IN VITRO ENRICHMENT OF PHOSPHORYLATED PROTEINS FROM SYNCHRONOUSLY EXCYSTED *CRYPTOSPORIDIUM PARVUM*

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Title

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MASTER OF SCIENCE

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ABSTRACT

Cryptosporidium is an enteric protozoan parasite that causes cryptosporidiosis and there is no effective drug treatment available. Two studies were conducted. In the first, a novel protocol was developed for the rapid *in vitro* excystation of *Cryptosporidium parvum*. Rapid excystation (release of infectious sporozoites from the environmental oocyst stage) is necessary to study molecular dynamics during the early stages of *Cryptosporidium* development. The developed excystation assay (Joshi assay) used an HCl (pH 2.5) oocyst pretreatment instead of the bleach pretreatment that is more frequently used in excystation assays. The Joshi assay achieved >90% excystation in 10 min. This rapid, reproducible, and synchronous *in vitro* excystation assay mimics the *in vivo* conditions in a mammal. In the second study, phosphorylated and non-phosphorylated proteins were selectively enriched from non-excysted and excysted *C. parvum* oocysts and separated using 2 dimensional gel electrophoresis. This is the first study that enriched phosphorylated proteins from *C.parvum.*

ACKNOWLEDGEMENTS

I truly thank my advisors, Drs. Eakalak Khan and John McEvoy for accepting me for the Master's program, providing funding and helping me attain the maturity level required as a Master's student. They are the best supervisors I could ever wish for. My special thanks goes to Dr. Steven Meinhardt (Associate Professor, Plant Pathology Department), for his never ending support in my proteomics work. He was always there to answer my questions, to train me with 2 dimensional gel electrophoresis techniques, allowed me to work in his lab and also gave me an opportunity to train 2 other graduate students in his lab. Besides helping me in my research, his precious advises, suggestions and culturally diverse talks gave me different perception towards life. I am thankful to Dr. Anna Grazul Bilska and Dr. Eugene Berry for being a part of my committee. Their time and comments are highly appreciated.

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DEDICATION

To my Mom *(Mrs. Anita Joshi),* Dad *(Mr. J. B. Joshi)* and Brother *(Rohit Joshi)*.

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LIST OF ABBREVIATIONS

C. parvum……...*Cryptosporidium parvum*

CHAPS……..Cholamidopropyl dimethylammonio propanesulfonate

DTT……Dithiothreitol

× g……..Gravitational force

HEPES……..Hydroxyethyl piperazineethanesulfonic acid

IEF……Iso Electric Focusing

IpG……Immobiline pH Gradient

µg…….Micro gram

mg……Mili gram

ml…….Mili litre

min……...Minute/s

NaTC……...Sodium Taurocholate

RPMI 1640……..Roswell Park Memorial Institute

RT……...Room Temperature

SDS PAGE…….Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis

Sec……..Second/s

W……Watt

CHAPTER 1: INTRODUCTION

1.1. General Introduction

This thesis focuses on *Cryptosporidium parvum,* an apicomplexan parasite that primarily infects epithelial cells of microvilli in the gastrointestinal tract of vertebrates, causing the disease cryptosporidiosis. Although the infection caused by *C. parvum* can be self-limiting for immunocompetent individuals, it can be chronic and even fatal for immunocompromised individuals. The transition from environmentally resistant oocysts to infectious sporozoites is critical to the initiation of infection; therefore, this thesis specifically addressed excystation, which is the release of motile sporozoites from oocysts. A novel, rapid *in vitro* excystation method was developed and used to study changes in protein phosphorylation during excystation. Comparing phosphorylated proteins pre- and post-excystation will help to understand cell signaling during excystation.

1.2. Background: *Cryptosporidium*

Cryptosporidium is a protozoan parasite that causes the diarrheal disease cryptosporidiosis in humans and other vertebrates. The disease is frequently self-limiting in immunocompetent hosts but can be lethal for those with a suppressed immune system. Moreover, there is currently no effective drug treatment available for immunocompromised individuals infected with *Cryptosporidium* (Blagburn and Soave, 1997). In humans, cryptosporidiosis can be severe in infants, elderly people, and people with AIDS, cancer or transplanted organs (Anonymous, 1982). The disease is more prevalent in underdeveloped parts of the world (Esteban et al., 1998).

Ernest Edward Tyzzer first described *Cryptosporidium muris* in the gastric glands of mice (Tyzzer, 1907), and subsequently described a second species, *C. parvum* in the small intestine of mice (Tyzzer, 1912). The public health significance of *Cryptosporidium* came to light when it was first associated with AIDS patients in 1982 (Anonymous, 1982). A waterborne cryptosporidiosis outbreak in Milwaukee, Wisconsin in 1993, which infected more than 400,000 individuals, established *Cryptosporidium* as a major waterborne parasite (Hoxie et.al, 1997).

The mechanism by which *Cryptosporidium* causes disease is poorly understood. *Cryptosporidium* has a monoxenous life cycle that includes both asexual and sexual stages. A thick walled oocyst protects the parasite in the environment. Oocysts excyst in the small intestine to release four sporozoites that invade epithelial cells of microvilli. Sporozoites mature into trophozoites, which further develop into type I meronts containing 8 merozoites. Merozoites released from type I meronts infect cells and develop into type I or type II meronts. Type II meronts contain four merozoites, which develop into micro- and macrogametocytes. Microgametes fuse with macrogametocytes to form zygotes, which undergo sporogony to produce oocysts with four sporozoites. Oocysts can be thin walled, in which case they contribute to autoinfection, or thick walled, in which case they are released back into the environment in feces.

1.3. Research Problem Statement

During excystation, *Cryptosporidium* rapidly transforms from an inactive, environmentally stable form (the oocyst) to an active, infective form (the motile sporozoite) in response to environmental stimuli. The mechanisms by which the parasite detects and responds to external stimuli during excystation are not known, but they are likely to involve a number of

signaling pathways. In order to study the proteins involved in these pathways, it is necessary to examine synchronized parasite populations at different stages of excystation. In most published *in vitro* excystation assays, oocysts excyst stochastically over a period of approximately 60 min, which makes the study of protein dynamics difficult. Also, the standard excystation assay used in most labs employs bleach as an oocyst pretreatment to stimulate excystation. Bleach has a pH of approximately 12.0, which does not mimic any *in vivo* conditions. In contrast to bleach, an HCl pretreatment at pH 2.5 mimics conditions in the stomach. Although Gut and Nelson (1999) reported rapid, synchronized oocyst excystation using an HCl pretreatment, others have failed to reproduce these findings. In order to study protein dynamics during excystation, there is a need to develop a reproducible, rapid excystation assay that mimics *in vivo* conditions. Phosphorylation is an important post-translational modification of proteins during cell signaling, and identifying proteins that are differentially phosphorylated can help to elucidate signaling pathways. To date, no studies have examined differential protein phosphorylation during *Cryptosporidium* excystation.

1.4. Research Benefits

The rapid and synchronous excystation of sporozoites *in vitro* under conditions similar to those in the host will make it possible to study the molecular mechanisms that underpin excystation. In addition, reducing the time required for *in vitro* excystation will result in a more efficient method for assessing viability.

Isolating and characterizing populations of phosphorylated and non phosphorylated proteins in of excysted and non-excysted oocysts is a necessary first step towards understanding

cell signaling during excystation. Further characterization of differentially phosphorylated proteins can lead to important new targets for drug development.

1.5. Research Approach

Published excystation protocols were modified and optimized to achieve >90% excystation rapidly. A novel excystation protocol was then used to study differential protein phosphorylation during excystation. Populations of phosphorylated and non-phosphorylated proteins were isolated, enriched, and subjected to two-dimensional (2-D) electrophoresis. A proprietary kit was used to enrich phosphorylated proteins, which; were then concentrated. Phosphorylated and non-phosphorylated proteins were separated by 2-D electrophoresis. Proteins were first subjected to isoelectric focusing (1^{st} dimension) before separation by molecular weight (2^{nd} dimension). As this was the first study to enrich phosphorylated proteins from *C. parvum,* several techniques were examined to determine the optimal conditions for enrichment.

1.6. Statement of Need

This study addresses statements of need

1. In order to study the molecular mechanisms underpinning the early stages of *Cryptosporidium* development, a synchronized *in vitro* excystation assay that mimics the host environment is needed.
2. In order to study the role of phosphorylated proteins in *Cryptosporidium* cell signaling during excystation, methods are needed to selectively enrich phosphorylated proteins from non-excysted and excysted oocysts, and to characterize the properties of phosphorylated and non-phosphorylated proteins.

1.7. Objectives

This study has following two objectives:

1. To develop a synchronous *in vitro* excystation assay for *C. parvum* that uses HCl rather than bleach as a pretreatment to trigger excystation.
2. To enrich the phosphorylated and non-phosphorylated proteins from excysted and non-excysted oocysts of *C. parvum*.

1.8. Thesis Organization

This thesis is divided into five chapters. Chapter 1 is the introduction; it includes background, research problem statement, statement of need, and thesis organization. Chapter 2 is a review of the literature that is relevant to the work. Chapter 3 deals with the development of a rapid *in vitro* synchronized excystation assay for *C. parvum*. Chapter 4 describes work to compare the characteristics of phosphorylated and non-phosphorylated proteins from excysted and non-excysted oocysts. Chapter 5 describes conclusions and recommendations for future work.

CHAPTER 2: LITERATURE REVIEW

2.1. Background Information About *Cryptosporidium*

In 1907, Ernst Edward Tyzzer discovered *Cryptosporidium* in the gastric glands of mice, described the asexual and sexual stages, and named the species *Cryptosporidium muris* (Tyzzer, 1907). In 1912, Tyzzer discovered a new species, *Cryptosporidium parvum*, located in the small intestine of laboratory mice. Oocysts of *C. parvum* were smaller than *C. muris*.

In 1955, Slavin identified a new species, *C. meleagridis*, in turkeys (Slavin, 1955). Along with *C. parvum* and *C. muris*, *C. meleagridis* is one of the few species described during the early years of *Cryptosporidium* research whose name is still in use today. *Cryptosporidium* was reported in a number of animals during this time, including cattle, pigs, sheep, horses, turkeys, monkeys, snakes, guinea pigs, and rabbits (Current, 1985). However, the veterinary and medical importance of *Cryptosporidium* was not recognized until the 1970s and 1980s. *Cryptosporidium* was first associated with the diarrheal disease cryptosporidiosis in cattle in the early 1970s (Panciera et al., 1971). Four cases of human cryptosporidiosis were reported between 1976 and 1979 in infants and immunosupressed adults (Nime et al, 1976; Meisel et al. 1976). In 1982, the CDC reported on 21 men with AIDS with untreatable, chronic, and in some cases lethal diarrheal disease caused by *Cryptosporidium* (Anonymous, 1982). This report marked the beginning of a new era of public health research on *Cryptosporidium*. A 1993 outbreak in Milwaukee, which affected 403,000 people and resulted in more than 100 deaths (Mac Kenzie et al., 1994), demonstrated the importance of water in the transmission of *Cryptosporidium*. Oocysts, the environmental stage of *Cryptosporidium*, are resistant to many disinfectants, including chlorine, and can survive for long periods in water (Keegan et al., 2008).

Cryptosporidium infects epithelial cells of the microvilli in the gastrointestinal tract of the vertebrates, and host specificity varies from narrow to broad. The severity and duration of infection depends on the immune status of the host. For immunocompetent hosts, the infection is usually self-limited and of mild or moderate severity; in contrast, immunosuppressed individuals can develop severe, chronic, and sometimes fatal diarrheal disease. The period between the ingestion of oocysts and shedding of new oocysts, the prepatent period, differs among *Cryptosporidium* species and hosts. Table 2.1 shows the experimentally determined prepatent period for *C. parvum* in different hosts.

Table 2.1. Prepatent Period of *C. parvum* Infecting Different Hosts (Fayer, 1997)

Host	Prepatent period (days)
Humans	4-22
Calves	2-7
Dogs	2-14
Pigs	3-6
Lambs	2-5

Each year, diarrheal disease kills 800,000 children under the age of five in Asia and sub-Saharan Africa (Liu et al., 2012). *Cryptosporidium* is second only to rotavirus as the leading cause of these deaths (Kotloff et al. in 2013).

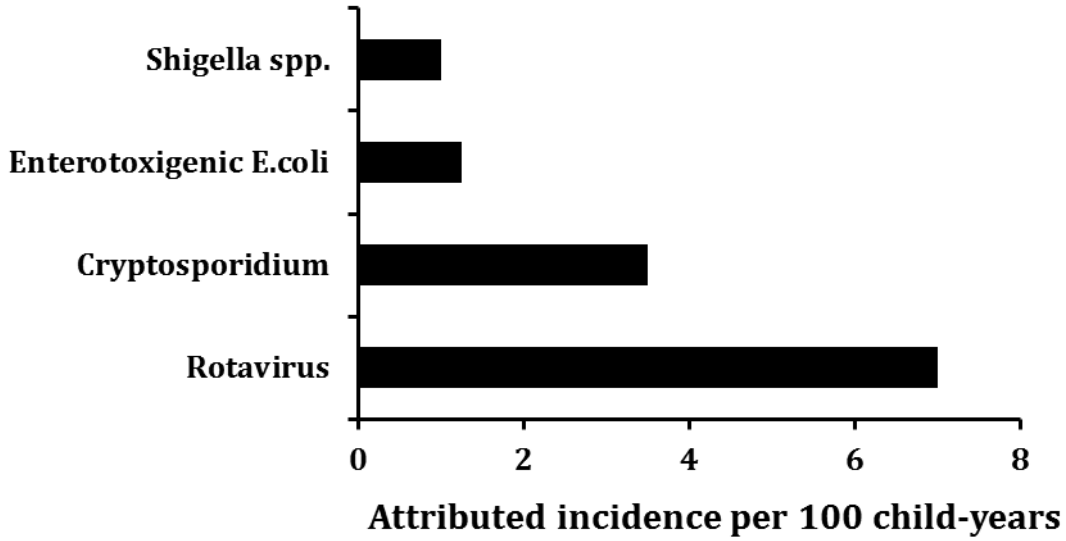


Figure 2.1. Cryptosporidiosis: Second Biggest Cause of Child Deaths and Diarrheal Diseases in Asia and Africa (Striepen, 2013).

Although, cryptosporidiosis has been reported in more than 40 countries, with higher incidences in developing countries, there is no fully effective drug or vaccine (Dillingham et al., 2002). In the USA, nitazoxanide (Alinia) is approved for children and immunocompetent individuals. Nitazoxanide is not an effective treatment for immunocompromised individuals because it requires an appropriate immune response (Gargala, 2008). The lack of an effective treatment or drug is due to the absence of basic research tools and infrastructure required to study the parasite. *Cryptosporidium* species cannot be cultured for very long *in vitro* and mice, which is used as a standard model to study the parasite, is not useful to study species that infect humans, e.g. *C. hominis* and *C. parvum* (Striepen, 2013).

Currently there are 25 recognized species of *Cryptosporidium* (Table 2). However, major studies (morphology, biochemistry, pathogenicity, biology, and prevalence) are conducted on *C. parvum* because it infects multiple hosts and produces enormous number of oocysts, which makes it easy to propagate (Huw et al., 2005). In addition, *C. parvum* is globally widespread and

is of medical and veterinary importance. Therefore, the species is used as a model to understand other species, which are not easily propagated. Hence, out of the all recognized species, the life cycle of *C. parvum* is the best studied.

Table 2.2. Recognized and Valid *Cryptosporidium* Species (Fayer et al., 1997)

S.no	Species	Host
1	*C. parvum*	Mammals
2	*C. hominis*	Humans
3	*C. muris*	Mammals
4	*C. andersoni*	Bovines, Bactrian camel
5	*C. felis*	Felids
6	*C. canis*	Canids, humans
7	*C. suis*	Pigs
8	*C. wrairi*	Guinea pigs
9	*C. baileyi*	Gallinaceous birds
10	*C. galli*	Chickens, finches
11	*C. meleagridis*	Turkeys
12	*C. serpentis*	Snakes, lizards
13	*C. saurophilum*	Lizards
14	*C. fragile*	Amphibians
15	*C. cuniculus*	Rabbits
16	*C. varanii*	Reptiles
17	*C. viatorum.*	Humans
18	*C. bovis*	Weaned calves and adult cattle

Table 2.2.Recognized and Valid *Cryptosporidium* Species (Fayer et al., 1997) *(continued)*

S.no.	Species	Host
19	*C. ryanae*	Weaned calves and adult cattle
20	*C. ubiquitum*	Ruminants, rodents, mates
21	*C. scrofarumm*	Pigs
22	*C. tyzzeri*	Rodents
23	*C. fayeri*	Marsupials
24	*C. macropodum*	Marsupials
25	*C. scophthalmi*	Fish

C. parvum oocysts can be transmitted zoonotically, anthroponotically, or indirectly via food and water (Peng et al., 1997). Both drinking and recreational water outbreaks are common. The flowchart in Figure 2.2 depicts the potential sources and routes of transmission of oocysts in the environment (Fayer, 1997).

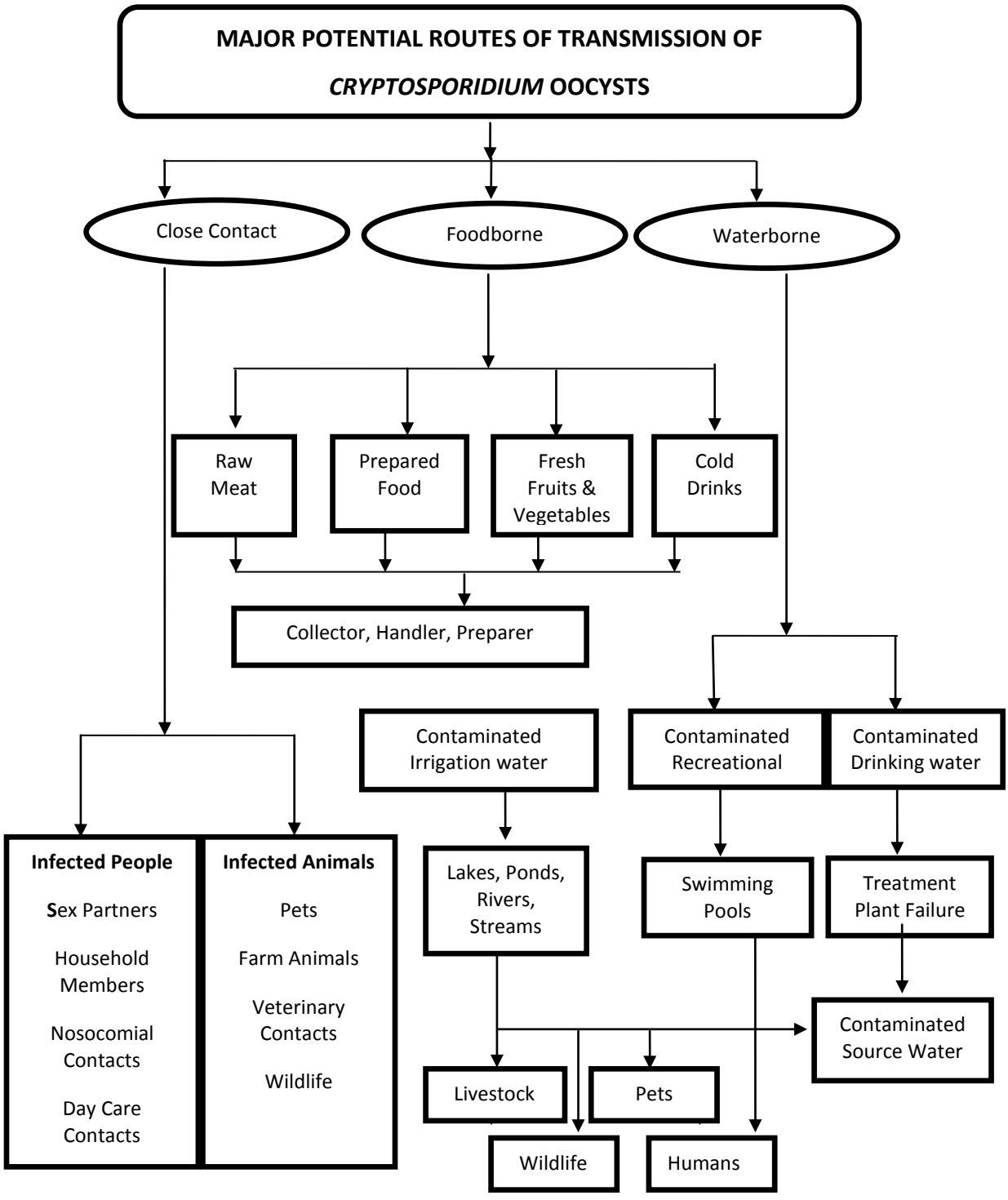


Figure 2.2. Schematic Representation of Transmission of *Cryptosporidium* (Fayer, 1997).

2.2. Morphology and Taxonomy

Oocysts of *Cryptosporidium* measure 4-6 µm in diameter and are spherical or ovoid in shape (Figure 2.3A). The sporozoites are encapsulated in a thick double layered wall that is resistant to the harsh environmental changes. On excystation, sporozoites exit from a door like opening called suture (Figure 2.3 B). Besides being extremely resistant to disinfectants (chlorine, chloramines, chlorine dioxide) in the drinking water, oocysts can survive and stay viable for infection for months in soil and surface waters (Siski et al., 2004 and Kato et al., 2004).

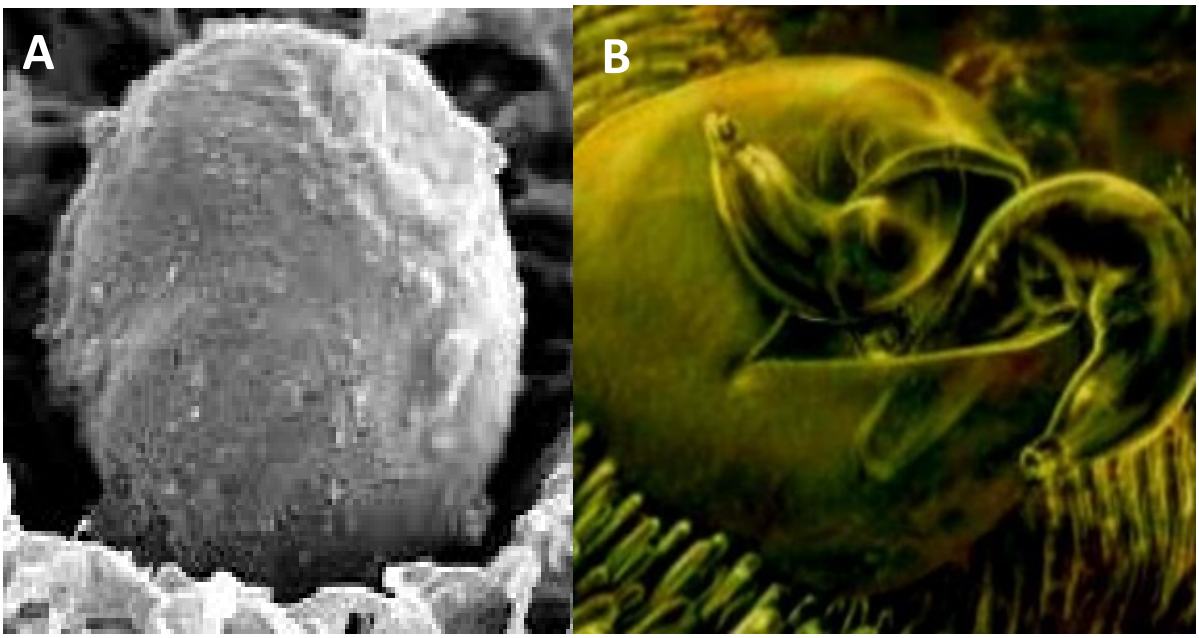


Figure 2.3. Scanning Electron Micrographs of a Non-Excysted [A] and Excysted [B] *Cryptosporidium parvum* Oocyst. (Images: By Dr. Udo Hertzel, University of Liverpool, and http://www.ksl.com/?nid=148&sid=1711437).

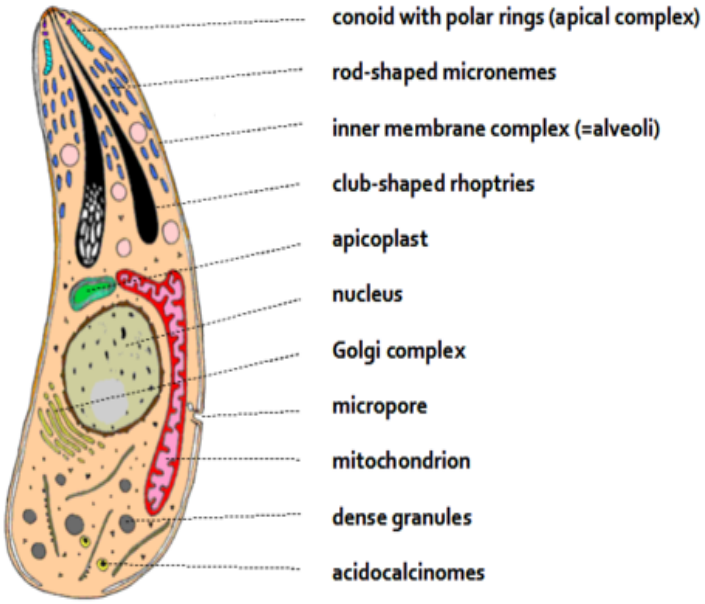


Figure 2.4. Schematic Drawing of a Typical Apicomplexan Sporozoite. A *Cryptosporidium parvum* Oocyst Contains 4 Sporozoites (Šlapeta and Adeline, 2011).

Cryptosporidium is a eukaryotic protozoan parasite in the phylum Apicomplexa. Apicomplexans have an apical complex composed of polar rings, rhoptries, micronemes and usually a conoid. *Cryptosporidium* oocysts are fully sporulated and immediately infective following shedding. Oocysts contain four sporozoites that exhibit gliding motility. They reproduce both asexually and sexually.

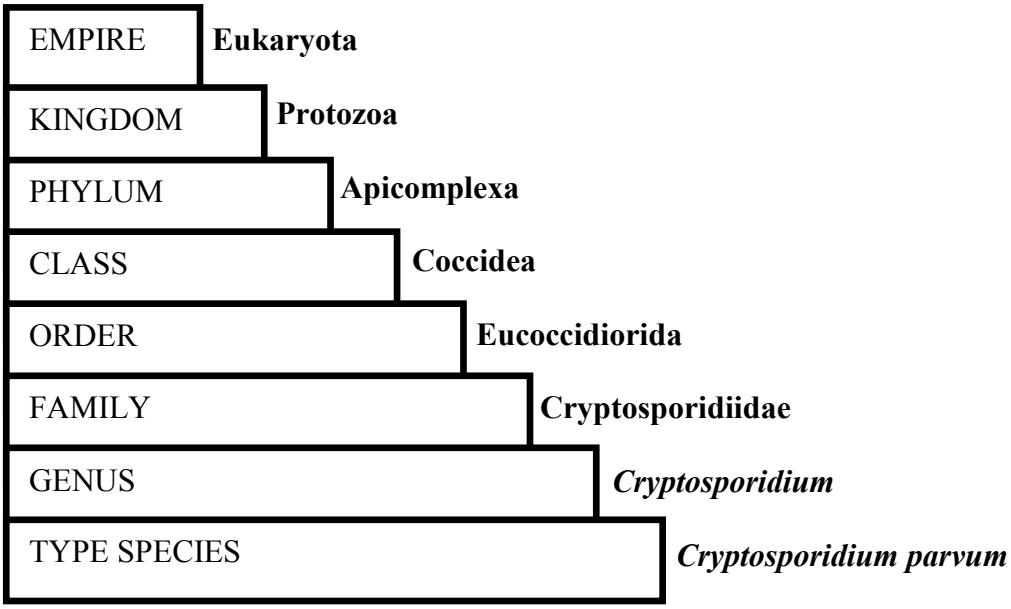


Figure 2.5. Taxonomic Classification of *Cryptosporidium* (Tyzzer, 1910, Levine, 1985, and Upton, 2010).

Once thought to be a coccidian, more recent molecular studies have shown *Cryptosporidium* to be an early-branching apicomplexan that is more closely related to gregarines, which are invertebrate parasites (Figure 2.6) (Carreno et al., 1999; Leander et al., 2003).

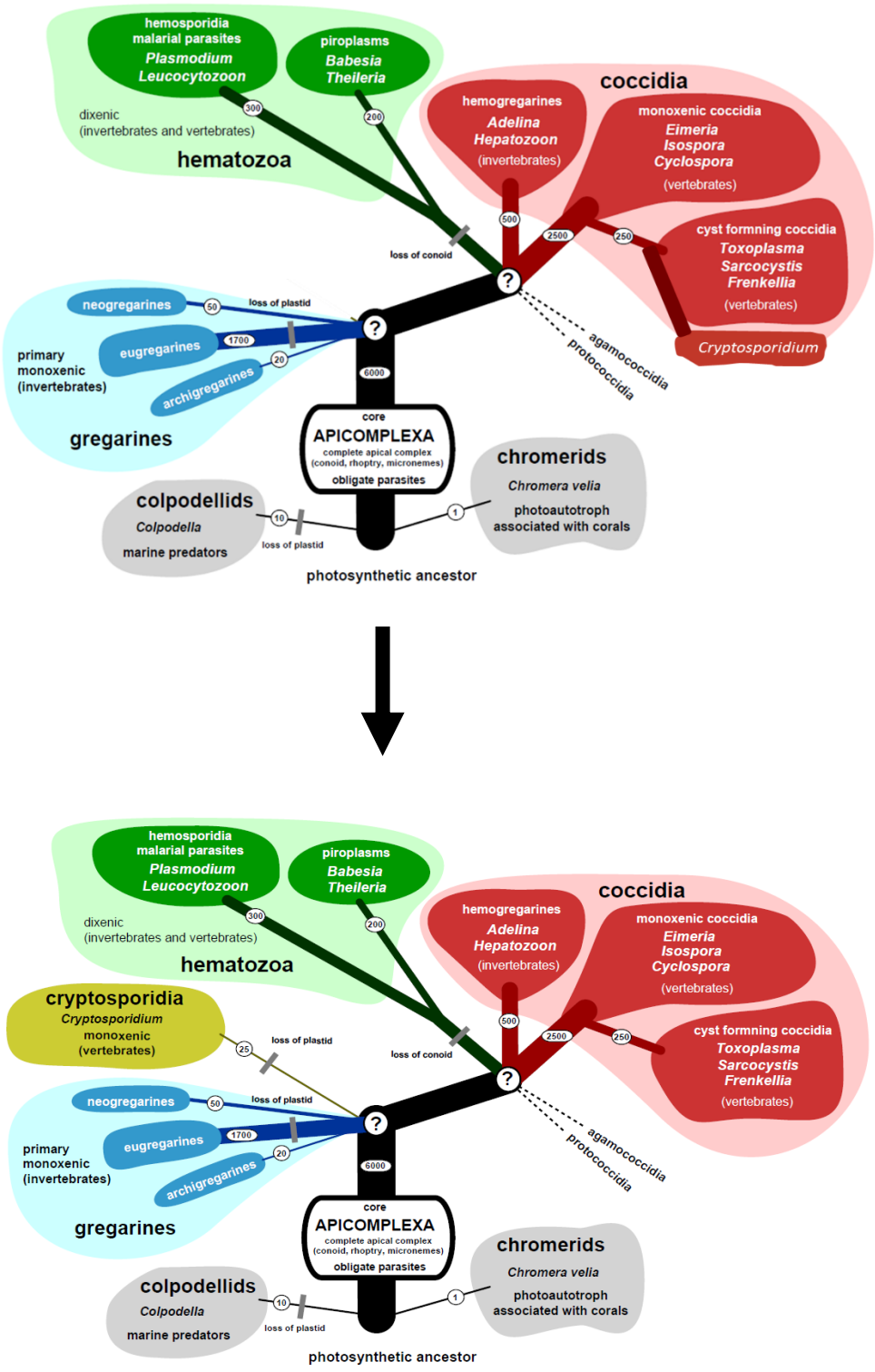


Figure 2.6. Revised Phylogenetic Tree of Apicomplexans. *Cryptosporidium* is more closely associated with gregarines than coccidia (Šlapeta and Adeline, 2011).

2.3. Life Cycle

The life cycle of *Cryptosporidium* is completed in a single host and has 6 major developmental stages: excystation, merogony, gametogony, fertilization and zygote development, formation of oocyst wall and sporogony (Tzipori and Ward, 2002).

2.3.1. The Oocyst

An infected host releases fully sporulated oocysts into the environment. The infectious dose can be as low as 10 oocysts depending on the species (Okhuysen, et al., 1991). An oocyst is encapsulated in a durable trilaminar wall, which protects the four sporozoites from harsh environmental conditions, thus, making the oocysts environmentally resistant for a prolonged time (Fayer, 2008).

2.3.2. Excystation, Attachment, and Invasion

Once ingested, viable oocysts excyst in the small intestine, releasing four spindle-shaped sporozoites through a suture in the oocyst wall. Sporozoites attach to the apical surface of host epithelial cells (Chen et al., 2002) and become enveloped by the host cell membrane to form an intracellular but extracytoplasmic parasitophorous vacuole (PV) (Huang et al., 2004). The lifecycle is diagrammatically presented in Figure 2.7 below.

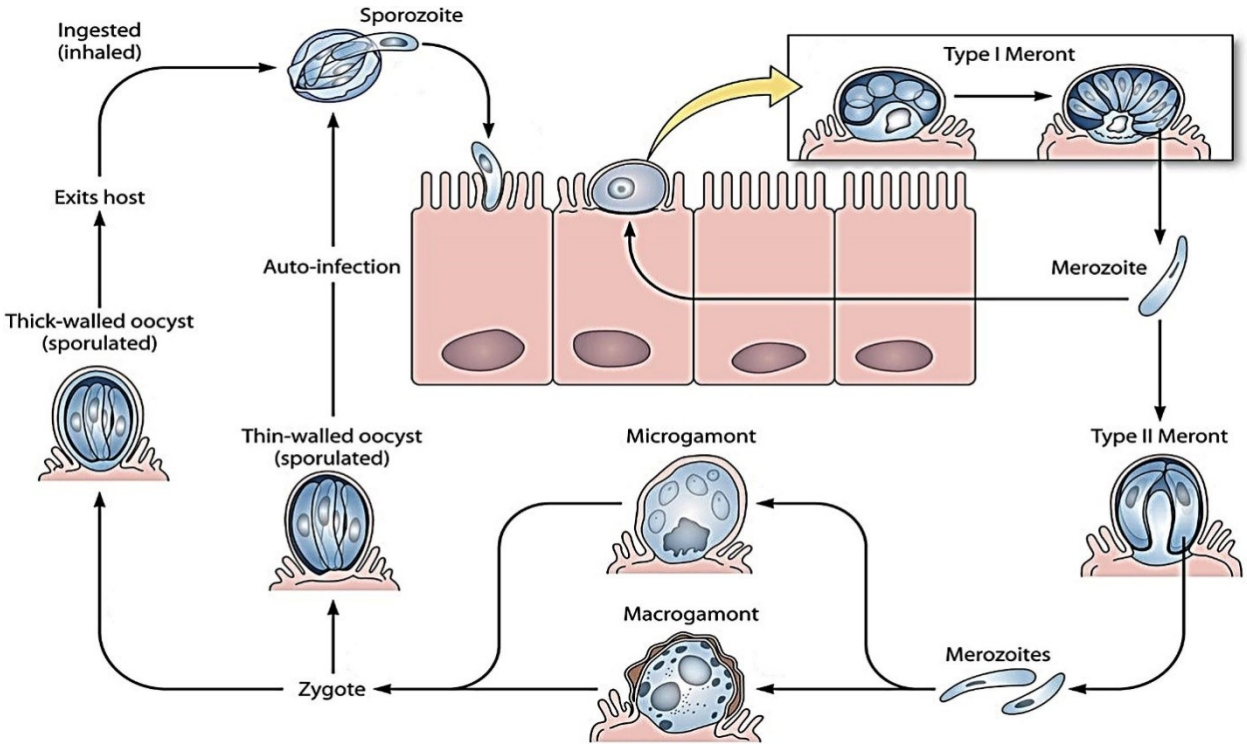


Figure 2.7. Lifecycle of *Cryptosporidium parvum* (Bouzid et al., 2013).

2.3.3. Asexual and Sexual Reproduction

Once inside the host cell, sporozoites mature into rounded trophozoites, which undergo three rounds of asexual replication (merogony) to produce eight merozoites (type I meront). Merozoites released form type I meronts are similar in structure and function to sporozoites and they invade other cells where they either develop into type I meronts, in a repeated cycle, or develop into type II meronts. Type II meronts undergo just two rounds of asexual replication to produce four merozoites. Following their release, these merozoites differentiate into microgamonts (male) and macrogamonts (female). Microgametes produced by microgamonts fertilize the macrogamont to form a zygote, which develops into either a thin or thick walled oocyst. The thin walled sporulated oocysts release sporozoites that can re-infect the epithelial cells in the intestinal tract. The thick walled oocysts are shed into the environment.

2.4. Excystation Mechanism

Although our understanding of the specific triggers and mechanisms of excystation remains crude (Huw et al., 2005), it is relatively straight forward to excyst *C. parvum* oocysts *in vitro* using a pretreatment in acid or bleach and a temperature of 37°C with bile salts and proteases (Fayer and Leek, 1984; Robertson et al., 1993 and Kato et al., 2001). However, studies also have shown that excystation can occur at extraintestinal sites such as the respiratory tract, lymph nodes, conjunctiva of the eye, testicle, ovary, uterus and, vagina (Travis et al., 1990; French et al., 1995; and Dunana et al., 1997). Considering that neither bile nor acidic conditions are found at these sites, other host-derived components might also trigger excystation.

Studies on effect of temperature have shown that in the absence of other host triggers, incubation of oocysts at 37°C produces better excystation than 4°C. (Reduker and Speer, 1985). It is hypothesized that the greater excystation at 37°C is because oocysts are more permeable at this temperature. In support, permeability of oocysts to DAPI (4`6-diamidino-2-phenyl-indole) was found to be greater at the higher temperature (Campbell et al., 1992). Exposure to an acidic environment also increases oocyst permeability by thinning the oocyst wall permeability (Jenkins, 2010). Increased oocyst wall permeability might facilitate signal transduction from external stimuli to sporozoites to initiate excystation.

Some studies have shown that parasite-derived protease play a role during excystation (Forney et al., 1996 and Ward et al., 1997). Snelling et al. (2007) demonstrated elevated expression of sporozoite proteins during excystation. These sporozoite proteins suggested a possibility of playing a crucial role in excystation because these proteins were detected in the organelles in the apical complex of the *C. parvum.* Okhuysen et al. (1994) found that arginine aminopeptidase, an integral membrane protein of sporozoites, was functionally associated with

sporozoites during excystation. Forney et al. (1996) reported increased protease activity during excystation, and later showed that serine and cysteine proteases played an important role during excystation.

2.5. Protein Phosphorylation and Cell Signaling

In accordance with central dogma, genes are transcribed to mRNA in the nucleus and translated to proteins in ribosomes in the cytoplasm. Proteins can subsequently undergo modification before being functional, and this is known as Post Translational Modification (PTM). During PTM, proteins may acquire several biochemical functional groups, such as phosphate, acetate, lipids, and carbohydrates. Besides the addition of functional groups, proteins may be enzymatically modified through the excision of amino acids. Knowledge of these chemical modifications – including temperospatial dynamics, regulation, and consequences – is necessary to understand functional proteomics. PTM can occur at any time during the life of a protein. For instance, folding immediately following translation stabilizes proteins, and other modifications can facilitate transport to specific compartments in the cell. Some PTMs can be reversible (Walsh, 2006). A major challenge in investigating PTMs is the development of specific detection and purification techniques; however, these technical hurdles are being overcome with refined proteomic techniques in immunodetection, phosphoprotein and phosphopeptide enrichment, mass spectrometry, and Eastern blotting (Jensen, 2004).

PTMs include phosphorylation, glycosylation, uniquitylation, and SUMOylation (Small Ubiquitin-like Modifier or SUMO) (Figure 2.8). This study focuses on protein phosphorylation.

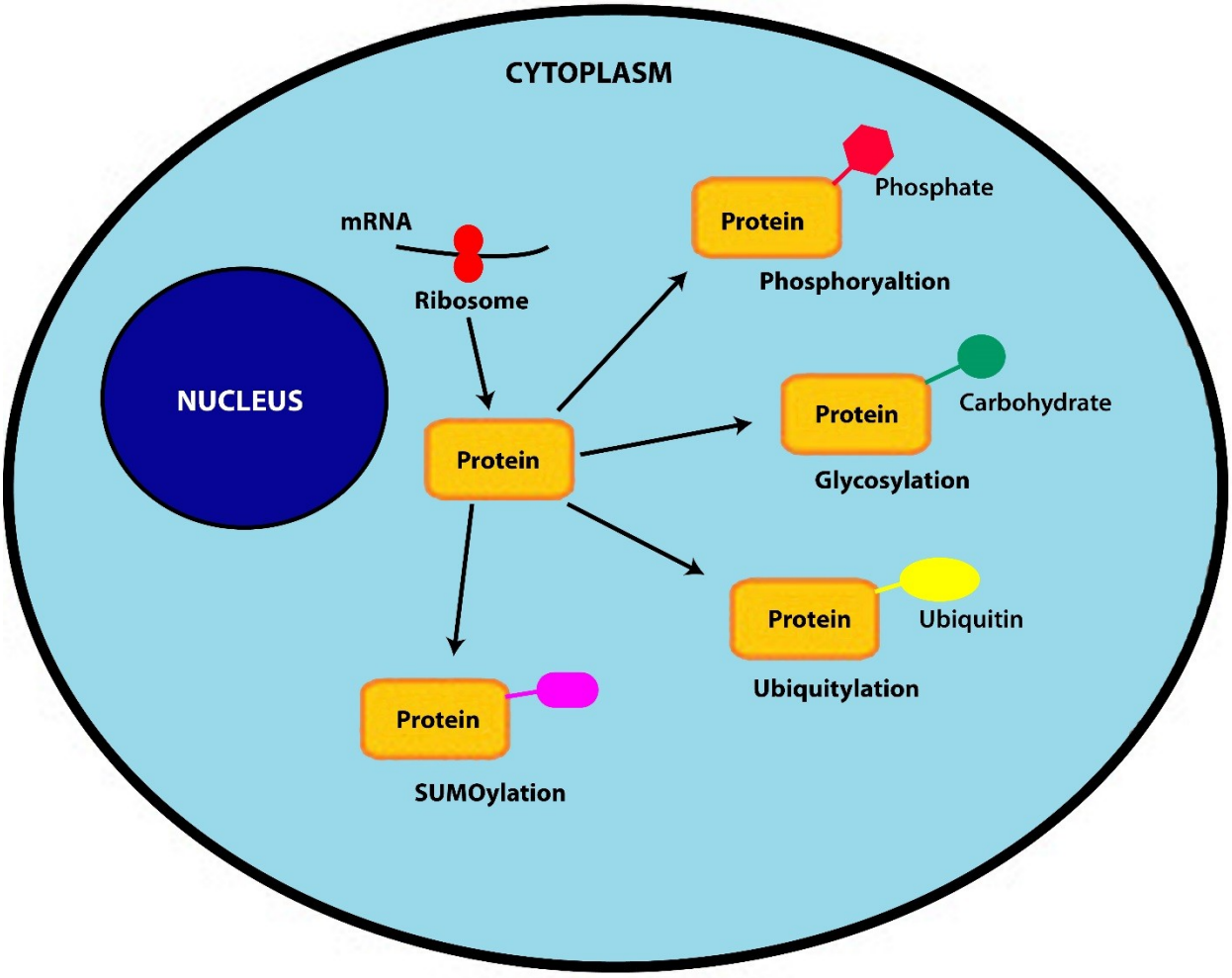
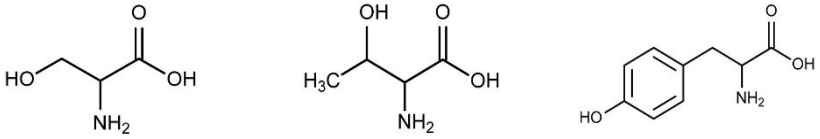


Figure 2.8. Cartoon Depicting Various Protein Post-Translational Modifications (Cohen, 2000).

Protein phosphorylation regulates several cellular processes such as the cell cycle, growth, apoptosis, and signal transduction pathways. Phosphoproteomics is the identification and characterization of phosphorylated proteins. Phosphorylation plays a key role in transmitting signals throughout the cell and in the regulation of protein functions. Although phosphorylation has been observed in both bacterial and eukaryotic proteins, it is more prevalent in eukaryotes. Reversible protein phosphorylation primarily on serine, threonine, or tyrosine residues is well understood (Cohen, 2000).



Serine Threonine Tyrosine

Figure 2.9. Amino Acids Involved in Phosphorylation.

In eukaryotic cells, phosphorylation potentially occurs on serine, threonine and tyrosine side chains (Figure 2.9). These amino acids have a nucleophilic hydroxyl (-OH) group that attacks the terminal phosphate group (γ-$PO3^{2-}$) of adenosine triphosphate (ATP), causing it to donate its phosphate group to the amino acid side chain. This process is facilitated by magnesium (Mg^{2+}), which chelates the γ- and β-phosphate groups in order to lower the threshold for transfer of phosphoryl to the nucleophilic (–OH) group. It is a unidirectional reaction due to the breakdown of the phosphate-phosphate bond from ATP to adenosine diphosphate (ADP) and the release of energy (Figure 2.10).

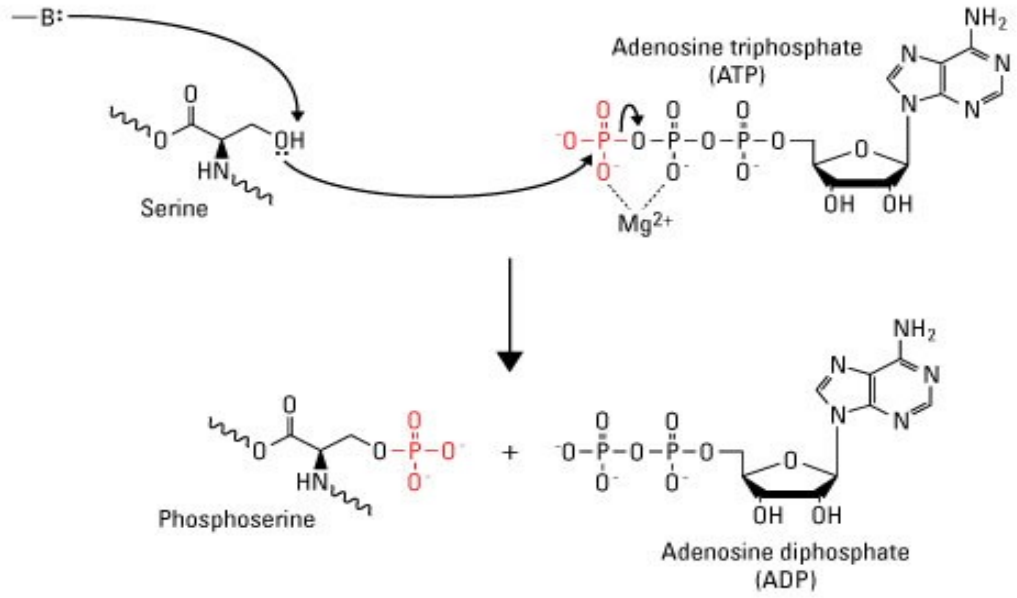


Figure 2.10. Mechanism Depicting Serine Phosphorylation. (Cohen, 2000). Enzyme-catalyzed proton transfer from the (–OH) group on serine stimulates the nucleophilic attack of the γ-phosphate group on ATP, resulting in transfer of the phosphate group to serine to form phosphoserine and ADP. (—B:) indicates the enzyme base that initiates proton transfer.

Protein phosphorylation is a reversible PTM wherein enzymes phosphorylate (kinases) and dephosphorylate (phosphatases) protein substrates (Figure 2.11). A network of protein kinases and phosphatases is responsible for switching between different pathways of cell division, development, and differentiation in response to growth factors or hormones.

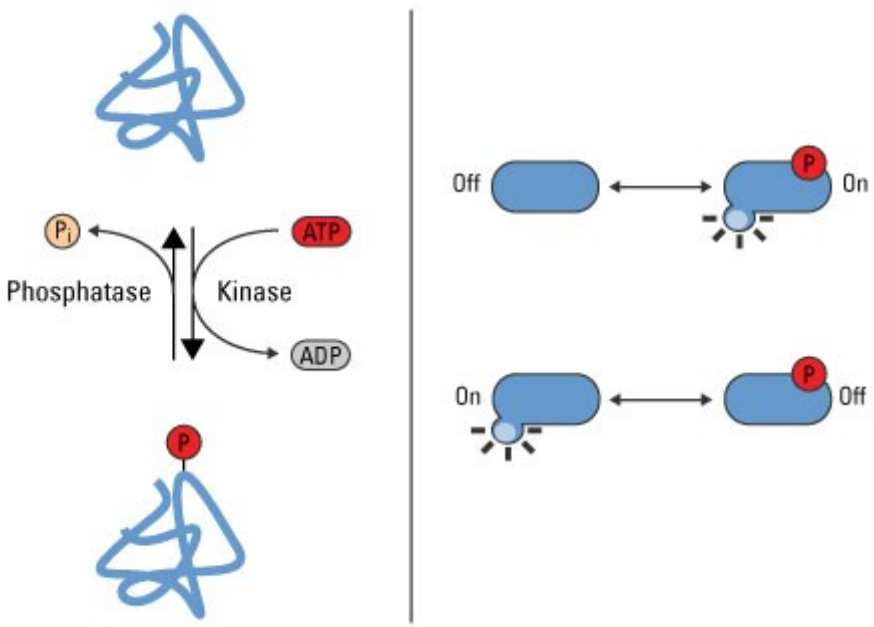


Figure 2.11. Phosphorylation Process: A Reversible PTM that Regulates Protein Function. (Cohen, 2000).

Left side: Protein kinases mediate phosphorylation at serine, threonine and tyrosine side chains, and phosphatases reverse protein phosphorylation by hydrolyzing the phosphate group.

Right side: Phosphorylation causes conformational changes in proteins that either activate (top) or inactivate (bottom) protein function.

The intensity and duration of phosphorylation-dependent signaling is regulated by three mechanisms (Walsh, 2006): removal of the activating ligand, kinase or substrate proteolysis, and phosphatase-dependent dephosphorylation. Protein phosphorylation, being a reversible PTM, is ideal for signal transduction, allowing cells to respond to any intracellular or extracellular stimuli. The signal transduction cascade involves a nexus of proteins that respond to physical sense cues by undergoing cleavage, ligand binding, or some other response. This sensing of the signal is then relayed to the second messenger and signaling enzymes. During phosphorylation, these receptors activate downstream kinases followed by phosphorylation and activation of downstream substrates and additional kinases until the specific response is achieved. Hence, with

this kind of signaling, a single molecule can activate a cellular program. For example, a growth factor can activate proliferation (Johnson and Lapadat, 2002).

CHAPTER 3: RAPID *IN-VITRO* EXCYSTATION OF *CRYPTOSPORIDIUM PARVUM*

3.1. Introduction

The protozoan parasite *Cryptosporidium parvum* causes the diarrheal disease cryptosporidiosis in animals and humans. Transmission can occur directly via a fecal-oral route or indirectly via contaminated food and water. First associated with human disease in 1976 (Nime et al., 1976), *Cryptosporidium* emerged as a serious human pathogen when it was identified as the cause of unrelenting diarrheal disease and death in people with AIDS (Anonymous, 1982). It is a frequent cause of waterborne disease outbreaks in the USA, Europe, and Australia (Robertson et al., 2002; Goh et al., 2004; Hunter et al., 2004b; Roy et al., 2004), and it is a major cause of death from diarrheal disease in children in Sub-Saharan Africa and Asia (Kotloff, 2013). Current anti-cryptosporidial agents are ineffective, particularly in immunocompromised persons, and the search for new targets is a major research priority. A focus of this search has been on critical early stages during infection, when motile sporozoites are released from oocysts to invade cells and commence replication. This process is repeated in a cycle of replication that is responsible for the pathology that defines the disease cryptosporidiosis. Understanding the mechanisms that underlie this cycle should yield promising new drug targets.

Following ingestion, infectious *Cryptosporidium* oocysts pass through the stomach and excyst in the small intestine to release four motile sporozoites. The acidic environment of the stomach primes oocysts for excystation, which is subsequently triggered by temperature, bile, enzymes, trypsin, and a higher pH in the small intestine (Fayer and Leek, 1984; Reduker and Speer, 1985; Current et al., 1990). Oocysts also can be excysted *in vitro* by mimicking conditions found in the gut.

In vitro excystation is used in research, industry, and regulatory settings as a reliable surrogate of viability: if an oocyst excysts, it is regarded as viable. However, viable oocysts may not be infective, so infecting laboratory animals remains the gold standard when determining the likelihood that oocysts could cause disease. Black et al. (1996) reported that *C. parvum* oocyst viability and infectivity are often overestimated by *in vitro* excystation assays. The advantages and disadvantages of the various viability and infectivity assays are listed in Table 3.1. Although *in vitro* excystation measures viability, not infectivity, it remains a useful indicator in resource-limited settings.

Table 3.1. Different Types of Viability Assays for *C. parvum* (AWWARF, 2000)

Assay	**Advantages**	**Disadvantages**
Animal infection	Measures infectivity	Expensive Labor intensive Requires animal holding facility Not all genotypes infect mice
Cell culture infectivity assay	No animals required. Measures infectivity	Expensive tissue culture Potential for contamination Difficult assessment of infection
In vitro excystation	No animals required Inexpensive Simple to use Expensive equipment not required Easy to analyze Rapid assessment of viability	Does not measure infectivity

Table 3.1. **Different types of viability assays for *C. parvum*** (AWWARF, 2000) *(continued)*

Assay	Advantages	Disadvantages
	Little expertise required No contamination issues	
Nucleic acid dyes	Simple to use Reliable	Does not measure infectivity Labor intensive (microscopy) Requires expensive equipment
Molecular techniques (E.g. RT-PCR)	Sensitive	Does not measure infectivity Requires molecular lab setup and expertise

Most *in vitro* excystation protocols require a short pre-treatment in acid or bleach for approximately 10 min (Current et al., 1990; Woodmansee et al., 1990). Bleach pretreatment is widely used because it has the added advantage of disinfecting the oocysts, which prevents bacterial contamination during cell culture. Bleach has been shown to remove the outer veil from the oocyst wall, which primes the oocyst for efficient *in vitro* excystation (Petry, 2004). Unlike bleach, acid pretreatment mimics the acidic environment of the stomach, which oocysts must pass through en route to excysting in the small intestine. Acid pretreatment is therefore more appropriate in studies of parasite molecular and cellular dynamics during excystation and early development. Following pretreatment, oocysts are incubated for an hour or more at 37°C in the presence of sodium taurocholate or bile. Because excystation occurs stochastically, sporozoites examined post excystation will not be well synchronized with respect to development. This can significantly complicate studies of molecular dynamics during early parasite development. Gut

and Nelson (1999) published a synchronized excystation assay, which reportedly achieved almost complete excystation in 3 min; however, this method has not been widely adopted, and some researchers have failed to replicate their findings (M. Kvac, personal communication).

The specific goal of this research was to identify *in vitro* conditions under which rapid excystation of oocysts could be achieved using an acid pretreatment step. The rationale is that attaining this goal will enable a more accurate assessment of gene and protein expression dynamics during the early stages of *Cryptosporidium* development. The approach was to compare the rapid excystation protocol reported by Gut and Nelson (1999) with a standard one-hour excystation protocol in order to identify factors that are essential for rapid excystation.

3.2. Materials and Methods

3.2.1. Chemicals and Reagents

Phosphate buffered saline (PBS) tablets, hydrochloric acid (HCl), sodium taurocholate (NaTC) powder and RPMI 1640 media (Roswell Park Memorial Institute) were purchased from Sigma Aldrich (St. Louis, MO, U.S.A). Hypochlorite (bleach) was purchased from VWR International, Inc. (West Chester, PA, USA). All solutions were filter sterilized and all procedures were performed in a laminar flow hood.

3.2.2. Source of Oocysts

Oocysts of the *C. parvum* Iowa isolate were purchased from Waterborne Inc. (6047 Hurst St., New Orleans, LA) and were stored at 4°C for less than 6 months. Oocysts were suspended in PBS with antibiotics (penicillin, streptomycin, gentamycin, amphotericin B) and 0.01% Tween.

3.2.3. Standard One-Hour *In Vitro* Excystation Assay

A 10 µl aliquot containing or 62,500 oocysts was transferred from a stock of 5×10^7 oocysts/8 ml to a microcentrifuge tube and pelleted by centrifugation at 20,000 × g for 5 min. The pellet was resuspended in freshly prepared 10% (v/v) ice-cold sodium hypochlorite and incubated for 10 min at 4°C in a refrigerator (bleach pretreatment). Bleach-treated oocysts were washed twice in PBS (20,000 × g for 5 min), resuspended in RPMI 1640 with 0.8% NaTC (excystation media), and incubated at 37°C for 60 min. Following the incubation, the suspension was pelleted at 20,000 × g for 5 min, the supernatant was removed and discarded, and the pellet was resuspended in PBS. A 5 µl aliquot was transferred to a slide and excysted versus non-excysted oocysts were viewed at 60× and 100× magnification under differential interference contrast (DIC) on an Olympus BX61 microscope.

3.2.4. Proposed Rapid *In Vitro* Excystation Assay

We developed a protocol for the *in vitro* excystation of *C. parvum* oocysts that met our requirement for a rapid assay with an acid pretreatment step (henceforth referred to as the Joshi assay). Briefly, a 10 µl aliquot containing 62,500 oocysts was transferred to a microcentrifuge tube and pelleted by centrifugation at 20,000× g for 5 min. The pellet was resuspended in 10 mM HCl (pH 1.5) and incubated in a water bath at 37°C for 10 min (acid pretreatment step, based on the protocol described by Gut and Nelson (1999). The suspension was centrifuged at 20,000 × g for 5 min and the pellet was resuspended in RPMI 1640 containing 0.8% NaTC (RPMI-NaTC; excystation media). The suspension was incubated at 37°C for 10 min in a water bath,

centrifuged at 20,000 × g for 5 min, the supernatant was removed, and the pellet was resuspended in PBS.

Variations of the Joshi assay were examined to determine how acid pretreatment, excystation media, excystation time, and excystation temperature affect excystation (Figure 3.1). A treatment with 10 mM HCl alone was carried out to determine the extent to which excystation occurs during the acid pretreatment step. Two excystation treatments (RPMI alone and RPMI-NaTC) were examined without the 10 mM HCl step to determine the extent to which the acid pretreatment step is necessary for efficient excystation. Excystation steps in all treatments were carried out at 4°C and 37°C for 10 min and 60 min. A 5 µl aliquot from each treatment was transferred to a glass slide and excysted versus non-excysted oocysts were examined for excystation using DIC microscopy at 60× and 100×.

3.2.5. Rate of Excystation Using the Joshi Assay

To accurately determine the rate of excystation over short time periods, it is necessary to be able to stop the excystation reaction instantaneously. Our preliminary studies showed that reducing the temperature is insufficient to stop excystation, so we used methanol, a common fixative, as an alternative approach to instantaneously stop all biological reactions in the cell. To test the efficacy of methanol, oocysts were treated with 10 mM HCl, as described earlier, centrifuged at 20,000 × g for 5 min, and resuspended in 16 µl of absolute methanol and 4 µl of RPMI-NaTC (80% methanol). As a control, the pellet was resuspended in 20 µl of RPMI-NaTC without methanol, as described previously.

To determine the rate of excystation using the Joshi assay, 10 mM HCl pretreated oocysts were incubated in 20 µl of RPMI-NaTC for 2, 4, 6, 8, 10 and 12 min. Excystation was stopped

by adding 80 µl methanol. An aliquot was analyzed by counting excysted versus non-excysted oocysts using DIC microscopy. All experiments were performed in triplicate.

3.2.6. Comparison of Excystation Achieved Using the One-Hour, Joshi, and Gut and Nelson Assays

Excystation achieved using the standard one-hour assay (3.2.3.) and the Joshi assay (3.2.4.) were compared to a rapid assay previously reported by Gut and Nelson (1999). For the Gut and Nelson assay, 62,500 oocysts were pelleted by centrifugation at 3,000× g for 1 min, suspended in 10 mM HCl, and incubated at 37°C for 10 min (acid pretreatment). The suspension was centrifuged at 3,000× g for 1 min and the pellet was resuspended in 2 mM NaTC in PBS and incubated for 10 min at 15°C, followed by a 3 min incubation at 37°C. Following incubation, the suspension was pelleted at 3,000 × g for 1 min, the supernatant was removed and discarded, and the pellet was resuspended in PBS.

3.2.7. Differential Interference Contrast (DIC) Microscopy

The wells of glass slides were outlined with a hydrophobic pen before adding a 5 µl aliquot of oocysts for analysis. A cover slip was added, sealed with transparent nail paint, and dried for 5 minutes before being viewed under differential interference contrast at 60× and 100× magnification using an Olympus BX 61 microscope. A total of 100 excysted versus non-excysted oocysts were counted (Figure 3.1).

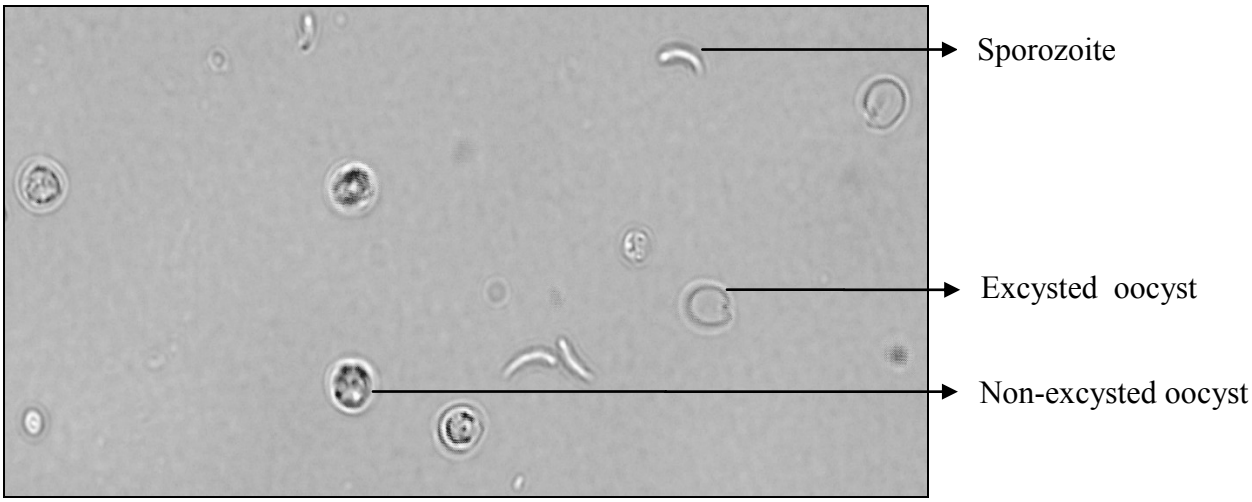


Figure 3.1. Differential Interference Contrast Microscopic Image of Excysted and Non-Excysted Oocysts of *C.parvum*.

3.3. Results and Discussion

3.3.1. The Joshi Assay

Figure 3.1 shows excystation achieved using the Joshi assay and variants of the Joshi assay. When excystation was performed at 37°C for 10 min and 60 min, the Joshi assay resulted in 97% and 98% excystation, respectively (Figure 3.1). When excystation was carried out at 4°C, excystation dropped to 10% and 33% after 10 min and 60 min, respectively. There was no excystation when the 10 mM HCl treatment was carried out alone, indicating that the acid pretreatment does not, by itself, result in excystation. However, the acid pretreatment is necessary for efficient excystation, as indicated by the absence of (10 min incubation; Figure 3.2A) or greatly reduced (60 min incubation; Figure 3.1B) excystation when this step was excluded. The importance of NaTC in the excystation media is demonstrated by the 10% reduction in excystation when only RPMI is used as the excystation media (Figure 3.2B). The Gut and Nelson protocol showed less excystation in our hands, which is in line with the experiences of others with this method (M Kvac, personal communication).

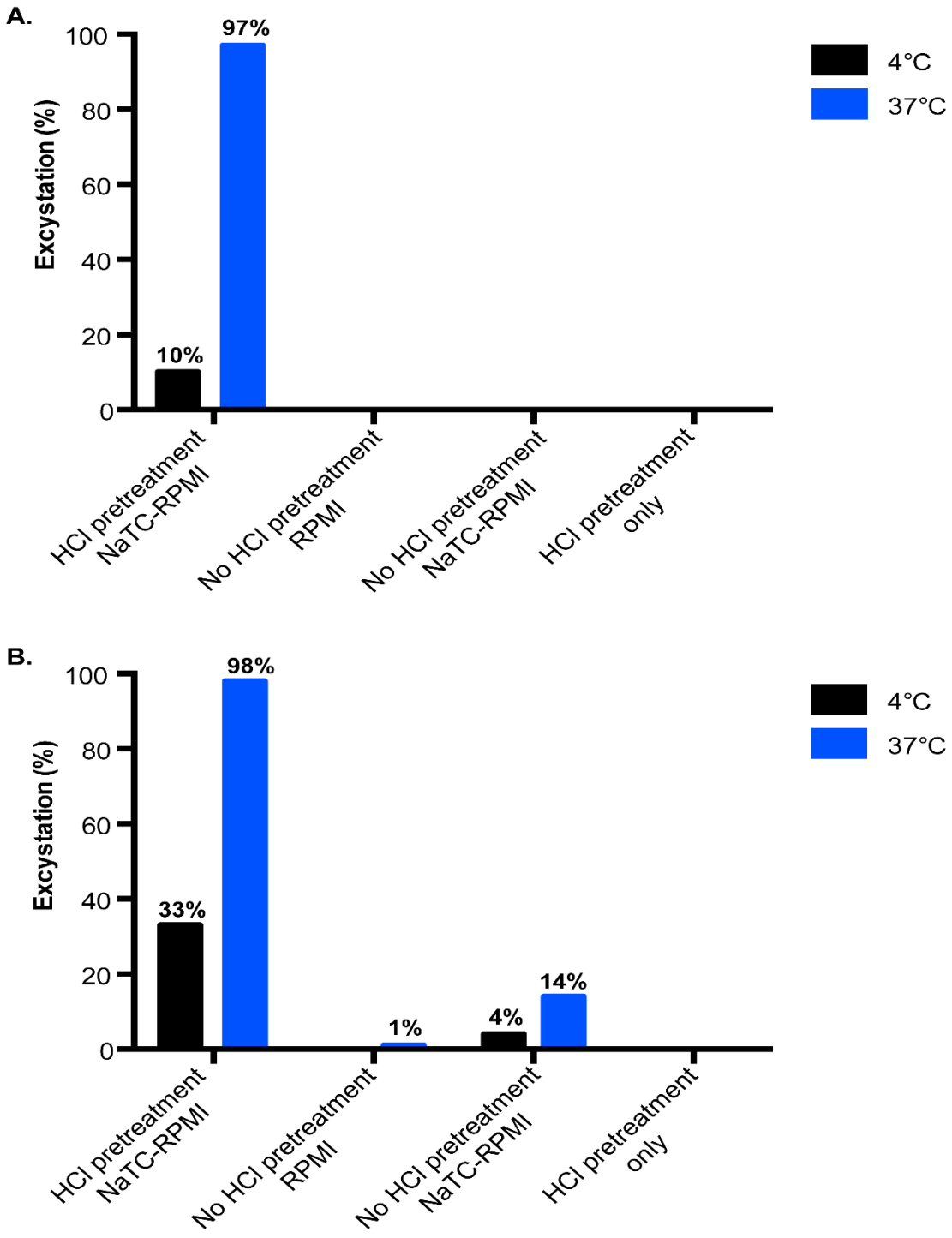


Figure 3.2. Excystation Achieved Using the Joshi Assay and Variants of the Joshi Assay, with Excystation Carried Out at 4°C and 37°C for 10 min [A] and 60 min [B].

3.3.2. Rate of Excystation Using the Joshi Assay

Although several studies have examined excystation in *Cryptosporidium*, none have examined the rate of excystation. Hence, this experiment was designed to examine the change in excystation of *C. parvum* oocysts with respect to incubation time in RPMI-NaTC. Figure 3.3 shows the rate and reproducibility of excystation using the Joshi assay. The percent excystation increased rapidly, reaching 90% by 8 min, 93% by 10 min, and 96% by 12 min. To verify the reproducibility of excystation, the experiment was repeated three times and precision was calculated from the standard deviation. The rate of excystation was highly reproducible (±0 to 2.8%) as shown in Figure 3.3.

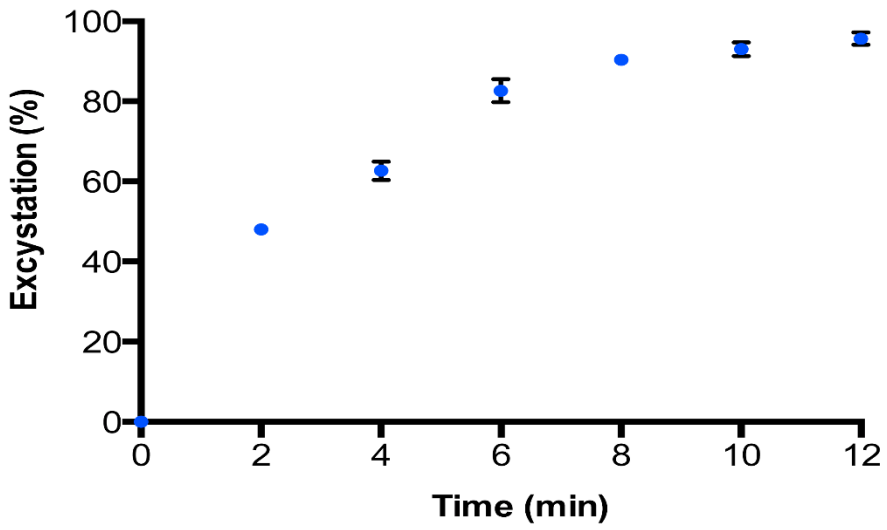


Figure 3.3. Rate of Excystation Using the Joshi Assay. Error bars indicate standard deviations.

3.3.3. Comparison of Excystation Achieved Using the One-Hour, Joshi, and Gut and Nelson Assays

Table 3.2 summarizes the features of the excystation assays that were compared. The standard protocol uses 10% hypochlorite (bleach) to remove the outer glycocalyx layer from oocysts prior to a 60 min excystation in RPMI-NaTC. As shown in Figure 3.4, this assay results in 94% excystation in our hands. However, a major drawback of this assay is that excystation is not well synchronized i.e. excystation occurs stochastically during the 60 min incubation. This lack of synchronization can present a problem in experiments that seek to quantify the molecular and cellular changes that take place during and after excystation. A second major drawback of the standard excystation assay is that the bleach treatment uses an alkaline environment to remove the oocyst glycocalyx, which does not reflect *in vivo* conditions. The Joshi and Gut and Nelson assays include an acid pretreatment (pH 2.5) that more closely mimics the acid environment in the stomach. Gut and Nelson report that complete excystation occurs within a 3 min incubation window, compared to 10 min for complete excystation using the Joshi assay. The Gut and Nelson assay also includes a 10 min incubation at 15°C, which the authors state is to allow oocysts to equilibrate in the media prior to excystation at 37°C. We have shown that excystation can occur at 4°C, albeit at a significantly reduced rate, and we expect that some excystation will occur during the 10 min incubation at 15°C. Therefore, we decided not to include the equilibration step in the Joshi protocol.

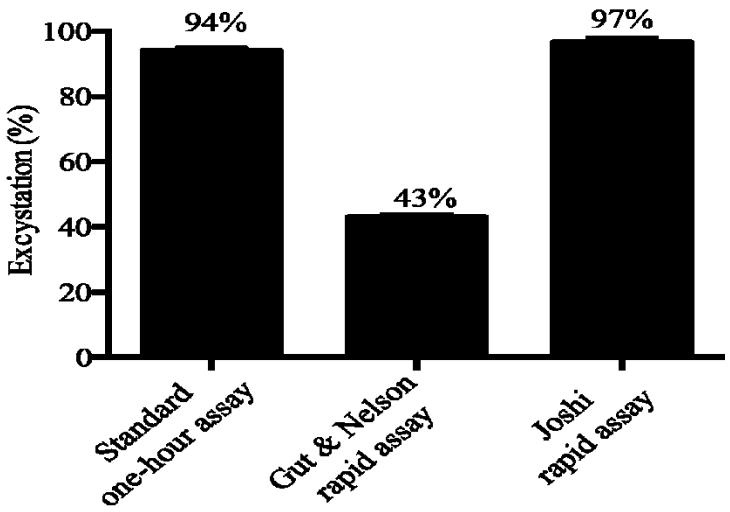


Figure 3.4. Oocyst Excystation Using Three Assays.

Table 3.2. Comparison of the Excystation Conditions in the Three Different Assays

Step	Standard Assay	Gut and Nelson Assay	Joshi Assay
Centrifugation time	5 min	1 min	5 min
Centrifugation speed	20,000 × g	3,000 × g	20,000 × g
Initial treatment	10% bleach	10 mM HCl	10 mM HCl
pH	12.6	2.5	2.5
Temperature	4°C	37°C	37°C
Excystation media	RPMI + 0.8% NaTC	PBS + 0.11% NaTC	RPMI + 0.8% NaTC
Incubation temp.	37°C	15, 37°C	37°C
Incubation time	60 min	10, 3 min	10 min
Incubation apparatus	Incubator	Thermo cycler	Water bath
Total time of assay	105 min	51 min	55 min
Percent excystation	94%	43%	97%

3.4. Summary

We developed a novel, rapid, reproducible excystation assay for *C. parvum* (Joshi assay). Factors required for the optimal excystation included a pretreatment of oocysts at 37° C for 10 min in HCl at pH 2.5 followed by incubation at neutral pH in the presence of NaTC. The Joshi excystation assay can be used to achieve well-synchronized sporozoites for downstream molecular analyses.

CHAPTER 4: ENRICHMENT AND 2-DIMENSIONAL ANALYSIS OF PHOSPHORYLATED PROTEINS OF *C. PARVUM*

4.1. Introduction

Eukaryotic cells can respond to their environment by modifying protein activities in response to specific signals. Protein phosphorylation cycles are a key component of the intracellular response to external signals (Kutuzov and Andreeva, 2008), and they play an important role in biological activities such as invasion and pathogenic capacity (Gonzalez, 2000). Kinases and phosphatases catalyze reversible protein phosphorylation during signal transduction, and these enzymes are considered promising targets for chemotherapeutic intervention and drug development (Gonzalez, 2000). However, while abundant information is available on phosphorylation in multicellular eukaryotes, little is known about unicellular parasites. Snelling et al. (2006) described the proteins expressed in the protozoan parasite *C. parvum* before and after oocyst excystation. They identified 303 proteins, of which 56 were hypothetical and 26 showed increased expression following excystation. However, the post-translational modification (PTM) of *C. parvum* proteins during the early stages of infection has yet to be studied.

This study was designed to explore the protein dynamics involved in excystation of *C. parvum* oocysts. Therefore, Joshi's rapid *in vitro* assay was employed to achieve synchronous excystation of the oocyst population followed by the enrichment and analysis of PTMs. The repertoire of phosphorylated and non-phosphorylated proteins expressed pre- and post-excystation was enriched using 2-dimensional gel electrophoresis.

4.2. Materials and Method

4.2.1. Chemicals, Test Kits, and Source of Oocysts

Phosphate buffered saline (PBS) tablets, hydrochloric acid (HCl), sodium taurocholate (NaTC) powder, RPMI 1640 media (Roswell Park Memorial Institute), hydroxyethyl piperazineethanesulfonic acid (HEPES), Tris-HCl, and coomassie brilliant blue G-250 were purchased from Sigma Aldrich (St. Louis, MO, U.S.A). Sodium hypochlorite (Bleach) was purchased from VWR. Cholamidopropyl dimethylammonio propanesulfonate (CHAPS), dithiothreitol (DTT), iodoacetamide, 7 cm immobilized pH gradient (IPG) ready strips (pH 4-7 and 3-10), and silver stain plus kit were purchased from Biorad (Hercules, CA).

Two dimensional clean up kit, IPG buffer (pH 3-11) and destreak rehydration solution were purchased from GE Healthcare Lifesciences (Pittsburg, PA). A coomassie plus (Bradford) assay kit, halt protease inhibitor cocktail (100×), halt phosphatase inhibitor cocktail (100×), phosphoprotein enrichment kit, gel code phosphoprotein staining kit, tris-glycine-SDS (10×) buffer were purchased from Pierce Thermo Scientific, (Rockford, IL). Two-dimensional (2D) quant kit was purchased from GE Healthcare (Piscataway, NJ).

The Iowa strain of *C. parvum* oocysts used during this study was purchased from Bunch Grass Farm, Deary, Idaho. Oocysts were stored in PBS and antibiotics (1000 IU Penicillin, 1000 µg Streptomycin) at 4°C until used.

4.2.2. Non-Excysted and Excysted Oocysts

For excysted and non-excysted oocyst treatments, 85×10^6 oocysts were washed three times with 50 mM HEPES. For the non-excysted oocyst treatment, oocysts were pelleted at

20,000 × g for 5 min and subjected to a total protein extraction as described below. For the excysted oocyst treatment, oocysts were excysted using a rapid method described in Chapter 3 (Joshi assay). Excysted oocysts were pelleted at 20,000 × g for 5 min and subjected to total protein extraction.

4.2.3. Total Protein Extraction

The oocyst pellet was suspended in 2 ml lysis buffer (50 mM Tris and 1% CHAPS) containing 20 µl Halt protease inhibitor cocktail and 20 µl Halt phosphatase inhibitor cocktail. The solution was vortexed for 60 s and incubated for 10 min at room temperature, with 60 s vortexing every 2 min during the incubation. The solution was subjected to five freeze-thaw cycles, each involving freezing in liquid nitrogen for 3 min and thawing in a water bath at 55°C for 6 min. After vortexing for 60 s, 300 µl aliquots were added to five 1.5 ml tubes, and a 200 µl aliquot was added to a single 1.5 ml tube. All six tubes were sonicated (Bioruptor UCD-200) at high power (250 W) with a 30 s cycle (15 s sonication, 15 s idle) for 20 min. Tubes were maintained on ice throughout the sonication to avoid overheating. After sonication, insoluble components were pelleted at 20,000× g for 40 min at 4°C (Eppendorf Centrifuge 5417R), washed 3 times with 50 mM HEPES, and stored at -80°C. The supernatant, which contained proteins, was used for phospho-protein enrichment.

4.2.4. Protein Assays

It was necessary to determine the concentration of protein in each sample in order to maintain consistency among treatments. Protein samples used in electrophoresis are often difficult to quantify due to the presence of interfering carrier ampholytes, detergents, and

reductants from sample preparation. Considering the solubilizing reagents used during protein enrichment, three different protein assays were employed to determine the protein concentration:

1. *Colorimetric Coomassie Plus Assay*. This is based on the modified Bradford assay. When coomassie dye binds to a protein in an acidic medium the absorption maximum shifts from 465 nm to 595 nm with a concomitant color change from brown to blue.

2. *Reducing Agent and Detergent Compatible (RC DC) assay.* This is a colorimetric assay based on the Lowry method. It is modified to be reducing agent and detergent compatible. Absorption is measured at 650-750 nm.

3. *2D Quant Protein Assay*. This assay is specifically used to quantify protein for electrophoresis applications because it precipitates and determines the protein concentration in the presence of salts (SDS, DTT, urea, thiourea, CHAPS, IPG Buffer, etc.). It is a colorimetric assay wherein copper ions bind specifically to the precipitated protein. Absorption is quantified at 480 nm.

4.2.4.1. Protein Assay Results

C. parvum protein concentrations were determined using three protein assays (Coomassie, RC DC and 2D Quant). All three assays failed to produce a linear response to standard curves prepared with bovine serum albumin (BSA) standards or protein extracted from excysted and non-excysted *C. parvum* oocysts.

As an alternative to the protein assay kits, protein concentration was determined using a Nanodrop, ND-1000 spectrophotometer. This approach measures absorbance at 280 nm using only 1-2 µl of the protein sample. Moreover, it is easier to perform and more time efficient than

the kits. Therefore, all the protein samples (non-excysted or excysted phosphorylated and non-phosphorylated protein) were quantified using the Nanodrop.

4.2.5. Phosphorylated Protein Enrichment

A Pierce phosphoprotein enrichment kit was employed to enrich phosphorylated proteins from the total protein sample obtained from non-excysted and excysted oocysts. The kit includes a column that contains a proprietary metal/buffer composition that specifically binds the phosphorylated proteins to produce enriched phosphorylated proteins in two hours.

Protein samples were diluted 1:1 using the Lysis/Binding/Wash Buffer with CHAPS before loading onto a phosphoprotein enrichment column. The column was inverted to suspend the resin slurry. The bottom of the column was snapped off and, placed in a 50 ml conical tube with a loose cap. The tube containing the column was centrifuged at 1000 × g for 1 min at 4°C and the storage solution was discarded. The column resin was equilibrated by adding 5 ml of Lysis/Binding/Wash Buffer with CHAPS, placed in a 50 ml conical tube, and centrifuged at 1000 × g for one minute at 4°C. The flow through was discarded; the column was plugged at the bottom and 4 mg of protein lysate was added. The column cap was tightened and the column was inverted several times to mix before placing on a rocker-shaker at 4°C for 30 min. The column was unplugged, placed in a new 50 ml conical tube, and centrifuged at 1000 × g for one minute at 4°C. The flow-through containing unbound phosphorylated protein was saved and stored at -80°C for further analysis. The column resin was placed in a new 50 ml conical tube, washed three times using 5 ml of Lysis/Binding/Wash Buffer with CHAPS, and centrifuged at 1000 × g for one minute at 4°C. The column was plugged and the column was transferred to a new 50 ml conical tube. One milliliter of elution buffer (without CHAPS) was added, and the column was

incubated at room temperature for 3 min with occasional agitation using a Stovall belly dancer shaker (Greensboro, NC, U.S). The plug was removed and the column was centrifuged at 1000 × g for one minute at 4°C. This step was repeated five times to give five elution fractions (5 ml total). The eluted fractions contained and enriched fraction of phosphorylated proteins.

Eluted phosphorylated proteins were further concentrated using the proprietary concentrators provided with the Pierce phosphoprotein enrichment kit. The upper chamber of the concentrator tube was filled with 5 ml of eluted sample and centrifuged at 4000 × g for 30 min at 4°C in a centrifuge with a swinging bucket (Beckman Coulter Allegra X-15R). The concentrated phosphorylated protein sample was pipetted from the upper chamber and stored in a separate 1.5 ml tube for protein concentration determination and purification for two-dimensional electrophoresis. The flow through collected in the lower chamber of the concentrator, which contained non-phosphorylated proteins, was stored at -80°C before analysis by two-dimensional electrophoresis.

4.2.6. Protein Purification

A 2D purification kit was employed to purify and precipitate 100 µg of protein sample (phosphorylated and non-phosphorylated) in a 1.5 ml microcentrifuge tube. This kit improved the protein quality by removing interfering substances such as detergents, salts, lipids, phenolics, and nucleic acids.

Three hundred microliters of precipitant (included with the kit) was added to four 1.5 ml tubes containing 100 µg protein. The solution was vortexed for 60 s and incubated on ice for 15 min. Co-precipitant (300 µl; included with the kit) was added and the solution was vortexed briefly before centrifuging at 14,000 × g for 5 min at 4°C. The supernatant was carefully

removed without disturbing the pellet. The centrifugation step was repeated to remove any visible liquid. The pellet was layered with 40 µl of co-precipitant and incubated on ice for 5 min, followed by centrifugation at 14,000 × g for 5 min at 4°C. The supernatant was carefully removed and the pellet was resuspended by vortexing for 10 s in 25 µl of de-ionized water. One milliliter of pre-chilled wash buffer and 5 µl wash additive were added, the suspension was vortexed for 60 s, and incubated at -20°C for 30 min. Tubes were vortexed for 60 s every 10 min during the incubation. The tubes were centrifuged at 14,000 × g for 5 min at 4°C to pellet the protein. The supernatant was discarded and the precipitate was allowed to dry briefly. The semi-dried precipitate in each tube was resuspended in 130 µl of 0.5% rehydration buffer for Isoelectric Focusing (IEF). The rehydration buffer comprised IPG buffer (pH 3-11) and a destreak rehydration solution. The protein-rehydration buffer solution was vortexed for 60 s and incubated at room temperature for 5 min followed by centrifugation at 14,000 × g for 5 min at 4°C to remove any insoluble material and reduce any foam.

4.2.7. Two-Dimensional Gel Electrophoresis

Proteins were first separated by IEF (first dimension) before Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE; second dimension).

4.2.7.1. Isoelectric Focusing

The protein sample precipitated from the 2D clean up kit was rehydrated in 130 µl of 0.5% rehydration buffer. The rehydration buffer comprised IPG buffer (pH 3-11) and a destreak rehydration solution. IPG buffer is an ampholyte-containing buffer that helps in solubilizing proteins and provides uniform conductivity. The destreak rehydration solution was used to

eliminate extra spots caused by nonspecific oxidation of proteins and to stabilize proteins throughout the two dimensional electrophoresis.

The protein-rehydration buffer solution was vortexed for 60 s and incubated at room temperature for 5 min, followed by centrifugation at 14,000 × g for 5 min at 4°C to remove any insoluble material and reduce any foam. One hundred and twenty five microliters of sample was loaded into the strip holder between the electrodes. Immobiline pH gradient (IPG) dry strips, 7 cm in length, with a linear (L) pH range of 4-7 and 3-10, were used to run the phosphorylated and non-phosphorylated protein samples. Table 4.1 shows the four samples analyzed and the amount of protein loaded for each.

Table 4.1. Amount of Protein Sample Loaded for IEF

Sample	Amount of Protein Loaded onto each IPG Strip	IPG Strip pH Range
1. Non-excysted phosphorylated	100 µg	4-7 L
2. Excysted phosphorylated	100 µg	4-7 L
3. Non-excysted non phosphorylated	100 µg	3-10 L
4. Excysted non-phosphorylated	100 µg	3-10 L

Note: L= Linear

The IPG strips were placed on top of the loaded samples with the gel side facing towards the sample, allowing it to absorb the protein sample. The strip was covered with 200 µl of mineral oil to avoid evaporation of the sample. The strip holders were placed in a parallel position matching the positive and negative electrodes on the Ettan IPGphor III system. The strips were run for 23 hours 35 minutes at a maximum of 5000 V for 12,603 volt-hours. The strips were stored at -80°C prior to SDS-PAGE (2^{nd} dimension).

4.2.7.2. Gel System and SDS PAGE

Three gel systems were tested: gradient gel, Shaegger's gel, and laemmli gel (Figure 4.1). Prepoured gradient gels (4-20%) resulted in the greatest resolution of protein spots, but were not cost effective.

A manually prepared Shaegger's gel (12%) resulted in good resolution of lower molecular weight protein spots. However, it was a complicated and tedious gel system, which required two different running buffers during electrophoresis. Moreover, it took more than 2 hours to run, causing the gel to overheat.

Manually prepared laemmli gels were the least complicated and least expensive of the three gel systems. Laemmli gels, which ran in an hour and didn't result in overheating, were better at resolving higher molecular weight proteins than lower molecular weight proteins. Unlike the Shaegger's gel system, the laemmli system uses one running buffer (Tris-Glycine-SDS) inside and outside the cassette during electrophoresis. Based on its performance and cost-effectiveness, the laemmli gel system was selected for SDS-PAGE.

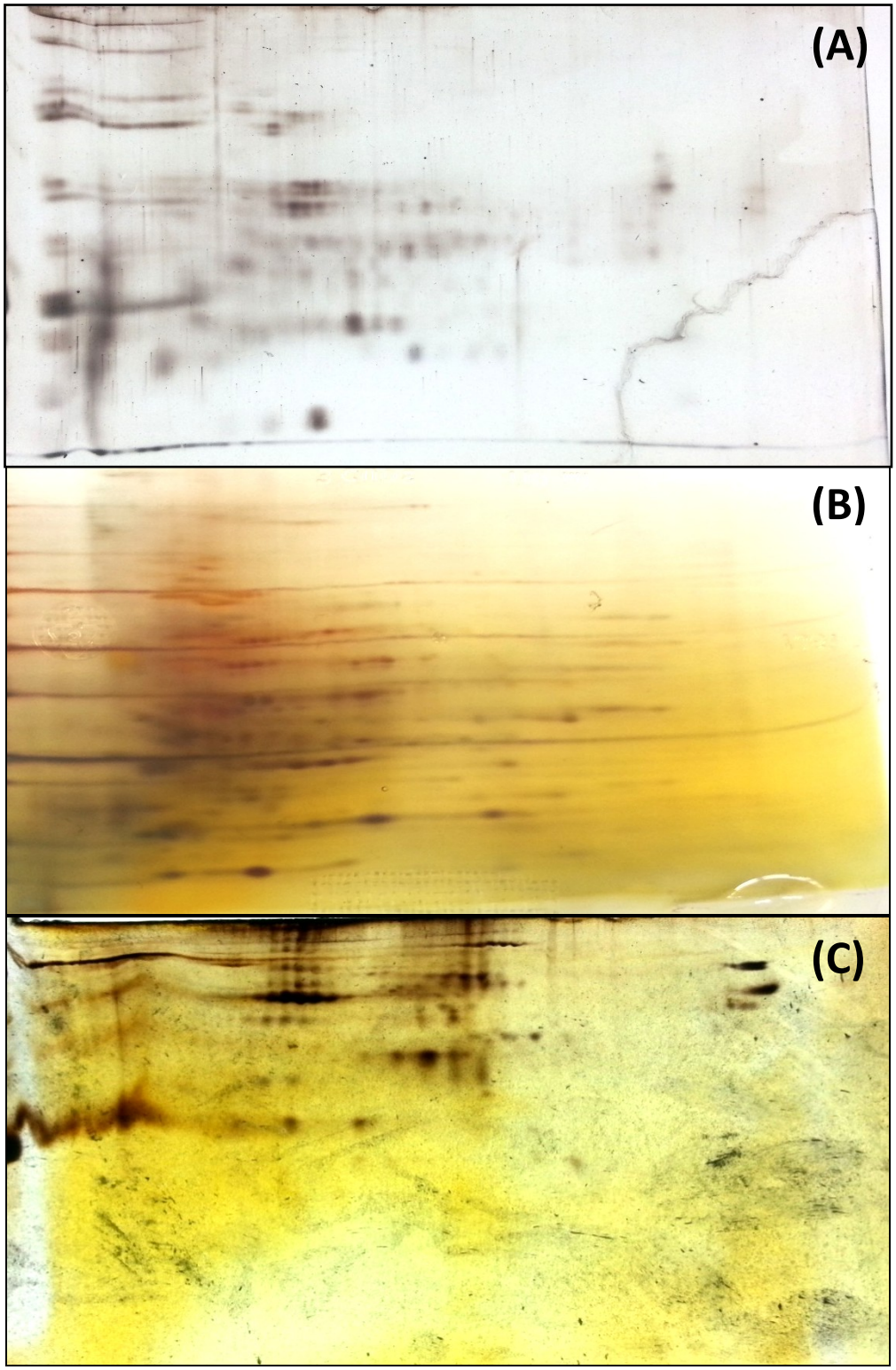


Figure 4.1. Comparison of Protein Spot Resolution Using 3 Different Polyacrylamide Gel Systems Stained with Silver Stain: A) Shaegger's (12%), B) Gradient (4-20%) and C) Laemmli (12%).

The laemmli gel used for SDS-PAGE was 1 mm thick. Twelve percent vertical gels were poured without a stacking gel. Each gel had two different sized wells; the smaller well was used for the molecular weight marker and the longer one was used for laying the IpG strip from IEF. Melted agarose was poured in the longer well followed by the addition of the focused IpG strip with its anode end towards the reference well. Tris Glycine SDS (1×) buffer was added to the gel system followed by the addition of 2 µl of dual colored molecular weight ladder. The gel was electrophoresed at 100V for an hour before staining.

Preliminary gels were run on a broader pH range of 3-11 to estimate the pH gradient of expressed proteins and the amount of protein to be loaded. The broader pH gradient permits identification of a pH range that is most suitable for the expressed proteins. A minimum of 100 µg of protein was required to observe protein spots adequately and the majority of proteins were observed in the acidic region of the pH gradient. Therefore, a pH range of 4-7 was selected for the phosphorylated protein study and a pH range of 3-10 was selected for the non-phosphorylated proteins.

4.2.8 Polyacrylamide Gel Staining

Three stains (phosphoprotein, colloidal coomassie, and silver) were tested. Gel code phosphoprotein stain, designed to specifically stain the phosphorylated proteins by hydrolyzing the phosphoester linkage of phosphoserine and phosphothreonine, was tested on a two-dimensional gel containing phosphorylated protein. However, protein spots were not visible in the sample or positive control with this stain.

Colloidal coomassie (G-250) is a dye commonly used for the direct visualization of proteins separated by SDS-PAGE. It binds to the protein to form a protein-dye complex, which

stabilizes the negatively charged anionic form of the dye to produce a blue color, even under acid conditions when most of the molecules in solution are in the cationic form. However, colloidal coomassie also failed to stain the protein spots.

Silver stain was employed because of its high sensitivity for proteins in polyacrylamide gels (30-40 fold more sensitive than the Coomassie stain). For silver staining, polyacrylamide gels are impregnated with soluble silver ion (Ag+) and developed by treatment with formaldehyde. Macromolecules in the gel promote the reduction of silver ion to metallic silver (Ag^0), which is insoluble and visible, allowing bands/spots containing protein or nucleic acid to be seen. The initial deposition of metallic silver promotes further deposition in an autocatalytic process, resulting in exceptionally high sensitivity. Both phosphorylated and non-phosphorylated protein spots were visible following silver staining, so this staining method was used for further studies.

Protein spots were visualized following a proprietary protocol provided by Biorad for the silver stain plus kit. Silver stained two-dimensional polyacrylamide gels were imaged manually using a Canon 5D III DSLR camera under trans-illuminating light. A comparison of the three staining methods is shown in Figure 4.2.

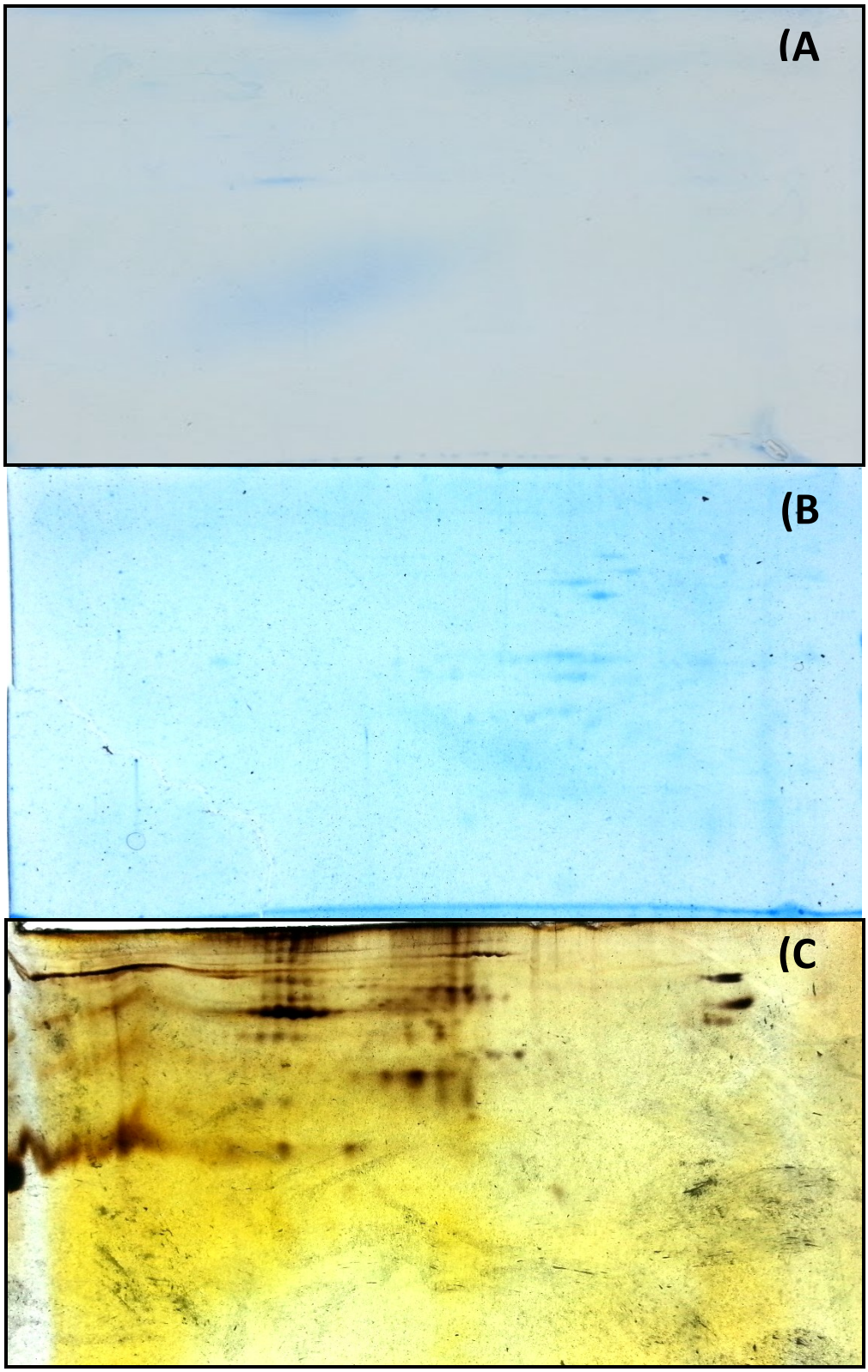


Figure 4.2. Comparison of Stains on a 12% Laemmli Gel A) Gel Code Phosphoprotein stained gel, B) Colloidal Coomassie stained gel and C) Silver stained gel.

4.2.9. Statistical Analyses

Protein concentration results from Nanodrop were statistically analyzed using Minitab software. Protein concentration data from 4 replicates were analyzed using the Analysis Of Variance (ANOVA) at 5% significance level. A follow up with enriched protein (total protein, phosphorylated protein and non-phosphorylated protein) comparison for both non-excysted and excysted condition was performed. ANOVA tested for our hypothesis that the obtained protein concentrations from total, phosphorylated and non-phosphorylated proteins were significantly different for the non-excysted and excysted conditions.

4.3. Results and Discussion

4.3.1. Protein Concentration

Total protein, phosphorylated protein, and non-phosphorylated protein were quantified in excysted and non-excysted oocysts. Figure 4.3 shows a comparison of the measured protein concentrations.

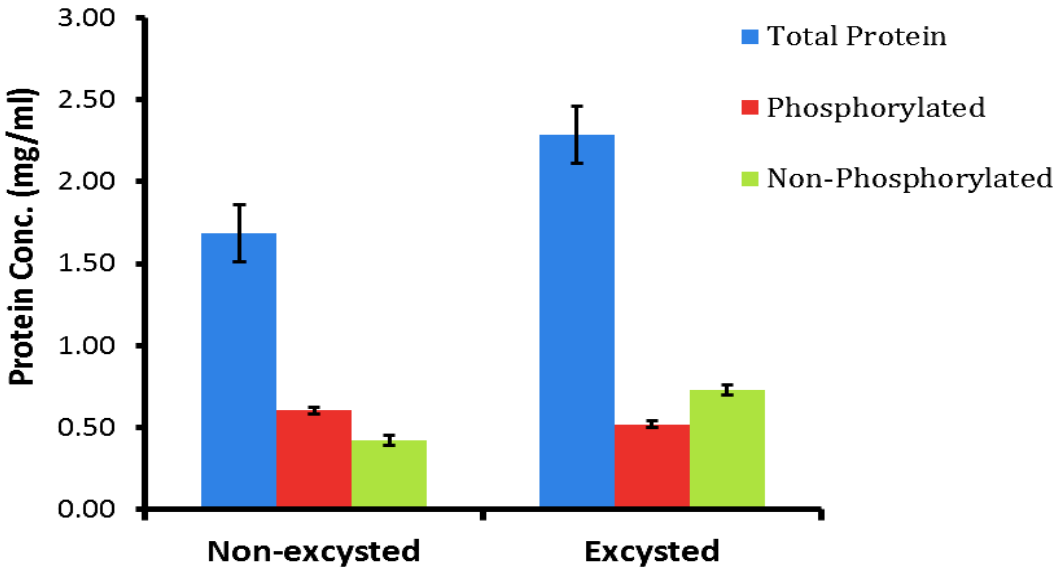


Figure 4.3. Protein Concentrations in Non-Excysted and Excysted Oocyts.

The total protein concentration for both the conditions (non-excysted and excysted oocysts) was considerably higher than the enriched phosphorylated and non-phosphorylated proteins. A higher protein concentration was also detected in the excysted oocysts compared to non-excysted oocysts, suggesting that protein expression increases during excystation. In contrast, there appears to be a slight decrease in proportion of phosphorylated proteins following excystation.

4.3.1.1. Statistical Analyses

The ANOVA test (one-way) was performed for the enriched total, phosphorylated and non-phosphorylated protein. As hypothesized the protein concentrations were significantly different for the non-excysted and excysted condition because the p-value for all the three protein concentrations were less than the significance level of 5%, i.e. p-value <0.05. Table 4.2 summarizes the results from the one way ANOVA test for the enriched total, phosphorylated and non-phosphorylated proteins. Detailed result obtained from ANOVA test can be viewed in the Appendix section.

Table 4.2. Summarized Statistical Analyses for Enriched Protein

Enriched Protein	Mean		Standard Deviation		p-value
	Non Excysted	Excysted	Non Excysted	Excysted	
Total Protein	1.6850	2.2850	0.1162	0.2310	0.004
Phosphorylated Protein	0.60500	0.52000	0.01915	0.02160	0.001
Non-Phosphorylated Protein	0.42500	0.72750	0.02380	0.03775	0.000

4.3.2. Two Dimensional Analyses

The first dimension of electrophoresis was the isoelectric focusing of phosphorylated and non-phosphorylated proteins from non-excysted and excysted oocysts. Figure 4.4 shows the IEF of phosphorylated proteins from non-excysted oocysts. The graph reflects the smooth rehydration and focusing (based on pI) of the protein sample on the IPG strip. Other treatments resulted in a similar IEF curve.

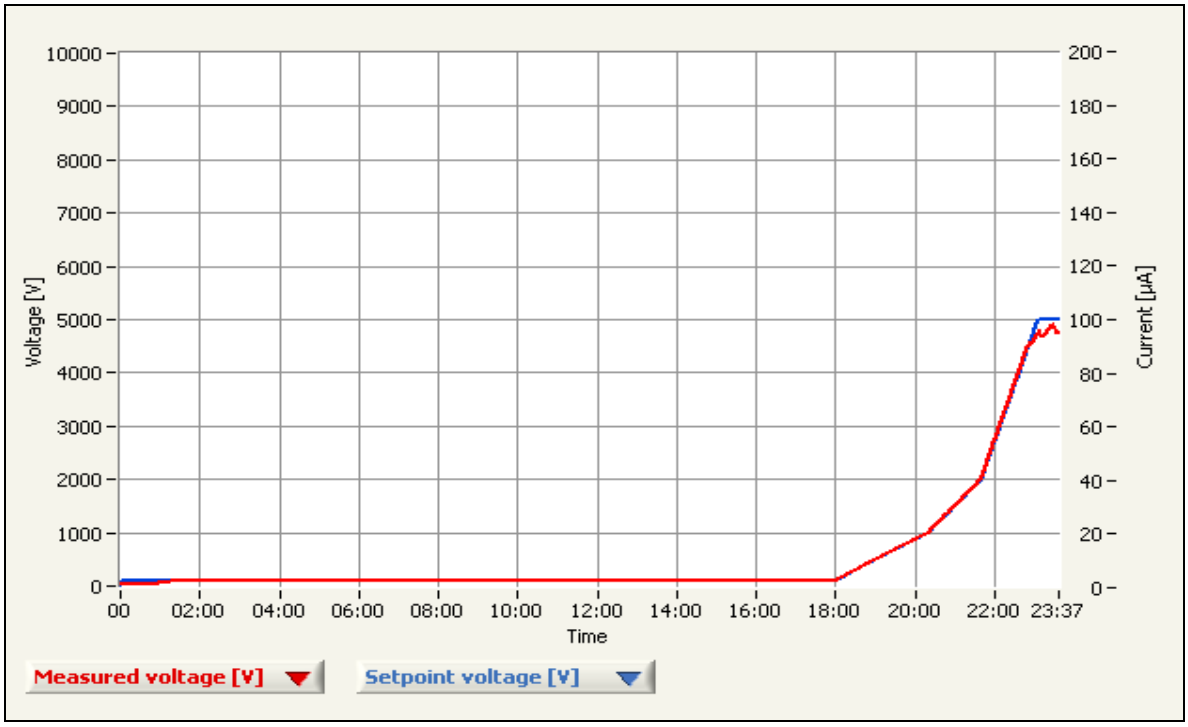


Figure 4.4. IEF Curve for Phosphorylated Proteins in Non-Excysted *C. parvum* Oocysts. The manually programmed set point voltage (blue line) was followed by the measured voltage (red line) smoothly.

Figure 4.5A shows the non-excysted phosphorylated proteins resolved in a 12% laemmli gel, and Figure 4.5B shows the excysted phosphorylated proteins resolved in a 4-20% gradient gel. Although these gels cannot be compared due to the difference in the gel percentage, excysted phosphorylated protein spots could only be observed clearly in the gradient gel. Hence, gradient gels were employed to visualize the highly resolved excysted phosphorylated proteins.

This study employed a comparative proteomics approach to visualize phosphorylated and non-phosphorylated protein expression from non-excysted and excysted *C. parvum* oocysts. Figure 4.5 (A and B) presents the 2 dimensional pattern of the expressed phosphorylated proteins obtained from non-excysted and excysted oocysts. In non-excysted oocysts (A), 46 phosphorylated protein spots were observed, compared to 36 protein spots in excysted oocysts (B). Since protein spots were observed throughout the gel within a pH range of 4-7, it indicates that majority of the phosphorylated proteins found in *C. parvum* are acidic, which could be due to the formation of acid-stable phosphomonoesters by serine, threonine and tyrosine. A greater number of phosphorylated protein spots were observed in the higher molecular weight region of the gels.

Besides enriching the phosphorylated proteins from the mixture of total protein, the phosphoprotein enrichment kit helped in separating the non-phosphorylated proteins. Figure 4.6 (C and D) show the non-phosphorylated protein pattern from non-excysted and excysted oocysts on a 12% laemmli gel. For the non-excysted oocysts, 73 non-phosphorylated protein spots were observed in the molecular weight range of 37-250; for excysted oocysts, 83 non-phosphorylated protein spots were observed in the molecular weight range of 15-150. No difference was observed between the two conditions; however, 14 common non-phosphorylated protein spots were observed on overlaid gel images.

Figures 4.5 and 4.6 show that for all treatments there were a greater proportion of non-phosphorylated proteins than phosphorylated proteins. Figures 4.7 and 4.8 (A, B, C and D) show the same gel images in Figure 4.5 and 4.6 with protein spots numbered. Table 4.3 summarizes the obtained results of the expressed phosphorylated and non-phosphorylated proteins from both non-excysted and excysted *C. parvum* oocysts.

Table 4.3. Summary of Expressed Phosphorylated and Non-Phosphorylated Proteins

Observed Protein Spots	Phosphorylated		Non-Phosphorylated	
	Non-Excysted	**Excysted**	**Non-Excysted**	**Excysted**
Gel Percentage	Laemmli (12%)	Gradient (4-20%)	Laemmli (12%)	Laemmli (12%)
pH Range	4-7	4-7	3-10	3-10
MW Range	15-150	15-150	37-250	15-150
Total Spots	46	36	73	83
Common spots	Not Comparable		14	

This is the first study that shows that we can successfully enrich and separate both phosphorylated and non-phosphorylated proteins from non-excysted and excysted *C. parvum* oocysts. To optimally enrich the phosphorylated and non-phosphorylated proteins from excysted and non-excysted oocysts, total protein can be extracted by sonication in Tris, CHAPS, and cocktail of protease and phosphatase inhibitors. Protein concentration can be determined using the Nanodrop spectrophotometer. A phosphoprotein enrichment kit (Thermo Scientific) can be used to separate phosphorylated and non-phosphorylated proteins and enrich phosphorylated proteins. Phosphorylated and non-phosphorylated proteins can be precipitated with a 2D clean up kit. For IEF, the first dimension of two-dimensional electrophoresis, IpG strips with a pH range of 4-7 are optimal for phosphorylated proteins, and strips with a pH range of 3-10 are optimal for non-phosphorylated proteins. For the second dimension, SDS-PAGE, 4-20% gradient gels are optimal followed by silver plus staining. To perform mass spectrometry, specific protein spots can be extracted from the silver stained gels and destained immediately or as soon as possible.

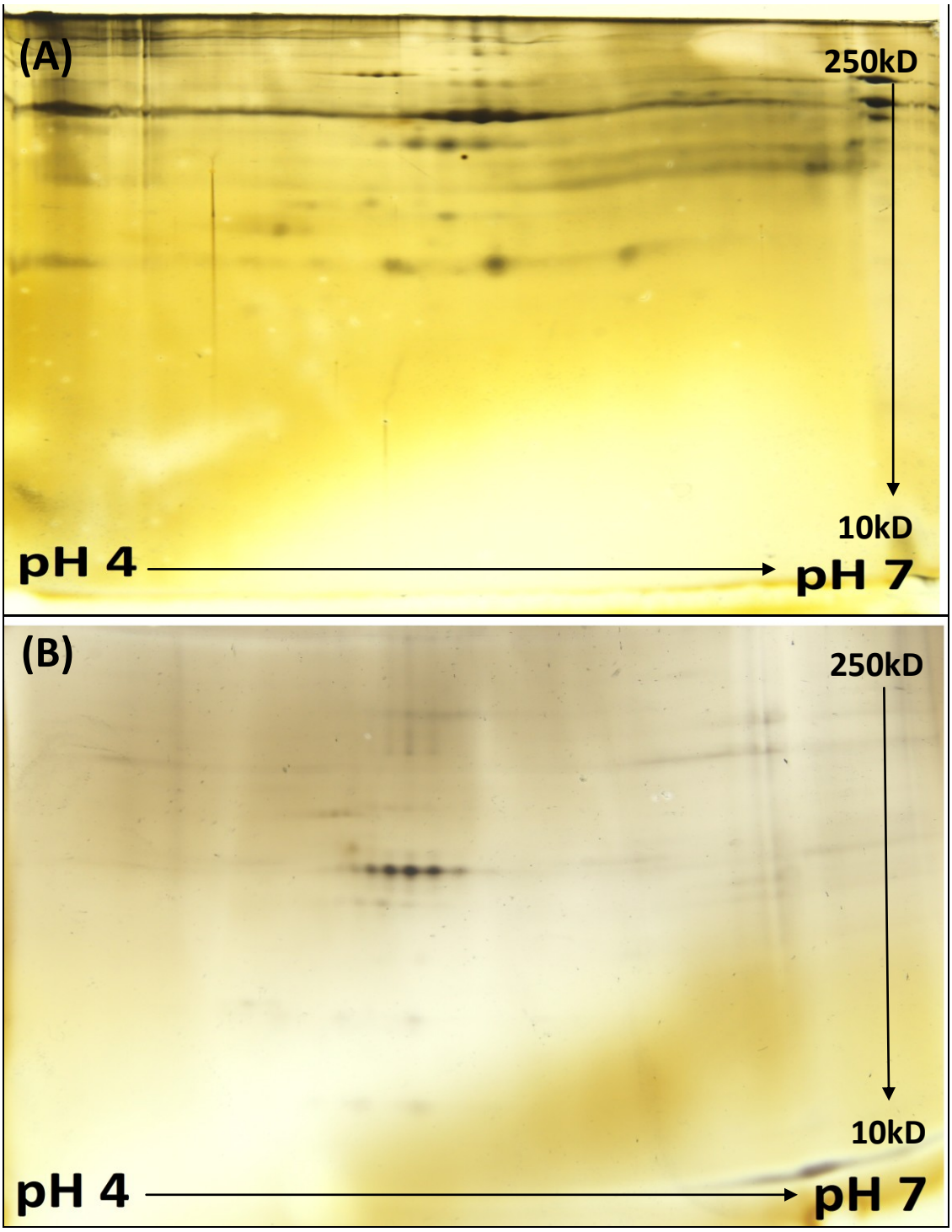


Figure 4.5. Two Dimensional View of Phosphorylated Proteins. (A) Phosphorylated protein gel of non-excysted oocysts (B) Phosphorylated protein gel of excysted oocysts.

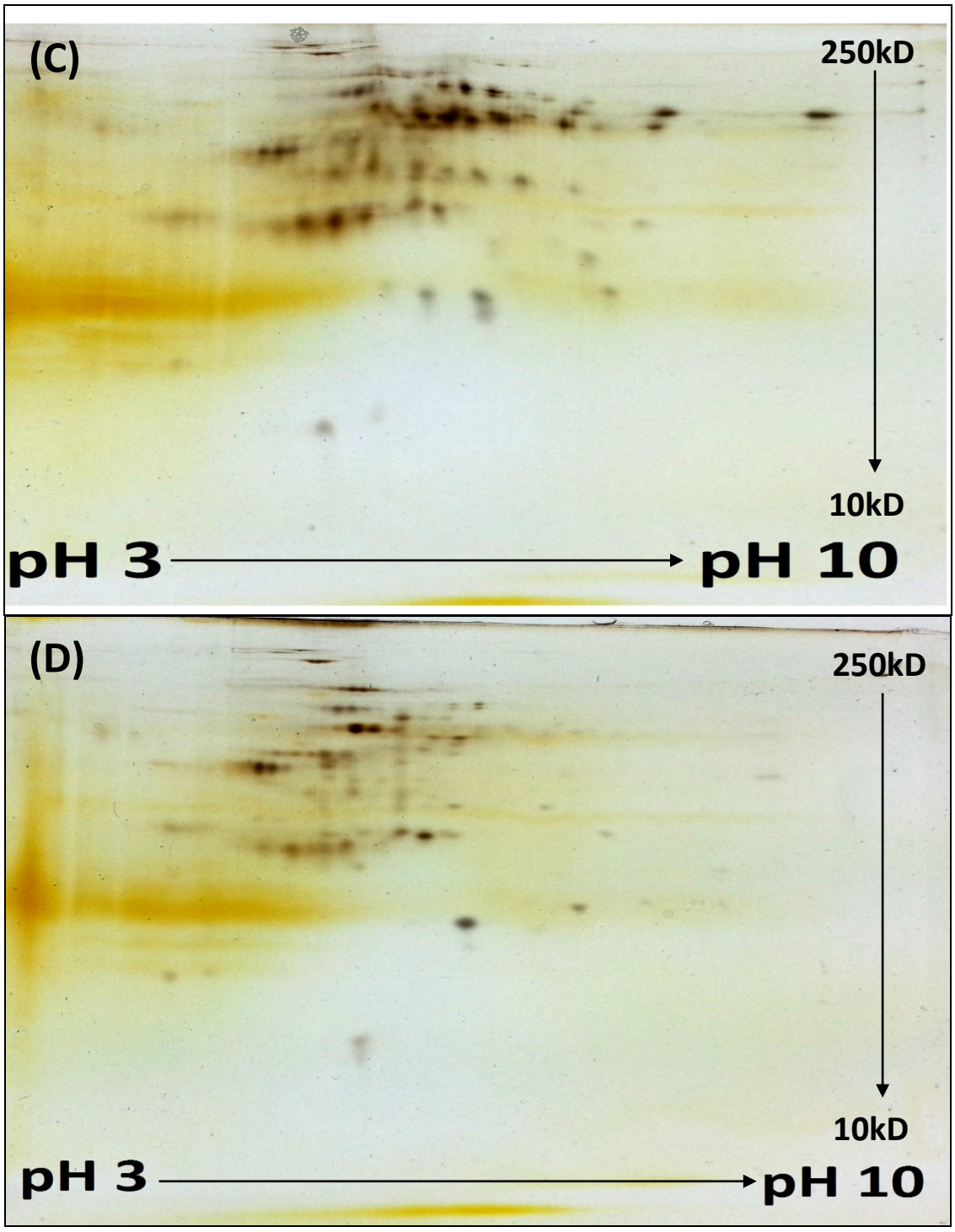


Figure 4.6. Two Dimensional View of Non-Phosphorylated Proteins. (C) Non-phosphorylated protein gel of non-excysted oocysts (D) Non-phosphorylated protein gel of excysted oocysts.

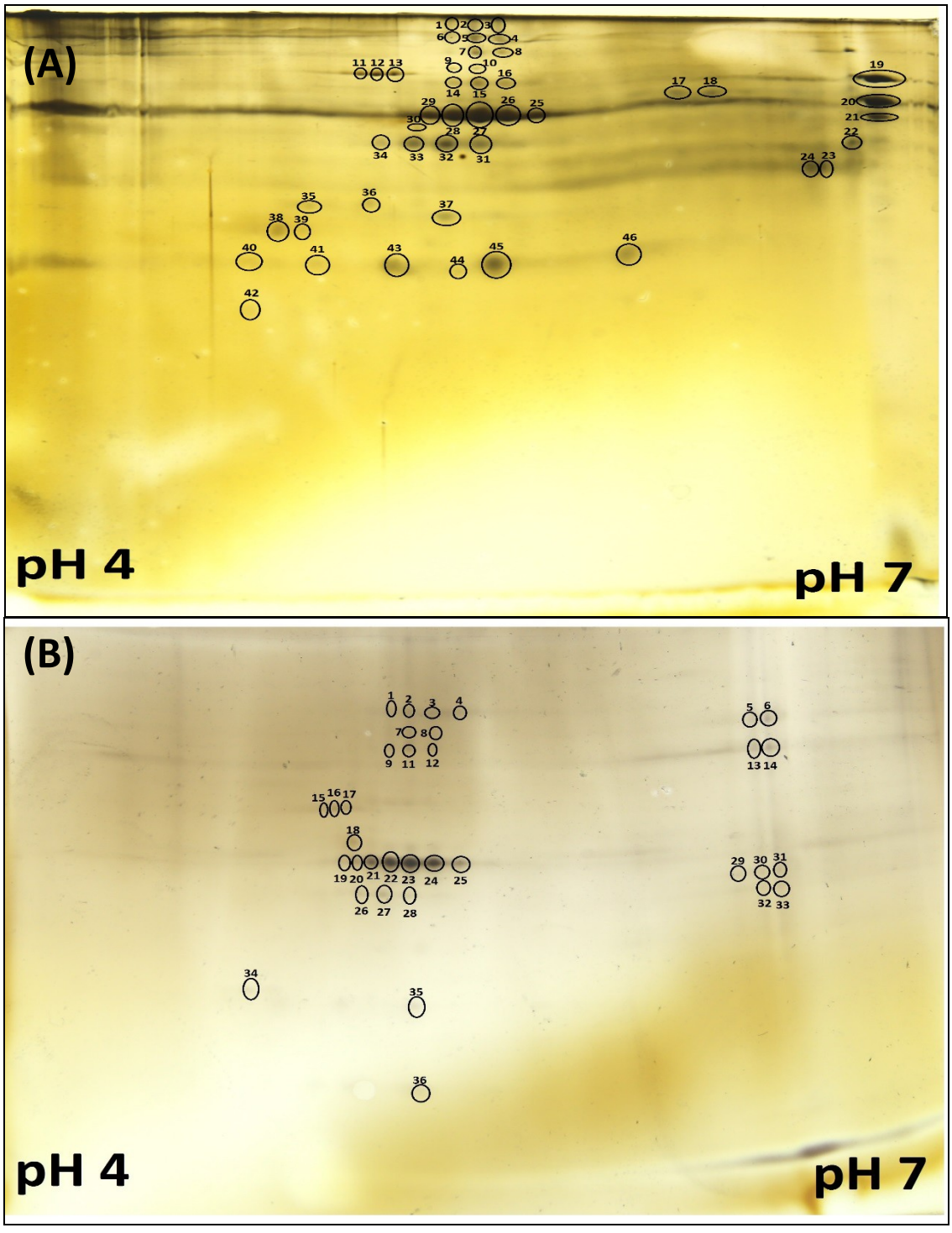


Figure 4.7. Phosphorylated Protein Spots Circled and Numbered. (A) Phosphorylated protein gel of non-excysted oocysts (B) Phosphorylated protein gel of excysted oocysts.

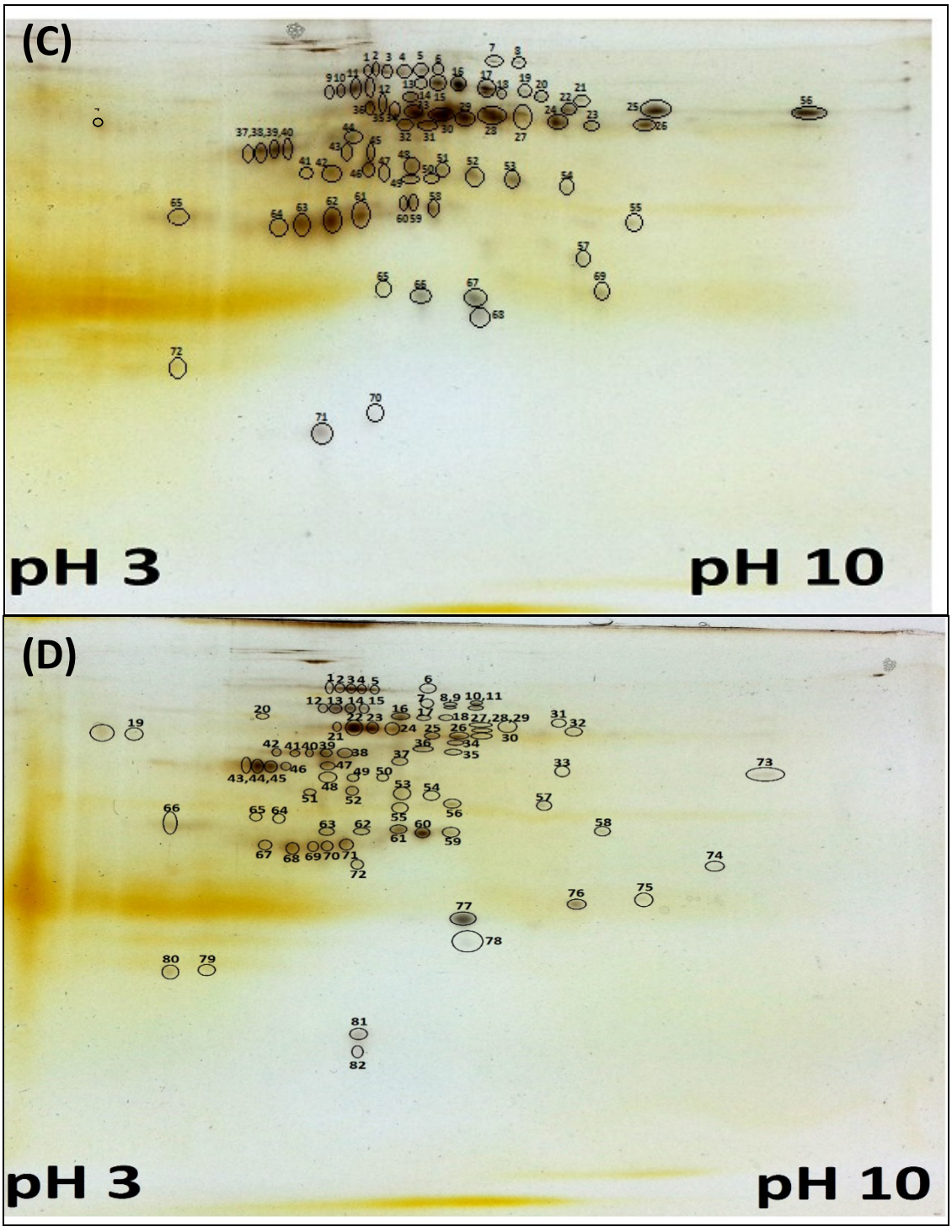


Figure 4.8. Non-Phosphorylated Protein Spots Circled and Numbered. (C) Non-phosphorylated protein gel of non-excysted oocysts (D) Non-phosphorylated protein gel of excysted oocysts.

4.4. Summary

This study was successful in enriching phosphorylated proteins from *C. parvum* oocysts. This is the first study to enrich and characterize phosphorylated and non-phosphorylated *C. parvum* proteins two dimensionally. The results show that changes in protein phosphorylation accompany excystation. Differential phosphorylation may play a role in cell signaling during excystation.

CHAPTER 5: CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE WORK

The scientific work presented in this thesis shows a novel, rapid method for the *in vitro* excystation of *C. parvum* oocysts (Chapter 3), and methods to enrich and characterize phosphorylated proteins from non-excysted and excysted oocysts (Chapter 4).

Experiments from the first section led to the development of a synchronous *in vitro* excystation assay (Joshi assay), which resulted in >90% excystation of *C. parvum* oocysts within 10 min. The rate of excystation was 27% per minute. In addition, optimal conditions required for excystation were investigated that best mimicked the *in vivo* environment. Oocysts did not excyst in HCl at pH 2.5, regardless of temperature; however, acid pretreated oocysts readily excysted in neutral pH media with sodium taurocholate, even at 4°C.

The performance of Joshi's *in vitro* excystation assay should be investigated using different species and strains of *Cryptosporidium*. This rapid assay provides reasonably synchronous excystation that can be used to study the sporozoite population from a genetic or proteomic perspective. Although differential microscopy was used in the current study to observe the oocysts, Scanning Electron Microscopy can add a different dimension to this work by identifying morphological differences after each step of *in vitro* excystation assay. From a public health perspective, techniques and assays involving *Cryptosporidium* should be standardized to enhance scientific consistency globally.

Excystation is a process whereby *Cryptosporidium* transforms from an inactive (oocyst) to an active (sporozoite) form. This study examined differential protein phosphorylation during this critical transformation in order to understand the underpinning signaling mechanisms. This was the first study to enrich phosphorylated proteins from non-excysted (inactive) and excysted

(active) stages, and to characterize phosphorylated and non-phosphorylated proteins two dimensionally. Optimal techniques required for the enrichment and separation of phosphorylated and non-phosphorylated proteins were reported. This work represents an important first step towards understanding the proteomic changes that drive excystation. Future research work can further characterize protein spots by mass spectrometry. Functions can be assigned to the identified proteins using the *Cryptosporidium* genome database (CryptoDB.org). Other work can explore participating components in phosphorylation, such as kinases and phosphatases. These studies should uncover promising targets for drug development to fight the disease cryptosporidiosis.

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APPENDIX

A.1. One-way ANOVA: Total Protein Versus Condition

```
Source     DF    SS      MS      F      P
Condition   1  0.7200  0.7200  21.54  0.004
Error       6  0.2006  0.0334
Total       7  0.9206

S = 0.1828   R-Sq = 78.21%   R-Sq(adj) = 74.58%

                           Individual 95% CIs For Mean Based on
                            Pooled StDev
Level      N    Mean   StDev   -+---------+---------+---------+--------
Excyst     4  2.2850  0.2310                      (------*-------)
Non-excyst 4  1.6850  0.1162   (------*-------)
                              -+---------+---------+---------+--------
                             1.50      1.80      2.10      2.40

Pooled StDev = 0.1828
```

A.2. One-way ANOVA: Phosphorylated Protein Versus Condition

```
Source     DF        SS        MS      F      P
Condition   1  0.014450  0.014450  34.68  0.001
Error       6  0.002500  0.000417
Total       7  0.016950

S = 0.02041   R-Sq = 85.25%   R-Sq(adj) = 82.79%

Level     N     Mean    StDev
Excyst    4  0.52000  0.02160
Non-excyst4  0.60500  0.01915

          Individual 95% CIs For Mean Based on Pooled StDev
Level     ---------+---------+---------+---------+
Excyst    (-------*------)
Non-excyst                          (------*------)
          ---------+---------+---------+---------+
                 0.525     0.560     0.595     0.630

Pooled StDev = 0.02041
```

A.3. One-way ANOVA: Non-Phosphorylated Protein Versus Condition

```
Source      DF        SS        MS       F      P
Conditions   1  0.183012  0.183012  183.78  0.000
Error        6  0.005975  0.000996
Total        7  0.188987

S = 0.03156   R-Sq = 96.84%   R-Sq(adj) = 96.31%
```

```
                                Individual 95% CIs For Mean Based on
                                Pooled StDev
Level       N    Mean    StDev   -+---------+---------+---------+--------
Excyst      4  0.72750  0.03775                                   (---*---)
Non-excyst  4  0.42500  0.02380    (--*---)
                                   -+---------+---------+---------+--------
                                  0.40      0.50      0.60      0.70

Pooled StDev = 0.03156
```