PROTEIN ENGINEERING AND FUNCTIONAL GENOMICS APPROACHES TO ALLEVIATE P AND N POLLUTION AS A RESULT OF ANIMAL AGRICULTURE

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PROTEIN ENGINEERING AND FUNCTIONAL GENOMICS APPROACHES TO ALLEVIATE P AND N POLLUTION AS A RESULT OF ANIMAL AGRICULTURE

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Animal agriculture may be a significant source of P and N pollution. In order to maximize animal growth, diets are often supplemented with inorganic P, depleting a nonrenewable resource. Furthermore, towards the same end, animal diets are often supplemented with a rich source of protein, generally soybean meal. This competes with human consumption of soy. The biotechnological application of phytase to supplement animal diets reduces manure P pollution and can completely replace the use of nonrenewable inorganic P. We attempted to increase the stability of *Escherichia coli* AppA2 phytase through *de novo* disulfide bond engineering to withstand the heat of feed pelleting. Though unsuccessful in our goal, we discovered disulfide bonds affect protein kinetics and other characteristics through not only modulation of stability, but also atom motility and position. We also explored potential additional benefits of phytase. Knowing AppA2 to possess a nucleotide binding site, we explored whether phytase could degrade DNA. Our studies failed to indicate substantial nuclease activity of phytase. Finally, towards the goal of alleviating animal agriculture N pollution in the form of poultry feathers and competition for human food sources, we studied the degradation of feather keratin by *Streptomyces fradiae* k11. By combining a functional genomics approach of draft genome sequencing and shotgun proteomics, comparative microbiological studies and mechanism driven experiments, we laid a new groundwork for continued research aimed at converting feather waste into a valuable protein resource.

BIOGRAPHICAL SKETCH

Jeremy D. Weaver attended high school at Lockport High School, in Lockport, NY. Immediately following, he enrolled as an undergraduate General Biology student in Cornell University College of Agriculture and Life Sciences. After his first year, he changed his major to Animal Science. He graduated with his Bachelors of Science degree in Animal Science in May, 2003. After graduation, he remained in the Animal Science Department at Cornell University as an employee of Dr. Dan Brown, with whom he had been conducting research in toxicology as part of his Federal Work Study program as a senior undergraduate student. During this time he decided to pursue graduate studies on phytase and protein engineering with Dr. Xingen Lei, also in the Department of Animal Science at Cornell University. In the summer of 2004, he was wed to Heeyoung Shim of Seoul, South Korea. Jeremy began his Masters of Science program with Dr. Lei in August, 2004. A short while later, on March 19, 2005, Jeremy and Heeyoung's son, Benjamin Jaeyoung Weaver, was born. Jeremy completed his Masters of Science degree in May, 2007 under the mentorship of Dr. Lei. On October 12, 2007, Jeremy and Heeyoung's daughter, Chloe Jina Weaver, was born.

I dedicate this dissertation to my truly amazing wife, Heeyoung,

and

to my nephew, Daniel, and sister-in-law, Heesuk, whom went to be with our Lord on August 26 and August 31, 2010

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LIST OF ABBREVIATIONS

2D-LC MS/MS	Two Dimensional Liquid Chromatography tandem Mass Spectrometry Mass Spectrometry
ATCC	American Type Culture Collection
BLASTP	Protein Basic Local Alignment Search Tool
Bp	Basepair
CDART	Conserved Domain Architecture Retrieval Tool
CDD	Conserved Domain Database
DLS	Dynamic Light Scattering
E	Glutamic Acid
EDTA	Ethylenediaminetetraacetic Acid
HAP	Histidine Acid Phosphatase
K	Lysine
Kb	Kilobases
Mb	Megabases
N	Nitrogen
P	Phosphorus
PAP	Purple Acid Phosphatase
PEG	Protein Encoding Gene
pNPP	Para-nitrophenylphosphate
Q	Glutamine
RAST	Rapid Annotation using Subsystem Technology
SCOP	Structural Classification of Proteins

SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
TEAB	Triethylammonium Bicarbonate
TMHMM	Transmembrane Hidden Markov Model
TMT	Tandem Mass Tags
VAST	Vector Alignment Search Tool
WT	Wild-type

CHAPTER ONE

INTRODUCTION

1.1 Animal Agriculture Contributes to Pollution

Intensive animal agriculture in confined areas leads to excessive manure phosphorus (P) and nitrogen (N) pollution of the surrounding soils and waterways. This leads to eutrophication resulting in hypoxia and decreased sunlight, leading to decreased aquatic animal numbers and environmental degradation (36). The US animal manure production is estimated to be 500 million tons manure/year (36), which can have a large impact on P and N pollution.

For example, animal manure on pasture and rangelands contributes 37% of the P pollution in the Gulf of Mexico (Fig. 1.1) (3), second only to crop cultivation, 27% of the P pollution in the Chesapeake Bay (25), and 29,000 metric tons of P annually in the North Carolina Coastal Plain (99). Likewise, animal agriculture contributes 5% of the N pollution in the Gulf of Mexico (3), 18% of the N pollution in the Chesapeake Bay (25), and 124,000 tons of N annually in the North Carolina Coastal Plain (99). Animal agriculture may contribute up to 94% and 72% of P and N water pollution in certain areas of the world (Table 1.1) (149).

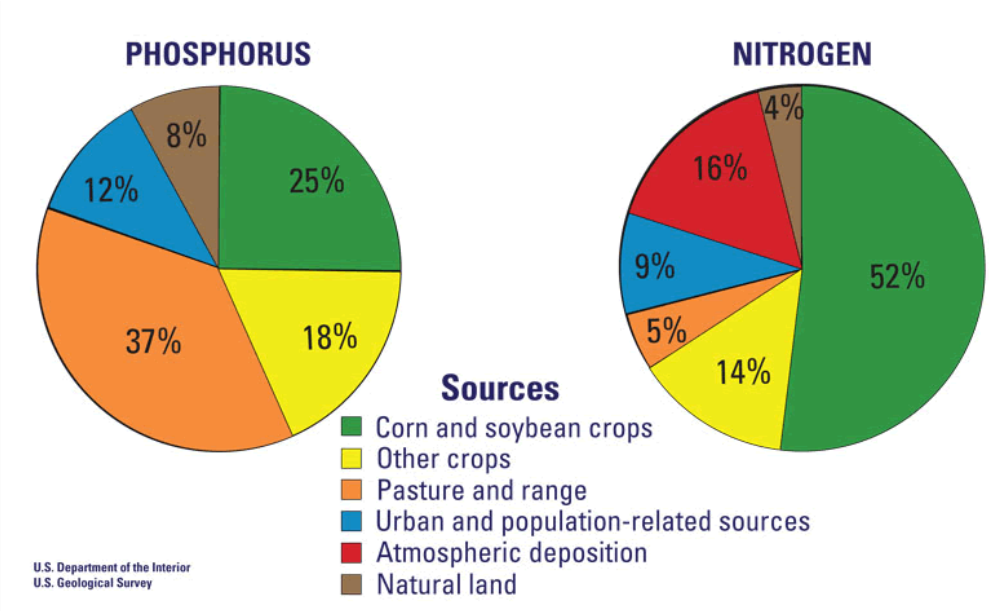


Figure 1.1 Source of P and N contamination in the Gulf of Mexico (3).

(http://water.usgs.gov/nawqa/sparrow/gulf_findings/primary_sources.html)

Table 1.1 Water P and N contamination worldwide (149).

Contaminant	Nation	% of water load
P	Thailand (pig waste)	61
	Vietnam (pig waste)	92
	China-Guangdong (pig waste)	94
	United States	32
N	Thailand (pig waste)	14
	Vietnam (pig waste)	38
	China-Guangdong (pig waste)	72
	United States	33

1.2 Animal Agriculture is Depleting Rock Phosphate Stores

Inorganic or rock phosphate, like dicalcium phosphate, is supplemented in animal diets to obtain maximal growth and feed efficiencies (1). The continued use of this nonrenewable resource poses a "potential phosphate crisis," and alternatives need to be researched (1).

1.3 Animal Agriculture Competes for Human Food Sources

In intensive animal agriculture, animal diets are often supplemented with rich sources of protein to improve animal growth and profitability. This is particularly true for poultry and swine diets, which are composted almost entirely of corn and protein-rich soybean meal. As these are also staple foods for human consumption, this creates competition between their use for direct human consumption and animal agriculture. The Food and Agriculture Organization estimates the global population of swine at 1 billion, and of poultry at 40 billion (149). Alongside, there is an estimated 2 billion sheep & goats and 1 billion cattle (149).

1.4 Phytases and Keratinases Alleviate These Problems

Biotechnological use of phytases added to animal diets is alleviating intensive animal agriculture P pollution and the heavy use of inorganic rock phosphate (41, 52, 70, 83). As most poultry and swine diets are pellleted for feeding ease, thermostable phytases which will endure the heat of the pelleting process (86).

Augmentation of animal diets with proteases may help alleviate N pollution from animal agriculture, but little exploration of potential proteases has been conducted (115, 116, 135). Protein sources for animal feed use which are not used for human consumption may help alleviate the competition for soybean meal between human consumption and animal production. One such source, feather waste from poultry production, is largely unused due to its poor digestibility (95, 116).

1.5 Research Aims

Towards these ends, we aimed to increase the thermalstability of phytase to resist heat denaturation by feed pelleting for use as an animal feed additive. We attempted this through the creation of *de novo* disulfide bond mutants of phytase via site-directed mutagenesis. We also explored other uses of phytases, particularly for the degradation of DNA. Finally, we hoped to isolate new enzymes of potential biotechnological application as animal feed additives for reducing waste N pollution and for the utilization of feather protein in animal diets. We used a functional genomics approach to study feather degradation by *Streptomyces fradiae* variant k11.

CHAPTER TWO

PHYTIC ACID AND PHYTASE

2.1 Phytic Acid Negatively Affects Animal Nutrition

Phytate (Fig. 2.1), the salt of phytic acid (*myo*-inositol 1,2,3,4,5,6-hexakis dihydrogen phosphate), is a natural phosphorus and energy storage compound in plants (133). Most seeds of cereals, legumes and oilseed crops, the primary feed source of simple-stomached animals, contain 1-2% phytic acid, representing 60-80% of their total phosphorus (133). During germination, the seedling relies on these stores for growth and development. However, these energy and phosphorus stores are largely unavailable to simple stomached animals, such as swine, poultry, fish and humans (24). In fact, phytates are antinutritive factors, due to their ability to bind positively charged divalent cations such as Ca^{2+}, Zn^{2+}, Fe^{2+} and Mg^{2+} (9).

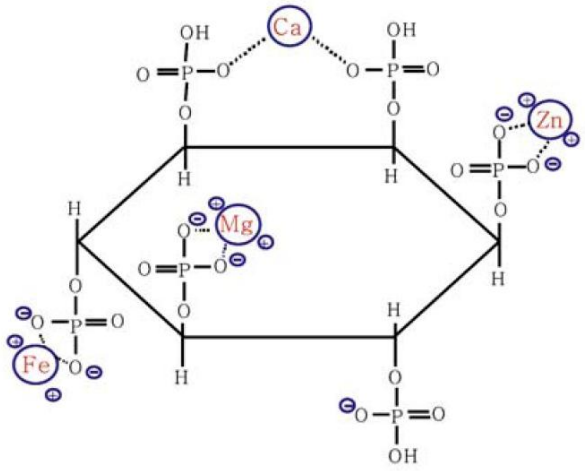


Figure 2.1 The structure of phytate showing bound divalent cations (118).

Phytate is also antinutritive due to its reduction in protein availability during digestion (141). This may be due to several factors, such as protein-phytate complexes naturally occurring in feed ingredients, the formation of protein aggregates around phytate, a negative impact of phytate on digestive enzymes, and an increase in endogenous amino acid losses (141). In order to reach optimal growth conditions for production, diets are usually supplemented with inorganic phosphorus. This practice causes excessive phosphorus in the waste, due to the undigested phytate. In areas of intensive agriculture, this can lead to algal blooms and eutrophication of water ways, damaging natural ecosystems.

2.2 Potential Solutions

Several approaches are possible to address the antinutritive effect of phytic acid in animal diets. One is through plant breeding to select for low-phytate plant lines. However, this would not eliminate the antinutritive effects of phytate for simple-stomached animals, and it would likely reduce the fitness of the plant; especially during germination when the plant is utilizing the phytate stores. Another approach is to decrease the phytic acid content of feed ingredients through chemical means (121). However, this would likely be expensive, time consuming and deleterious to the nutritional quality of the feed.

Phytases are phosphohydrolases that catalyze the breakdown of phytic acid into *myo*-inositol and free phosphate (87). Phytases can be found in plants, microbes, and animals (121). Phytases added to the feed make the phosphorus and bound divalent cations in the digestive tract available to the animal, thus replacing the need for inorganic phosphorus supplementation (87). It has been proposed to inoculate the digestive tract of production animals with phytase producing microbes. However, this

may lead to a worsening of the eutrophication problem, if phytase producing bacteria pass into the environment through the feces where they can hydrolyze phytate to release phosphorus on which the algae can thrive. Also proposed was the expression of phytase by genetically modified production animals. This was accomplished with swine (43). This approach, however, will likely face great resistance from the consumers, who will baulk at the idea of eating pork from a genetically modified pig. Of greatest interest is the addition of overexpressed microbial phytases to the diet of production animals. In this case, the phytase will be active in the digestive tract of the animal, mainly in the stomach, but will not likely pass into the feces in active form (177).

2.3 Phytase Supplementation Alleviates Phosphorus and Phytic Acid Problems

The use of phytases is the most promising solution to the antinutritive effects of phytate. The phytase will be active in the digestive tract of the animal, mainly in the stomach, but will not likely pass into the feces in active form (Yi and Kornegay 1996). Phytase supplementation will make available the phytic acid-P, entirely replacing the need for rock phosphate supplementation and alleviating the potential phosphate crisis (10), reduce manure P content 50% (85), and increase the availability of divalent cations , improving animal nutrition (84).

2.4 Phosphatases and Phytases

Traditionally, phosphatases have been classified into three classes: Protein phosphatases, acid phosphatases and alkaline phosphatases (164) (Fig. 2.2 and 2.3). As this scheme is slightly outdated, I have included a more current classification based

on structure in the Appendix. Phytases exist in each of these three traditional phosphatase classes, and so can initially be classified accordingly. Phytases in the acid phosphatases can further be classified into two additional groups based on structure, the histidine acid phosphatases (HAP) and the purple acid phosphatases (PAP).

2.4.2 Protein phosphatases

The protein phosphatases are traditionally composed of serine and threonine protein phosphatases. These phosphatases are metalloenzymes (12). For the purposes of this dissertation, I have included in this class the dual specificity phosphatase-like (DSP) phytases, such as that from *Selenomonas ruminantium* (27) (Fig. 2.2). Although this phytase does not share structural homology and the metal cofactor requirement of the serine and threonine protein phosphatases, it is a phosphotyrosine protein phosphatase (The serine and threonine protein phosphatases belong to the fold Another Three Helical Bundle in the class All Alpha Proteins of SCOP, the Structural Classification of Proteins (110), while the phosphotyrosine protein phosphatases make up two folds in the class Alpha and Beta Proteins (a/b); see the Appendix). The DSP phytases also could be grouped similar to the low molecular weight acid phosphatases of the acid phosphatases in the traditional classification, as these are also phosphotyrosine protein phosphatases. However, the DSP phytases do not share the characteristics of the low molecular weight acid phosphatases either (see *2.3.4 Acid phosphatases: Low molecular weight acid phosphatases*), so I have grouped them arbitrarily with the protein phosphatases in the traditional classification. The DSP phytases are also referred to as PTP phytases, or phosphotyrosine phosphatase phytases. The pH

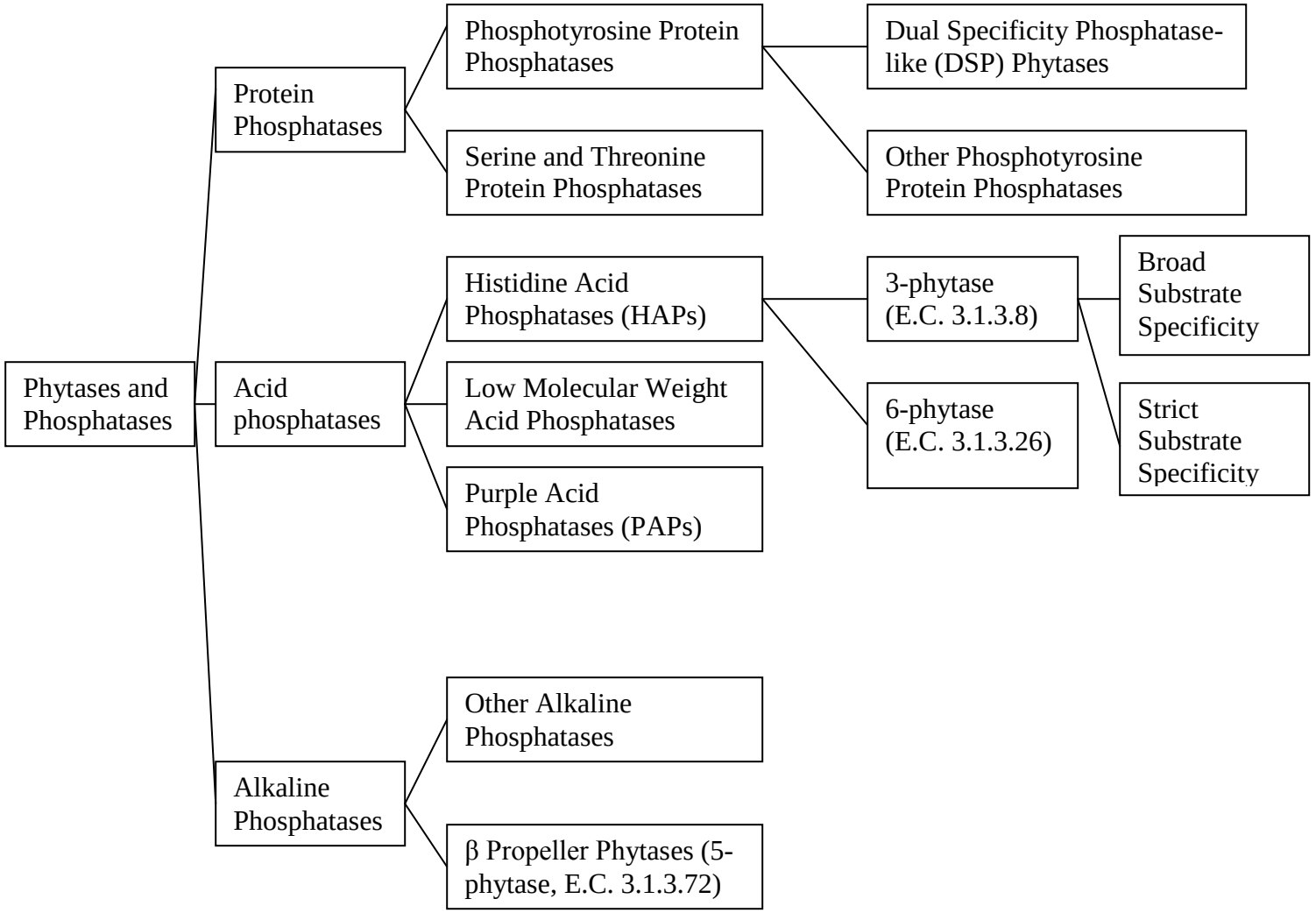
optimum of *S. ruminantium* phytase is 4 – 5.5 (175), and it has a calculated molecular weight of 37kDa (167).

2.4.3 Acid phosphatases: HAP

Acid phosphatases can be classified into three groups: the high molecular weight acid phosphatases, low molecular weight acid phosphatases and the purple acid phosphatases. The high molecular weight acid phosphatases generally have a molecular weight of approximately 40 – 60 kDa. This group is also known as the histidine acid phophatases (HAP, Fig. 2.2). The HAP are composed of one α/β domain and one α domain. The HAP are characterized by a conserved RHGXRXP motif and a conserved HD motif in the catalytic site, as well as a two-step mechanism (109, 158, 163). Non-phytase examples of the HAP are the *Escherichia coli* periplasmic acid phosphatase, and the human prostatic and lysosomal acid phosphatases (143). The HAP subclass of phytases are the most well studied and largest group of phytases. Reflecting this, the International Union of Pure and Applied Chemistry and the International Union of Biochemistry (IUPAC-IUB) has assigned two Enzyme Committee (EC) numbers to the subclasses of the HAP, the 3-phytases (E.C. 3.1.3.8) and 6-phytases (E.C. 3.1.3.26).

The 3-phytases, also called 1-phytases, initially hydrolyze the C_1 or C_3 carbon of phytic acid (*myo*-inositol-1,2,3,4,5,6-hexakis dihydrogen phosphate). These phytases are usually of microbial origin. The 3-phytases can be further subdivided into two classes of enzymes, those with a strict substrate specificity for phytate, such as *Aspergillus niger* PhyA, and those with a broad substrate specificity, such as *A. niger* PhyB. PhyB's substrates include para-nitrophenylphosphate (pNPP), fructose 1,6-bisphosphate, and glucose 6-phosphate, which are hydrolyzed ten times faster than

Figure 2.2 Classification of phosphatases and phytases. Phosphatases have traditionally been classified into three groups, the protein phosphatases, acid phosphatases and alkaline phosphatases. Phytases exist in all three groups, with the acid phosphatases having two groups of phytases within it, the histidine acid phosphatases/phytases and the purple acid phosphatases. This simple scheme of three classes of phosphatases may now be outdated, as some phosphatases, such as the recently identified β propeller phytases (62, 69), do not fit the traditional requirements of this classification. For the purposes of this dissertation, I have listed the β propeller phytases as a subclass of the alkaline phosphatases due to their near neutral to slightly alkaline pH optimums, and the dual specificity phosphatase-like phytases as part of the protein phosphatases. The β propeller phytases are metalloenzymes, like other alkaline phosphatases, but do not share similar protein folds, catalytic mechanisms, nor other characteristics of alkaline phosphatases. The dual specificity phosphatase-like phytases are phosphotyrosine protein phosphatases, whereas the protein phosphatases were traditionally considered to be serine and threonine protein phosphatases.


Phytases and Phosphatases
Protein Phosphatases
Acid phosphatases
Alkaline Phosphatases
Phosphotyrosine Protein Phosphatases
Serine and Threonine Protein Phosphatases
Histidine Acid Phosphatases (HAPs)
Low Molecular Weight Acid Phosphatases
Purple Acid Phosphatases (PAPs)
Other Alkaline Phosphatases
β Propeller Phytases (5-phytase, E.C. 3.1.3.72)
Dual Specificity Phosphatase-like (DSP) Phytases
Other Phosphotyrosine Protein Phosphatases
3-phytase (E.C. 3.1.3.8)
6-phytase (E.C. 3.1.3.26)
Broad Substrate Specificity
Strict Substrate Specificity

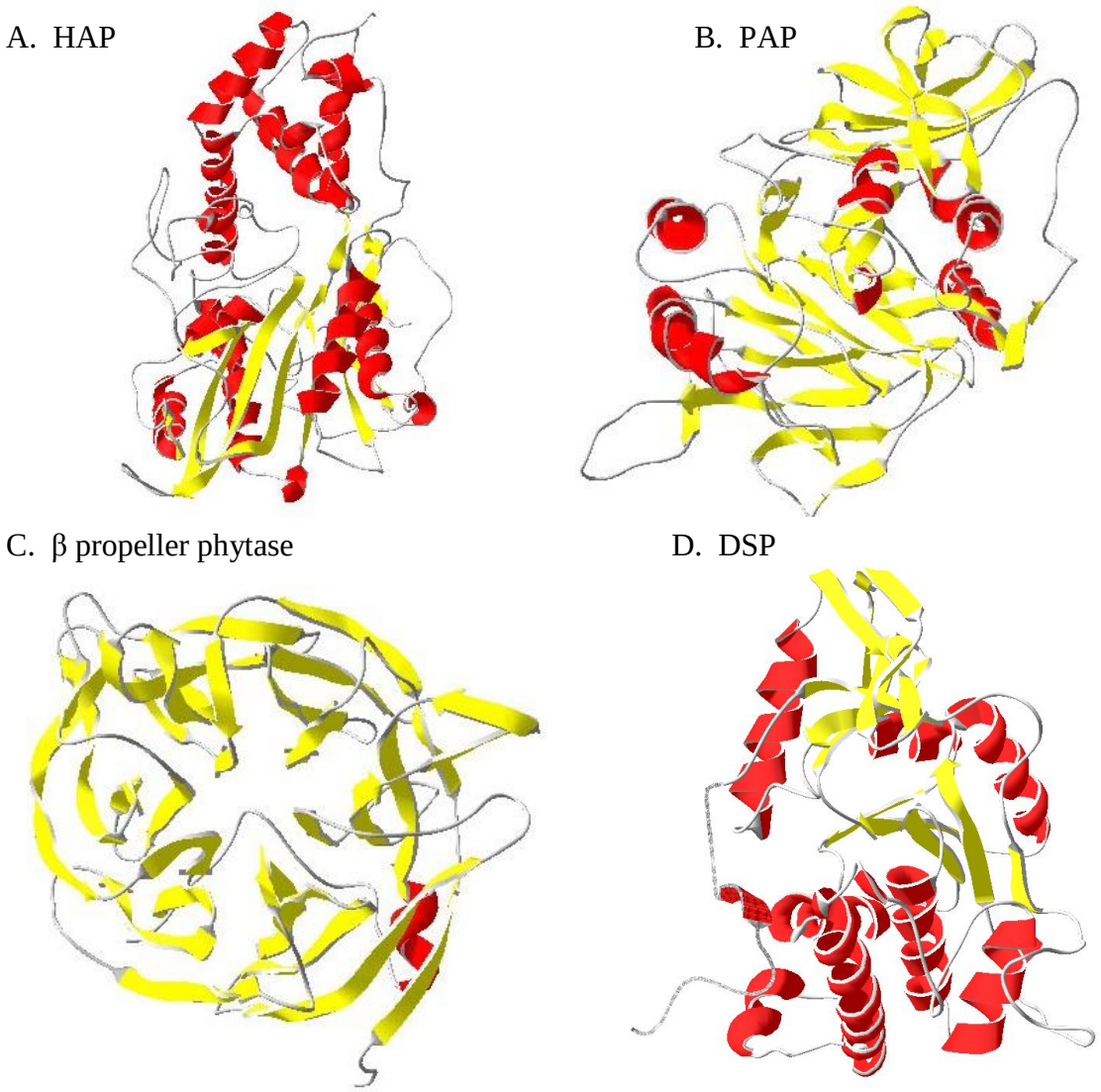


Figure 2.3 Representative structures of the four major types of phytases. Structures are shown as ribbon diagrams with α-helices in red and β-sheets in yellow. (A) HAP (histidine acid phosphatase) is a monomer of *Aspergillus niger* PhyB, (B) PAP (purple acid phosphatase) is a monomer of kidney bean purple acid phosphatase, (C) β propeller phytase is a *Bacillus amyloliquefaciens* phytase, and (D) DSP (dual specificity phosphatase) is a *Selenomonas ruminantium* phytase. All images were prepared using Deep View (Swiss pdb Viewer) (Guex and Peitsch 1997). PDB files used were 1QFX, 1KBP, 1POO and 1U26, respectively.

phytate. PhyA also has activity on these substrates, but to a much lesser extent, having the hightest activity on phytate. The other subclass of HAP, the 6-phytases, are also called 4-phytases. They initially hydrolyze the C_6 carbon of phytate. These phytases are usually of plant or gram-negative bacterial origin. Examples of the 6-phytases are maize phytase (102) and *E. coli* AppA (44) and AppA2 (138).

2.4.4 Acid phosphatases: Low molecular weight acid phosphatases

The second group of acid phosphatases is the low molecular weight acid phosphatases. These phosphatases dephosphorylate the phosphotyrosyl group of proteins, and generally have a low molecular weight of approximately 18 kDa (Shin et al. 2001). This group does not contain any phytases. Although DSP phytases are also phosphotyrosine protein phosphatases, they are do not share structural homology with the low molecular weight acid phosphatases. The DSP phytases are also approximately twice as large as the low molecular weight acid phosphatases. For the purposes of this dissertation, I have arbitrarily classified the DSP phytases with the protein phosphatases. For a more updated classification scheme based on structure, see the Appendix.

2.4.5 Acid phosphatases: PAP

The third and final group of acid phosphatases is the purple acid phosphatases (109), otherwise known as "tartrate-resistant" acid phosphatases, which fall under E.C. 3.1.3.2. These phosphatases are homodimeric metalloenzymes with dinuclear Fe-Fe or Fe-Zn active centers (Fig. 2.3). Non-phytase examples of this class of phosphatases are Bovine Spleen PAP and Pig PAP, both of which have an active center containing

two Fe(II) atoms. The phytase subclass of the PAP includes plant phytases, such as the Kidney Bean PAP and soybean PAP (GmPhy), with active containing an Fe(III) atom and a Zn(II) atom (54). Also belonging to this group are fungal phytases, like that of *A. niger* pH 6.0 optimum acid phosphatase (108, 160).

2.4.6 Alkaline phosphatases

Alkaline phosphatases traditionally are dimeric proteins with approximately 60 kDa subunits, of which each subunit contains two Zn^{2+} ions and one Mg^{2+} ion in the active center (143). A typical representative of this group is the *E. coli* alkaline phosphatase (28, 29). For the purposes of this dissertation, I will classify a newly identified (1998) group of phytases, the β propeller phytases (62, 69), as alkaline phosphatases, as they do not fall under any group of phosphatases thus described. This group of phytases has near neutral or slightly alkaline pH optimums generally of 7.0 – 8.0 (118)and are metalloenzymes, similar to other alkaline phosphatases. However, the β propeller phytases differ in their active center, catalytic mechanism, and overall protein fold from other alkaline phosphatases.

The β propeller phytases are also known as 5-phytases (E.C. 3.1.3.72) and as alkaline or neutral phosphatases. They have a strict substrate specificity for phytate, even greater so than that of the strict substrate specificity class of 3-phytases. The β propeller phytases have less than or equal to 6% activity for other substrates as compared with their activity for phytate (118). In fact, for most other substrates, they have no activity. The β propeller phytases generally have a high melting temperature (T_m, the temperature at which the protein unfolds) as compared with other phytases (48, 69). Examples of these phytases are PhyC from the gram positive bacteria

Bacillus subtilis and some phytases from plants, such as that from *Lilium longiflorum* (140).

β propeller phytases are characterized by a six-bladed scaffold composed of 6 β sheets (Fig. 2.3). In the active form, these phytases contain 6 calcium ions, which are critical for thermostability and catalysis (143). These phytases possess two phosphate binding sites, the cleavage site and the affinity site (49, 61-63). The affinity site holds one phosphate group from phytate, while the cleavage site removes the phosphate group from the neighboring carbon of phytate. This results in the hydrolysis of alternate phosphate groups to produce a final product of *myo*-inositol 1,3,5 or 2,4,6-triphosphate, with only three of the phosphate groups liberated (143). The β propeller phytases, like the 3-phytases, initiate hydrolysis at the C_3 carbon of phytate (63). Unlike the HAP and PAP, β propeller phytases are highly specific for the calcium-phytate complex (117), which forms at alkaline pHs. The HAP and PAP catalyze the hydrolysis of Na-phytate which is prevalent in acidic pHs.

2.5 Phytase Protein Engineering and Research Challenges

One of the big frontiers in phytase research is the identification or engineering of new phytases. Currently, several wild type (WT) enzymes are on the market, such as *A. niger* PhyA, *E. coli* AppA2 and *Peniophora lycii* phytase. Current and future research looks to identify new phytases which may have better characteristics than the currently marketed ones for the desired applications, or to engineer existing phytases to incorporate desired characteristics.

Phytases for the feed industry ideally would possess numerous characteristics: they would perform in the pH of the digestive tract, whether for swine, poultry, or another species; they would high catalytic activities since they are only in the site of

activity, generally the stomach, for short periods of time; be resistant to proteolytic enzymes such as pepsin found in the gastric environment; and be able to withstand heat up to 80°C with steam produced by the feed pelleting process so that the enzyme can be incorporated into easily handled feed pellets (87). A phytase that can withstand long term storage and variable transport temperatures and conditions is also desired.

Several different enzymes are likely to be needed for different applications. For example, phytases with different optimal pHs may be specific for use in poultry where the crop pH is around 6.5 versus the acidic swine stomach pH. Also different enzymes may be used for aquatic animals that have lower body temperatures than swine or poultry, in which case enzymes with lower optimal temperature would be desired. Enzymes are now being considered for other applications as well, such as use for increasing the bioavailability of soil phytate phosphorus for plants.

Many new phytases are being discovered with improved characteristics, such as high catalytic activity (55, 64, 68). New phytases of higher thermostability are also being identified (26, 184).

Another approach to obtain phytases with desirable characteristics is protein engineering of already identified phytases. Several attempts have been successful. Diversa Corporation succeeded in increasing the thermal stability of *E. coli* AppA (40) as did Roche Vitamins (81, 82). Roche Vitamins was also successful in engineering increased resistance to proteolytic degradation (170). The pH profiles of some phytases have also been modified (67, 157). Through engineering, phytases, such as *A. niger* PhyB, which have some desirable characteristics but not others, can be modified to further improve their usefulness. PhyB, for example, is relatively thermostable compared to other HAP, has a high catalytic activity, and is able to remove all six phosphate groups from the substrate phytate, unlike most HAP that can only remove five. These characteristics all made PhyB useful in industrial application (169-171).

However, PhyB's pH profile peaks at 2.5 and is very narrow. It quickly loses activity by pH 3.5. This narrow pH profile and its lack of proteolytic resistance limit its use for animal agricultural purposes. First, engineering of PhyB to produce a broader pH profile, and second possibly increasing proteolytic resistance, would make this quite an attractive enzyme for agriculture.

2.6 Thermostable Phytase Needed

Simple-stomached animal feeds are often pelleted for ease of handling, and can reach temperatures of 80°C for 10-15 minutes (87). At this temperature, current phytases are denatured, preventing their widespread use in the US.

2.6 Methods Used to Increase Phytase Stability

Previous attempts have been made to increase the stability of phytases to resist the heat of feed pelleting. Several cases have met with varied levels of success, employed different approaches and worked with different phytases. Random mutagenesis followed by screening was performed on *E. coli* AppA through gene site saturation mutagenesis (40), and on *E. coli* AppA2 through error-prone PCR (65) followed by assembly of mutations (66). Some rational approaches have also been successful, such as rational substitutions in *A. niger* PhyA based on structural comparison to the homologous but more stable *A. fumigatus* Afp phytase (183). The consensus concept of phytase engineering has worked very well (80-82)(80). Finally, accidental discoveries of improved thermostability have been made while designing mutations aimed at other purposes (139).

2.7 Disulfide Bonds Can Stabilize Proteins

Disulfide bonds are generally believed to stabilize proteins by reducing the entropy of unfolding. The role of structural disulfide bonds in protein stability has been extensively studied, including their natural conformations (147) and bond characteristics (129, 130). Protein engineers have long been attempting to increase protein stability by engineering of de novo disulfide bridges . Although attempts have had mixed results, many have been successful (47, 59, 100, 111, 122, 162). In extreme cases proteins have been hyperstabilized by DB engineering, such as with T4 lysozyme, (101) and thermolysin-like protease (100). Several programs have been created to assist in choice of sites for engineered DB to enhance stability (30, 32, 124, 146).

2.8 Aims

In order to address the problem of heat denaturation of phytase during feed pelleting, thereby enhancing the effectiveness and use of phytase as a feed supplement, we aimed to engineer disulfide bonds into the *E. coli* AppA2 phytase (138).

CHAPTER THREE

IMPACT OF DISULFIDE BRIDGE DELETION OR ADDITION IN *ESCHERICHIA COLI* PHYTASE

3.1 Summary

De novo disulfide bridges have been documented to increase the thermostability of proteins, but little attention has been devoted to their effect on catalytic kinetics. Aiming to increase the stability of *Escherichia coli* AppA2 phytase, we first created four single DB deletion mutants to assess their impacts, then created nine *de novo* DB mutants. However, only Q134C/A202C marginally increased the stability, whereas other mutants showed unimproved or decreased stability. Yet, we found all but two mutants with affected catalytic efficiency. Moreover, at 37°C, ss2- (C133/C408S), ss3- (C178S/C188) and A21C/W40C maintained the same thermostability as WT while showing decreased activity, and altered pH profile and optimal temperature. Additional mutations in T33C/L170C to counteract the structural effects of the *de novo* DB on atom displacement and motility suggest the DB effect on biochemical characteristics is mediated by not only stability, but also alterations in atom motility and displacement of atom coordinates.

3.2 Introduction

Disulfide bridges (DB) are classified into three groups: structural, catalytic, and allosteric (144). Structural DB are not catalytically involved but aid protein structure formation and maintenance. Catalytic DB are responsible for thiol-disulfide

interchange reactions of enzymatic function. Allosteric DB regulate enzyme activity by conferring conformational changes to the protein upon formation or breakage of the disulfide bond.

The role of structural DB in protein stability has been extensively studied, including their natural conformations (147) and bond characteristics (129, 130). Traditional dogma stated DB stabilize proteins by reducing the entropy of the unfolded state of the protein (14, 126, 182). Protein engineers have long been attempting to increase protein stability by engineering of *de novo* DB. However, the outcome of such efforts has been mixed (47, 59, 100, 111, 122, 162). In extreme cases, proteins have been hyperstabilized by DB engineering, such as with T4 lysozyme, (101) and thermolysin-like protease (100). Several programs have been created to assist in choice of sites for engineered DB to enhance stability (30, 32, 124, 146).

Our objective was to increase the thermostability of *Escherichia coli* AppA2 phytase via engineering of *de novo* DB. AppA2 is an industrial enzyme used for phosphorus nutrition of simple-stomached production animals (87), which needs to be stabilized to endure the heat generated during the feed pelleting process. AppA2 contains four structural DB, follows Michaelis-Menten kinetics, and is 98% homologous at the amino acid level to AppA (138), which has had its crystal structure determined (92), making it ideal for DB studies. Previous work with homologous phytases has shown the DB are critical for folding and catalytic activity by chemical reduction of all bridges simultaneously, but has not deciphered the individual contributions of the four bridges (145, 159, 161, 165).

In order to decipher the individual roles of the four native DB and to aid in selection of sites for *de novo* DB engineering, we first created four single DB deletion mutants and characterized them. We then created nine *de novo* DB mutants aimed at increasing the thermostability of AppA2. Finally, we created three more mutants

building on T33C/L170C in order to gain insight into the mechanism by which DB affected the phytase biochemical characteristics.

3.3 Experimental Procedures

3.3.1 AppA2 mutant construction, expression and purification

All reagents were from Sigma-Aldrich or Fisher Scientific unless otherwise stated. Site-directed mutagenesis of *E. coli appA2* in the pGAPZαA vector (Invitrogen Corporation, Carlsbad, CA) and sub-cloning in *E. coli* were performed using the QuikChange Multi or II XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions.

DB deletion mutants (Fig 3.1) were created by mutating one Cys of each bridge to Ser. DB addition mutants were created by mutating two residues to Cys. Possible mutation sites were identified using an in-house script to generate a list of possible incorporation sites with Cα and Cβ distances less than 6.5 Å apart based on a homologous crystal structure (PDB ID 1DKQ) (92). Of the 110 possibilities, nine sites were chosen to incorporate a DB based on structural analysis (Fig 3.1). Several strategies were employed in site selection. Generally, sites chosen were close to the surface of the protein. We preferred DB spanning approximately 20 or more amino acid residues. More sites were selected in the α-domain than the αβ-domain since it generally has a higher B-factor. Several mutations were designed to connect loops or the β-hairpin to adjacent structures.

Three mutations were independently added to T33C/L170C based on structural analysis in order to explore the mechanisms by which DB affected the protein. T33C/L170C was chosen because it was one of the four mutations causing a

pleiotropic effect. For two of the others, ss2- and ss3-, it would have been hard to counter deletion of a DB. The third, A21C/W40C, was in the conserved region of the phytases, so neighboring mutations would have affected directly the catalytic site. The mutation of the smaller L217 to a larger M was added to T33C/L170C/ to fill the hole created by mutation of the larger L residue to smaller Cys residue at position 170. A25insG (insert G after A25) and T26G were added to T33C/L170C to increase the flexibility of the loop believed to be constrained by T33C/L170C by use of G, the least constrained amino acid.

E. coli transformants were plated on LB medium containing 25 μg mL^{-1} zeocin. Plasmid preps were performed using the Wizard Plus DNA Purification System (Promega Corporation, Madison, WI). All constructs were confirmed by DNA sequencing. Cloning and constitutive expression in *Pichia pastoris* X-33 was carried out as described previously (67). The supernatant expressed proteins were purified by ultrafiltration and sequential ion-exchange chromatography as previously described to achieve near homogeneity (confirmed by SDS-PAGE, data not shown) (66). Purified proteins were used for all characterization.

3.3.2 Thiol titration study

Purified proteins (30μg) were denatured by boiling for 10 minutes at 100°C in a denaturing solution containing 2% SDS, 2 mM EDTA and 80 mM disodium phosphate pH 8.8 (50). The denatured proteins were assayed with 10 mM 5,5'-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent) in 100 mM disodium phosphate pH 8.8 (35). After a 15 minute incubation at 25°C, the absorbance at 412 nm was measured. The free thiols were calculated using the molar extinction coefficient, ε = 13,600 $M^{-1}cm^{-1}$ after subtraction of the WT blank.

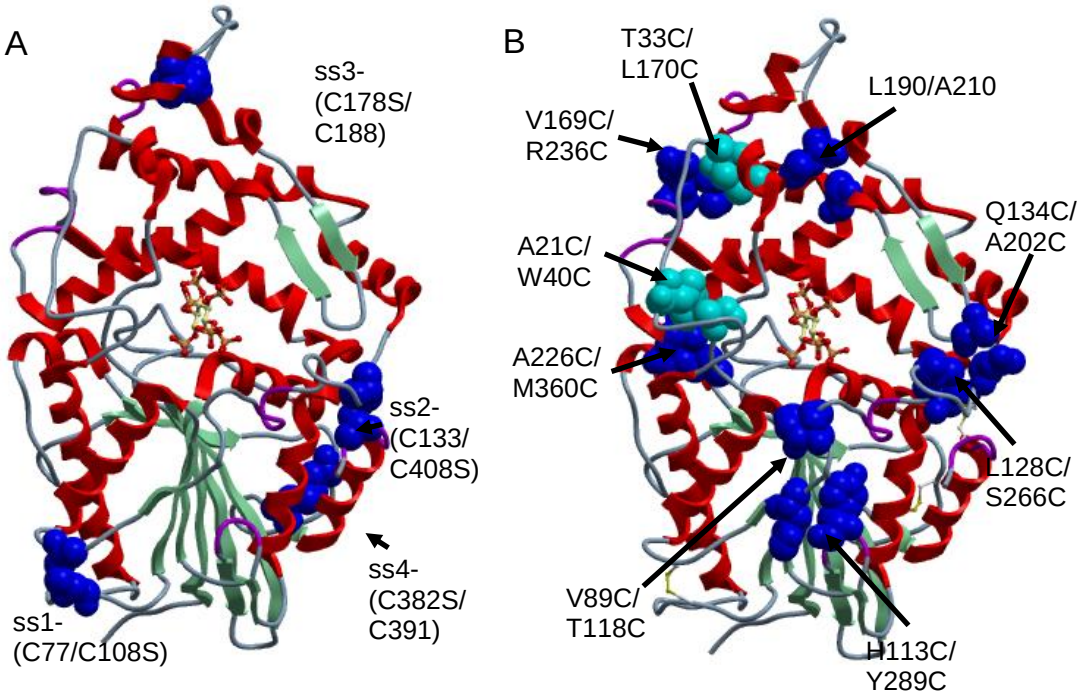


Figure 3.1 Crystal structure of *E. coli* phytase showing sites of mutations. Mutation site residues are shown in blue space filling, alpha-helices in red, beta-sheets in green. Proteins are positioned so the viewer is looking into the active site. (A) Sites of disulfide bridge deletion mutants. (B) Sites of disulfide bridge addition mutants. The phytic acid substrate is shown in CPK ball-and-stick in the active site. Mutation site residues for A21C/W40C and T33C/L170C are shown in light blue. Molecular graphics images were produced using the ICM-Browser (Molsoft L.L.C., San Diego, CA).

3.3.3 Characterization

One unit (U) of phytase activity is defined as 1 μmol orthophosphate released per minute with Na-phytate (Dodecasodium salt of phytic acid from rice, P-3168) as substrate. The colormetric phytase assay was performed at 37°C in 0.2 M Gly-HCl buffer pH 3.5 unless otherwise stated with a 5.4 mM final Na-phytate concentration as described previously (23, 51) For all assays, n = 3.

The kinetic parameters (K_m, V_{max}, k_{cat}, k_{cat}/K_m) of all enzymes (except ss2- which had too little activity to characterize) were determined in 0.2 M glycine-HCl pH 3.5 at 37°C with 9 substrate concentrations ranging from 10 μM to 6 mM using 1, 2 and 3 minute time points at 0.2 U/mL reaction mixture. Protein concentrations were determined by the absorbance at 280 nm and respective extinction coefficients for each enzyme (50,460 $M^{-1}cm^{-1}$ for WT) as calculated using ProtParam (167). Unglycosylated molecular weights were used for parameter calculations. The Michaelis-Menten equation was fit to the data for K_m and V_{max} using Kaleidagraph (v. 3.5, Synergy Software, Reading, PA).

All DB deletion and addition mutants were further characterized. The thermostability was tested in 25 mM Gly-HCl pH 3.5 by heating 1.7 mL of enzyme (5 μg/mL) for 10 minutes at each of 25, 37, 45, 55, 65, 75 and 85°C. Enzymes were then placed on ice 30 minutes, followed by dilution in 0.2 M Gly-HCl pH 3.5 and assay of residual activity at 37°C.

For optimal temperature determination, the enzymes were assayed in 0.2 M Gly-HCl pH 3.5 at 25, 37, 45, 55, 65, 75 and 85°C (addition mutants only). The pH profile at 37°C was determined from pH 2-3.5 in 0.2 M Gly-HCl and pH 3.5-6.5 in 0.2 M citrate.

3.3.4 Statistical analysis

Data were analyzed by JMP Statistical Discovery Software (release 7.0; SAS Institute Inc., Cary, NC) using Dunnett's Method to compare means to the control (WT or T33C/L170C in the case of the mechanism study). Significance was set at a P value of 0.05. Data are presented as the mean ± standard error of the mean.

3.4 Results

3.4.1 Thiol titration

All engineered disulfide bridges were confirmed to have been formed by the absence of free thiols. Disulfide bridge deletion mutants ss2- and ss3- contained one free thiol, whereas ss1- and ss4- were found to have no free thiols.

3.4.2 Kinetics

Among all the DB deletion and addition mutants, all but two (A226C/M360C and V89C/T118C, which were equal to WT) had a decreased kinetic efficiency (k_{cat}/K_m) compared to WT at 37°C (Table 3.1). Among the DB deletion mutants, ss3- and ss2-, which had too little activity to characterize kinetically, had the greatest impact. Mutant ss2- had only 1% the specific activity of WT (at 5.4mM substrate concentration), as compared to 6% specific acitivity of WT for ss3-. Among DB addition mutants, A21C/W40C, T33C/L170C and V169C/ R236C had the greatest impact on the kinetic efficiency.

Table 3.1. Kinetic parameters of all mutants at 37°C[1].

		K_m (μM)	V_{max} (μM min^{-1} mg^{-1})	k_{cat} (sec^{-1})	k_{cat}/K_m (x10^6 M^{-1} sec^{-1})
	WT	**94±4**	**623±11**	**463±8**	**4.95±0.13**
Deletion	ss1-	152*±16	99*±3	74*±2	0.49*±0.04
	ss2-	NA[2]	NA	NA	NA
	ss3-	209*±15	66*±2	49*±1	0.24*±0.01
	ss4-	105±14	285*±18	212*±13	2.07*±0.18
Addition	L190C/A210C	111±3	468*±4	349*±3	3.15*±0.07
	Q134C/A202C	164*±14	654*±5	487*±4	3.01*±0.23
	H113C/Y289C	78±4	413*±3	307*±2	3.93*±0.15
	V89C/T118C	64±5	435*±8	324*±6	5.06±0.28
	L128C/S266C	102±3	281*±2	209*±2	2.05*±0.05
	V169C/R236C	127±3	96*±0	71*±0	0.56*±0.01
	A226C/M360C	84±1	558*±3	415*±2	4.93±0.03
	A21C/W40C	44*±1	65*±1	48*±1	1.10*±0.02
	T33C/L170C	152*±9	193*±5	143*±4	0.95*±0.03
Mechanism	T33C/L170C/ L217M	141±9	225†±6	168†±4	1.2†±0.05
	T33C/L170C/ A25insG	124±4	218†±3	162†±2	1.31†±0.02
	T33C/L170C/ T26G	135±4	70†±1	52†±1	0.38†±0.01

[1]Mutants highlighted in grey had the greatest impact on the protein characteristics, including kinetics, thermostability, optimal temperature and pH profile.

[2]Mutant ss2- had too little activity to characterize (1% specific activity of WT, as compared to 6% specific acitivity of WT for ss3-).

*****Denotes significant difference ($P \leq 0.05$) from WT using Dunnett's Method.

†Denotes significant difference ($P \leq 0.05$) from T33C/L170C using Dunnett's Method.

Mutants T33C/L170C/L217M and T33C/L170C/A25insG had a significantly higher ($P \leq 0.05$) V_{max}, k_{cat} and k_{cat}/K_m than T33C/L170C at 37°C, whereas T33C/L170C/T26G had significantly lower ($P \leq 0.05$) values than T33C/L170C for the same three parameters.

3.4.3 Thermostability

Only one mutant, Q134C/A202C, showed a greater stability than WT, and this only at 45°C and 65°C (Fig 3.2 and 3.3). Although all other deletion and addition mutants showed decreased stability at all or some of the higher temperatures (≥45°C), ss1-, ss2-, ss3-, A21C/W40C, H113C/Y289C and Q134C/A202C did not have different stability (residual activity) from WT at 37°C.

3.4.4 pH profile & optimal temperature

Only mutants ss2-, ss3-, A21C/W40C and T33C/L170C had differences in optimal temperature and pH profile from that of WT (Fig. 3.4 and 3.5). All other DB deletion and addition mutants had profiles similar to WT. Mutants ss2-, ss3-, and T33C/L170C had optimal temperatures shifted downwards from 55°C of WT to 45°C, and, along with A21C/W40C showed greater relative activity at sub-optimal temperatures than WT. Mutant ss3- showed a decrease in relative activity at temperatures above optimum.

All four mutants, ss2-, ss3-, A21C/W40C and T33C/L170C, also had decreased relative activity at the more acidic pHs (2-3), while ss2- and A21C/W40C also had decreased relative activity at slightly acidic pHs (4.5-6).

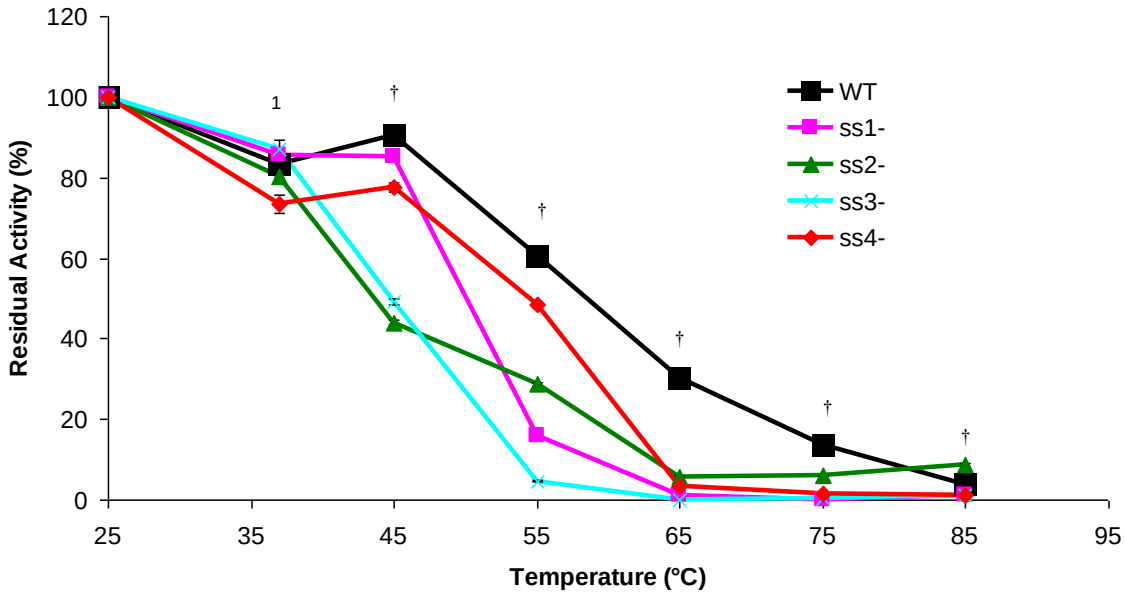


Figure 3.2 Thermostability of disulfide bridge deletion mutants compared to WT. Judged by residual activity at 45°C, mutants ss2- and ss3- had the greatest effect on the thermostability of the protein. Dunnett's Method was used to compare means to the WT.

[1]ss4- was different ($P \leq 0.05$) from WT at 37°C.

[†]All mutants were different ($P \leq 0.05$) from WT at the given temperature.

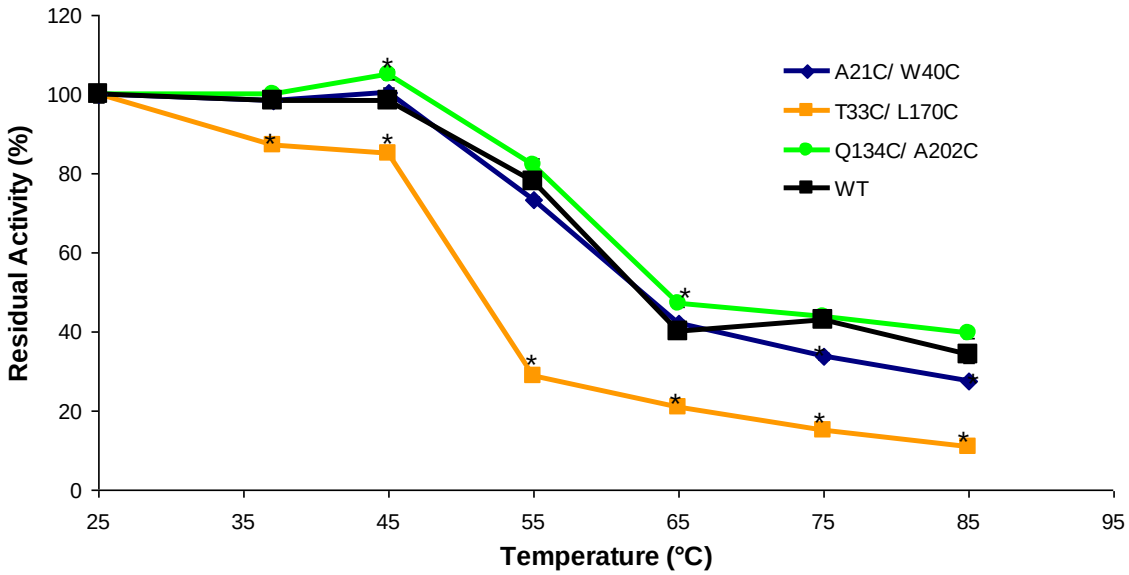


Figure 3.3 Thermostability of disulfide bridge addition mutants compared to WT. All deletion and addition mutants (not shown) but one (Q134C/A202C) showed decreased thermostability compared to WT. Note A21C/W40C had the same residual activity as WT at 37°C. Dunnett's Method was used to compare means to the WT.

* Indicates different ($P \leq 0.05$) from WT at this temperature.

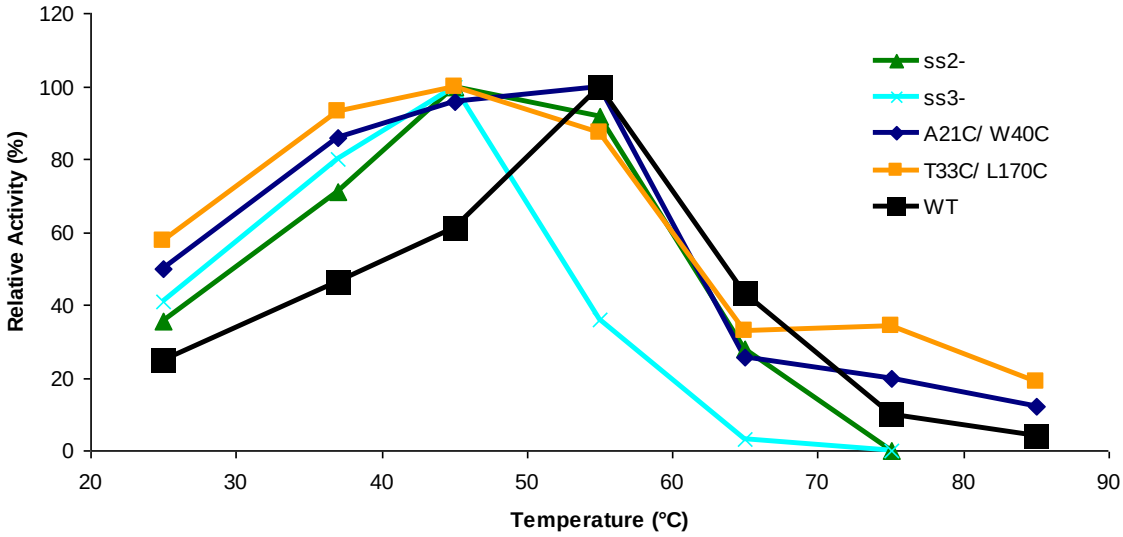


Figure 3.4 Optimal temperature of select mutants. Mutants ss2- and ss3- shifted the optimal temperature down from 55°C to 45°C. A21C/W40C and T33C/L170C displayed higher relative activities at lower temperatures than WT. All other mutants had similar profiles as WT.

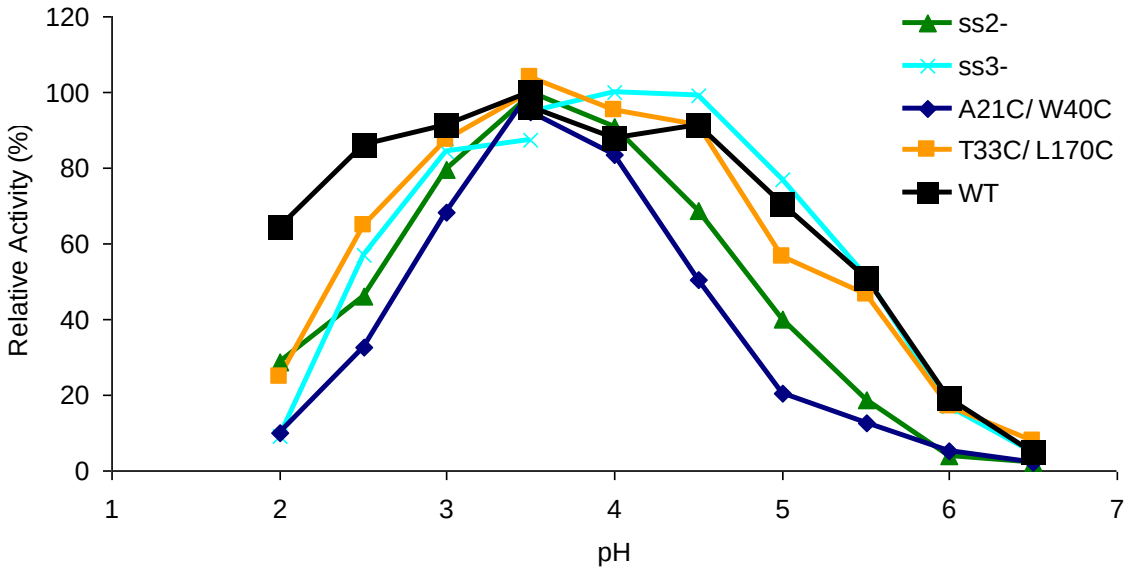


Figure 3.5 pH profile of select mutants at 37°C. Mutants ss2- and A21C/W40C had decreased relative activities at both low and high pHs, while ss3- and T33C/L170C had decreased relative activities at low pHs. All other mutants had similar profiles as WT.

3.5 Discussion

As expected, the four DB deletion mutants displayed reduced thermostability compared to the WT at ≥45°C ($P \leq 0.05$). However, at 37°C, ss1-, ss2- and ss3- did not have reduced thermostability compared to WT. The DB addition mutants showed a similar trend. Only one of the nine DB addition mutants, Q134C/A202C displayed marginally increasd thermostability over the WT, whereas the rest had reduced thermostability compared to WT at least at higher temperatures ($P \leq 0.05$). This highlights the difficulty in designing stabilizing DB. Many factors are influenced by the *de novo* DB, including the entropy and enthalpy of the folded and unfolded state of the protein, as well as the solvent (14).

We also observed nearly a universal effect on catalytics kinetics by either DB deletion or addition. All of the DB deletion mutants and seven of the nine addition mutants had decreased kinetic efficiency (k_{cat}/K_m) compared to WT at 37°C. All of the DB deletion and addition mutants had altered V_{max} and k_{cat}. Although there is a lot of discussion regarding how DB may affect protein stability (14), there is a paucity of discussion about how DB may affect catalytic kinetics. Li et al. reported the independent addition of two different DB did not change the catalytic kinetics of glucoamylase (91). However, seeing as how DB can have significant effects on the protein, this may be an unusual case.

Firstly, DB may affect catalysis through the stability of the protein. If the protein denatures, it can no longer perform as a catalyst, and therefore catalytic kinetics could be reduced. A *de novo* DB may affect catalysis if it were placed in such a way as to interfere with ligand binding. However, we will not focus on that as none of the DB addition mutants in our case fit that criterion.

DB may also affect catalysis by placement (or perhaps displacement) of amino acid residues or their side chains. An example would be an allosteric DB affecting catalysis by affecting topology. It has been shown that deletion of a WT DB causes small shifts in the coordinates of atoms to reduce the packing defect caused by the mutation (34). Likewise, engineering of a *de novo* DB in a protein may disrupt favorable enthalpic interactions in the native state, causing residues to shift to minimize the energy of the native state, and therefore confering sub- Å shifts in the coordinates of amino acid residues, which are more pronounced (can be a few Å) in the region of the DB (125). A *de novo* DB may also confer displacement of backbone atoms, as it confines residues to a certain distance through the covalent DB or places restraints on phi and psi angles, causing downstream perturbations of phi and psi angles in other residues. For example, DB engineering has been observed to cause a rigid-body rotation of 5.1° in the amino terminal domain relative to the carboxy terminal (58) These associated displacements may affect the catalytic and/or binding site, and thus affecting the catalytic kinetics.

Alternatively, DB may also affect catalytic kinetics by affecting residue mobility. If a residue(s) require a certain degree of flexibility or rigidness for optimal catalytic performance, and the motility is affected by the engineering of a *de novo* DB, catalysis will be impaired.

Intuitively, one might think that introduction of a DB would decrease motility. Normal mode analysis of bovine pancreatic trypsin inhibitor showed decreased motility of cystine sulfur atoms upon formation of a DB (156). Reduction of the DB resulted in an average of 45% increase in local flexibility, but generally did not affect the overall flexibility (averaged over all the atoms), with one exception exhibiting a 13% increase. Likewise, a *de novo* DB was shown to inhibit a transient cleft formation in cytochrome b_5 by decreasing the motility of the local residues, as probed

by a Trp four amino acids away from one of the involved Cys using molecular dynamics simulations, time-resolved fluorescence and 1H NMR experiments (150, 151). A similar restriction of cleft formation has been performed in T4 lysozyme (58).

On the contrary, a T4 lysozyme mutant with an engineered DB between residues 9 and 164 did not increase rigidity of the folded protein as measured by temperature factors of the crystal structures of the WT and mutant. Both sites of the Cys mutation were already flexible in the WT. At one mutation site, the temperature factors were similar to WT, whereas the other site had increased temperature factors over the WT (125). Another T4 lysozyme mutant with an engineered DB between residues 21 and 42, which increased the melting temperature by 11°C, had increased mobility (disorder) in the vicinity of the DB, again determined by temperature factors of the crystal structures (58).

Some mutants, ss2-, ss3-, A21C/W40C and T33C/L170C, also had effects beyond catalytic kinetics. These mutants had effects on the optimal temperature and/or the optimal temperature profile, as well as the pH profile, whereas all other mutants only affected the catalytic kinetics. Mutant ss2- destroyed the nonconsecutive DB between C133 and C408. The effects of ss2- can be rationalized by the classical theory of stabilization of proteins by DB, which is that they reduce the entropy of the unfolded state, thereby favoring folding, and that this effect is increased by increasing loop size between the involved cystines (14). Mutant ss3- destroyed a DB with only a 10 residue loop (C178 to C188), but which is flexible (as shown by high temperature factors or absence in highly homologous structures (92)). Although constraining a flexible region decreases the native state entropy, it seems to be outweighted by the decrease in entropy of the unfolded state and the fact that the flexible loop residues are able to pack with little loss of enthalpic interactions once constrained (14). A21C/W40C and T33C/L170C were both in the vicinity of a mobile loop (residues

20-25) proposed to initiate substrate binding and shift conformation upon that binding (92) (Fig. 3.6).

Note that of these four mutants with pleiotropic effects, three, namely ss2-, ss3- and A21C/W40C (as well as three other mutants, ss1-, H113C/Y289C and Q134C/A202C that only affected kinetics) did not have different thermostability (residual activity) from WT at 37°C, at which the kinetics were determined, but had ≤22% the kinetic efficiency of WT. These mutants did not begin to unfold until temperatures ≥45°C. Thus, the kinetic effects must be due to either displacement or alterations in motility.

We used T33C/L170C to further explore the mechanism by which one of these pleiotropic *de novo* DB affected the biochemical characteristics. Possible mechanisms by which T33C/L170C affected the biochemical characteristics were reduced stability, changed motility of nearby residues important for catalytic function, and displacement of residues due to the mutations. Since T33C/L170C has a reduced stability at 37°C from the WT, this stability was known to affect the catalytic kinetics. To determine if reduced motility was a factor, we designed mutants T33C/L170C/A25insG (insertion of a Gly between residues 25 and 26) and T33C/L170C/T26G aimed at increasing the motility of loop 20-25 (Fig 3.6) through use of Gly, the least constrained residue. To determine if displacement was a factor, we mutated the smaller L217 to larger Met in mutant T33C/L170C/L217M to try and fill the hole created by the mutation of L170 to the smaller Cys (Fig 3.6). If reduced motility and atom displacement had an adverse effect on kinetics of T33C/L170C, we would have expected these counteracting mutations in to improve kinetics.

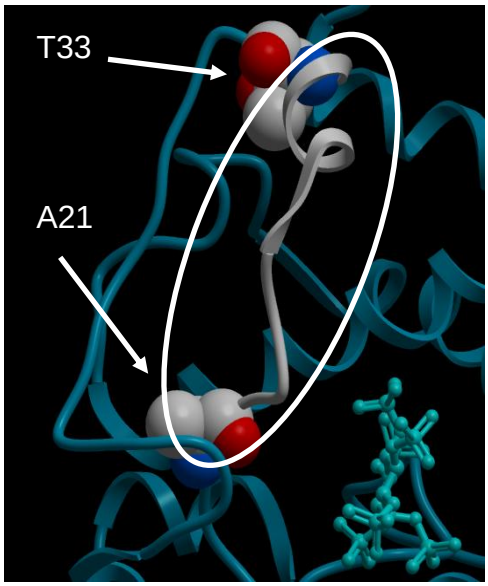


Figure 3.6 Crystal structure showing loop 20-25 and mutation sites for A21C/W40C and T33C/L170C. The viewer is looking into the active site, with phytic acid shown in ball-and-stick, residues 21 and 40 in space filling, and loop 20-25 in white. The mutations A25insG (addition of a Gly between residues 25 and 26) and T26G in T33C/L170C were between T33 and loop 20-25, and were aimed at increasing the motility of loop 20-25. The mutation L217M in T33C/L170C was aimed at filling the hole created by mutation of L170 to Cys.

Both T33C/L170C/A25insG and T33C/L170C/L217M resulted in similar improvements in all the kinetic parameters, resulting in an increased k_{cat}/K_m over T33C/L170C of 38% and 26%, respectively (Table 3.1). This suggests both an effect of reduced mobility and displacement of atoms by the *de novo* DB on the kinetics. Also, the DB T33C/L170C affected the mobility of residues several amino acids away, as the compensatory mutation was at residue 26, while the DB was at residue 33. Mutant T33C/L170C/T26G decreased the catalytic kinetics. We deem this due to the breakage of a hydrogen bond between T33 and M29 which is apparently critical for the functioning of loop 20-25. On a side note, the mutation A25insG may be able to be used to increase the activity of WT AppA2.

Protein engineers have used engineered DB for other purposes besides increasing thermostability, such as to tether molecules together through an intermolecular DB. Several examples exist, such as linking two identical functions, such as with iron-superoxide dismutase (17), or to colocalize different functions together, as in antibody-directed enzyme prodrug therapy (ADEPT) (137). An antibody targeting a certain tissue is linked to an enzyme that activates a prodrug to focus drug therapy on specific tissues. In such cases, researches should be careful to check the biochemical characteristics of the linked enzymes to ensure the *de novo* DB has not negatively impacted them, thus defeating the purpose of the design.

In conclusion, catalytic kinetics and other biochemical characteristics of enzymes may be affected by native and *de novo* DB. Just as *de novo* DB may affect protein stability through a myriad of factors, so may DB affect biochemical characteristics. These effects arise from not only alterations in enzyme stability, but also seem to be due to alterations in atom mobility and perturbation of atom coordinates.

CHAPTER FOUR

THE POTENTIAL NUCLEASE ACTIVITY OF HISTIDINE ACID PHYTASES

4.1 Summary

Several cases exist in which a phosphomonoesterase has been shown to possess phosphodiesterase activity. The histidine acid phytase (HAP) *Escherichia coli* AppA, a phosphomonoesterase, was shown to bind strongly with Cibacron Blue, indicating it possess a nucleotide binding site. Furthermore, the αβ domain superficially resembles the αβ domain of restriction endonucleases. Since AppA homologous phytases *E. coli* AppA2 and *Aspergillus niger* PhyA are supplemented to animal feeds for poultry and swine, we explored whether these enzymes act on DNA, which contains a phosphodiester bond, thus aiding in the digestion of ingested DNA in animals receiving phytase supplemented diets. Structural examinations, bioinformatics searches and *in vitro* assays all indicate HAP to not possess nuclease activity. This is corroborated by *in vivo* evidence showing a lack of greater DNA degradation in the digesta of swine supplemented with HAP versus animals on a control diet.

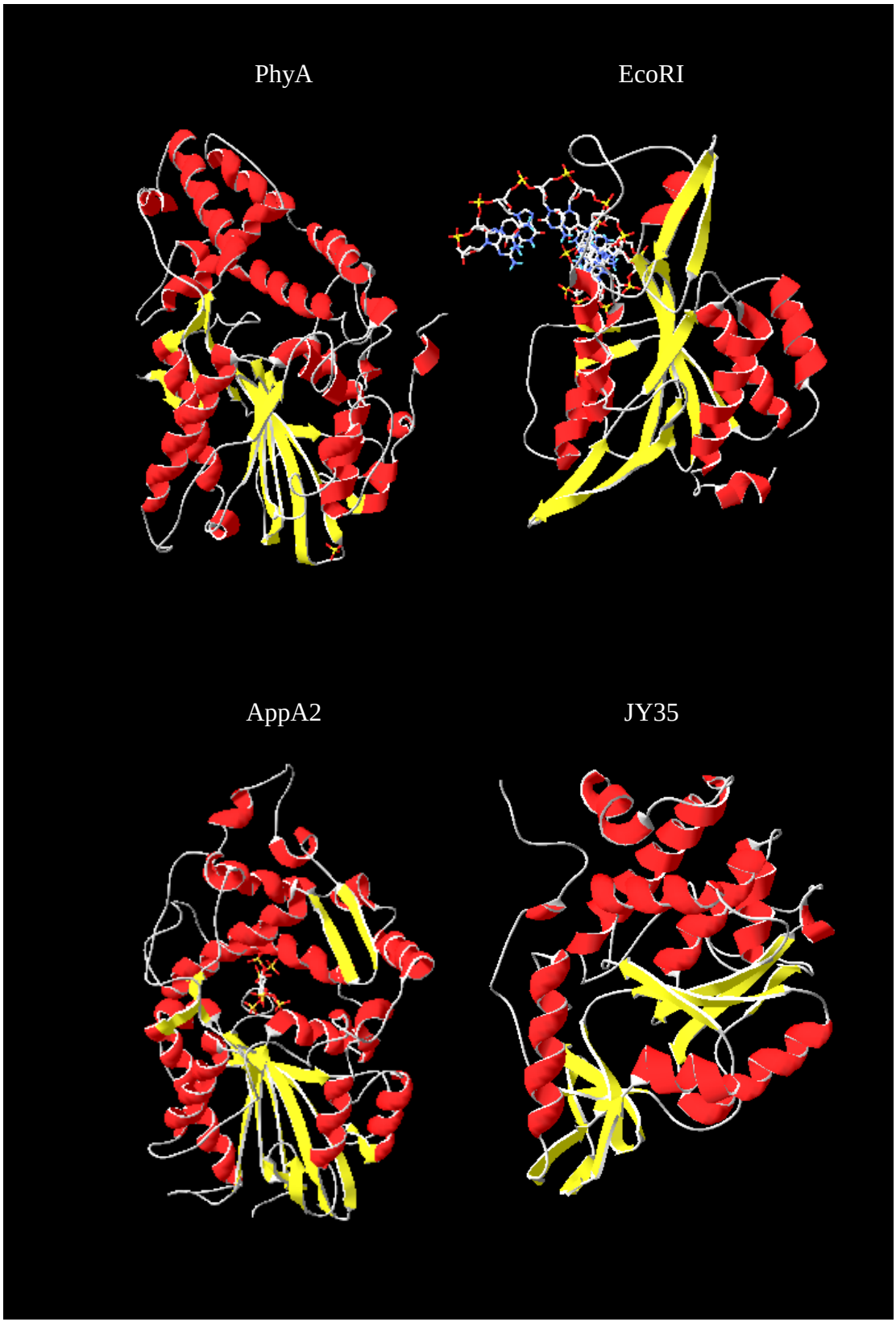
4.2 Introduction

Generally, enzymes acting on phosphoric ester bonds are classified under the Enzyme Commission into those acting on phosphomonoesters (EC 3.1.3), phosphodiesters (EC 3.1.4) or phosphotriesters (EC 3.1.8). Some enzymes possessing either phosphomonoesterase or phosphodiesterase activity have been found to also possess

the other activity, although to a lesser degree. Rat alkaline phosphatase, primarily a phosphomonoesterase, was shown to possess approximately $1/8^{th}$ the phosphodiesterase activity as its phosphomonoesterase activity (134). Through kinetic inhibition studies, the two activities were shown to originate from the same active site. *E. coli* alkaline phosphatase, a phosphomonoesterase, was also shown to possess phosphodiesterase activity (114). Sequence homology showed the alkaline phosphatase to be distantly related to the *E. coli* alkaline phosphodiesterase/nucleotide pyrophosphatase (NPP). The phosphodiesterase activity was confirmed due to the alkaline phosphatase through copurification of the activities, inhibition studies and mutational studies. Conversely, phosphodiesterases, like mammalian NPP, have been shown to possess phosphomonoesterase activity from the same active site as the phosphodiesterase activity (42). The phosphodiesterase activity ranges from 10^2 to 10^8 times that of the phosphomonoesterase activity (180).

Phytases are phosphomonoesterases (EC 3.1.3.2, 8, 26, and 72) with preferential activity towards phytic acid (87). Phytases, generally of the HAP class such as *E. coli* AppA and AppA2 and *A. niger* PhyA, are supplemented to poultry and swine diets to improve the phosphorus bioavailability to the animals (2, 10, 60, 148). *E. coli* AppA was found to bind with Cibacron Blue F3GA, a dye used for probing nucleotide binding sites in proteins (31). Cibacron Blue F3GA had a strong inhibition (K_i = 0.35 μM) of the phosphomonoesterase activity of AppA (31), indicating AppA to possess a nucleotide binding site. This paired with a superficial observation that the αβ domain of AppA superficially resembles the αβ domain of restriction endonucleases (Fig. 4.1), and the possession of phosphodiesterase activity by phosphomonoesterases and *vice versa*, led us to explore the possibility of DNA degradation by these phytases.

Figure 4.1 Crystal structures of phytases and the restriction endonuclease EcoRI. PhyA and AppA are both HAP. Their αβ domain superficially resembles the αβ domain of EcoRI, with the β-sheets sandwiched inbetween two large α-helices on one side and three large α-helices on the other. JY35, which bears no resemblance to the αβ domain of restriction endonucleases, represented here by EcoRI, is a phytase of the dual specificity fold. Structures represented are PDB files 1IHP, 1QRI, 1DKQ and 1U24 for PhyA, EcoRI, AppA and JY35, respectively. Images were produced with DeepView (45).


PhyA
EcoRI
AppA2
JY35

4.3 Experimental Procedures

4.3.1 Structural comparison and bioinformatic analysis

The arrangement of α-helices was analyzed using DeepView and the protein data bank (PDB) files for PhyA (1IHP), AppA (1DKQ), *Selenomonas ruminantium* JY35 (1U24) and EcoRI (1QRI) (45). BLASTP, CDD, CDART, and VAST were used for bioinformatic searches relating HAP to nuclease activity .

4.3.2 In vitro characterization

PhyA and AppA2 were heterologously expressed in *Pichia pastoris* and purified using two steps of ion exchange chromatography as described previously (166). *S. ruminantium* JY35 phytase (27, 174) was also expressed and purified in a similar manner. Phytase activity was measured as described using a colorimetric assay (51). One unit is equivalent to the hydrolysis of 1 μmol phosphate from phytic acid per minute at 37°C. Nuclease activity was measured by monitoring the degradation of high molecular weight herring sperm DNA or torula yeast RNA at 37°C. DNA degradation was measured by the decrease in fluorescence of the DNA-ethidium bromide complex on an FL600 fluorescent plate reader (Biotek Instruments) (105, 106), or visualized by agarose gel electrophoresis with ethidium bromide staining. For all nuclease assays, RQ1 DNase (Promega) served as a positive control. Nuclease dependency on metal ions was checked by the addition of 2mM final concentration of $CaCl_2$, $MgCl_2$, $MnCl_2$, and $ZnS0_4$. Dose dependency of the observed nuclease activity was checked using three orders of magnitude of purified phytase in the assay as above. Inhibition kinetics were measured with a constant phytic acid concentration while

varying the concentration of DNA. Kinetic constants for phytic acid were measured and IC50 values were calculated for DNA inhibition of phytic acid.

4.3.3 Effect of high-level phytase supplementation on digesta DNA concentration in growing pigs

Stomach digesta contents were obtained from growing pigs fed a nutritionally adequate corn-soybean meal basal diet (n=13) or supplemented with 2,500 units/kg of AppA2 (OptiPhos, JBS United) (n=12). The pig feeding trial was conducted as described (38). Briefly, Yorkshire x Hampshire x Landrance crossbred weanling pigs were housed and the Cornell University Swine Farm. All experiments were approved by the University Institutional Animal Care and Use Committee. At 5 weeks, 5 pigs were slaughtered per group, and then the rest were slaughtered at 10 weeks on the diet. Digesta contents were collected immediately after slaughter, frozen and lyophilized.

A crude DNA was extracted from 500mg digesta dry matter for quantification using the ethidium bromide fluorescent assay above. The digesta was extracted twice with 5mL 10 mM Tris-HCl, 5 mM EDTA, pH 8.0 at 4°C for 5 min. on a wheel at 10RPM. After centrifugation at 4°C, 30,000 x g for 30 min., the DNA in 7 mL of clarified supernatant was precipitated with 8.0 mL isopropanol and 700 μL 3.0 M Na-Acetate, pH 5.2 at -20 °C for 2 hrs. The DNA pellet was redissolved in 10mM Tris-HCl pH 8.0, 1mM EDTA and quantified immediately.

4.4 Results

4.4.1 Structural comparison and bioinformatic analysis

A closer inspection of the arrangement of α-helices in HAP and restriction endonucleases showed the two classes of proteins to have a different arrangement of helices (Fig. 4.2). All bioinformatic searches failed to uncover a structural relationship between HAP and nucleases.

4.4.2 In vitro characterization

AppA2 failed to show a dose response curve for degradation of DNA (Fig. 4.3). JY35 also showed a disappearance of DNA, although at a very extended time (Fig. 4.4). The presence of divalent metal cations improved the activity. PhyA and JY35 failed to show a degradation of DNA in a continuously monitored fluorescent assay (Fig. 4.5). The three phytases failed to show typical inhibition curves for DNA inhibition of phytase activity (Fig. 4.6). Kinetic values for the three phytases did not point to a specific type of inhibition by DNA, as the values for the three phytases all displayed different responses to the presence of DNA (Table 4.1).

4.4.3 Effect of high-level phytase supplementation on digesta DNA concentration in growing pigs

In animals on diet for 5 weeks, it appeared the supplementation of phytase decreased the amount of free DNA in the stomach (Fig. 4.7). However, in animals on diet for 10

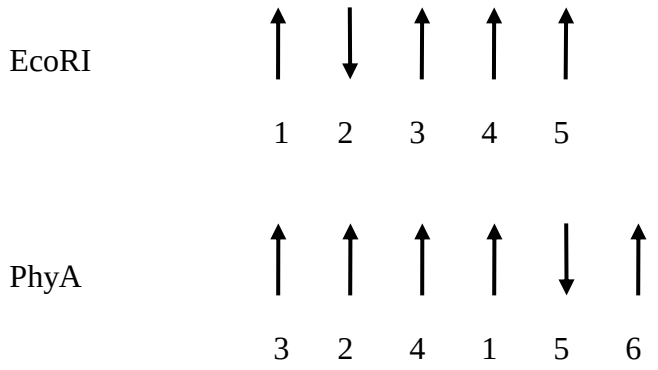


Figure 4.2 Arrangement of α-helices in EcoRI and PhyA. Numbers indicate order in appearance from the N-terminus, and arrowheads point in the direction of the C-terminus.

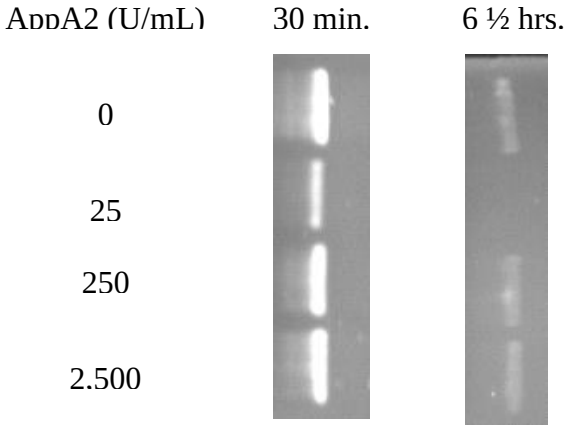


Figure 4.3 Lack of dose dependent degradation of DNA by AppA2.

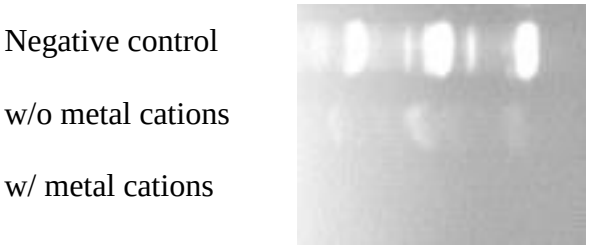


Figure 4.4 Effect of divalent metal cations on DNA degradation by JY35. The assay was conducted at 37° for 2 ½ days.

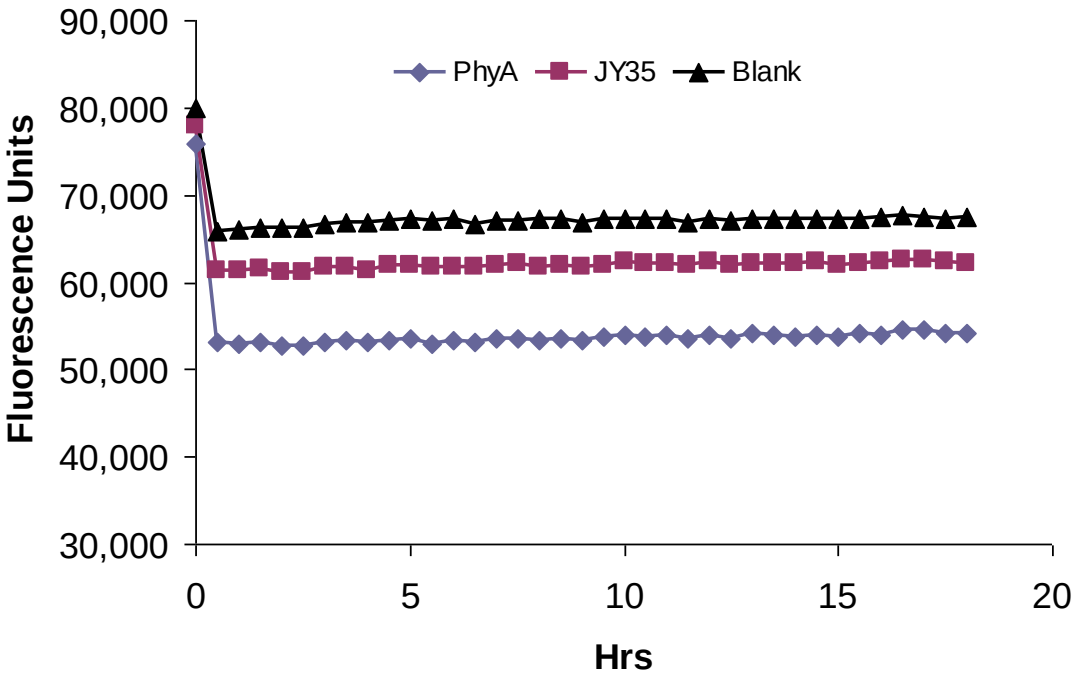


Figure 4.5 Absence of nuclease activity for PhyA and JY35 over 18hrs. The fluorescence of the DNA-ethidium bromide complex was monitored in a kinetic read at 37°C with herring high molecular weight sperm DNA as the substrate.

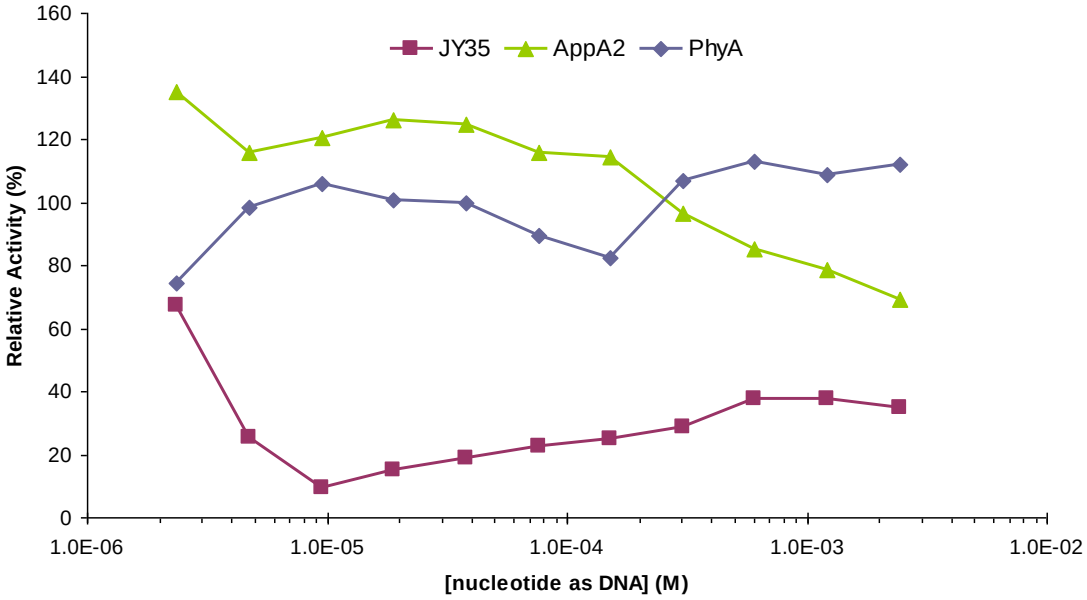


Figure 4.6 Inhibition curve of phytase activity by DNA. DNA concentrations ranged from 2.4μM to 2.4mM. Activities are relative to 100% activity in the absence of DNA, respective to enzyme. None of the enzymes displayed a typical sigmoidal inhibition curve.

Table 4.1 Kinetic constants for phytic acid of three phytases in the presence and absence of DNA.*

Sample	V_{max} **(μM/min)**	K_m **(μM)**
PhyA	61±3)	14±1
PhyA & DNA	74±0	15±1
AppA2	37±3	22±2
AppA2 & DNA	72±2	85±12
JY35	72±7	491±76
JY35 & DNA	130±6	209±24

*PhyA, and AppA2 were assayed in 1,212 μM DNA, the IC50 value for DNA inhibition of phytase activity as calculated from the inhibition curve (Fig. 4.6) for AppA2, while JY35 was assayed in its IC50 value of 3.3 μM DNA.

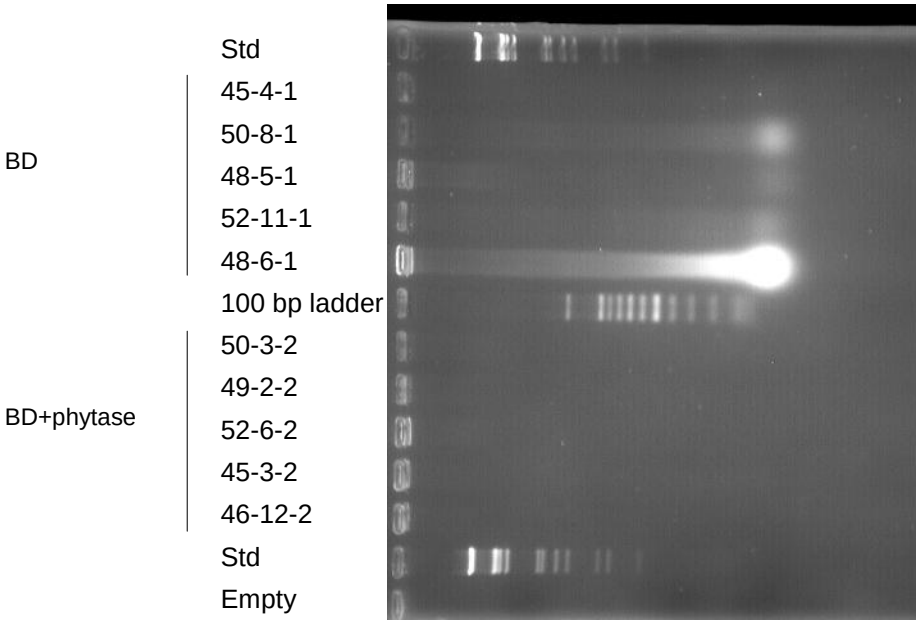


Figure 4.7 Stomach digesta DNA at 5 weeks of diet. BD = Basal diet, Std = DNA standard. Individual pig identification numbers are indicated.

weeks, it appeared the supplementation of phytase increased the amount of free DNA in the stomach (Fig. 4.8).

4.5 Discussion

HAP AppA2 and PhyA failed to demonstrate nuclease activity. Bioinformatics searches using CDD, CDART, VAST and BLASTP failed to uncover any structural link to nucleases. Furthermore, the superficial resemblance between HAP and restriction endonucleases was, on closer inspection, not a true resemblance. The HAP showed inconsistent degradation of DNA, sometimes displaying no activity at all (Fig. 4.5). To confirm the lack of significance between the superficial resemblance of HAP to restriction endonucleases, we tested JY35 for nuclease activity. As JY35 is not a HAP and bears no resemblance to restriction endonucleases, its demonstration of DNA degradation can be attributed to either contamination or the phytase active site also being able to catalyze the cleavage of phosphodiester bonds in DNA. The apparent increase in activity in the presence of divalent cations lends support to the existence of contaminating nucleases, as neither JY35 nor HAP contain divalent metals in their structure. This effect was also seen on the degradation by PhyA preparations (data not shown), again indicating contamination in the preparation.

If the HAP or JY35 possessed nuclease activity in the same active site as phytase activity, the phytase activity should be able to be hindered by the presence of DNA competing for the same active site. However, the phytases showed no typical inhibition curve of phytase activity by DNA, again supporting contamination of the preparations (Fig. 4.6). To further this notion, we measured the K_m and V_{max} of the phytases for phytic acid in the presence of DNA at an IC50 concentration. As PhyA showed no decrease in phytase activity in the presence of DNA, we used the same

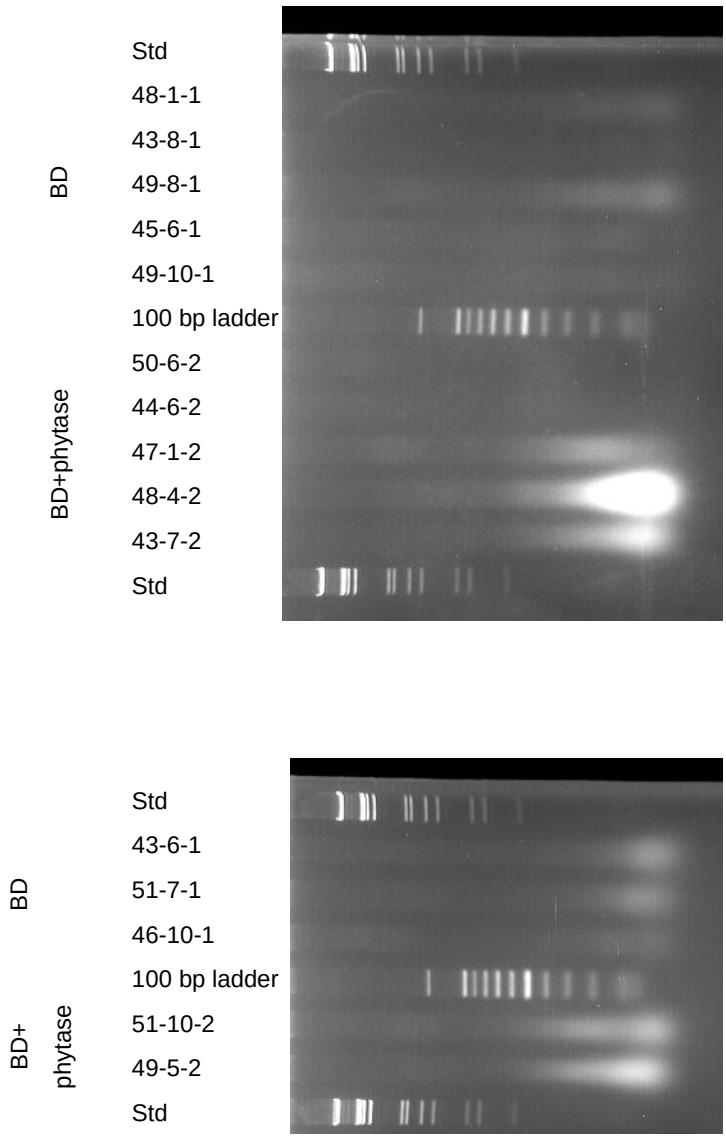


Figure 4.8 Stomach digesta DNA at 10 weeks of diet. BD = Basal diet, Std = DNA standard. Individual pig identification numbers are indicated.

DNA concentration as for its homologue, AppA2. If DNA were competitively binding to the same active site, the presence of DNA would raise the K_m but have not effet on the V_{max}. AppA2 was the only phytase to show an increase in K_m, while JY35 even decreased (Table 4.1). However, AppA2 also increased in V_{max}, which is unexpected. Thus every *in vitro* assay fails to demonstrate nuclease activity for the HAP.

Likewise, the *in vivo* data fails to support a nuclease activity for HAP. Although after 5 weeks of diet, the phytase supplemented group had less free stomach digesta DNA than the basal, this phenomenon was reversed after 10 weeks of diet. Although we tried to control confounding factors through fasting and refeeding just prior to slaughter, it should be noted the *in vivo* data may be confounded by the length of time digesta was in the stomach before slaughter. Differences could arise from competition for feed upon refeeding in a group penned setting, affecting time of ingestion and amount of feed ingested, as well as time of slaughter. Thus no consistent evidence could be through bioinformatics, *in vitro* or *in vivo* for the possession of nuclease activity by HAP. The ability of AppA to bind Cibacron Blue F3GA is attributed to the remnants of a dinucleotide fold present in the structure of the phosphoglycerate mutase-like superfamily of proteins, which have no apparent nucleotide requirement (20). This superfamily includes the HAP family of proteins, and thus AppA.

CHAPTER FIVE

FEATHER DEGRADATION

5.1 Feather waste has the potential to be a high protein feed additive

Feathers represent 5-7% of the body weight of domestic chickens, which results in large amounts of annual feather waste production worldwide from the poultry industry (46, 119). This waste product possesses enormous potential as a protein supplement, with feather meal being 96-98% crude protein (127). However, this protein has poor bioavailability (123). Hydrothermal treatment of ground feather is currently used to overcome this low bioavailability (78, 107, 123). However, the feather meal product still has poor bioavailability, essential amino acids are degraded during the hydrothermal treatment, and the treatment process is energetically expensive (78, 107, 123). Therefore, the ability of certain microbes to degrade feather has garnered the attention of biotechnologists whom hope to turn feather waste into a highly available protein supplement by keratin digestion (119).

5.2 Other applications of proteases

Proteases are one of the most important classes of industrial enzymes (72, 79). Keratinophylic microbial proteases find application in fields other than animal nutrition. They may also be used to convert feather into nitrogenous fertilizers, biodegradable films or plastics, glues and foils (46). Proteases are also are used detergents, meat processing, leather industry (8). Keratinases also show promise in the degradation of prions for the sterilization of slaughterhouses and medical

equipment (76). The isolation of protease-resistant enzymes from keratin-degrading microbes may also be of interest (90).

5.3 Purified keratinolytic proteases lack the effectiveness of the host in keratin degradation

The field of keratin degradation has largely gone unchanged for decades, with the focus being to isolate proteases from the keratin-degrading microbes (119). Characterized keratinases largely belong to the serine or metallo-proteases (46). Many of them share high homology with proteases from nonkeratin-degrading microbes (46). However, keratinophylic microbial cell-free filtrates and the purified keratinases largely lack the efficacy of keratin degradation possessed by the host microbe (46, 74, 113). Most purified keratinases cannot completely hydrolyze native keratin. This is also true for the secreted proteases already characterized from *Streptomyces fradiae* var. k11 (89, 103).

5.4 Reduction of keratin disulfide bonds is critical to degradation

β-keratin contains a high proportion of disulfide bonds, accounting for its rigid and durable structure (33). Many purified keratinases poorly degrade keratin due to this extensive disulfide bonding, commonly resulting in 10% degradation (15, 173). Reduction of keratin disulfide bonds has been proposed to be the key step in allowing protease digestion of the keratin (132). Several researches have found cell cultures from keratin-degrading microbes to possess free thiols from the reduction of disulfide bonds (16, 73, 131). Furthermore, the inability to degrade keratin by some non-keratinolytic subtilisins with nearly identical sequences to known keratinases is

attributed to the lack of disulfide reducing ability of the host (37). These non-keratinolytic subtilisins were found to also degrade keratin in the presence of non-proteolytic *Bacillus subtilis*, which served as a source of redox potential (37). In fact, conventional serine and cysteine proteases like subtilsin, chymotrypsin and papain, which selectively cleave proteins at hydrophobic residues, have been shown capable of keratin degradation with the aid of a reductant (132). A strong correlation between the extracellular free thiol concentration and the amount of feather in the medium has been demonstrated (16). According to Ramnani and Gupta (132), "Thus it can be put forth that all enzymatic keratinolysis from any organism essentially needs to be assisted by a suitable redox."

5.5 Different sources of reducing power exist

The mechanism of keratin disulfide bond reduction may be enzymes, secreted reductants, or from some action of the cell. In one report on *Stenotrophomonas,* a secreted disulfide reductase-like protein increased the activity of keratinase upon codigestion of keratin by 50-fold (173). The filamentous fungi *Microsporum gypseum* excreted sulfite into the medium (74). In neutral and alkaline mediums, the sulfite reduces disulfide bonds, producing sulfate, and allowing attack by the proteases. *Bacillus* cells were found to attach to the surface of the keratin and hypothesized to produce a continual supply of reductant (131). *S. fradiae,* proteases failed to produce free thiol groups from keratin, whereas the culture was able to do so (112). This led to the hypothesis that keratin is reduced by a reducing mechanism on the mycelial surface of *S. fradiae* (113). Furthermore, no sulfite excretion could be detected in *S. fradiae*. Thiosulfate was excreted into the medium as a waste product, but this is not able to reduce the disulfide bridges (74, 75). Kunert

concluded in *S. fradiae* the "keratin was most likely denatured by direct reduction of cystine bridges" (74). Research with *S. pactum* also demonstrated its mycelial surface could reduce tetrazolium salts, whereas the cell-free filtrate did not (16). Furthermore, reduction of oxidized glutathione was not accomplished by the cell-free filtrate nor the cellular homogenate, but was done so by washed cells, and a sodium azide (to kill the cells) treated culture was unable to degrade keratin, leading to the conclusion that metabolically active cells were needed for reducing power (16).

5.6 *Streptomyces* genomes

Four complete *Streptomyces* genome sequences are currently available: *S. coelicolor* A3(2) (13), *S. avermitilis* MA-4680 (53), *S. griseus* subsp. *griseus* NBRC 13350 (57) and *S. scabiei* 87.22 (176). There are an additional 20 *Streptomyces* draft genome sequences (NCBI), none of which are for *S. fradiae*. Anderson and Wellington found residues 158-276 of the 16s rRNA sequence variable enough among streptomycetes to allow phylogenetic classification (5). Based on this region, the pathogen *S. scabiei* is most closely related to *S. fradiae* var. k11 among the streptomycetes with complete genome sequences (Fig. 5.1).

5.7 Research Aims

No previous genomics or systems biology approach has been conducted for the study of keratin degradation. We had three aims in our research. Our first aim was to generate a draft sequence of the genome of *S. fradiae* var. k11. Secondly, we hoped to determine the identity and role of proteins secreted during growth on feathers.

```
S_scabiei          TCTAATACCGGATAC-GACACTCTCGGGCATCCGATGAGTGTGGAAAGCTCCGGCGGTGA 59
S_fradiae_k11      TCTAATACCGGATAC-GACCACTTCAGGCATCTGATGGTGGTGGAAAGCTCCGGCGGTGC 59
S_coelicolor       TCTAATACCGGATACTGACCCTCGCAGGCATCTG-CGAGGTTCGAAAGCTCCGGCGGTGA 59
S_avermitilis      TCTAATACCGGATAA-TACTCTCGCAGGCATCTG-TGAGGGTTAAAAGCTCCGGCGGTGC 58
S_griseus          TCTAATACCGGATAA-CACTCTGTCCCGCATGGGACGG-GGTTAAAAGCTCCGGCGGTGA 58
                   **************   **     *  ****  *  *    *  ***************

S_scabiei          AGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGAGGTAACGGCTCACCAAGGCGACGACG 119
S_fradiae_k11      AGGATGAGCCCGCGGCCTATCAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGACG 119
S_coelicolor       AGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGAGGTAATGGCTCACCAAGGCGACGACG 119
S_avermitilis      AGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGAGGTAGTGGCTCACCAAGGCGACGACG 118
S_griseus          AGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGGGGTAATGGCCTACCAAGGCGACGACG 118
                   ************************ ******* ****  ***  ***************
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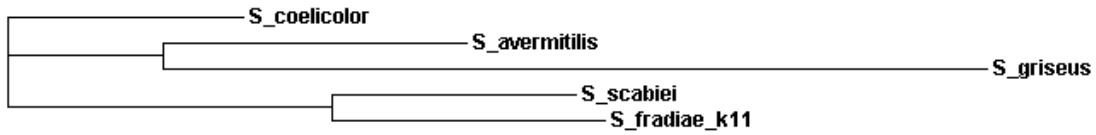


Figure 5.1 Phylogenetic relationship of *S. fradiae* var. k11 and streptomycetes with complete genome sequences. Residues 158 to approximately 276 of the 16s rRNA sequence of *S. scabiei*, *S. coelicolor*, *S. avermitilis* and *S. griseus* were aligned with the corresponding region of *S. fradiae* var. k11 16s rRNA using ClustalW2 (77). The branch length in the phylogram tree is relative to the relational distance between the species.

Finally, we hoped to gain insight into the mechanism by which *S. fradiae* var. k11 dealt with the disulfide bonds of keratin.

5.8 Significance of Research

In spite of the extent of research on keratin-degrading microbes and their proteases, only one is being commercialized for the improvement of feather waste for feed supplementation (15, 19, 46, 56, 94, 95, 113, 119, 119, 142, 154, 172, 179). Furthermore, this enzyme is highly homologous with subtilisins from nonkeratin-degrading bacteria (93), meaning this is not the key component conferring keratin-degrading ability to its host.

S. fradiae has long been known as one of the most potent degraders of keratin (46, 113, 178)(89, 113, 178). Although proteases have already been purified from *S. fradiae*, they are far from having the efficacy in keratin degradation of the bacteria itself (88, 103). We hope to elucidate the key components involved in the initial degradation of keratin from *S. fradiae* var. k11.

The proteases isolated from keratinophylic bacteria also show promise in the degradation of prions, the infectious form of which forms β-keratin rich plaques (46, 76). Prions are the infectious agents responsible for transmissible spongiform encephalopathies, such as mad cow disease.

In addition, our genomic approach has the potential to identify new proteins. Our preliminary proteomic data showed only approximately 20% of the proteins secreted by *Streptomyces fradiae* var. k11 after induction with feather were able to be identified based on the current complete *Streptomcyes* genomes. *Streptomcyes* are well known for their extensive ability to utilize diverse substrates for growth (98).

Finally, in conjunction with other studies, this proposal has the possibility of revealing new antibiotics. *Streptomyces* are the major source of antibiotics in use in medical and veterinary practice, with the production of over 500 antibiotics attributed to *Streptomyces* (98). A current genomic initiative at the Broad Institute is to sequence the genomes of approximately twenty *Streptomycetes* for comparative genomics and antibiotic discovery. Since *Streptomyces fradiae* is not included in their list of organisms for which to sequence genomes, our genomic sequence may prove useful for such efforts.

CHAPTER SIX

INSIGHT INTO THE MECHANISM OF KERATIN DEGRADATION BY STREPTOMYCES FRADIAE VAR. K11 THROUGH FUNCTIONAL GENOMICS

6.1 Summary

Our study is the first functional genomics approach to study the mechanism of feather degradation by one of the most effective feather-degrading microbes, *Streptomyces fradiae* var. k11. Due to our inability to analyze proteomic data using existing complete *Streptomyces* genomes, a low quality draft genome was produced. Illumina paired-end sequencing followed by Velvet assembly produced 5,806 contigs (5.3 Mb), from which a draft genome consisting of 3.3 Mb in 2,207 contigs was annotated with the RAST server. A 2D gel analysis followed by tandem MS/MS for protein identification of secreted proteins newly induced or upregulated ≥2 fold by growth on feather versus a control showed numerous proteases and peptidases. Further quantitative 2D-LC MS/MS proteomics of membrane and cytosolic proteins utilizing isobaric TMT tags revealed several other pathways significantly upregulated by growth in the presence of feather versus a control.

6.2 Introduction

The study of microbial keratin degradation has largely been pursued since the 1960s (112, 113). These studies have risen out of the unusual ability of keratinases to degrade a highly durable, insoluble substrate, as well as the desire to turn the feather

byproduct of the poultry industry into a valuable protein feed supplement(46). Feathers are 97% protein, yet are a poor protein source for production animals due to their poor digestion (119). The US poultry industry produces >1 million metric tons of feather waste per year, much of it ending up in landfills. Much of the research on keratin degradation has focused on the isolation of keratin degrading microbes, and the purification and characterization of their keratinases (proteases) (4, 15, 18, 21, 22, 39, 56, 73, 88, 95, 97, 104, 113, 135, 136, 152, 154, 155, 168, 172, 178). More recently, there is evidence suggesting the key step in keratin degradation is the reduction of keratin disulfide bonds (16, 46, 132, 173). *Streptomyces fradiae* has repeatedly been isolated and studied as one of the most efficient keratin degrading microbes (74, 75, 89, 113, 178). However, the mechanism by which it reduces the disulfide bonds of keratin is largely unexplained (16, 74, 75). We aimed to discover the mechanism by which *S. fradiae* reduces the keratin disulfide bonds through a functional genomics approach.

6.3 Experimental Procedures

6.3.1 Strain

Streptomyces fradiae var. k11 was acquired from our collaborators in China (89). *Streptomyces avermitilis* (ATCC #31267) and *Streptomyces violaceoruber* (ATCC #BAA-471) were acquired from the American Type Culture Collection. *Streptomyces coelicolor* was acquired from the John Innes Center.

6.3.2 Draft genome

Genome sequencing was conducted by the method of whole genome shotgun sequencing. *S. fradiae* var. k11 was grown in gause media (89) supplemented with 0.2% agar. Genomic DNA was prepared by standard methods of protease digestion, chloroform extraction and ethanol precipitation (128), then further purified by a silica-based column for sequencing (Promega, Madison, WI). Sequencing was carried out at the Cornell DNA Sequencing Center on an Illumina/Solexa Genome Analyzer, first using a single-end read with a length of 86 bases, then a second round of sequencing using a paired-end read with a length of 86 bases. Genome assembly was conducted by the Cornell Computational Biology Service Unit using Velvet for assembly of contigs (181). The assembled contigs were submitted to RAST for automatic annotation (11). The annotated *S. fradiae* k11 genome was compared to the completely sequenced genomes of *S. avermitilis* and *S. coelicolor* using the SEED Viewer and its corresponding annotations for the genomes (120). The SEED Viewer's sequence based comparison was used for analysis of orthologs between the genomes.

6.3.3 2D gel proteomics of secreted proteins

S. fradiae var. k11 was grown in LB (Luria-Bertani) broth or Gause media with 3% whole feathers for 72 hrs (89). A control strain, *S. avermitilis* was also grown in the same conditions. The proteins in the cell-free culture filtrate were precipitated by trichloroacetic acid and redissolved for 2D (isoelectric focusing and size separation by SDS-PAGE) gel analysis at the Cornell Proteomics and Mass Spectrometry Core Facility. Gels were stained with Colloidal Coomassie Blue (Invitrogen) and images

captured with a Typhoon 9400 at excitation wavelength 633nm. Images were analyzed using Progenesis SameSpots using nonlinear dynamics.

6.3.4 Quantitative 2D-LC MS/MS proteomics of membrane and cytosolic proteins utilizing isobaric TMT tags

A time course of protein expression was analyzed by culturing *S. fradiae* k11 in ISP1 media with or without 0.3% whole feathers for 6, 24 and 72 hrs. at 30°C 250RPM. Membrane and cytosolic proteins were isolated immediately after each time point. Crude filtration was performed using a vacuum filter to pass the culture through glass wool in a funnel to separate whole feathers and feather pieces. Cells were pelleted at 10,000 x g 4°C 5 min, washed with 25mL ddH2O and repelleted. Then the pellet was vortexed in 4.95mL lysis buffer (50mM Hepes pH 7.4, 100mM NaCl, 5mM EDTA, 1mM sodium pyrophosphate, 1mM sodium orthovanadate, 10mM NaF, 10µg/mL leupeptin, 10µg/mL aprotinin) and 50µL 100mM PMSF in EtOH (final 1mM PMSF, added fresh) before sonication at ~20 watts using a 4s burst for 10 min. on ice. Debris was pelleted at 10,000 x g 4°C 10 min. The supernatant with membrane rafts was recentrifuged at 14,000 x g 4°C 10 min., after which the clarified supernatant was centrifuged at 100,000 x g 4°C 30 min. (33,000RPM in 70.1 Ti rotor) to pellet membrane rafts. The pellet was washed with 1mL ice-cold 100mM Na2CO3 pH 11.5, and recentrifuged an additional 15 min. The pellet was completely dissolved in 600µL of solvation buffer (2% SDS, 6M urea, 5mM EDTA in 0.1M TEAB (triethylammonium bicarbonate)). Protein concentration was measured with the BCA protein assay kit (Thermo Scientific), then 100µg of protein was aliquoted into a tube and frozen at -20°C. After all time points were collected, samples were reduced and alkylated with iodoacetamide followed by overnight digestion with trypsin (Promega).

Peptides were labeled with TMT six-plex isobaric tags (Thermo Scientific) according to the manufacturer's instructions. Samples were mixed in equal volumes and subjected to SCX/nanoLC-MS/MS anlaysis at the Cornell Proteomics and Mass Spectrometry Core Facility. Peptide mass fingerprints were searched against a custom database composed of the draft *S. fradiae* k11 genome using Mascot. The significance threshold was set at 0.05 and the ion score cut-off was at 15. Peptide ratios between the feather induced and non-induced cultures respective to time point were bias-corrected. Quantitation was based on unique peptides.

6.4 Results

6.4.1 Draft Genome

The single-end read resulted in 424Mb which assembled into 5.37Mb with 79-fold average depth of coverage, but the mean contig size of 489bp was too short for annotation (Table 6.1). The paired-end read resulted in nearly a doubling of the mean contig size, and assembled into 5.32Mb of an expected 8-10Mb genome (13, 53). After submission to RAST, ambiguous nucleotide stretches were removed to create scaffolds. A total of 3.32Mb of the assembled genome was annotated, with a mean contig size of 1.5kb. The remaining contigs (mean size 0.6kb) were too short for annotation as they lacked complete open reading frames.

The *S. fradiae* k11 genome shares only a portion of the conserved core genome shared by *S. avermitilis* and *S. coelicolor*, yet it has a similar number of unique proteins (Fig. 6.1). This is in contrast to the expected conservation of the core genome as depicted in Figure 6.2. *S. avermitilis* has orthologs for 68.9% of the *S. fradiae* k11

Table 6.1. Genome assembly statistics.

	Single-end Read	Paired-end Read	Scaffolds	Annotated
Contigs	10,983	5,806	5,825	2,207
Mean Contig Size (bp)	489	916	913	1,505
Largest Contig (Kb)	3.8	20.9	20.9	20.9
Total Sequence Size (Mb)	5.37	5.32	5.32	3.32

The *S. fradiae* k11 draft genome is much smaller than the completely sequenced genomes of *S. avermitilis* and *S. coelicolor* (Table 6.2). It has a much larger number of contigs than the complete genomes, but with a similar GC content. The *S. fradiae* k11 draft genome has roughly half the RNAs and protein encoding genes (PEG), which are on average a third shorter than the PEGs of *S. avermitilis* and *S. coelicolor*. Likewise, the subsystem coverage is roughly a third that of *S. avermitilis* and *S. coelicolor*. The *S. fradiae* k11 genome mean intergenic distance is greater, and correspondingly, the coding density is less.

Table 6.2. Comparison of *S. fradiae* k11 draft genome with *S. avermitilis* and *S. coelicolor* complete genomes and SEED subsystem statistics.

Genome Statistics	***S. fradiae* k11**	***S. avermitilis***	***S. coelicolor***
Genome Size (bp)	3,321,597	9,119,895	9,054,847
Number of Contigs	2,207	2	3
Number of Coding Sequences	3,987	7,792	8,154
Number of RNAs	47	108	106
Coding Density (%)	75.3	86.8	88.5
Mean Intergenic Distance (bp)	206	155	128
GC content (%)	72.3	70.7	72.1
Mean AA Length	208	337	326
SD of AA Length	164	307	246
Subsystem Statistics			
Subsystem Coverage (%)	15	24	24
Number of Subsystems in Genome	204	345	350
Protein Encoding Genes			
In subsystem	596	1843	1905
Non-hypothetical	578	1727	1798
Hypothetical	18	116	107
Not in subsystem	3391	5949	6249
Non-hypothetical	1958	3950	4465
Hypothetical	1433	1999	1784

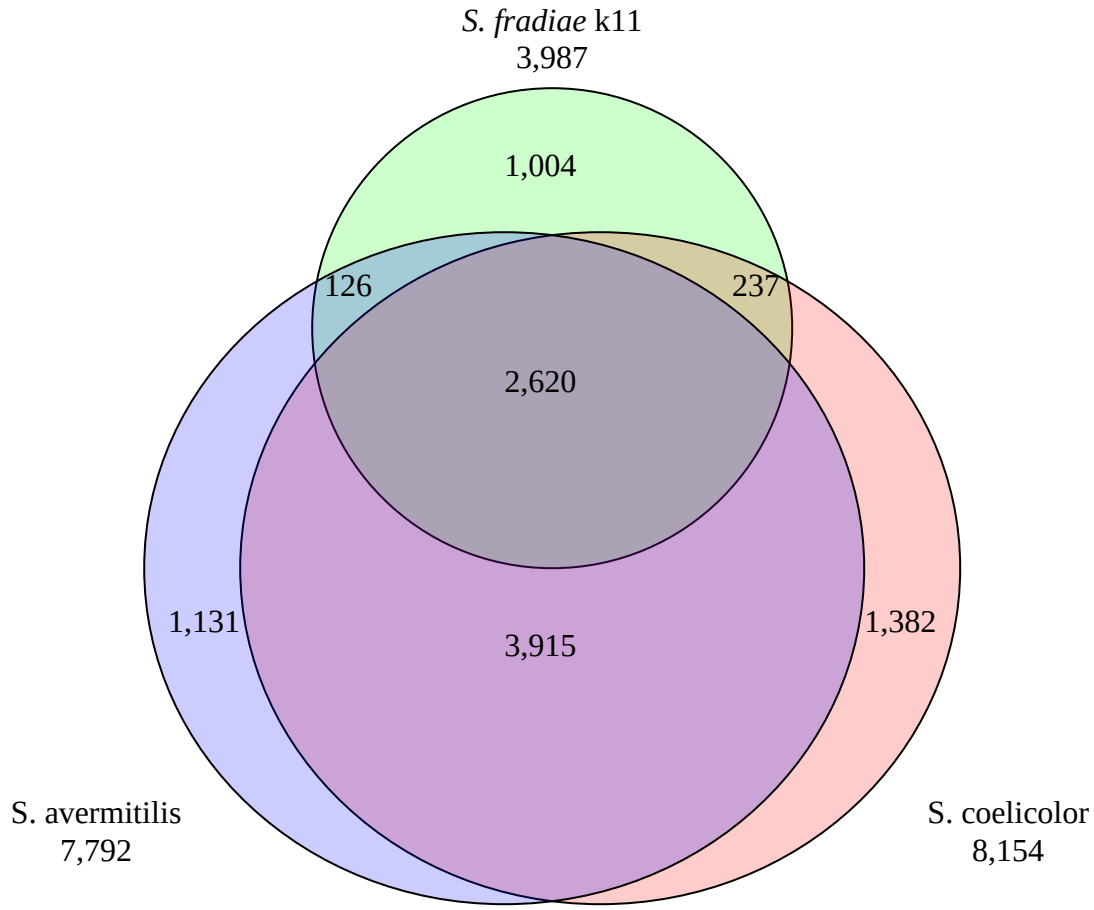


Figure 6.1 Orthologs shared between and unique PEGs of *S. fradiae* k11, *S. avermitilis* and *S. coelicolor*. The total number of PEGs is indicated under the respective organism.

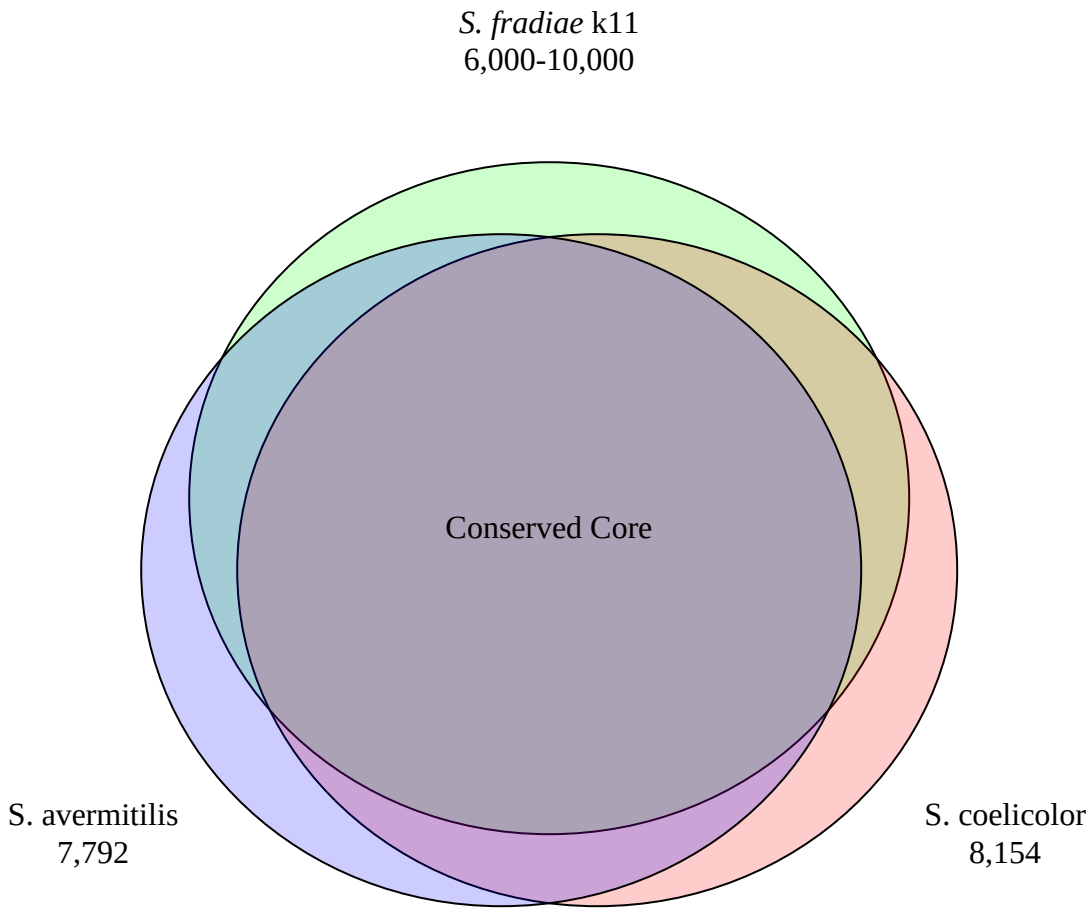


Figure 6.2 Expected distribution of orthologs shared between and unique PEGs of *S. fradiae* k11, *S. avermitilis* and *S. coelicolor.* The total number of PEGs is indicated under the respective organism.

PEGs, while *S. coelicolor* has orthologs for 71.7% of the *S. fradiae* k11 PEGs, with an average protein identity of 69.5 and 69.4% respectively.

Comparison of the SEED subsystems shows *S. fradiae* k11 to contain far fewer subsystems (Table 6.3). Focus is placed on the comparison of the *S. avermitilis* and *S. coelicolor* genomes. The number of subsystem feature counts present in each category in the *S. avermitilis* genome is compared to the number of respective subsystem feature counts present in the *S. coelicolor* genome. Note the extreme case for sulfur metabolism, for which *S. avermitilis* contains 60 feature counts, while *S. coelicolor* contains only 34 feature counts.

Those subsystems thought to be related to the degradation of feather are explored in more detail (Table 6.4). PEG involved in protein utilization, specifically proteases of various types, show no great difference in number between *S. avermitilis* and *S. coelicolor*. On the contrary, striking differences arise between *S. avermitilis* and *S. coelicolor* regarding N and S metabolism. *S. avermitilis* possesses 16 PEG related to nitric oxide synthase, while *S. coelicolor* has none. Also of striking contrast, as already mentioned, are the greater number of *S. avermitilis* PEG related to S metabolism. Specifically, *S. avermitilis* possesses 5 more galactosylceramide and sulfatide metabolism PEG, and 20 PEG related to organic sulfur assimilation compared to 1 for *S. coelicolor.*

6.4.2 2D gel proteomics of secreted proteins

The gel from *S. avermitilis* was completely different and could not be aligned to those of *S. fradiae* var. k11 (Fig. 6.3). Between the *S. fradiae* var. k11 gels from cultures with and without feather, 261 spots were found to be unmatched or upregulated by at least two-fold in either of the cultures. Upon peptide sequence

Table 6.3. Comparison of *S. fradiae* k11 with *S. avermitilis* and *S. coelicolor* SEED subsystems feature counts.

	***S. fradiae* k11**	***S. avermitilis***	***S. coelicolor***	***S.a./S.c.***
Phages, Prophages, Transposable elements	0	1	1	1.0
Secretion systems	0	0	0	NA
Plant-Prokaryote DOE project	0	53	49	1.1
Cofactors, Vitamins, Prosthetic Groups, Pigments	89	248	273	0.9
Cell Wall and Capsule	37	94	111	0.8
Potassium metabolism	4	13	12	1.1
Photosynthesis	0	0	0	NA
Plasmids	0	0	0	NA
Miscellaneous	6	9	14	0.6
Membrane Transport	15	41	64	0.6
RNA Metabolism	11	86	86	1.0
Nucleosides and Nucleotides	36	104	113	0.9
Protein Metabolism	52	212	209	1.0
Cell Division and Cell Cycle	26	33	34	1.0
Motility and Chemotaxis	3	2	1	2.0
Regulation and Cell signaling	0	48	63	0.8
Secondary Metabolism	0	0	9	0.0
DNA Metabolism	24	90	113	0.8
Virulence	13	56	74	0.8
Fatty Acids, Lipids, and Isoprenoids	27	145	171	0.8
Nitrogen Metabolism	13	34	32	1.1
Dormancy and Sporulation	2	2	2	1.0
Respiration	82	119	119	1.0
Stress Response	46	134	130	1.0
Metabolism of Aromatic Compounds	11	41	45	0.9
Amino Acids and Derivatives	188	473	446	1.1
Sulfur Metabolism	7	60	34	1.8
Phosphorus Metabolism	7	39	40	1.0
Carbohydrates	146	510	583	0.9

*S.a./S.c. is the ratio of S. avermitilis to S. coelicolor subsystem feature counts. Ratios with ≥ 50% difference are highlighted.

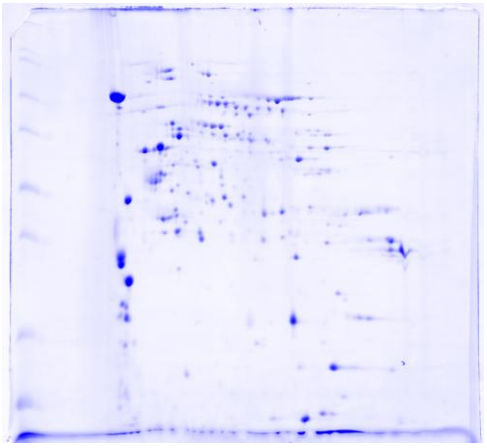
Table 6.4 Comparison of *S. fradiae* k11 with *S. avermitilis* and *S. coelicolor* SEED subsystem feature counts related to protein, N and S metabolism.

	***S. fradiae* k11**	***S. avermitilis***	***S. coelicolor***	***S.a./S.c.***
Protein Metabolism	52	212	209	1.0
Protein processing and modification	12	18	17	1.1
Signal peptidase	2	5	5	1.0
Peptide methionine sulfoxide reductase	3	3	3	1.0
Protein degradation	16	23	25	0.9
Metalloendopeptidases (EC 3.4.24.-)	1	0	1	NA
Putative TldE-TldD proteolytic complex	3	2	2	1.0
Aminopeptidases (EC 3.4.11.-)	2	3	4	0.8
Proteasome archaeal	7	7	6	1.2
Dipeptidases (EC 3.4.13.-)	0	0	1	NA
Proteolysis in bacteria, ATP-dependent	3	11	11	1.0
Nitrogen Metabolism	13	34	32	1.1
Allantoin Utilization	0	5	6	0.8
Nitric oxide synthase	0	16	0	NA
Nitrosative stress	2	1	1	1.0
Ammonia assimilation	5	8	7	1.1
Nitrate and nitrite ammonification	6	4	18	0.2
Sulfur Metabolism	7	60	34	1.8
Inorganic Sulfur Assimilation	0	19	15	1.3
Sulfur Metabolism - no subcategory	7	21	18	1.2
Thioredoxin-disulfide reductase	5	9	11	0.8
Galactosylceramide and Sulfatide metabolism	2	12	7	1.7
Organic sulfur assimilation	0	20	1	20.0
Taurine Utilization	0	4	0	NA
Alkanesulfonate assimilation	0	10	0	NA
Alkanesulfonates Utilization	0	6	1	6.0

*S.a./S.c. is the ratio of S. avermitilis to S. coelicolor subsystem feature counts. Ratios with ≥ 50% difference are highlighted.

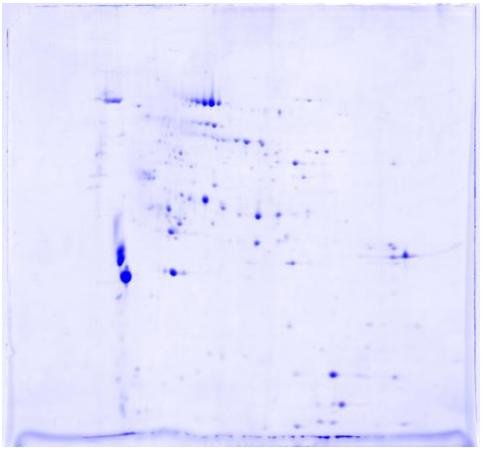
***S. fradiae* var. k11**

Gause + feather



***S. fradiae* var. k11**

LB



S. avermitilis

LB

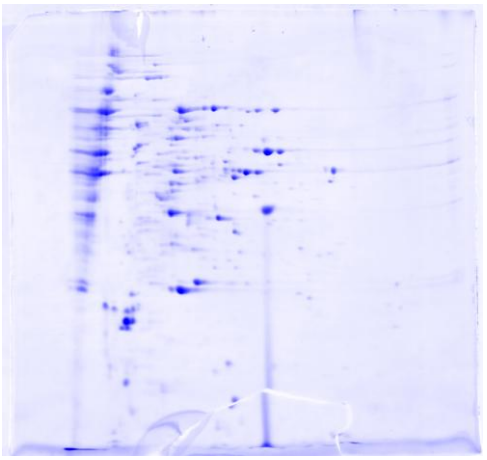


Figure 6.3 2D gels. Supernatant proteins from *S. fradiae* var. k11 grown in LB broth or Gause media with 3% whole feathers for 72 hrs, and from *S. avermitilis* grown in LB for 72 hrs, were separated on a 2D gel by isoelectric focusing and size separation (SDS-PAGE), then stained with Colloidal Coomassie Blue (Invitrogen).

searches in Mascot against the published complete *Streptomyces* genomes, only approximately 20% of the proteins in the spots could be identified. Thus the *S. fradiae* k11 draft genome was used as the database for Mascot search. All but 4 of the proteins could then be identified. The presence of feather in the culture induced 72 new spots, or 29 unique top protein (most abundant in a spot) hits due to protein redundancy in different spots, and 124 upregulated spots, or 49 unique top protein hits due to protein redundancy in different spots. Considering only top protein hits for each spot induced or upregulated by the presence of feather, among the 29 new unique spots, there were 7 proteases/peptidases and 8 P acquisition related proteins. There were 49 unique proteins identified as upregulated ≥2-fold by feather, among them 7 proteases/peptidases and 5 P acquisition related proteins.

6.4.3 Quantitative 2D-LC MS/MS proteomics of membrane and cytosolic proteins utilizing isobaric TMT tags

A total of 460 unique proteins were identified, of which 10.2% were predicted to have ≥1 transmembrane helix by TMHMM (71). Only proteins upregulated ≥2-fold by induction with feather as compared to the control culture at the respective time point were considered in the analysis. At 6 hrs., 26 proteins were induced by feather as compared to the control without feather, among them those involved in energy status maintenance and nitrate reduction. By 24 hrs., the response was much greater in terms of differences from the control without feather, with 66 proteins upregulated ≥2-fold. There were again responses from proteins that are identified to be involved with energy status, oxygen stress/sensing, transcription factors and secretion systems. At 72 hrs., there were 44 proteins upregulated ≥2-fold by feather versus the control without feather, including responses from proteins that are identified to be involved

with secretion systems, protein synthesis machinery (tRNA sythesis and ribosomal proteins), amino acid metabolism and energy status.

6.5 Discussion

We successfully assembled a low quality draft genome of *S. fradiae* k11 using Illumina sequencing to serve as a proteomics database. Most of the proteins in the proteomic experiments were able to be identified using this draft genome, as compared to only 20% using the *S. avermitilis* genome. However, in terms of genomes, this one is very poor. We suspect to have only approximately a third to half the genome, as compared to the size of the *S. avermitilis* and *S. coelicolor* genomes. Around 2Mb of our assembled contigs were not even annotated by RAST, so we know there is more than 2Mb of the *S. fradiae* k11 genome missing from our draft. Furthermore, *S. fradiae* k11 lacks much of the conserved genome core shared between *S. avermitilis* and *S. coelicolor*. And the 3.3Mb that is annotated has a lower coding density, smaller PEG length, and higher intergenic distance as compared to *S. avermitilis* and *S. coelicolor*, which are very close to each other in these respects. All these data point to the fact that there are still many PEG within the 3.3Mb which have not been annotated. This is likely due to the large number of contigs, so PEG are split between contigs, thus not annotated. The smaller PEG length is of concern in that it indicates miscalled open reading frames, which could transcend to misannotation. The Broad Institute has been carrying out a project to sequence Streptomycete genomes. They are using pyrosequencing (Roche 454 GS FLX) sequencing for *de novo* sequencing of the genomes. This results in less genome coverage, but greater read lengths, which aids assembly. For future studies with *S. fradiae* k11, we will pursue

pyrosequencing to vastly improve the draft genome quality. This will in turn lead to better annotation and serve as a better proteomics database.

Since the *S. fradiase* k11 draft genome is known to be missing so much of the genome, it futile to compare its genome to that of *S. avermitilis* or *S. coelicolor*. However, we have found *S. avermitilis* to occasionally degrade feather in culture as well, albeit at a much slower rate that *S. fradiae* k11 (data not shown). Thus the difference in feather degradation by *S. avermitilis* and *S. fradiae* k11 may be due at least in part to expression differences, rather than genomic differences. Thus genomic comparisons between *S. avermitilis* and *S. coelicolor* may provide insight into the mechanism of feather degradation by Streptomycetes. Though *S. fradiae* has been repeatedly reported to efficiently degrade feathers (75, 89, 113, 178), other Streptomycetes have also been reported to degrade feathers (15, 22, 152-154, 172).

First of all, caution must be taken on the comparison of subsystems in the SEED for *S. avermitilis* and *S. coelicolor,* as roughly only a quarter of their genomes are in the subsystems. After review of the subsystem feature counts in *S. avermitilis* and *S. coelicolor,* a striking difference appears on the number of S metabolism related PEG. The number of proteases between *S. avermitilis* and *S. coelicolor,* which are potential candidates for efficient feather degradation, are similar. In fact, *S. coelicolor* has a greater number of proteases/peptidases than *S. avermitilis.* However, *S. avermitilis* has a far greater number of PEG in the S metabolism subsystem. As feather contains a high number of disulfide bonds, it contains a lot of S. This difference is particularly in the organic S assimilation subsystem. This findings correlate with the literature, in that the reduction of disulfide bonds is thought to be the limiting step in proteolysis of keratin. Also, *S. avermitilis* contains a greater number of N metabolism PEGs, particularly those related to nitric oxide synthase. The possible role of these proteins in feather degradation may be explored in future studies.

As expected, feathers greatly induce the secretion of proteases by *S. fradiae* k11 into the media, as evidenced by the 2D gel data. We also found proteins related to P scavenging to be highly induced by feather. However, it is hard to decipher whether this is due to the presence of feather or the change from rich media (without feather) to minimal media (with feather) for the cultures. This was done to maximize expression of feather degrading related genes, but may cause confounding results. Thus rich media was used for the quantitative TMT proteomics. The response to feather in the media is not fully engaged in a short time, as there were many more proteins induced at 24 hrs. by the presence of feather than at 6 hrs. The responses at these times of energy metabolism and oxygen stress may relate to the bacteria's mechanism of disulfide bond reduction. This will need to be explored in future genetic studies. Also of interest is the induction of transcription factors. Studies of these transcription factors, which may regulate the response to feathers, will be very valuable for the elucidation of the response of *S. fradiae* k11 to feathers. By 72 hrs., it is evident that the bacteria is harvesting the protein from the feathers, as evidenced by the increased amino acid metabolism and protein synthesis.

Being the first functional genomics approach to the study of keratin degradation, this research will lay a necessary groundwork and provide direction for future genetic studies to unravel the mystery of keratin degradation by *S. fradie* k11.

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CHAPTER SEVEN

CONCLUSION

We aimed to address the issues of animal agriculture P and N pollution, the depletion of nonrenewable P, and the competition between animals and humans for protein sources. We took a biotechnological approach to accomplish this. We hoped to address the P issue through engineering of a thermostable phytase via disulfide bond engineering. The aim of increasing the thermostability of phytase via this means largely failed. However, unexpected findings related to basic protein structure and function were uncovered. Previous research on disulfide bond engineering has focused on the effect of de novo disulfide bonds on protein stability, while their effect on other biochemical characteristics has been underreported. We detail the effects of de novo disulfide bonds on not just protein stability, but also on the kinetics, pH and optimal temperature profiles, even under conditions when stability is not affected. This will be of particular interest to those trying to engineer disulfide bridges in proteins in order to increase protein stability, tether proteins together, or any other reason. More broadly, this work relates to all interested in protein structures, including those studying protein folding, structure prediction and analysis.

We also explored a potential alternative use of phytase in the degradation of ingested DNA. We excluded this possible additional benefit of phytase supplementation on digestion.

To address the issue of N pollution, we took a functional genomics approach to elucidate the mechanism of *S. fradiae* k11 feather degradation. We have laid a critical groundwork and given direction to future studies of keratin degradation,

particularly the reduction of keratin disulfide bonds. Of more immediate use, we have identified a large number of potential biotechnologically interesting enzymes. *S. fradiae* k11 produces a plethora of proteases/peptidases, the largest class of industrially utilized enzymes. Furthermore, since *S. fradiae* k11 secretes many proteases, other enzymes it secretes are likely to be fairly resistant to proteolysis. Among those of biotechnological interest are phytase, and xylanases and cellulases, which may find use in ethanol production, particularly in that from cellulosic materials. Of potential medical interest, is the production of antibiotics by *S. fradiae* k11. This was the goal behind the Broad Institute's streptomycete genome sequencing project. *S. fradiae* is known to produce neomycin, one of the commonly used antibiotics in topical ointments such as Neosporin. As the genome of *S. fradiae* was not sequenced by the Broad Institute, obtaining a more complete genome may aid in the comparative genomics project and search for cryptic antibiotics.

Through follow up genetic studies, feather waste has the potential of becoming a high protein feed additive for alleviating the competition over protein sources between animal agriculture and human consumption. The next step towards this goal will be to obtain a better draft genome, followed by genetic studies.

APPENDIX

Table A.1 Structural classification of phosphatases and phytases. Data was generated from SCOP database 1.75 with SCOPM 1.101 (6, 7, 96, 110). The outline levels represent the class, fold, superfamily, family, and protein domain. All folds possessing a phosphatase are displayed. Only those parts of the hierarchy containing phytases show superfamily, family, and protein domain details. The four classes of phytases are highlighted in gray. Note, purple acid phosphatases appears twice in the hierarchy, as they also contain an IgG fold.

- **I.** All alpha proteins
  - A. Another 3-helical bundle
  - B. Four-helical up-and-down bundle
  - C. KaiA/RbsU domain
  - D. Acid phosphatase/Vanadium-dependent haloperoxidase
  - E. alpha-alpha superhelix
- **II.** All beta proteins
  - A. Immunoglobulin-like beta-sandwich
    1. Immunoglobulin
    2. Fibronectin type III
    3. Purple acid phosphatase, N-terminal domain
       - a. Purple acid phosphatase, N-terminal domain
         - i. Purple acid phosphatase, N-terminal domain
  - B. C2 domain-like [49561]
  - C. SMAD/FHA domain
  - D. Concanavalin A-like lectins/glucanases
  - E. PDZ domain-like
  - F. 6-bladed beta-propeller
    1. Thermostable phytase (3-phytase)
       - a. Thermostable phytase (3-phytase)
         - i. Thermostable phytase (3-phytase)
- **III.** Alpha and beta proteins (a/b)
  - A. MurF and HprK N-domain-like
  - B. HAD-like
  - C. Phosphotyrosine protein phosphatases I-like

D. (Phosphotyrosine protein) phosphatases II
    1. (Phosphotyrosine protein) phosphatases II
        a. Dual specificity phosphatase-like
        b. Higher-molecular-weight phosphotyrosine protein phosphatases
        c. Myotubularin-like phosphatases
        d. Myo-inositol hexaphosphate phosphohydrolase (phytase) PhyA
            i. Myo-inositol hexaphosphate phosphohydrolase (phytase) PhyA
        e. Mcobacterial PtpB-like
E. Rhodanese/Cell cycle control phosphatase
F. Ribonuclease H-like motif
G. Phosphoglycerate mutase-like
    1. Phosphoglycerate mutase-like
        a. Cofactor-dependent phosphoglycerate mutase
        b. Histidine acid phosphatase
            i. Prostatic acid phosphatase
            ii. Phytase (myo-inositol-hexakisphosphate-3-phosphohydrolase)
            iii. Glucose-1-phosphatase
        c. 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase, phosphatase domain
H. Alkaline phosphatase-like
I. SurE-like
J. ComB-like

IV. Alpha and beta proteins (a+b)

A. SH2-like
B. PHP14-like
C. Profilin-like
D. DNase I-like
E. PP2C-like
F. Metallo-dependent phosphatases
    1. Metallo-dependent phosphatases
        a. Purple acid phosphatase-like
            i. Plant purple acid phosphatase, catalytic domain
            ii. Mammalian purple acid phosphatase
        b. Protein serine/threonine phosphatase
        c. DR1281-like
G. YopH tyrosine phosphatase N-terminal domain

V. Multi-domain proteins (alpha and beta)

A. Carbohydrate phosphatase

VI. Coiled coil proteins

A. Antiparallel coiled-coil

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