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Does SABP2 Exist As a Dimer?

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Does SABP2 Exist As a Dimer?

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ABSTRACT

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Salicylic acid binding protein 2 (SABP2) is one of the key enzymes in salicylic acid-dependent plant defense pathway. SABP2 is a 29 kDa protein present in extremely low abundance in plants and it catalyzes the conversion of signaling molecule methyl salicylate into salicylic acid. Although it has been shown that 6x His-tagged SABP2 over expressed in *E. coli* is a homodimer, its exact conformation in *planta* is still unknown. Therefore, we proposed to determine if SABP2 exist as a dimer and/or monomer under natural condition. To verify the exact conformation of native SABP2 protein in plant, SABP2 was purified from wild type tobacco using a 5-step purification protocol. Analysis of purified SABP2 in gel filtration and immunoblot assay suggested that SABP2 exists as a monomer in tobacco plant. Studies on SABP2 conformation will give us insight into the structure and functional relationship of this protein in salicylic acid-dependent disease resistance pathway.

DEDICATION

To my late father and to my family members.

ACKNOWLEDGEMENTS

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CHAPTER 1

INTRODUCTION

Since the dawn of evolution, plants have been playing key role in survival of life on earth. Plants have developed special mechanisms to use the solar energy and convert it into food, making them as primary producer in the food web. Like all other animals, humans are dependent on plants for food. As the population of earth is increasing day by day, the necessity of food is growing but the landmass required to grow food crops is limited. It is well anticipated that in near future the world population will surpass the limit of food production. In order to overcome the food scarcity new techniques have evolved. Plant breeding techniques are being used for production of improved crop yield. Application of pesticides and artificial fertilizer has been also shown to increase the crop yield. Development of new agricultural techniques (harvesting, irrigation, soil plough) also has a positive effect on production rate. But all of these techniques have limitations. Plant breeding techniques can be very time consuming; residuals from pesticides and inorganic fertilizer pollute natural environment and can be detrimental to living organisms including human (Zahm and Blair 1992; Calaf and Roy 2007; Pimentel et al. 2007). So a different approach has to be made to increase the food production in an environmentally healthy way. Besides food, plants are also important in medicinal drugs, paper industry, clothing industry, prevention of soil erosion, and oxygen production. Recently, biochemical and molecular engineering techniques have been applied to produce increased amount of fuel that will be the sole source of fuel in near future because the stock of fossil fuel is limited (Simon et al. 2010).

Every year significant amount of crop yield is affected because plants are constantly exposed to various abiotic (drought, heat, snow, temperature, salinity, etc.) and biotic stresses (pathogen attack). Biotic stress is caused by pathogen and can cause huge loss of crop production. For example, in 1993 cassava mosaic disease (CMD) caused by cassava mosaic Gemini virus resulted in a decline of 90% cassava production in east Africa (Thresh et al. 1994). Some plants have developed elaborate strategies to overcome some of these stresses. Further studies are required to fully understand the mechanisms through which plants defend themselves against a broad range of pathogen. In order to gain a deeper understanding of these mechanisms, it is important to study the pathogenesis processes as well as molecular function of key defense pathways. Studies of biochemical and physiological pathways in plants will help us to understand the biological mechanism through which plants restrict pathogen infection and gradually achieve innate immunity. Once we know the proper function of biological molecules, it may be feasible to manipulate the production levels of crop plants in an environmentally healthy way.

Plant Defense Mechanism

The ability to discriminate between self and non-self is a key feature of all living organisms and forms the basis for the activation of innate defense mechanisms against microbial infections. Both plants and animals have innate immune system but due to lack of circulatory system (which is found in animals), plant immune systems are different from animals. Therefore, plants recognize the foreign molecules (usually from pathogen) and activate local defense mechanism at the site of infection (Dangl and Jones 2001; Ausubel 2005; Chisholm et al. 2006).

Many plants are resistant to most species of microbial invaders. This is known as "special resistance" (Gomez-Gomez and Boller 2002).

As plants are constantly exposed to microorganisms, they regularly evolve alternate way to resist microbial growth. Pathogens must enter the cell to cause infection. Generally they penetrate plant cell wall. Once they are in the extracellular area, plant cells can recognize the microbial compounds (known as virulence molecules) that trigger plant basal defense, such as viral proteins, bacterial flagellin, lipopolysaccharides, peptidoglycans, and fungal chitin. These molecules are known as Pathogen Associated Molecular Patterns (PAMPs), which initiate PAMPs triggered immunity (Okazakia et al. 1996; Zipfel and Felix 2005; Boller and Felix 2009) to activate the immune system. Plants recognize these virulence molecules by a specific set of receptors known as pathogen or Pattern Recognition Receptors (PRRs). Once the virulence molecules are recognized, plants secret a number of secondary metabolites including phytoalexins (Thordal 2003). However, some pathogens can overcome this basal defense and continue infection (Van der Biezen and Jones 1998; Jones and Dangl 2001). Plants have developed the alternate way of defense by expressing a set of genes (known as R genes or resistance genes) to encode specific intracellular receptor proteins, called R proteins (Iriti and Faoro 2007). The pathogen derived molecules that are recognized by the R proteins are called avirulence (Avr) proteins (Jones and Dangl 2006). The interaction between the Avr proteins and R proteins results in hypersensitive response (HR) at the infection site (Hammond-Kosack and Jones 1996). This interaction causes the plants to synthesize hormones that play the central role in plant defense signaling. However, this increase in hormone synthesis can result in massive changes in plant cellular homeostasis, redox changes, oxidative burst, and programmed cell death (Lamb and Dixon 1997). R proteins have been shown to govern plant–pathogen interactions in a variety of

host plants, directing response toward a broad diversity of pathogens including bacteria, fungi, oomycetes, nematodes, viruses, and insects (Parker 2003). Interaction between R proteins and avr proteins results in activation of plant defense mechanism that includes transcriptional activation of defense genes, production of lytic enzyme, or anti-microbial proteins, anti-microbial secondary metabolites (Buchter et al. 1997), allosteric enzyme activation, initiating cell wall reinforcement by deposition of callose and lignin (Yang et al. 1997), production of reactive oxygen intermediates (Bolwell et al. 2002). In addition, increased level of hormone accumulation occurs at the site of infection such as salicylic acid (SA), jasmonic acid (JA), ethylene, and zibberelic acid followed by the late expression of pathogenesis-related genes (*PR* genes) (Gozzo 2003; Iriti and Faoro 2007). As a result the infected cells undergo a massive biochemical and physiological changes that ultimately causes hypersensitive cell death (also known as Programmed Cell Death (PCD)) (Lam and Lawton 2001). Hypersensitive response (HR) leading to programmed cell death (PCD) limits the spreading of pathogen near the site of infection.

A signal from the infected part spreads throughout the plant and induces subtle changes in cellular environment and subsequent gene expression in uninfected parts of plant. As a result, the systemic parts of the plant induce disease resistance capacity by production of phytoalexins and PR proteins (Van Loon 1997; Neuhaus 1999; Van Loon and Van Strien 1999). Two types of systemic induction have been reported (Kloepper et al. 1992; Vallad and Goodman 2004). Induced systemic resistance (ISR) is usually caused in SA-independent manner by *Rhizobacterium* sp. (Pieterse et al. 1998) and is mediated by jasmonic acid (JA) and Ethylene (ET). ISR is not accompanied by *PR* gene expression. The resistance governed by ISR is not

broad based and is only active against a few necrotrophic agents (Bostock 2005). Conversely, systemic acquired resistance (SAR) requires the accumulation of salicylic acid (SA) in both infected and systemic parts (Van Loon 1987; Kloepper et al. 1992). SAR is accompanied by induction of PR proteins and the defense response is long-lasting and broad-based compared to ISR (Durrant and Dong- 2004; Bostock et al. 2005). Moreover, SAR is effective against a broad range of pathogens including fungi, bacteria, viruses, nematodes, and even insect herbivores (Metraux 2002; Vallad and Goodman 2004).

Signaling Pathways

Various signaling molecules have been shown to play important role in disease resistance signaling in plants. Three endogenous plant signaling molecules, salicylic acid (SA), jasmonic acid (JA), and ethylene (ET), are involved in plant defense (Dong 1998; Thomma et al. 2001). These signaling molecules are involved in 2 major defense signaling pathways: an SA-dependent pathway that requires SA and an SA-independent pathway that involves JA and ET. These signaling pathways interact with each other in a complex regulatory network (Barbara and David 2002).

Salicylic Acid Mediated Defense

Salicylic acid is an important phenolic phytohormone that is synthesized in plants. SA has several roles in plants including seed germination, leaf and flower senescence, cell growth, respiration, and most importantly in plant defense signaling (Raskin 1992; Vlot et al. 2009).

(A) *Biosynthesis of Salicylic Acid.* In plants 2 pathways for SA biosynthesis are known. In one pathway biosynthesis of SA starts with shikimic acid pathway. Shikimic acid pathway produces chorismate, which is converted into SA (Figure 1). In cell the main chorismate pool is in the chloroplast. The isochorismate synthase 1(ICS1) catalyzes the conversion of chorismate to isochorismate (Marcus et al. 2007). In the final step isopyruvate lyase (IPL)

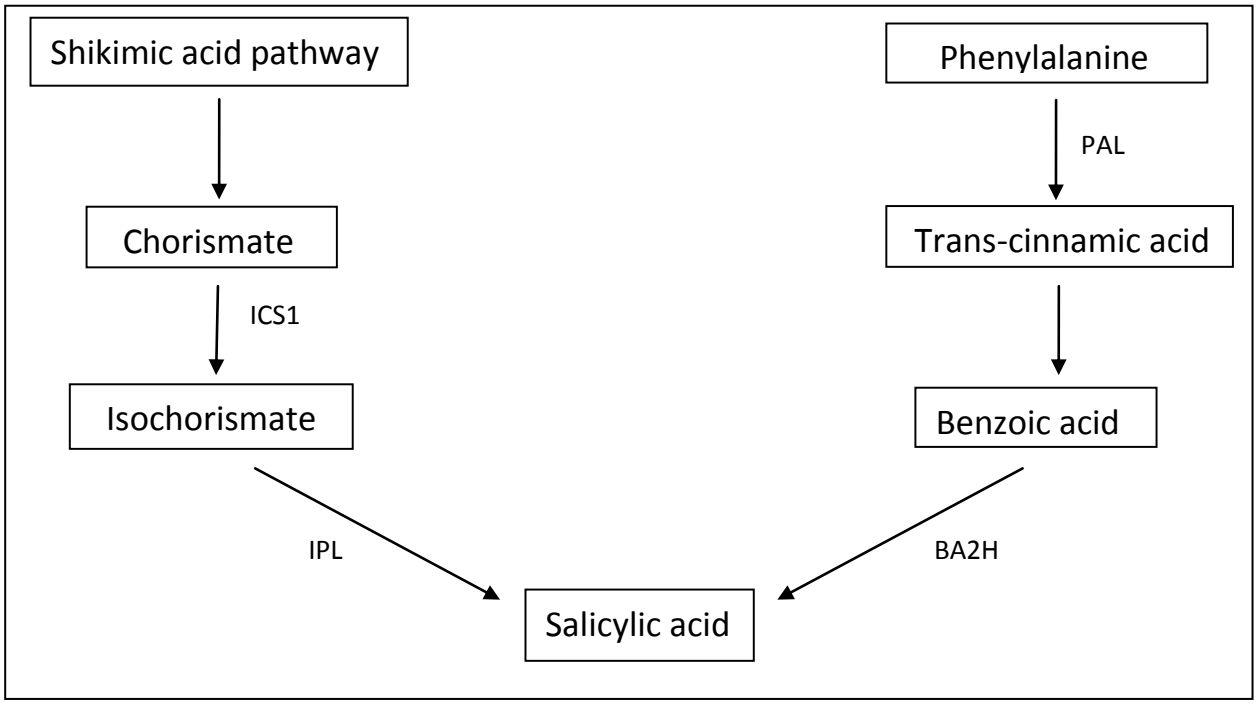


Figure 1. Biosynthetic pathways of Salicylic Acid in plants. Salicylic acid is synthesized from Chorismate via Isochorismate Synthase (ICS) and from Phenylalanine via Phenyl Ammonia Lyase (PAL).

catalyzes the conversion of isochorismate to SA. An alternate pathway has been known in tobacco, synthesizing SA from phenylalanine via benzoic acid (Ogawa et al. 2005) (Figure 1). The first reaction is catalyzed by phenylalanine ammonia lyase (PAL) that catalyzes the conversion of phenylalanine into trans-cinnamic acid. This trans-cinnamic acid then converts

into benzoic acid. Finally, benzoic acid 2-hydroxylase (BA2H) catalyzes the conversion of benzoic acid to SA (Ward et al. 1991). However in arabidopsis plants, about 90% of endogenous SA is synthesized via isochorismate pathway catalyzed by isochorismate synthase 1 (ICS1) (Wildermuth et al. 2001).

(B) *Salicylic Acid Dependent Defense*. Generally plants have low basal levels of SA. But during infection, the level rises up to 20-50 folds (Malamy et al. 1990). This accumulation occurs not only in the infected tissues but also systemically throughout the whole plant (Malamy et al. 1990; Durrant and Dong 2004). This increased level of SA needs to be transported to the cytoplasm where it leads to activation of defense responses. Because SA is a polar molecule, it cannot pass through the hydrophobic chloroplast membranes. It has been shown that Salicylic Acid Methyl Transferase (SAMT) converts SA into its lipid soluble form methyl salicylate (MeSA) (Chen et al. 2003). The lipid soluble MeSA can be easily diffused through chloroplast membrane. When MeSA arrives in cytoplasm, Salicylic Acid Binding Protein 2 (SABP2) converts the MeSA into SA (Kumar and Klessig 2003).

(C) *Salicylic Acid and Local Defense.* The increased level of SA changes the redox potential of the cytoplasm. As a result, reduction happens in 2 cysteine residues, Cys 82 and Cys 216 of NPR1(nonexpressor of pathogenesis-related genes 1) oligomer, which causes the monomerization of NPR1 protein (Mou et al. 2003; Tada et al. 2008) (Figure 2). The NPR1 is an important protein regulator of systemic acquired resistance (SAR). This protein can also regulate disease resistance both in monocot (Rice) and dicot (Arabidopsis, Tobacco) plants and shares a

conserved signal transduction pathway controlling NPR1 mediated pathway (Chern et al. 2001). NPR1 monomer migrates to the nucleus to induce defense gene expression (Kinkema et al. 2000; Mou et al. 2003). It has been shown that NPRI monomer interacts with TGA transcription factors that are subclass of leucine zipper family (Zhang et al. 1999; Fan and Dong 2002). This interaction activates the TGA transcription factors to bind the enhancer region of PR genes promoter to induce the expression of pathogenesis-related proteins (PR proteins) (Fan and Dong 2002). PR proteins then promote local immune response which restricts the pathogen infection in necrotic lesions.

(D) *Salicylic Acid and Systemic Acquired Resistance*. Besides developing local resistance, SA also triggers the systemic resistance in plants. Initially it was believed that SA is the signal for SAR. However, recent studies have shown that MeSA, a lipid mobile form of SA, is the signal for SAR (Park et al. 2007). Moreover, increased level of MeSA was extracted from phloem exudates of infected plants (Park et al. 2007), suggesting that MeSA diffuses across the cells and reaches the upper systemic tissue through phloem. SABP2 catalyzes the conversion of MeSA into SA in the infected tissue. As a result, the SA level rises in the cytoplasm. SA has a high binding affinity to SABP2 at KD of 90nM (Kumar and Klessig 2003; Forouhar et al. 2005). When the SA level reaches the maximum in tissue, it binds to the active site of SABP2 and inhibits its esterase activity (Forouhar et al. 2005). As a result, the conversion of MeSA into SA ceases, resulting in increased MeSA levels that travel through phloem to the systemic tissue where SABP2 converts MeSA into SA (Figure 2) leading to activation of defense responses. This phenomenon is known as systemic acquired resistance (SAR).

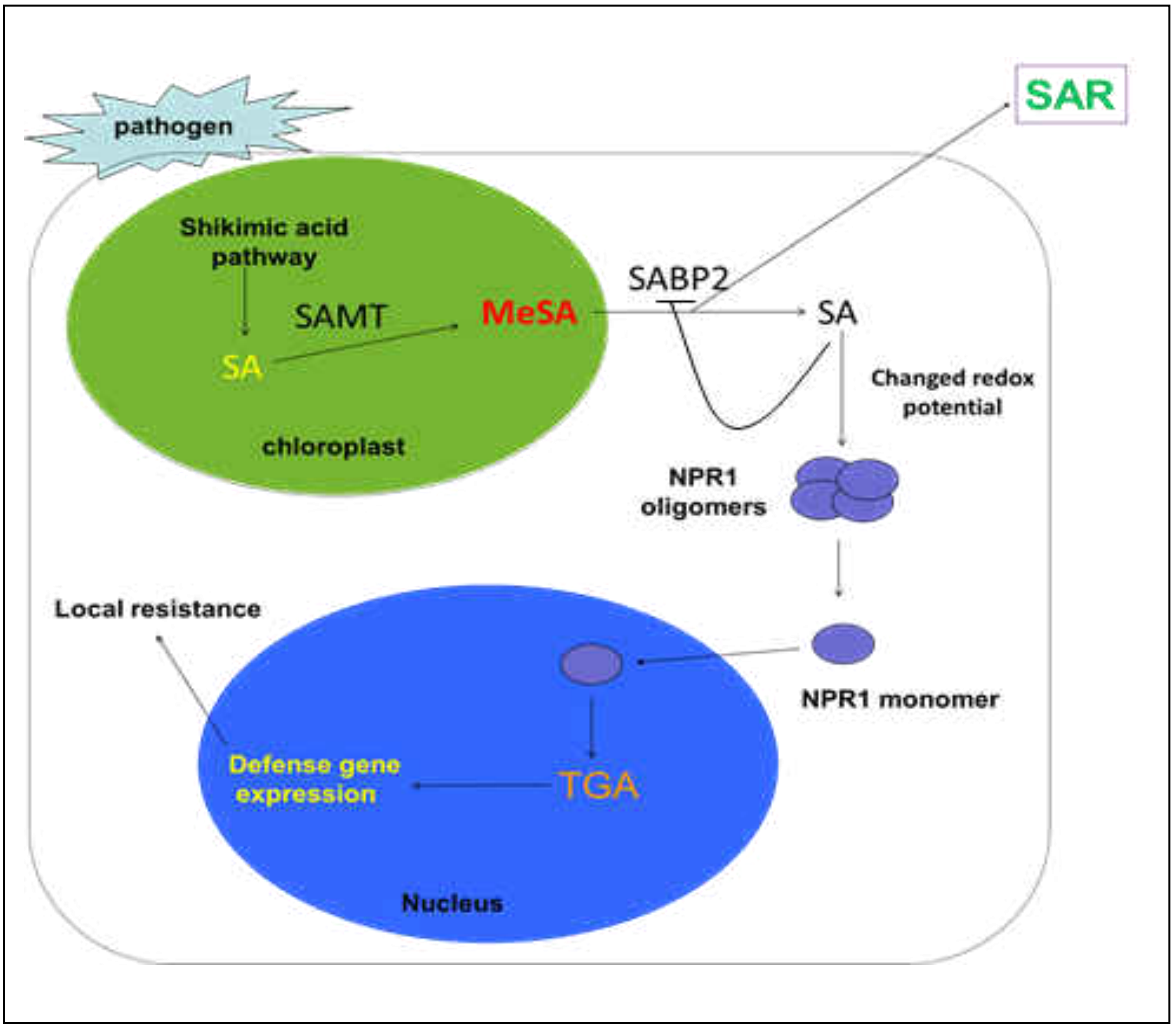


Figure 2. A Simplified Pathway for Salicylic Acid Mediated Defense Signaling. Pathogen infection results in increased level of SA synthesis in chloroplast. Salicylic acid methyl transferase (SAMT) methylates SA to MeSA that diffuses to the cytoplasm. Salicylic acid binding protein 2 (SABP2), an esterase, converts cytoplasmic MeSA into SA. As a result, cellular redox potential changes lead to induction of defense related genes. In cytoplasm SA feedback inhibit SABP2's esterase activity, resulting in accumulation of MeSA, which is required for developing SAR in systemic tissues.

SAR is characterized by the expression of PR proteins and enhanced resistance to secondary infection. PR protein does not express in the healthy plant, making it a useful marker for SAR development (Durrant and Dong 2004). This SAR is a long-lasting and broad-based immunity in

systemic tissue not only against the inoculated pathogen but also against a wide spectrum of other pathogens (Ryals et al. 1996; Sticher et al. 1997). Tobacco plants expressing bacterial *nahG* gene (nahG converts SA into catechol) showed increased disease susceptibility and failed to develop SAR after TMV infection (Gaffney et al. 1993; Delaney et al. 1994) suggesting SAR is mediated by SA. Salicylic acid synthetic analogs such as BTH and INA (which have been used in agriculture to enhance plant natural innate immunity) have been shown to induce *PR* genes and develop SAR (Gorlach et al. 1996).

Jasmonic Acid Dependent Defense

Jasmonate, which is an intermediate of Jasmonic acid (JA) biosynthesis, is a signaling molecule in plants (Mur et al. 2006). It has an important role in regulation of herbivory and wound responses in plants (John et al. 2007). JA also has a role in pollen and seed development (Reymond et al. 1998; Li et al. 2001), fruit ripening, root growth, tendril coiling, and disease resistance in plants (Creelman and Mullet 1997). Studies showed that JA mediates ISR, which is triggered by nonpathogenic bacteria and is effective against a broad spectrum of pathogens (Saskia et al. 2000; Martin and Richard 2002). Studies have also showed that *Arabidopsis thaliana* are impaired in JA production such as jasmonic acid resistant1(*jar1*) plants exhibit enhanced susceptibility to a variety of pathogens including fungal pathogens *Alternaria brassicicola*, *Botrytis cinerea*, and *Pythium sp*., and the bacterial pathogen *Erwinia carotovora* (Thomma et al. 1998; Stintzi et al. 2001). These pathogens rapidly kill the host cells to obtain nutrient and thus are called necrotrophs (Jackson et al. 1996). Overexpression of JA in plant shows increased resistance against some pathogens. As for example, *Arabidopsis thaliana* plants

overexpressing the *JMT* gene that encodes an enzyme that catalyzes the production of methyl jasmonate (MeJA) from JA constitutively express *PDF1.2* gene and exhibit enhanced resistance to *B. cinerea* (Seo et al. 2001).

Ethylene Dependent Responses

Ethylene is a very important hormone and it has significant roles in fruit ripening, controlling the initiation of changes in color, aromas, texture, flavor, and other biochemical and physiological attributes (Michael et al. 1996). In addition, ethylene also has a role in plant defense (Feys and Parker 2000; McDowell and Dangl 2000; Glazebrook 2001; Thomma et al. 2001). When a plant interacts with a pathogen, ethylene is rapidly biosynthesized (Yang 1985; Lotan and Fluhr 1990). Ethylene induces a set of genes called ethylene response genes (Van loon and Antoniw 1982; Eyal et al. 1992) that is a sign of host reaction to pathogenic invasion (Meins and Ahl 1989; Bol et al. 1990). The role of ET in plant defense is controversial as it induces resistance in some interactions (Thomma et al. 1999; Norman et al. 2000) but promotes disease susceptibility in others (Bent et al. 1992; Lund et al. 1998; Hoffman et al. 1999). As for example, *Arabidopsis thaliana* ethylene insensitive 2 (*ein2*) mutant plants show increased susceptibility to *B. cinerea* (Thomma et al. 1999) but show decreased symptoms after infection with virulent strains of *P. syringae* or *Xanthomonas campestris pv. Campestris* (Bent et al. 1992). It has been shown that expression of several JA-dependent defense genes (i.e. *PDF1.2, THI2.1, HEL, and CHIB*) also requires the expression of *EIN2* (Penninckx et al. 1998; Thomma et al. 1999), suggesting that both ET and JA signaling pathways are interlinked. Moreover, both ET and JA

signaling pathways are required for induction of induced systemic resistance (ISR) that is triggered by root colonizing bacterium *P. fluorescens* (Pieterse et al. 1999).

Crosstalk among Signaling Pathways

All of these 3 (SA, JA, and ET) pathways have synergism as well as antagonism among themselves to protect plants from pathogen. Salicylic acid has a role of defense against biotrophic pathogens, while JA and ethylene induce defense against necrotrophic pathogens and insects (Glazebrook 2005). Besides SA, JA, and ET, there are other defense signaling molecules that interact with each other to fine tune the defense response in plants. As for example, nitric oxide (NO) is a signaling molecule that is involved in signaling processes in plants (Delledonne et al. 1998). Studies have shown that SA is not required for NO signaling process in plants (Wendehenne et al. 2004; Grun et al. 2006). Tobacco plants expressing bacterial *nahG* gene that degrades SA into catechol and shows local and systemic resistance when the plants were induced with NO. Similarly, SA-dependent SAR has been shown to be arrested when the plants were treated with NO scavengers (Song and Goodman 2001).

SA and JA pathways have been shown to be mutually antagonists, and recent studies have confirmed the antagonistic effect of SA on JA signaling in *Arabidopsis thaliana* (Mur et al. 2006). Arabidopsis plants impaired in SA accumulation such as *eds4* and *pad4* mutant exhibit enhanced responses to inducers of JA-dependent gene expression (Gupta et al. 2000). Antagonistic effect of JA on SA is also observed in tobacco plants, where JA inhibits the expression of SA-dependent genes (Niki et al. 1998). Studies have also revealed the synergistic effect between SA and JA pathways. Microarray analysis of defense inducing treated

Arabidopsis thaliana plants showed that more than 50 defense-related genes are co-induced by JA and SA (Schenk et al. 2000), suggesting that these 2 signaling molecules regulate these genes.

On the other hand, SA and ET signaling pathways have been shown to interact with each other via both positive and negative regulation. Studies have shown that accumulation of SA is dependent of ET synthesis when tomato plants were infected by *X. campestris pv. vesicatoria* (O'Donnell et al. 2001). Genetic data also suggest that ET signaling pathway can negatively affect SA-dependent responses (Lawton et al. 1994).

In contrast, studies have revealed the positive interactions between JA and ET signaling pathways. As for example, when *Arabidopsis thaliana* plants were infected with *A. brassicicola*, both JA and ET signaling were required for the expression of defense-related gene *PDF1.2* (Penninckx et al. 1996). Microarray data from *Arabidopsis thaliana* revealed that almost half of the genes that are induced by ET treatment were also induced by JA treatment (Schenk et al. 2000), suggesting the synergistic interaction between these 2 pathways. Other plant hormones have also been shown to interact with SA signaling pathway. It has been shown that giberellic acid regulates the disease resistance by adjusting SA-JA equilibrium in plant (Navarro et al. 2008). Another growth hormone, auxin, has been shown to enhance susceptibility to pathogen, but it had no effect on SA-mediated defense responses (Wang et al. 2007).

Salicylic Acid Binding Proteins and Plant Defense

SA-mediated defense is a major defense mechanism in plants that contributes to the development of systemic acquired resistance (SAR). In the past, significant progress has been made to understand the salicylic acid signaling in plants. Studies have been done to investigate the proteins that bind to SA in defense pathway. Discovery of Salicylic acid binding proteins has opened a new era to understand plants' natural defense against pathogens (Kumar and Klessig 2003). The first SA binding protein discovered is SABP (a tetramer, Mr of 240 kDa) that is a catalase, and it reversibly binds SA with a K_d of 14 μM (Chen et al. 1993 a; Chen et al. 1993 b). Upon infection when the SA level increases in cells, SA inhibits the H_2O_2 degrading activity of SABP. As a result, reactive oxygen species like H_2O_2 accumulate in the cells causing hypersensitive response (HR) that contributes to apoptotic cell death in order to restrict pathogen invasion (Conrath et al. 1995; Chen et al. 2003). Continuous efforts to isolate SA effector proteins in tobacco have led to discovery of another enzyme, salicylic acid binding protein 2 (SABP2) that has a higher affinity for SA (K_D of 90nM) (Du and Klessig 1997; Kumar and Klessig 2003). Another SA binding protein was identified that is a chloroplast carbonic anhydrase (SABP3) (Slaymaker et al. 2002). It has been shown that SABP3 has antioxidant properties and may have a role in hypersensitive response in tobacco (Slaymaker et al. 2002).

Tobacco SABP2 and Its role in Plant Defense

SABP2 plays an important role in conversion of SA from MeSA both in infected and systemic leaves (Kumar and Klessig 2003). SABP2 silenced plants via RNA interference exhibit suppressed local resistance to tobacco mosaic virus (TMV) and failed to develop SAR (Kumar and Klessig 2003). SABP2 is a soluble protein and expresses in a very low levels in plants. It has a molecular weight (Mr) of 29 kDa, and it is a member of α/β hydrolase super family, and has esterase activity (Forouhar et al. 2005). Recently, a combination of enzymology, biochemistry, and biophysics was used to study SABP2 structure and function. It was showed by X-ray crystallography that SA binds in the active site of SABP2 (Forouhar et al. 2005).

Structure of SABP2

There are 2 domains in the SABP2 structure, i.e. core domain and cap domain. The core domain contains 6 central parallel β sheets with 6 α helices, while the cap domain contains 3 standards antiparallel β sheets and 3 α helices (Figure 3). Moreover, X-ray crystallography structure revealed that recombinant SABP2 overexpressed in *E. coli* is a homodimer (Forouhar et al. 2005). During dimerization the cap domain of one monomer contacts with the core domain of others. The dimer interface is far away from the active site of SABP2 suggesting no effect of dimerization on its active site (Forouhar et al. 2005).

The Active Site of SABP2

As a member of α/β hydrolase super family, the active site of SABP2 contains a catalytic triad, Ser-81, His-238, and Asp-210. These residues are conserved among the members of this super family (Forouhar et al. 2005). The active site is in the core domain of SABP2 and the cap

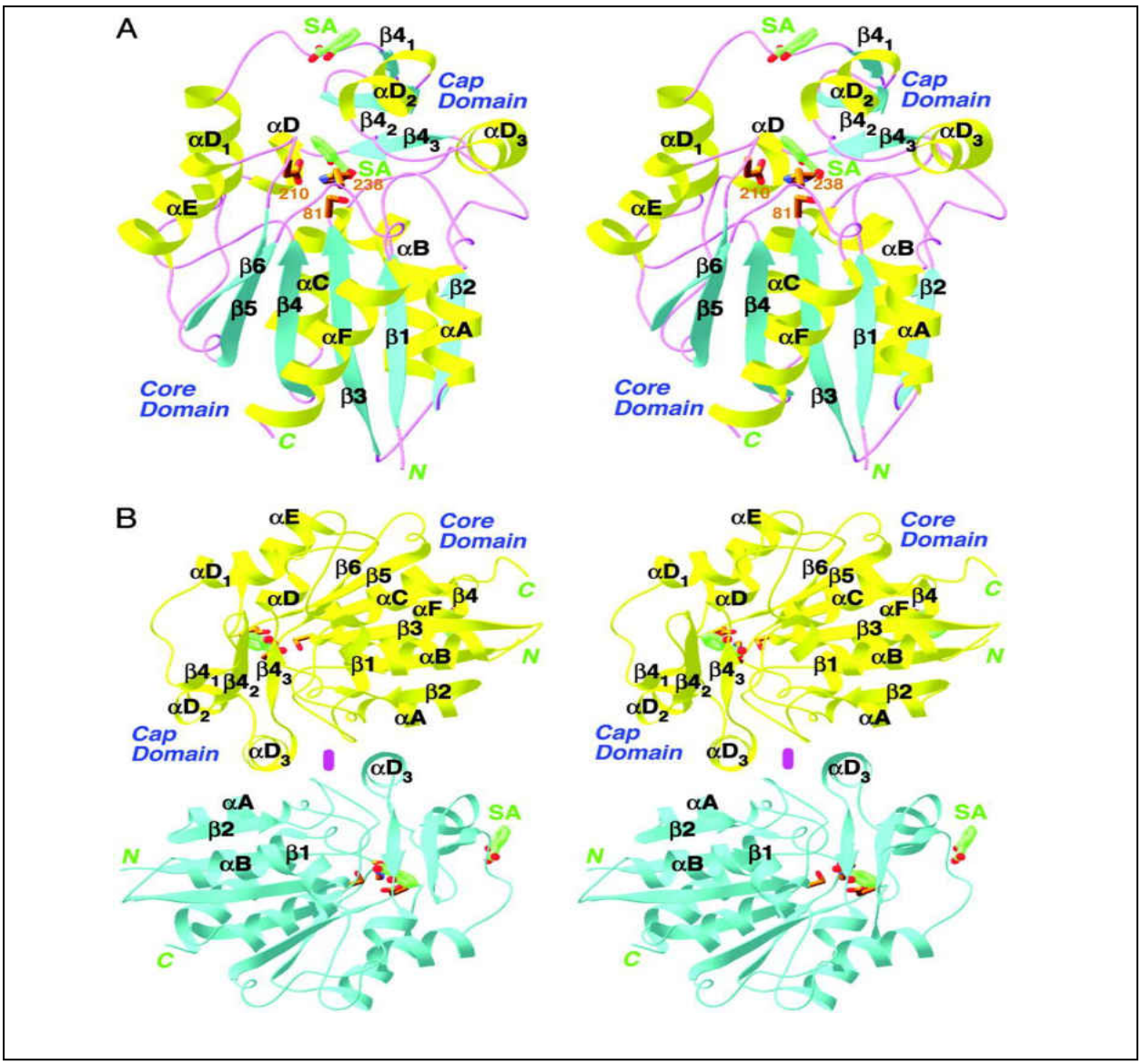


Figure 3. Three Dimensional Structure of RecSABP2 Purified from E. coli. A. SABP2 in complex with SA. B. SABP2 dimer (Forouhar et al. 2005. Proc Natl Acad Sci USA 102(5): 1773-8. Copyright (2005) National Academy of Sciences, U.S.A.)

domain covers the exposed side of the active site (Figure 3). The active site of SABP2 is too small to fit both MeSA and SA, hence SA may compete with MeSA to bind with SABP2 and therefore SA is a potent product inhibitor of SABP2's MeSA esterase activity. It was shown that

SA inhibits SABP2's esterase activity even in very low concentrations (Forouhar et al. 2005) which is consistent with the fact that SABP2 has a high affinity for SA. It was also shown that SABP2 hydrolyzed more than 90% of MeSA into SA in competitive binding assay (Forouhar et al. 2005) showing its specificity.

Dimerization and Its Effect

Self-association of proteins is a common phenomenon in biology. Protein dimerization is very important in case of enzyme regulation, ion channels, receptors, and transcription factors (Xenarios et al. 2002; Alm and Arkin 2003). Dimerization can confer several structural and functional advantages to proteins including improved stability and control over the accessibility and specificity of the substrate in active sites (Marianayagam et al. 2004). Protein dimerization has an immense role in biology. According to Brenda enzyme database (http://www.brenda.uni-koeln.de/) out of 452 human enzymes, only one-third (141) are monomers and the rest are either dimers (125) or higher oligomers indicating the importance of protein oligomerization.

Dimerization can function as a mechanism for sensing protein concentration. Increase in protein concentration above the oligomerization threshold can stimulate enzyme activity. In addition to response to protein concentration, dimerization can also regulate enzyme activity. Dimerization can generate new intermolecular interfaces for allosteric regulation. As for example, in lower vertebrate oxygenation dissociates haemoglobin tetramer into dimer producing a site for oxygen store because the dimer has high affinity for oxygen (Bonafe et al. 1999). Dimerization can activate an enzyme. As for example, caspases are important enzymes during apoptotic process. Enzyme assay and gel filtration analysis have showed that under physiological

condition caspase-9 is an inactive monomer, but during apoptosis the caspase-9 oligomerize brings its local concentration above dissociation constant (Kd) resulting the activation of caspase-9 (Renatus et al. 2001). Conversely, dimerization can also inhibit an active monomeric enzyme. As for example, receptor-like protein tyrosine phosphatase-α exists on the cell surface predominantly as a weak homodimer because part of one monomer blocks the active site of others (Jiang et al. 2000). Oligomerization in cell surface receptors is very common in signal transduction pathways, such as an enzyme can bind an agonist to transfer a signal across the cell membrane (Hebert and Bouvier 1998). The mechanism of dimerization can be very specific to the protein involved. Although dimerization can occur via covalent interactions such as disulphide-bonded metabotropic glutamate receptor 1 (Kunishima et al. 2000), most of the G-protein receptors dimerize via non-covalent interactions (Breitwieser 2004).

In addition, Dimerization might also provide a mechanism by which to cluster downstream signaling components and thereby enhance signaling (Woolf and Linderman 2003). Protein dimerization is also required for the assembly of proteins into membrane channels for the controlled transport of molecules across the cell membrane (Agre and Kozono 2003). Protein oligomerization is mostly important for the assembly of the protein complexes during gene expression. As for example, the Jun-Fos heterodimer, which controls the transcription of several genes during mitotic signaling, exhibits greater transcriptional activation when bound by NFAT (Beckett 2001). Moreover, the requirement of monomerization of NPR1 oligomer during SA signaling pathway also suggests the importance of protein dimerization for its activity.

Importance of Research

SABP2, an important tobacco enzyme, plays key role in salicylic acid mediated plant defense pathway by catalyzing the conversion of MeSA into SA that is required for downstream defense responses. However, every enzyme has its specific structure that modulates the active site for substrate recognition and specificity. Conformational changes can change function of enzymes. In order to enhance the disease resistance capacity by genetic engineering in economically important plants, it is important to investigate the natural conformation of SABP2. Moreover, study of SABP2 structure may enable us to determine if SABP2 undergoes conformational changes following pathogen infection. Knowledge gathered from this research may lead us to generate crop plants with enhanced disease resistance against pathogens.

Hypothesis

Upon infection, SABP2 plays an important role by catalyzing the conversion of MeSA into SA in order to induce SAR in plant. Dimerization may have a significant effect on SABP2 activity in SA pathway. It has been shown that recombinant SABP2 overexpressed in *E. coli* is a homodimer at pH 7.5-8.0 (Forouhar et al. 2005). Moreover, partially purified SABP2 from natural sources suggested that it may be a monomer at physiological concentrations (Du and Klessig 1997, Kumar and Klessig 2003), but its exact conformation in plant is still unknown. The research was designed to determine if SABP2 exists as a dimer in plant under natural condition. Based on previous information, we hypothesized that under natural condition SABP2 exists as a dimer and/or monomer in tobacco plants.

CHAPTER 2

MATERIALS AND METHODS

Plant Materials

The wild type tobacco plant *Nicotiana tabacum* cv. Xanthi nc (NN), wild type Arabidopsis plant *Arabidopsis thaliana* (Col-0), and transgenic *Arabidopsis thaliana* overexpressing 6x myc-SABP2 were used for this study. Seeds were sown in soil containing peat moss (Fafard Canadian growing mix F-15, Agawam, MA). The soil was autoclaved for 20 min before sowing the seeds. The plants were grown at 22ºC in a growth chamber (PGW36, Conviron, Canada) with a 16-hour day cycle. After 2 weeks of plantation the seedlings were transferred to 4 x 4 inch flats, and transferred to 8 inch pots after 4 weeks. Six to 8 week-old plants were used for all experiments.

Chemicals and Reagents

Sodium dodecyl sulfate (SDS), ß-mercaptoethanol (ß-ME), tetramethylethylenediamine (TEMED), ammonium persulfate (APS), bovine serum albumen (BSA), bovine thrombin, coomassie brilliant blue R-250, ponceau-S, ethylenediaminetetraacetic acid (EDTA), TRIS base, phenylmethylsulfonyl fluoride (PMSF), glycine, glycerol, methanol, imidazole ($C_3H_4N_2$), Tween-20, Triton X-100, N,N-Bis(2-hydroxyethyl)glycine (Bicine), magnesium chloride ($MgCl_2$), sodium chloride (NaCl), sodium phosphate monobasic (NaH_2PO_4), sodium phosphate dibasic (Na_2HPO_4), benzamidine-HCl, ammonium sulfate ($(NH_4)_2SO_4$), sodium citrate ($Na_2HC_6H_5O_7$), and all other standard chemicals were purchased from Fisher Scientific, Pittsburgh, PA.

Polyvinylpolypyrrolidone (PVPP) was purchased from Acros Organics, Audubon Park, NJ. The Mini Protean 3 cell, 30% acrylamide, Bradford's reagent, prestained low molecular weight marker, 10x SDS loading buffer, SDS dye, and Mini trans blot system were purchased from Bio-Rad, Hercules, CA. Polyvinylidene fluoride (PVDF) membranes were purchased from Millipore, Billerica, MA. Butyl sepharose column, hitrap desalting column, Q sepharose column, mono Q column, and Superdex-75 column were purchased from Amarsham Biosciences, Piscataway, NJ. Bicinchoninic acid (BCA) protein assay and Pierce ECL western blotting substrate were purchased from Thermo Scientific, Rockford, IL. Kodak developer and fixer replenisher were purchased from Sigma-Aldrich, St. Louis, MO. 6xHis-tag SABP2 protein was expressed in BL21 (DE3). Rabbit polyclonal SABP2 antibodies and monoclonal anti-rabbit, IgG γ chain specific secondary antibodies and tobacco mosaic virus (TMV) were available in-house.

Other Materials

One ml syringes (BD syringes, NJ), pestle grinder (Fisher Scientific), cheese cloth and miracloth (Fischer Scientific), Spectrophotometer, high speed centrifuge (Beckman, model J2-21 or Sorvall RC5B), SYNERGY HT Multi-Mode Microplate Reader (Biotek), and AKTA purifier 10 (GE) system were used for this research.

Methods

Purification of Recombinant SABP2 Expressed in E. coli

In order to characterize SABP2, recombinant 6x his-tagged SABP2 cloned in pET21 (his-tag at "C" terminal) and pET58 (his-tag at "N" terminal) was expressed in *E. coli*. The his-tagged SABP2 was purified as described elsewhere (Forouhar et al. 2005). Briefly, an overnight culture inoculated from a single colony was grown at 37ºC in 3 ml LB medium containing 100 µg/ml Ampicillin. The overnight culture was diluted 100 times in fresh 50 ml LB medium (containing 100 µg/ml Ampicillin) and was further incubated at 37ºC until the OD_{600} = 0.5. Protein expression was induced overnight at 17ºC after addition of 1mM IPTG (final concentration). The *E. coli* cells were harvested by centrifuging at 3,000 rpm for 20min at 4ºC. Cells were sonicated 6 times for 15 sec each with 15 sec interval, at 20% amplitude. The cell lysate was then centrifuged at 14,000 rpm at 4ºC for 10 min. The supernatant containing soluble proteins was mixed with nickel resin for 1 hour at room temperature and washed with binding buffer. The bound SABP2 was eluted with 250mM imidazole at 4°C. The eluted protein was concentrated and desalted using PD-10 desalting column. Desalted SABP2 was further purified on a Q-sepharose column. The bound proteins were eluted using linear gradient of ammonium sulfate in 10mM bicine, pH 8.5. The eluted fractions containing SABP2 were concentrated and desalted using PD-10 column. Purity of recSABP2 was confirmed by 12% SDS-PAGE analysis. Protein concentration was determined using BCA protein assay reagents following manufacturer's instructions. Purified proteins were stored at 4°C until use.

Treatment of recSABP2 with Detergents, Heat, Reducing Agents and Salt

In order to characterize recSABP2, purified SABP2 from E. coli was treated with various reducing agent, temperature, salt, and detergent conditions. To investigate the effect of temperature on SABP2, recSABP2 was mixed with native sample buffer (lacking SDS and reducing agents) and was incubated for 5 min at different temperatures (55°C, 60°C, 65°C, 70°C, 75°C, and 80°C). After incubation the samples were analyzed in a 10% native-PAGE.

To determine the effect of mild detergent on SABP2, recSABP2 was mixed with sample buffer (without reducing agents) containing various concentrations (final conc.) of Triton X-100 (0.3%, 0.2%, 0.1%, 0.05%, and 0.01%) and was incubated in room temperature for 15 min. After incubation the samples were analyzed in both 12% SDS-PAGE and 10% native-PAGE.

To determine the effect of long term-incubation of SABP2 in detergent and salt, recSABP2 was incubated at 4°C for 4 weeks with either different final concentrations of SDS (0.2%, 0.1%, 0.05%, and 0.01%) or with different final concentrations of NaCl (1 M, 500 mM, and 300 mM). The samples were analyzed in 10% native-PAGE.

In order to investigate the effects of various conditions with SDS, reducing agent and temperature, recSABP2 was treated with combination of SDS, β-ME and temperature. Samples were treated with SDS (0.2% final conc.), β-ME (50 mM) and either incubated at room temperature for 5 min or were boiled for 5 min. The samples were analyzed in both 12% SDS-PAGE and 10% native-PAGE.

Removal of His-tag from recSABP2

Bovine thrombin was used to cleave the N-terminus 6x his-tag and associated extra 7 amino acids (total 13 amino acids) at thrombin cleavage site available in recSABP2-58 construction. Reaction tubes were prepared with 10 µg of recSABP2 and 0.1 U of Thrombin. The reaction volume was made 50 µl for each reaction tube by adding de-ionized water. The reaction tubes were incubated at room temperature for 2 hours following addition of 1 mM PMSF to stop the reactions. The cleavage was observed in both 12% SDS-PAGE and 10% native-PAGE.

Induction of c-myc SABP2 Expression in Transgenic Arabidopsis thaliana

Four-week-old wild type and transgenic Arabidopsis thaliana plants were used for these experiments. In order to induce the expression of c-myc tagged SABP2, transgenic arabidopsis plants were foliar sprayed with 0.03 mM β estradiol (diluted in 0.01% Tween 20). Leaves were harvested after 24 hour of β estradiol treatment.

Isolation of Total Protein from Arabidopsis and Detection of myc-SABP2

Leaves from wild type (Col-0) and transgenic Arabidopsis plants were harvested and washed with de-ionized water. Leaves were homogenized in protein extraction buffer (50mM Tris-HCl pH 7.5, 150 mM NaCl,10% glycerol, 0.1% Triton-X – 100, 1mM PMSF, 1X complete protease inhibitor cocktail). The homogenate were centrifuged at 10,000 rpm for 20 min at 4°C. The supernatants were saturated with 80% ammonium sulfate. The homogenate were incubated

for an additional 20 min on ice followed by centrifugation at 10,000 rpm for 20 min at 4°C. The pellets were resuspended in protein extraction buffer. Resuspended fractions (100 µl) were desalted using Sephadex G-25 spun column pre-equilibrated with 100 µl of 10mM Bicine buffer, pH 8.0. Total protein contents in the desalted fractions were quantified using Bradford's reagent. Desalted proteins were either stored at -20°C or were used immediately for experiments. Total proteins were resolved in 12% SDS-PAGE for denaturing condition and in 4-20% Tris-Acetate gradient gel (Invitrogen) for non-denaturing condition, following Western blot using monoclonal c-myc antibody.

TMV Induced Expression and Detection of SABP2 in Tobacco

In order to induce the expression of SABP2 in tobacco, 6-weeks-old wild type tobacco plants Nicotiana tabacum cv. Xanthi nc (NN) were infected with tobacco mosaic virus (TMV). Two lower leaves, 2 middle leaves, and 2 upper leaves were inoculated with TMV concentrations 1.0, 0.75, and 0.5 µg/ml respectively. Carborundum was dusted evenly to the upper surface of the leaves, and TMV diluted in phosphate buffer was rubbed gently with a piece of cheesecloth. The TMV infected plants were kept separately from uninfected plants but were exposed to regular water and light conditions. Total protein extraction was performed similarly as earlier described for Arabidopsis plants. Total proteins were resolved in 12% SDS-PAGE following Western blot using SABP2 polyclonal antibody.

Purification of Natural SABP2 from Tobacco

In order to investigate the natural conformation, SABP2 was purified from wild type tobacco *Nicotiana tabacum cv. Xanthi* nc (NN). Six-8-weeks old plants were used for this experiment. Full grown leaves were harvested, deveined, and washed with de-ionized water. All steps were carried out at 4°C. Leaves (468g) were homogenized in 3 volume of ice cold protein extraction buffer (buffer A) [20 mM Sodium Citrate/5 mM $MgSO_4$/ 1 mM EDTA, pH 6.3/14 mM 2-mercaptoethanol/0.1 mM phenylmethylsulfonylflouride (PMSF)/1mM benzamidine-HCl] with 1.5% (wt/wt) polyvinylpolypyrrolidone (PVPP) using a warring blender. The homogenate was filtered through 4 layers of cheesecloth and one layer of miracloth. The filtrate was centrifuged (GS3 rotor, Sorvall) at 10,000 rpm for 20 min. Supernatant containing soluble proteins were used for ammonium sulfate precipitation and fractionation.

Ammonium Sulfate Fractionation

Supernatant were slowly saturated with 50% ammonium sulfate (313 g per litre) by adding powdered ammonium sulfate while slowly stirring. Saturated supernatant was further incubated on ice for 20 min. After incubation, the homogenate was centrifuged (SS 34 rotor, Sorvall) at 10,000 rpm for 20 min. The pellet was stored at -80°C until use. The proteins in supernatants were further precipitated using ammonium sulfate to a concentration of 75% ammonium sulfate (176 g per litre) as described above. Saturated supernatant was further incubated on ice for 20 min followed by centrifugation (SS 34 rotor, Sorvall) at 10,000 rpm for 20 min. The pellets were either stored at -80°C or were used for further purification. The presence of SABP2 was determined by Western blot using SABP2 polyclonal antibody.

Purification of 50-75% Ammonium Sulfate Fraction by Hydrophobic Interaction Chromatography

Ammonium sulfate fractions containing SABP2 were purified using hydrophobic interaction column (HIC). Ammonium sulfate pellets (50-75%) were resuspended in buffer B (10 mM bicine/ pH 8.5/ 14 mM 2-mercaptoethanol/ 0.1 mM PMSF/ 1 mM benzamidine-HCl) and were centrifuged at 10,000 rpm for 10 min. The protein contents in supernatants were determined using Bradford's reagent. The supernatants containing total 88 mg of proteins were loaded on a Fast Flow Butyl Sepharose column (HiScreen Butyl FF, column volume 9.7 ml) pre-equilibrated with 1.5 M ammonium sulfate in buffer B. Loosely bound proteins were washed with buffer B. The bound proteins were eluted with a linear gradient of decreasing concentration of ammonium sulfate (1.5 -0 M) in buffer B. The presence of SABP2 in the eluted fractions was determined by Western blot using SABP2 polyclonal antibody.

Purification using Q Sepharose

Fractions containing SABP2 from Butyl Sepharose column were further purified in an anion exchange column. Fractions were pooled, concentrated, and desalted by using buffer B in a Hitrap desalting column (column volume 10 ml). Desalted fractions containing proteins (51 mg) were applied to a Q Sepharose column (column volume 5 ml) pre-equilibrated with buffer B. The bound proteins were eluted with a linear gradient of 0-200 mM ammonium sulfate in buffer B. The presence of SABP2 in the eluted fractions was determined by Western blot using SABP2 polyclonal antibody.

Purification using Mono Q

Fractions containing SABP2 from Q Sepharose column were further purified in another anion exchange column. Fractions were pooled, concentrated, and desalted by using buffer B in a Hitrap Desalting Column (column volume 10 ml) (GE Healthcare). Desalted fractions containing 10 mg proteins were applied to a Mono Q column (Mono Q 5/50 GL, GE Healthcare). The bound proteins were eluted with a linear gradient of 0-200 mM ammonium sulfate in buffer B. The presence of SABP2 in the eluted fractions was determined by Western blot using SABP2 polyclonal antibody and a 12% SDS-PAGE were run to determine purification of SABP2.

Purification using Superdex-75

Fractions containing SABP2 from Mono Q column were further purified in a size exclusion column. The 0.2 ml of fraction #15 containing highest amount of SABP2 (0.6 mg) was loaded to a Superdex 75 column (Superdex 75 10/300 GL, GE Healthcare) pre-equilibrated with buffer C (150 mM ammonium sulfate in buffer B). The proteins were eluted with buffer C. The column was calibrated with protein standards according to manufacturer's instruction (Sigma). Four different protein standards were used to calibrate the column. Each standard was loaded as a volume of 50 µl: Blue dextran (2,000 kDa, 2 mg/ml), BSA (66 kDa, 10 mg/ml), carbonic anhydrase (29 kDa, 3 mg/ml), and cytochrome c (12.4 kDa, 2 mg/ml). Purified recSABP2-21 (2 mg/ml) was also used to determine its conformation. The presence of SABP2 in the eluted fractions was determined by Western blot using SABP2 polyclonal antibody.

Polyacrylamide Gel Electrophoresis and Western Blot Analysis

SDS-PAGE

SDS-PAGE was performed according to Laemmli's protocol (Laemmli, 1970). Unless stated, each sample for SDS-PAGE was mixed with 2x SDS sample buffer containing β-ME, boiled for 5 minutes, and centrifuged at 21130 x g for 10 minutes at room temperature. Protein separation on SDS-PAGE was performed at 20mA constant current for 60 minutes. Buffers used were prepared as described in Appendix B.

Native-PAGE

Native-PAGE was performed according to Laemmli's protocol (Laemmli 1970) except all the buffers and gels were prepared without SDS and reducing agents. Unless stated, all samples for native-PAGE were mixed with 2x sample buffer containing no SDS or reducing agents and instead of boiling samples were incubated on ice for 5 minutes following centrifugation at 21130 x g for 10 minutes at 4°C. Protein separation on native-PAGE was performed at 4°C at 10mA constant current for 3 hours. Buffers used were prepared as described in Appendix B.

Western Blot Analysis

Western analysis was performed using standard protocol after electrophoresis gels were incubated in transfer buffer (Appendix B) for 15 minutes. Prior to transferring proteins from gel

to PVDF membrane, the PVDF membranes were soaked in 100% methanol for 15 seconds following wash with de-ionized water for 2 minutes and washed with transfer buffer for 5 minutes. The PVDF membrane and gel were sandwiched between pre-soaked sponges and Whatman papers. Protein transfers were carried out at constant 100V for 1 hour at 4°C. After transfer, the membranes were placed in 100% methanol for 10 seconds and were let dry on Whatman paper. After the membranes dried completely, they were again soaked in 100% methanol for 10 seconds following rinse with de-ionized water. The membranes were stained with Ponceau-S and photographed to verify equal loading and transfer of proteins. The membranes were washed 3 times with 1x Phosphate buffer saline (PBS) (Appendix B). The blots were probed with monoclonal c-myc or rabbit polyclonal SABP2 primary antibodies (1:1000) in 5mL of blocking buffer (Appendix B) overnight at 4°C on a shaker. The blots were washed 3 times for 5 minutes each with 1x PBS, 1x PBS with 3% tween 20, and 1x PBS sequentially. The blots were then probed with either anti-mouse IgG Fc specific goat or anti-rabbit secondary antibodies with HRP conjugate diluted at 1:5000 for 30 minutes at room temperature and washed sequentially as described earlier. The signals on membranes were developed with ECL reagent (Thermo Scientific), and protein expression was analyzed by using x-ray films as described by the manufacturer.

CHAPTER 3

RESULTS

Purification of recSABP2 Overexpressed in E. coli

RecSABP2 (6x his-tagged) was expressed in *E. coli* and was purified using Ni-NTA affinity column. SDS-PAGE analysis confirmed the purification of recSABP2. Samples were mixed with 2x SDS sample buffer, boiled for 5 min, and centrifuged at 14,000 rpm for 10 min prior to load on gel. SABP2 (29 kDa) (Lane 2, Input); SABP2 was eluted in fraction # 5-13 (Figure 4, lanes 6-14). Most of the other *E. coli* proteins except SABP2 came out in flow through (Figure 4, lane 3). Fraction # 5-7 contained highest amount of SABP2 (Figure 4, lanes 6-8).

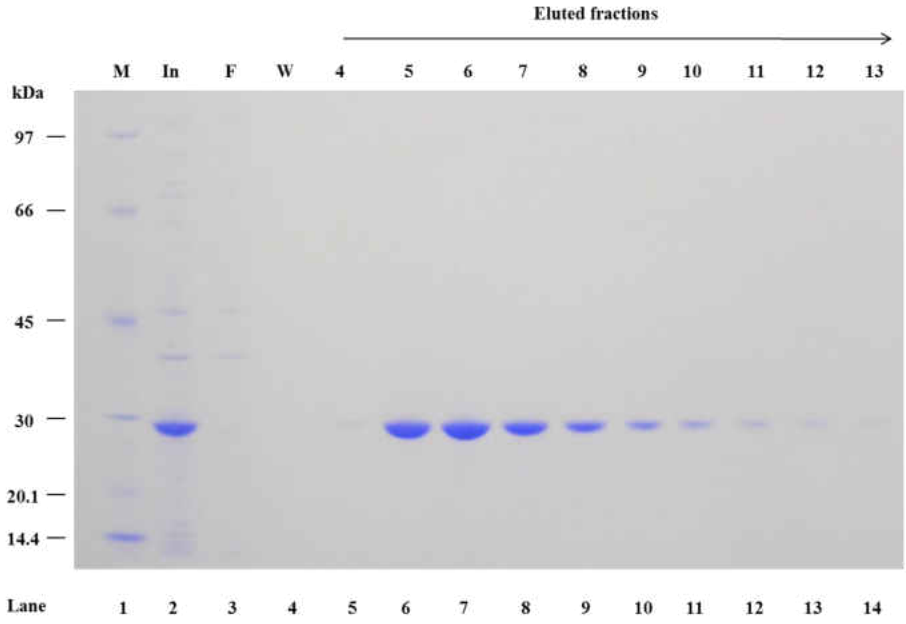


Figure 4. Purification of RecSABP2 expressed in *E. coli*. (M) low molecular weight marker, (In) input to the column, (F) flow through, (W) wash, samples 4-13 (lane 5-14) are eluted fractions from column.

Effect of Temperature on SABP2 Conformation

In order to investigate the effect of temperature on SABP2, purified SABP2 from *E. coli* was mixed with 2x native sample buffer. Samples were incubated for 5 min at different temperatures (55°C, 60°C, 65°C, 70°C, 75°C, and 80°C). Samples were centrifuged for 10,000 rpm for 10 min at 4°C prior to loading on a 10% native-PAGE. Very low amount of SABP2 was visible in the heat treated samples (Figure 5, lanes 4-15) compared to the untreated samples (Lane 2 & 3). Above 60°C (Figure 5, lanes 6-15) no SABP2 was seen. The effect of heat was similar on both SABP2-21 and SABP2-58, except that SABP2-21(with 13 extra amino acids) runs faster than SABP2-58 (with 25 extra amino acids) in native gel.

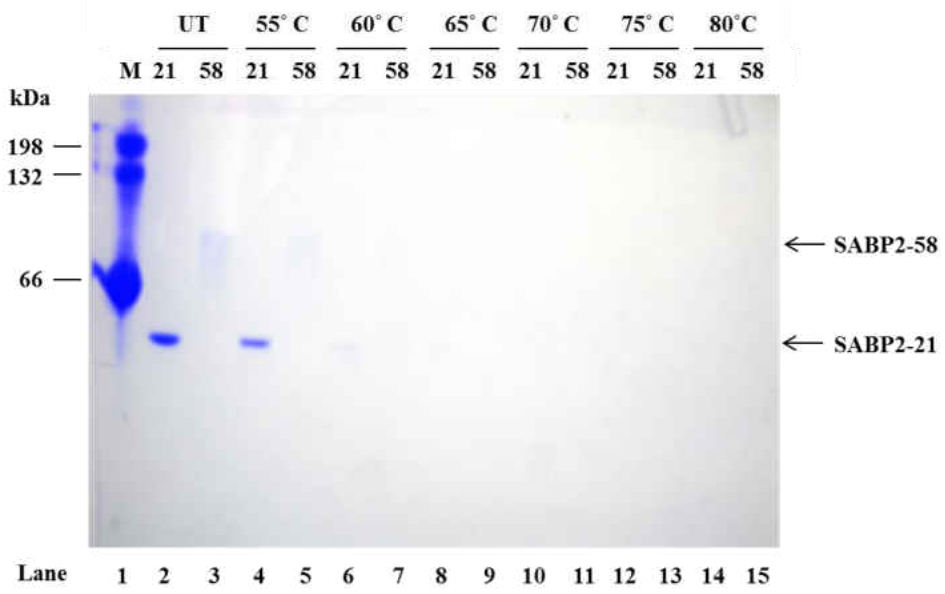


Figure 5. Effect of temperature on recSABP2. (M) BSA, (UT) samples without heat treatment (were incubated on ice), (21) SABP2-21, (58) SABP2-58.

Effect of Mild Detergent on SABP2

In order to investigate the effect of non-ionic detergent on SABP2, purified SABP2 from *E. coli* were incubated with various concentrations of Triton X-100 (0.3%-0.01%) for 15 min either at room temperature for SDS-PAGE (Figure 6) or on ice for native-PAGE (Figure 6). Samples were mixed with 2x native sample buffer and were centrifuged at 14,000 rpm for 10

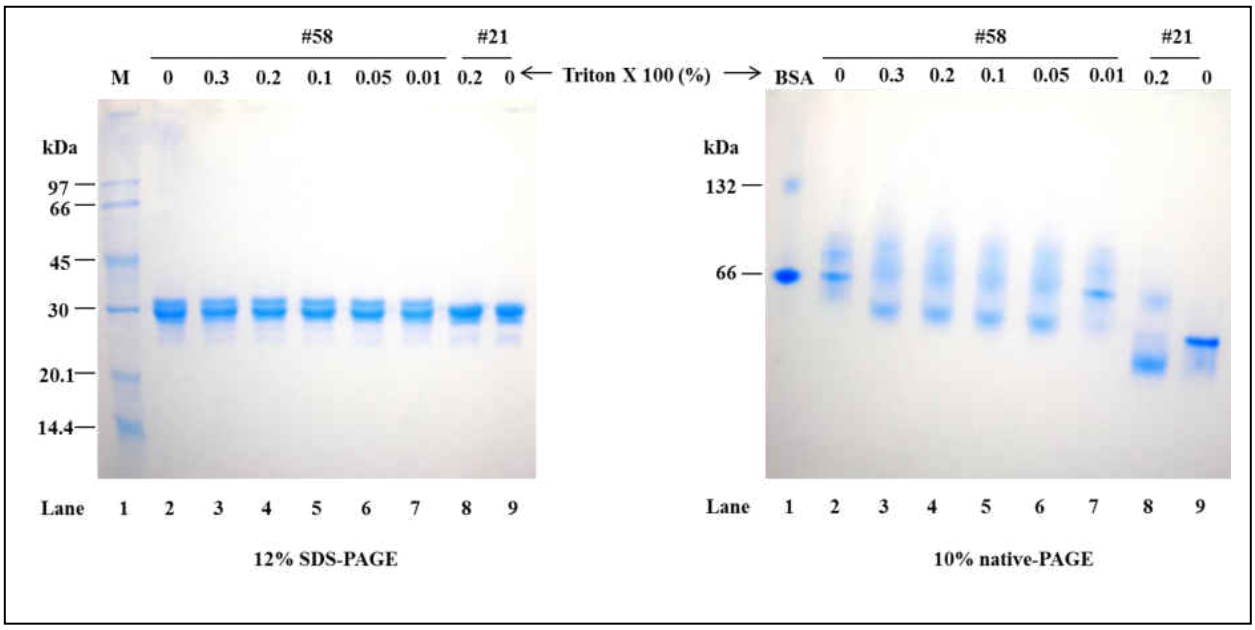


Figure 6. Effect of Triton X-100 treatment on SABP2. (M) low molecular weight marker; (#21) SABP2-21; (#58) SABP2-58; BSA (bovine serum albumin). In native-PAGE, SABP2 runs differently in the presence of Triton X-100 (lane # 3-8) compared to SDS-PAGE.

min either at room temperature for SDS-PAGE (Figure 6) or at 4°C for native-PAGE (Figure 6). SABP2 (#21 & 58) ran as a ~ 30 kDa band under denaturing condition (Figure 6, SDS-PAGE), but under non-denaturing condition SABP2-58 ran around 66 kDa (Figure 6, native-PAGE, lane #2) while SABP2-21 runs faster than SABP2-58 (Figure 6, native-PAGE, lane 9). In the

presence of more than 0.01% of Triton X-100, both SABP2-21 & SABP2-58 runs faster (Figure 6, native-PAGE, lanes 3-6 & 8) than the untreated samples (Figure 6, native-PAGE, lane 2 & 9). In the presence of 0.01% Triton X-100 SABP2-58 runs similarly as untreated samples (Figure 6, native-PAGE, lane 7).

Effect of SDS and NaCl on recSABP2

In order to investigate the effect of SDS and salt on SABP2, purified SABP2 was incubated with various concentrations of SDS and NaCl for 4 weeks at 4°C. Samples were mixed with 2x native sample buffer and were centrifuged for 14, 000 rpm for 10 min at 4°C. Proteins were separated on a 10% native-PAGE (Figure 7). When SABP2 was incubated in more than

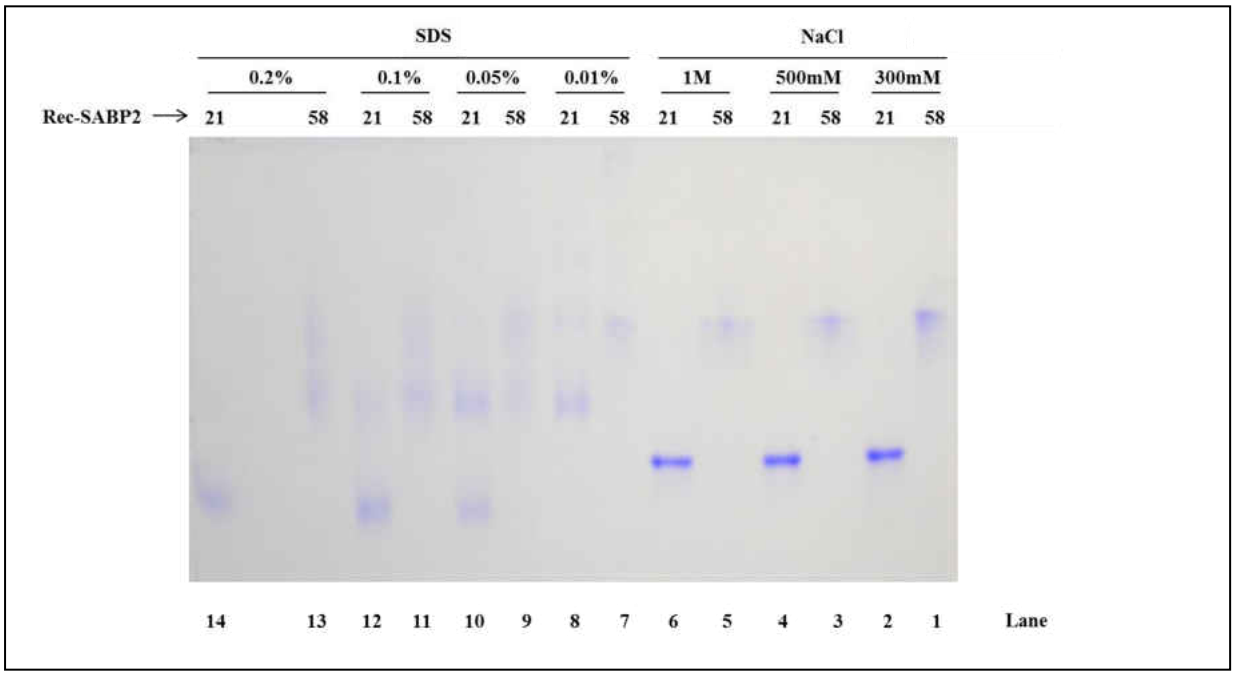


Figure 7. Effect of long-term storage of SABP2 with SDS and NaCl. (21) SABP2-21, (58) SABP2-58. Amount of SABP2 is decreasing from low to high NaCl concentration, suggesting that SABP2 is precipitating in high NaCl concentration. Compared to NaCl treatment, SABP2 runs faster in the presence of SDS (lane # 9-14).

0.01% SDS, both SABP2-21 & SABP2-58 run faster in native gel (Figure 7, lanes 9-14) and the effect is severe in 0.2% SDS (Figure 7, lanes 13 & 14). When SABP2 was incubated with NaCl, the amount of SABP2 band appear to decrease as the concentration of NaCl increases (0.3-1.0 M), suggesting that SABP2 may be precipitating in high salt concentration (Figure 7, lanes 1-6). SABP2 (both #21 & #58) were running faster in the presence of SDS than in the presence of NaCl (Figure 7, lanes 7-14).

Effect of SDS, Heat and β-ME on recSABP2

In order to investigate the combined effects of SDS, reducing agents and temperature on SABP2, purified SABP2 from *E. coli* was treated with 2x sample buffer containing no SDS or 0.2% SDS, and no β-ME or 50 mM β-ME. Samples were either incubated for 5 min at room temperature or were boiled for 5 min. Following treatment, all samples were centrifuged at 14,000 rpm for 10 min. Proteins were separated either in a 12% SDS-PAGE or in a 10% native-PAGE. SABP2 runs faster in the presence of SDS (Figure 8 A, lanes 4, 6, 9, & 10) even without heat and reducing agent treatment (lane 6). When the samples were heated in the absence of SDS, protein aggregation leading to formation of higher oligomeric forms of SABP2 (Figure 8 B, lanes 7 & 8) and most of SABP2 started precipitating as seen by decreasing amounts in gel (Figure 8 A & B, lanes 7 & 8). This precipitated SABP2 was partially recovered by resuspending in sample buffer containing SDS (Figure 8 A & B, lanes 12 & 14). When SABP2 was treated with only 2-ME (50mM), it did not show any significant effect (Figure 8 A & B, lane # 5) compared to the untreated sample (Figure 8 A & B, lane #3).

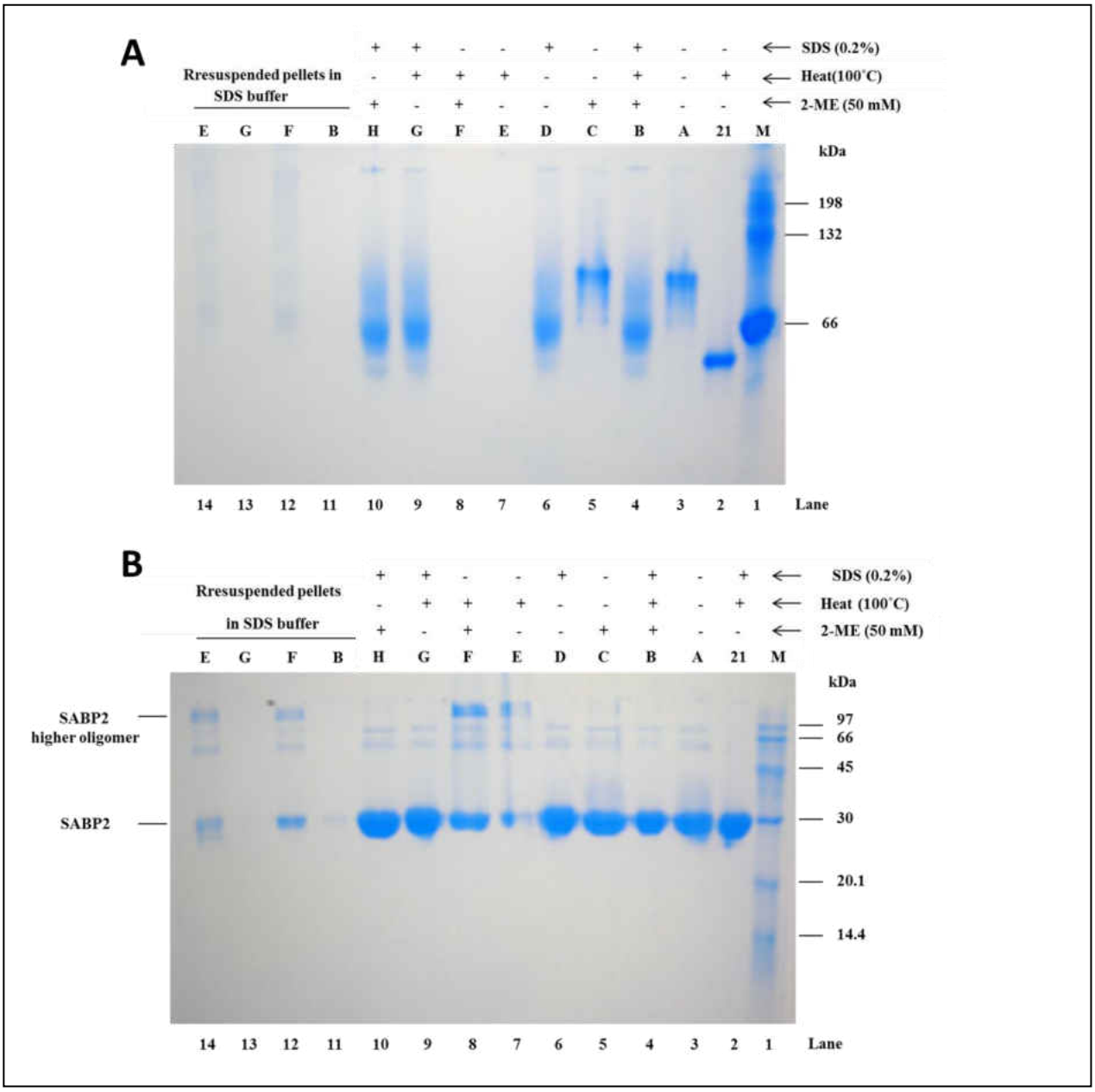
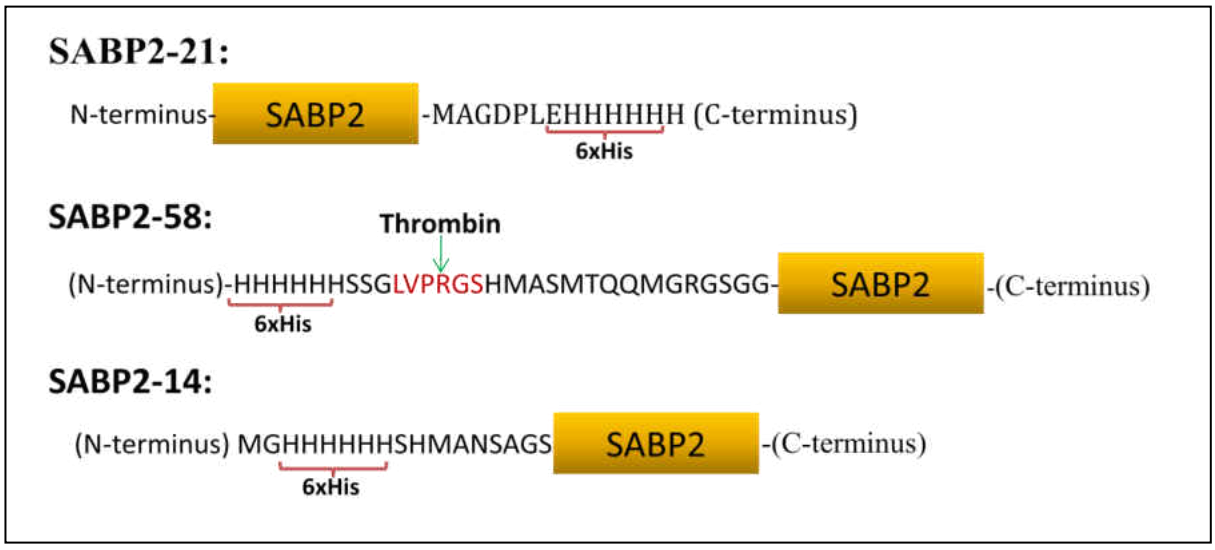


Figure 8. Effect of SDS, Heat, and β-ME on RecSABP2. (A) 10% native-PAGE and (B) 12% SDS-PAGE. M, low molecular weight marker for SDS-PAGE and BSA for native-PAGE. (Sample 21) recSABP2-21, (samples A-H) recSABP2-58. (Lanes # 11-14) pellets from respective samples were resuspended in 2x SDS sample buffer containing 0.2% SDS and 50 mM β -ME. In SDS-PAGE, most of the SABP2 were found around 29 kDa (monomer) and some higher oligomeric forms were also found in absence of SDS in sample buffer (lanes # 7 and 8). In native-PAGE, SABP2 were found around 60 kDa (dimer). When treated with SDS, SABP2 runs differently in native PAGE (lanes # 4, 6, 9 and 10). Note: in native-PAGE, SABP2-58 runs differently than SABP2-21 due to extra 14 amino acids at N-terminus.

Removal of His-tag from recSABP2

Some degradation was observed when purified recSABP2-58 was stored at 4°C for extended period (2 months or more). To verify the cleavage, recSABP2 (both freshly prepared and ~ 2 months old) were incubated with thrombin for 2 hours at room temperature. Effect of thrombin cleavage was observed in both 12% SDS-PAGE and 10% native-PAGE. Thrombin cleaved N terminus 6x his-tag and 7 extra amino acids from freshly prepared SABP2-58 (Figure 9). The cleavage was observed both in SDS-PAGE and in native-PAGE compared to the sample without thrombin treatment (Figure 9, lanes 4 & 5). But thrombin could not cleave in 2 months old SABP2-58 samples (Figure 9, lanes 6 & 7). Thrombin could not cleave in SABP2-14 that also has N-terminus his-tag but without any thrombin cleavage site, showing the specificity of thrombin (Figure 9, lanes 2 & 3). In native-PAGE, 2 months old SABP2-58 runs as a lower molecular weight than freshly prepared SABP2-58, suggesting self-cleaving of SABP2-58 over time (Figure 9, native-PAGE, lanes 4 & 6). No self-cleavage was observed for SABP2-21 (Figure 9, lanes 8 & 9).



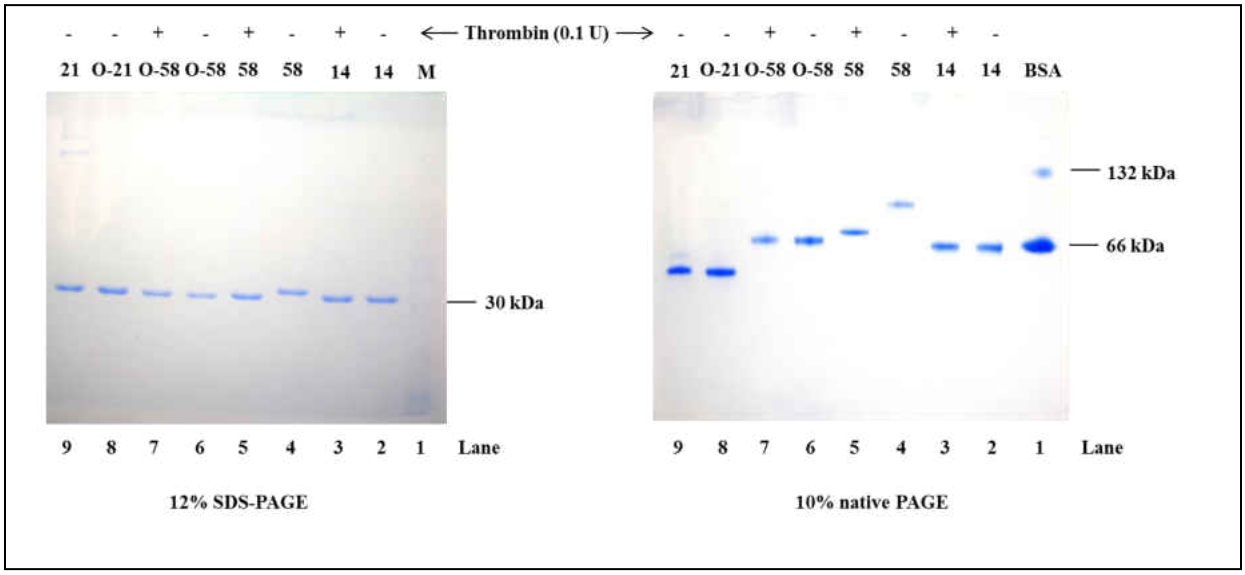
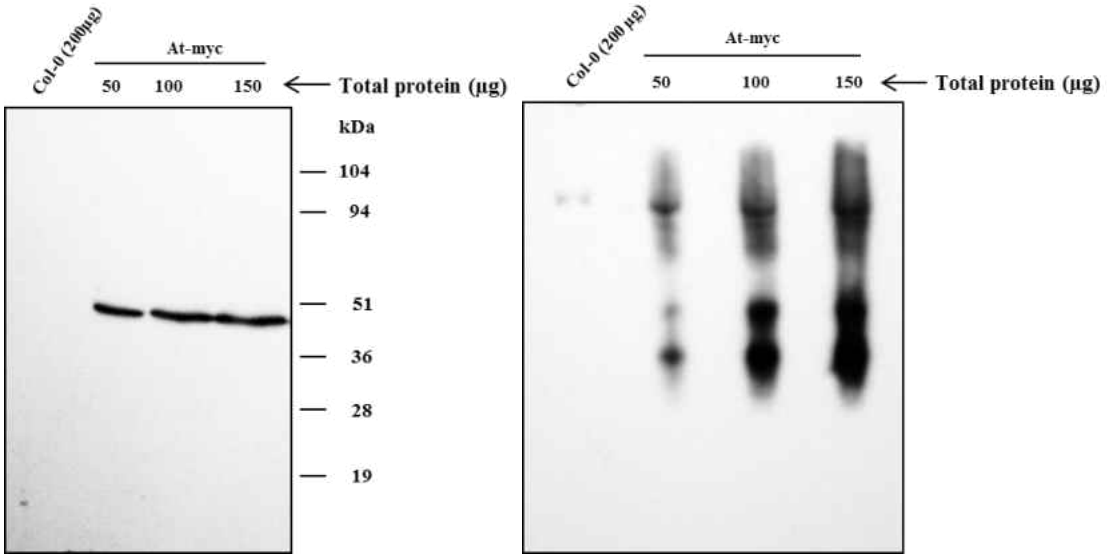


Figure 9. Removal of His-tag from recSABP2. (M) low molecular weight marker; (14) freshly prepared SABP2-14; (58) freshly prepared SABP2-58; (O-58) 2 months old SABP2-58; (21) freshly prepared SABP2-21; (O-21) 2 months old SABP2-21. Thrombin cleaved 13 extra amino acids (N-terminus) from freshly prepared SABP2-58 (lane # 5), but not from 2 months old SABP2-58 (lane # 6). No self-cleavage was observed in SABP2-21 (lane # 8).

MycSABP2 Expression in Arabidopsis

In order to investigate the tobacco SABP2 expressed in Arabidopsis plants, transgenic Arabidopsis plants were foliar sprayed with 0.03 mM β estradiol and were incubated for 24 hours to induce the expression of myc tagged SABP2. The leaves were harvested and total proteins were extracted as described earlier. MycSABP2 was detected by Western analysis using monoclonal c-myc antibodies. Wild type Arabidopsis (Col-0) plants were used as control for mycSABP2 expression. MycSABP2 was expressed in estradiol treated plants and the protein was detected in total protein as low as 50µg (Figure 10 A & B), but in the control Arabidopsis plants no SABP2 was detected even from 200µg of total protein (Figure 10 A & B, Col-0). Under

denaturing condition mycSABP2 runs around 50 kDa (Figure 10 A), but under non-denaturing condition mycSABP2 shows various oligomeric forms (Figure 10 B).



(A) (B)

Figure 10. MycSABP2 Expression in Arabidopsis. (A) Western analysis after 12% SDS-PAGE. (B) Western analysis after non-denaturing electrophoresis in 4-20% Tris-acetate gradient gel. (At-myc) Arabidopsis plants expressing mycSABP2; (Col-0) control plants. In SDS-PAGE, mycSABP2 was detected around 50 kDa (A), while under non-denaturing condition multiple bands were observed (B), suggesting oligomeric structures of mycSABP2.

TMV Infection Results Increased SABP2 Expression in Tobacco

To induce expression of native SABP2, wild type tobacco (*Nicotiana tabacum cv. Xanthi* nc (NN) was infected with TMV (Kumar and Klessig 2003). Two lower leaves, 2 middle leaves, and 2 upper leaves were inoculated with TMV concentrations 1µg/ml, 0.75 µg/ml and 0.5 µg/ml respectively. Twelve days post-inoculation leaves were harvested and total protein was extracted as described earlier. SABP2 expression was detected by Western blot analysis using rabbit

polyclonal SABP2 antibody (Figure 11). Plants without TMV treatment were used as negative control and recSABP2 was used as positive control. SABP2 was detected in TMV treated plants (Figure 11, lanes 2-4) but very small amounts of SABP2 was detected in untreated plants (lane 1). Treatment of plants with 0.75µg/ml TMV resulted maximum expression of SABP2 (lane 3). Besides bands around 28 kDa, higher oligomeric forms of SABP2 (~ 94 kDa) were also observed in TMV treated plants (lanes 2-4) compared to untreated plants (lane 1).

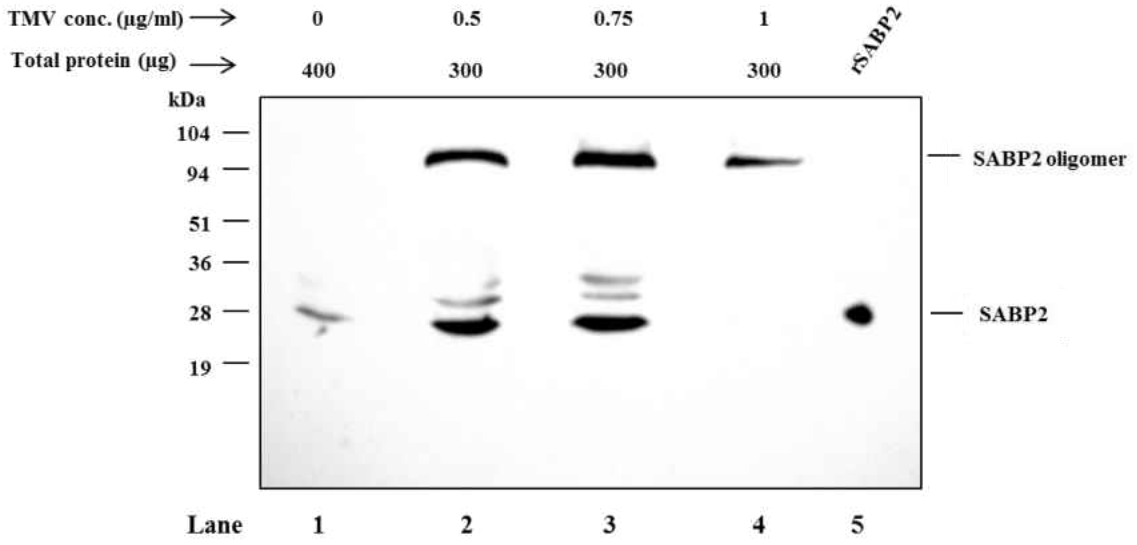


Figure 11. TMV Induced Expression of SABP2. (rSABP2) recombinant SABP2. SABP2 was detected after 12 days of TMV infection [lanes # (2-4)] with a higher oligomeric form around 94 kDa. Very few amount of SABP2 was detected in control plants without TMV infection (lane #1).

Purification of Native SABP2 from Tobacco

Extraction and Ammonium Sulfate Fractionation

In order to investigate the structure of native SABP2, purification of SABP2 from tobacco leaves using conventional chromatographic techniques was undertaken as described by (Kumar and Klessig 2003). Tobacco *Nicotiana tabacum* cv nc (Xanthi NN) leaves were homogenized in protein extraction buffer and SABP2 was fractionated as 50-75% ammonium sulfate (Figure 12) as described earlier. After every step of purification, the presence of SABP2 was confirmed by Western analysis using polyclonal SABP2 antibodies and SDS-PAGE analysis

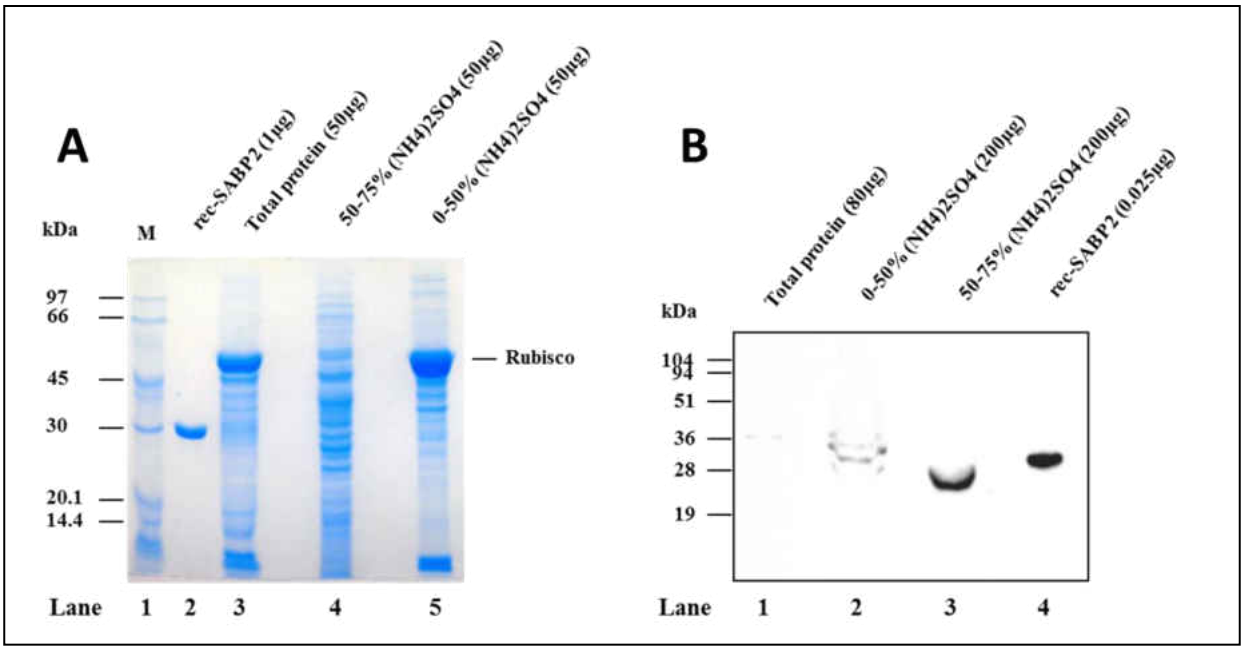


Figure 12. Ammonium Sulfate Fractionation of SABP2. (A) Protein profile of ammonium sulfate fractions in 10% SDS-PAGE (Coomassie stain). (B) SABP2 (29 kDa) was detected in 50-75% ammonium sulfate fraction by SABP2 polyclonal antibody; recSABP2 as positive control. Note: recSABP2 size is slightly higher than native SABP2 due to extra 13 amino acids.

was performed to verify purification of SABP2. SABP2 was readily detected in 50-75% ammonium sulfate fraction (Figure 12 B, lane 3) but not in 0-50% ammonium sulfate (Figure 12 B, lane 2). No SABP2 could be detected in total extract (Figure 12 B, lane 1). A Large amount of protein was fractionated as 0-50% ammonium sulfate including the Rubisco (Figure 12 A, lane 5).

Purification using Butyl Sepharose Column

Fifty to 75% ammonium sulfate fraction containing SABP2 was further purified using a Butyl Sepharose Column as described earlier. Eluted fractions were subject to immunoblot using SABP2 antibody to select the fractions for further purification. In butyl sepharose column, most of the proteins eluted as a wide peak and were collected as fraction #10-39 (Figure 13). Fraction

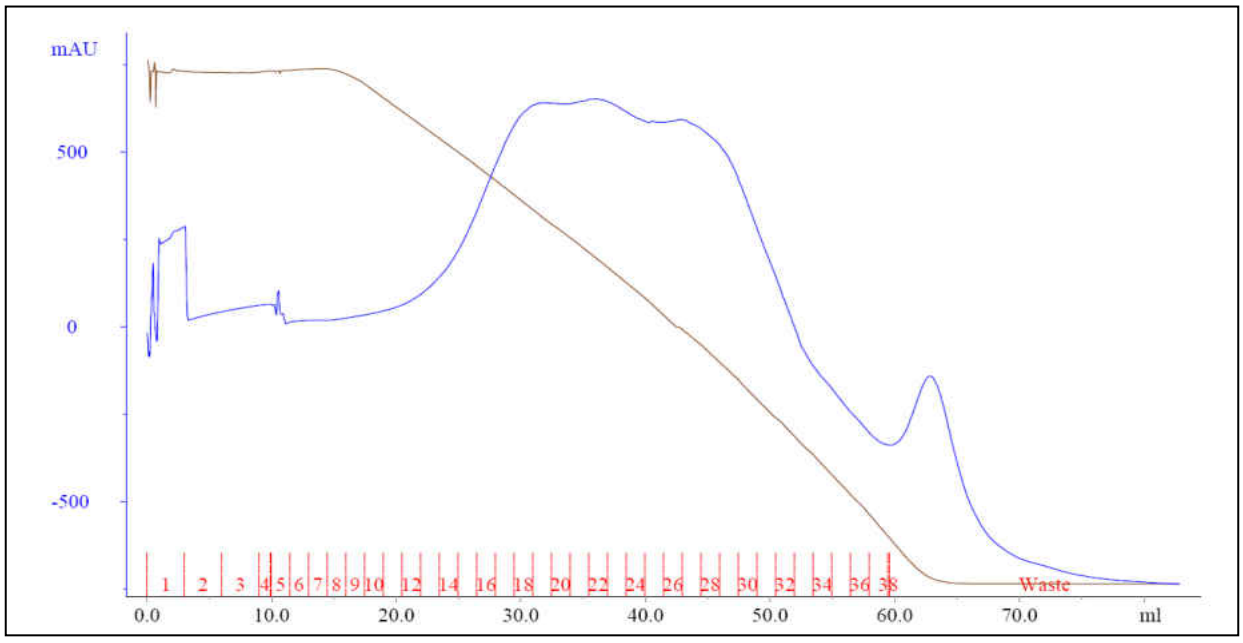


Figure 13. Chromatography Profile of Protein in Butyl Sepharose Column. (Blue line), absorbance of protein at 280 nm; (brown line), salt conductivity; (red line) collected fractions (1ml). Fractions # (7-39) were used for gel and Western blot analysis (Figure 14).

#7-39 were analyzed by SDS-PAGE and Western blot (Figure 14). SABP2 (29 kDa) was detected in eluted fractions (#23-29) (Figure 14 C).

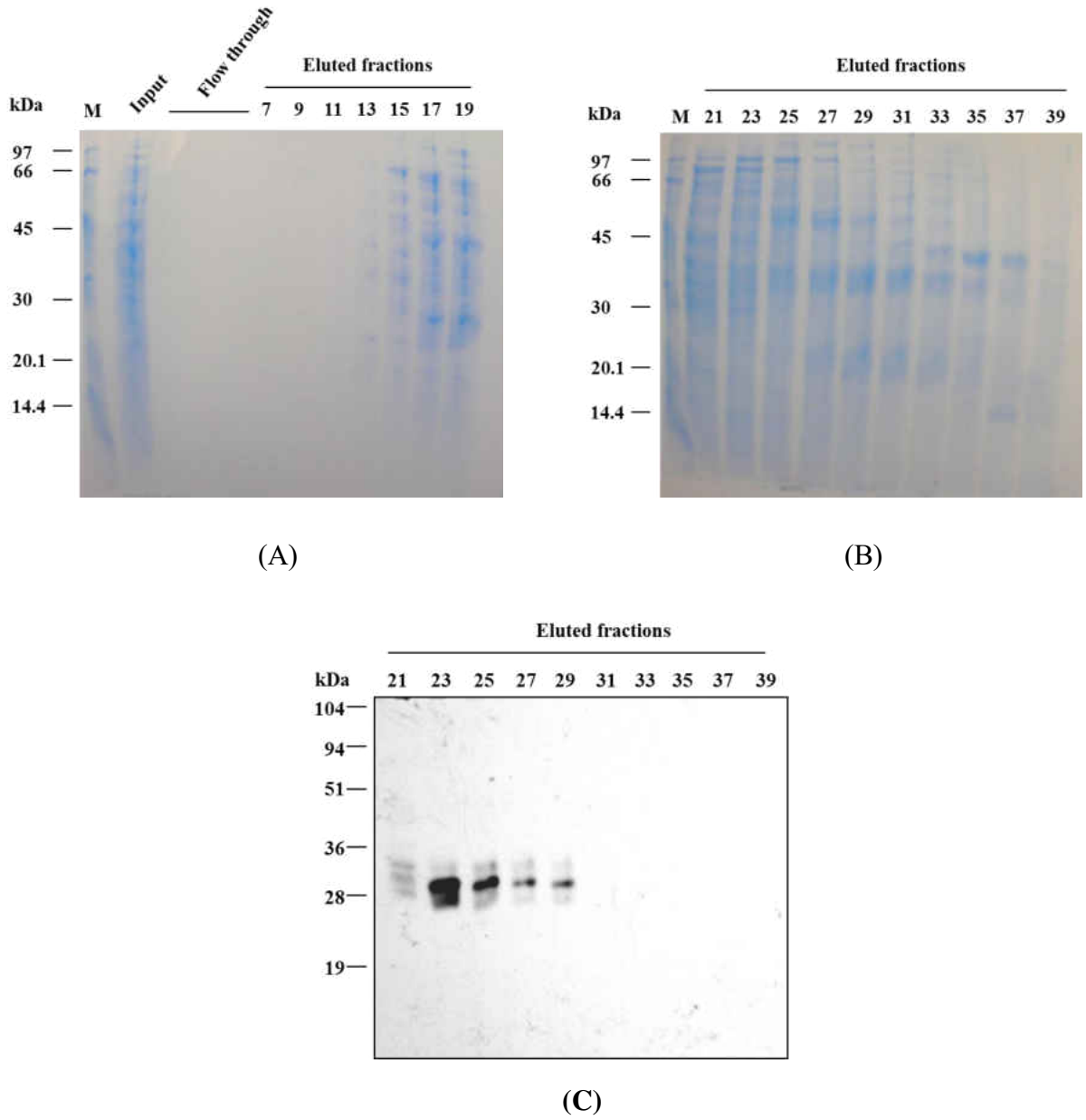


Figure 14. Purification of SABP2 in Butyl Sepharose Column. (A) and (B) Protein profile of Butyl Sepharose eluted fractions # (7-39) in SDS-PAGE (coomassie stain); (M) low molecular weight marker. (C) SABP2 (29 kDa) was detected in fractions # (23-29) by SABP2 polyclonal antibody.

Purification using Q Sepharose Column

Butyl Sepharose purified fractions (# 22-30) containing SABP2 were pooled, concentrated by ammonium sulfate precipitation, desalted, and further purified using a Q Sepharose column as described earlier. In Q sepharose column, a sharp peak was observed around 18 ml (Figure 15). Fractions #13-23 containing most proteins were analyzed by SDS-PAGE and Western blot (Figure 16). SABP2 (29 kDa) was detected in fraction #17-20 (Figure 16 B). Purification was verified by SDS-PAGE, which indicated that some other proteins were also eluted along with SABP2 (Figure 16 A).

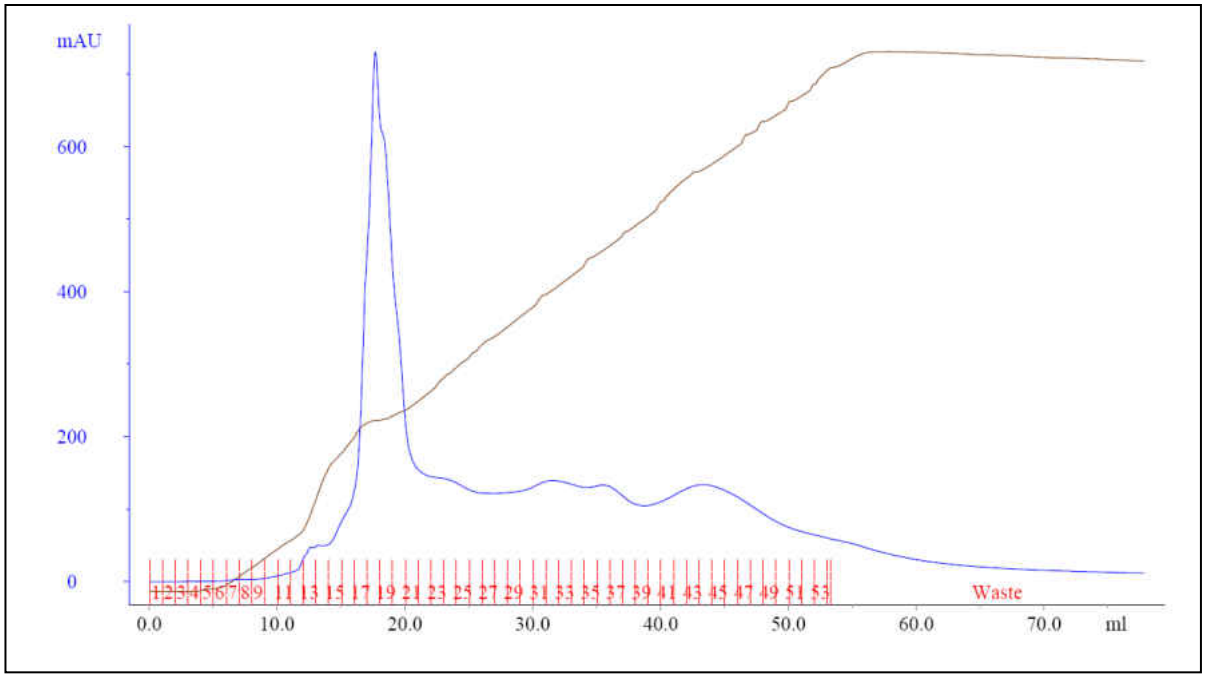


Figure 15. Chromatography Profile of Protein in Q Sepharose Column. (Blue line) absorbance of protein at 280 nm; (brown line) salt conductivity; (red line) collected fractions (1 ml). Fractions # (13-23) were used for gel and Western blot analysis (Figure 16).

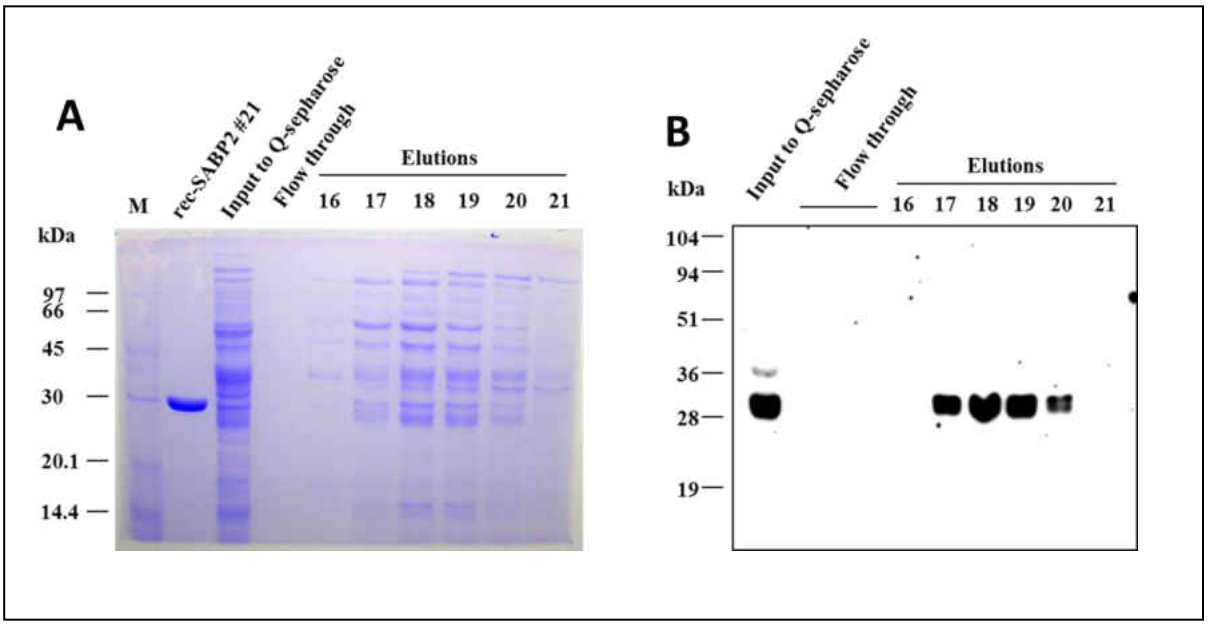


Figure 16. Purification of SABP2 in Q Sepharose Column. (A) Protein profile of Q Sepharose eluted fractions # (16-21) in SDS-PAGE (coomassie stain); (M) low molecular weight marker. (B) SABP2 (29 kDa) was detected in fractions # (17-20) by SABP2 polyclonal antibody.

Purification using Mono Q Column

Q Sepharose purified fractions (# 17-20) containing SABP2 were pooled, concentrated by ammonium sulfate precipitation, desalted, and further purified on a Mono Q column as described earlier. In Mono Q column most proteins eluted around 4 ml showing a sharp peak collected as fractions #12-20 (Figure 17). The eluted fractions were analyzed by Western blot. SABP2 (29 kDa) was detected in fractions #13-17 where fraction #15 had maximum amount of SABP2 (Figure 18 B). The purification of SABP2 was analyzed by SDS-PAGE where SABP2 eluted with other proteins (Figure 18 A).

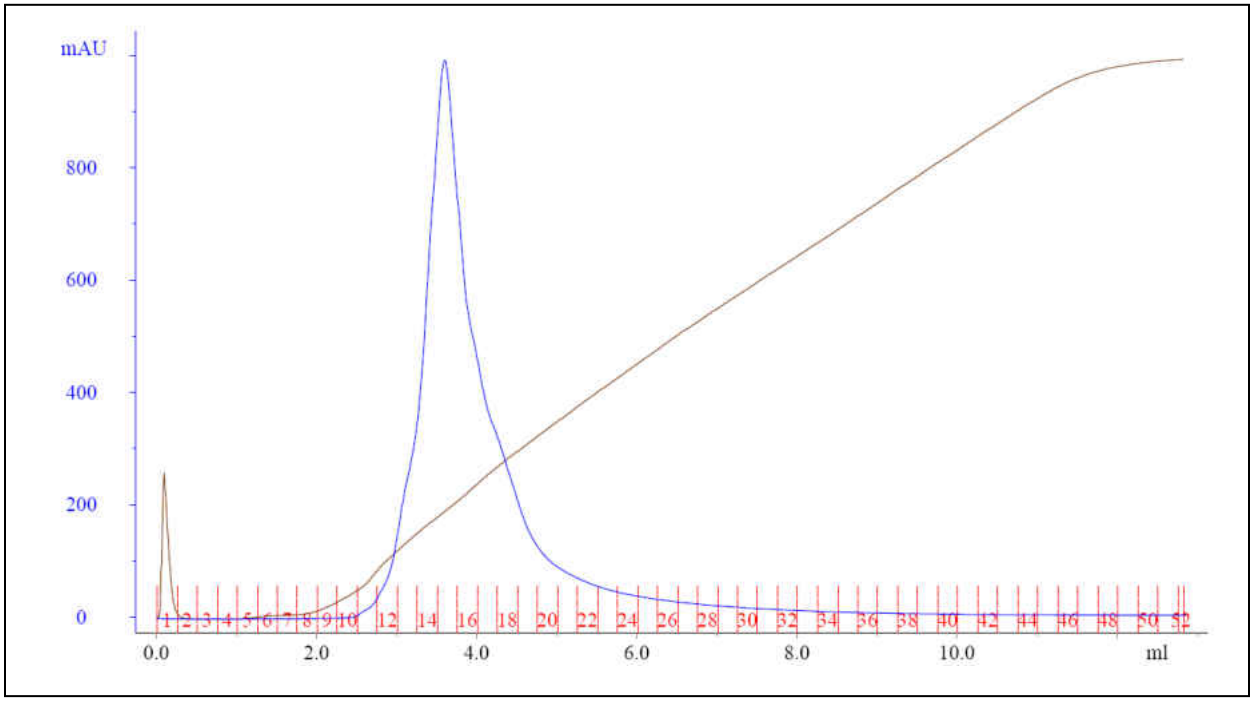


Figure 17. Chromatography Profile of Protein in Mono Q Column. (Blue line) absorbance of protein at 280 nm; (brown line) salt conductivity; (red line) collected fractions (0.25 ml). Fractions # (10-22) were used for gel and Western blot analysis (Figure 18).

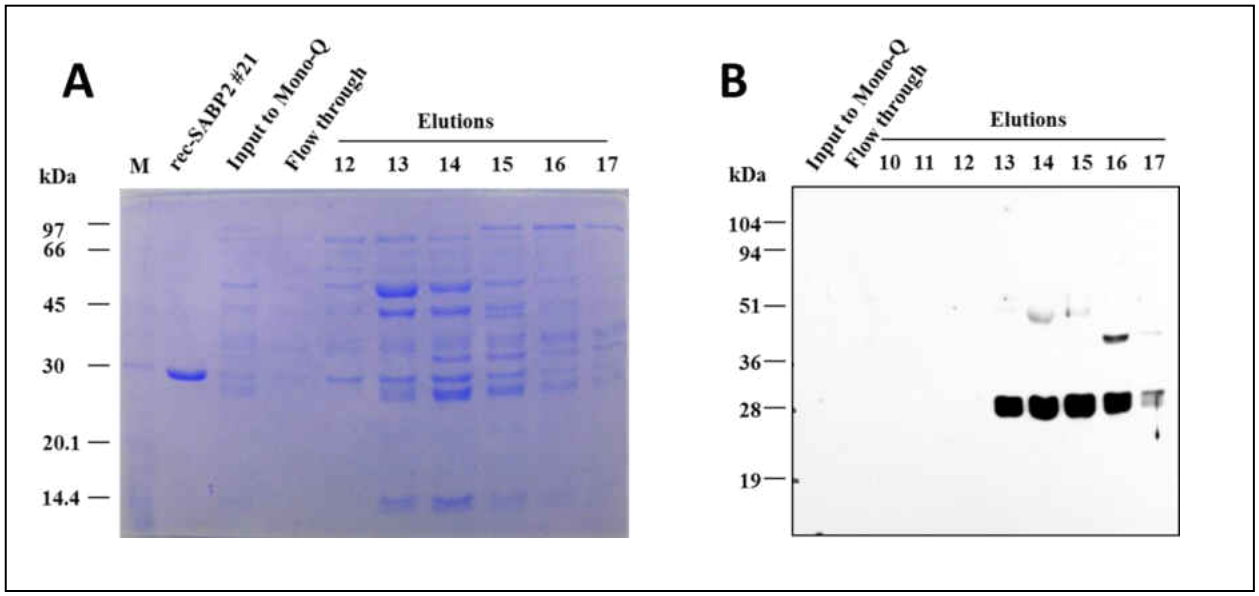


Figure 18. Purification of SABP2 in Mono Q Column. (A) Protein profile of Mono Q eluted fractions # (12-17) in SDS-PAGE (coomassie stain); (M) low molecular weight marker. (B) SABP2 (29 kDa) was detected in fractions # (13-17) by SABP2 polyclonal antibody.

Purification using Superdex 75 Column

Mono Q purified fraction (# 15) (0.2 ml) containing highest amount of SABP2 was loaded on a Superdex 75 column and was purified as described earlier. In order to calibrate the Superdex 75 column, mixture of 4 protein standards was run (Figure 19). The elution volume of blue dextran (2,000 kDa) was 7.6 ml, which was used as column void volume (Vo) (Figure 19). The elution volumes of other 3 standards , bovine serum albumin (9.75 ml), carbonic anhydrase (12 ml), and cytochrome c (13 ml) (Figure 19), were used to calculate the retention co-efficient (Kav) of the standards (Table 1). RecSABP2-21 (400 mg) was applied to the column that gave an elution volume of 10.6 ml (Figure 20). The mono Q purified proteins eluted between fractions #29-51 (Figure 21). Eluted fractions were analyzed using Western blot. Native SABP2 was detected in fraction # 46 & 47 (Figure 22 B), which gave an elution volume (Ve) of about 12 ml (Figure 21). The purification was analyzed using SDS-PAGE followed by silver stain where SABP2 eluted as 29 kDa protein along with another protein around 35 kDa (Figure 22 A). A standard curve was obtained by plotting the Kav values of the standards against their log 10 molecular weight (Figure 23). The Kav values for recSABP2-21 and native SABP2 were plotted to obtain estimated log 10 molecular weight (Figure 23). The molecular weights of recSABP2-21 and native SABP2 were calculated according to inverse log table. The estimated molecular weight of recSABP2-21 was calculated as 57.5 kDa (dimer) and for native SABP2 was 28.8 kDa (monomer).

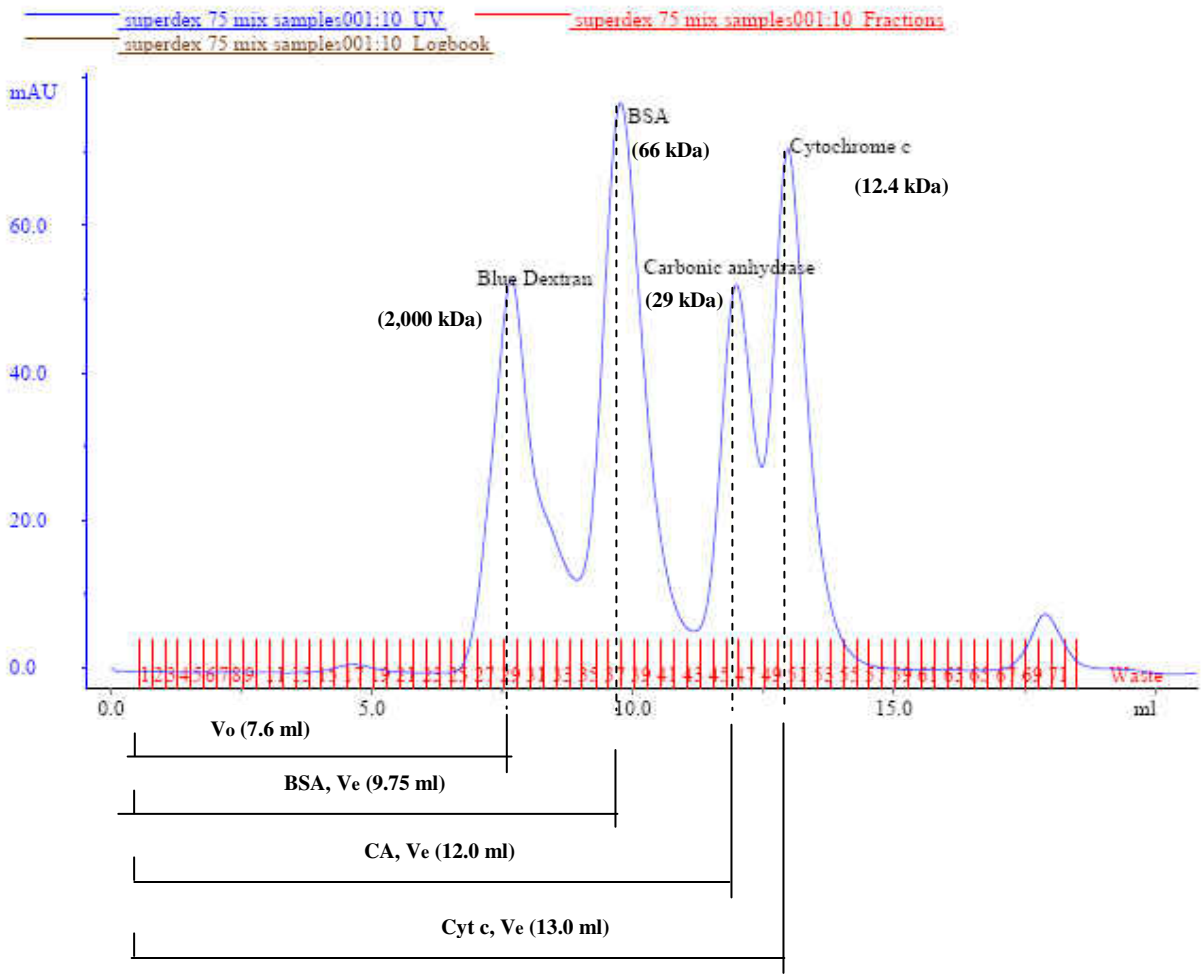


Figure 19. Chromatography Profile for Mixed Standards in Superdex 75 Column. 50 µl of each standard (blue dextran, 2,000 kDa; BSA, 66 kDa; carbonic anhydrase, 29 kDa and cytochrome c, 12.4 kDa) were mixed together and a total volume of 200 µl was loaded on column. (Blue line) absorbance of protein at 280 nm; (red line) collected fractions (0.25 ml). Void volume (Vo), elution volume (Ve).

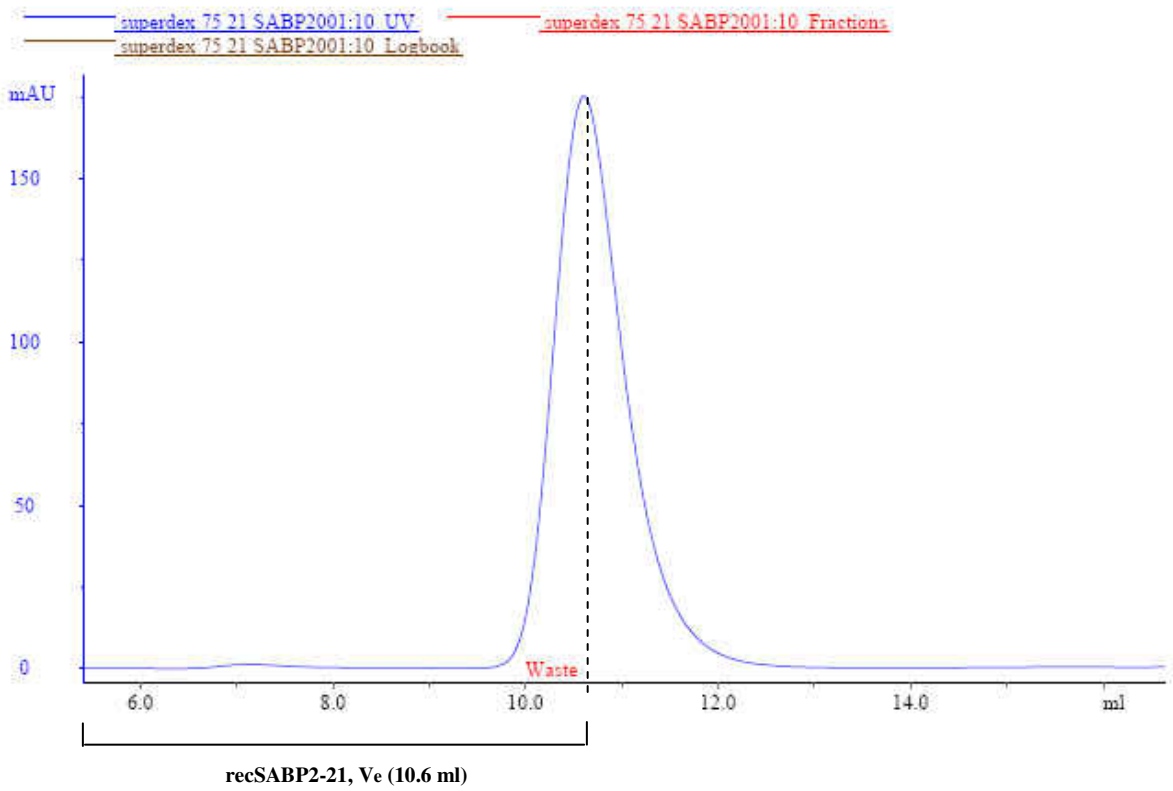


Figure 20. Chromatography Profile of RecSABP2-21 in Superdex 75 Column. 200 µl (400 µg) of Q sepharose purified recSABP2-21 was loaded on the column. (Blue line) absorbance of protein at 280 nm; (red line) collected fractions (0.25 ml). The elution volume (Ve) for recSABP2-21 is 10.6 ml.

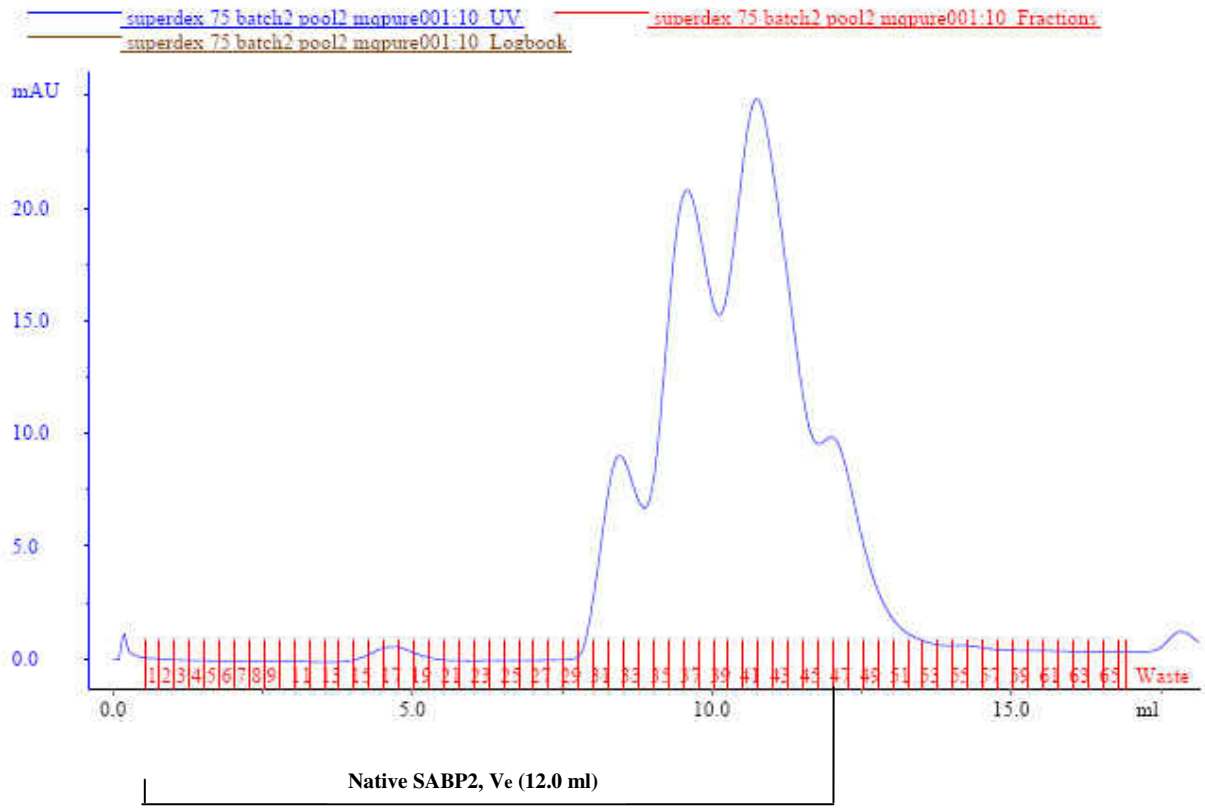
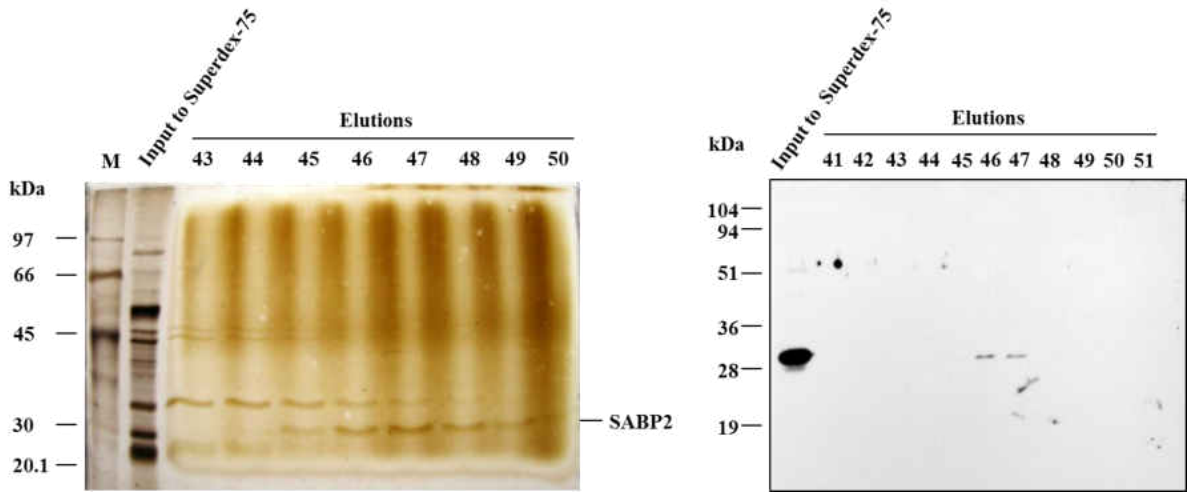


Figure 21. Chromatography Profile of Protein in Superdex 75 Column. 200 µl (0.6 µg) of Mono Q purified fraction #15 was loaded on the column. (Blue line) absorbance of protein at 280 nm; (red line) collected fractions (0.25 ml). SABP2 elution volume (Ve) is 12 ml. Fractions # (29-51) were used for gel and western blot analysis (Figure 22).



(A) (B)

Figure 22. Purification of SABP2 in Superdex 75 Column. (A) Protein profile of Superdex 75 eluted fractions # (43-50) in SDS-PAGE (silver stain); (M) low molecular weight marker. (B) SABP2 (29 kDa) was detected in fractions # (46-47) by SABP2 polyclonal antibody. Note: the signal of SABP2 eluted fractions (B) is week compared to input in column because SABP2 became diluted 25 fold during elution.

Table 1. Column Void Volume (Vo), Elution Volume (Ve) and Retention co-efficient (Kav) Values for Standards and Samples in Superdex 75.

	Ve (ml)	**Vo (ml)**	**Vc (ml)**	**Kav = Ve-Vo/Vc-Vo**
BSA	9.75	7.6	24	0.131
Carbonic anhydrase	12	7.6	24	0.268
Cytochrome c	13	7.6	24	0.329
recSABP2-21	10.6	7.6	24	0.183
natural SABP2	12	7.6	24	0.268

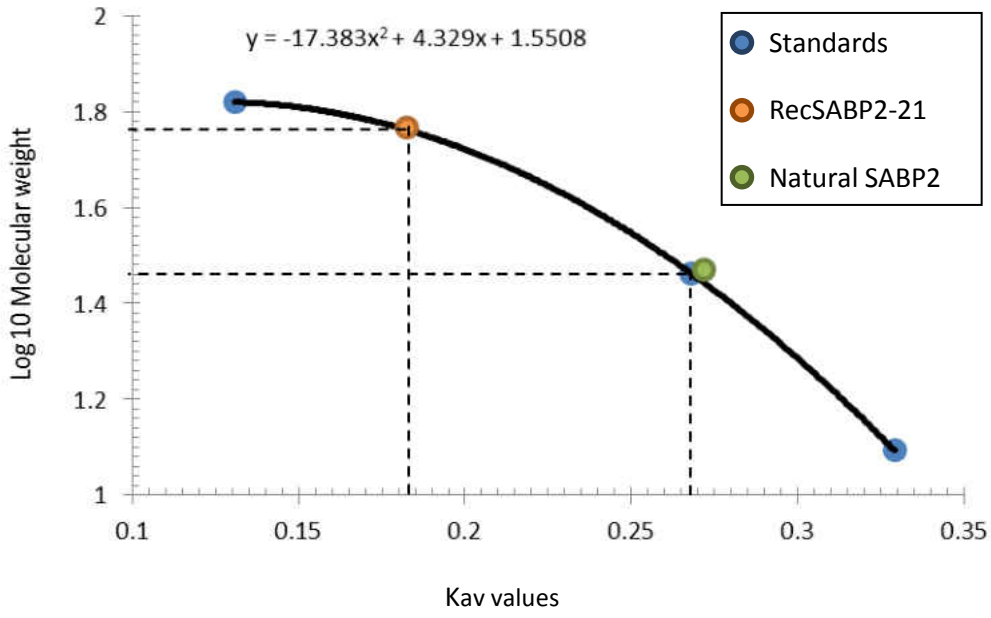
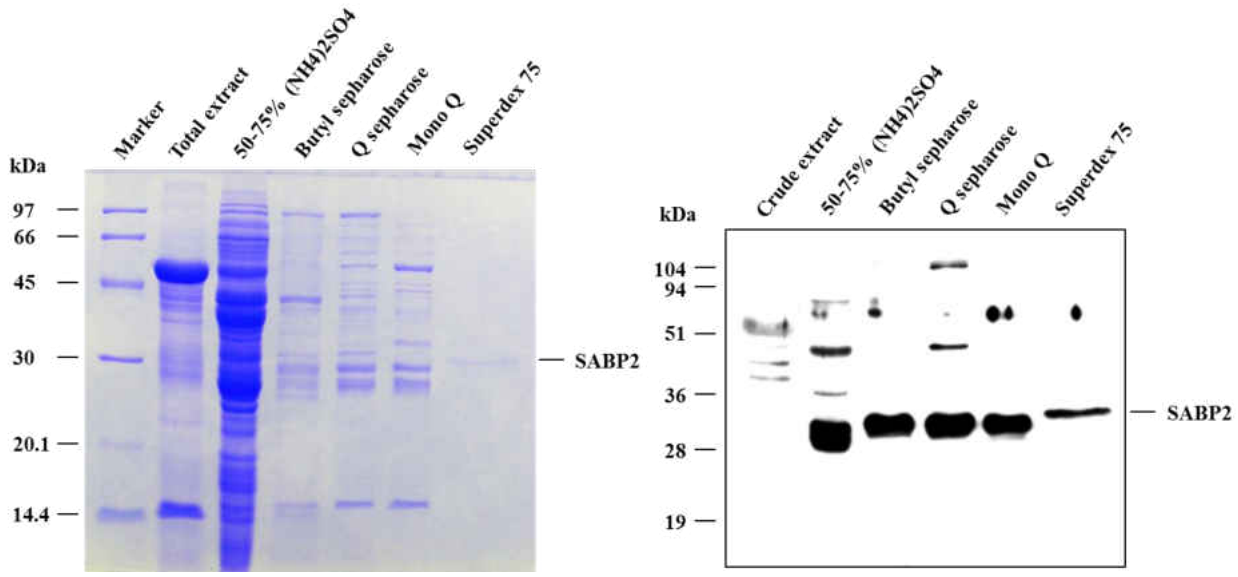


Figure 23. Calibration Curve for Superdex 75 Column. Standard curve obtained by plotting the Kav values of the standards against their log 10 molecular weight. Log 10 molecular weight of unknown samples (recSABP2-21 and native SABP2) were determined using the equation of the polynomial curve. The log 10 molecular weight of recSABP2-21 is 1.76 (approximate molecular weight 57.5 kDa) and for natural SABP2 is 1.46 (approximate molecular weight 28.8 kDa).

Protein Purification Profiles of Tobacco SABP2

The purification profile was analyzed by SDS-PAGE and western blot. Purification was performed using a 5-step protocol described earlier. Protein was separated as a highly concentrated form in 50-75% ammonium sulfate but became diluted many times during purification in chromatography columns (Figure 24 A). After final purification in Superdex 75 column, SABP2 was detected as a single band around 29 kDa both in coomassie blue stained gel (Figure 24 A) and Western blot (Figure 24 B).



(A) (B)

Figure 24. Protein Profiles from the Purification of Tobacco SABP2. (A) SDS-PAGE analysis of fractions from each step of the SABP2 purification protocol. Aliquots from pooled fractions containing highest amount of SABP2 were analyzed by 12% SDS-PAGE stained with Coomassie blue. (B) Western blot analysis of fractions from each step of the SABP2 purification protocol using SABP2 polyclonal antibody.

CHAPTER 4

DISCUSSION

Among various signaling pathways in plant, SA signaling pathway has been studied extensively. The role of SA in development of SAR has been the major focus for these studies. Genetic and biochemical studies have provided us a plethora of information about SA mediated defense pathway. Many biological components in this pathway have been revealed, but it is far from complete. SABP2 is a 29 kDa protein that plays a pivotal role in SA signaling pathway. SABP2 has an esterase activity that converts the lipid soluble MeSA into SA in order to enhance the SA level in cytoplasm for downstream defense signaling (Malamy et al. 1990; Metraux et al. 1990; Rasmussen et al. 1991). Increased level of SA in cytoplasm feedback inhibits SABP2's esterase activity, which is required for induction of SAR in systemic tissues (Park et al. 2007). SABP2 has been overexpressed in *E. coli* and characterized (Forouhar et al. 2005), which showed that recSABP2 is a homodimer and the dimer has the same ability as for monomer to bind with SA (Forouhar et al. 2005). Moreover, it has been also shown that recSABP2 has high affinity for SA and could convert MeSA into SA (Forouhar et al. 2005). This leads us to investigate if SABP2 exists as a monomer or dimer in plants under natural condition. Previous study suggested that SABP2 may be a monomer in physiological concentrations in tobacco leaves (Kumar and Klessig. 2003). Based on previous information, we hypothesized that under natural condition SABP2 may exist as dimer and/or monomer in plant. We overexpressed the recSABP2 in *E. coli* and characterized the recSABP2 to gather information that would be helpful in characterizing SABP2 from plant sources. Based on the results obtained, it can be inferred that SABP2 dimer is sensitive to treatment with detergents. When SABP2 was incubated with SDS

(either in the presence or absence of reducing agent and heat), its electrophoretic mobility changes (Figure 8 A) suggesting the chaotropic effect of SDS on SABP2. Similarly, when SABP2 was incubated with SDS for a long time, it ran faster in native gel may be due to the extra negative charges due to binding of SDS and the effect is more visible in higher SDS concentrations (0.2%) (Figure 7). Treatment of SABP2 with non-ionic detergent (Triton X-100) showed similar results where almost all the proteins denatured upon treatment with 0.05% Triton X-100 (Figure 6, native-PAGE). In addition, it was also observed that SABP2 precipitates when incubated at 55°C or higher (Figures 5 and 8 A) even in the presence of reducing agent (Figure 8 A, lane# 8), and SDS can partially recover the precipitated proteins (Figure 8, lane # 12 and 14). On the other hand, treatment of SABP2 with salt (NaCl) results in precipitation of SABP2 in a concentration dependent manner (Figure 7). On the other hand, in attempt to answer the question if SABP2 exists as a dimer in plants, we overexpressed myc-SABP2 in *Arabidopsis thaliana* because the natural expression of SABP2 in tobacco plants is in very negligible amounts, which makes it difficult to identify this protein. The myc-SABP2 overexpressed in Arabidopsis showed a single band around 50 kDa in SDS-PAGE (Figure 10 A) suggesting the monomeric form of myc-SABP2, because myc tag (6x) added an additional 60 amino acids to native SABP2 (29 kDa). But, when the plant extract from myc Arabidopsis were subject to non-denaturing electrophoresis in a 4-20% gradient gel following Western blot, myc-SABP2 showed multiple bands, suggesting the oligomeric structures of myc-SABP2 (Figure 10 B). However, under non-denaturing electrophoresis, proteins can run differently due to possible interactions with other proteins. Moreover, a large myc tag may induce the change in the protein structure of SABP2. In another experiment, SABP2 expression was induced by infecting the wild type tobacco plants with tobacco mosaic virus (TMV). After 12 days of infection, increased amount of SABP2

resulted in an easy detection in Western blot analysis using SABP2 antibody (Figure 11). Interestingly, in Western blot another band around 94 kDa was also detected by SABP2 antibody, suggested that it could be higher oligomeric form of SABP2 (Figure 11). However, this high molecular weight structure could be due to the amount of SABP2 expressed because some proteins tend to aggregate in high concentration. Or it could be another protein having some similarity to SABP2 that is induced by TMV infection. Moreover, upon infection plants activate their defense mechanism and we know that SABP2 is a key component of SA signaling pathway. Therefore, upon infection, more SABP2 was synthesized to convert the increasing amount of MeSA into SA. This higher concentration of cellular SABP2 could induce conformational changes in SABP2, which may be unlikely to happen under natural conditions (Figure 11, lane 1). Besides, during disease resistance signaling SABP2 may interact with other proteins to enhance downstream signaling. This result leads us to further investigate if SABP2 exists as dimer under natural conditions. In order to determine the exact conformation of SABP2 under natural conditions, purification of SABP2 from its natural source tobacco was undertaken. Results from characterization of recSABP2 indicated that detergents (even mild detergent) have denaturing effect on native structure of SABP2. Therefore, detergent was excluded from protein extraction buffer used in SABP2 purification from tobacco. Previously it has been shown that SABP2 precipitates between 50-75% ammonium sulfate (Kumar and Klessig 2003). We took advantage of this information and we fractionated SABP2 as 50-75% ammonium sulfate (figure 12 B), removing most of the proteins from 0-50% ammonium sulfate (Figure 12 A). Further purifications were carried out using an AKTA purifier 10 system. SABP2 was purified from 468 g of wild type tobacco leaf tissue using a 5-step protocol (Figure 24 A) and after every step SABP2 was detected by Western blot analysis using SABP2 antibody (Figure 24 B). Purified

SABP2 (29 kDa) was detected in silver stain (Figure 22 A), commassie stain (Figure 24 A), and in Western blot (Figure 24 B). At final purification step in Superdex 75 column SABP2 eluted in fraction # 46 and 47 (Figure 22 B) with an elution volume (Ve) of 12 ml (Figure 21). The recSABP2-21 eluted with an elution volume (Ve) of 10.6 ml (Figure 20). Previously, the column was calibrated with 4 standards (Blue dextran, BSA, Carbonic anhydrase, and Cytochrome c). Blue dextran (2,000 kDa) was used to obtain the void volume (Vo) of the column (Figure 19). The elution volume (Ve) of BSA (66 kDa), Carbonic anhydrase (29 kDa), and Cytochrome c (12.4 kDa) (Figure 19) were used to obtain a standard curve by plotting the gel phase distribution coefficient or retention coefficient (Kav) of the standards (Table 1) against the logarithms of their molecular weights (Log 10 molecular weight) (Figure 23). Retention coefficients (Kav) were calculated by the formula: Kav = Ve-Vo/Vc-Vo, where Ve is elution volume, Vo is column void volume, and Vc is total column volume (24 ml for Superdex 75 10/300 GL). The estimated log 10 molecular weight of eluted natural SABP2 and recSABP2-21 were determined by plotting their Kav values in the equation for the standard curve (Figure 23). The estimated molecular weight (anti-log 10 molecular weight) of recSABP2-21 was determined as 57.5 kDa (dimer), which is consistent with the previous results (Forouhar et al. 2005). On the other hand, the estimated molecular weight of native SABP2 was determined as 28.8 kDa, suggesting that native SABP2 is a monomer, which is also consistent with previous suggestions that SABP2 may be a monomer at physiological concentrations (Kumar and Klessig 2003). Our finding suggests that native SABP2 purified from tobacco leaves exists as a monomer.

Future Directions

To further understand the structural variation of SABP2, it will be important to isolate SABP2 from tobacco plants infected with TMV. It will be interesting to see if SABP2 undergoes conformational changes following TMV infection. Moreover, structural determination can lead us to investigate changes in catalytic function of this protein during resistance response.

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APPENDICES

APPENDIX A – Abbreviations

SABP2	- Salicylic acid binding protein 2
CMD	- Cassava mosaic disease
PRRs	- Pattern recognition receptors
PAMPs	- Pathogen-associated molecular patterns
R protein	- Resistance protein
Avr	- Avirulence
PAL	- Phenyl ammonia lyase
ICS 1	- Isochorismate synthase 1
BA2H	- Benzoic-2-hydroxylase
HR	- Hypersensitive response
PCD	- Program cell death
SA	- Salicylic acid
JA	- Jasmonic acid
ET	- Ethylene
ISR	- Induced systemic resistance
SAR	- Systemic acquired resistance
SAMT	- Salicylic acid methyl transferase
MeSA	- Methyl salicylate
NO	- Nitric oxide

TMV	- Tobacco mosaic virus
PR	- Pathogenesis-related
BTH	- Benzo-(1, 2, 3)-thiadiazole-7-carbothioic acid S-methyl ester
INA	- 2, 6-dichloro-isonicotinic acid
ROI	- Reactive oxygen intermediates
NPR1	- Non-expresser of pathogenesis-related protein 1
IPL	- Isopyruvate lyase
NFAT	- Nuclear factor of activated T-cells

APPENDIX B – Buffers and Reagents

Protein Extraction Buffer (buffer A) (1L)

Sodium Citrate (7.44g), M.W. = 372.24g/L, final concentration = 20mM

MgSO4 (1.23g). M.W. = 246.48g/L, final concentration = 5mM

EDTA (0.42g), M.W. = 416.20g/L, final concentration = 1mM

Adjust pH to 6.3

Stored at 4ºC until use.

Prior to grinding plant tissue add 1ml of ß-ME (14.4mM final concentration), 1ml of PMSF(100mM) (0.1mM final concentration), 0.15g of benzamidine HCl (156.61g/mol, final concentration 1mM) and 15g of 100% PVPP (1.5% wt/vol) to 1L of buffer.

Bicine buffer (buffer B) (1L)

Bicine (1.63g), M.W.= 163.2g/mol, final concentration = 10Mm

Adjust pH to 8.5 with 1 N NaOH

1ml of ß-ME (14.4mM final concentration), 1ml of PMSF(100mM) (0.1mM final concentration), 0.15g of benzamidine HCl (156.61g/mol, final concentration 1mM).

10x Phosphate Buffer Saline (10x PBS)

Sodium Chloride (76g), M.W. = 58.44g/mol, final concentration = 1.3M

Sodium Phosphate dibasic (10g), M.W. = 141.96g/mol, final concentration = 70mM

Sodium Phosphate monobasic (4.1g), M.W. = 119.96g/mol, final concentration = 30mM

For 1x PBS (1 L), dilute 100mL of 10x PBS in 900mL of water.

For 1x PBS (1 L) with 3% Tween 20, dilute 100mL of 10x PBS in 870mL, then add 30mL of tween 20.

Western Blotting Blocking Buffer (100mL)

1x PBS buffer, 100mL

Dry Milk (1g), final concentration = 1%

BSA (3g), final concentration = 3%

4x SDS-PAGE Separating gel Buffer (500mL)

Tris base (90.85g), M.W. = 121.1g/mol, final concentration = 1.5M

Adjust pH to 8.8

Add SDS (0.2g), final concentration = 0.04%

4x SDS-PAGE Stacking gel Buffer (500mL)

Tris base (30.28), M.W. = 121.1g/mol, final concentration = 0.5M

Adjust pH to 6.8

Add SDS (0.2g), final concentration = 0.04%

10x SDS-PAGE Running Buffer (1 L)

Tris base (30g), M.W. = 121.1g/mol

Glycine (144g), M.W. 75.07g/mol

SDS (10g)

10x Western Blotting Transfer Buffer (1L)

Tris base (30.3g), M.W. = 121.1g/mol, final concentration = 125mM

Glycine (72.06g), M.W. = 75.07g/mol, final concentration 960mM

For western, 1x transfer buffer is prepared by mixing 100mL of 10x transfer buffer, 100mL of 100% methanol, and 800mL of cold water.

Ammonium Persulfate (20% in 1mL)

Dissolve Ammonium persulfate (20g) in 1mL of water

2x SDS-PAGE Loading Dye (100mL)

1M Tris-Cl, pH 6.8 (10mL), final concentration = 100mM

SDS (0.4g), final concentration = 0.4%

Glycerol (20mL), final concentration, 20%

Bromophenol blue (0.2g), final concentration = 0.2%

Add 5mL of ME before use.

Ponceau S Stain (100mL)

Ponceau S (0.1g), final concentration = 0.1%

Acetic acid (5mL), final concentration = 5%

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