

CHARACTERIZATION OF THE ZPS1P CELL WALL PROTEIN FROM
Saccharomyces cerevisiae

By

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by

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This document is dedicated to my fiancé and my family for all their help and support during the last few years.

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I would like to thank my future husband and my family for their moral support, my lab mates for their continuous help and friendship, and my mentor, Dr. Thomas Lyons, for his endless guidance and patience.

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Abstract of Thesis Presented to the Graduate School
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CHARACTERIZATION OF THE ZPS1P CELL WALL PROTEIN FROM
Saccharomyces cerevisiae

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Fungal cell wall proteins are involved in establishing infection through interaction with host ligands and by mediating morphological changes that enhance pathogenicity. In recent years, research has focused on a family of fungal cell wall proteins that are structurally related to zinc-dependent metalloproteases of the M35 clan. Members of this protein family include Zps1p from *Saccharomyces cerevisiae*, Pra1 from *Candida albicans*, CpAspf2 from *Coccidioides posadasii*, Aspnd1 from *Aspergillus nidulans*, and Aspf2 from *Aspergillus fumigatus*. The proteins from *C. albicans* and *Aspergillus spp.* are known cell-surface antigens during fungal infections, and both Pra1 and Aspf2 bind specific ligands within mammalian hosts. Although expression of these proteins during fungal infection is well documented, their biological function remains unknown. In this thesis, we report preliminary work toward characterization of Zps1p from *S. cerevisiae*. Results indicate expression of *ZPS1* to be regulated in response to zinc- and iron-limitation, as well as extracellular pH. In addition, we present the partial purification of

recombinant Zps1p from bacterial inclusion bodies. Analysis of Zps1p is intended to provide the framework for future expression, purification, and characterization of the Zps1p-like proteins from the medically important fungi *C. albicans* and *Aspergillus spp.*

CHAPTER 1 INTRODUCTION

Many fungi are responsible for both superficial and systemic infections in man. Immunocompromised individuals are susceptible to fungal infections caused by a variety of pathogens, including *Candida albicans* and several *Aspergillus* species. Relevant diseases caused by these species include candidiasis, aspergilloma, invasive aspergillosis, and allergic bronchopulmonary aspergillosis (ABPA). Although these mycoses are well documented, many factors contributing to fungal pathogenesis are still not well understood. Efforts to better understand virulence factors often focus on components of the fungal cell wall.

The fungal cell wall is a complex mixture of carbohydrates (80 to 90%), proteins (6 to 25%), and minor amounts of lipid (1 to 7%).¹ As the outermost part of the cell, the wall initiates physical interaction between the microorganism and the environment, including the host. The host-parasite interaction, resulting in adhesion, is the first critical step in establishing infection and modulation of the host immune response. In addition, the cell wall mediates fungal cell-cell adhesion (flocculation), a first step in the morphological change from a unicellular yeast to growth as multicellular filaments (mycelia or hypha). Formation of mycelia enhances pathogenicity, allowing the invasion of host tissues, and is influenced by environmental variables including extracellular pH^{2,3} and nutritional status.⁴ For these reasons, fungal cell wall proteins (CWPs) have been of heightened interest. Not only are CWPs involved in intercellular binding, many possess enzymatic activity involved in cell wall biosynthesis and maintenance, and acquisition of

extracellular nutrients.¹ When the actions of CWPs negatively impact the viability of the host, the proteins are considered virulence factors that advance the establishment of infection. Due to their accessibility at the cell surface, and their critical role in intercellular interactions, CWPs are ideal targets for the development of antifungal drugs.

A family of cell wall proteins from various fungi has been the focus of much research in recent years. Members of this family include Zps1p from *Saccharomyces cerevisiae*, Pra1 from *Candida albicans*, CpAspf2 from *Coccidioides posadasii*, and Aspnd1 and Aspf2 from *Aspergillus nidulans* and *Aspergillus fumigatus*, respectively. These CWPs share a number of key structural features, have high sequence homology, and exhibit significant similarity to a family of zinc-dependent metalloproteases of the M35 clan, known as the aspzincins⁵ (Figure 1-1). At present, the biochemical function of these proteins remains unknown.

The focus of this research is to characterize the Zps1p cell wall protein from the yeast *Saccharomyces cerevisiae*. This document entails the preliminary work toward characterization of Zps1p through study of its structure, function, and gene regulation in *S. cerevisiae*. Analysis of Zps1p is intended to provide the framework for future expression, purification, and characterization of the Zps1p-like proteins from the medically important fungi *C. albicans* (Pra1) and *Aspergillus spp.* (Aspnd1 and Aspf2). The relevance of the Zps1p-like proteins is discussed below.

Zps1p-like Proteins from *Candida albicans* and *Aspergillus spp.*

The homologues from *A. fumigatus* and *A. nidulans* are known as Aspf2⁶ and Aspnd1,⁷ respectively. In *C. albicans*, the homologue is known by many names: Pra1 (pH regulated antigen),⁸ FBF (fibrinogen binding factor),⁹ FBP1 (fibrinogen binding protein),¹⁰ and mp58 (58-kDa fibrinogen-binding mannoprotein).¹¹

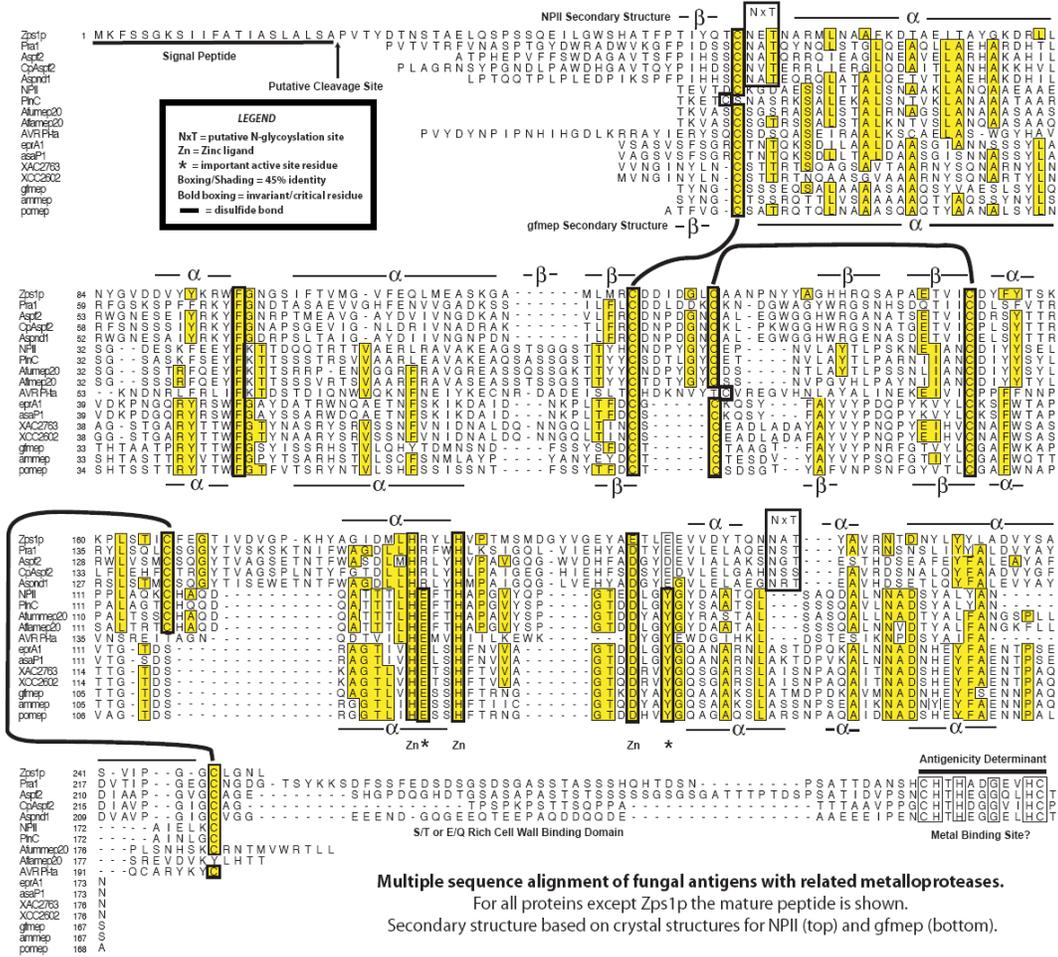


Figure 1-1. Multiple sequence alignment of fungal cell wall proteins with related metalloproteases. Aspf2, Aspd1, and Pra1 are all secreted proteins with four N-glycosylation sites (Asn-X-Ser/Thr) and eight cysteine residues perfectly conserved, suggesting a similar function.^{6;7;11} Both Pra1 and Aspf2 contain a serine- and threonine-rich region of potential O-glycosylation close to the C-terminus, a purported cell wall binding domain (CWBD).¹² Studies have shown that Pra1 does indeed contain O-linked sugar moieties.¹¹ Aspd1 lacks this Ser- and Thr- rich region. However, the C-terminal region of Aspd1 is glutamate- and glutamine-rich, the purpose of which is unclear. Although their biochemical function remains unknown, these three proteins from *Candida* and *Aspergillus* are immunodominant antigens in fungal infections.

Purification and characterization of various *Aspergillus spp.* cell wall proteins for use in immunodiagnosis of ABPA and other diseases led to the identification of AspF2 and Aspnd1 as proteins that elicit a strong immune response.^{6;7;13;14} These discoveries were made by immunoblotting water-soluble extracts of *Aspergillus spp.* with sera from patients with ABPA. Sera from those infected with different forms of aspergillosis contain elevated levels of immunoglobulin G (IgG) and immunoglobulin E (IgE) antibodies specific for *Aspergillus* antigens, including Aspnd1 and AspF2. These antigens are consistently recognized by serum samples from aspergilloma patients, but not with sera from control or healthy individuals.^{7;14} Deglycosylated forms of the purified proteins remain reactive to the antibodies, suggesting the N-glycosidic groups are not required for recognition by the aspergillosis serum samples tested. These data indicate that the epitopes recognized are located mainly in the polypeptide region.^{7;13} This hypothesis is further supported by antibody reactivity with the recombinant forms of Aspnd1 and AspF2 which, when over-expressed in the prokaryote *E. coli*, lack the glycosidic moieties.^{15;16} Furthermore, all reactivity was abolished following protease treatment.^{7;13}

During characterization of the Aspnd1 antigen from *A. nidulans*, Calera *et al.* observed sera that reacted with Aspnd1 also consistently reacted with antigens from *A. fumigatus*. Therefore, they tested the reactivity of purified anti-Aspnd1 specific IgG with several different *A. fumigatus* antigens including AspF2 (also known as gp55). The various antigens reacted with the anti-Aspnd1, suggesting a close relationship between the immunodominant antigens from the two *Aspergillus* species, possibly through the existence of common peptide epitopes.⁷ Likewise, Banerjee *et al.* detected binding of

anti-Aspf2 antibodies by Aspnd1. The relatedness of Aspnd1 and Aspf2 is supported through analysis of their primary structure. The similarity between the two proteins suggests they share several epitopes and should therefore elicit the formation of IgG and IgE antibodies able to recognize both antigens. To answer this question, Banerjee *et al.* compared the reactivity of purified Aspnd1 and Aspf2 toward IgE antibodies from ABPA serum. The mean IgE binding of purified Aspf2 was almost three fold higher than binding of Aspnd1. Differences in Aspnd1 and Aspf2 binding to IgE may result from differences in posttranslational modifications or the tertiary structures of these proteins.⁶ Comparison of IgE binding by both native and recombinant forms of these antigens indicates that the recombinant forms most likely have structures functionally comparable to the native proteins, since IgE binding is dependent on proper three-dimensional structure.^{15;16}

Similarly, Pra1 from *C. albicans* elicits a strong immune response with sera from patients infected with candidiasis. Antibodies against Pra1 are present in sera from patients with systemic candidiasis,^{17;18} and Pra1 itself has been detected in the cell wall of clinical isolates of *C. albicans*.¹⁷ Deglycosylation of Pra1 did not affect reactivity with anti-Pra1 antibodies, again suggesting the epitopes recognized are located mainly in the polypeptide region. An extensive epitope-scanning study, employing a complete set of overlapping dodecapeptides deduced from the Pra1 sequence, identified several immunoreactive continuous B-cell epitopes within the protein sequence. Six regions of elevated reactivity were identified, including four internal regions and both the amino and carboxy termini of the mature polypeptide. Of these regions, the C-terminal domain was

highly reactive towards the anti-Pra1 antibodies and therefore subjected to further epitope mapping.¹⁸

Analysis of the epitopic region at the C-terminal domain of the Pra1 polypeptide identified the nonapeptide ²⁹⁰HTHADGEVH²⁹⁸ as the minimal region required to retain antibody-binding activity. Researchers further probed the significance of this epitopic region by synthesizing a synthetic peptide corresponding to the last ten amino acid residues at the C-terminus. The synthetic peptide, coupled to keyhole limpet hemocyanin (KLH), was used to immunize two mice. The serum samples obtained from the two immunized mice were able to recognize Pra1 from cell wall extracts of *C. albicans* with high specificity. Interestingly, this C-terminal sequence (CHTHxxGxxHC) is conserved in both Aspnd1 and Aspf2, as are the four internal epitope regions (Table 1-1). The conservation of linear epitopes within this family of cell wall proteins from various fungal pathogens provides additional support suggesting their role in the host-parasite interaction.¹⁸

Table 1-1. Sequence homology of five of the identified IgG epitopes.¹⁸

Pra1 epitope sequence	Antigen Sequence	
	Aspnd1	Aspf2
LRFGSK	LRWGNE	LRWGNE
RKYF	RKYF	RKYF
NDGWAGYW	LEGWGGHW	LEGWGGHW
DVYA	EVYA	EAYA
HTHADGEVH	HTHEGGELH	HTHEGGQLH

Not only do these fungal antigens interact with antibodies within the host, but Aspf2 and Pra1 also bind specific host ligands. Fungal adhesion to host cells and tissues initiates establishment of infection and is considered a potential virulence factor. Aspf2

binds the extracellular matrix protein laminin,^{6;19} and Pra1 binds the serum protein fibrinogen.^{9;11}

Proteins in the extracellular matrix (ECM) are known to bind to *A. fumigatus* conidia (an infectious airborne form of the fungus). Binding of conidia to ECM ligands is believed to be a crucial first step initiating aspergillosis, and specific recognition of these ligands may greatly influence pathogenicity.^{20;21} One component of the ECM is laminin, a multidomain glycoprotein and major component of the basement membrane. Interaction of laminin with cell surface ligands facilitates cell-cell adhesion, cell migration and cell differentiation.¹ As reported by Banerjee *et al.*, laminin shows a dose-dependent interaction with Aspf2. Both native and recombinant Aspf2 demonstrate high binding affinity to laminin, with greater affinity observed by the native protein. With specific binding to laminin, and significant homology to Pra1 (which binds fibrinogen), the involvement of Aspf2 in fungal adherence to the ECM may play an important role in establishing pathogenicity.⁶

Blood serum proteins (e.g., serum albumin, transferrin, fibrinogen, complement fragments C3d and iC3b) are additional targets for fungal binding. Interactions of *C. albicans* with fibrinogen have been well characterized.¹ In 1987, Bouali *et al.* identified a fibrinogen binding factor (FBF) on the surface of *C. albicans* germ-tubes and mycelium, the fungal forms most often found in infected tissues.⁹ Five years later, in 1992, Casanova *et al.* identified this FBF as a 58-kDa fibrinogen-binding mannoprotein (mp58), which is now known to be Pra1. Binding of Pra1 to fibrinogen is apparently specific, since binding to other mammalian proteins tested (laminin, fibronectin, C3d, type IV collagen) was not observed. O-deglycosylated Pra1 was unable to interact with

fibrinogen, implying this carbohydrate domain may play a role in binding. The *in vivo* production of Pra1 during candidiasis and its ability to bind fibrinogen suggest a role in infection.^{1;11}

Another factor supporting an active role of Pra1 in candidiasis is its differential expression in response to pH.⁸ The ability of *C. albicans* to grow and differentiate over a broad pH range is critical for its survival in a variety of environments and host tissues (*e.g.*, blood, pH ~ 7.2; vaginal tract, pH ~ 4.5).³ Extracellular pH is an environmental signal that regulates the yeast-to-mycelia transition *in vivo*, a morphological change that greatly enhances invasion of host tissues.² In *C. albicans*, gene expression is regulated by the Rim101p transcription factor in response to alkaline pH.²² Studies have shown the *C. albicans* Rim101p pH response pathway to be required for several host-pathogen interactions, and therefore essential for pathogenesis.²³ Pra1 is a Rim101p target gene maximally expressed at neutral pH, with no detectable expression below pH 6.0. However, ambient pH is not the sole factor influencing expression. When cultured in rich medium (YPD) buffered at pH 7.0, no Pra1 production was detected. This result implies partial regulation by nutritional status, a hypothesis that remains to be tested.⁸

Although the effect of nutritional status on Pra1 expression has not been assessed, the nutrient regulation of the *Aspergillus spp.* antigens has been investigated. Researchers recognized that production of Aspnd1 and AspF2 only occurred when the fungi are grown in certain conditions, especially in Czapek-Dox (CD) medium (3g NaNO₃, 0.5g MgSO₄·7H₂O, 0.5g KCl, 55mg FeSO₄, 1g KH₂PO₄, and 30g sucrose per liter). Therefore, the various components of CD medium were tested to determine which is influencing antigen production. Elucidation of the regulatory elements responsible for

Aspnd1 and Aspf2 expression may provide clues to their function and potential roles in virulence.²⁴

Variations of CD medium were quantitatively and qualitatively tested against a control medium, AMM (1% glucose, 0.6% NaNO₃, 0.052% MgSO₄, 0.052% KCl, 0.15% KH₂PO₄, and traces of FeSO₄ and ZnSO₄), known not to stimulate Aspnd1 or Aspf2 production under normal conditions. The type and amount of carbon or nitrogen source did not affect antigen production, nor did addition of iron to the CD medium. However, addition of μ -molar concentrations of zinc eliminated antigen synthesis in CD, while removal of zinc from AMM medium induced antigen production. Addition of other divalent metals (Co²⁺, Ni²⁺, Cu²⁺, Ca²⁺) had no inhibitory effects, with the exception of Cd²⁺ and Mn²⁺ (only slight inhibition). Currently, researchers are attempting to identify and characterize potential zinc response elements (ZREs) in the promoter regions of *ASPND1* and *ASPF2*.²⁴ Detected in the promoter region of *ASPND1* are at least five potential PacC binding sites.⁷ PacC is a pH responsive transcription factor in *Aspergillus spp.* and is homologous to *C. albicans* Rim101p.²⁵ The presence of putative PacC sites suggests possible regulation of Aspnd1 expression in response to ambient pH.

Regulation of Aspnd1 and Aspf2 expression by zinc deficiency may play an important role in pathogenesis. During infection, the host environment is one of nutritional limitation. In efforts to starve invading pathogens, part of the acute-phase response of the human immune system is to redistribute micronutrients like iron and zinc to the liver.²⁶ Therefore, this regulation may illustrate the role of zinc's nutritional status as a signal for fungal pathogens of a host environment, initiating transcription of genes involved in zinc acquisition and transport or commencement of pathogenesis. Bacterial

hemolysins offer precedent for the metalloregulation of virulence factors.²⁷ The partial regulation of Pra1 expression by nutritional status may also have important implications in pathogenesis. The effect of zinc limitation on Pra1 expression may be worth investigation, since zinc deficiency has been shown to induce mycelium formation in several dimorphic yeasts.^{4;28}

Zps1p from *Saccharomyces cerevisiae*

Within the family of cell wall proteins, the homologue from *Saccharomyces cerevisiae* is Zps1p (Yol154w). Although *S. cerevisiae* is typically nonpathogenic, it is able to infect immunocompromised individuals²⁹ and colonize complement factor five-deficient mice.³⁰ Zps1p is a secreted cell wall protein,³¹ with two putative N-glycosylation sites and six cysteine residues conserved with respect to Pra1, Aspnd1, and Aspf2. Unlike the homologues from *Candida* and *Aspergillus spp.*, Zps1p has a truncated C-terminal region lacking the potential cell wall binding domain and the conserved antigenicity determinant. Like its related fungal antigens, the function of Zps1p is also unknown. Disruption of the *ZPS1* gene failed to reveal any strong phenotype and resulted in a viable strain, indicating that Zps1p is non-essential.³² Although the function of Zps1p is unknown, many research groups have provided information about its regulation. Interestingly, Zps1p expression is regulated by some of the same factors as the other fungal antigens, including zinc limitation and extracellular pH.

In *S. cerevisiae*, the transcription factor Zap1p is activated by zinc deficiency.³³ DNA microarray data has shown Zap1p regulates expression of 46 genes in *S. cerevisiae* under zinc deficient conditions. Of these genes, one of the most heavily induced is *ZPS1*. This result was confirmed by measuring zinc-regulation of a *ZPS1-lacZ* reporter

construct, resulting from fusion of the *ZPS1* promoter region (-1000 bp to ATG) to the *lacZ* reporter gene. Zap1p activates gene transcription during zinc deficiency by binding to a zinc response element (ZRE) upstream of the target gene's start codon. The consensus ZRE recognized by Zap1p is ACCTTNAAGGT. Within the *ZPS1* promoter region are two putative ZREs between -300 and -340 bp upstream of the start codon: ACCTTCAGGGT (-328 to -318) and ACCCTGAAGGT (-313 to -303). DNA microarray data indicated that *ZPS1* was induced 14 fold by zinc deficiency, while *ZPS1-lacZ* fusion constructs were 10 times more inducible.³⁴

Expression of *Zps1p* is also affected by alkaline pH. This regulation is dependant on the *S. cerevisiae* Rim101p transcription factor, which is homologous to the Rim101p and PacC transcription factors of *Candida* and *Aspergillus*, respectively. Using the *ZPS1-lacZ* construct, researchers observed a 100-fold increase in expression at pH 8 compared to pH 4, while alkaline induction did not occur in a *rim101Δ* strain. In addition, *ZPS1* is more highly expressed in yeast strains harboring a hyperactive allele of Rim101p at pH 4.³⁵ This direct regulation of *ZPS1* expression by Rim101p is intriguing, for not only is Rim101p structurally similar to Zap1p, but these two proteins also interact *in vivo*,³⁶ suggesting they may co-regulate *ZPS1* expression.

Potential regulation of *ZPS1* by environmental iron status has also been implied in work studying iron-regulatory systems in yeast.³⁷ In *S. cerevisiae*, iron homeostasis is regulated by the Aft1p transcription factor in response to low-iron conditions.³⁸ In addition, *S. cerevisiae* contains a homologue of Aft1p, known as Aft2p, which regulates transcription of many of the same genes as Aft1p during iron deficiency.³⁷ In strains harboring a hyperactive allele of Aft2p, activation of *ZPS1* was increased over 8 fold

when compared to wild type strains. This effect is dependent on Zap1p, suggesting Aft2p activity affects zinc metabolism.³⁷ The data obtained through study of *ZPS1* regulation further support its similarity to the fungal antigens from *C. albicans* and *Aspergillus spp.*

Zinc-dependent Metalloproteases of the M35 Clan

Based on sequence comparison and structural predictions, the Zps1p-like proteins show similarity to zinc-dependent metalloproteases of the M35 clan⁵ (known as the aspzincins). Included in this subfamily of secreted metalloendopeptidases (MEPs) are deuterolysin (neutral proteinase, NP11, aspzincin) from *Aspergillus oryzae*,³⁹ penicilloysin (PlnC) from *Penicillium citrinum*,⁴⁰ mep20 from both *Aspergillus fumigatus* and *Aspergillus flavus*,⁴¹ and the AVR Pi-ta avirulence determinant from *Magnaporthe grisea*.⁴² Many of these species are known pathogens. This protein family is characterized by a leader sequence directing the protein into the secretory pathway, a long pro-peptide that is cleaved during secretion, a mature polypeptide that contains three disulfide bonds, and two highly conserved motifs: HExxH and GTxDDxxYG.⁴³ A crystal structure of deuterolysin,⁴³ supported by site directed mutagenesis studies,³⁹ indicated the two histidine residues of the HExxH motif, the second aspartate residue of the GTxDDxxYG motif, and two water molecules were the zinc binding ligands. The conserved glutamate is a catalytic residue, promoting the nucleophilic attack of a water molecule on the carbonyl moiety of the substrate. The conserved tyrosine residue interacts with the second zinc bound water molecule, possibly stabilizing the transition state by hydrogen bonding interactions.⁴³ Figure 1-2 shows the crystal structure of the active site residues.

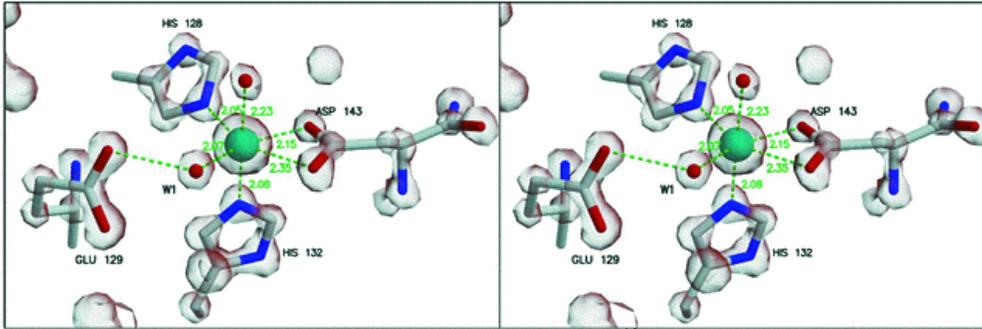


Figure 1-2. Active-site residues of deuterolysin.⁴³

More distantly related members of the M35 clan include GfMEP from *Grifola frondosa*, PoMEP from *Pleurotus ostreatus*,⁴⁴ AmMEP from *Armillariella mella*,⁴⁵ eprA1 from *Aeromonas hydrophila*,⁴⁶ asaP1 from *Aeromonas salmonicida*,⁴⁷ XAC2763 from *Xanthomonas axonopodis*, and XCC2062 from *Xanthomonas campestris*.⁴⁸ Many of these species are also pathogenic. Furthermore, AmMEP from the edible mushroom *A. mella* is known to hydrolyze fibrinogen.⁴⁵ The crystal structure of GfMEP has been solved. Despite one less disulfide bond, GfMEP possesses a near identical fold and active site as deuterolysin, suggesting a conserved mechanism.⁴⁹

The substrate specificities of deuterolysin, PlnC, and mep20 are toward basic polypeptides. Both deuterolysin and PlnC show high activities on the basic nuclear proteins histone, protamine, and salmine, but very low activities on milk casein, hemoglobin, albumin, and gelatin.³⁹ Further analysis of deuterolysin's substrate specificity indicates high proteolytic activity toward the peptide bonds next to pairs of basic residues.⁵⁰ GfMEP and PoMEP have strict specificity toward acyl-lysine bonds, also basic in nature.⁴⁴ Analysis of the GfMEP structure reveals an electrostatically negative region that attracts a positively charged lysine side chain of a substrate.⁴⁹

Comparison of the Zps1p-like Proteins and the M35 Metalloproteases

Although Zps1p and the related fungal antigens possess similarities to the M35 clan (*i.e.*, secretory signal, conserved cysteine residues), they differ in their most highly conserved motifs. Figure 1-3 illustrates the basic structural features of the Zps1p-like proteins and the M35 proteases.

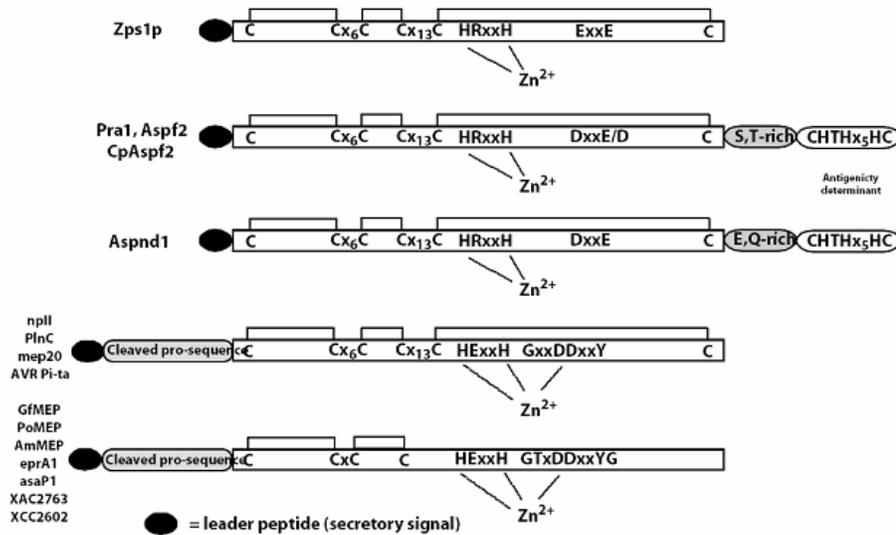


Figure 1-3. Basic structural features of the Zps1p-like proteins and the metalloproteases in the M35 clan.

The Zps1p-like proteins lack the HExxH and GTxDDxxYG motifs found in the metalloproteases. However, they contain highly conserved HRxxH and D/ExxD/E motifs, which may serve as functional replacements enabling metal binding and potentially proteolytic activity. Figure 1-4 compares the known active site structure of the metalloproteases in the M35 clan with a possible structure in the Zps1p-like proteins.

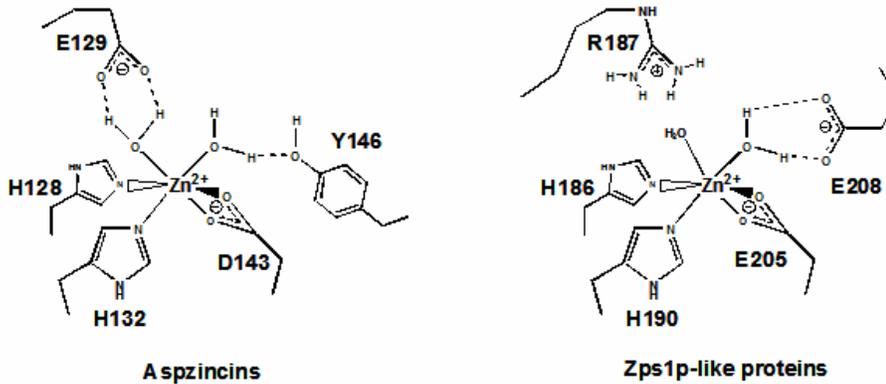


Figure 1-4. Active site structures. On the right is the known active site of the aspzincins, deduced from the crystal structures of deuterolysin⁴³ and *Gf*MEP.⁴⁹ On the left is a possible structure of an active site within the *Zps1p*-like proteins.

Despite their similarity to the M35 metalloproteases, it is quite possible that the *Zps1p*-like proteins do not act as metal-binding proteins or possess proteolytic activity. However, the HRxxH and D/ExxD/E motifs highly conserved within the *Zps1p* family of cell wall proteins may act as peptide binding ligands, enhancing potential virulence within a host. The significance of these motifs can be thoroughly probed through study of purified *Zps1p* structure and function.

CHAPTER 2 RESULTS AND DISCUSSION

Regulation of *ZPS1* Gene Expression

ZPS1 regulation in *S. cerevisiae* was studied using the *ZPS1-lacZ* reporter construct. β -Galactosidase activity, reported in Miller units, was measured as a function of growth condition. To confirm the dependence of Zap1p on *ZPS1* regulation, we monitored the responsiveness of the *ZPS1-lacZ* reporter to zinc deficiency. Simultaneously, we further probed the apparent regulation by iron status by measuring activity of the *ZPS1-lacZ* reporter in response to growth under iron deficient conditions and combined zinc- and iron-limitation (Figure 2-1).

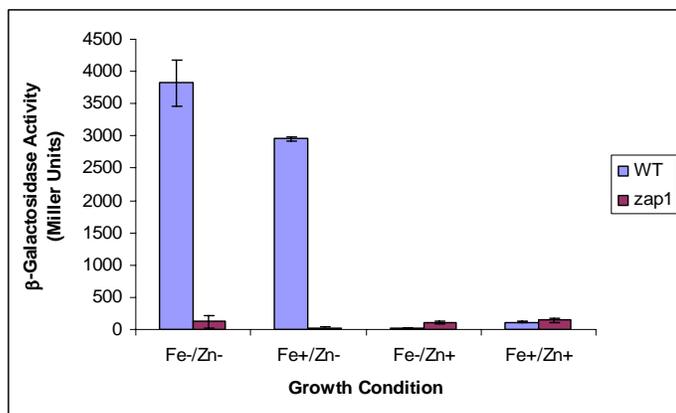


Figure 2-1. Zinc and iron responsiveness of the *ZPS1-lacZ* reporter. β -Galactosidase activity in wild-type cells and *zap1* mutant cells grown in CSD with or without 10 μ M iron and/or zinc added.

The *ZPS1-lacZ* reporter construct was indeed regulated by zinc in a Zap1p dependent manner, with no detectable expression in a *zap1* knockout strain. Induction was only observed when the yeast were grown under zinc-deficient conditions, with no measurable increase in *ZPS1-lacZ* activity when the yeast were grown under solely iron

deficient conditions. When the yeast were grown under both zinc- and iron-limitation, a significant increase in *ZPS1-lacZ* activity was observed. As the literature previously suggests, the increased *ZPS1* expression by iron-deficiency may be due to Aft2p.³⁷ To further investigate this hypothesis, future work may involve monitoring *ZPS1* expression in strains lacking Aft2p, Aft1p, or both.

Previously, reports have described *ZPS1* regulation by Rim101p in response to alkaline pH.³⁵ Therefore, we attempted to study *ZPS1-lacZ* activity in response to iron and/or zinc deficiency at both acidic and alkaline pH. Under standard growth conditions, the Chelex-treated synthetic defined medium (CSD) used to limit zinc and iron availability is at pH 4 (optimal for yeast growth). When the CSD medium was buffered to pH 8.0, the metals in the medium became insoluble and precipitated out of solution. Therefore we were limited to monitoring the effects of Rim101p on *ZPS1-lacZ* expression at acidic pH (Figure 2-2). When compared to wild type yeast, strains lacking Rim101p exhibit a significant decrease in *ZPS1-lacZ* activity, which remained a function of zinc-deficiency. This result suggests that Rim101p affects *ZPS1* expression even at acidic pH, possibly by enhancing Zap1p regulation of this gene. These observations further support the hypothesis that Zap1p and Rim101p co-regulate *ZPS1*.

Partial Purification of Zps1p from Inclusion Bodies

We are currently attempting to purify recombinant Zps1p from *Escherichia coli* for use in characterizing Zps1p structure and function. Although Zps1p is not native in *E. coli*, expression of yeast proteins in bacterial systems has several advantages, including high yield and lack of glycosylation moieties, which may complicate protein purification.

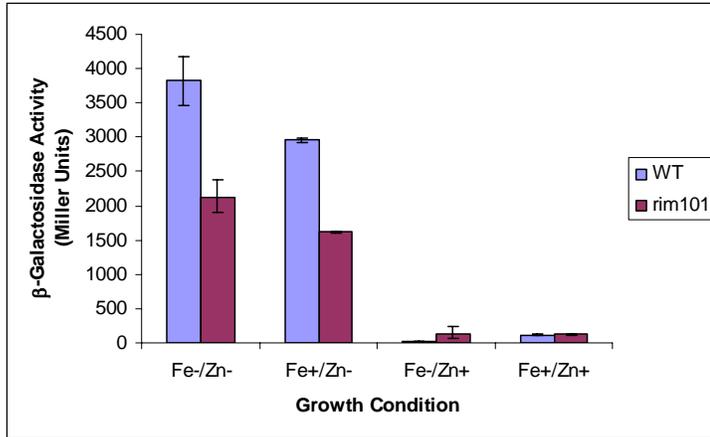


Figure 2-2. Zinc and iron responsiveness of the *ZPS1-lacZ* reporter. β -Galactosidase activity in wild-type cells and *rim101* mutant cells grown in CSD (pH 4.0) with or without 10 μ M iron and/or zinc added.

As described in the Materials and Methods section, *ZPS1* (lacking the leader peptide sequence) has been cloned by the polymerase chain reaction (PCR) and inserted into the pET-22b(+) expression vector for isopropyl- β -D-thiogalactopyranoside (IPTG) inducible expression by bacteriophage T7 RNA polymerase in BL21(DE3) *E. coli*. When expressed, the mature form of Zps1p should have an apparent molecular weight of \sim 25.5 kDa. Approximately eight hours of induction by IPTG is required for optimum Zps1p yield (Figure 2-3).

Following large scale induction (as described in Methods section), the resulting pellet was thawed and resuspended in 20 mL of cold 50 mM Tris(hydroxymethyl)aminomethane (Tris), buffered at pH 7.4, containing 1 mM phenylmethanesulfonyl fluoride (PMSF), a protease inhibitor used to prevent degradation of Zps1p. The cells were lysed using several cycles of French press at 4oC. Multiple rounds of French press were required to adequately break the large cell pellet resulting from 8 h of growth. The resulting lysate was centrifuged at 19,000 rpm for 20 min at 4oC and the supernatant was decanted and saved.

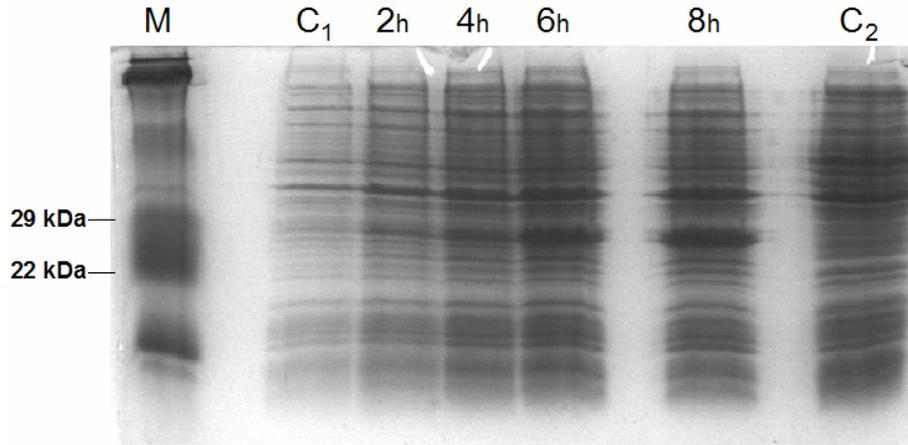


Figure 2-3. SDS-PAGE analysis of *E. coli* transformants containing the pET-22b(+)-*ZPS1* expression vector not induced (lane C) or induced with IPTG for the number of hours indicated (lanes 2 - 8 h). For comparison, products from non-induced cells after 8 h growth are also shown (lane C₂). In lane M is a molecular weight marker.

At this time, the soluble (supernatant) and insoluble (pellet) components of the lysate were analyzed by SDS-PAGE to determine the location of Zps1p (Figure 2-4). Zps1p was present in the insoluble fraction, indicating the protein accumulates as inclusion bodies (dense aggregates of misfolded polypeptide). Formation of recombinant Zps1p inclusion bodies is not unexpected, given that expression of recombinant Aspnd1p in *E. coli* also results in inclusion body formation.¹⁵

To solubilize the inclusion bodies, 5 mL of 50 mM Tris (pH 7.4) containing 8 M Urea was used to denature the Zps1p aggregates by gentle mixing overnight. Next, we attempted to refold the denatured protein by single-step dilution. This entailed slowly (> 24 h) dripping 50 mL of buffer into the sample so to gradually decrease the concentration of Urea to ~ 0.7 M. Dilution was followed by dialysis to remove all traces of the denaturant and the sample was centrifuged at 8,000 rpm for 20 min at 4°C to collect any insoluble material. The soluble and insoluble components were analyzed for Zps1p

content by SDS-PAGE (Figure 2-5). The results indicated Zps1p was successfully solubilized, with only trace amounts in the insoluble fraction.

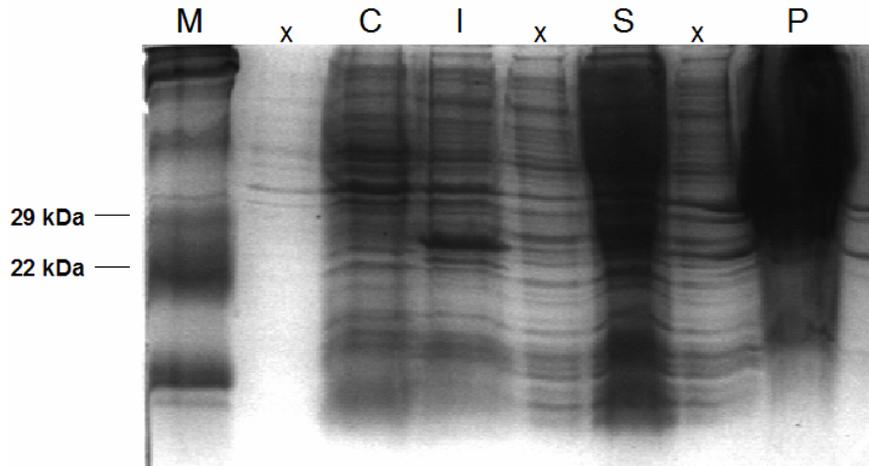


Figure 2-4. SDS-PAGE analysis of soluble and insoluble components of the cell lysate obtained from breakage of *E. coli* expressing Zps1p. Lane M, molecular weight marker; Lane C, non-induced *E. coli* (8 h growth); Lane I, IPTG induced *E. coli* (8 h growth); Lane S, soluble fraction; Lane P, insoluble fraction. In attempts to load the maximum sample volumes to each lane, runoff into neighboring lanes occurred (Lanes X).

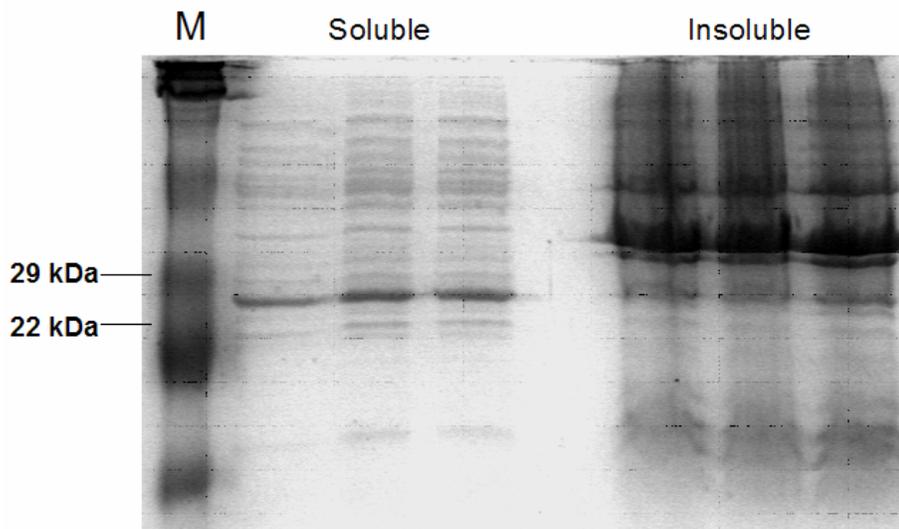


Figure 2-5. SDS-PAGE analysis of the soluble and insoluble products obtained after solubilization and refolding of the inclusion body pellet. Lane M, molecular weight marker.

Next, we attempted to purify the soluble Zps1p by size-exclusion chromatography (SEC). The protein sample was concentrated, applied to a column containing Sephadex G-75 (Sigma) size-exclusion resin (molecular weight cutoff *ca.* 80 kDa), with a bed volume of approximately 310 mL. The protein was eluted using 50 mM Tris (pH 7.4). After elution of the void volume, 2 mL fractions were collected and analyzed for protein using the method of Bradford.⁵¹ The Bradford protein assay indicated that the protein eluted as one major peak (fractions 9 – 12) shortly after collection of the void volume. Fractions 9 – 12 were combined and the content was analyzed by SDS-PAGE (Figure 2-6).

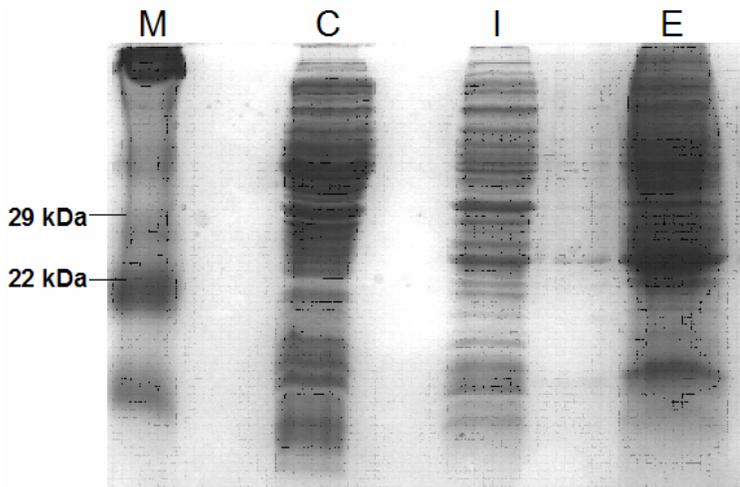


Figure 2-6. SDS-PAGE analysis of the major protein peak collected after SEC (combined fractions 9 – 12). Lane C, non-induced *E. coli* (8 h growth); Lane I, IPTG induced *E. coli* (8 h growth); Lane E, protein eluate from SEC column.

The results indicated poor separation of Zps1p from contaminating proteins. The lack of separation may result from aggregation of Zps1p with other peptides, possibly due to unfavorable disulfide bridging involving one of Zps1p's six cysteine residues.

Therefore, use of a reducing agent such as dithiothreitol (DTT) during the refolding,

concentrating, and chromatographic steps may prove effective in decreasing unfavorable disulfide bond formation.

To reduce the concentration of contaminating proteins that could unfavorably interact with Zps1p forming aggregates, a purification strategy was adapted from a published method.⁵² This method involves washing the inclusion body pellet with the detergent sodium deoxycholate (DOC) to remove impurities. First, the frozen cell pellet obtained after IPTG induction was thawed and resuspended in 20 mL of cold Buffer A (5% Glycerol, 50 mM NaCl, 0.5 mM EDTA, 50 mM Tris-HCl) containing 1 mM PMSF and 0.1 mM DTT. The cells were lysed using several cycles of French press at 4°C. Next, DOC was added to the lysate to give a concentration of 0.2% (approximately 240 µL of a 20% DOC stock), which is used to help liberate slightly insoluble proteins. The solution was mixed well, allowed to stand for 10 min at room temperature, and centrifuged at 13,000 rpm for 10 min at 4°C. The supernatant was decanted and saved for future analysis (Supernatant 1).

Following collection by centrifugation, the inclusion body pellet appears as a white bull's-eye, which is the inclusion body protein, surrounded by a brownish layer. The brownish layer consists of contaminating cellular debris that can be effectively solubilized by washing the pellet with 2% DOC. Therefore, the inclusion body protein was washed by resuspending the pellet in 18 mL of Buffer A (containing 1 mM PMSF and 0.1 mM DTT) and 2 mL of 20% DOC. The solution was allowed to stand for at least 10 min at room temperature before being centrifuged at 13,000 rpm for 10 min at 4°C. The supernatant was decanted and saved for future analysis (Supernatant 2). The remaining pellet was washed one additional time and, after centrifugation, the

supernatant was decanted and saved for future analysis (Supernatant 3). At this time, the washed inclusion body pellet was solubilized by resuspending in 5 mL Buffer A containing 8 M Urea and gently agitated overnight at 4°C. Prior to refolding by single-step dilution, the protein purity was assessed by SDS-PAGE to determine the effectiveness of the DOC wash (Figure 2-7). The gel showed few major bands, one being Zps1p, thus demonstrating the value of the DOC wash in purifying the inclusion body protein.

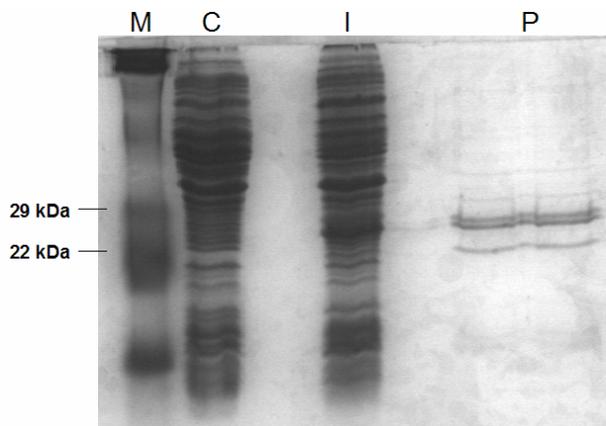


Figure 2-7. SDS-PAGE analysis of the washed inclusion body pellet, solubilized in Buffer A containing 8 M Urea (Lanes P). Lane M, molecular weight marker; Lane C, non-induced *E. coli* (8 h growth); Lane I, IPTG induced *E. coli* (8 h growth).

Next, we attempted to refold the solubilized inclusion body protein by single-step dilution using Buffer A. As before, this procedure involved slowly decreasing the concentration of Urea by dilution followed by dialysis. In an attempt to discourage unfavorable disulfide bridging, 0.1 mM DTT was added to the buffer during the refolding process. After dialysis, the sample was centrifuged at 8,000 rpm for 20 min at 4°C to pellet insoluble materials, and the supernatant was decanted and concentrated. The supernatant and pellet collected after refolding were analyzed by SDS-PAGE for protein content, as were the soluble fractions (Supernatant 1 – 3) collected after each treatment

with DOC (Figure 2-8). Unfortunately, refolding was unsuccessful and Zps1p was present in the insoluble fraction.

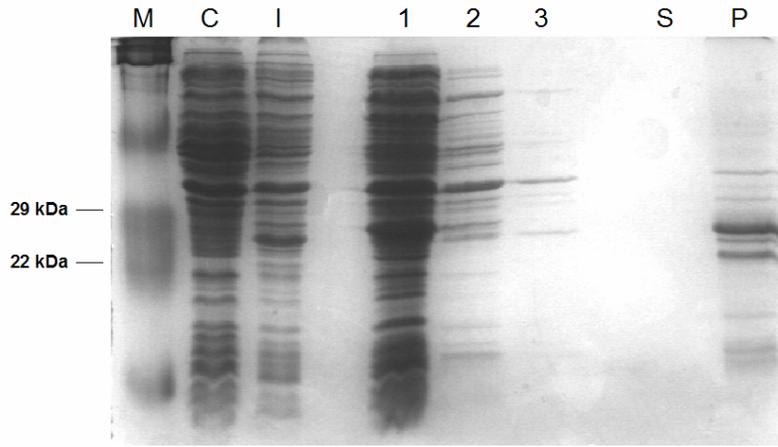


Figure 2-8. SDS-PAGE analysis of the soluble and insoluble products obtained after solubilization and refolding of the inclusion body pellet. Also shown are the supernatant fractions collected after each purification step. Lane M, molecular weight marker; Lane C, non-induced *E. coli* (8 h growth); Lane I, IPTG induced *E. coli* (8 h growth); Lane 1, Supernatant 1 (post-lysis); Lane 2, Supernatant 2 (after first DOC wash); Lane 3, Supernatant 3 (after second DOC wash); Lane S, soluble fraction (after refolding); Lane P, insoluble fraction (after refolding).

It is unclear why the solubilized inclusion body protein failed to refold despite its improved purity and the addition of DTT (to prevent unfavorable disulfides). One possible explanation is that the rate of dilution was accelerated due to poor control of the flow rate. It is critical that the rate of dilution is slow. At high denaturant concentrations, the unfolded protein is well solvated and flexible. Rapidly altering solvent dynamics toward an aqueous environment forces the protein to collapse into a compact and rigid structure. Unfortunately, the resulting structure is often misfolded or aggregated and therefore insoluble. Gradual dilution allows for refolding at intermediate concentrations of urea, where the denaturant concentration is low enough to force protein molecules to collapse, yet allowing flexible motion enabling proteins to reorganize their structures and stay in solution. Therefore, it may be beneficial to alter the refolding strategy so to

provide a slower and more controlled rate of denaturant dilution. Alternative refolding strategies include, but are not limited to: one-step dialysis, step-wise dialysis, and buffer-exchange by gel filtration.⁵³ Although expressing recombinant Zps1p from *E. coli* is advantageous due to high protein yield, it is possible the protein will not properly refold after solubilization from inclusion bodies. If future efforts to refold and purify Zps1p from *E. coli* inclusion bodies are unsuccessful, it may be necessary to purify Zps1p directly from *S. cerevisiae*.

CHAPTER 3 CONCLUSIONS

The work presented represents initial steps toward characterization of Zps1p from *S. cerevisiae*. *ZPS1* expression is regulated by extracellular pH and zinc-deficiency, environmental signals known to elicit Zps1p-like antigen production in *Candida* and *Aspergillus spp.*, respectively. These results suggest that the regulation, and consequently function, of the cell wall proteins is conserved among these fungi. This hypothesis is supported by their high sequence homology. Because of their localization within the fungal cell wall and the observed binding of Pra1 and Aspnd1 to host molecules, the Zps1p-like proteins in *C. albicans* and *Aspergillus spp.* are believed play a role in establishing infection. The Zps1p-like proteins may function as virulence factors by mediating critical host-parasite interactions or through involvement in morphological processes. Therefore, future characterization of Zps1p may include investigating the protein's potential role in fungal cell-cell adhesion (flocculation) or adherence to host ligands (*e.g.*, ECM or serum proteins).

The partial purification of recombinant Zps1p from bacterial inclusion bodies is an important first step toward characterization of Zps1p. Once purified, a wealth of information can be obtained by studying both Zps1p structure and function. Due to similarities between the Zps1p-like proteins and zinc-dependent metalloproteases, future work using purified Zps1p ought to include metal binding studies and testing for proteolytic activity towards a variety of substrates.

CHAPTER 4 MATERIALS AND METHODS

Growth Media

For standard growth of *E. coli*, LB medium was used. The recipe per liter is 10 g NaCl, 10 g Bactotryptone, and 5 g Yeast Extract. When required, ampicillin was added to a final concentration of 200 µg/mL. In preparation of plates, 15 g of agar was added per liter.

YPD medium was used for routine, non-selective yeast growth. The recipe per liter is 10 g Yeast Extract, 20 g Bactopeptone, and 20 g Dextrose. In preparation of plates, 15 g of agar was added per liter.

For maintenance of recombinant yeast strains, selective (SD) medium was used. The base recipe per liter is 5 g (NH₄)₂SO₄, 20 g Dextrose, and 1.7 g Yeast Nitrogen Base without amino acids or (NH₄)₂SO₄ (Difco; Sparks, MD). To satisfy the auxotrophic strains used in this study, the medium was supplemented with 0.1 g L-Histidine, 0.1 g L-Leucine, and 0.1 g L-Lysine per liter. Although the strains required Uracil, this was omitted from the medium for selective growth. This medium will be referred to as SD-Ura. For plates, 15 g of agar was added per liter.

To limit zinc and iron availability, Chelex-treated synthetic defined medium (CSD) was used. The recipe per liter, using H₂O at 18 MΩ purity, is 20 g Dextrose, 5.1 g Yeast Nitrogen Base without amino acids or divalent cations or potassium phosphate (Bio101; Vista, CA), and 0.1 g each of L-Histidine, L-Leucine, and L-Lysine. Again, for selective purposes, Uracil was omitted from the medium. To remove metals from the

media, 25 g of Chelex-100 ion exchange resin (Sigma) was added, and the mixture was stirred for a minimum of 2 h. After removal of the resin, 10 mL of potassium phosphate monobasic (100 g/L) was added and the pH was adjusted to 4.0 using HCl. Next, divalent metal ions were added to the medium to the following concentrations (as recommended by Bio101): 0.4 mg/L MnSO₄, 0.04 mg/L CuSO₄, 100 mg/L CaCl₂, and 500 mg/L MgSO₄. The resulting solution was filter sterilized into a polycarbonate flask washed with Acationox detergent (Baxter Scientific Products; McGraw Park, IL). The resulting solution contains residual zinc and iron at concentrations less than 100 nM (approximate value), and is referred to as CSD-Ura(-Zn/-Fe). For zinc or iron replete medium, the desired metal is added back to the medium to a final concentration of 10 μM.

Solutions and Buffers for Yeast Transformations and β-Galactosidase Assays

10x TE (250 mL):

100 mM Tris and 10 mM EDTA pH 7.5. Sterilize.

LiTE solution (50 mL):

5 mL sterile 10x TE, 5 mL sterile 1 M Lithium Acetate, 40 mL sterile H₂O.

PEG-LiTE solution (50 mL):

5 mL sterile 10x TE, 5 mL sterile 1 M Lithium Acetate, 40 mL sterile 44% (w/v) PEG-3350

Carrier DNA (10 mL):

100 mg salmon testes DNA and 10 mL ultrapure H₂O. Shear DNA by drawing the mixture up into a 10 mL syringe with an 18g needle 15 times, boil, and restore volume to 10 mL. Store as 1 mL aliquots at -20°C.

Z-buffer, pH 7.0 (1 L):

0.06 M Na₂HPO₄, 0.04 M NaH₂PO₄-H₂O, 0.01 M KCl, 0.001 M MgSO₄.

Bacterial and Yeast Strains

Listed below are the bacterial and yeast strains used in this work.

Table 4-1. Yeast strains used for the work described.

Yeast Strain	Mutation	Source	Genotype
BY4742	Wild type	-	MAT α ; <i>his3</i> ; <i>leu2</i> ; <i>ura 3</i> ; <i>lys2</i>
Y11367	<i>zap1</i>	EUROSCARF	MAT α ; <i>his3</i> ; <i>leu2</i> ; <i>ura 3</i> ; <i>lys2</i> <i>zap1::kanMX4</i>
Y10936	<i>rim101</i>	EUROSCARF	MAT α ; <i>his3</i> ; <i>leu2</i> ; <i>ura 3</i> ; <i>lys2</i> <i>rim101::kanMX4</i>

Table 4-2: Bacterial strains used for the work described.

<i>E. coli</i> Strain	Genotype
TOP 10	F ⁻ <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) ϕ 80 <i>lacZ</i> Δ M15 Δ <i>lacX74</i> <i>deoR</i> <i>recA1</i> <i>araD139</i> Δ (<i>ara-leu</i>)7697 <i>galU</i> <i>galK</i> <i>rpsL</i> (Str ^R) <i>endA1</i> <i>nupG</i>
BL21(DE3)	F ⁻ <i>ompT</i> <i>hsdS_B</i> (r _B ⁻ m _B ⁻) <i>gal</i> <i>dcm</i> (DE3)

Yeast Transformations

Using the lithium acetate method, yeast strains of interest were transformed with a plasmid containing the *ZPS1-lacZ* fusion (with Ura⁺ selection), which was previously constructed³⁴ in YEp353⁵⁴ by gap repair.⁵⁵ This was accomplished by growing the yeast in 5 mL YPD at 30°C at 250 rpm overnight. The following day, 300 µL of the overnight culture was transferred to a new tube containing 5 mL YPD and incubated for 2 hr. at 30°C at 250 rpm. Next, the cells were harvested by centrifugation at 3500 rpm for 3 min and the supernatant was decanted. The remaining cell pellet was washed by adding 5 mL LiTE solution and vortexing. Again, the cells were harvested by centrifugation (as described above). The cells were resuspended in residual LiTE solution by vortexing and 50 µL of the cell suspension was transferred into a sterile 1.5 mL centrifuge tube. Added to the centrifuge tube containing cells were 2 µL (~ 400 µg) of plasmid DNA containing the *ZPS1-lacZ* fusion and 10 µL (~ 10 µg) of salmon sperm carrier DNA (boiled for 5 min and flash cooled on ice prior to use). Next, 500 µL of PEG-LiTE solution was added. The mixture was vortexed briefly and incubated at 30°C at 250 rpm for 30 – 45 min. After incubation, the sample was heat shocked for 10 – 15 min at 42°C. The cells were pelleted at 4000 rpm for 1 min in a microcentrifuge. The supernatant was aspirated and 500 µL LiTE solution was added to the pellet and subsequently vortexed. Finally, 50 – 200 µL of transformant was plated on SD-Ura plates to (select for the YEp353 plasmid) and incubated at 30°C for 3 – 5 days. Plates which grew colonies were stored at 4°C for future use.

β -Galactosidase Assays

A single colony of yeast transformed with the *ZPS1-lacZ* fusion plasmid was transferred to 5 mL of SD-Ura and incubated at 30°C at 250 rpm overnight. This liquid culture was used to inoculate metal-free 14 mL polystyrene tubes containing 5 mL of CSD-Ura with the appropriate combinations of zinc and iron as follows: -Zn/-Fe, 45 μ L cell culture; -Zn/+Fe, 30 μ L cell culture; +Zn/-Fe, 30 μ L cell culture; +Zn/+Fe, 20 μ L cell culture. The cultures were grown for 12 h and then stored on ice for approximately 20 min. Next, the cells were harvested by centrifugation for 3 min at 3500 rpm at 4°C. The supernatant was discarded and the resulting pellet was washed by adding 5 mL cold Z-buffer and vortexing. The cells were harvested by centrifugation (as above) and the supernatant was discarded.

The cells were resuspended in 2 mL cold Z-buffer and 1 mL of the cell suspension was transferred to a 5 mL glass assay tube containing 50 μ L CHCl_3 and 50 μ L 0.1% SDS. The contents of the tube were vortexed to permeabilize the cells and then incubated at 30°C for 10 min to equilibrate. After incubation, the tube was vortexed vigorously for 3 sec and its contents (principally CHCl_3) were allowed to settle for approximately 10 se. before transferring 100 μ L of the suspension to a 96-well plate (in triplicate). The assay reaction was initiated by adding 20 μ L of 4 mg/mL *o*-nitrophenyl- β -D-galactopyranoside (ONPG). The sample was mixed and the reaction was allowed to proceed until the darkest samples were an intense yellow color. The reaction was stopped by adding 50 μ L of 1 M Na_2CO_3 and the reaction time (min) was noted. The absorbance at 420 nm was measured (reference wavelength, 600 nm) using a SAFIRE microplate reader (Tecan) and XFLUOR software. The absorbance at 600 nm (OD_{600}) of

the remaining cell suspension (from above) was also measured using a BIO-RAD SmartSpec™ 3000 bench-top spectrophotometer. β -galactosidase activity was measured in Miller Units using the method of Guarente,⁵⁶ and activity units were calculated as follows: $(\Delta A_{420} \times 1000)/(\text{min} \times \text{mL of culture used} \times \text{OD}_{600})$.

Cloning of *ZPS1* and Construction of an *E. coli* Expression Plasmid

The gene that encodes the mature form of Zps1p (lacking the signal peptide) was PCR cloned from *S. cerevisiae* strain BY4724 genomic DNA using forward and reverse primers containing *Nde* I and *EcoR* I restriction sites, respectively. The primers were obtained from Integrated DNA Technologies, Inc. (Coralville, IA) and were designed as follows:

ZPS1 for: 5'- AAC TTT AAG AAG GAG ATA TAC ATA

TGC CTG TCA CTT ACG ACA CCA A -3'

ZPS1 rev: 5'- CAA GCT TGT CGA CGG AGC TCG AAT

TCT TAC AAG TTA CCT AGA CAG C -3'

The PCR reaction was catalyzed using Taq DNA polymerase and the thermocycling conditions employed were as follows: one cycle at 95oC for 3 min; and 25 cycles at 95oC for 30 sec, 50oC for 30 sec, 72oC for 1.5 min; and a final extension at 72oC for 8 min.

The PCR product was digested for 4 h at 37oC using the restriction enzymes *Nde* I and *EcoR* I (New England Biolabs; Beverly, MA). The pET-22b(+) vector was obtained from Novagen (La Jolla, CA). The pET-22b(+) plasmid was also digested using *Nde* I and *EcoR* I (as described above). Following restriction digestion, the cut PCR and pET-22b(+) samples were subjected to agarose gel electrophoresis (0.8% agarose) and

purified using the QIAquick Gel Extraction Kit, following the manufacture's protocol (QIAGEN Inc.; Valencia, CA). These purified samples were subsequently used to ligate the cloned ZPS1 gene into the pET-22b(+) vector between the Nde I and EcoR I restriction sites using T4 DNA ligase (New England Biolabs), incubated overnight at 16°C. The ligation product was used to transform electrocompetent *E. coli* TOP10 cells by electroporation following standard procedures.⁵⁷ The *E. coli* transformant (10 – 150 μ L) was plated on LB agar plates containing ampicillin (for plasmid selection) and incubated at 37°C overnight. Plates that grew colonies were stored at 4°C for future use. To obtain large quantities of the pET-22b(+)-ZPS1 construct, a single colony from the transformation product was used to inoculate 5 mL of LB medium containing ampicillin. The cells were grown at 37°C at 250 rpm overnight. Using the Promega (Madison, WI) Wizard Plus Miniprep DNA purification system, the pET-22b(+)-ZPS1 plasmid was purified from the overnight culture according to the manufacture's directions. Using the purified plasmid, the sequence of the cloned ZPS1 gene was confirmed by the ICBR DNA sequencing core laboratory at the University of Florida.

Expression of Zps1p in *E. coli*

To obtain Zps1p using the T7 expression system, the pET-22b(+)-ZPS1 plasmid was transformed into BL21(DE3) *E. coli* by electroporation using standard methods. The *E. coli* transformant (10 – 150 μ L) was plated on LB agar plates containing ampicillin and incubated at 37°C overnight. Plates that grew colonies were stored at 4°C for future use. A single colony from the transformation product was used to inoculate 15 mL of LB medium containing ampicillin. The cells were grown at 37°C at 250 rpm overnight and 10 mL of culture was used to inoculate 1 L of LB medium containing ampicillin. The 1

L culture was incubated at 37°C at 250 rpm for approximately 2 h until reaching an OD₆₀₀ of 0.4 – 1.0. At this time, Zps1p expression was induced by addition of IPTG (Isopropyl-β-D-thiogalactoside) to a final concentration of 1 mM. The culture was then incubated at 30°C at 250 rpm for 8 h. Finally, the cells were harvested by centrifugation (3000 rpm for 15 min at 4°C) and washed two times using 50 mM Tris (pH 7.4). The resulting pellet was stored frozen at -20°C overnight.

Estimation of Protein Purity by SDS-PAGE

SDS-PAGE gels containing 14% (w/v) polyacrylamide were prepared and analyzed by standard methods. Samples were prepared by adding equal volumes of 2x Laemmli sample buffer, boiling for 10 min, followed by centrifugation at 14,000 rpm in a microfuge for 1 min to pellet any insoluble debris. The gels were run at 70 V, using a Tris-glycine electrode buffer. All gels were stained with Coomassie blue.

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