

The Characterization of Two Novel PTP-like Enzymes From *Bdellovibrio bacteriovorus* and
Pseudomonas syringae pv. *tomato* DC3000

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Abstract

Recently, a new group of phytate degrading enzymes has been isolated from anaerobic bacteria from the rumen. This enzyme group is characterized by a protein tyrosine phosphatase (PTP) -like active site signature sequence. Searches of genetic databases have identified over 40 homologues to this group of phytate-degrading enzymes in a wide variety of bacterial species. To further our understanding of this enzyme group, two new representative genes from *Bdellovibrio bacteriovorus* and *Pseudomonas syringae* pv. *tomato* DC3000 were overexpressed and their recombinant gene products examined. *B. bacteriovorus* is a bacterium that preys on other Gram negative bacteria while *P. syringae* DC3000 is a phytopathogen that infects a variety of plant species. A combination of biochemical, kinetic and site-directed mutagenesis analyses demonstrated that these enzymes dephosphorylate phytic acid (InsP₆) following a classical PTP mechanism, and have a high specificity for InsP₆. The biological function of these enzymes was studied by over producing HopAO1 and HopAO1 C378S in *Saccharomyces cerevisiae*. HopAO1 inhibited growth of the yeast while the catalytically inactive HopAO1 C378S mutant had no effect. Future studies with this system will shed light on the biological function of HopAO1, PhyAbb and other PTP-like phytate degrading enzymes.

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List of Abbreviations

ADP Adenosine 5' –Diphosphate
ATP Adenosine 5' –Triphosphate
bp Base Pairs
EDTA EthyleneDiaminetetra-acetic acid
HPIC High Performance Ion-Pair Chromatography
InsP₆ *myo*-Inositol Hexakisphosphate
IPP Inositol Polyphosphate
IPPase Inositol Polyphosphatase
IPTG Isopropyl β-D-thiogalactopyranoside
M_r Molecular Weight
PCR Polymerase Chain Reaction
PhyAme PTP-Like IPPase from *Megasphaera elsdenii*
PhyAsl PTP-Like IPPase from *Selenomonas lacticifex*
PhyAsr PTP-Like IPPase from *Selenomonas ruminantium*
PhyAsrl PTP-Like IPPase from *Selenomonas ruminantium* subsp. *lactilytica*
PhyBsrl PTP-Like IPPase from *Selenomonas ruminantium* subsp. *lactilytica*
pNPP para-nitrophenyl phosphate
PTP Protein Tyrosine Phosphatase
SDS-PAGE Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis

Chapter One

Literature Review

1.1 *myo*-Inositol Hexakisphosphate

1.1.1. Structure and Chemistry

Inositol is a cyclic carbohydrate with a hydroxyl group attached to each of the ring carbons. Also known as *myo*-inositol (hexahydroxycyclohexane), it is the first inositol characterized, and was initially isolated from muscle tissue by Scherer in 1850. This finding led to 80 years of intense interest on the natural occurrence, properties, derivatives, and stereoisomers of cyclitols (Loewus and Murthy, 2000). It was Scherer who coined the term inositol from the Greek word for muscle (Posternak, 1965). Interest in inositol research has seen a resurgence over the past 20 years due in large part to the recognition of critical roles played by phosphoinositides and inositol polyphosphates in signal transduction (Irvine and Schell, 2001; Shears, 2004; Toker, 2002; Toker and Cantley, 1997; Vanhaesebroeck *et al.*, 2001). *myo*-inositol plays a key role as the central component for biosynthesis of a wide variety of compounds including inositol phosphates, phosphatidylinositides, glycosylphosphatidylinositols, inositol esters, and ethers (Murthy, 2006).

The numbering of *myo*-inositol is dependent upon the chirality of the molecule. Numbering of carbon 1 (C-1) relies upon the unique characteristics of C-2. The C-2 hydroxyl moiety exists as the sole axial group amongst the carbon ring at physiological pH (Agranoff, 2009). Thus, if the carbon ring is numbered clockwise from C-2, the assignment of carbon 1 is 1L and if the carbon ring is numbered counter-clockwise from C-2 it is assigned 1D (Loewus and

Murthy, 2000). Generally, the abbreviation (Ins) should be regarded as *myo*-inositol with the numbering in the D- configuration, unless otherwise specified as the L- configuration. *myo*-inositol contains a plane of symmetry that crosses through carbons C-2 and C-5 (Murthy, 2006). The remaining four carbon atoms exist as two prochiral pairs, C1/C3 and C4/C6.

The molecular structure of *myo*-inositol hexakisphosphate (commonly known as phytic acid; InsP₆) was solved in the early 1900's via empirical determination of the molecular composition and further verified in the late 1960's with the use of ³¹P-NMR (Johnson and Tate, 1969). A further study using ³¹P-NMR found InsP₆ to exist in a chair conformation with five equatorial phosphate groups and one axial in the C-2 position (Costello *et al.*, 1976) as shown in figure 1.1. ¹³C NMR, ³¹P NMR and Raman spectroscopy confirmed that the 5-equatorial/1-axial chair conformation of InsP₆ is the dominant conformer in aqueous solutions at a pH below 9.2 and the 5-axial/1-equatorial confirmation is the predominant conformer at a pH of above 9.6 (Isbrandt and Oertel, 1980). In the pH range of 9.2-9.6, both conformations exist in dynamic equilibrium.

Twelve proton dissociation sites exist on InsP₆, six of which were determined to be strongly acidic (pK_a values of 1.8); three sites are slightly acidic (pK_a values between 5.7 and 7.6); and the remaining three groups are very weakly acidic (pK_a values between 9.2 and 9.6) (Isbrandt and Oertel, 1980). These pK_a values are dependent upon the concentration of the counterion present. Over a broad pH range, InsP₆ contains several negative charges that attract positively charged species thus acting as a strong chelator. The conformational changes of InsP₆ and the large negative charge associated with this substrate has implications for binding

interactions between InsP₆ and enzymes acting on this substrate as well as various cations (Murphy *et al.*, 2008).

1.1.2. Occurrence and Distribution of *myo*-Inositol Hexakisphosphate

InsP₆ is ubiquitous in eukaryotic species, and is usually the most abundant inositol phosphate found in cells (Sasakawa *et al.*, 1995). InsP₆ was initially discovered in 1872 in wheat endosperm that contained a calcium/magnesium salt of organic phosphate (Pfeffer, 1872). More recently, InsP₆ has been extensively studied in plants. InsP₆ is the most prevalent IPP found in mature seeds (Lott *et al.*, 2000) and accounts for 50-80% of the total phosphorus in seeds (Cosgrove, 1966). InsP₆ has also been found in vegetative tissues (Campbell *et al.*, 1991; Roberts and Loewus, 1968), spores (DeMaggio and Stetler, 1985), and in pollen (Helsper *et al.*, 1984; Jackson and Linskens, 1982). However, it wasn't until the 1970's when organic phosphates were first discovered in red blood cells of birds, reptiles, and fish (Bartlett, 1976, 1978; Isaacks *et al.*, 1978). Heslop *et al.* (1985) then identified higher order IPPs, including InsP₆, in mammalian GH₄ pituitary cells by labeling [³H] inositol (Heslop *et al.*, 1985). Phosphorylated inositols including InsP₆ have not been found in either bacteria or archaea (Roberts, 2006).

1.1.3. Physiological Functions of InsP₆

InsP₆ is the most abundant cellular inositol phosphate (Raboy, 2003). As mentioned, InsP₆ is the major source of phosphorus found in seeds, and is also found in other tissues such as pollen, roots, tubers and turions (Cosgrove, 1980; Raboy, 1997). The most obvious function for InsP₆ in these tissues is phosphorus storage, storage and retrieval of minerals and regeneration of inositol during development and germination. In the late 1980s and early 1990s, it became clear

that InsP₆ was ubiquitous amongst eukaryotic species, and in most cases the most abundant inositol phosphate in cells (Sasakawa *et al.*, 1995). Initially, it was suggested that the sole function of InsP₆ was a phosphate storage molecule, and played a role in sequestering amino acids, cations and *myo*-inositol. This was based primarily on observations that during seed germination and plant development, plants depend on InsP₆ as a source of these essential nutrients. However more recently, the biological role of InsP₆ includes DNA repair (Hanakahi *et al.*, 2000), RNA processing (Macbeth *et al.*, 2005), mRNA export (Stewart, 2007; York *et al.*, 1999), apoptosis (Majerus *et al.*, 2008), pathogenicity (Chatterjee *et al.*, 2003; Lupardus *et al.*, 2008), translation (Bolger *et al.*, 2008), vacuole biogenesis (Dubois *et al.*, 2002), and ribosome biogenesis (Horigome *et al.*, 2009). The importance of InsP₆ has been reinforced through studies with mice deficient in enzymes responsible for InsP₆ biosynthesis. These animals were shown to contain lethal phenotypes in embryo (Frederick *et al.*, 2005; Verbsky *et al.*, 2005b).

1.1.4. Nutrition and Environment

Since InsP₆ accounts for up to 90% of the phosphorus content in grains and seeds, it represents a significant component in the grain based diets of humans and animals reared in intensive livestock operations. Consequently, InsP₆ has been shown to have profound effects on human health and the environment. InsP₆ is a chelator of mineral cations and may inhibit proper absorption of dietary minerals. This has had a substantial effect on iron and zinc deficiencies in the developing world (Mendoza *et al.*, 1998; Raboy, 2001). Also, due to the high electronegative nature of InsP₆, it has been shown to bind to enzymes and inhibit activity (Caldwell, 1992). Non-ruminant (i.e., monogastric) animals, such as poultry, swine, and fish are incapable of metabolizing InsP₆. InsP₆ excreted from these animals contributes considerably to pollution due

to eutrophication of aquatic ecosystems (Jongbloed and Lenis, 1998). The addition of InsP₆ degrading enzymes to livestock feed has been used successfully since the early 1990's to ameliorate environmental problems. Also, deployment of crops low in InsP₆ has alleviated some of the nutritional problems associated with high InsP₆ intake (Abelson, 1999; Lott *et al.*, 2000).

1.2. Phytases

Organisms rely on the phosphorylation and dephosphorylation of phosphate monoesters as part of many critical processes including energy metabolism, metabolic regulation, and signal transduction. Due to the abundance of InsP₆ among the plant and animal kingdoms and the importance of this molecule, a distinct class of enzymes has evolved which hydrolyze this highly electronegative molecule. Inositol hexakisphosphate phosphohydrolases (more commonly known as phytases) are the enzymes primarily responsible for the degradation of InsP₆ to lower IPPs and inorganic monophosphate(s).

1.2.1. Sources of Phytases

Microbial Sources

Most of the studies on phytases thus far have been from fungi, markedly from the *Aspergillus* species (Konietzny and Greiner, 2002). Microorganisms used in food fermentation have also been studied for their ability to degrade phytate during the fermentation process including yeast such as *S. cerevisiae* (Nakamura *et al.*, 2000) and *Schwanniomyces castellii* (Lambrechts *et al.*, 1992). Phytate degrading enzymes have also been discovered in bacteria such as *Pseudomonas* sp. (Richardson and Hadobas, 2001), *Bacillus subtilis* (Kerovuo *et al.*, 1998; Powar and Jagannathan, 1982; Shimizu, 1992), *B. amyloliquefaciens* (Kim *et al.*, 1998),

Klebsiella sp. (Greiner *et al.*, 1997; Tambe *et al.*, 1994), *E.coli* (Greiner *et al.*, 1993), *Enterobacter* (Yoon *et al.*, 1996), *Selenomonas ruminantium*, *Megasphaera elsdenii*, *Prevotella* sp., and *Mitsuokella multiacidus* (Yanke *et al.*, 1998). With the use of PCR, a diverse group of microbial phytases have been discovered in the ruminal fluid of cattle (Nakashima *et al.*, 2006). These microorganisms are likely responsible for the ability of ruminants to derive a significant portion of their phosphorus requirements from InsP_6 (Nakashima *et al.*, 2006). More recently, phytase probes were used for bioinformatic studies and carried out on microbial genomes in the NCBI and CAMERA databases and found a variety of species encoding phytases (Lim *et al.*, 2007).

Plant Sources

Phytases occur mostly in grains, seeds and pollen of higher plants such as cereals, legumes, oilseeds, and nuts: however, low phytase activity has been demonstrated in the roots of plants as well (Konietzny and Greiner, 2002). Phytase activity during germination is responsible for generating free phosphate, minerals, and *myo*-inositol for the purpose of development and growth (Reddy *et al.*, 1989). Phytase activity has been found in maize (Laboure *et al.*, 1993), barley (Greiner *et al.*, 2000b), rye (Greiner *et al.*, 1998), spelt (Konietzny *et al.*, 1995), canola seed (Houde *et al.*, 1990), and lily (Scott and Loewus, 1986). Difficulties purifying plant phytases due to co-purification of other phosphatases (Laboure *et al.*, 1993) has led to only a fraction of phytases discovered in comparison with microbial phytases.

Animal Sources

Phytate degrading enzymes were detected in 1908 in the liver and blood of calves (McCollum and Hart, 1908). The adverse nutritional consequences of diets rich in phytate has

stimulated studies examining phytase activity in the gastrointestinal tract of various animals (Konietzny and Greiner, 2002). Phytase activity has been detected in the mucosa extracts of the small intestine of rats, rabbits, guinea-pigs, chicken, and humans (Bitar and Reinhold, 1971; Cooper and Gowing, 1983; Iqbal *et al.*, 1994). More recently, phytate degrading activity was identified in rat hepatic tissue, in which it was localized in the endoplasmic reticulum (Craxton *et al.*, 1997). This phytase, known as multiple inositol polyphosphate phosphatase (MIPP), contains an 18 amino acid region that aligned with 60% identity to phytase A from *Aspergillus* (Craxton *et al.*, 1997). Homologues of MIPP were also cloned from mice and humans (Chi *et al.*, 1999).

1.2.2. Classes of Phytases

1.2.2.1. Histidine Acid Phosphatases

Members of this class of enzymes all share a highly conserved active site motif (RHGXRXP) that is involved in the hydrolysis of phosphomonoesters (Ullah *et al.*, 1991). Mutagenesis studies (Ostanin *et al.*, 1992; Ostanin and Van Etten, 1993) and X-ray crystallographic structures of transition state complexes (Lim *et al.*, 2000; Liu *et al.*, 2004) have helped explain the mechanisms involved for hydrolysis. HAPs follow a two-step mechanism for dephosphorylation of phytic acid. Firstly, the guanidinium group of Arg in the tripeptide RHG of the active site interacts with the phosphate group of the substrate, thus making it more susceptible for nucleophilic attack (Mullaney and Ullah, 2003). In this instance, the active site histidine is responsible for forming a phosphohistidine intermediate by acting as a nucleophile and an upstream aspartic acid residue acts as the general acid by protonating the leaving group.

Phytases can be classified into three groups based on their pH optima for catalysis; acidic, neutral, or basic phosphatases. Most studies on phytases thus far have dealt with the study of acidic phosphatases, due to the recent interest in identifying enzymes capable of hydrolyzing phytic acid within the digestive tract of monogastric animals (Mullaney and Ullah, 2003). pH optima for HAPs are generally found at pH 2.5 and/or pH 4.5-6 (Konietzny and Greiner, 2002; Mullaney and Ullah, 2003; Oh *et al.*, 2004) placing HAPs in the acid phosphatase group. However, recently a novel alkaline phosphatase known as L1ALP was described in *Lilium longiflorum*. This enzyme contains the RHGXRXP signature sequence commonly found in HAPs but has an optimal activity against InsP₆ at a pH of 8 (Mehta *et al.*, 2006). This suggests a misnomer in the name histidine acid phosphatase.

Interestingly, not all HAPs contain catalytic activity against phytic acid. The specific activity for phytic acid varies extensively among HAP phytases. One class of HAP phytase showed a low specific activity against phytic acid, yet showed a broad specificity for a variety of phosphorylated substrates. (Mullaney and Ullah, 2003). In another class, the HAP phytases showed a high specific activity against phytic acid, however this class was restricted in its ability to dephosphorylate the same range of substrates (Mullaney and Ullah, 2003). It was determined that the catalytic centers showed little variation, however the substrate specificity site showed a marked difference depending on the substrate. X-ray crystallographic analysis showed that PhyA from *Aspergillus niger* NRRL 3135, which is widely known for its strong specific activity against phytic acid, revealed that the enzyme's substrate specificity site was attuned to the negative charge associated with phytic acid (Kostrewa *et al.*, 1999). PhyB from *A. niger* NRRL 3135, was determined to contain a broader substrate specificity due to a more electrostatically neutral substrate specificity site at physiological pH (Kostrewa *et al.*, 1999; Wyss *et al.*, 1999).

1.2.2.2. β Propeller Phytases

β propeller phytases (BPPs) are a recently discovered class of phosphatase that contains a novel mechanism for catalyzing reactions. The first representative BPPs were cloned from two different Gram positive *Bacillus* strains. PhyC was cloned from *Bacillus subtilis* (Kerovuo *et al.*, 1998), and TS-Phy was cloned from *Bacillus amyloliquefaciens* (Kim *et al.*, 1998). More recently, a protein (PhyS) from the Gram negative bacterium *Shewanella oneidensis* was characterized and shown to contain two BPP domains (Cheng and Lim, 2006). Also, genome sequence analysis has revealed BPP-like sequences are widely distributed in the genomes of numerous bacteria (Cheng and Lim, 2006).

Similarly, these BPPs contain an active site sequence motif RHGXRXP common with HAPs (Kerovuo *et al.*, 1998; Kim *et al.*, 1998). However, a feature common to BPPs and not HAPs is the dependence on Ca^{2+} ions for stability and activity, thus suggesting an alternative mode of phytate hydrolysis (Kerovuo *et al.*, 2000a). The calcium ions enable the binding of substrate by providing a favorable electrostatic environment (Oh *et al.*, 2001). The three dimensional structure of the *B. amyloliquefaciens* BPP revealed a form similar to a propeller with six blades (Ha *et al.*, 2000). Data suggests that BPPs contain two phosphate binding sites, a cleavage site, and an affinity site (Shin *et al.*, 2001). The cleavage site being responsible for hydrolysis and the affinity site facilitates binding of phosphorylated substrates.

BPPs differ in their biochemical properties in comparison to HAPs. The BPPs from the *Bacillus* species have optimal activity against InsP_6 in the pH in the range of 7-8. The difference in optimal pH of the BPPs in comparison with the acidic phytases is likely due to the protonation

of calcium binding residues at lower pH resulting in the inefficient binding of Ca^{2+} (Oh *et al.*, 2001).

1.2.2.3. Purple Acid Phosphatases

The first purple acid phosphatase (PAP) characterized was from germinating soybeans that were isolated from cotyledons (Hegeman and Grabau, 2001). This phytase, known as GmPhy, contains the PAP active site motif and belongs to a class of metalloenzymes that have been widely studied (Klabunde *et al.*, 1994). Another PAP was cloned from *Aspergillus niger* (Ullah and Cummins, 1988). This enzyme, known as Apase6, only contains a limited ability to utilize phytic acid as a substrate (Ullah and Cummins, 1988). More recently, searches of genetic sequence databases have revealed the existence of PAP-like sequences in bacteria, fungi, plants, and animals (Schenk *et al.*, 2000). These enzymes contain conserved motifs in positions relative to members of a dimetal-containing phosphoesterase (DMP) family of kidney bean PAPs (Mullaney and Ullah, 1998). The active site of PAPs contain seven invariant residues that are responsible for the coordination of a binuclear Fe(III)-Me(II) centre, in which Me can be Fe, Mn, or Zn (Klabunde *et al.*, 1996; Schenk *et al.*, 1999; Strater *et al.*, 1995). The exact mechanism for catalyzing the release of a phosphate molecule is currently poorly understood: however, recent mutagenesis experiments have begun to elucidate the roles key residues play within the active site (Funhoff *et al.*, 2005; Truong *et al.*, 2005).

Similar to HAPs, PAPs are enzymes that are most active in acidic environments. The PAP from kidney beans has a optimal pH between 4.5 and 5 (Hegeman and Grabau, 2001), while the PAP from *A. niger* is most active at a pH of 6 (Mullaney and Ullah, 1998).

1.2.2.4. Protein Tyrosine Phosphatase-Like Inositol Polyphosphatases

Protein tyrosine phosphatases (PTP) are a superfamily of enzymes that are responsible for the removal of phosphates from tyrosine-phosphorylated residues. Proteins from this superfamily have been identified in a wide range of prokaryotes; these enzymes serve as regulators of cellular function like their better-studied eukaryotic counterparts (Kennelly and Potts, 1999; Shi *et al.*, 1998).

Recently, a novel group of PTP-like enzymes was discovered with the cloning of a gene coding for a phytate degrading enzyme (PhyAsr) from the anaerobic bacterium *Selenomonas ruminantium* (Puhl *et al.*, 2007; Yanke *et al.*, 1998). The enzyme has a highly conserved PTP-like active site signature sequence (HCEAGVGR) but an overall primary sequence identity with other PTPs and inositol polyphosphatases (IPPases) of less than 20% (Puhl *et al.*, 2007). PhyAsr has considerable activity against InsP₆ *in vitro*, and contain very little activity against other phosphorylated substrates (Puhl *et al.*, 2007). This enzyme is the first example of a PTP-like protein with a catalytic preference for InsP₆ rather than phosphopeptides.

Subsequently, additional PTP-like IPPases have been cloned and characterized from the following anaerobic bacteria: *S. ruminantium subsp. lactilytica* (Puhl *et al.*, 2008a), *lacticifex* (Puhl *et al.*, 2008b) and *Megasphaera elsdenii* (Puhl *et al.*, 2009). These PTP-like IPPases, like PhyAsr, show *in vitro* activity against InsP₆, with little to no activity against other phosphorylated substrates (Puhl *et al.*, 2009; Puhl *et al.*, 2008a; Puhl *et al.*, 2008b). These enzymes can be classified as acidic phosphatases, as they show optimal InsP₆ hydrolysis in the pH range of 4.5-5.

As mentioned, these enzymes contain a PTP-like active site signature sequence (C(X)₅R) conserved amongst the PTP superfamily. The structures of two PTP-like IPPases have been solved: PhyAsr (Chu *et al.*, 2004; Puhl *et al.*, 2007; Gruninger *et al.*, 2008) and phytase A (PhyAmm) from *Mitsuokella multacida* (Gruninger *et al.*, 2009) and shown to contain two domains, one which contains the PTP-like fold. The active-site signature sequence forms a P-loop at the base of the substrate binding pocket consistent with other PTPs (Chu *et al.*, 2004; Puhl *et al.*, 2007; Gruninger *et al.*, 2008; Gruninger *et al.*, 2009). The depth of the binding pocket is an important determinant for PTP substrate specificity (Denu and Dixon, 1998). For example, the phosphoinositide phosphatase PTEN and closest structural homologue to PTP-like IPPases, has a wider opening to facilitate the large phosphorylated inositol group of its substrate (Lee *et al.*, 1999). Consistent with this, PhyAsr and PhyAmm contain pockets that are deeper than PTEN (14Å), which is believed to be due to the insertion of the small β domain and an extended α helix (Chu *et al.*, 2004). PhyAsr was shown to initiate hydrolysis at the 3-phosphate position of InsP₆ in which substantial P-loop conformational changes have yet to be reported (Puhl *et al.*, 2007). In comparison, PTP and PTP-like enzymes generally undergo substrate-induced conformational changes in the TrpProAsp (WPD)-loop in which the conserved general acid Aspartic acid residue resides (Zhang, 1998; Zhang, 2003).

PTP and PTP-like activity is accomplished via a two step general acid-general base mechanism for dephosphorylation. The catalytically required invariant Cysteine residue, shown to be required for PTP activity by empirical studies employing chemical modification and site-directed mutagenesis (Zhou *et al.*, 1994) acts as a thiolate and forms a phospho-cysteine intermediate (Cirri *et al.*, 1993; Zhang *et al.*, 1994; Zhou *et al.*, 1994). The conserved arginine residue acts by stabilizing the scissile phosphate through the action of the guanidinium group by

stabilizing the highly negative charge of the phosphate group and coordinating the oxygen atoms into an optimal orientation (Barford *et al.*, 1994; Zhang *et al.*, 1994). Other main chain nitrogens in the P-loop also help in stabilizing the negative charge (Barford *et al.*, 1994). These PTP-like enzymes have also demonstrated the use of a general acid-base mechanism of dephosphorylation by an Asp residue located upstream of the PTP signature sequence. Again, mutational studies have shown that this general acid is required for proper hydrolysis in a *Yersinia* PTP (Zhang *et al.*, 1994). Hydrolysis is accomplished by protonation of the Asp residue at an appropriate pH. Studies on the conserved Asp in dual specificity phosphatases have been associated with activation of a water molecule, believed to be responsible for the hydrolysis of the phosphocysteine intermediate (Denu and Dixon, 1998). This conserved Asp residue has also shown to be important for proper catalysis in low-molecular weight PTPs from yeast (Wu and Zhang, 1996) and mammalian PTP1B (Lohse *et al.*, 1997). The discovered PTP-like IPPases are shown in Figure 1.2.

An alignment of the amino acid sequences of the characterized PTP-like IPPases is shown in Figure 1.2. The PTP-like IPPases studied thus far are group studied thus far are from the Veillonellaceae family including *Selenomonas*, *Mitsuokella*, and *Megasphaera*. A search of GenBank using the PhyAsr sequence as a probe has identified over 40 homologues in Clostridial species, GenBank identified over 40 homologues which include: the anaerobic Gram-positive Clostridiales species (i.e., anaerobic Gram-positive bacteria) as well as an assortment of Gram-negative bacteria, including plant pathogens (*Acidovorax*, *Pseudomonas*, and *Xanthomonas*), a predacious bacterium (*Bdellovibrio*), a human pathogen (*Legionella*), and a member of the fruiting, gliding bacteria (*Stigmatella*).

1.2.2.4.1. PTP-Like IPPase Homologue from *Bdellovibrio bacteriovorus*

Bdellovibrio bacteriovorus is a flagellated small Gram-negative actively motile bacterium that preys on other Gram-negative bacteria (Sockett and Lambert, 2004). Bdellovibrios satisfy their nutritional needs by penetrating the periplasmic space of other Gram-negative bacteria where they feed on biopolymers, replicate and subsequently burst free from the cell envelope (Sockett and Lambert, 2004; Stolp and Starr, 1963). Bdellovibrios have been shown to prey on plant, animal, and human pathogens (Martin, 2002) and are ubiquitous in nature inhabiting a wide variety of environments including aquatic habitats, terrestrial habitats, and even mammalian intestines (Rendulic *et al.*, 2004). Upon entering the periplasmic space, *B. bacteriovorus* can form a structure known as a bdelloplast which allows the predatory cell to remain dormant without affecting the viability of the host (Rendulic *et al.*, 2004). Recently, the genome of *B. bacteriovorus* was sequenced and has revealed a large complement of degradative enzymes (Rendulic *et al.*, 2004). Study of these enzymes will give insight into the evolutionary adaptations involved amongst these predatory cells and their prey (Rendulic *et al.*, 2004).

The large difference in ecosystems inhabited by anaerobic ruminant bacteria and *Bdellovibrio* suggests the enzymatic function of these of degradative enzymes have evolved independently. Bioinformatic analysis has revealed that the novel PTP-like IPPase from *B. bacteriovorus* shares 31% identity with PhyAsr from *S. ruminantium*. This novel PTP-like IPPase from *B. bacteriovorus* is the first enzyme of this class to be characterized from a free-living bacterium.

1.2.2.4.2. The PTP-like IPPase Homologue HopAO1

Pseudomonas syringae pv *tomato* DC3000 is an exceptionally well characterized phytopathogen noted for its extensive array of effector molecules that are mobilized by a type three secretion system. The extensive body of literature published on this bacterium is in part due to its ability to transfect both potato plants and the genetically amenable *Arabidopsis thaliana*. In the past decade, many effector proteins have been well characterized and due to the availability of the draft nucleotide sequence, more are awaiting confirmation. HopAO1, previously known as HopPtoD2, is one of these effectors. HopAO1 is a 468 amino acid long polypeptide with a predicted molecular weight of 51.4 kDa. BlastP search results show that HopAO1 contains an amino terminal domain (of approximately 200 amino acids) which shares similarities with several AvrPphD homologues found in other *P.syringae* pathovars and other plant pathogens (Espinosa *et al.*, 2003). Interestingly, other AvrPphD homologues share similarity throughout the entire sequence, whereas HopAO1 is restricted to the N-terminus. The C-terminus domain of HopAO1 shares similarities with proteins from the PTP superfamily and more specifically PTP-like IPPases. BlastP search reveals the putative PTP-like IPPase from *Acidovorax avenae* subsp. *citrulli* shares the highest similarity (*E*-value $2e-56$) and a putative PTP from *Xanthomonas campestris* pv. *vesicatoria*, and a putative PTP from *Bdellovibrio bacteriovorus* with *E*-values of $4e-37$ and $1e-28$ respectively. These sequence similarities are a result of the invariant residues (HCX₅RS/T) that are responsible for the catalytic activity of PTPs (Denu *et al.*, 1996) and PTP-like IPPases (Chu *et al.*, 2004; Puhl *et al.*, 2007).

Espinosa *et al* (2003) and Bretz *et al* (2003) independently determined that HopAO1 contained activity against segments of tyrosine phosphorylated peptides. Both groups used the

commercially available phosphotyrosine peptides derived from the EGF receptor and the insulin receptor (Espinosa *et al.*, 2003; Bretz *et al.*, 2003). Due to solubility problems, cell lysate from *E.coli* over-expressing HopAO1 was used to determine phosphatase activity (Espinosa *et al.*, 2003; Bretz *et al.*, 2003). The activity was comparable with the well known PTPs PTP-1 β and the leukocyte antigen related (LAR) protein (Espinosa *et al.*, 2003; Bretz *et al.*, 2003). In order to demonstrate the PTP activity was the result of HopAO1 within the cell lysate, the nucleophilic cysteine residue was mutated to a non-functional serine (Espinosa *et al.*, 2003; Bretz *et al.*, 2003). As expected, the activity levels were near background indicating a PTP mechanism for the removal of phosphates from the peptides and demonstrating that HopAO1 was responsible for the perceived activity (Espinosa *et al.*, 2003; Bretz *et al.*, 2003).

1.3. Enzymatic Action of IPPases and Virulence on Host Plants and Animals

The plant pathogen *Xanthomonas oryzae* pv. *oryzae* is a well known phytopathogen that causes bacterial leaf blight, a serious disease of rice. It has recently been reported that the virulence activity of this pathogen is due in part to an open reading frame (*phyA*) encoding a 373-amino acid (aa) polypeptide PhyA containing a 28-aa predicted signal peptide (Chatterjee *et al.*, 2003). BLAST search showed significant similarities with conserved hypothetical proteins in both *Xanthomonas axonopodis* pv. *citri* and *Xanthomonas campestris* pv. *campestris* and limited homology to secreted phytases from the *Bacillus* species (Chatterjee *et al.*, 2003). Chatterjee *et al* (2003) determined that *Xanthomonas oryzae* pv. *oryzae* mutants deficient in PhyA were less virulent than wild-type and when the sole source of phosphate was InsP₆, these mutants were unable to grow properly. Thus, the group suggest that PhyA from *Xanthomonas*

oryzae pv. *oryzae* likely targets InsP₆ and this mode of action promotes virulence on host plant cells through a signaling pathway yet to be determined (Chatterjee *et al.*, 2003).

Salmonella Dublin is an enteric pathogen that uses a Type Three Secretion System (TTSS) during infection to inject the effector protein SopB into host cells (Norris *et al.*, 1998). SopB is homologous to mammalian inositol polyphosphate 4-phosphatases and contains *in vitro* activity against Ins(1,3,4,5,6)P₅ and the phosphatidylinositides PI(3,4,5)P₃ and PI(3,4)P₂ (Norris *et al.*, 1998). Cells expressing active site mutant SopB proteins (i.e. catalytic Cys changed to Ser) were shown to contain less virulence *in vivo* in comparison to wild type cells, suggesting the enzyme promotes virulence by subverting cellular inositol phosphate signaling pathways (Norris *et al.*, 1998). Interestingly, SigD from *Salmonella typhimurium*, a homologue of SopB (93% identity), activates the Akt pro-survival pathway *in vivo* by localizing Akt to membranes with high concentrations of PI(3,4,5)P₃ and/or PI(3,4)P₂ and triggers cytoskeleton rearrangements (Steele-Mortimer *et al.*, 2000). It was postulated that since the activation of the Akt pro-survival pathway is both necessary and sufficient for eukaryotic cell survival (Datta *et al.*, 1999) that epithelial cells would survive for a sufficient intracellular time frame allowing for multiplication of the *Salmonella* pathogen (Knodler *et al.*, 2005).

1.4. Study Objectives

The focus of this study is to expand our knowledge of the PTP-like IPPase group by characterizing two additional representative homologues found in the database. As mentioned, the novel PTP-like IPPase from *Bdellovibrio bacteriovorus* contains 31% identity with PhyAsr despite the significant differences associated with the organisms expressing the PTP-like IPPase gene. HopAO1 from *Pseudomonas syringae* pv. *tomato* DC3000, despite lower sequence

identity with PhyAsr (21%), HopAO1 appears to contain more similarities with the previously characterized PTP-like IPPases than that shared with other members of the PTP superfamily. Additionally, another goal of this study was to develop a system to determine the biological role of these newly characterized phosphatases.

These putative PTP-like IPPases from *B. bacteriovorus* and *P. syringae* pv. *tomato* DC3000 were chosen in this study largely based on the novel aspects of the organisms that express them. There has yet to be a PTP-like IPPase characterized from a free-living organism or a plant pathogen such as *B. bacteriovorus* or *P. syringae* pv. *tomato* DC3000 respectively. Due to the previous work done with HopAO1 and the less than convincing results suggesting that HopAO1 works on a phosphopeptide substrate (Espinosa *et al.*, 2003; Underwood *et al.*, 2007), I suggest that HopAO1 targets an IPP due to the sequence similarities with other PTP-like IPPases.

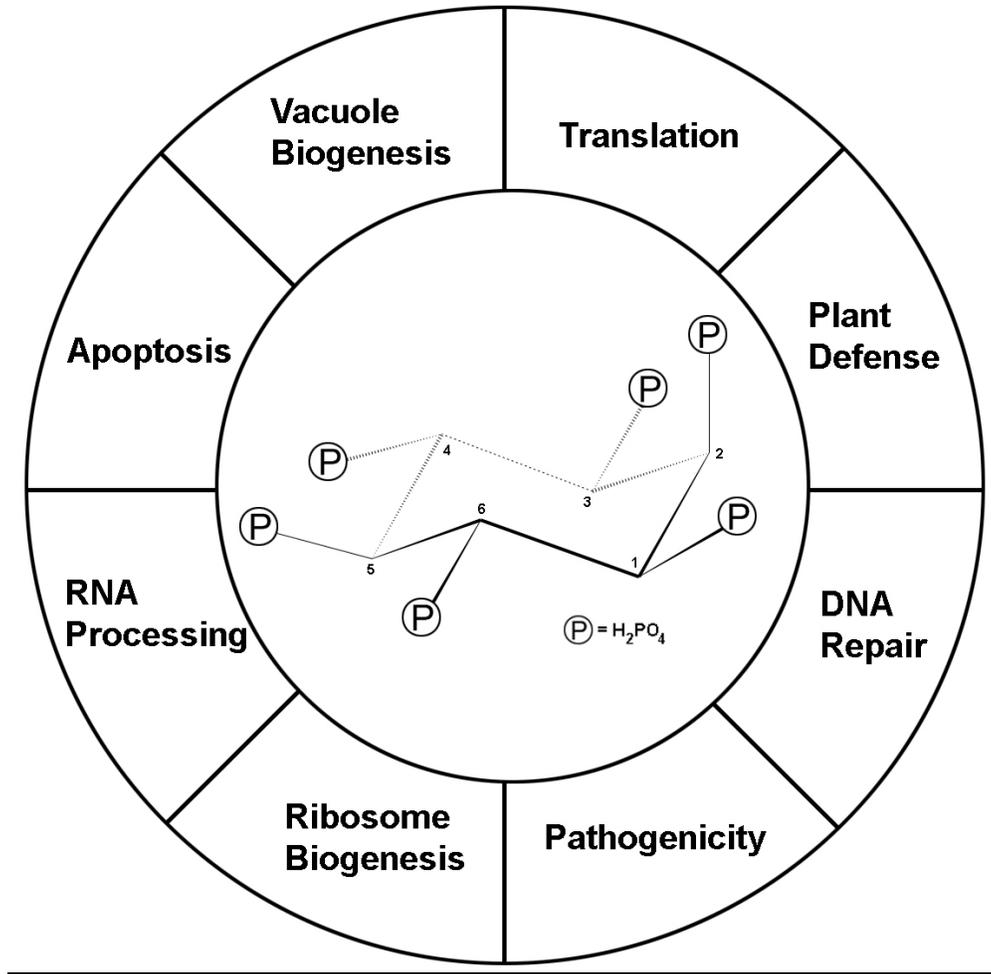


Figure 1.1. The most energetically favorable conformation of InsP₆ displaying the one axial and five equatorial phosphates. Numbering corresponds to the D-configuration. Also included are some of the processes in which InsP₆ has been shown to participate.

```

*           *           *
PhyAsr      : LREKTAGP--IYDVDLRQESHGYLDG-IPVSWYGERDWANLGKSSQHEALADERHRLHAAL:176
PhyAsl      : LRQQAEGP--VYIIDLRQESHGILNG-DAVSWYGKRDWGNLGRNQRAVQSDERRRLKAAL:171
PhyAmm      : LKQQAKGP--IYIMDLRQETHGVFNG-NAVSWYGLRDWGNLGNKKAQEVLDKENSRLNAAR:472
PhyBsl      : ---KADEP--LWVIDLRQESHGFVNG-EAVSWHGPNNAAANAGKDAPAVEAETARLQAVL:141
PhyAsrl     : IRTAAGSDAVIYVVDLRKESHGFVNGDIPVSQYMKKNRGNVKLKAAAVKQVEGKWLQSLV:157

*           *           *
PhyAsr      : HKTVYIAPLGKHKLPEGGE--VRRVQKVTQEQEVAAEAGMRYFRIAATDHVWPTPENIDR:234
PhyAsl      : GKIQYVAPLNKHKLPSSGK--AERITQAMTEEQLVTQSGLKYVRITATDHVWPAPECIDQ:229
PhyAmm      : GKSLIVAELEKDKMPIDPK--PVKIESVMTEQQLVEKNGLHYRRIAATDHIWPSAANIDE:530
PhyBsl      : GHESHSIPMGTSDTASGMQPFALTALVRTATTERQEACRNGYGYVRIAATDMQWPEPQIDA:201
PhyAsrl     : GKELTFVPMGKTDTKLFPA-CSVKVEKVETEALASRLGMRYKRILITDQMAPTDEEVDA:216

*           <=>           *           *
PhyAsr      : FLAFYRTLPOD-AWLHFHCEAGVGRTTAFMVMTDMLKNPSVSLKDILYRQHEIGGFYYGE:293
PhyAsl      : FIRLYRQLPPK-AWLHFHCQAGVGRTTTTYMALYDMMRNPDVTLKDILYRQHEIGGTYLGY:288
PhyAmm      : FINFTRIMPAN-AWLHFHCQAGAGRTTAYMAMYDMMKNPDVSLGDILSRQYLLGGNYVAY:589
PhyBsl      : FLTIFYRSLAPDSGWLHFHCHAGCGRTTTTFMALYEMLRFPGRSLDHVLAHQRTIGGADLAI:261
PhyAsrl     : FMAFYKSLPKN-AWLHFHCHAGHGRTTTFAVFYDILSNPAVAMDDIVARQYALGGTNLFA:275

*           *           *
PhyAsr      : FPIKTKDK-DSWTKKYREKIVMIEQFYRYVQENRADGYQTPWSVWLKSHPAKA:346
PhyAsl      : EGAKHQDRTEGWKSVYYADKAEMIQSFYRYVQONHADGYKVLWSEWLKQLQAGKV:342
PhyAmm      : EIAPKPKPD--QWKADYYHQKAHMIKQFYRYVQENHADGFKTSWSQWLAHQDV--:640
PhyBsl      : PGSR-----YDSLKLFHQYVQENYAAGFTESWSSWLARH-----:295
PhyAsrl     : PGKKDN-----WKGKEIRKRAEQIRKFYAYVQANRSNOYAQTFSAWVKAQR---:321

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Figure 1.2. Amino acid sequence alignment of the characterized and published PTP-like IPPases to date. Shading is determined according to alignment consensus as given by GENE DOC (black = 100%; dark grey = 80%; light grey = 60%) with similarity groups DN, EQ, ST, KR, FYW, and LIVM enabled. The protein abbreviation, source, and NCBI accession number are as follows: PhyAsr, *Selenomonas ruminantium*, AAQ13669 (residues 120-346); PhyAsl, *Selenomonas lactificifex*, ABC69367 (residues 115-342); PhyAmm, *Mitsuokella multacida*, ABA18187 (residues 416-640); PhyBsl, *Selenomonas lactificifex*, ABC69361 (residues 88-295); PhyAsrl, *Selenomonas ruminantium* subsp. *lactylitica*, ABC69359 (residues 98-321). Double sided arrow represents the PTP active site signature sequence.

Chapter Two

Phytase A from *Bdellovibrio bacteriovorus* is a PTP-like IPPase

2.1. Introduction

myo-inositol 1,2,3,4,5,6-hexakisphosphate (InsP₆) is the most abundant inositol polyphosphate (IPP). InsP₆ and other IPP derivatives have been implicated in the regulation of protein expression and have been shown to participate in the regulation of mRNA export and protein expression (York *et al.*, 1999; Macbeth *et al.*, 2005), dsDNA break repair (Hanakahi *et al.*, 2000), regulation of telomere length (York *et al.*, 2005), and vacuole and ribosome biogenesis (Dubois *et al.*, 2002; Horigome *et al.*, 2009). Phytases are a group of enzymes that catalyze the release of orthophosphate from InsP₆ (Mullaney and Ullah, 2003). Recently, a novel group of phytases was shown to contain a PTP-like active site signature sequence (HC(X)₅R) common to members of the PTP-superfamily. Thus far, the only characterized PTP-like inositol polyphosphatases (IPPases) have been from anaerobic bacteria (Puhl *et al.*, 2009, Puhl *et al.*, 2008a, Puhl *et al.*, 2008b, Puhl *et al.*, 2007). In vitro, these enzymes have been shown to hydrolyze InsP₆ to less phosphorylated IPPs; thereby increasing the availability of free inorganic phosphate (Puhl *et al.*, 2009, Puhl *et al.*, 2008a, Puhl *et al.*, 2008b, Puhl *et al.*, 2007).

The PTP-like IPPases have been shown to contain significant sequence similarities and share many biochemical characteristics such as optimum temperatures and pH (Puhl *et al.*, 2009, Puhl *et al.*, 2008a, Puhl *et al.*, 2008b, Puhl *et al.*, 2007). Interestingly, BlastP searches have uncovered rather diverse groups of bacteria that contain putative PTP-like IPPases and are not associated with the rumen. These include the mammalian pathogens *Clostridium botulinum* and *Legionella pneumophila*, the plant pathogens *Pseudomonas syringae* and *Xanthomonas*

campestris and *Bdellovibrio bacteriovorus*, a bacterium that preys on other Gram-negative bacteria.

Bdellovibrios were first isolated in soil and are largely found in wet aerobic environments (Stolp and Starr, 1963). These predatory bacteria penetrate the periplasmic space of other Gram-negative bacteria where they multiply and finally burst from the cell envelope (Sockett and Lambert, 2004; Stolp and Starr, 1963). The presence of a PTP-like IPPase in *B. bacteriovorus* raises interesting questions about the role of this enzyme in the biology of this aerobic free-living bacterial species, particularly since this species infects the periplasmic space of other Gram-negative bacteria where InsP₆ is not likely to be encountered (Irvine and Schell, 2001). Therefore, it is possible that this putative PTP-like IPPase is an archaic protein no longer vital to the propagation of *Bdellovibrio bacteriovorus*, or that this secreted enzyme is scavenging for extra-cellular phosphate. This putative PTP-like IPPase (PhyAbb) contains significant activity against InsP₆ and contains similar biochemical characteristics to the other characterized PTP-like IPPases. Site-directed mutagenesis studies support the hypothesis that PhyAbb is a novel PTP-like IPPase that uses a PTP-like mechanism to hydrolyze InsP₆.

2.2. Materials and Methods

Sequence analysis

Sequence comparisons were determined by BlastP (Altschul *et al.*, 1990). These sequences were then aligned with ClustalW (Higgins *et al.*, 1994) following the default settings using Blossum62. Sequence alignments were prepared using GeneDoc (Nicolas *et al.*, 1997).

Cloning of the Bdellovibrio bacteriovorus putative PTP-like IPPase

Total DNA from *Bdellovibrio* was isolated as previously described (Priefer *et al.*, 1984). The sequence coding the mature *Bdellovibrio* putative PTP-like IPPase product (NP 968118) was amplified from the genomic DNA using PCR and the primers shown in Table 2.1. The *NdeI* restriction site was designed into the primers for easy introduction into the pET28 vector. The PCR product was initially cloned into dephosphorylated *EcoRV* digested pBluescript II SK (+) (Stratagene, La Jolla, CA, USA). The resulting construct was digested with *NdeI* and the resulting fragment coding for the *B. bacteriovorus* PTP-like IPPase (PhyAbb) was gel purified (MinElute Gel Extraction Kit; Qiagen Inc.; Mississauga, ON), ligated into similarly digested pET28b vector (Novagen, San Diego, CA, USA) and transformed into DH5 α cells (Novagen, San Diego, CA, USA) according to manufacturer's protocol. *Bdellovibrio bacteriovorus* PhyAbb mutants (Asp177Asn and Cys206Ser) were produced via counter PCR amplification from the expression construct mentioned above as previously described (Street *et al.*, 1991). Constructs were verified with automated cycle sequencing at the University of Calgary Core DNA and protein services facilities. Sequence data was analyzed with SEQUENCHERTM version 4.0 (Gene Codes Corp. Ann Arbor, MI) and MacDNAsis version 3.2 (Hitachi Software Engineering Co., Ltd., San Bruno, CA).

Protein production and purification

Escherichia coli BL21 (DE3) cells (Novagen, San Diego, CA, USA) were transformed with the putative PTP-like IPPase expression construct known as *phyAbb* and mutants *phyAbbD177N* and *phyAbbC206S*. Protein over-expression was accomplished according to the pET Systems Manual (Novagen, San Diego, CA, USA.). Cell cultures were grown to an OD₆₀₀

of 0.7 at 37°C and subsequently induced by the addition isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1mM. Cells were grown for 8 hours at room temperature with shaking at 225rpm. Induced cells were harvested, re-suspended in lysis buffer: 25mM KH₂PO₄ (pH 7.5), 100mM NaCl, 25mM imidazole, and 5mM β-mercaptoethanol (BME), and lysed by sonication with a Branson (Danbury, CT) model 450 sonifier. Cellular debris was removed via centrifugation (20,000 x g). The recombinant 6xHis tagged PhyAbb was purified to homogeneity by binding cell lysate to the metal chelating affinity Ni²⁺ NTA resin (Bio-Rad, Mississauga, ON), washed in lysis buffer and eluted in lysis buffer supplemented with 400mM imidazole. Protein was purified further by size exclusion chromatography (SEC) with Sephacryl S-200 (Pharmacia, Mississauga, ON) into the following storage buffer: 25mM Tris (pH 7.5), 100mM NaCl, 5mM β-mercaptoethanol and 10% glycerol. Purified protein was then flash frozen and stored at – 80 °C until further use. Protein purity was confirmed by visualizing samples on a (12/5)% (w/v) SDS –polyacrylamide gel (SDS-PAGE; (Laemmli, 1970). The protein bands were visualized by Coomassie Brilliant Blue R-250 staining. Protein concentrations were determined by Bradford analysis with BSA as a standard.

Enzyme assays

Enzyme assays were carried out at 50°C unless specified otherwise. Assays consisted of 600 μl buffered substrate solution and 150 μl of a 99.4 nM protein solution. The buffered substrate solution contained 50 mM sodium and 2 mM sodium phytate in a 50 mM sodium acetate buffer (pH 4.0). Ionic strength (*I*) was held constant at 50 mM with the addition of NaCl, or when examining the effects of *I*, varied from 0.05 to 0.8 M. To determine the effect of pH, activity was measured at the following pH ranges with overlapping buffer systems: [50 mM]

glycine (pH 3), formate (pH 3-4), acetate (pH 4-6), and Tris-HCl (pH 6-7). Activity was also determined over a temperature range between 25°C and 60°C in 5°C increments. Kinetic parameters were determined by running the standard assay with sodium phytate concentrations ranging from 0.006 – 0.800 mM. Sodium phytate was replaced with other phosphoester containing substrates for the determination of substrate specificity. The phosphoester containing substrates examined are listed in Table 2.2.

2.3. Results

Sequence analysis

The PhyAbb protein contains homologues in GenBank that are PTP-like IPPases and putative PTP-like IPPases (Figure 2.1). Thus, due to sequence similarities with other PTP-like IPPases, the ORF of this putative PTP-like IPPase is designated *phyAbb* (GeneBank accession number NC 005363). The *phyAbb* coding sequence encodes a 293 amino acid polypeptide (PhyAbb) that contains a PTP-like active site signature sequence (HCRAGKGR) common to members of this newly discovered group of enzymes. The sequence also includes a putative signal peptide sequence suggesting that it is secreted. The homologues with the highest sequence identity to PhyAbb include putative PTP-like IPPases (*Stigmatella aurantiaca* and *Clostridium perfringens*; 35% and 31% identity respectively), and previously characterized PTP-like IPPases (*Selenomonas ruminantium*: PhyAsr and *Mitsuokella multacida*: PhyAmm; both 31% identity) (Gruninger *et al.*, 2009; Puhl *et al.*, 2007).

Expression and purification of the putative PTP-like IPPase

Upon induction of expression in *E. coli* BL21 (DE3) cells carrying pET28b::*phyAbb*, overproduction of a polypeptide with an M_r of approximately 34,000 was observed by SDS PAGE (data not shown). This is consistent with the predicted molecular mass of the recombinant protein ($M_r = 33,549$). Purification of PhyAbb via Ni^{2+} -affinity chromatography and size-exclusion chromatography yielded protein preparations with >99% homogeneity as determined by SDS PAGE and Coomassie Blue-250 staining (data not shown).

Enzymatic activity and substrate specificity

Sequence alignments with other PTP-like IPPases shows that PhyAbb is homologous with these proteins, and thus PhyAbb was initially characterized against InsP_6 . PhyAbb can hydrolyze InsP_6 , and displayed a maximum specific activity of 310 U mg^{-1} . The activity against InsP_6 falls into the range of previously described phytases, from 0.5 U mg^{-1} in mung bean (Mandel *et al.*, 1972) to greater than 800 U mg^{-1} in *E. coli* (Golovan *et al.*, 2000; Greiner *et al.*, 1993; Wyss *et al.*, 1999). Storage of PhyAbb for periods of 16 hours at 4°C or freezing at -80°C had no effect on phytase activity (Data not shown). The use of fresh or previously frozen enzyme in assays had no effect on the activity against InsP_6 (Data not shown).

In order to establish optimal conditions, ionic strength (I), temperature, and pH profiles were determined for InsP_6 hydrolysis. The hydrolysis of InsP_6 is dependent upon I (Figure 2.2) as the optimal I is low in relation to other PTP-like IPPases (Puhl *et al.*, 2009; Puhl *et al.* 2008a; Puhl *et al.*, 2008b; Puhl *et al.*, 2007). As seen, the optimal I is 50 mM (which is the lowest I possible under these experimental conditions) and as the I is increased relative activity drops. Thus, I is was maintained at 50 mM in subsequent assays.

PhyAbb was tested for its ability to hydrolyze InsP₆ at varying pH. The enzyme displays optimal activity at an acidic pH of 4.0 (Figure 2.3). As seen, on either side of pH 4.0, the relative activity steadily declines, and no activity is detectable below pH 3.0 or higher than pH 7.0. The optimal temperature for hydrolysis of InsP₆ was also determined. Maximal phytase activity occurred at 50°C and drops appreciably on either side of the optimum (Figure 2.4).

Due to sequence similarities with other PTP-like IPPases, and the fact that these enzymes belong to the PTP superfamily, mutational analysis was conducted on residues responsible for catalysis. Sequence alignment suggests that Cys206 and Asp177 function as the thiolate that forms the phospho-intermediate and the general acid of catalysis respectively. No detectable phytase activity was observed for active site mutants PhyAbb(Cys206Ser) and PhyAbb(Asp177Asn).

The ability of PhyAbb to hydrolyze various phosphorylated compounds was determined in order to characterize its substrate specificity (Table 1.2). PhyAbb showed significant activity against only InsP₆. Interestingly, despite the active site signature sequence common to all members of the PTP superfamily, PhyAbb was unable to hydrolyze pNPP, a common PTP substrate, to any appreciable extent.

To study the enzyme-substrate affinities, kinetic parameters of PhyAbb against InsP₆ were determined. The initial velocity of the reaction as a function of the concentration of substrate is consistent with a classical Michaelis-Menton enzyme mechanism (data not shown). The apparent k_{cat} and K_{m} were determined to be 124 s⁻¹ and 574 μM respectively. The resulting catalytic efficiency ($k_{\text{cat}} K_{\text{m}}^{-1}$) was determined to be 216 mM⁻¹ s⁻¹.

2.4. Discussion

Sequence Analysis

I have cloned a putative PTP-like IPPase gene from *B. bacteriovorus* (*phyAbb*). The amino acid sequence of this enzyme contains substantial similarities with the other PTP-like IPPases studied to date (Puhl *et al.*, 2009; Puhl *et al.* 2008a; Puhl *et al.*, 2008b; Puhl *et al.*, 2007; refer to chapter 3). This sequence conservation is most notable in the region containing the PTP-like active site signature sequence. The well-characterized PTP-like IPPases PhyAsr, and PhyAmm have been shown to contain a PTP-like core structure and catalytic mechanism similar to members of the PTP superfamily (Gruninger *et al.* 2009; Puhl *et al.* 2007; Chu *et al.* 2004). Thus the sequence identity, conservation of the active site signature sequence, and similar ability to dephosphorylate InsP₆ suggest a similar three dimensional structure and common mechanism of hydrolysis.

Enzymatic activity and substrate specificity

Recombinant PhyAbb displayed a maximum activity of 310 U mg⁻¹ against InsP₆ which falls into the range of previously described phytases such as PhyAsr, PhyAsl, PhyAme, and PhyAmm which display specific activities of 668 U mg⁻¹, 433 U mg⁻¹, 269 U mg⁻¹, and 836 U mg⁻¹ respectively (Gruninger *et al.* 2009, Puhl *et al.* 2008a; Puhl *et al.* 2008b; Puhl *et al.* 2007). The activity is significantly greater than that of HopAO1 from *Pseudomonas syringae* pv. *tomato* DC3000 (refer chapter 3) however, the apparent K_m for HopAO1 is much smaller suggesting a stronger affinity for substrate. The catalytic constant for PhyAbb was found to fall into the range of PhyAme (k_{cat} 122 s⁻¹), PhyAsl (k_{cat} 256 s⁻¹), and PhyAsr (k_{cat} 264 s⁻¹) (Puhl *et al.* 2009;

Puhl *et al.*, 2008a; Puhl *et al.*, 2007) and was significantly larger than PhyAsrI (k_{cat} 2.9 s⁻¹) and PhyBsl (k_{cat} 18 s⁻¹) (Puhl *et al.*, 2008a; Puhl *et al.*, 2008b).

As with other PTPs and PTP-like enzymes, *I* appears to have an effect on the *in vitro* capabilities of this enzyme to hydrolyze InsP₆. The substantial inherent charge of IPP substrates, especially InsP₆, and the positively charged binding pocket of the enzyme influences the dependence of salt over certain *I* ranges (Puhl *et al.* 2009; Puhl *et al.*, 2007). Influences to salt tolerances were reported amongst other PTP-like IPPases from *S. ruminantium*; PhyAsr (Chu *et al.*, 2004; Puhl *et al.*, 2007), *S. ruminantium subsp. lactilytica* (Puhl *et al.*, 2008a), *lacticifex* (Puhl *et al.*, 2008b) and *Megasphaera elsdenii* (Puhl *et al.*, 2009). However, PhyAbb differs significantly in its optimum *I*. It has been reported that the ionic strength of rumen fluid is between 0.085 to 0.15 M (Koppolu and Clements, 2004; Wohlt *et al.*, 1973) which indicates a likely adaptation to the rumen environment. Hence, it is reasonable to assume that PhyAbb contains a low *I* due to environmental conditions in which the *in vivo* catalysis occurs which is likely an external environment due to the presence of a signal peptide.

Based on their pH optima for catalysis, phytases can be divided into three major groups, acid, alkaline, and neutral (Konietzny and Greiner, 2002). Due to an interest in identifying enzymes that could hydrolyze InsP₆ in the digestive tract of monogastric animals, most studies thus far have focused on acidic phytases (Mullaney and Ullah, 2003). Acid phytases include enzymes belonging to the HAP, PAP, and PTP superfamily classes of phosphatases (Mullaney and Ullah, 2003; Puhl *et al.*, 2007; Puhl *et al.*, Puhl *et al.*, 2008a; Puhl *et al.*, 2008b; Puhl *et al.*, 2009). PhyAbb displays an optimum pH of 4.0 categorizing it as an acidic phosphatase. This is similar to the other characterized PTP-like IPPases from *S. ruminantium*; PhyAsr (Puhl *et al.*,

2007), *S. ruminantium subsp. lactilytica* (Puhl *et al.*, 2008a), *lactificifex* (Puhl *et al.*, 2008b) and *Megasphaera elsdenii* (Puhl *et al.*, 2009). Again, due to environments in which the ruminant organisms reside, the temperature optima of hydrolysis are at or near 37°C (Puhl *et al.*, 2008b; Puhl *et al.*, 2007). However this is not always the case, the optima temperature is elevated to approximately 50°C in certain instances (Puhl *et al.*, 2009; Puhl *et al.*, 2008a). PhyAbb contains a temperature optima of 50 °C, similar to the PTP-like IPPase enzymes from *S. ruminantium subsp. lactilytica* (Puhl *et al.*, 2008a), and *Megasphaera elsdenii* (Puhl *et al.*, 2009). It is possible that this is not a result of the natural *in vivo* habitat temperatures but rather that these enzymes are resistant to higher temperatures and the elevated kinetics of the *in vitro* reaction has led to increased hydrolysis.

PhyAbb appears to behave similarly to other PTP-like IPPases characterized thus far with respect to substrate specificity and the inability to dephosphorylate substrates other than IPPs (Puhl *et al.*, 2009; Puhl *et al.*, 2008a; Puhl *et al.*, 2008b; Puhl *et al.*, 2007). However, this is unlike many HAP acid phytases that display a wide range of specificity towards substrates with phosphate esters (Konietzny and Greiner, 2002). This along with the activity and kinetic data reported in this Chapter strongly suggests that PhyAbb dephosphorylates its substrates in a manner similar to the other PTP-like IPPases and likely favors InsP₆ as a substrate.

Table 2.1. PCR and counter PCR primers used in this experiment. Restriction sites are indicated in bold. 5' phosphorylation for counter PCR is indicated by PO₄.

Primer Designation	Primer Sequence
PhyAbb_NdeI_For	CAT ATG CAA AAA TCC GTA AGC CTC ACT
PhyAbb_NdeI_Rev	CAT ATG TCA TCT TAA CAC CCA CTC ACC
PhyAbb_C206S_ATWF	PO ₄ – CGT GCC GGC AAGGGC CG
PhyAbb_C206S_ATWR	GGA CCC ACG TAA AAG TGA GA
PhyAbb_D177N_ATWF	PO ₄ – CAT GTG CGT CCG GTG CAT TC
PhyAbb_D177N_ATWR	CGC AGA CTG CCA CTG GTT A

Table 2.2. Substrate specificity of PhyAbb. Determination of relative activity was achieved by taking the hydrolysis of InsP₆ as 100%. Substrate concentrations were all tested at 2 mM. N.D.A stands for no detectable activity.

Substrate	Specific activity (U mg⁻¹)	Relative activity
InsP ₆	310.78	100.00
ATP	1.65	5.31 x 10 ⁻¹
D-fructose 1,6 diphosphate	3.63 x 10 ⁻¹	1.17 x 10 ⁻¹
α-Naphthyl phosphate	2.01 x 10 ⁻¹	6.47 x 10 ⁻²
pNPP	1.37 x 10 ⁻¹	4.41 x 10 ⁻²
Phenolphthalein diphosphate	9.30 x 10 ⁻²	2.99 x 10 ⁻²
ADP	7.30 x 10 ⁻²	2.35 x 10 ⁻²
O-phospho-L-tyrosine	4.08 x 10 ⁻²	1.31 x 10 ⁻²
O-nitrophenyl β-D-galactopyranoside	N.D.A	N.D.A
O-phospho-L-threonine	N.D.A	N.D.A
D-fructose 6-phosphate	N.D.A	N.D.A
D-ribose 5-phosphate	N.D.A	N.D.A
D-glucose 6-phosphate	N.D.A	N.D.A
D-glucose 1-phosphate	N.D.A	N.D.A

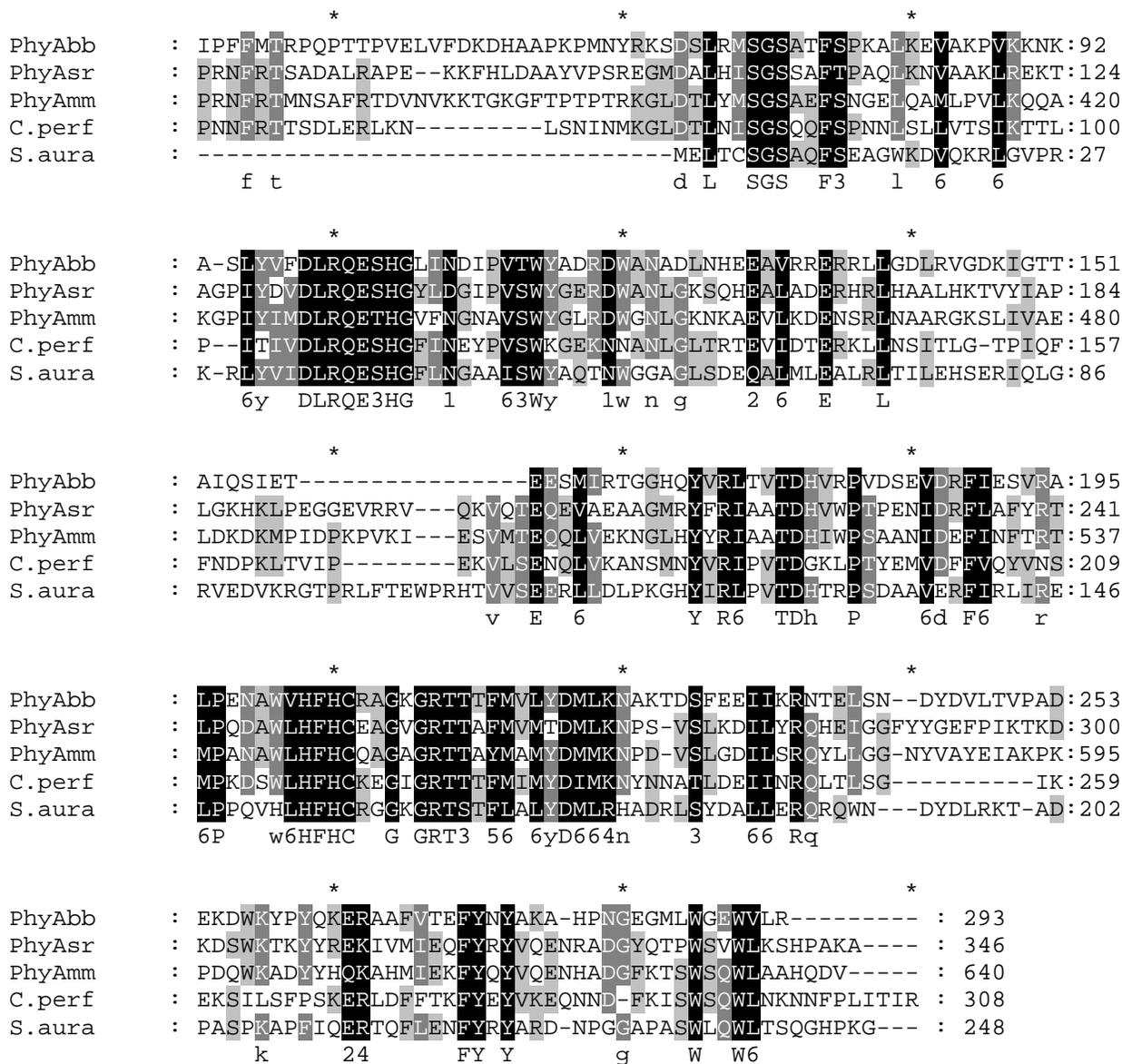


Figure 2.1. Amino acid sequence alignment of the putative PTP-like IPPase (PhyAbb) from *Bdellovibrio bacteriovorus* and its GeneBank homologues. Shading is determined according to alignment consensus as given by GENE DOC (black = 100%; dark grey = 80%; light grey = 60%) with similarity groups DN, EQ, ST, KR, FYW, and LIVM enabled. The protein abbreviation, source, and NCBI accession number are as follows: PhyAbb, *Bdellovibrio bacteriovorus*, NP 968118 (residues 33-293); PhyAsr, *Selenomonas ruminantium*, AAQ13669 (residues 65-346); PhyAmm, *Mitsuokella multacida*, ABA18187 (residues 361-640); Putative PTP-like IPPase, *Clostridium perfringens*, YP696211 (residues 41-308); Putative PTP-like IPPase, *Stigmatella aurantiaca*, ZP01462826 (residues 1-248).

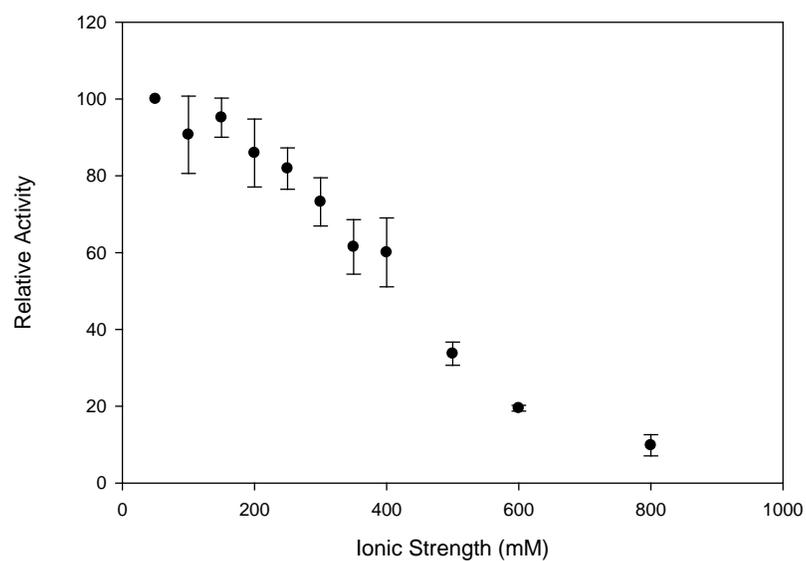


Figure 2.2. Effect of I on PhyAbb activity against InsP_6 . I was controlled by varying NaCl concentrations. Data represents mean values with error bars representing standard deviations between five independent trials.

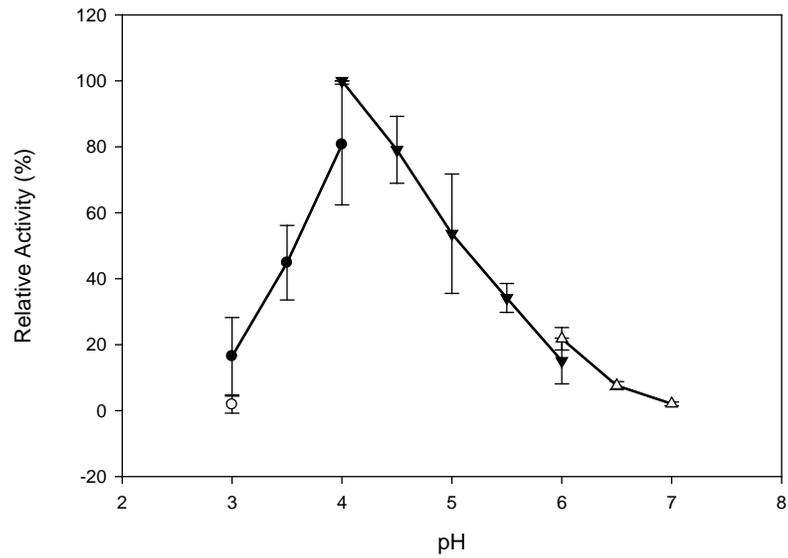


Figure 2.3. Effects of pH on PhyAbb activity against InsP₆. Phosphatase assay was performed according to standard protocol with 2 mM sodium phytate, over a range of 3 to 7. Buffers used include: glycine (open circles), formate (closed circles), acetate (closed triangles), and Tris-HCl (open triangles). Data represents mean values with error bars representing standard deviations between five independent trials

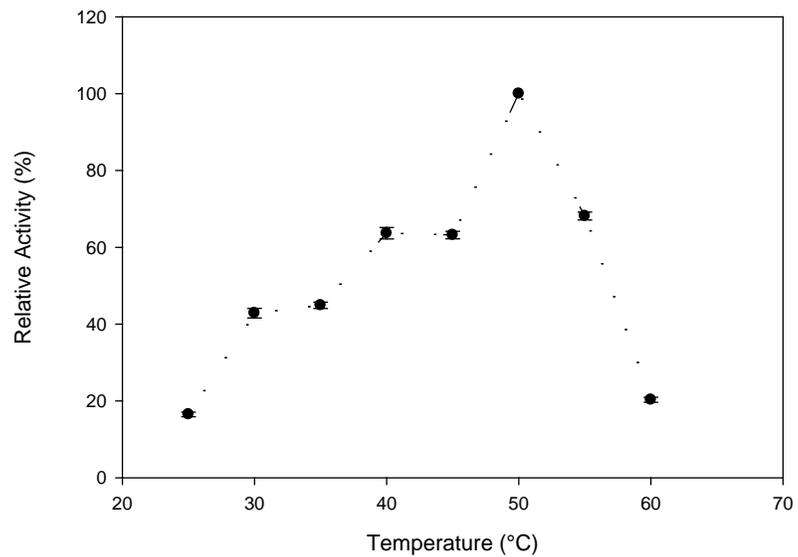


Figure 2.4. Effects of temperature on PhyAbb activity. Standard phosphatase assays were performed with 2 mM sodium phytate. Temperature was increased by 5°C increments from 25°C to 60°C. Data represents mean values with error bars representing standard deviations between three independent trials

Chapter Three

HopAO1 from *Pseudomonas syringae* pv. *tomato* DC3000 is a PTP-like IPPase

3.1. Introduction

The phytopathogenic bacterium *Pseudomonas syringae* pv. *tomato* DC3000 has been studied extensively due to its ability to translocate effector proteins into the cytosolic milieu of plant cells and its ability to cause necrosis on the susceptible *tomato* and *Arabidopsis* plants. However, upon infection in non-host plants such as tobacco, the plant responds to infection by eliciting the hypersensitivity response (HR) (Fouts *et al.*, 2003). The HR is a rapid, localized event established by the plant cell in order to prevent the spreading of the pathogen to healthy tissue. Interestingly, it has been shown that the expression of various effector proteins in yeast from *P. syringae* can greatly reduce the yeast cell's ability to grow and in certain cases leads to cell death (Munkvold *et al.*, 2008).

A newly discovered effector protein from *P. syringae* pv. *tomato* DC3000 known as HopAO1 (Petnicki-Ocwieja *et al.*, 2002) has the ability to circumvent the HR in host-cultivars. HopAO1 (previously known as HopPtoD2) is located within a cluster of hypersensitivity response and pathogenicity (*hrp*)-genes that is positioned within a pathogenicity island (Pai), but not linked to the Hrp Pai responsible for encoding the type three secretion system (TTSS) apparatus (Alfano *et al.*, 2000; Badel *et al.*, 2002). HopAO1 contains an N-terminal domain which shares similarities with AvrPphD (Espinosa *et al.*, 2003). This domain is responsible for the translocation into host plant cells, and the secretion signal is located in the first 50 amino acids, while the effector activity is localized at the C-terminus (Mudgett and Staskawicz, 1999; Guttman and Greenberg, 2001). HopAO1 contains a conserved protein tyrosine phosphatase

(PTP) active site signature sequence in its C-terminal domain (HC(X)₅R) (Espinosa *et al.*, 2003). The observation that HopAO1 will dephosphorylate phosphotyrosine peptides has led to speculation that a classic PTP mechanism contributes to virulence in host plant cells (Bretz *et al.*, 2003; Espinosa *et al.*, 2003). HopAO1 knock-out and active site Cys378Ser mutants lack PTP activity and have reduced ability to promote virulence (Bretz *et al.*, 2003; Espinosa *et al.*, 2003), further supporting the case for a classic PTP mechanism responsible for virulence. The catalytic mechanism for members of the prokaryotic PTP superfamily have been studied extensively and appear to mimic their better characterized eukaryotic counterparts as regulators of cellular function (Kennelly and Potts, 1999; Shi *et al.*, 1998).

Despite the recent evidence suggesting HopAO1 functions as a PTP, database searches show that HopAO1 is much more closely related to a recently discovered group of phytases that are distantly related to the PTP superfamily and have a catalytic preference for inositol polyphosphates (IPP; Puhl *et al.*, 2009). These PTP-like IPPases have been characterized in anaerobic bacterial species such as *Selenomonas ruminantium* (PhyAsr; Puhl *et al.*, 2007), *Selenomonas ruminantium subsp lactilytica* (PhyAsrl; Puhl *et al.*, 2008b), *Selenomonas lacticifex* (PhyAsl and PhyBsl; Puhl *et al.*, 2008a) and *Megasphaera elsdenii* (PhyAme; Puhl *et al.*, 2009). Enzymes capable of hydrolyzing one of the most abundant IPPs, *myo*-inositol hexakisphosphate (more commonly known as phytic acid - InsP₆), are collectively termed phytases. Phytases hydrolyze InsP₆ in a sequential stepwise manner, yielding products that which can become substrates in further hydrolytic reactions (Konietzny and Greiner, 2002). The importance of InsP₆ has been studied extensively over the past 50 years and has been implicated in cell wall integrity and vacuole biogenesis (Dubois *et al.*, 2002), export of mRNA (York *et al.*, 1999), translation (Bolger *et al.*, 2008), phosphate regulation (Auesukaree *et al.*, 2005), telomere length

(York *et al.*, 2005), and most recently it has been shown to affect ribosome biogenesis (Horigome *et al.*, 2009).

Several pathogens including *Pseudomonas* sp., *Salmonella* sp. and *Xanthomonas* sp. are known to inject IPPase virulence factors using a type three secretion system (Anderson *et al.*, 2006, Norris *et al.*, 1998, Chatterjee *et al.*, 2003). The plant pathogen *Xanthomonas oryzae* pv. *oryzae* requires a secreted phytase for optimal growth and virulence suggesting that InsP₆ may play a role in inhibiting pathogenesis (Chatterjee *et al.*, 2003). With HopAO1, previous studies have suggested a protein-protein enzymatic mechanism (Bretz *et al.*, 2003; Espinosa *et al.*, 2003) where a tyrosine phosphorylated substrate is hydrolyzed. Our goal is to provide evidence suggesting that not only does HopAO1 catalyze reactions with phosphorylated protein substrates, but also hydrolyses InsP₆ to a greater extent.

3.2. Materials and Methods

Sequence analysis

Sequence comparisons were determined by BlastP (Altschul *et al.*, 1990). These sequences were then aligned with ClustalW (Higgins *et al.*, 1994) following the default settings using Blossum62. Sequence alignments were prepared using GeneDoc (Nicolas *et al.*, 1997).

Cloning and construction of the HopAO1 expression construct

Total DNA from *P. syringae* pv. *tomato* DC3000 was isolated as described previously (Priefer *et al.*, 1984). The region coding the mature *P. syringae* pv. *tomato* DC3000 HopAO1 (HopA01; GenBank accession number NC 794465) was amplified from genomic DNA using PCR with the primers shown in table 3.1. The *Nde*I restriction site was designed into the

forward primer while the *XhoI* restriction site was designed into the reverse primer to facilitate easy introduction into the pET28b expression vector (Novagen, San Diego, CA). The PCR product was digested with *NdeI* and *XhoI* and ligated into similarly digested pET28b vector pDNA. HopAO1 mutants C378S and D348N were produced via counter PCR amplification from the expression construct mentioned above as previously described (Street *et al.*, 1991). Constructs were verified with automated cycle sequencing at the University of Calgary Core DNA and protein services facilities. Sequence data was analyzed with SEQUENCHER™ version 4.0 (Gene Codes Corp. Ann Arbor, MI) and MacDNAsis version 3.2 (Hitachi Software Engineering Co., Ltd., San Bruno, CA).

HopAO1 production and purification

Escherichia coli BL21 (DE3) cells (Novagen, San Diego, CA) were transformed with the HopAO1 expression construct. Cultures were grown to an OD₆₀₀ of 0.6 at 37°C, then transferred to 25°C until an OD₆₀₀ of 0.85 was reached. The cells were incubated at 19°C for 5 minutes and induced with IPTG to a final concentration of 0.5mM. Incubation was continued for 5 hours at 19°C. Induced cells were harvested by centrifugation and resuspended in lysis buffer: 50 mM KH₂PO₄ (pH 7.5), 350 mM NaCl, 6 mM β-mercaptoethanol (BME), 10% glycerol and 2 mM imidazole. Cells were lysed by sonication with a Branson (Danbury, CT) model 450 sonifier and cellular debris was removed via centrifugation (20,000 x g for 1 hour). The protein was purified to near homogeneity by batch binding cell lysate with Ni²⁺ NTA (Bio-Rad Laboratories Ltd Mississauga, ON) for 1h and contaminating protein were washed away with 100 mL of lysis buffer three consecutive times. Bound protein was then rapidly eluted in 50 mM Tris (7.5), 350 mM NaCl, 6 mM BME, 10% glycerol, and 400 mM imidazole. Protein was immediately buffer

exchanged and purified further via size exclusion chromatography with Sephacryl S-200 (Pharmacia (GE Healthcare) Baie d'Urfe, QU) into a final buffer of 25 mM Tris (7.5), 350 mM NaCl, 6 mM BME, and 10% glycerol. Protein homogeneity was confirmed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE; Laemmli, 1970) with 12% (w/v) polyacrylamide gels stained with Coomassie Brilliant Blue R-250. Protein concentrations were determined by Bradford analysis (Bradford, 1976).

Assay of enzymatic activity

All activity measurements were determined at 25°C with the exception of the experiment dealing with effects of temperature on activity. In this experiment temperature was varied between 5°C and 55°C. Standard assay mixtures consisted of 600 µL buffered substrate solution and 150 µl of a 373 nM enzyme solution. The buffered substrate solution contained 50 mM imidazole (pH 7) and 2 mM sodium phytate. Ionic strength (*I*) was held at 200 mM with NaCl except for the assays examining effects of ionic strength which varied from 75 to 600 mM with addition of NaCl. For determining pH effects, phytase activity was measured in the pH range from 5 to 8 with the following buffer systems: 50 mM sodium acetate (pH 5-6), 50 mM imidazole (pH 6-7), 50 mM Bis-Tris Propane (pH 6.5-7.5), and 50 mM Tris (pH 7-8). Kinetic parameters were determined with the standard assay and a variable concentration (0.025 – 2 mM) of InsP₆.

The substrate specificity of HopAO1 was determined by substituting sodium phytate in the standard phosphatase assay with 2 mM of phosphoester containing substrates: pNPP, O-phospho-L-tyrosine, ATP, D-fructose 1,6 diphosphate, α -Naphthyl phosphate, Phenolphthalein diphosphate, ADP, O-nitrophenyl β -D- galactopyranoside, O-phospho-L-threonine, D-fructose

6-phosphate, D-ribose 5-phosphate, D-glucose 6-phosphate, D-glucose 1-phosphate. Hydrolysis of the phosphorylated peptides was measured according to the manufacturer's instructions for the commercial PTP assay kit (Sigma-Aldrich, St-Louis, MO). A 2.48 μM solution of HopAO1 was used in the assays for determining substrate specificity due to the low levels of activity of HopAO1 on the tested substrates.

Following an incubation period (10 minutes for sodium phytate and 30 minutes for all other substrates), the reactions were stopped and the liberated phosphate was quantified using a modified ammonium molybdate method (Yanke *et al.*, 1999) or a modified Heinonen and Lahti method (Heinonen and Lahti, 1981) as described previously (Puhl *et al.*, 2009). Activity (U) was expressed as μmol phosphate liberated per min. The steady-state kinetic constants (K_M , k_{cat}) were calculated from Michaelis-Menton plots. The data was analyzed with non-linear regression using SIGMA-PLOT 8.0 (Systat Software Inc.; Point Richmond, CA).

Preparation of individual myo-inositol phosphate isomers

The isomers D-Ins(1,2,4,5,6)P₅, D-Ins(1,2,5,6)P₄, and D-Ins(1,2,6)P₃ from InsP₆ were generated by phytases from *Aspergillus niger*, *Escherichia coli*, and *rye*. These isomers along with the InsP₅ generated from HopAO1 were prepared as described previously (Greiner *et al.*, 2002a; Greiner *et al.*, 2002b).

Identification of products formed from hydrolysis

Dephosphorylation pathways were determined by Ralf Greiner (Max Rubner Institute, Federal Research Institute of Nutrition and Food, Karlsruhe, Germany) according to the following methods. Phosphatase assays were run following standard procedure, and were

stopped periodically and resolved on a High-Performance Ion Chromatography system (HPIC) using a Carbo Pac PA-100 (4 x 250 mm) analytical column (Dionex; Sunnyvale, CA) along with a gradient of 5-98% HCl (0.5 M, 0.8 ml/min) as described previously (Skoglund *et al.*, 1998). Upon reaction completion, eluants were mixed in a post-column reactor with 0.1% Fe(NO₃)₃ in a 2% HClO₄ solution (0.4 ml/min) (Phillippy and Bland 1988). Combining these flow rates equated to 1.2 ml/min. *myo*-inositol monophosphates were generated by incubating 1.0 U of enzyme with a limiting amount (0.1 μmol) of InsP₆ to a final volume of 500 μl of 50 mM NH₄-acetate. Identification of end products was accomplished using a gas chromatograph coupled with mass spectrometry as described previously (Greiner *et al.*, 2002a; Greiner *et al.*, 2002b).

3.3. Results

Sequence analysis

A 1.5 kbp DNA fragment was isolated and cloned from *Pseudomonas syringae* pv *tomato* DC3000 (GenBank accession number: NC 004578). The complete *hopAO1* open reading frame (ORF) encodes a 468 amino acid polypeptide (HopAO1) that contains the PTP-like active site signature sequence (HCNGGRGR). Several homologues, with significant sequence identities, were identified with BLAST and all of which are of bacterial origin (Figure 3.1). These homologues include two putative PTP-like IPPases from *Acidovorax avenae* subsp. *citrulli* and *Xanthomonas campestris* pv. *vesicatoria* (37% and 31% sequence identities respectively) and two characterized PTP-like IPPases *Bdellovibrio bacteriovorus* (PhyAbb: Chapter 2 of this thesis) and *Selenomonas ruminantium* (PhyAsr: Puhl *et al.*, 2007) (22% and 21% sequence identity respectively). The human protein phosphatase and tensin homologue (PTEN), a known

PTP, was also used in the alignment. However, in this case, sequence identity between HopAO1 and PTEN is very low (3%). The percent identities represent total alignments.

Expression and purification of HopAO1

Previous work was done with crude extracts from *E.coli* cells over-producing HopAO1 (Espinosa *et al.*, 2003; Bretz *et al.*, 2003). Upon induction with IPTG, overproduction of a polypeptide with a M_r of around 52,000 was observed by SDS-PAGE. This is consistent with the molecular weight predicted from the sequence of the recombinant protein ($M_r = 53,543$). Induction of HopAO1 for periods of time longer than 7 hours and/or at temperatures greater than 20°C resulted in reduced protein yield as a result of the formation of inclusion bodies. Purification of HopAO1 with Ni²⁺-NTA resin followed by size exclusion chromatography yielded >99% homogenous protein as determined by SDS-PAGE and Coomassie Blue-250 staining (data not shown).

Enzymatic activity and substrate specificity

Due to C-terminal sequence similarities with PhyAsr (a known IPPase from *Selenomonas ruminantium*), activity of HopAO1 was initially investigated against InsP₆. HopAO1 can hydrolyze InsP₆, and displayed a maximum specific activity of 9.64 U mg⁻¹. The *I*, temperature and pH profiles for the hydrolysis of InsP₆ by HopAO1 were determined in order to establish optimal conditions. The temperature optimum for HopAO1 appears to reside at 25°C. HopAO1 hydrolysis of phytate decreases rapidly above 30°C and the enzyme is inactive above 40°C (Figure 3.2). The rate of InsP₆ hydrolysis is dependent upon *I* (Figure. 3.3). The *I* optimum is between 175 and 225 mM, therefore further studies were conducted with an *I* of 200 mM. HopAO1 displayed optimal activity at a neutral pH 7 in imidazole buffer (Figure 3.4).

Interestingly, it appears that the choice of buffer is important for optimal activity; HopAO1, as HopAO1 shows much lower activity in Tris at pH 7 than in imidazole or Bis Tris propane buffers (Figure 3.4). HopAO1 activity on phytate rapidly declines on either side of the pH optimum.

Residues responsible for catalysis were mutated in order to determine whether HopAO1 follows a PTP-like mechanism for the hydrolysis of InsP₆. Enzymes belonging to the PTP superfamily have been shown to follow an acid-base mechanism of dephosphorylation. The sequence of HopAO1 in relation to other recently characterized PTP-like IPPases suggests that Cys378 and Asp348 function as the thiolate that forms the phospho-intermediate and the general acid of catalysis respectively. Upon mutation to the most structurally conservative residues possible, the Cys378Ser mutant and the Asp348Asn mutant displayed no observable activity against InsP₆.

The exact physiological substrate(s) for HopAO1 have yet to be elucidated. Thus, we tested the ability of HopAO1 to hydrolyze a variety of phosphorylated substrates in order to characterize its specificity. Compounds hydrolyzed by HopAO1 are shown in Table 3.2. HopAO1 has a narrow substrate specificity as it has very little activity against some of the commonly tested phosphatase substrates. Of the commonly used phosphatase substrates, pNPP and O-phospho-L-tyrosine, yielded specific activity levels of $1.36 \times 10^{-4} \text{ U mg}^{-1}$ and $1.18 \times 10^{-4} \text{ U mg}^{-1}$ respectively. As for the phosphorylated peptides previously assayed with crude cell lysates (Espinosa *et al.*, 2003; Bretz *et al.*, 2003), HopAO1 displayed the following levels of activity: $1.43 \times 10^{-4} \text{ U mg}^{-1}$ and $1.67 \times 10^{-4} \text{ U mg}^{-1}$ against fragments of the insulin receptor and EGF receptor, respectively.

In order to elucidate the enzyme-substrate affinities, we determined the catalytic characteristics of recombinant HopAO1 against InsP₆. The initial velocity of the reaction as a function of the concentration of substrate is consistent with classical Michaelis-Menton enzyme mechanism (Figure 3.5). The apparent k_{cat} and K_M for InsP₆ were determined to be 1.8 s⁻¹ and 62.5 μM, respectively. The resulting catalytic efficiency (k_{cat}/K_M) was 28.5 mM⁻¹ s⁻¹. This catalytic efficiency falls into the range of some of the PTP-like IPPases examined in our laboratory thus far (Puhl *et al.*, 2008a; Puhl *et al.*, 2008b)

InsP₆ hydrolysis pathway

In order to determine the dephosphorylation pathway of InsP₆ by HopAO1, purified enzyme was incubated with excess InsP₆ for 30 minutes, 60 minutes, and 24 hours. The reaction was stopped and the resulting IPPs were resolved by isomer specific HPIC. Following 15 minutes of incubation with enzyme, the quantity of InsP₆ was almost completely hydrolyzed to the D/L-I(1,3,4)P₃. Trace amounts of D/L-Ins(1,2,4,5,6)P₅, D/L-Ins(1,2,3,4,6)P₅, D/L-Ins(1,2,4,5)P₄, and D/L-Ins(1,2,3,4)P₄ were also observed after 15 minutes. This suggests that InsP₆ is hydrolyzed via the following pathways: major pathway (D/L-I(1,2,3,4,6)P₅, D/L-I(1,2,3,4)P₄, and D/L-I(1,3,4)P₃) and minor pathway (D/L-Ins(1,2,4,5,6)P₅, D/L-Ins(1,2,4,6)P₄). A schematic diagram of the hydrolysis pathway is shown in Figure 3.6.

3.4. Discussion

Genetic analysis of HopAO1

The predicted gene product from *hopAO1* shares 35% sequence similarity to PhyAsr (Chu *et al.*, 2004, Puhl *et al.*, 2007), 19% with PhyAsrl (Puhl *et al.*, 2008a), and 48% similarity with a

putative PTP product from *Acidovorax avenae* subsp. *citrulli*. Conservation of sequence similarities were confined to the C-terminus region that contained the PTP-like active site signature sequence. The sequence similarities shared by these PTP-like IPPases and HopAO1 suggests these enzymes adopt a similar three-dimensional structure and thus a common mechanism for catalysis of substrate. N-terminal sequence similarities with other TTSS avirulent proteins (Espinosa *et al.*, 2003) suggests a mechanism for secretion common to effectors released by the TTSS.

Substrate specificity and enzymatic activity

The sequence similarities with known PTP-like IPPases, suggests that the C-terminal PTP region of HopAO1 is an IPPase. HopAO1 hydrolyzes InsP₆ with a specific activity of 9.64 U mg⁻¹, low in comparison with PhyAsr, which at 668.11 U mg⁻¹ has the greatest activity towards InsP₆ by any PTP-like IPPase characterized to date (Puhl *et al.*, 2007). Comparable specific activities have been reported for PTP-like IPPases from *S. ruminantium* subsp. *lactilytica* (PhyAsr1, 16.23 U mg⁻¹; Puhl *et al.*, 2008b) and *Selenomonas lacticifex* (PhyBsl, 12.7 U mg⁻¹; Puhl *et al.*, 2008a). In comparison, HAP phytases show a range of activities from 0.5 U mg⁻¹ in mung bean (Mandel *et al.*, 1972) to 3457 U mg⁻¹ for AppA from *Citrobacter braakii* (Kim *et al.*, 2003). Despite the low activity levels against InsP₆ compared to other phytases, activity against the traditional PTP substrates such as *o*-phospho-L-tyrosine and pNPP and the phosphorylated peptides were approximately 70,000 fold less, indicating a strong tendency to favor the inositol polyphosphates. HopAO1 has activity against phosphorylated peptides (Bretz *et al.*, 2003; Espinosa *et al.*, 2003) and its ability to hydrolyze the same substrates was retested with purified

enzyme. HopAO1 showed very little activity towards these phospho-peptides when compared with InsP₆.

The effect of *I* on HopAO1 activity also falls into the range of previously characterized PTP-like IPPases (Puhl *et al.*, 2008a; Puhl *et al.*, 2008b). These results imply that electrostatic interactions are important for HopAO1 activity and are likely due to the strong electronegative charge associated with InsP₆ (Puhl *et al.*, 2007) which is comparable to the other described PTP-like IPPases. Interestingly, the optimal temperature for HopAO1 activity against InsP₆ is considerably lower than any other PTP-like IPPases characterized to date. The optimum temperature for hydrolysis of InsP₆ of the other PTP-like IPPases ranges from 40 to 60°C (Puhl *et al.*, 2009, Puhl *et al.*, 2008a, Puhl *et al.*, 2008b, Puhl *et al.*, 2007). This low optimal temperature for HopAO1 activity likely reflects the environment in which this enzyme facilitates infection in host plant cells.

Hydrolysis pathway

All characterized IPPases to date remove phosphate groups from InsP_x to an InsP_{x-1} derivative in which the product can be used as the substrate in the following round of hydrolysis (Konietzny and Greiner, 2002). However, few enzymes are capable of removing phosphate from InsP₁ (Konietzny and Greiner, 2002). It has been suggested that PhyAsr acts in a processive manner and possesses a standby site in which reorientation of substrate is mediated between successive hydrolysis reactions (Chu *et al.*, 2004). However, experimental evidence has shown that PhyAsr acts as a distributive enzyme which is inconsistent with the standby site facilitating processive degradation of InsP₆ (Puhl *et al.*, 2007). Most of the known non PTP-like IPPases initiate hydrolysis at the D-3 (L-1) or D-4 (L-6) phosphate positions, with a few initiating at the

D-6 position (Konietzny and Greiner, 2002). This is also the case with the PTP-like IPPase PhyAsr which catalyzes the removal of the 3-phosphate preferentially (Puhl *et al.*, 2007). However variations do exist, such as the recently characterized novel PTP-like IPPase from *S. ruminantium* subsp. *lactilytica* which has demonstrated specificity for the 5-phosphate position (Puhl *et al.*, 2008a). Unlike other PTP-like IPPases, HopAO1 almost completely hydrolyzes InsP₆ after 15 minutes into an InsP₃ substrate without significant amounts of other intermediates. HopAO1 initially catalyzes the removal of the 5-phosphate and the only InsP₃ remaining upon hydrolysis is D-Ins(1,3,4)P₃. This suggests that HopAO1 prefers higher order inositol polyphosphates. Interestingly, HopAO1 is capable of cleaving the axial 2-phosphate from InsP₆ which is novel for this class of enzymes and is possibly related to its *in vivo* activity.

A number of pathogens secrete inositol phosphatase virulence factors into host cells. The *Salmonella sp.* effector protein SopB/SigD promotes cytoskeleton rearrangements that accompany bacterial internalization and activates the Akt pro-survival pathway *in vivo* by an unknown mechanism (Norris *et al.*, 1998, Steele-Mortimer *et al.*, 2000, Knodler *et al.*, 2005). The plant pathogen *Xanthomonas oryzae* pv. *oryzae* requires a secreted phytase for optimal growth and virulence suggesting that InsP₆ may play a role in inhibiting pathogenesis (Chatterjee *et al.*, 2003). Transgenic *Arabidopsis thaliana* yielding lower levels of InsP₆ are more susceptible to infection from *P. syringae* pv. *tomato* DC3000 than their wild-type counterparts (Murphy *et al.*, 2008). InsP₆ can act as both second messengers (Lemtiri-Chlieh *et al.*, 2000, Lemtiri-Chlieh *et al.*, 2003) and as a protein structural cofactor (Tan *et al.*, 2007), therefore the role in basal defense is likely mediated by one or both of these activities. It was determined that InsP₆ is as important for mediating plant defense responses as salicylic acid (Murphy *et al.*,

2008) which is an essential signaling molecule for initiating resistance (Delaney *et al.*, 1994; Gaffney *et al.*, 1993).

A phospholipase C and phospholipase D dependent pathway that generates InsP₂, InsP₃, and phosphatidic acid is up-regulated upon pathogen infection in *Arabidopsis* and ultimately leads to the hypersensitive response (HR) (Andersson *et al.*, 2006). Hydrolysis of InsP₂ and InsP₃ would inhibit the HR response in infected cells and promote pathogen infection. The use of inositol phosphatase during infection suggests that inositol phosphates are involved in signaling events associated with disease resistance and that pathogens have evolved mechanism to specifically target these important second messengers. The IPPases from different pathogenic bacteria supports the suggestion that HopAO1 most likely hydrolyzes some form of IPP or phosphatidyl inositol polyphosphate that is crucial for eliciting plant defense responses.

Recently, effectors from *P. syringae* pv. *tomato* DC3000 have been over-produced in *S. cerevesiae* (Munkvold *et al.*, 2008). Interestingly, growth of cells over-producing HopAO1 was greatly hampered in comparison to the negative control lacZ (Munkvold *et al.*, 2008). This result suggests that HopAO1 is functioning on similar substrates in both systems. Thus, due to the efficacy of working in yeast in comparison to plant cells, the relatively low k_{cat} associated with HopAO1 activity against InsP₆, and the abolishment of activity with the active site mutant, this system may prove beneficial for the elucidation of a biological substrate through substrate trapping experiments.

Table 3.1. PCR and counter PCR primers used in this experiment. Restriction sites are indicated in bold. 5' phosphorylation for counter PCR is indicated by PO₄.

Primer Designation	Primer Sequence
HopAO1_NdeI_For	ATG CCA TAT GAA TCC CCT GCA AC TAT T
HopAO1_XhoI_Rev	ATG CCT CGA GTC ATT CTA ACG CTA TTT TTG
HopAO1_C378S_ATWF	PO ₄ – CTA ACG GCG GTC GGG GC
HopAO1_C378S_ATWR	AGT GCA CCA CTA GCG ACT C
HopAO1_D348N_ATWF	PO ₄ – AAC CAT AAC AGG CCT AGT
HopAO1_D348N_ATWR	CGC AGA CTG CCA CTG GTT A

Table 3.2. The phosphoester containing substrates that were hydrolyzed by HopAO1 and the maximum observed rates of hydrolysis. ATP, D-fructose 1,6 diphosphate, α -Naphthyl phosphate, Phenolphthalein diphosphate, ADP, O-nitrophenyl β -D- galactopyranoside, O-phospho-L-threonine, D-fructose 6-phosphate, D-ribose 5-phosphate, D-glucose 6-phosphate, D-glucose 1-phosphate were also tested and contained no observable activity.

Substrate	Specific Activity	
	U/mg	%
*InsP ₆	9.64	100
†PTP Substrate 1	1.43 x 10 ⁻⁴	< 0.1
†PTP Substrate 2	1.67 x 10 ⁻⁴	< 0.1
†O-phospho-L-tyrosine	1.18 x 10 ⁻⁴	< 0.1
†pNPP	1.36 x 10 ⁻⁴	< 0.1

*10 minute incubation time with 373 nM enzyme concentration

†30 minute incubation time with 2.48 μ M enzyme concentration

```

*           *           *
HopAO1      : -GLSDLKLAGCERIS--SVEQVKSIRAALGGGPI TVLDDLREESHAI VNGLPITLRGPMDW :
A. aven     : -GLSLQLSGSERIT--SVEQVRAIRQACGDAPLVVVDLRQESHAVADGHSLTWRGPMDW :
PhyAbb      : ---DSLIRMSGSATFSPKALKEVAKPVKKNKAS-IYVFDLRQESHGLINDIPVWYADRDW :
PhyAsr      : EGMDALHISGSSAFTPAQLKNVAALKREKTAGEIYDVDLRQESHGYLDGIPVSWYGERDW :
X. camp     : TGWKNLHVSGSGSIA--SIGQITRLRPS-KERPVVVLDAREESHAI VGGYPGIEWRTPNWW :
PTEN        : ----- :

*           *           *
HopAO1      : ANAGLSQVDGAARE SAMITELKRTKSLTLVDANYVKGKSNPQTTELKNLNVRSEREVVT :
A. aven     : GNVGLGTAATAREAEQLEELRRQGNVATHADHVKGKSDEPALRRLDTTLARSEQEIVE :
PhyAbb      : ANADLNHEEA VRRERR-----LLGDLRVGDKIG----TTAIQSIETTESMIR :
PhyAsr      : ANLGKSQHEALADERHRLHAALHKTVYIAPLGKHKLP EGGE----VRRVQKVQTEQEVAE :
X. camp     : GNAGKSRDEALADEQQRIQALKSQETVHIFHRKDVKSEARNPRGATLSKPLIFSEEELVR :
PTEN        : -----LCAERHYDTAKFNCRVA :

*           *           *
HopAO1      : EACATYRRVAITDHNRPSPPEATDELVDIMRHCLQANESLVVHCNCGRGRTTTFAMIMVD-M :
A. aven     : AACADYRRIVAVTDHLRPSRGEVDQFIDLVRG-LPDGAGLHVHCNCGRGRTTTFMVLYD-M :
PhyAbb      : TGCHQYVRLTVTDHVRPVDSEVDRFIESVRA-LPENAWVHFHCRAGKGRITTFMVLYD-M :
PhyAsr      : AACMRYFRIAATDHVWPTPENIDRFLAFYRT-LPQDAWLHFHCEACVGRITTFAMVMTD-M :
X. camp     : AACAKYVRLTVTDHLSPRADDIDAFIAMERE-MAHDERLHVHCGMGLGRITTFIVMHD-I :
PTEN        : QYPFEDHNPPQLELTKPFCELDL-----QWLSEDDNHVAAIHCKAGKGRITGVMTICAYLLH :

*           *           *
HopAO1      : LKNARNHSAETLITRMAKLSYDYNMTD-LGS--ISALKRPFLEDRLKFLQAFHDYARNN- :
A. aven     : PRNAREAGADALMARQSR LGMDYNRCA-YRP--PDACG-----FQVKFSERTLAVVRTS- :
PhyAbb      : LKNAKTDSFEELIKRNTELSNDYDVLTVPADEKD-WKYPYQKERA AFVTEFYNYAKAH- :
PhyAsr      : LKNPSVSLKDI IYRQHEIGGFYYGEFP-IKTKDKDSWTKYREKIVMIEQFYRYVQENR :
X. camp     : LRNAAMLSFDDIERQRKFNPRSLDN-NKD-VSDKGRSEFRNERSEFLPLFYEYAKQN- :
PTEN        : RGKFLKAQEALDFYGEVTRDKKGVTI PSQRRYVYYSYLLKNHLDYRPVALLFHKMMFE :

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Figure 3.1. Amino acid sequence alignment of the *Pseudomonas syringae* pv. *tomato* DC3000 PTP-like IPPase HopAO1 and its GenBank homologues. Shading is determined according to alignment consensus as given by GENE DOC (black = 100%; dark grey = 75%; light grey = 50%) with similarity groups DN, EQ, ST, KR, FYW, and LIVM enabled. The protein abbreviation, source, and NCBI accession number are as follows: HopAO1, *Pseudomonas syringae* pv. *tomato* DC3000, NP_794465 (residue numbers 219-450); Putative PTP-like IPPase, *Acidovorax avenae* subsp. *citrulli*, YP971831 (residues 71-296); PhyAbb, *Bdellovibrio bacteriovorus*, NP968118 (residues 66-279); PhyAsr, *Selenomonas ruminantium*, AAQ13669 (residues 95-327); Putative PTP-like IPPase, *Xanthomonas campestris* pv. *vesicatoria*, YP361664 (residues 109-340); PTEN, *Homo sapiens*, AAD13528 (residues 70-201).

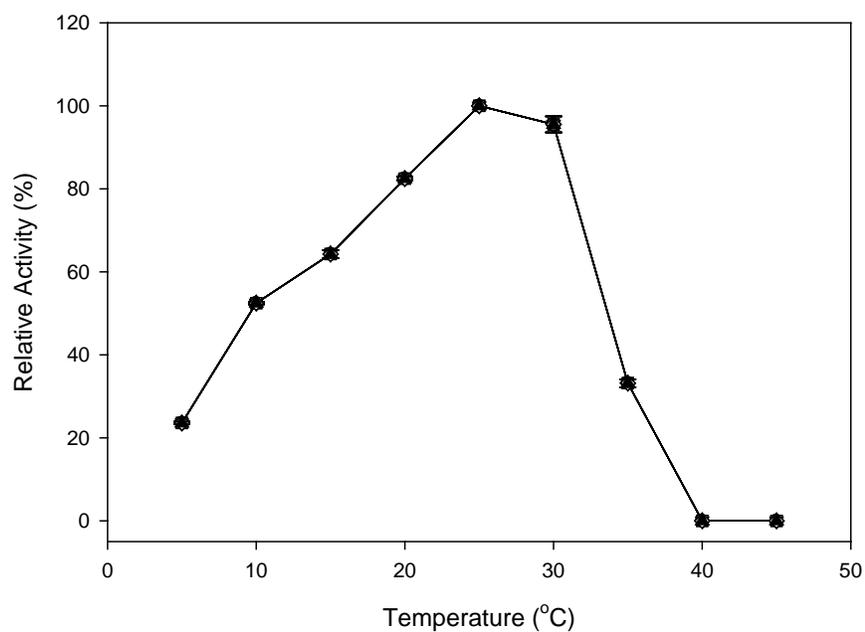


Figure 3.2. Effects of temperature on HopAO1 activity. Standard phytase assays were performed with 2mM sodium phytate with temperature increments from 5 to 45 °C. Data represents mean values with error bars representing standard deviations between three independent trials

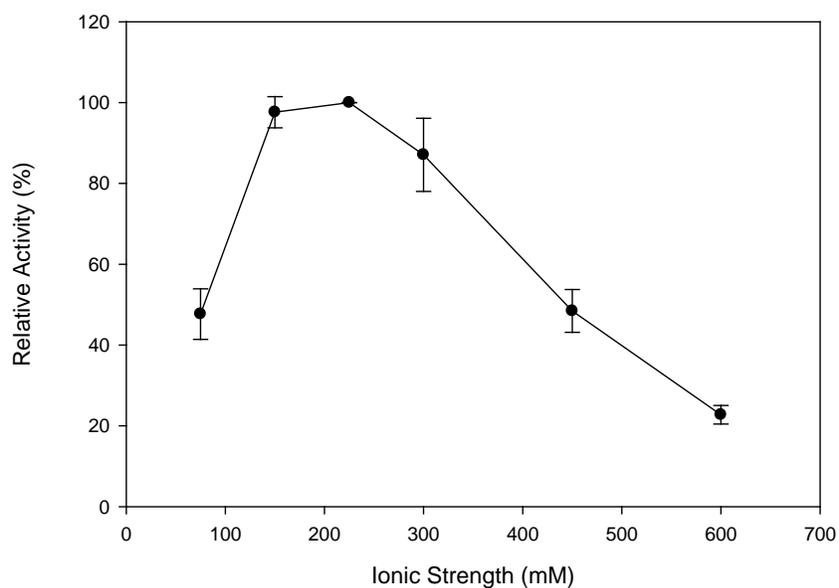


Figure 3.3. Effect of I on the activity of HopAO1. Standard phytase assays were run with 2 mM sodium phytate at room temperature under varying NaCl concentrations. The data represents the mean values with error bars representing standard deviations for three independent experiments.

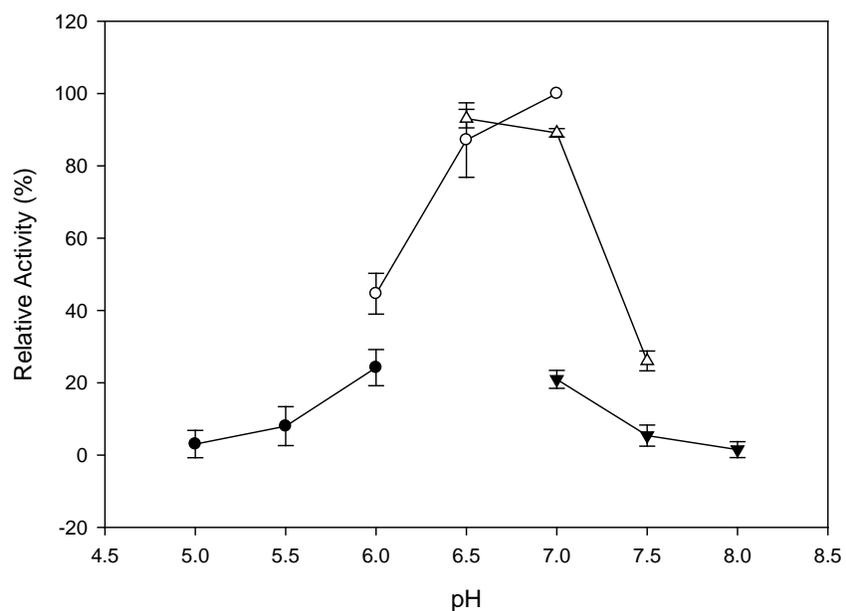


Figure 3.4. Effect of pH on HopAO1 activity. Standard phytase assays were run with 2 mM sodium phytate at room temperature over a pH range of 5 to 8. Buffers used include: acetate (closed circles), imidazole (open circles), Bis Tris propane (open triangles) and Tris (closed triangles). The data represents mean values with error bars representing standard deviation for three independent experiments.

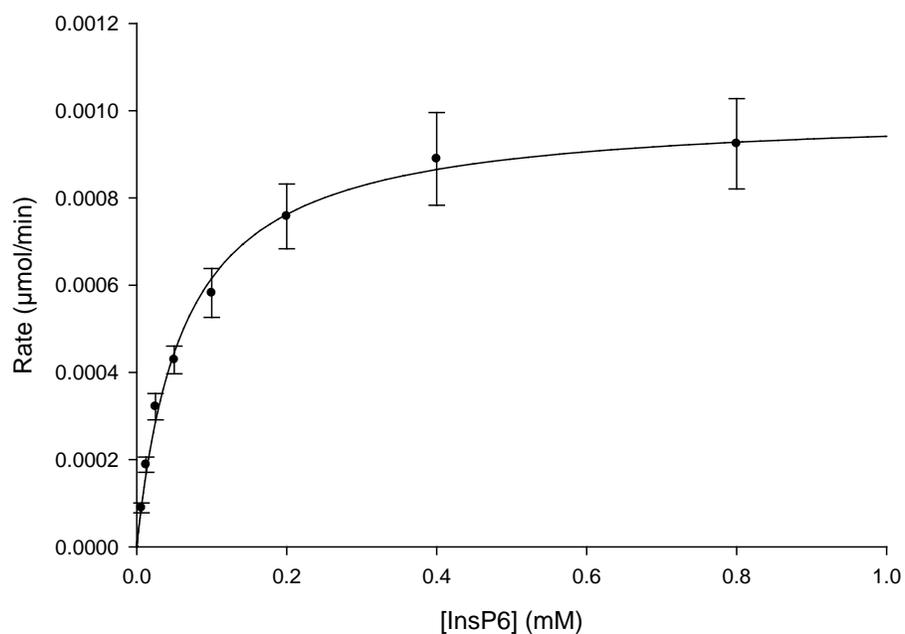


Figure 3.5. Steady state kinetic analysis of HopAO1 activity towards InsP₆. Initial rate data was fit using a classical Michaelis-Menton equation (Sigma Plot 8.0). The data represents mean values with error bars representing standard deviation for three independent experiments.

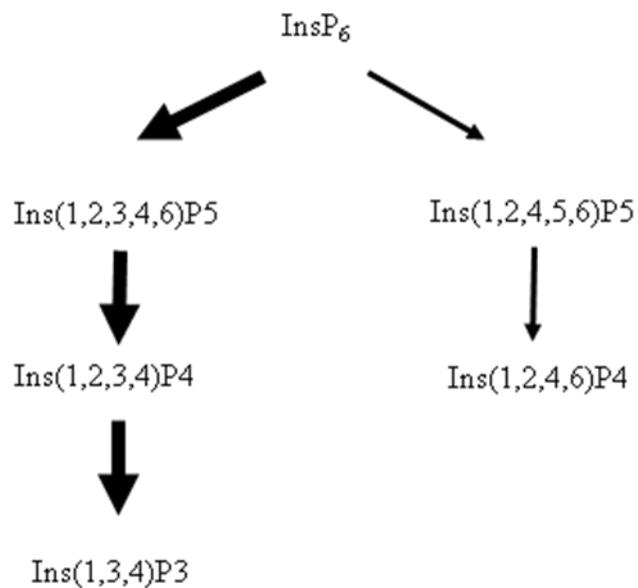


Figure 3.6. The dephosphorylation pathways of InsP_6 by HopAO1 as determined by high-performance ion pair chromatography (HPIC) and kinetic analysis. The larger arrows indicate the major pathways, while the narrow arrow represents the minor pathway.

Chapter Four

Effects of the over-expression of the PTP-like IPPases HopAO1 from *Pseudomonas syringae* pv. *tomato* DC3000 and PhyAsr from *Selenomonas ruminantium* on *Saccharomyces cerevisiae* growth

4.1. Introduction

Pseudomonas syringae pv. *tomato* DC3000 is a well characterized plant pathogen that utilizes the type three secretory system (TTSS) to facilitate transfer of virulent effectors into host cells. It has been established that HopAO1, a *P.syringae* DC3000 effector protein, functions by dephosphorylating a substrate within host cells and disrupting the host cell from initiating an innate immune response (Espinosa *et al.*, 2003; Bretz *et al.*, 2003). *In vitro* experiments performed on HopAO1 have shown that it contains very low levels of activity against tyrosine phosphorylated peptides (Espinosa *et al.*, 2003; Bretz *et al.*, 2003; Refer to Chapter 3). However, the enzyme contains substantially more activity against InsP₆ (Refer to Chapter 3). Despite these recent findings, the *in vivo* substrate has yet to be determined.

Plant cells are not the easiest model system to use for determining the *in vivo* effects caused by virulent effector molecules due to slow growth and the difficulties in producing effectors transiently. Recently researchers have attempted to explain the function(s) of the *P.syringae* DC3000 repertoire of effectors by using a simpler model system. Munkvold et al (2008) used *Saccharomyces cerevesiae* due to the efficacy of a well-established and robust system in which heterologously expressed proteins can be studied, and the belief that these effector proteins are likely manipulating a substrate or system that is universal amongst eukaryotic cells. For example, the effector protein YopE from *Yersinia* sp. was shown to perturb

actin dynamics in both animals and yeast (Lesser and Miller, 2001; Pawel-Rammingen *et al.*, 2000). When over-expressed in *S. cerevisiae*, HopAO1 was shown to cause a phenotypic effect on the growth of *S. cerevisiae* (Munkvold *et al.*, 2008) thus substantiating the claim that HopAO1 is likely working on a similar substrate in both yeast and host plant cells.

Due to the 70,000 fold larger specific activity against InsP₆ *in vitro* in comparison with the PTP phosphopeptides and the biological importance of this substrate in both plants and yeast, I propose that HopAO1 is most likely subverting plant apoptotic pathways by functioning on InsP₆ or a phosphorylated *myo*-inositol derivative. In an attempt to identify the biological substrate, I cloned HopAO1 into *S. cerevisiae* along with the well characterized PTP-like IPPase PhyAsr. We found that when HopAO1 is over-expressed, it significantly hampers *S. cerevisiae* growth. However, over-production of the catalytically inactive mutant hopAO1C378S yields similar results to the negative control lacZ. PhyAsr also appears to inhibit *S. cerevisiae* growth, however, not to the same extent as HopAO1.

4.2. Materials and Methods

Plasmids, Strains, and Media

Yeast expression constructs were prepared by ligating selected IPPase coding sequences (CDS) into pYES2/CT (Invitrogen, Canada Inc., Burlington ON). Expression constructs were prepared in *Escherichia coli* DH5 α cells. Bacterial strains were grown in Luria Bertani (LB) broth at 37°C at 200 RPM. Ampicillin was added at a final concentration of 100 μ g/ml to select for pYES2/CT expression constructs.

Saccharomyces cerevisiae INVSc cells (Invitrogen) were grown initially in Yeast Peptone Dextrose (YPD) media containing 2% carbon source. Upon transformation, yeast cells were grown in minimal medium lacking uracil (SC-U) in order to maintain the plasmid. Yeast was grown at 30°C with shaking at 200 RPM in all cases.

DNA manipulations

DNA manipulations were carried out following standard techniques (Sambrook *et al.*, 1989). The primers used in this study (Table 4.1) were synthesized by Integrated DNA Technologies (IDT, San Diego, CA). Phusion high-fidelity DNA polymerase (Finnzymes, New England Biolabs, Pickering ON) was used to amplify the IPPase CDSs. Nucleotide sequence analysis was performed by automated cycle sequencing at the University of Calgary Core DNA and protein service facilities. Sequence data was then analyzed with SEQUENCHER™ version 4.0 (Gene Codes Corp. Ann Arbor, MI) and MacDNAsis version 3.2 (Hitachi Software Engineering Co., Ltd., San Bruno, CA).

Forward and reverse primers were designed with restriction sites to facilitate ligation into appropriately cleaved pYES2/CT. Primers were designed to incorporate flanking *HindIII* sites into *hopAO1*, *hopAO1C378S*, *phyAsr* and *phyasrC252S* CDSs. The resulting PCR products were initially cloned into pBlueScript SK II+ (Stratagene, La Jolla CA). *HindIII* DNA fragments containing IPPase CDS were ligated into similarly digested pYES2/CT. These constructs were further verified with automated cycle sequencing.

Yeast Inhibition Assays

IPPase constructs and the pYES2/CT::lacZ control were transformed into *S. cerevisiae* INVSc cells by using an adapted lithium acetate protocol according to the instructions from the manufacturer (Invitrogen). Transformants were plated on to a minimal agar medium supplemented with 2% dextrose and lacking uracil, (SC-U) for selection of the plasmid. The plates were incubated at 30°C until colonies were visible (approximately 24 hours). Cells from colonies were grown in SC-U broth for 24 hours at 30°C.

The effect of production of HopAO1, HopAO1 C378S, PhyAsr, PhyAsr C252S, and LacZ on *S. cerevisiae* cells was examined by monitoring culture growth. Cells were grown overnight in SC-U. Cells were harvested by centrifugation and re-suspended to an optical density (OD at 600 nm) of approximately 0.4 in induction medium: SC-U supplemented with 1% raffinose and 2% galactose. Cultures were incubated at 30°C and 200 RPM and growth was monitored by measuring optical density (600 nm) over a 24 h period (i.e., readings were taken at 1, 2, 4, 6, 8, 10, 12, 16, 20, and 24 h time points). Cells were also plated on SC-U (1% raffinose, 2% galactose) induction media to test for colony formation.

4.3. Results

Phosphatase activity of HopAO1 affects the growth of yeast cells

The effect of over producing HopAO1 in *S. cerevisiae* cells was examined in yeast strains transformed with the PTP-like IPPase CDSs under the control of the galactose inducible *GALI* promoter. The *lacZ* gene was also expressed as a negative control in order to show that the growth inhibition was not a result of over-production of a protein, but rather related to enzyme

activity. Yeast strains expressing *hopAO1*, *hopAO1C378S*, or the *lacZ* gene were grown under conditions supporting repression (i.e., dextrose as the carbon source) or induction (i.e., galactose as the carbon source). The repressing medium was used in order to ensure that there was no leaky expression of the recombinant yeast. It was observed that all three recombinant strains grew equally well on the repression medium suggesting that there is no leaky expression of HopAO1 (Figure 4.1). Upon induction with 2% galactose, the growth of the recombinant yeast differed significantly from the yeast grown on repression medium (Figure 4.2). The recombinant strains producing *lacZ* and the catalytically inactive HopAO1 C378S grew at the same rates under inducing conditions (Figure 4.2). While protein production affected the growth of all recombinant yeast strains, over production of HopAO1 had the greatest impact on yeast culture growth. After 24 hours, the optical density of yeast cultures producing HopAO1 was only 33% of that achieved by the other two cultures (Figure 4.2).

Phosphatase Activity of PhyAsr on Yeast Growth

Due to sequence similarities of HopAO1 with other characterized PTP-like IPPases, *phyAsr* from *S. ruminantium* was over-expressed, under the control of the GAL1 promoter, to determine the effect of this enzyme on the growth of *S. cerevisiae*. Upon induction with 2% galactose, cells over-producing the inactive mutant *PhyAsr* C252S and the negative control *LacZ* had a similar effect on *S. cerevisiae* growth (Figure 4.3). Production of recombinant wild-type *PhyAsr* delays growth and reduces culture yield in relation to cultures producing *LacZ* or *PhyAsr* C252S (Figure 4.3).

4.4. Discussion

Previous studies have failed to show a link between PTP activity and *in vivo* mechanisms of action of HopAO1 (Espinosa *et al.*, 2003; Bretz *et al.*, 2003). Due to a C-terminal domain containing a PTP active site signature sequence (HC(X)₅R), it was originally assumed that HopAO1 was subverting plant defense responses by acting on a phosphorylated tyrosine containing polypeptide. Crude extract containing HopAO1 was shown to have low level activity against fragments of tyrosine phosphorylated peptides of both the epidermal growth factor receptor and the insulin receptor (Espinosa *et al.*, 2003; Bretz *et al.*, 2003, Refer Chapter 3). In comparison, purified recombinant HopAO1 contains nearly 70,000 fold more activity against InsP₆ than both the tyrosine phosphorylated peptide fragments mentioned above (Refer Chapter 3). Furthermore, given that the MAPK cascade is activated in part due to tyrosine phosphorylation, Espinosa *et al.* (2003) tested the ability of HopAO1 to interact directly with two MAPKs. The group selected the salicylic acid-induced protein kinase (SIPK) and the wound-induced protein kinase (WIPK) for testing via standard yeast two hybrid assays (Espinosa *et al.*, 2003). However, HopAO1 failed to interact with either of these proteins suggesting an alternative substrate through which virulence is facilitated on host plants.

Due to the high sequence similarities with previously characterized PTP-like IPPases, and the significantly higher levels of activity against InsP₆ in comparison to some of the common PTP substrates (Refer Chapter 3), I hypothesized that HopAO1 targets InsP₆ *in vivo*. InsP₆ is the most abundant inositol polyphosphate in eukaryotic cells (Shears, 2001) and is the precursor molecule of diphosphoinositol polyphosphates, a class of inositols that contain one or more pyrophosphate moieties located on the inositol ring (Menniti *et al.*, 1993; Stephens *et al.*, 1993).

Interestingly, it has been reported that yeast defective in producing the inositol pyrophosphate-forming enzyme InsP₆ kinase (KCS1) display defective vesicular endocytosis (Saiardi *et al.*, 2002), slow cell growth (Saiardi *et al.*, 2000), sensitivity to environmental stresses (Dubois *et al.*, 2002), and abnormal ribosomal functions (Saiardi *et al.*, 2004). Thus, due to the high activity of HopAO1 against InsP₆ (Refer Chapter 3), one could argue that depleted stores of InsP₆ would result in the lack of growth experienced in yeast strains over-expressing HopAO1.

Reduced expression of a 5/6 kinase, which catalyzes the production of InsP₆, results in HEK293 cells that are more prone to TNF- α mediated apoptosis (Verbsky and Majerus, 2005). Cells over-expressing the 5/6 kinase were able to counteract the effects of not only TNF- α mediated apoptosis, but also Fas-induced apoptosis (Verbsky and Majerus, 2005). Experimental evidence suggests that this is a result of not the protein kinase activity of 5/6 kinase, but that of the inositol kinase activity of this enzyme (Verbsky and Majerus, 2005). In contrast, there is some evidence that inositol pyrophosphates may play a role in causing human cells to become more prone to apoptosis (Morrison *et al.*, 2001). Identifying genes involved in interferon β -induced apoptosis, Morrison *et al.* (2001) found that the pyrophosphate containing InsP₇ producing enzyme inositol hexakisphosphate kinase 2 was a possible candidate. A reasonable explanation is that production of InsP₇ causes depletion of InsP₆ stores, which results in sensitization of cells to apoptosis (Majerus and Verbsky, 2005). Thus, it is likely that the balance between InsP₆ and inositol pyrophosphates may act as a molecular switch controlling apoptosis (Majerus and Verbsky, 2005).

Recent studies of *S. cerevisiae* have shown that InsP₆ metabolism is linked to ribosomal biogenesis (Horigome *et al.*, 2009). The protein Rrs1 is essential to the assembly of the 60S

ribosomal subunit (Tsuno *et al.*, 2000, Morita *et al.*, 2002). The mutant *rrs1-1* contains a nonsense mutation at codon 114, and this mutant grows extremely slow at temperatures below 18°C (Horigome *et al.*, 2009). Horigome *et al.* (2009) attempted to gain a greater understanding into the processes involved in ribosomal biogenesis by isolating temperature-sensitive alleles that suppress the cold sensitivity inherent of the *rrs1-1* mutant. Evidence suggested that the mutant *KCS1* gene, which encodes an InsP₆ kinase, suppressed the cold sensitivity of *rrs1-1* (Horigome *et al.*, 2009). *Kcs1* is known to phosphorylate InsP₆ to diphosphoinositol polyphosphates, and it appears that when this protein is non functional, it helps restore the ribosomal biogenesis activity of the *rrs1-1* mutant (Horigome *et al.*, 2009). Thus, it appears that since the *kcs1* mutant is incapable of producing diphosphoinositol polyphosphates, levels of InsP₆ remain higher implicating this molecule in ribosomal biogenesis. Therefore, the activity of HopAO1 against InsP₆ may prevent the yeast cells from properly forming ribosomal subunits leading to cellular death.

A possible explanation for the higher growth rates of *S. cerevisiae* over-expressing phyAsr compared to cells over-expressing hopAO1 is likely a result of the environments in which these two enzymes generally function. HopAO1 contains an optimal *in vitro* activity against InsP₆ at a pH of 7.0, while PhyAsr is an acid-phytase which contains an optimal *in vitro* activity at a pH of 5.0 (Puhl *et al.*, 2007). The relative *in vitro* activity of PhyAsr at pH 7.0 is approximately 12% in relation to the optimal pH of 5.0 (Puhl *et al.*, 2007). The optimal *in vitro* activity is also affected significantly due to temperature. As seen in Chapter 3 of this thesis, HopAO1 contains an optimal activity at standard ambient temperature and maintains similar activity at 30°C. However, the optimal *in vitro* activity of PhyAsr is 55°C which falls in the range of the other previously characterized PTP-like IPPases (Puhl *et al.*, 2009, Puhl *et al.*,

2008a, Puhl *et al.*, 2008b, Puhl *et al.*, 2007). Thus, it is possible that the *in vivo* conditions of yeast growth are not conducive for PhyAsr activity.

Table 4.1. Primers used in this study. Restriction sites are indicated in bold.

Primer Designation	Sequence
HopAO1_HindIII_For	GCA AGC TTA TGA ATC CCC TGC AAC CTA T
HopAO1_HindIII_Rev	GCA AGC TTT CAT TCT AAC GCT ATT TTT G
HopAO1_C378S_HindIIIF	GCA AGC TTA TGA ATC CCC TGC AAC CTA T
HopAO1_C378S_HindIIIR	GCA AGC TTT CAT TCT AAC GCT ATT TTT G
PhyAsr_HindIII_For	GCA AGC TTA AAA TGG CCA AGG CGC C
PhyAsr_HindIII_Rev	GCA AGC TTT TAC GCC TTC GCC GGA TG
PhyAsr_C252S_HindIIIF	GCA AGC TTA AAA TGG CCA AGG CGC C
PhyAsr_C252S_HindIIIR	GCA AGC TTT TAC GCC TTC GCC GGA TG

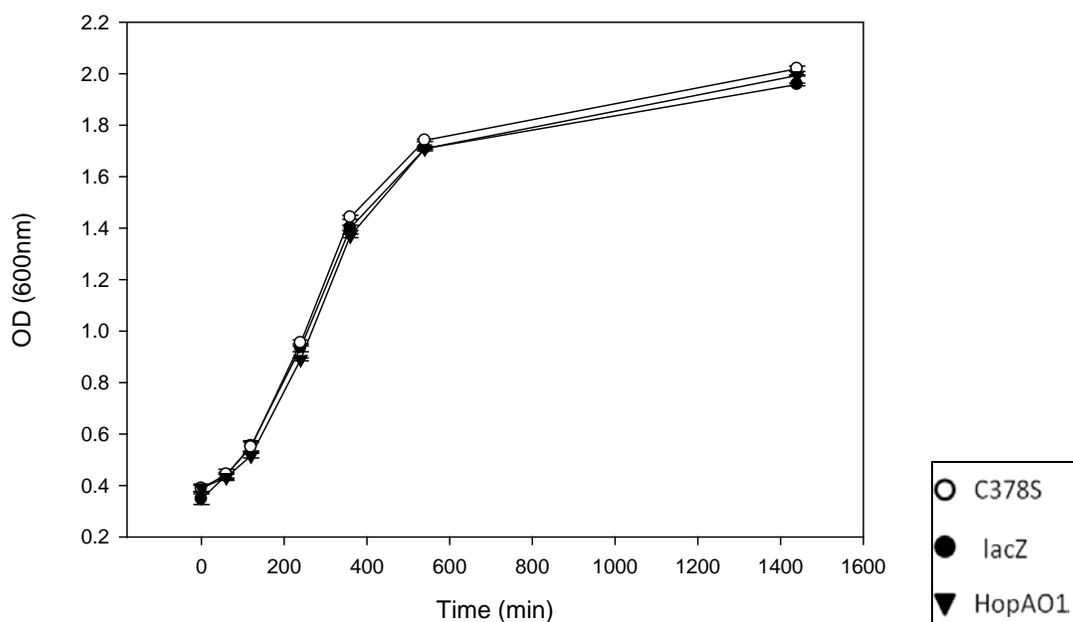


Figure 4.1. A time course for yeast growth under conditions repressing foreign gene expression. Cells were grown in SC-U medium supplemented with 2% glucose. Cells were grown at 30°C and 200 RPM shaking. The data represents mean values with error bars representing standard deviations of three separate trials.

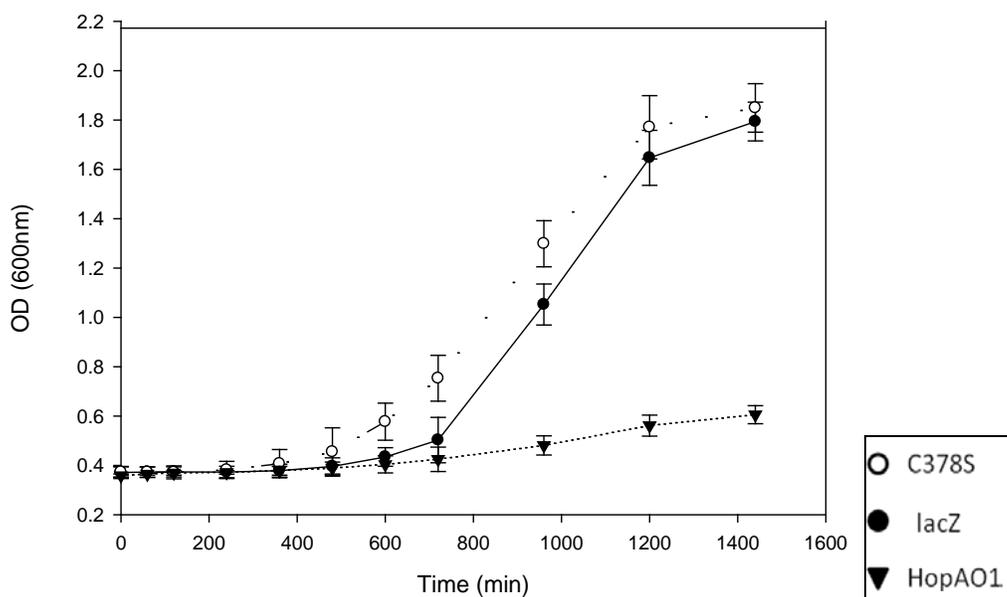


Figure 4.2. A time course for yeast growth under conditions promoting expression of foreign genes. Cells were grown in SC-U medium supplemented with 2% galactose. Cells were grown and induced at 30°C and 200 RPM shaking. The data represents mean values with error bars representing standard deviations of three separate trials.

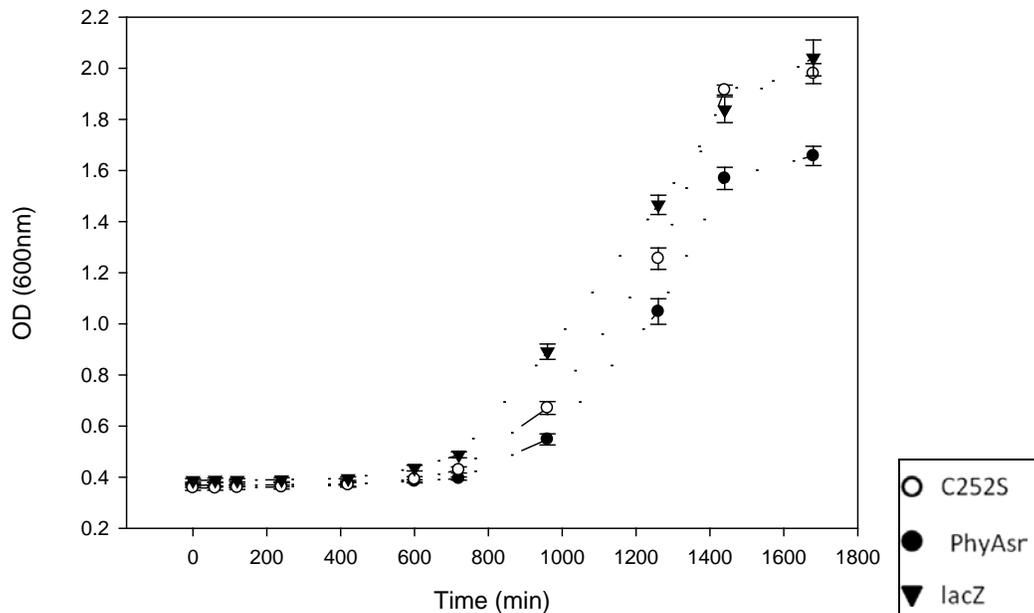


Figure 4.3. A time course for yeast growth under conditions expressing foreign genes. Cells were grown in SC-U medium supplemented with 2% galactose. Cells were grown and induced at 30°C and 200 RPM shaking. The data represents mean values with error bars representing standard deviations of three separate trials.

General Discussion

myo-inositol hexakisphosphate phosphohydrolases, or more commonly known as phytases, are a class of enzymes that contain the capability to remove at least one phosphate group from phytic acid *in vitro* (Haefner *et al.*, 2005). In most cases, the final inositol polyphosphate product is InsP₁ (Casey and Walsh, 2004; Sajidan *et al.*, 2004; Wyss *et al.*, 1999) or InsP₃ (Hara *et al.*, 1985; Kerovuoto *et al.*, 2000b; Quan *et al.*, 2004). Phytases are common in nature and have been discovered in animals, plants, and microorganisms. Phytate degrading enzymes have been reported in calves (McCollum and Hart, 1908), birds, fish, and reptiles (Rapoport *et al.*, 1941), and more recently in maize (Hubel and Beck, 1996), rice (Hayakawa *et al.*, 1989; Maugenest *et al.*, 1999), wheat (Nakano *et al.*, 1999), and soybean (Hamada, 1996). Recently, a new class of phytase (i.e., PTP-like inositol polyphosphatases) has been described that contains an active site common to protein tyrosine phosphatases (PTPs) (Puhl *et al.*, 2009, Puhl *et al.*, 2008a, Puhl *et al.*, 2008b, Puhl *et al.*, 2007) indicating that dephosphorylation occurs via a classical PTP mechanism. The work described in this thesis involves the cloning of genes encoding putative PTP-like inositol polyphosphatases (IPPases) from *Pseudomonas syringae* pv. *tomato* DC3000 and *Bdellovibrio bacteriovorus*, and the subsequent over production, purification, and biochemical characterization of these recombinant proteins.

Prior to this work, HopAO1 was reported as belonging to the PTP superfamily and thus, was believed to be active against tyrosine-phosphorylated peptides. Initial studies were conducted with phosphotyrosine-containing peptide fragments derived from both the insulin receptor and EGF receptor (Espinosa *et al.*, 2003; Bretz *et al.*, 2003). Bretz *et al.* (2003) concluded that *E.coli* extracts containing HopAO1 were capable of hydrolyzing the insulin

receptor substrate at rates two to threefold faster than the control extracts and comparable to the well known PTP leukocyte antigen related protein (LAR) against similar substrates. With this information, Espinosa *et al* (2003) hypothesized that HopAO1 was most likely hydrolyzing a substrate along the MAPK cascade. HopAO1 was co-produced with a constitutively active MAPK kinase known as NtMEK2, which is capable of eliciting a HR when expressed in tobacco (Espinosa *et al.*, 2003). Interestingly, in these cells, the HR was suppressed which led Espinosa *et al* (2003) to conclude that HopAO1 works on a phosphotyrosine-containing polypeptide downstream of NtMEK2. My research determined that recombinant HopAO1 does in fact contain activity against these phosphotyrosine-containing peptides; moreover, it is approximately 70,000 fold more active against InsP₆ than the phosphopeptides. These results suggest that HopAO1 may interfere with host immune responses through its activity against InsP₆ or lower IPPs.

Sequence Analysis

PTP-like IPPases have only recently been described; however, work done in this study has increased the understanding of the biochemistry of these novel proteins. Identification of residues responsible for catalysis is of utmost importance for determining the function and mechanism of action of a particular enzyme. The active site signature sequence of PTP-like IPPases is invariant among the cloned and characterized representatives studied thus far. However, regions flanking the active site are responsible for the largest degree of variability amongst the PTP superfamily of proteins (Andersen *et al.*, 2001; Mauro and Dixon, 1994; Tonks and Neel, 2001). The differences in these regions are likely contributing to the different pH, *I*, and temperature profiles between HopAO1 and the characterized PTP-like IPPases. This may

also explain the different *in vivo* effects on growth observed between over-expressing HopAO1 and PhyAsr in *S. cerevisiae*. Further mutational analysis of the non-catalytic regions may elucidate the functional variability of these novel enzymes and help determine *in vivo* mechanisms.

All previously characterized PTP-like IPPases contain a predicted N-terminal signal peptide (Puhl *et al.*, 2009, Puhl *et al.*, 2008a, Puhl *et al.*, 2008b, Puhl *et al.*, 2007) suggesting that these enzymes are secreted. This is supported by the observation that PhyAmm and PhyAsr are both localized to the outer membrane of *M. multacidus* and *S. ruminantium* respectively (D'Silva *et al.*, 2000). Similarly, PhyAbb from *B. bacteriovorus* contains a predicted signal peptide, and likely secretes the enzyme into extracellular space. In contrast, HopAO1 contains an N-terminal domain which shares sequence similarities to AvrPto another translocated effector protein (Espinosa *et al.*, 2004) which suggests that this domain is likely responsible for recognition from the TTSS apparatus and its release into the cytosol of plant cells.

Biochemical Characteristics

Lack of significant sequence similarity between HopAO1 and the described PTP-like IPPases is likely the contributing factor to the differences associated with the biochemical characteristics associated with these enzymes. The PTP-like IPPases described thus far in the literature displays optimal activity against InsP₆ under acidic conditions (i.e., pH 4.5-5), and their activity sharply declines above pH 6 (Puhl *et al.*, 2009, Puhl *et al.*, 2008a, Puhl *et al.*, 2008b, Puhl *et al.*, 2007). Optimum *in vitro* HopAO1 activity lies in the neutral pH range with little activity below pH of 6. This is consistent with the *in vivo* environment in which HopAO1 functions. The differences associated with the *in vitro* biochemical characteristics amongst these

enzymes are likely an adaptation to their natural *in vivo* biological functions. PhyAbb from *B. bacteriovorus* has an optimal pH of 4 that lies in the range of the previously reported PTP-like IPPases. However, unlike the previously characterized PTP-like IPPases, PhyAbb does not function within the digestive tract of ruminants. The *in vivo* substrate of PhyAbb has yet to be determined. The predatory nature of *B. bacteriovorus* against other Gram negative bacteria and inherent lack of InsPs in prokaryotes leads to interesting questions about the *in vivo* mechanisms associated with this PTP-like IPPase.

Another common characteristic among the enzymes studied is the dependence of *I* on activity *in vitro*. Contributing to this effect are the electrostatic interactions that are likely to occur between the highly electronegative substrate and the positively charged binding pocket associated with PhyAsr (Chu *et al.*, 2004). The optimal *in vitro I* for the previously described PTP-like IPPases is in the range of 100-400mM (Puhl *et al.*, 2009, Puhl *et al.*, 2008a, Puhl *et al.*, 2008b, Puhl *et al.*, 2007). The optimal *I* for HopAO1 is 175mM placing it in the range of the previously described PTP-like IPPases. Interestingly, despite greater sequence similarities between PhyAbb and the previously described PTP-like IPPases, PhyAbb contains a much lower optimal *I* indicating that these enzymes rely on electrostatic interactions to varying degrees.

InsP₆ Hydrolysis Pathway

Perhaps the most interesting aspect of the hydrolysis pathway of HopAO1 against InsP₆ is the ability to dephosphorylate the 2-axial position. This has yet to be observed in PTP-like IPPases and in all cases the end product of hydrolysis has been shown to be Ins(2)P₁ (Puhl *et al.*, 2009, Puhl *et al.*, 2008a, Puhl *et al.*, 2008b, Puhl *et al.*, 2007). Therefore it is reasonable to assume that the virulence factor associated with HopAO1 is possibly due to this 2-axial

phosphate hydrolysis and may explain the differences associated with cellular growth upon over production of HopAO1 and PhyAsr in *S. cerevesiae* (Refer to Chapter 4). Also, all of the previously described PTP-like IPPases appear to hydrolyze InsP₆ down to a mono-phosphorylated InsP₁ (Puhl *et al.*, 2009, Puhl *et al.*, 2008a, Puhl *et al.*, 2008b, Puhl *et al.*, 2007). Whereas HopAO1 completely hydrolyzes InsP₆ in 15 minutes, however the end product is Ins(1,3,4)P₃ (Refer to Chapter 3). It is possible that the variable regions amongst the PTP-like IPPases are responsible for the specificity for certain phosphate groups. Further mutational studies may help elucidate the function of these regions.

Future Directions

The tight regulation of signaling cascades occurring within eukaryotic cells is largely a result of phosphorylation and dephosphorylation events. The MAPK cascade is an intricate mechanism used by eukaryotes to establish an early on-set defense response to pathogens. In order to understand finer details of the MAPK cascade, we must be able to decode the mechanisms involved in the phosphorylation cascade. This includes determining the phosphorylated/dephosphorylated substrates involved. Naturally, it is much easier to determine the substrate of a kinase than that of a phosphatase. Kinases act directly on their targets resulting in the addition of a phosphate group. Thus, using techniques such as radioactive labeling or chemiluminescence will point directly to the substrate involved in the phosphorylation reaction (Blanchetot *et al.*, 2005). However, in the case of phosphatases, one requires the ability to detect the removal of a phosphate from the substrate in question and subsequent detection of the substrate. With wild-type enzymes, this proves very difficult due to the speed at which catalysis occurs, and also due to the hydrolytic promiscuity of many PTPs (Blanchetot *et al.*, 2005).

In the case of PTPs and PTP-like IPPases, a PTP signature sequence exists which contains a cysteine residue that is responsible for generating a phospho-cysteine intermediate, and an upstream aspartic acid residue (located in the conserved general acid loop) leads to activation of a water molecule for subsequent hydrolysis. Analyses in which these essential residues are mutated generally results in a significant decrease in phosphatase activity (Puhl *et al.*, 2009, Puhl *et al.*, 2008a, Puhl *et al.*, 2008b, Puhl *et al.*, 2007, refer Chapters 2 and 3). Due to the blockage of catalysis the enzyme can form a complex with the substrate and this enzyme-substrate complex in many cases is stable enough to further purify (Blanchetot *et al.*, 2005). Other parameters that contribute to the trapping experiment include little if any activity towards the substrate (low k_{cat}), a strong affinity for the physiological substrate (small K_m), and the ability to retain as much structural integrity as possible (Blanchetot *et al.*, 2005). Fortunately in our case HopAO1(C378S) and HopAO1(D348N) contain no measurable activity towards the hypothesized substrate and as mentioned the wild-type enzyme contains the smallest K_m amongst the other PTP-like IPPases characterized to date.

Since HopAO1 appears to effect the growth of *S. cerevisiae* (Munkvold *et al.*, 2008), it is assumed that HopAO1 is affecting the same process in yeast as it is in host plants. Due to the much shorter generation times for yeast, and the lack of effect on yeast cell growth by the HopAO1(C378S) mutant, one should be able to trap the substrate *in vivo*. Thus, assuming this HopAO1 mutant irreversibly binds its physiological substrate, and taking advantage of the multi His tag located on the C-terminus of the recombinant enzyme, purification of the HopAO1(C378S)-substrate complex can be performed on Ni-NTA resin. Scaling up this process should generate enough binding to elucidate the *in vivo* substrate by mass spectrometry (Blanchetot *et al.*, 2005). This should answer the question whether HopAO1 is functioning on a

protein substrate, an inositol polyphosphate/ derivative of inositol polyphosphate, or a phosphorylated compound different from the aforementioned.

The PTP-like IPPases characterized in this study furthers our understanding of this unique class of enzyme; however, it also extends the application perspective of phytases. Conventionally, phytases have been used to hydrolyze InsP_6 as a means of reducing phosphate pollution, increasing the nutritional availability of free phosphorus, and reducing the nutrient mineral loss due to chelation of this highly electronegative molecule (Wodzinski and Ullah, 1996). Another possible application for this new class of enzyme is the generation of specific IPPs. InsP_6 hydrolysis pathway has shown that HopAO1 hydrolyzes the axial 2-phosphate position first making it novel amongst the PTP-like IPPases. By combining the activities of IPPases and IPP kinases, the production of many different derivatives of IPPs can be generated which may prove beneficial for pharmaceuticals and reduce the costs of expensive synthetic IPPs.

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