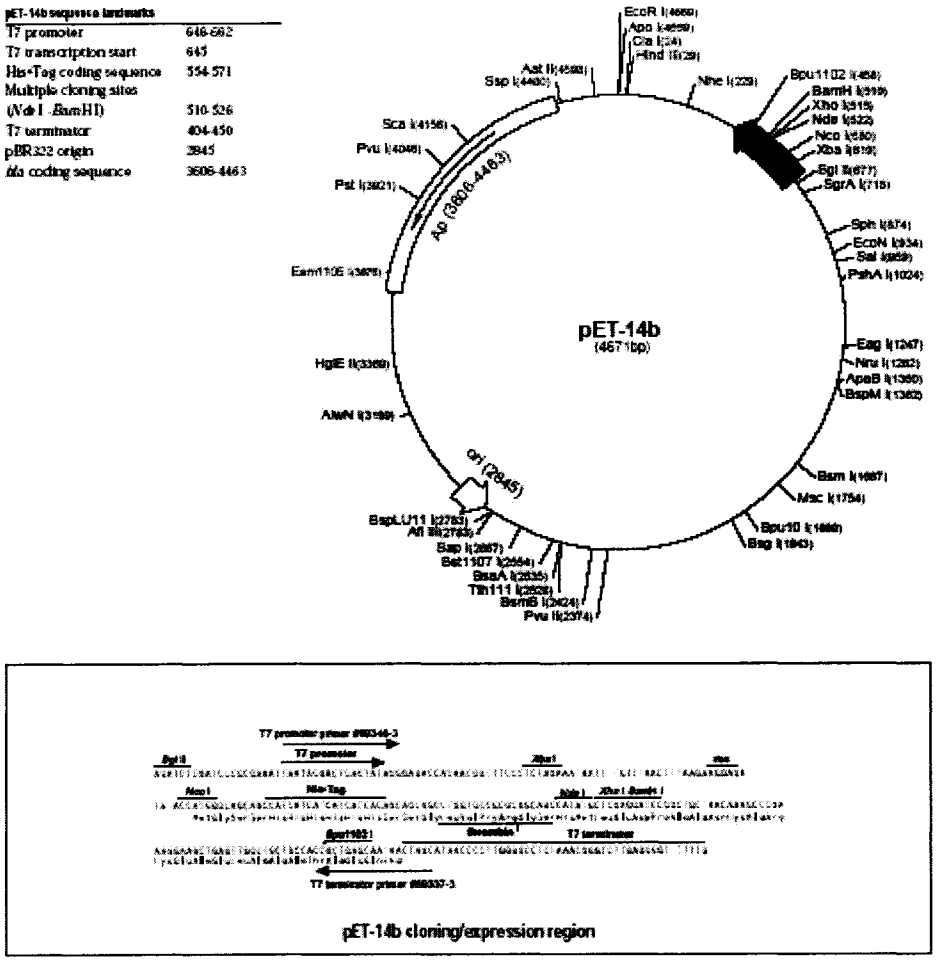

Figure 2.1. pET-14b Vector Map.

2.1.1.1.2 Bacterial strain

The bacterial strain used to transform the SH2 gene was BL21 (DE3) pLysS Competent Cells (Stratagene, USA), which are chemically competent *E.coli* cells used for tight control of protein expression in T7 RNA Polymerase-based systems. The cells are resistant to the lytic bacteriophages T1 and T5. The BL21 (DE3) pLysS strain is a derivative of *E.coli* B. It is deficient in both *lon* and *ompT* proteases, resulting in superior isolation of intact recombinant proteins. The host is a lysogen of λDE3, and therefore carries a chromosomal copy of the T7 RNA polymerase gene that is controlled by the

lacUV5 promoter. BL21 (DE3) pLysS uses the T7 RNA promoter to control protein expression. The strain carries a low-level expression plasmid that expresses the T7 lysozyme gene at nominal levels. T7 lysozyme binds to T7 RNA polymerase, thus inhibiting transcription by this enzyme. Upon IPTG induction, overproduction of the T7 RNA polymerase effectively shuts down any low level inhibition by T7 lysozyme. BL21 (DE3) pLysS strain is resistant to chloramphenicol.

2.1.1.1.3. Media

LB agar and LB broth EZMix were purchased from Sigma Aldrich (USA). The culture medium was prepared as recommended by Sigma and the agar plates were prepared in the presence of ampicillin and chloramphenicol.

2.1.1.2. Method

The SH2 gene ligated to pET-14b was transformed into BL21 (DE3) pLysS strain of *E. coli* using the heat shock method. 50 μL of these cells were put in an Eppendorf tube and incubated on ice for 10 min to thaw. The DNA for SH2 gene was added to these tubes and placed in a water bath at 42 °C for 45 s. The tubes were placed back on ice for 2 min to avoid damage to the cells. 1 mL of LB broth was added to these tubes and incubated at 37 °C for 1 h. 200 μL of this culture was plated using a sterile plater on three different prewarmed agar plates. These agar plates had ampicillin (50 μg/mL) and chloramphenicol (34 μg/mL) as antibiotic resistance. The plates were incubated at 37 °C overnight.

The following day, a few colonies were picked from each plate and added to a falcon tube containing 4.9 mL of fresh LB broth in the presence of ampicillin (50 μg/mL) and chloramphenicol (34 μg/mL). These tubes were incubated at 37 °C at 240 rpm

overnight. The following day, the tubes that had turned cloudy were stored in 50 % sterile glycerol for testing for protein overexpression.

2.1.2. Protein Expression

2.1.2.1. Materials

2.1.2.1.1. Isopropyl-β-D-Thiogalactopyranoside (IPTG)

IPTG (FW 238.3 g/mol), purchased from Sigma Aldrich (USA), is an artificial inducer of the *lac* operon. It induces the activity of beta-galactosidase by strongly binding and inhibiting the *lac* repressor (Sambrook and Russel, 2001). A synthetic analog of galactose, IPTG cannot be hydrolyzed and broken down by the cell. Hence, its concentration remains constant during an experiment.

2.1.2.2. Method

A 100 mL overnight bacterial culture was prepared with the glycerol stock of the colony picked from the agar plate in the presence of ampicillin (50 μg/mL) and chloramphenicol (34 μg/mL). Following day, a fresh 500 mL culture was inoculated with the overnight culture in the presence of antibiotics at the same concentration. The culture was grown till OD_{600} = 0.5; the log phase of growth for *E. coli*; at which point the protein overexpression was induced with 0.4 mM IPTG. This concentration was empirically determined. 2 mL aliquots were taken from the culture before induction; and every hour post induction for 4 hours. These aliquots were centrifuged at 14,000 rpm, the supernatant was discarded and the cell pellets were stored at -80 °C for analyzing the protein expression level using SDS-PAGE. At the end of 4 h the cells were harvested by centrifuging them at 6500 rpm for 10 min. These cells were stored at -80 °C till further use for purification.

2.1.3. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

2.1.3.1. Materials

2.1.3.1.1. Sodium Dodecyl Sulfate

SDS (FW 288.38 g/mol) was ordered from Sigma Aldrich (USA). SDS (CH_3-$(CH_2)_{10}$-$CH_2OSO_3^-Na^+$) is an anionic detergent that binds strongly to a protein in a specific mass ratio of 1.4:1 and in doing so denatures the protein. Thus, SDS confers a negative charge to the polypeptide in proportion to its length; i.e., the denatured polypeptides become "rods" of negative charge cloud with equal charge or charge densities per unit length.

2.1.3.1.2. Dithiothreitol (DTT)

DTT (FW 154.25 g/mol, $C_4H_{10}O_2S_2$), purchased from Sigma Aldrich (USA), reduces any disulfide bonds present holding together the protein tertiary structure.

2.1.3.1.3. Ammonium Persulfate (APS)

APS (FW 228.20 g/mol, $(NH_4)_2S_2O_8$) was ordered from Sigma. Polymerization of acrylamide and bisacrylamide monomers is induced by ammonium persulfate (APS), which spontaneously decomposes to form free radicals. A 10% w/v fresh solution was prepared by dissolving APS in dI water.

2.1.3.1.4. N,N,N',N'-Tetramethylethylenediamine (TEMED)

TEMED (FW 116.21 g/mol, $C_6H_{16}N_2$) was purchased from Sigma Aldrich (USA). It is a free radical stabilizer, which is generally included to promote polymerization.

2.1.3.1.5. Protein molecular weight marker

The molecular weight of the proteins can be determined by comparing against a number of standard proteins, known as molecular mass marker. This marker was ordered from Fermentas.

2.1.3.1.6. Loading buffer

The protein sample to be analyzed on SDS-PAGE is mixed with 3X SDS-PAGE sample buffer (250 mM Tris-Cl, pH 6.8, 5% SDS, 0.25% bromophenol blue, 25% glycerol) and DTT (1/20 v/v) is added freshly. The ionisable tracking dye, bromophenol blue, allows the electrophoretic run to be monitored, and glycerol gives the sample solution density thus allowing it to settle down to the bottom of the loading well.

2.1.3.1.7. Staining and destaining solutions

The gel was stained with Coomassie blue, ordered from Sigma. The gels were stained in staining solution (45% methanol, 45% dI water, 10% acetic acid, and 0.1% w/v Coomassie blue dye) overnight before washing them in a destaining solution (45% methanol, 45% dI water, and 10% acetic acid).

2.1.3.2. Method

The 2 mL aliquots stored after overexpression were thawed. 50 μL of 3X sample loading buffer was added to the cells in the presence of DTT. These samples were boiled at 100 °C for 5 min along with the protein molecular weight marker. The samples were allowed to cool to room temperature before loading them onto the SDS-PAGE. The gel electrophoresis tank was filled with 200 mL of running buffer. The gels were run at a constant voltage of 200 V and current of 50 mA till the tracking dye reached the end of the gel. The gel was removed and put in a staining solution on a rocker to stain overnight.

The gel was next washed in a destaining solution, which removed the unbound background dye from the gel leaving stained proteins visible as blue bands on a clear background. The molecular weight of the proteins was determined by comparing against a number of standard proteins, known as molecular mass marker.

2.1.4. Immunoblot Analysis

2.1.4.1. Materials

2.1.4.1.1. Nitrocellulose membrane

Nitrocellulose membrane is a high-quality membrane ideal for blotting of proteins and nucleic acids. This membrane was ordered from Invitrogen (Catalog no. LC2000). This membrane with pore size of 0.2 μm is especially suitable for proteins with molecular weight less than 20 kDa.

2.1.4.1.2. Anti·His HRP conjugates

Anti·His HRP Conjugates were ordered from Qiagen (USA) and consist of mouse monoclonal IgG1 Anti·His Antibodies coupled to HorseRadish Peroxidase (HRP). They can be used for direct detection of any protein with an accessible 6xHis tag by chromogenic or chemiluminescent methods. They eliminate the need for secondary antibodies, thereby saving time and expense in blotting and ELISA procedures. The conjugates are available in highly specific Penta·His, Tetra·His, and RGS·His forms. Anti·His HRP conjugates are supplied with a specially formulated Blocking Reagent and Blocking Reagent Buffer to ensure optimal specificity and sensitivity.

2.1.4.1.3. 6xHis protein ladder

The 6xHis Protein Ladder serves as a molecular weight standard, and as a positive control for dot blotting. The 6xHis Protein Ladder consists of five 6xHis-tagged proteins

ranging from 15 to 100 kDa. Each of the proteins that make up the 6xHis Protein Ladder is tagged at the N-terminus with the $(His)_6$ sequence; therefore, they can be detected by any of the QIA*express* detection reagents including the Anti·His HRP conjugate.

2.1.4.1.4. 4-chloro-1-naphthol

4-chloro-1-naphthol (FW 178.61 g/mol, $C_{10}H_7ClO$) was ordered from Qiagen. It is a substrate, which upon reacting with HRP produces a blue colored insoluble precipitate.

2.1.4.2 Method

The 2 mL aliquot collected 4 h post induction with IPTG was centrifuged at 14,000 rpm and the supernatant was discarded. The resulting cell pellet was diluted by adding varying amounts of dilution buffer for denaturing conditions (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris, pH 8.0), ranging from 20 μL to 200 μL. 2 μL of each of these dilutions were blotted onto the nitrocellulose membrane. The membrane was allowed to dry for 30 min. The membrane was washed twice with TBS buffer and incubated for 1 h in Anti·His HRP conjugate blocking buffer supplied by the manufacturer. It was washed with TBS-Tween/Triton buffer followed by TBS buffer. The membrane was then incubated in the Anti·His HRP conjugate solution (1:1000 dilution of Anti·His HRP conjugate in blocking buffer) overnight. Next day it was washed with TBS-Tween/Triton buffer. The membrane was stained with HRP staining solution. This solution was prepared by dissolving 18 mg 4-chloro-1-naphthol in 6mL methanol. 24 mL 1x Tris saline (10X Tris saline: 9% w/v NaCl in 1M Tris-Cl, pH 8.0) was added to the methanol followed by 60 μL hydrogen peroxide. This staining solution is stable only for a short

period. The membrane was incubated in this staining solution until a signal was clearly visible.

A similar procedure was followed for a negative control which comprised of the culture aliquot collected before induction with IPTG. The manufacturer supplied 6xHis protein ladder was used as a positive control.

2.1.5. Cell Lysis

2.1.5.1. Materials

2.1.5.1.1. Sonicator

The cells were lysed using Sonicator® 3000 from Misonix Inc. and was provided with a microtip used to generate high frequency sound waves which shear the cell walls.

2.1.5.1.2. Protease inhibitor cocktail

A protease inhibitor cocktail consisting of AEBSF (4-(2-aminoethyl) benzenesulfonyl fluoride), phosphoramidon, pepstatin A, bestatin, and E-64 was added to these suspensions at a ratio of 1 mL per 20 g wet cell paste to limit proteolysis during purification.

2.1.5.2. Method

The cells harvested after overexpression were freeze thawed and 3 mL of resuspension buffer (20 mM Tris-HCl, pH 8.0) was added per 100 mL of cell culture. This suspension comprised of the soluble proteins was sonicated on ice for 30 s with 30 s interval in between four times. The resulting lysate was centrifuged at 10,000 rpm at 4 °C for 30 min. The supernatant was discarded and 3 mL of isolation buffer (2 M urea, 20 mM Tris-HCl, 0.5 M NaCl, and 2% Triton X-100, pH 8.0) per 100 mL cell pellets was added to the inclusion bodies. The suspension was sonicated and centrifuged. This was

repeated twice to isolate the inclusion bodies. These inclusion bodies were stored at -80 °C in the presence of 50 μL protease inhibitor for further use in purification.

2.1.6. Protein Purification

2.1.6.1. Materials

2.1.6.1.1. ÄKTA *prime*

ÄKTA *FPLC*, shown in Figure 2.2, is a fully automated liquid chromatography system designed for research scale purification of proteins. The system simplifies the transition from laboratory to full scale production.

ÄKTA *FPLC* features:

- Flow rates up to 20 mL/min and pressures up to 5 MPa.
- One working platform for all liquid chromatography techniques suitable for protein purification, from micro-gram to gram scale.
- Method templates as a basis for creating customized methods.
- Continuous monitoring of A_{280}.

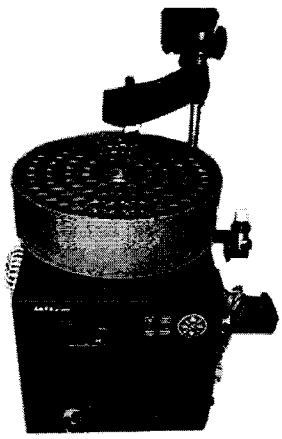

Figure 2.2. AKTA FPLC System.

2.1.6.1.2. HisTrap™ HP columns

Histidine tagging is the most used form of tagging recombinant proteins. HisTrap HP columns, shown in Figure 2.3, are prepacked with Ni Sepharose High Performance can also be used for the purification of tagged proteins containing shorter or longer polyhistidine tags, such as $(histidine)_4$ or $(histidine)_{10}$. The shorter $(histidine)_4$ will bind weakly and the longer $(histidine)_{10}$ will bind strongly compared with $(histidine)_6$. This difference in binding strength can be used during purification, since $(histidine)_{10}$ binds strongly, a higher concentration of imidazole can also be added to the lysed cells and used during the binding and washing steps before elution. This can facilitate the removal of contaminants which may otherwise get co-purified with the tagged target protein.

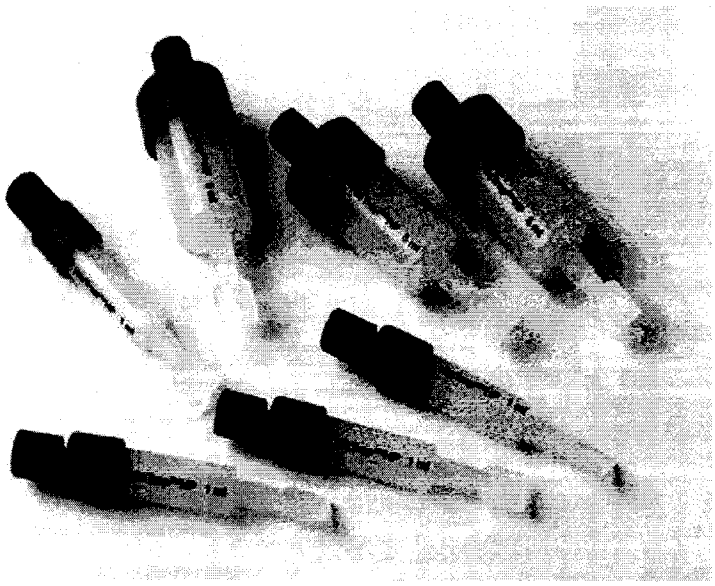

Figure 2.3. 1 mL and 5 mL HisTrapTM Columns.

2.1.6.2. Method

2.1.6.2.1. Purification of the SH2 protein expressed as inclusion bodies

The inclusion bodies isolated after cell lysis were thawed. They were dissolved in 4 mL solubilization buffer (6 M GdnHCl, 20 mM Tris-HCl, 0.5 M NaCl, 10 mM imidazole, and 1 mM 2-mercaptoethanol, pH 8.0) on a rocker for 1 h. This sample was filtered through 0.45 μm filter using a syringe. The column was equilibrated with 50 column volumes of solubilization buffer. The A_{280} was monitored, and upon reaching a steady baseline the column equilibration was stopped. At this time, the sample was loaded onto the column and washed with the solubilization buffer. The increase in A_{280} indicated the unbound host cell proteins being washed off the column. The column was washed until a steady baseline was attained. The protein bound onto the column was washed with 50 column volumes of refolding buffer (6 M urea, 20 mM Tris-HCl, 0.5 M NaCl, 5 mM imidazole, and 1 mM 2-mercaptoethanol, pH 8.0). On-column refolding of the protein was attempted by running a linear gradient of 6 M-0 M urea. This refolding was achieved by running 50 column volumes of a linear gradient of refolding buffer and wash buffer (20 mM Tris-HCl, 0.5 M NaCl, 10 mM imidazole, and1 mM 2-mercaptoethanol, pH 8.0). The final elution step was performed by running a linear gradient of 0.01 M-0.5 M imidazole. The target protein was eluted by running 100 column volumes of a gradient of wash buffer and elution buffer (20 mM Tris-HCl, 0.5 M NaCl, 0.5 M imidazole, and 1 mM 2-mercaptoethanol, pH 8.0). The A_{280} was monitored and fractions were collected upon observing absorbance peaks. These fractions were stored at 4 °C after adding 0.1% w/v sodium azide to avoid bacterial growth and later analyzed using SDS-PAGE.

2.1.6.2.2. Purification of the SH2 protein expressed in soluble fraction

The supernatant was collected and stored after lysing and centrifuging the cells in resuspension buffer. This supernatant was filtered through a Whatman filter paper and a second filtration step was done using a 0.45 μm filter. 20 mM imidazole and 1 mM 2-mercaptoethanol was added to this soluble fraction to minimize the binding of host cell proteins to the column. The column was equilibrated by running 50 column volumes of binding buffer (20 mM Tris-HCl, 0.5 M NaCl, 20 mM imidazole, and 1 mM 2-mercaptoethanol, pH 8.0). Upon reaching a steady baseline, the sample was applied onto the column and washed with the binding buffer. The column was washed with 50 column volumes of wash buffer. The elution was done by running a linear gradient of 0 M-0.5 M imidazole. The fractions were collected by observing A_{280} and stored as described earlier.

2.1.7. Dialysis

2.1.7.1. Materials

2.1.7.1.1. Dialysis tubing

The dialysis tubing (Spectrum®) with a molecular weight cut-off (MWCO) of 10000 daltons was chosen to dialyze the 16734 dalton SH2 domain. The dry tubing was pre-treated by soaking and rinsing in ddI water at 70 °C thrice before use.

2.1.7.2. Method

The purified protein fraction was dialyzed against a buffer (20 mM sodium phosphate, 10 mM NaCl, and 1 mM DTT, pH 7.8) to get rid of the imidazole and other salts used in the purification process. The ratio of protein to buffer was 1:300. The dialysis was done with three buffer changes. The first two buffer changes were at the end

of four hours, and finally the protein was dialyzed overnight. Upon completion, the buffer and protein were both stored at 4 °C.

2.1.8. Limited Proteolysis

2.1.8.1. Materials

2.1.8.1.1. Restriction grade thrombin

Restriction-grade thrombin (Catalog no. 69671-3) was purchased from Novagen. Thrombin specifically cleaves the amino acid sequence Leu-Val-Pro-Arg↓Gly-Ser in His tagged proteins, thereby separating the His tag from the recombinant protein. The kit also supplied a 48 kDa cleavage-control protein to be used as a positive control which upon cleavage with thrombin yielded 35 kDa and 13 kDa protein fragments.

2.1.8.2. Method

The thrombin was diluted in a 1:25 ratio using a thrombin dilution buffer (50 mM sodium citrate, pH 6.5, 200 mM NaCl, 0.1% PEG-8000, and 50% glycerol). The reaction mixture comprised of 5 μL 10X Thrombin Cleavage Buffer (200 mM Tris-HCl pH 8.4, 1.5 M NaCl, and 25 mM $CaCl_2$), 2 μL diluted thrombin, 20 μL purified protein and the volume was brought up to 50 μL by adding dI water. A positive control supplied by Novagen was used to test the activity of the thrombin. This control was a 48 kDa protein; which upon incubation with thrombin was cleaved into a 35 kDa and a 13 kDa fragments. The positive control was carried out in a similar manner by using the cleavage control protein. The negative control comprised of all the reagents except the diluted thrombin. The temperature at which the reaction was carried out was determined empirically. The reaction mixture was incubated at 4 °C for 16 h. Aliquots were collected at 2h, 4h, 8h, and 16h intervals to monitor the time required for cleavage. These aliquots were treated

with 3X sample loading buffer and heated at 100 °C for 3 min to completely denature the thrombin and inhibit any further reaction.

2.2. Biophysical Techniques

2.2.1. Ultraviolet/Visible Spectroscopy

Ultraviolet/visible spectroscopy involves the absorption of ultraviolet/visible light by a molecule causing the promotion of an electron from a ground electronic state to an excited electronic state. A schematic block diagram of a UV spectrometer is shown in Figure 2.4.

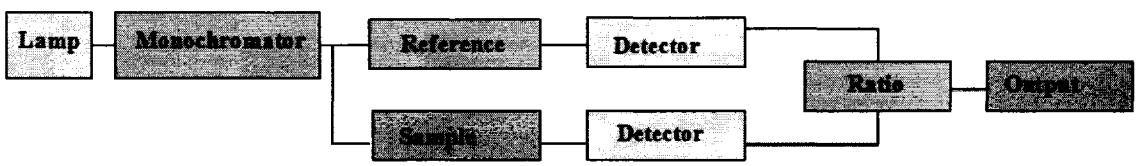

Figure 2.4. Schematic diagram of UV Spectrometer.

This instrument works on the Beer-Lambert's law, $A = \epsilon bc$, where A is absorbance, ϵ is the extinction coefficient with units of L/mol-cm, b is the path length of the sample (cm), which is the path length of the cuvette in which the sample is contained, and c is the concentration of the protein in solution, expressed in mol/L.

UV spectroscopy determined the concentration of the purified protein that was used for further characterization using CD spectroscopy and DSC. The absorbance was noted at a wavelength of 280 nm to calculate the protein concentration (Gill and von Hippel, 1989).

2.2.2. Capillary Electrophoresis (CE)

As the name suggests, CE involves electrophoresis of simple molecules, organic and inorganic ions, peptides, proteins, carbohydrates and nucleic acids in a narrow bore

capillary (typically 50 μm internal diameter). CE was used to demonstrate the purity of the recombinant protein as well as to monitor the proteolytic cleavage of the $(His)_6$ tag using thrombin over a period of 16 hours. This approach was used keeping in mind the pharmaceutical application of CE, which is to demonstrate the purity of antibiotic, or the presence or amount of impurities. The basic instrumentation consists of four parts as shown below. A narrow diameter capillary is required for the separation, a high voltage power supply is required to drive the separation, a detector is required to detect the presence and amount of analyte and a safety interlock-equipped enclosure is used to protect the operator from high voltage (not shown). A schematic block diagram of CE is shown in Figure 2.5.

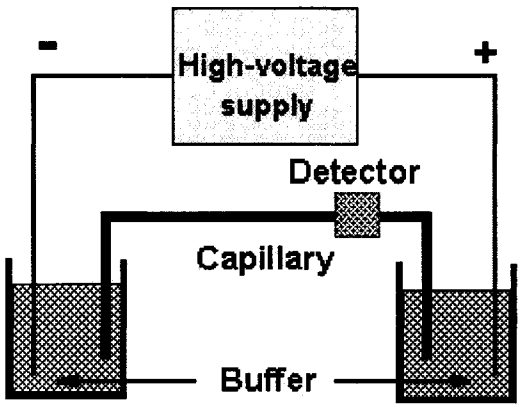

Figure 2.5. Schematic Diagram of Capillary Electrophoresis.

Either pressure or vacuum is applied to the sample and 10 - 100 nL is injected, or an electrical current is applied through the sample and only the charged molecules enter the capillary. The resulting migration of a charged particle in CE is a sum of the electrophoretic flow, which is determined by the size and charge on the molecule, and the electroosmotic flow, which is determined by the charge on the inner surface of the

capillary. One advantage of capillaries is that they reduce problems resulting from heating effects. Because of the small diameter of the capillary there is a large surface-to-volume ratio, which gives enhanced heat dissipation. This heat dissipation helps to eliminate both convection currents and zone broadening owing to increased diffusion caused by heating. It is therefore not necessary to include a stabilizing medium in the capillary and allows free-flow electrophoresis.

The capillary length plays no role in separation efficiency but has an important role in migration time and high separation efficiencies are best achieved through the use of high voltages. There is a practical limit to the amount of voltage that can be applied, as determined by the capillary length. Voltages in the range of 10 to 50kV with capillaries of 50 to 100 cm are commonly used.

A high voltage is applied across the capillary and component molecules in the injected sample migrate at different rates along the length of the capillary tube. Electrophoretic migration causes the movement of charged molecules in solution towards an electrode of opposite charge. Owing to this electrophoretic migration, positive and negative sample molecules migrate at different velocities. The higher the positive charge on a molecule, the faster it travels towards the cathode, with highly negative charged molecules being the slowest to migrate. As the separated molecules approach the cathode, they pass through a detector window where they are detected by an ultraviolet monitor that transmits signal to a recorder, integrator (mass spectroscopy in some cases) or computer. The signal plotted as absorbance against time is called an electropherograph.

A neutral capillary was rinsed with citrate-MES buffer, pH 6.0 for 10 minutes. 10kV voltage was applied for 10 minutes across the capillary to rinse out all the

contaminants. Pressure was applied at 5 psi for 3 seconds to inject 2nL of the tensin-SH2 protein into the capillary. Separation was achieved by applying a voltage of 10kV for 1h. The source filter was set to 214 nm.

2.2.3. Circular Dichroism Spectroscopy

CD measures the interaction of circularly polarized light with molecules. Circularly polarized light comes in left- and right-handed forms, like screw threads. These forms interact equally with non-chiral chromophores, but differently when the chromophore has a right- or left-handed form. The result is a small difference in the extinction coefficients for left- and right-polarized light, which varies with wavelength.

In proteins, the chiral arrangement of peptide bonds in secondary structures such as α-helix and β-sheet leads to characteristic CD spectra at UV wavelengths. Alteration of the relative orientations, because of conformational or structural variations, causes changes in the CD spectrum.

2.2.3.1. Determination of protein secondary structure by circular dichroism

Secondary structure can be determined by CD spectroscopy in the far-UV spectral region (190-250 nm). At these wavelengths the chromophore is the peptide bond, and the signal arises when it is located in a regular, folded environment. Alpha-helix, beta-sheet, and random coil structures each give rise to a characteristic shape and magnitude of CD spectrum. The approximate fraction of each secondary structure type present in any protein can thus be determined by analyzing its far-UV CD spectrum as a sum of fractional multiples of such reference spectra for each structural type.

Like all spectroscopic techniques, the CD signal reflects an average of the entire molecular population. Thus, while CD can determine that a protein contains about 50%

alpha-helix, it cannot determine which specific residues are involved in the alpha-helical portion. Far-UV CD spectra require 200 µl of solution containing 50 µg/ml to 1 mg/ml protein, in any buffer which does not have a high absorbance in this region of the spectrum. (High concentrations of DTT, histidine, imidazole, or chloride ions for example, cannot be used in the far-UV region).

2.2.3.2. Information about protein tertiary structure from circular dichroism

The CD spectrum of a protein in the near-UV spectral region (250-350 nm) can be sensitive to certain aspects of tertiary structure. At these wavelengths the chromophores are the aromatic amino acids and disulfide bonds, and the CD signals they produce are sensitive to the overall tertiary structure of the protein. Signals in the region from 250-270 nm are attributable to phenylalanine residues, signals from 270-290 nm are attributable to tyrosine, and those from 280-300 nm are attributable to tryptophan. Disulfide bonds give rise to broad weak signals throughout the near-UV spectrum. If a protein retains secondary structure but no defined three-dimensional structure (e.g. an incorrectly folded or "molten-globule" structure), the signals in the near-UV region will be nearly zero. On the other hand, the presence of significant near-UV signals is a good indication that the protein is folded into a well-defined structure. The near-UV CD spectrum can be sensitive to small changes in tertiary structure because of protein-protein interactions and/or changes in solvent conditions. The signal strength in the near-UV CD region is much weaker than that in the far-UV CD region. Near-UV CD spectra require about 1 ml of protein solution with concentration ranging from 0.25 mg/ml to 2 mg/ml.

2.2.3.3. Spectral interpretation and deconvolution

2.2.3.3.1. Interpretation of the spectra

The relationship between the secondary structure of the protein and the CD spectrum has been realized quite early and was published in 1969 by Greenfield and Fasman. The data obtained after performing far-UV CD experiments can be compared with the standard spectra of poly-L-lysine, shown in Figure 2.6, to study the structure of the polypeptides.

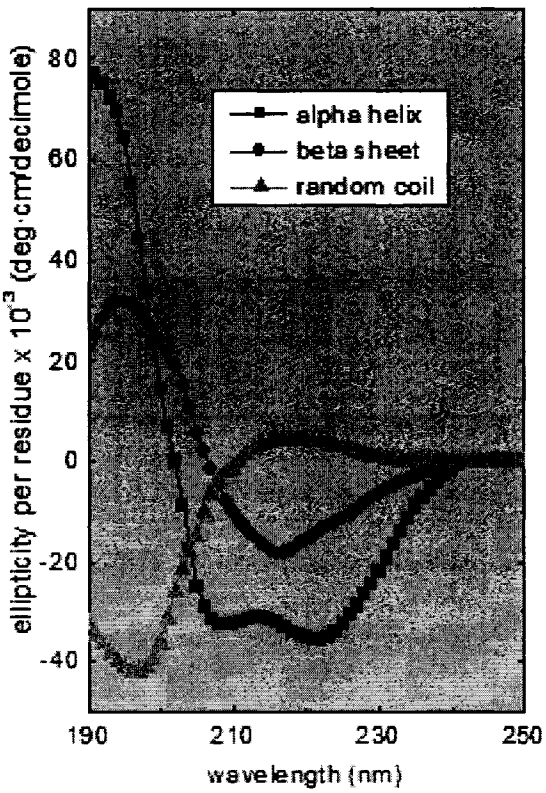

Figure 2.6. Actual Fit of a Sample Data Against the Fasman Poly-lys Standards (Greenfield and Fasman, 1969).

2.2.3.3.2. Deconvolution

The CDPro software suite, which includes programs CDSSTR, CONTIN, and SELCON3 (Sreerama and Woody, 2000) was used to deconvolute the far-UV CD spectra into contributions from the α helix, β sheet, β turn, and coil. The software programs were used as recommended over the wavelength range of 195-240 nm, which included the significant part of the data from the CD spectrum of the tensin-SH2 protein. The chosen reference set, consisting of the far-UV CD spectra of 43 proteins of known secondary structure content, was the best option among all reference sets available in the software suite, and was also the recommended option in the software for the input data in various experiments.

All CD studies of recombinant tensin-SH2 domain contained the His-tag. The experiments were done at 22-24 °C with a Jasco J-810 CD spectropolarimeter (Japan). The step size was 0.1 nm; scanning speed, 0.3 nm s^{-1}; response time, 0.3 s; number of scans, 60 (far-UV) or 150 (near-UV). Far-UV measurements were taken in the range 190-260 nm at 25 °C in a 0.1 cm path length cuvette. The protein concentration was 0.12 mg mL^{-1} in 25 mM sodium phosphate, 10 mM NaCl, 1mM β-mercaptoethanol, pH 7.8. Near UV spectra were taken in the range 250-360 nm in a 1 cm path length cuvette. The protein concentration was 1 mg mL^{-1} in the same buffer as above. Buffer baselines were collected with the same instrument settings and subtracted from the respective sample spectra. Quartz cuvettes filled with the protein sample were placed in the sample chamber, purged of air with nitrogen. This purging was done to avoid the far-UV light absorption by molecular oxygen, which masks the CD signal.

2.2.4. Differential Scanning Calorimetry (DSC)

DSC is used to investigate protein stability and is combined with other biophysical methods to link thermodynamics, structure, and function.

A protein in aqueous solution is in equilibrium between the native (folded) conformation and its denatured (unfolded) conformation. The stability of the native state is based on the magnitude of the Gibbs free energy (ΔG) of the system and the thermodynamic relationships between enthalpy (ΔH) and entropy (ΔS) changes. A positive ΔG indicates the native state is more stable than the denatured state – the more positive the ΔG, the greater the stability. For a protein to unfold, stabilizing forces need to be broken. Conformational entropy overcomes stabilizing forces allowing the protein to unfold at temperatures where entropy becomes dominant. DSC measures ΔH of protein unfolding because of heat denaturation. The higher the transition midpoint (T_m), the more stable the protein at lower temperatures. During the same experiment DSC also measures the change in heat capacity (ΔCp) for protein denaturation. Heat capacity changes associated with protein unfolding are primarily because of changes in hydration of side chains that were buried in the native state but become solvent exposed in the denatured state. Many factors are responsible for the folding and stability of native proteins, including hydrophobic interactions, hydrogen bonding, conformational entropy, and the physical environment.

The data from a typical DSC experiment is called a thermogram. Protein unfolding is recognized as a sharp endothermic peak centered at a characteristic temperature called the transition midpoint (T_m). T_m, ΔH and ΔCp of the transition are

calculated by fitting the data to a two-state transition model using non-linear least squares regression analysis.

2.2.5. Mass Spectrometry (MS)

Two advances in the MS of peptides and proteins were announced independently towards the end of 1980s and, with ongoing developments, are still at the center of current methodologies. Both developments were the refinement and application of ionization techniques, namely Electrospray Ionization (ESI) and Matrix-assisted Laser Desorption and Ionization (MALDI), and both increased the sensitivity and upper mass limit of peptides or proteins amenable to mass spectrometry. Such was the impact of these techniques that the originators were awarded Nobel Prize in 2002.

Although the process of ionization is not fully understood, ESI is simple in practice in that the method only requires a solution of the analyte to be sprayed across an electric field. The resulting analyte ions are sampled into a mass analyzer and the sample usually observed as protonated molecules in the positive ion detection mode. Although initially used to measure intact proteins, ESI was also found to detect lower molecular mass peptides with newfound sensitivity. One advantage of the technique is that as the molecular mass of the peptide increases, so do the dimensions of the molecule and the number of basic sites within the molecule; hence, higher mass samples can collect more protons per molecule. Thus, a peptide of molecular mass 500 daltons maybe only slightly protonated, whereas a protein of mass 20 kDa may average 20 protons per molecule and the sample ions would appear at mass-to-charge 500 and 1000, respectively, in an electrospray mass spectrum.

Atoms can be deflected by magnetic fields - provided the atom is first turned into an ion. Electrically charged particles are affected by a magnetic field although electrically neutral ones are not affected. The sequence of events is as follows:

Stage 1: Ionization

The atom is ionized by knocking one or more electrons off to give a positive ion. This is true even for chemical species which one would normally expect to form negative ions (e.g. chlorine) or never form ions at all (e.g. argon). Mass spectrometers always work with positive ions.

Stage 2: Acceleration

The ions are accelerated so that they all have the same kinetic energy.

Stage 3: Deflection

The ions are deflected by a magnetic field according to their masses. The lighter they are, the more deflected they are. The amount of deflection also depends on the number of positive charges on the ion - in other words, on how many electrons were knocked off in the first stage. The more the ion is charged, the more it gets deflected.

Stage 4: Detection

The beam of ions passing through the machine is detected electrically. A schematic of mass spectrometer is shown in Figure 2.7.

The protein was dialyzed in carbonate buffer (25mM sodium carbonate, 10mM NaCl, and pH 9.6) before sending the sample for mass spectrometry at UIUC. This buffer was used to avoid the ionization of phosphate ions when subjected to electron spray ionization.

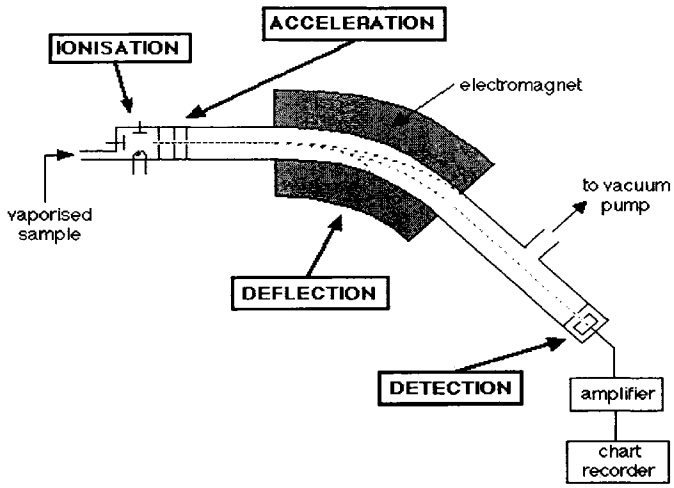

Figure 2.7. Schematic Diagram of Mass Spectrophotometer.

CHAPTER 3

RESULTS

3.1. Tensin-SH2 Domain Overexpression

The SH2 gene ligated to pET-14b was transformed into BL21 (DE3) pLysS strain of *E. coli* using the heat shock method. 50 µl of BL21 (DE3) cells were put in an Eppendorf tube and incubated on ice for 10 minutes to thaw. The DNA encoding SH2 gene was added to these tubes and placed in a water bath at 42 °C for 45 s. These tubes were placed back on ice for 2 min to avoid damage to the cells. 1 ml of LB was added to these tubes and incubated at 37 °C for 1 h. 200 µl of this culture was plated using a sterile plater on three different prewarmed agar plates. These agar plates had ampicillin (50 µg/ml) and chloramphenicol (34 µg/ml) as antibiotic resistance. The plates were incubated at 37 °C overnight. The following day, a few colonies were picked from each plate and added to a falcon tube containing 4.9 ml of fresh LB broth in the presence of ampicillin (50 µg/ml) and chloramphenicol (34 µg/ml). These tubes were incubated at 37 °C at 240 rpm overnight. The following day, the tubes that had turned cloudy were stored in 50 % sterile glycerol to test for protein overexpression.

A 100 ml overnight bacterial culture was prepared with the glycerol stock of the colony picked from the agar plate in the presence of ampicillin (50 µg/ml) and

chloramphenicol (34 μg/ml). The following day, a fresh 500 ml culture was inoculated with the overnight culture in the presence of antibiotics at the same concentration. The culture was grown till OD_{600} = 0.5; the log phase of growth for *E. coli*; at which point the protein overexpression was induced with 0.4 mM IPTG. This concentration was empirically determined. 2 ml aliquots were taken from the culture before induction; and every hour post induction for 3 h. At the end of 3 h the cells were harvested by centrifuging at 6500 rpm for 10 min. These cells were stored at -80 °C till further use for purification. The selected aliquots were analyzed on a 12% SDS-PAGE to analyze the overexpression as shown in Figure 3.1

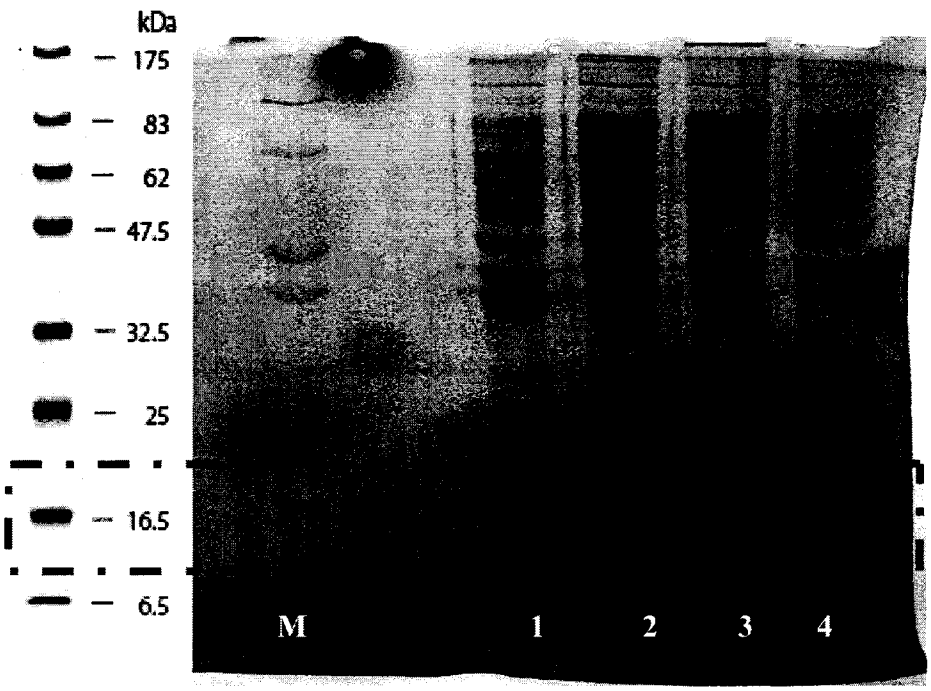

Figure 3.1. 12% SDS-PAGE Showing the Overexpression of SH2 Domain in *E. coli.* Lane M: Protein Marker; Lane 1: Aliquot 2 h Post Induction; Lane 2: 3 h Post Induction; Lane 3: 4 h Post Induction; and Lane 4: Aliquot Pre-induction with IPTG.

3.2. Immunoblot Analysis

Chromogenic detection was used to view the presence of His-tagged target protein. This method employs the use of a substrate 4-chloro-1-naphthol which upon reacting with HRP produces a blue colored insoluble precipitate. A small test was done to confirm this color change wherein solutions of HRP at varying concentrations were reacted with 4-chloro-1-naphthol and the reaction was observed (Figure 3.2A). In this experiment, 6x-His tagged ladder was used as a positive control. This ladder is a mixture of five different His-tagged proteins. An uninduced sample was collected and used as a negative control (Figure 3.2B). The dot blot and detection protocol for SH2 domain was followed from the Qiagen manual. Different dilutions of the overexpressed protein after 3h were made and blotted onto the nitrocellulose membrane (Figure 3.2C). The controls were not used on this membrane as they had been used before and because of the lack of space on the membrane.

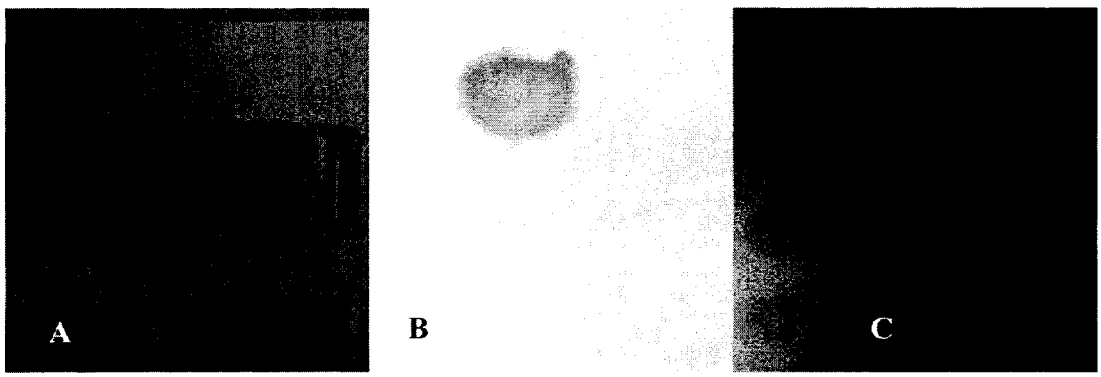

Figure 3.2. Immunodetection. (A) The reaction Between Varying Concentrations of HRP with 4-chloro-1-naphthol (B) The result of the Positive and Negative Controls in the Dot Blotting Technique (C) The Detection of Different Dilutions of the SH2 Protein on a Nitrocellulose Membrane.

3.3. Protein Purification

3.3.1. Protein Purification Protocol for Inclusion Bodies

Resuspension buffer was added to cells harvested 3 h post induction with IPTG. They were sonicated for 30s X 3; with 30 s interval between each pulse. The cells were centrifuged at 10000 rpm for 20 min and the resuspended pellet was sonicated in isolation buffer as before. The resuspended pellet was again centrifuged at 10000 rpm and this was repeated twice. The resulting inclusion bodies were solubilized in solubilization buffer and loaded onto the HisTrap column. The bound protein was washed on-column using refolding buffer. On-column refolding of the protein was done by running a linear 6-0M gradient of urea. The unbound proteins were washed off using wash buffer and eluted using a linear gradient of 0 M-0.5 M imidazole. Fractions of eluted protein were collected by monitoring the absorbance at 280 nm; an inbuilt feature in the AKTAprime. These fractions were analyzed on a SDS-PAGE for yield as well as purity as shown in Figure 3.3.

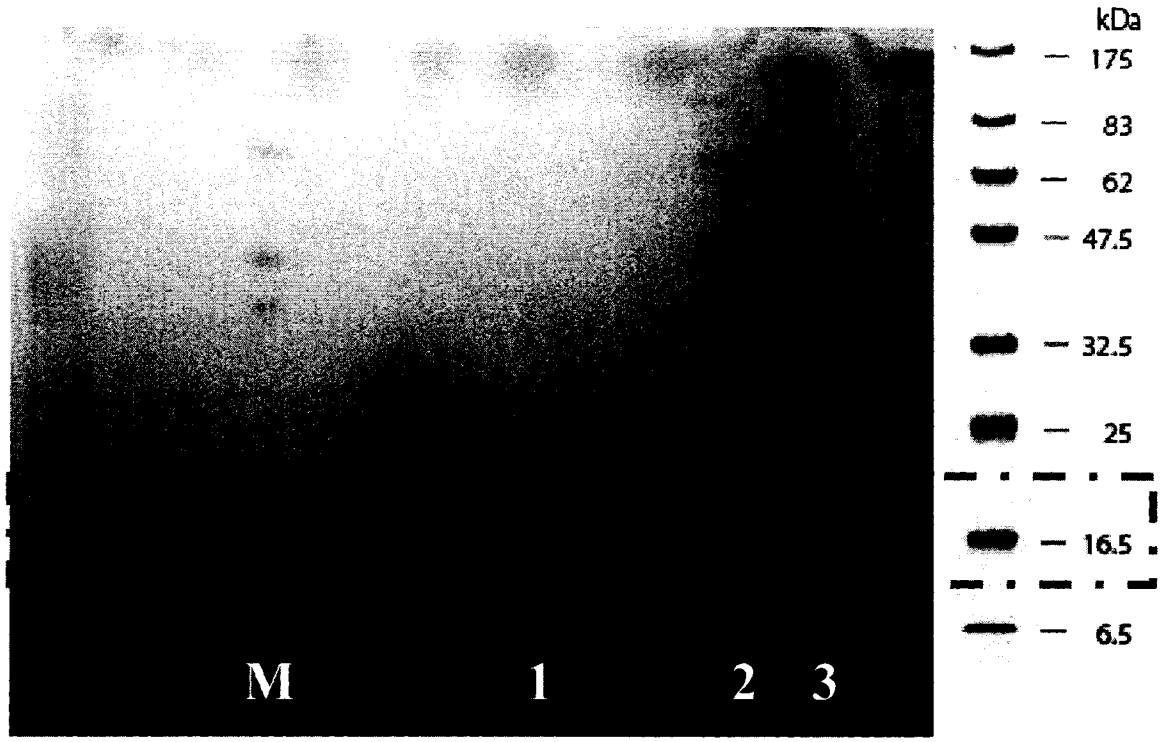

Figure 3.3. SDS-PAGE Showing Analysis of Purified Fractions. Lane M: Protein Molecular Weight Marker; Lane 1: Purified SH2 Domain; Lane 2: Purified SH2 Domain with Contaminants; Lane 3: Overexpressed SH2 Domain

3.3.2. Determining the Solubility of Overexpressed Protein

Bacterial cells collected 3 h post induction were lysed by sonication on ice for 40 s, and the resulting suspensions were centrifuged at 10,000 rpm for 15 min at 4 °C. Thereafter, small amounts of the resulting cell pellet and supernatant were analyzed by SDS-PAGE, as shown in Figure 3.4, to check for the amount of protein in expressed in the soluble fraction and in the inclusion bodies.

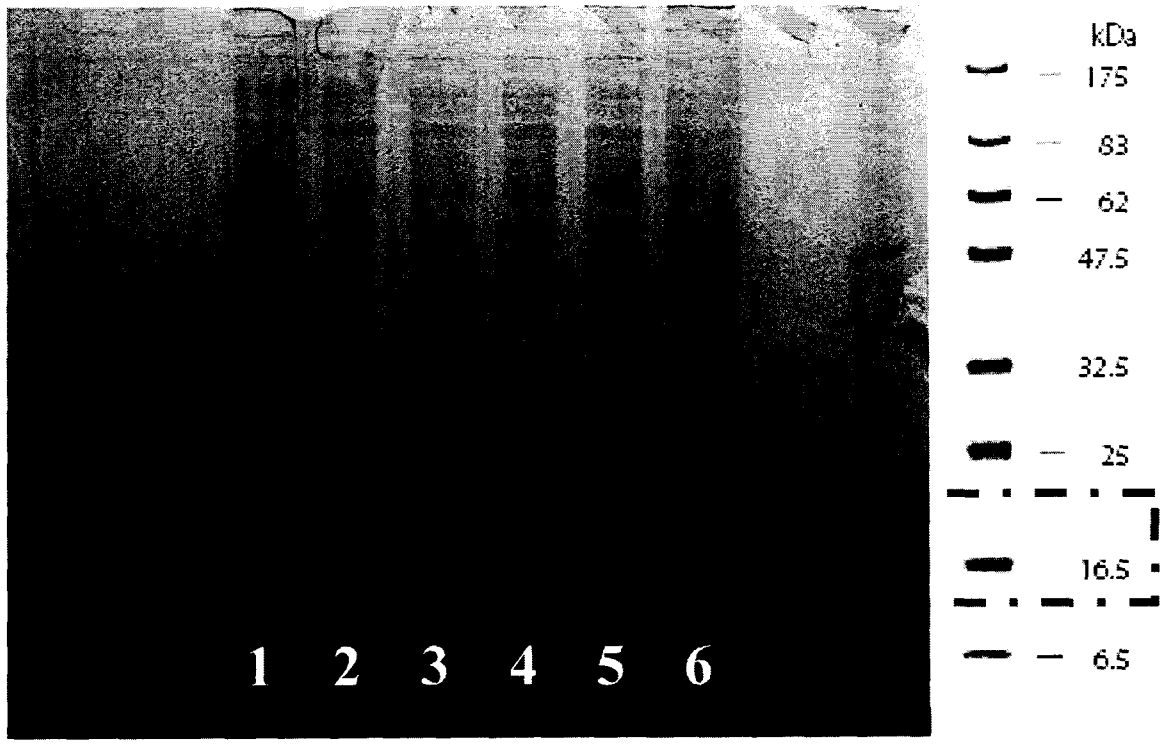

Figure 3.4. 12% SDS-PAGE Showing the Protein Expressed in Soluble Fraction and Inclusion Bodies. Lane 1: Cell Pellet (Inclusion Bodies); Lanes 2, 3, 4, and 5: Supernatant (Soluble Fraction); Lane 6: Spillover from Lane 5.

3.3.3. Protein Purification of the Soluble Fraction

The cell pellets harvested after 3 h post induction were added to resuspension buffer and sonicated for 30s X 4; with 30s interval in between. The suspension was centrifuged at 10000 rpm for 20 minutes. The supernatant was collected and filtered through a Whatman filter paper followed by a 45 μm filter using a syringe. This soluble fraction was loaded onto the HisTrap column and washed with 20 column volumes of binding buffer. The unbound proteins were washed off until the absorbance at 280 nm reached zero. Next, the column was washed with 20 column volumes of wash buffer to elute the weakly bound proteins off the column. The protein bound onto the column was eluted using a 0 M-0.5 M linear gradient of imidazole. The fractions collected at various

time intervals were analyzed on a SDS-PAGE to determine the purity and yield as shown in Figure 3.5.

Figure 3.5. 12% SDS-PAGE Analysis Showing Fractions Collected from Soluble Fraction Purification. Lane 1: Overexpressed SH2 Protein; Lanes 2, 3, 4, and 5: Purified SH2 Protein Fractions.

3.4. Initial Protein Characterization

The amino acid sequence of the tensin-SH2 domain was used to determine initial characterization parameters like molecular mass, isoelectric point (pI) and extinction co-efficient. The isoelectric point determination was necessary to determine the buffer compatibility so as to keep the protein soluble. The extinction co-efficient was necessary to determine the protein concentration. This analysis was carried out using the ExPASy (**Ex**pert **P**rotein **A**nalysis **Sy**stem) server of the Swiss Institute of Bioinformatics (SIB). The calculated molecular mass of this protein was 16734 daltons (da) and the isoelectric

point was 9.15 with the His-tag. The extinction coefficient of the SH2 domain with His-tag was 10930 $M^{-1}cm^{-1}$.

3.5. Limited Proteolysis

3.5.1. Analysis by SDS-PAGE

His-tag is about 20 amino acids in length; which implies that the cleaved protein is about 2 kDa less in molecular weight than the uncleaved fraction. The molecular weight of thrombin is 35 kDa. A 1:25 dilution of the thrombin was made using the dilution buffer supplied by Novagen. This reaction mixture was incubated at room temperature on a rocker. Aliquots were collected after 2, 4, 8 and 16 h and the reaction was stopped by heating the aliquot at 100 °C for 2 min and adding the SDS-PAGE loading buffer. These aliquots as well as the controls were analyzed on a 12% SDS-PAGE as shown in Figure 3.6.

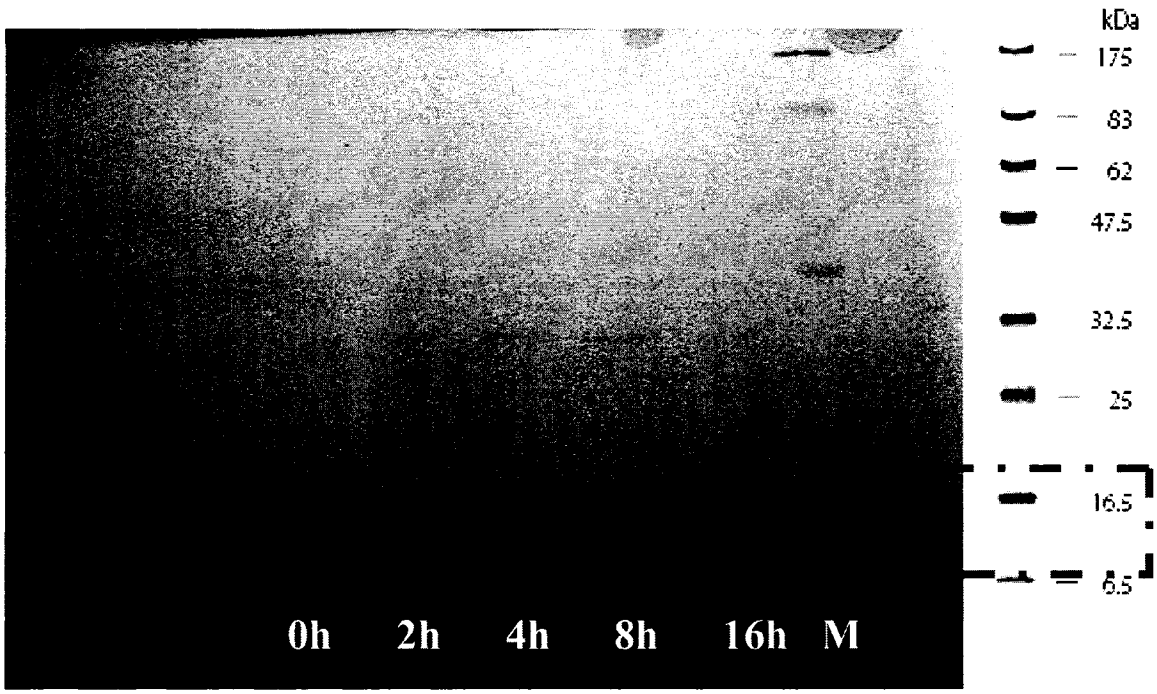

Figure 3.6. 12% SDS-PAGE Showing Thrombin Cleavage Analysis.

3.5.2. Analysis by Capillary Electrophoresis

Tensin-SH2 domain and thrombin were analyzed on the CE individually for purity and migration time. The SH2 protein sample used to demonstrate the cleavage of His-tag using CE was prepared as described in 2.1.8.2. The protein was in 25 mM sodium phosphate, 10 mM NaCl, 1 mM β-mercaptoethanol, and pH 7.8. Aliquots were collected at 8 and 16 h intervals and boiled at 100 °C for 5 min to inhibit thrombin activity. SDS-PAGE loading buffer was avoided to inhibit the reaction because of the possible damage to the capillary. The resulting electropherograms are shown in Figure 3.7A, Figure 3.7B, Figure 3.7C, and Figure 3.7D.

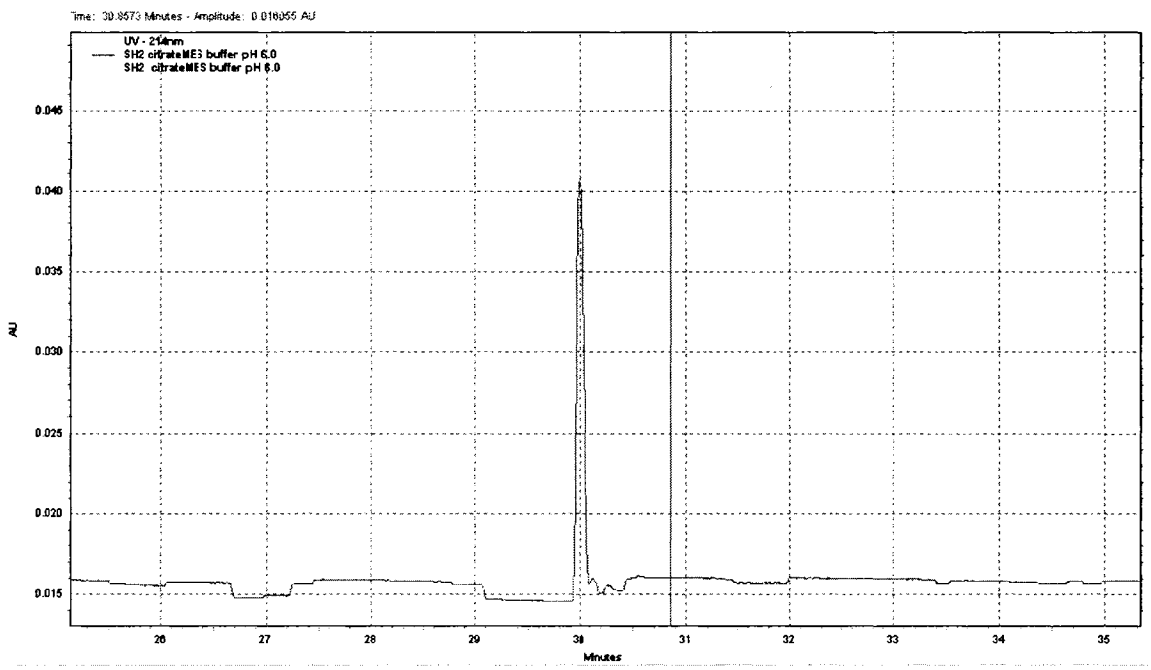

Figure 3.7A. Electropherogram Showing Pure SH2 Domain.

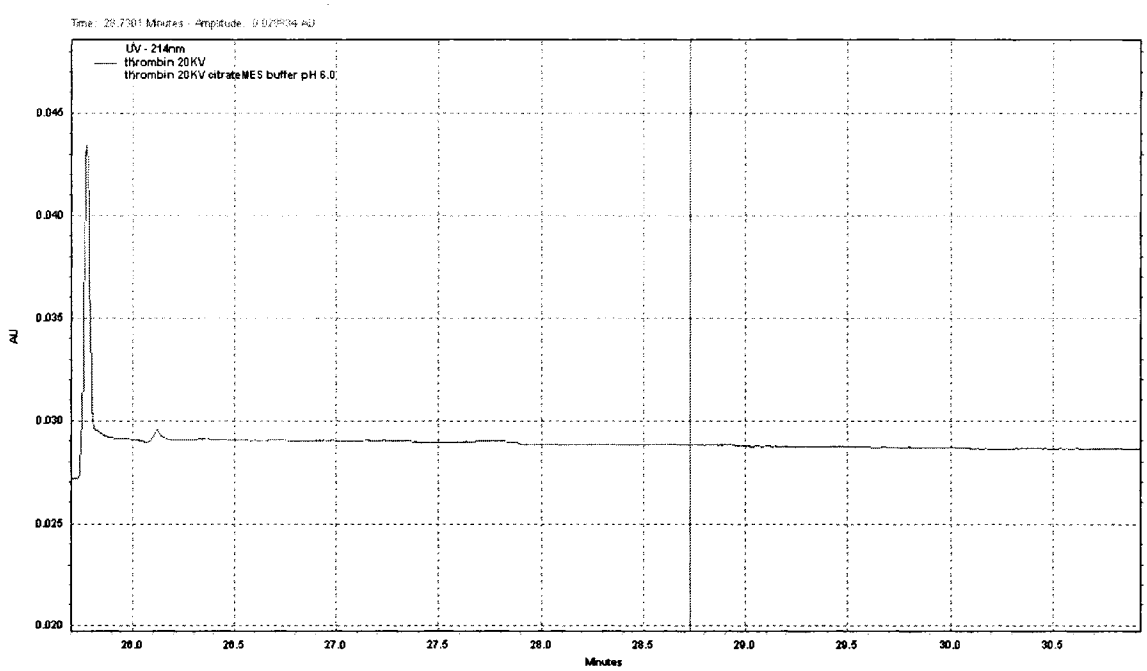

Figure 3.7B. Electropherogram Showing Pure Thrombin.

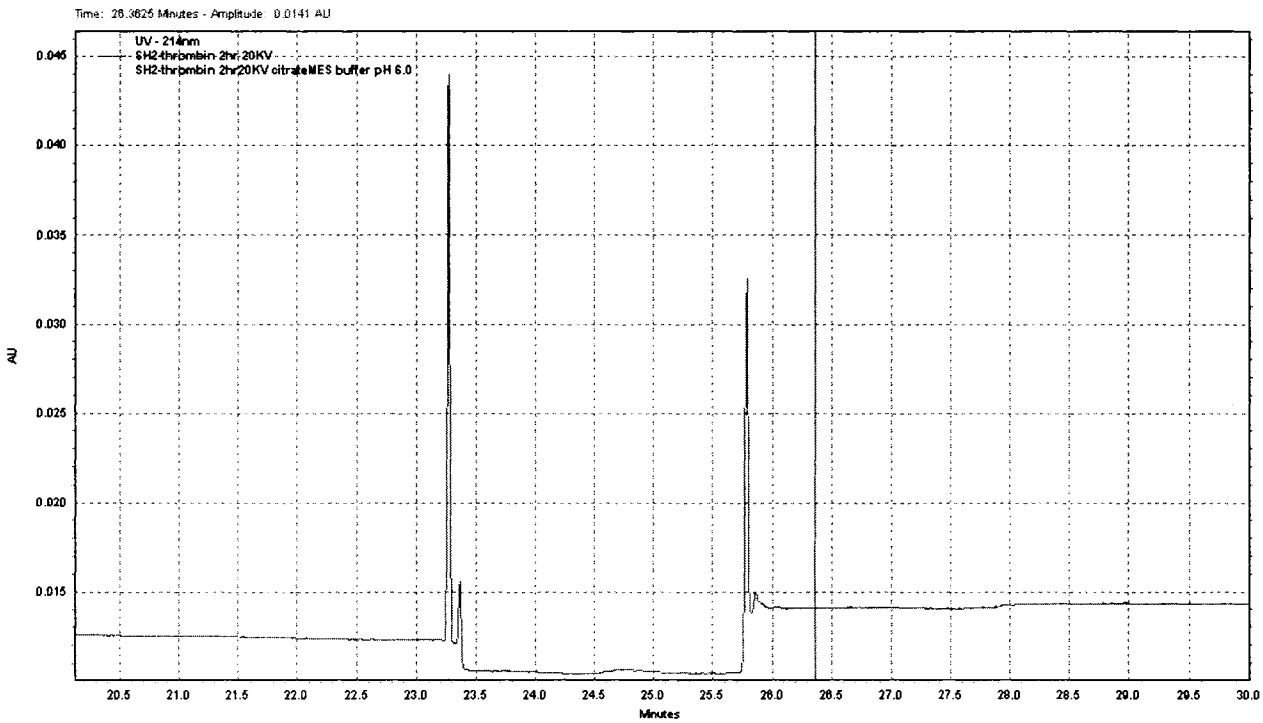

Figure 3.7C. Electropherogram Showing Thrombin Cleavage After 8h.

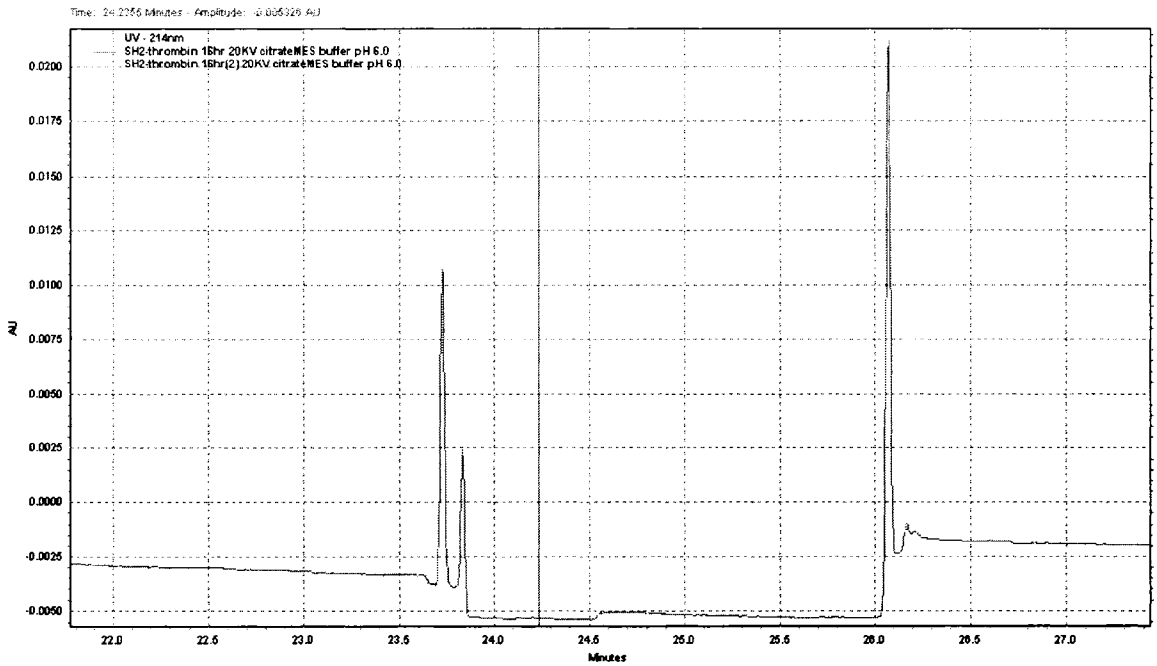

Figure 3.7D. Elelctropherogram Showing Thrombin Cleavage After 16h.

3.6. Secondary Structure Determination

3.6.1. Secondary Structure Determination at Optimal pH Value

Figure 3.8 shows SH2 secondary structure in 0 M and 6 M Guanidine HCl. The protein was in 25 mM sodium phosphate, 10 mM NaCl, 1 mM β-mercaptoethanol, and pH 7.8. Data were not collected below 213 nm for 6 M spectra because of interference by chloride ions. All spectra are baseline subtracted. The secondary structure content of the tensin-SH2 domain, obtained by deconvoluting the far-UV CD spectra using CDPro is given in Table 3.1.

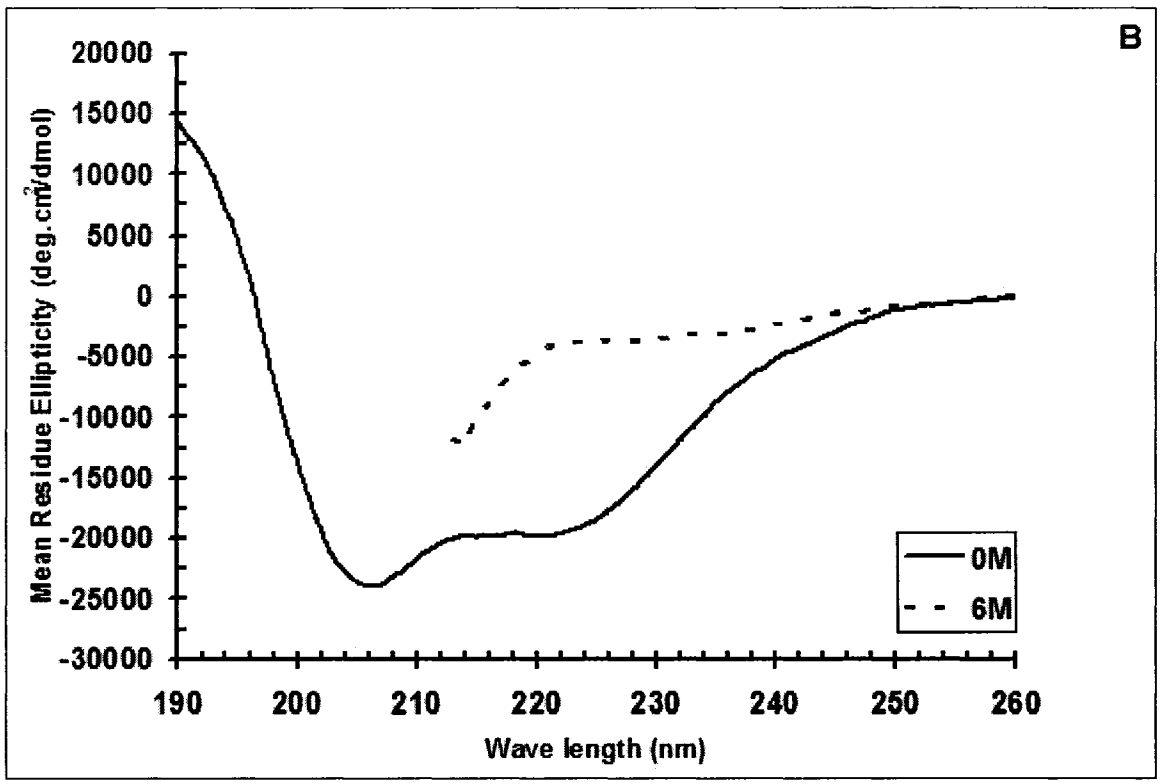

Figure 3.8. SH2 Domain in 0 M at pH 7.8 or 6 M GdnHCl.

Table 3.1. Estimates of Secondary Structure by Deconvolution in 20 mM phosphate buffer, 10 mM NaCl 1mM β-mercaptoethanol, and pH 7.8.

	SH2 domain at pH 7.8			
	α helix	β sheet	coil	turn
CONTINLL	0.212	0.214	0.319	0.254
SELCON3	0.243	0.214	0.313	0.244
CDSSTR	0.245	0.206	0.317	0.229
Best estimate	0.23± 0.02	0.21± 0.01	0.32± 0.01	0.24± 0.01

3.6.2. Secondary Structure Determination at Extreme pH Values

To observe the secondary structure over an extreme pH range, the protein sample was dialyzed in a buffer containing 25 mM sodium phosphate, 10 mM NaCl, 1 mM β-mercaptoethanol, and pH 12. The resulting spectrum is shown in Figure 3.9.

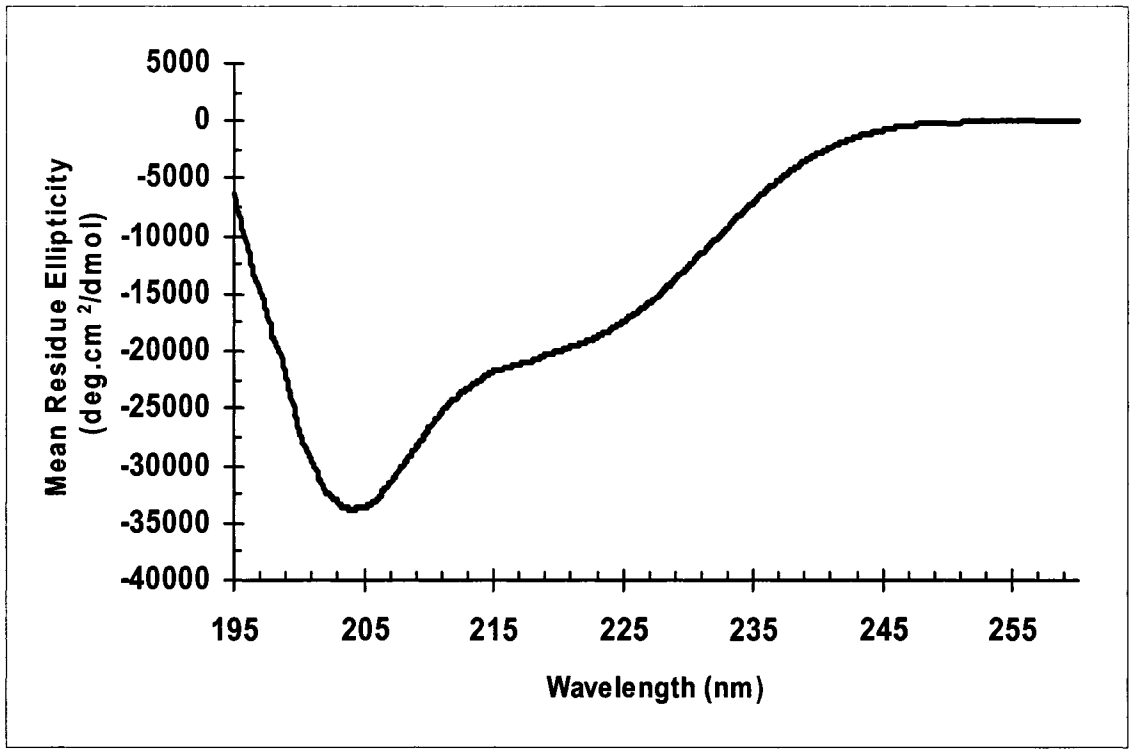

Figure 3.9. Far-UV CD Spectra of SH2 Domain at pH 12.0.

Similarly, the secondary structure was determined in a buffer containing 25 mM sodium phosphate, 10 mM NaCl, 1 mM β-mercaptoethanol, and pH 2.0. as shown in Figure 3.10.

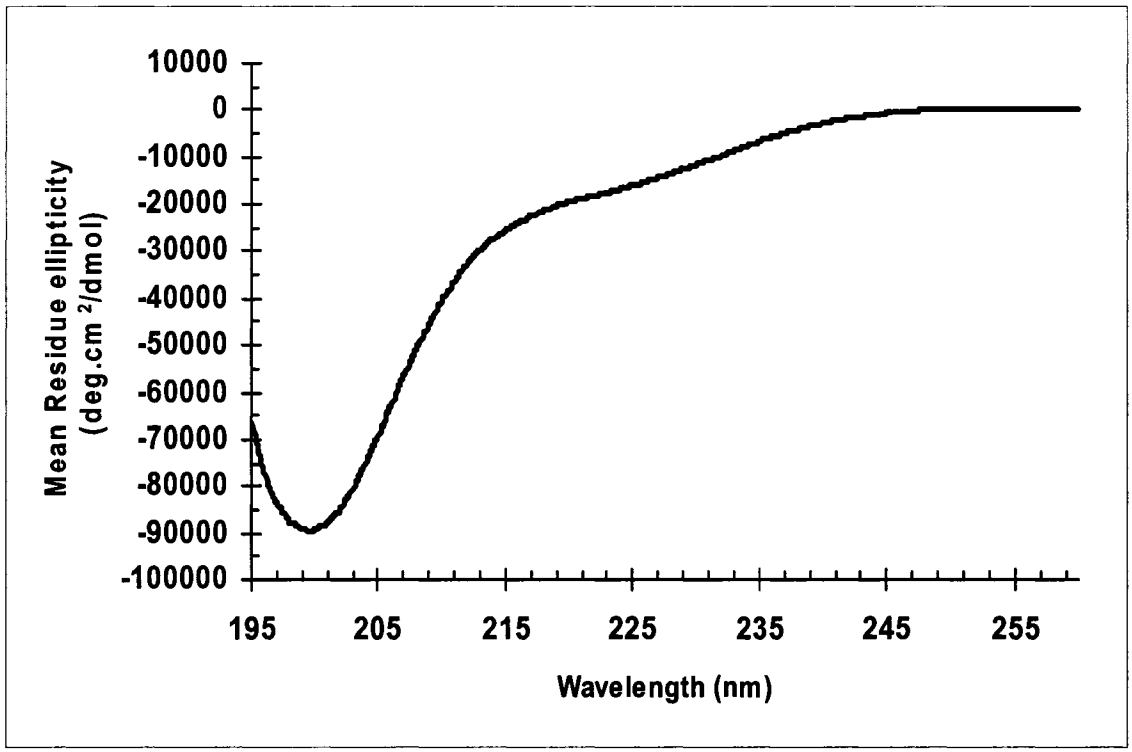

Figure 3.10. Far-UV CD Spectra of SH2 Domain at pH 2.0.

The above sample at pH 2.0 was redialyzed in 25 mM sodium phosphate, 10 mM NaCl, 1mM β-mercaptoethanol, pH 7.8 to observe the effect of change in pH over the secondary structure as shown in Figure 3.11.

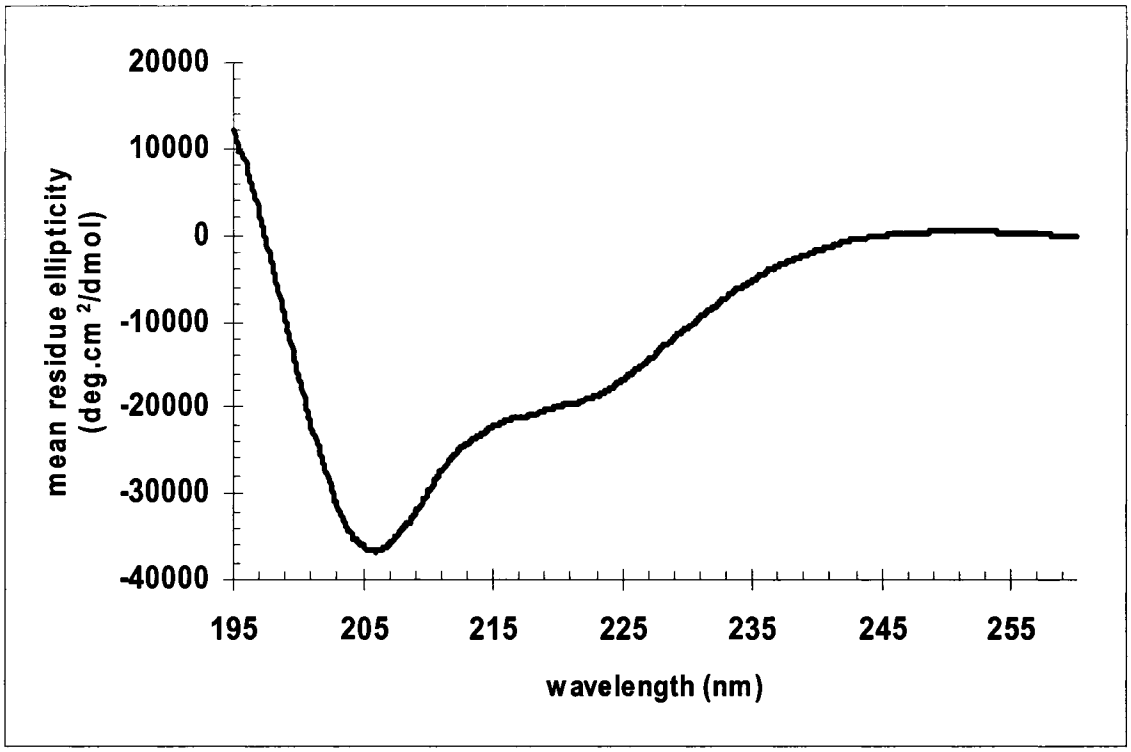

Figure 3.11. Far-UV CD Spectra of SH2 Domain After Readjusting the pH Value from 2.0 to 7.8.

Figure 3.12 shows an overlay of the secondary structure analysis of the tensin-SH2 domain at different pH values.

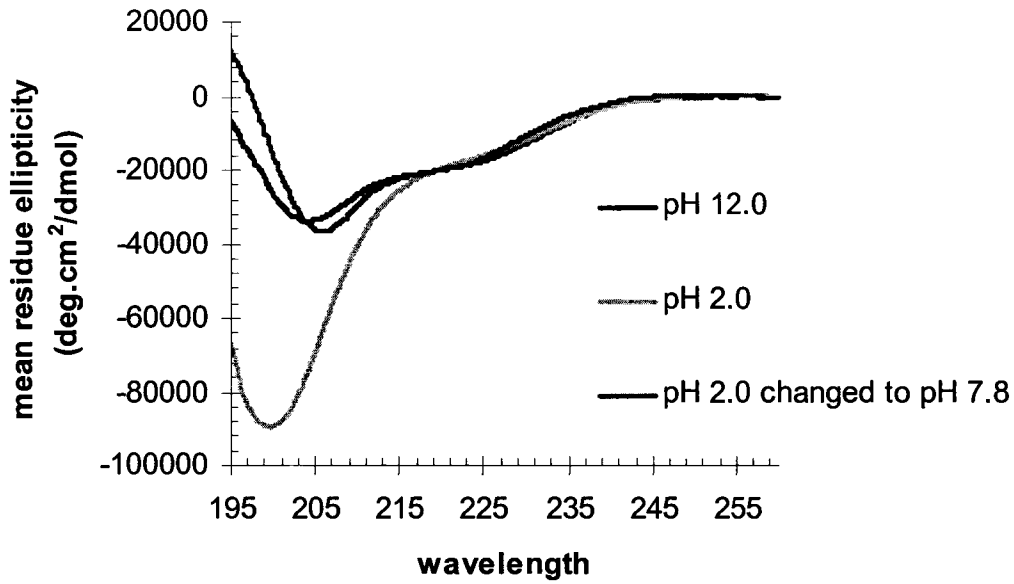

Figure 3.12. Overlay Showing Secondary Structure Determination at Different pH Values.

3.6.3. Sequence Alignment by Homology Modeling

Figure 3.13 shows SH2 domain of human tensin aligned with other homologs.

This amino acid sequence alignment was done using Genedoc (Zhao, 2006).

```
tensin  DTSKYWYKPEISREQAIALLK--DQEPGAFIIRDSHSFRGAYGLAMKVSSPPPTIMQQNK
1BHF    LEPEPWFFKNLSRKDAERQLLAPGNTHGSFLIRESESTAGSFSLSVRDFDQ--NQGEVVK
1D4W    MDAVAVYHGKISRETGEKLLLA-TGLDGSYLLRDSESVPGVYCLCVLY-------HGYIY
1P13    --AEEWYFGKITRRESERLLLNPENPRGTFLVRESETTKGAYCLSVSDFDN--AKGLNVK

tensin  KGDMTHELVRHFLIETGPRGVKLKGCPNEPNFGSLSALVYQHSIIPLALPCKLVIPNRD
1BHF    HYKIRNLDNGGFYIS-----------PRITFPGLHELVRHYTNASDGLCTRLSRPCQT
1D4W    TYRVSQTETGSWSAETAPGV-------HKRYFRKIKNLISAFQKPDQGIVIPLQYPVEK
1P13    HYKIRKLDSGGFYIT-----------SRTQFSSLQQLVAYYSKHADGLCHRLTNVCP-
```

Figure 3.13. Human Tensin-SH2 Domain Sequence Alignment with Other SH2 Domains of Lck, SLAM, and Src.

3.7. Tertiary Structure Determination

The near-UV CD spectra of tensin-SH2 domain was obtained in 25 mM sodium phosphate, 10 mM NaCl, 1mM β-mercaptoethanol, pH 7.8 as shown in Figure 3.14.

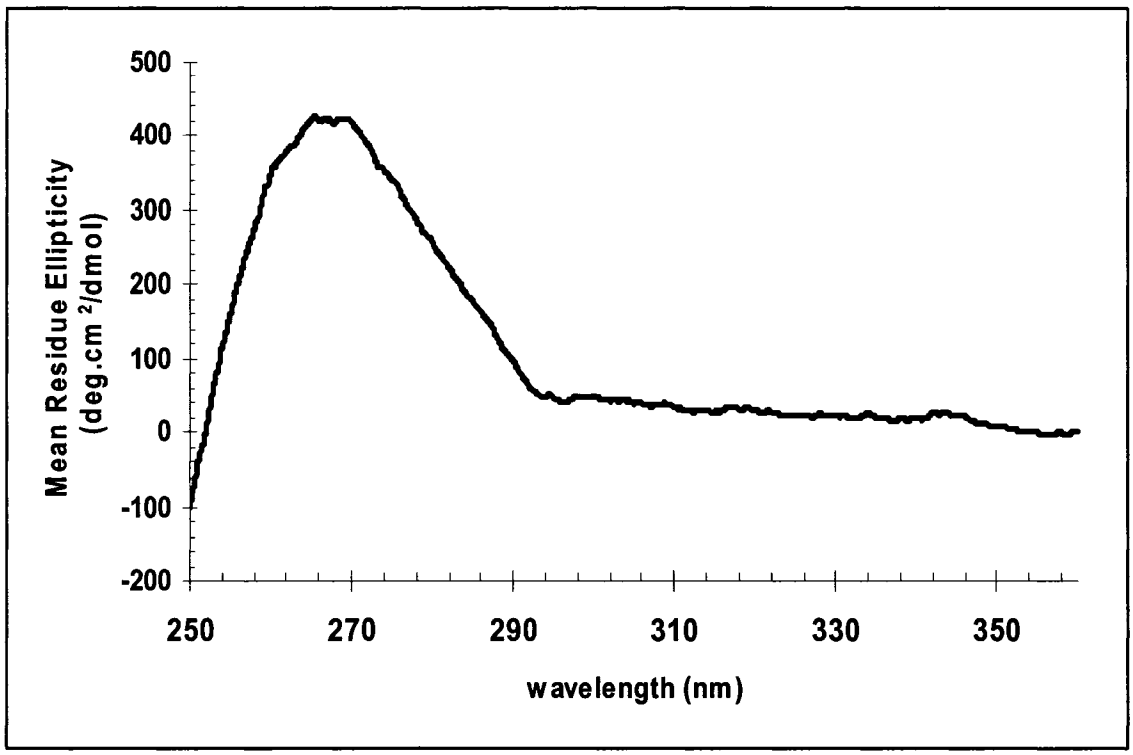

Figure 3.14. Near-UV CD Spectra of Tensin-SH2 Domain.

3.8. Biophysical Characterization

3.8.1. Mass Spectrometry

The SH2 sample was dialyzed in 25 mM sodium carbonate, 10mM NaCl, 1 mM β-mercaptoethanol, pH 9.6 and sent to University of Illinois at Urbana-Champaign (UIUC) Protein services facility for mass spectrometry analysis. The choice of buffer was based on the fact that phosphate ions ionize upon undergoing electrospray ionization (ESI) thereby interfering with the detection process. The exact molecular mass of the protein is shown in Figure 3.15.

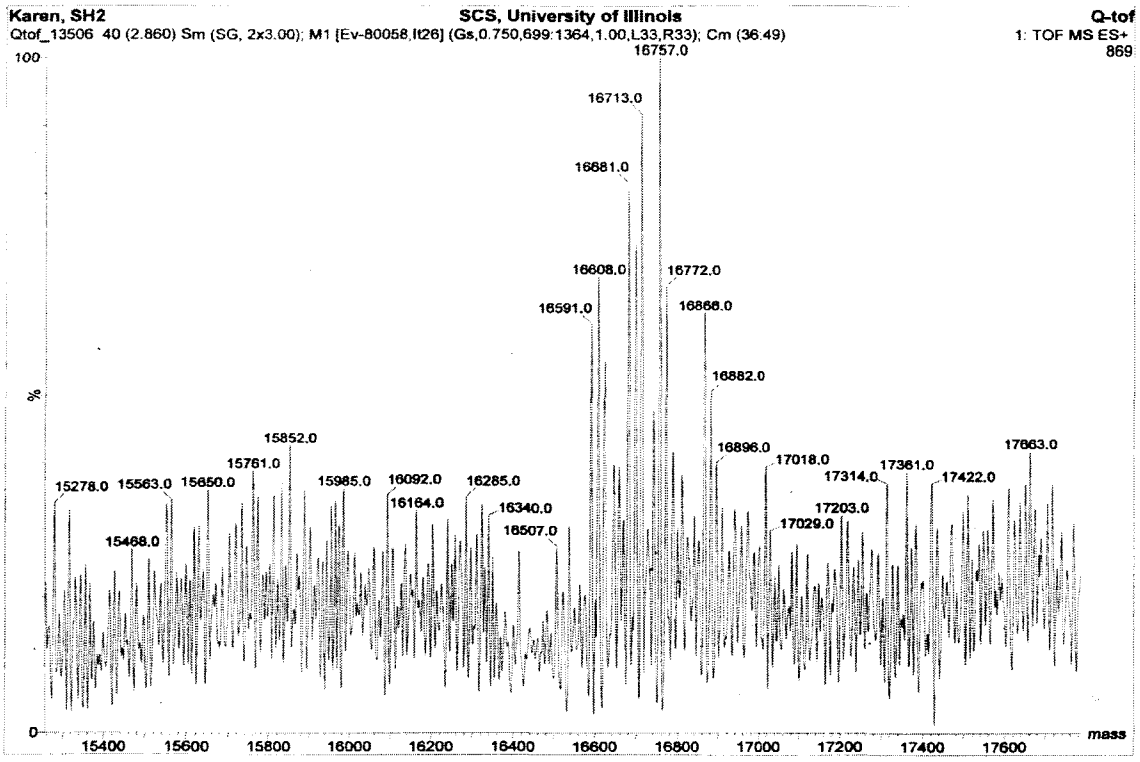

Figure 3.15. Mass Spectrometry Result for Tensin-SH2 Domain Showing the Expected Peak at 16757 Daltons.

3.8.2. Guanidine HCl (GdnHCl) Induced Denaturation Monitored by CD

Chemical denaturation of the SH2 domain was monitored by measuring the change in ellipticity at 220 nm as a function of increase in the concentration of GdnHCl. Ellipticity at 220 nm as a function of guanidine concentration is shown in Figure 3.16.

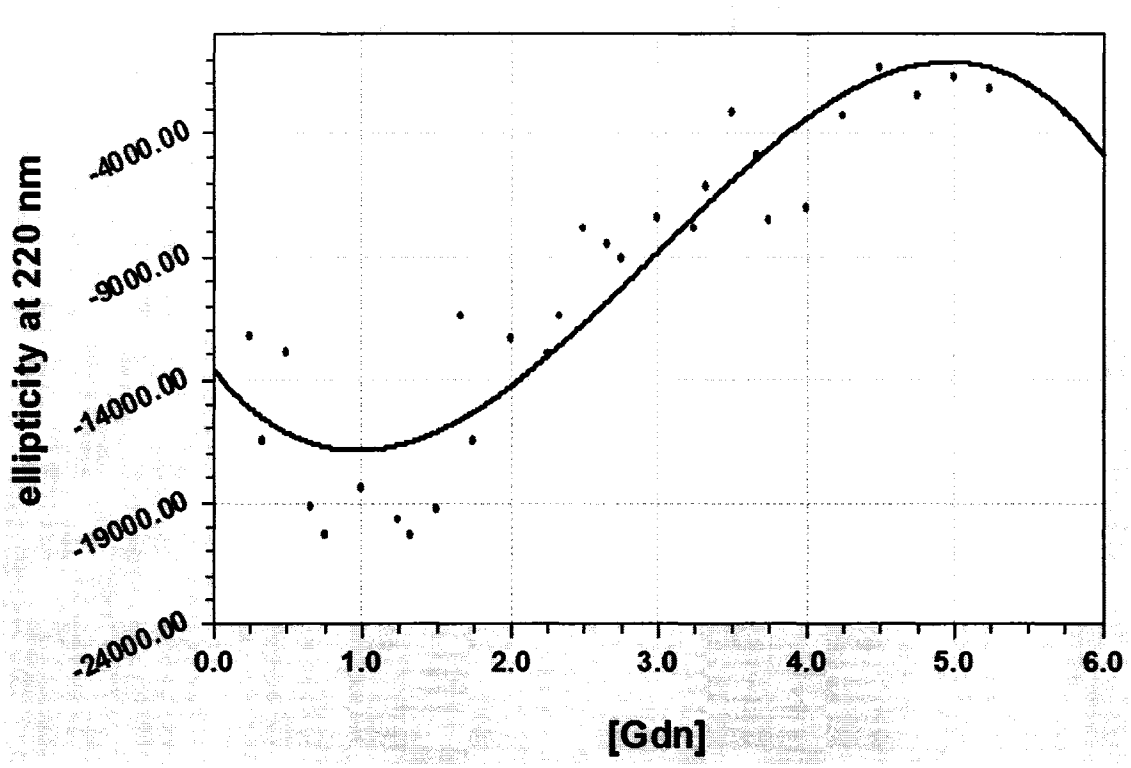

Figure 3.16. Change in Ellipticity at 220 nm as a Function of GdnHCl Concentration for Tensin-SH2 Domain at pH 7.8.

3.8.3. Heat Induced Denaturation Monitored by DSC

The calorimetric profile of the tensin SH2 domain is shown in Figure 3.17. The data were obtained under reducing conditions (1 mM β-mercaptoethanol); tensin being an intracellular protein.

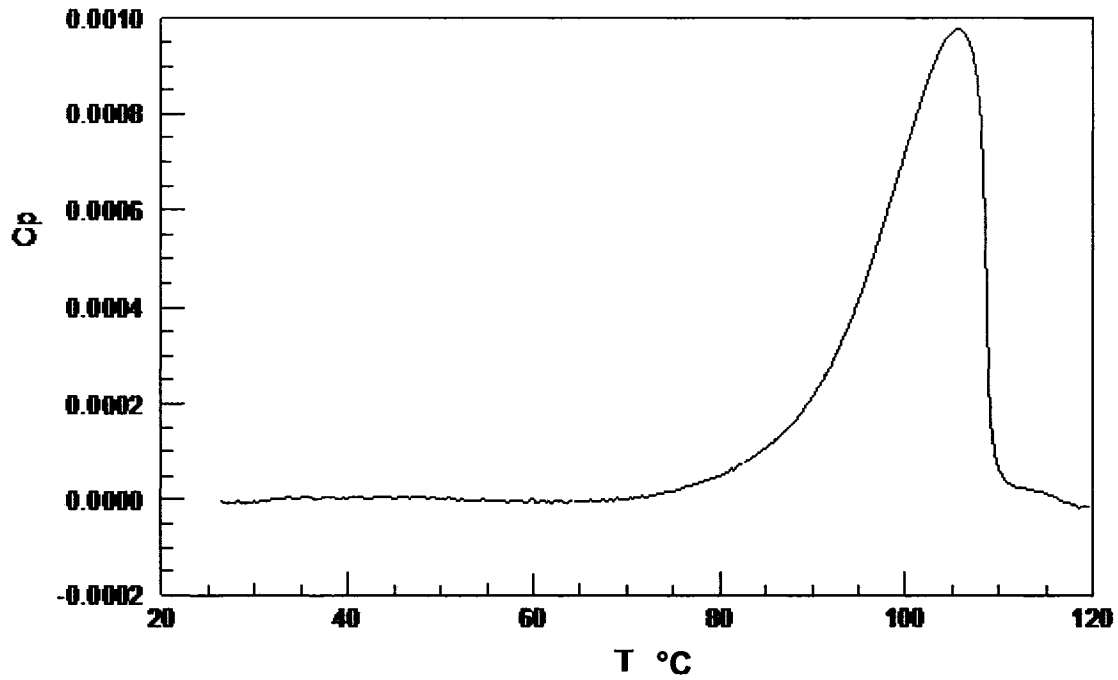

Figure 3.17. Calorimetric Profile of Tensin-SH2 Domain.

CHAPTER 4

DISCUSSION

SDS-PAGE analysis of cells harvested 4 h post induction revealed a significant amount of target protein being expressed. Immunblot analysis confirmed the presence of His-tag in the overexpressed SH2 protein. The initial purification protocol for proteins expressed in inclusion bodies resulted in little or no target protein. Consequently, the cells harvested after overexpression were sonicated and the resulting pellets and supernatant analyzed on a SDS-PAGE. This revealed that a large amount of the SH2 domain was expressed in soluble fraction. Subsequently, the purification protocol was modified and the soluble fraction of the protein was loaded onto the column which resulted in an improved yield of 0.2 mg/ml of the target protein.

SDS-PAGE analysis of the His-tag removal shows that the His-tag was cleaved only after 16 h, suggesting that no cleavage took place in 8 h, or it was not sufficient to be detectable under the used staining conditions. To confirm this finding, a novel approach of CE migration time was used. The electropherogram for the SH2 protein showed a peak of 0.025 AU_{214} after 30 min whereas thrombin showed a peak of 0.017 AU_{214} after 25.5 min. The aliquots collected after 2 and 4 h intervals showed no increase in the number of peaks as it should have been in case of appreciable cleavage being taken

place. The aliquot analyzed after 8 h showed thrombin with a peak of 0.022 AU_{214} after 25.5 min; and two other peaks of 0.027 AU_{214} at 23.3 min and 0.005 AU_{214} at 23.4 min. The first peak of 0.027 AU_{214} is the uncleaved SH2 domain as the amplitude is appreciably close to the pure SH2 domain electropherogram. The second closely following peak of 0.005 AU_{214} is the SH2 domain with the cleaved His-tag. This argument is supported by the electropherogram after 16 h; where thrombin is seen as having a peak of 0.026 AU_{214} at 26.1 min as before preceded by two peaks of 0.026 AU_{214} at 23.7 min and 0.0075 AU_{214} at 23.8 min. The 50% increase in the amplitude of the closely following second peak is attributed to more cleavage been taken place which results in the increase in the absorbance of the His-tag cleaved SH2 domain on the electropherogram.

SH2 domains belong to the $\alpha+\beta$ structure class. The three-dimensional structures of all known SH2 domains are similar; having a large anti-parallel β sheet flanked by two α helices on either side (Kuriyan and Cowburn, 1993, 1997). The CD spectra of $\alpha+\beta$ proteins typically show two minima, around 208-210 nm and 220 nm, and one maximum around 190-195 nm (Greenfield, N.J., 1996). The far-UV CD spectrum of the tensin SH2 domain at pH 7.8 has minima at 208 nm and 220 nm and a maximum at 195 nm. The minima are attributable to helical structure (Dafforn et al., 2004). The figure also shows the SH2 domain in 6 M guanidine. There is evidence for some residual structure. The SH2 domain spectrum in 6 M guanidine looks less like a random coil than the tensin-C2 domain spectrum, apparently indicating that the thermostability of the SH2 domain is greater than that of the C2 domain.

The far-UV CD spectrum of tensin-SH2 domain at pH 7.8 is similar to STAT3-SH2 domain (Haan et al., 1998) and Btk SH2 domain (Tzeng et al., 2000) obtained at physiological pH. The common feature of all the spectra is a shoulder at 220 nm and minima at 208-210 nm which are attributable to helical structures; which implies that tensin-SH2 domain is also a part of $\alpha+\beta$ structure class like other SH2 domains. The far-UV CD spectrum of the STAT3-SH2 domain retains its secondary structure at pH 4.5; however tensin-SH2 domain unfolds atleast partially at pH 2.0 losing the secondary structure. As the pH decreases, there is a change in the net ionization state of acidic side chains which results in a net increase in the charge on the surface of the protein. So at low pH values, the positive charges repel each other by electrostatic interactions and this destabilizes the folded conformation of the protein.

Figure 4.1 shows the structure of two SH2 domain templates and the tensin model colored by secondary structure. Table 4.1 gives the quantitative measurement of the structures. RMSD of backbone between reference proteins and tensin model are calculated, the highest value is 1.64 Å (between 1D4W and 1P13), still low and thereby close to average protein structure characterization. Figure 4.1 shows that the SLAM, Src, and tensin-SH2 domain have one large anti-parallel β-sheet, flanked by two α-helices on either side of it (Zhao, 2006).

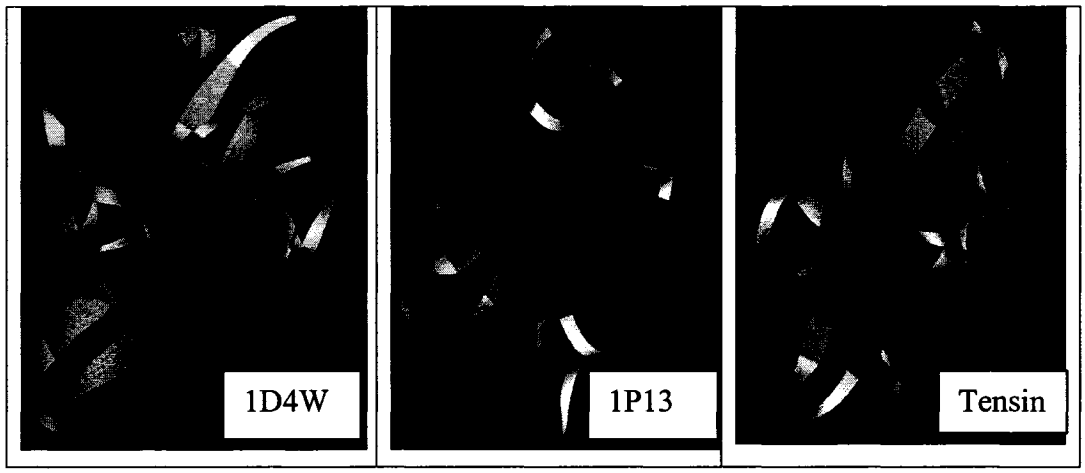

Figure 4.1. Ribbon Diagram of SH2 domain of 1D4W, 1P13 and Tensin. Red, α-helix; Blue, β-sheets; Green, Turns; White, Irregular Structure.

Table 4.1. RMSD of backbone between reference proteins and tensin in Å.

	1D4W	**1LKK**	**1P13**
1D4W	-	0.85	1.64
1LKK	-	-	1.42
1P13	-	-	-
Tensin	0.95	0.56	0.55

The far-UV spectra of SH2 domain at pH 12 show a shift in the minima from 210 to 205 nm but retain most of the secondary structure. SH2 domain has a pI of 9.15; it being a basic protein it was not entirely surprising that it retains its secondary structure at a high pH value in the basic range. The far-UV CD spectra at pH 2.0 closely resembles that of a typical random coil; indicating that the protein loses all or most of its secondary structure in the acidic pH range. The far-UV spectra of the protein at pH 2.0 upon changing its pH to 7.8 reveals a shoulder at 220 nm and a minima at 205 nm typical of $\alpha+\beta$ proteins. This data indicate that the SH2 protein folding-unfolding process on change in pH value is reversible.

The SH2 domain with His tag consists of 150 amino acid residues, and the calculated p*I* is 9.15. The experimental pH of 7.8 was chosen for a positive net charge and solubility. There are ten aromatic residues in this domain: five Phe, one Trp, and four Tyr. The spectra of both domains indicate aromatic asymmetry and thereby indicate that at least some of the molecules are folded or partly folded under the conditions of data collection.

The calculated molecular mass of SH2 domain is 16734 da. Mass spectrometry analysis revealed a mass of 16757 da. The difference of 23 da can be attributed to a sodium ion whose molecular mass is exactly 23 da being bound to the protein during one of the several dialysis steps done to get rid of excess salt from the purified protein.

Chemical denaturation of the domains was monitored by measuring the change in ellipticity at 220 nm on increasing the concentration of GdnHCl. Ellipticity at 220 nm as a function of guanidine concentration is shown for SH2 domain. For comparison, the midpoint concentration of guanidine denaturation of the Btk SH2 domain is 2.2 M (Tzeng et al., 2000), while that of the STAT3 SH2 domain is 1.7 M (Haan et al., 1999). The denaturation temperatures as determined by change in ellipticity at 222 nm are 56 °C for the Btk SH2 domain (Tzeng et al., 2000), 57 °C for the p85 PI-3 kinase SH2 domain (Williams and Shoelson, 1993) and 43 °C for the STAT3 SH2 domain (Haan et al., 1999). The midpoint concentration of guanidine denaturation for SH2 domain is approximately 3 M, which is significantly higher than other known SH2 domains indicating that tensin-SH2 domain is highly thermostable.

As seen in the calorimetric profile of SH2 domain, the heat absorption peak rises somewhat gradually at first, the maximum occurs around 105 °C, and the peak falls

sharply. The transition temperature is high and can probably be attributed at least in part to a relatively small heat capacity change on denaturation: the SH2 module is small, and the increase in heat capacity is correspondingly small, broadening the ΔG versus T curve, decreasing the cold denaturation temperature, and increasing the heat denaturation temperature. The heat capacity profile resembles that displayed by other proteins (Biswas and Kayastha, 2002). There was no evident precipitation after heating the protein to 125 °C. Nevertheless, some aggregation may have occurred on unfolding. There was no endothermic peak in a subsequent scan of the sample (data not shown) indicating that unfolding was irreversible under the conditions of the experiment.

CHAPTER 5

CONCLUSIONS AND FUTURE WORK

Optimal expression level was achieved for tensin-SH2 domain using *E. coli* as a host organism. The protein was soluble and stable; as is evident from the near-UV CD and DSC studies. The SH2 domain folds into a compact structure on its own; without the need for upstream or downstream domains of tensin. Electropherogram showed the protein to be of high purity; which should be able to crystallize without much difficulty. Conditions can be seeked for growing crystals of the tensin-SH2 domain (>0.1 mm in its smallest dimension) so that the long-range order will be sufficient to yield an atomic resolution structure. Crystal Screen Kits can be purchased from Hampton Research for this purpose. Initial crystallization conditions of tensin-SH2 domain will be sought using these kits, testing a wide range of recombinant protein concentration, pH, salt concentrations, and precipitants. When crystals are obtained, information from Crystal Screen will be used to optimize crystallization conditions to produce crystals suitable for X-ray diffraction analysis. X-ray diffraction experiments can eventually be done at the Center for Advanced Microstructures and Devices (CAMD), LSU, Baton Rouge or Brookhaven National Laboratory (BNL), Upton, New York.

The chemical and heat denaturation studies show that the tensin-SH2 domain is highly thermostable; much more than some other known SH2 domains. Characterization of thermodynamics of binding can be done using Isothermal titration calorimetry. Purified human tensin-SH2 domain can be used to raise antibodies in rabbits. Then the antibodies can be tested for cross-reactivity with mouse tensin. Further the antibodies can be used to label focal adhesions in cultured mouse cells. Focal adhesions are a marker of cell attachment. More detailed knowledge of the structure and function of tensin will accelerate acquisition of more detailed knowledge of other focal adhesion components, advancing the development of molecular models of cell attachment and migration. Such knowledge is of interest tobasic science as well as medicine. Moreover, it could also provide a model for nanotechnology development, providing inspiration for the design of novel types of molecular recognition and functionality, and materials design and fabrication.

Although biomimetic nanotechnology is in its infancy, with no applications yet reaching commercialization, the barriers in some cases lie mainly in scaling up production processes to industrial levels. Dropping in scale from hundreds of nanometers to 10 nm brings researchers to the realm of large molecules. Organisms build structures with proteins, so a second major biomimetic approach uses natural or designed recombinant proteins to create nanostructures. For one thing, naturally occuring proteins can form repetitive, crystalline structures to serve as substrates for arrays of nanomachines or for nanoelectronics. Other researchers are experimenting with proteins in a far more complex way; using their ability to specifically bind with each other and with inorganic materials as a way to build new materials. One of the characteristics of

biologically produced nonliving materials, such as abalone shell and spider silk, is a hierarchical structure. That is, structures exist not just at the macroscopic level and the crystalline level, but at many scales in between. This structuring often imparts remarkable characteristics to a material, such as silk's great strength. If researchers can design appropriate new proteins, they could be used to produce similarly complex artificial materials in an industrial process. Protein-based techniques are at an intermediate stage; neither entirely a pure research subject, nor one verging on commercial application. For the most part, these techniques aim at using biologically based processes to produce artificial structures that could, in principle, be built by entirely inorganic means.

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