

Effect of Metabolic Enzymes on Amylopectin Content and Infectivity of
Cryptosporidium parvum

By

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ABSTRACT

Many parasites belonging to the phylum Apicomplexa have the ability to cause illness in both humans and other animal species. The majority of these apicomplexans contain amylopectin granules for use as energy throughout their life cycles. It has been hypothesized that amylopectin granules in Apicomplexan protozoa are used as an energy source to aid the parasites in surviving in the environment in the oocyst stage, to allow latent stages to excyst and release infective stages, to allow them to be mobile to access infective sites, invade host cells, and to convert to other life stages to continue their life cycle.

The objective of this project was to determine if parasite glycolytic enzymes: alpha-amylase, amyloglucosidase, enolase, lactate dehydrogenase, and phosphorylase could be used to decrease amylopectin stores in *Cryptosporidium parvum* oocysts/sporozoitcs and thereby reduce infectivity and potentially be used as a control method in both freshly excreted oocysts and those that have been surviving in the environment. In addition, glycolytic enzymes and substrates: glucose, glucose-1-phosphate, and glycogen synthase were investigated to determine if they can be used to increase amylopectin stores and thus increase infectivity to aid in detection and storage of oocysts.

Oocysts of *Cryptosporidium parvum* were suspended in a solution containing 1mg/ml glycolytic enzymes or substrates (with the exception of glucose used at 0.05M and glycogen synthase at 1U/ml) and electroporated to allow the enzymes to enter the oocysts. The oocysts were incubated at 37°C for 1 hour to allow the enzyme or substrate treatments to react with amylopectin granules. The oocysts were incubated on HCT-8 cells for 24 hours to allow infection. Real-time PCR and immunohistochemistry were performed to determine the effect of the enzymes on infectivity of sporozoites released from treated oocysts. In addition, an amylopectin assay and excystation assay was performed to determine if the enzymes were able to degrade amylopectin and if a decrease in amylopectin reduced the amount of energy available for excystation, respectively.

Alpha amylase and amyloglucosidase had the greatest impact on reducing both the amount of amylopectin and the infectivity of fresh oocysts with reductions of 99.6% and 99.7% in infective oocysts, respectively ($p < 0.05$). These results suggest that amylopectin may indeed be an important factor that parasites use to infect animals, although further research is needed. In stored oocysts, enzymes significantly reduced amylopectin content but not infectivity. Data shows that in fresh oocysts, amylopectin content, excystation, and infectivity are directly correlated with a decrease in amylopectin correlating to decreased excystation and infectivity. In contrast, there was no direct correlation for stored oocysts.

When glucose, glucose-1-phosphate, or glycogen synthase was used to increase infectivity, results show that glycogen synthase had little effect, but glucose and glucose-1-phosphate significantly increased amylopectin content, excystation, and infectivity. In

conclusion, amylopectin may be an important polysaccharide store of *Cryptosporidium* parasites to cause infection by allowing excystation of the oocysts to release the infective sporozoites. Thus this research may add to further study of the role of amylopectin in infectivity of *C. parvum*, the ability to create a vaccine or control method based on amylopectin metabolism, and to increase detection and storage of oocysts.

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I. INTRODUCTION

Cryptosporidium has caused many outbreaks of intestinal illness via food, water, and person-to-person contact. This parasite is also responsible for economic losses in cattle or other livestock production. It has been hypothesized that amylopectin stores in other Apicomplexans such as *Toxoplasma* and *Eimeria* allow the parasite to survive in the environment, access, attach, infect host cells, and developing into other life cycle stages. While amylopectin has been found in *Cryptosporidium*, limited research has been performed to determine if it has the same function as found in other Apicomplexans or if change in the storage of this polysaccharide could affect infectivity. Therefore, developing a better understanding of amylopectin biogenesis in *Cryptosporidium* is worthy of further exploration, since compounds which inhibit this process could define potential anti-parasitic drugs since glycolytic enzymes in *Cryptosporidium* are more similar to plant glycolytic enzymes than human enzymes (Karkhanis et al., 1993, Guerardel et al., 2005).

One functional area to focus anti-cryptosporidial drug discovery efforts is energy metabolism (Strong and Nelson, 2000). Recent studies in apicomplexan parasites show that it is possible to use inhibitors targeted specifically to the enzymes involved in carbohydrate or energy metabolism of the parasite as potential drugs for treatment (Yang and Parmley, 1995). The tailored physiology of *C. parvum* and *C. hominis* indicates the potential for attractive therapeutic targets, including: essential transport systems, components of glycolysis, starch, and amylopectin metabolism (Xu, 2004).

Although clearly speculative, inhibition of these or similar polysaccharide hydrolytic enzymes might well disrupt *C. parvum* energy metabolism by limiting the

supply of glucose available to the anaerobic glycolytic pathway, thereby reducing substrate-level phosphorylation (Strong and Nelson, 2000). Thus the inhibition of these enzymes may aid in control methods, while increasing precursors of amylopectin metabolism may aid in detection and increased time of storage of *C. parvum*. In addition, research of *C. parvum*'s metabolism may aid the adaptation of vaccine development strategies. The purpose of this research was to exploit the energy metabolism of *C. parvum* by changing amylopectin content and infectivity of this parasite. In addition, it will be determined if amylopectin content affects excystation related to infectivity (Woodmansee, 1987).

II. LITERATURE REVIEW

CRYPTOSPORIDIUM

Cryptosporidiosis, caused by the coccidian protozoa *Cryptosporidium parvum*, has been gaining more prominence in the last few years. This parasite has caused many problems in food and water due to its ability to survive in the environment and the ability to resist commonly used water treatment processes. The thick oocyst wall and the microscopic size of the *C. parvum* oocysts (between 4 and 6 μm) protect the infective sporozoites, and contribute to its resistance to environmental stress and water treatments (Haas and Aturaliye, 1999). Due to the hardness of the organism, water treatment disinfectants such as chlorine and chloramines would require doses not within an acceptable usage range for water treatment facilities to achieve any significant inactivation (Haas and Aturaliye, 1999). In addition, there is potential for *C. parvum* oocysts to pass through water treatment filters if a high amount of effluent is present, such as after a flood. The largest waterborne outbreak of 403,000 people in Milwaukee was potentially related to flood waters causing untreated water to enter the water treatment facility causing an increase of effluent (MacKenzie et al., 1995). Since *Cryptosporidium* is commonly found in water and responsible for many waterborne outbreaks there is potential for food borne outbreaks due to irrigation water, improper GAPs, SSOPs and GMPS of farm workers and food handlers, and use of non-potable processing water, among other factors. Due to an increased number of illnesses and few drugs available, increased research is needed to create a vaccine, effective drugs, or a control method to prevent increased illness from this parasite.

AMYLOPECTIN IN PROTOZOA

Amylopectin Characterization

Parasites of the phylum Apicomplexa include important pathogens responsible for widespread disease of considerable medical, veterinary, and economic importance (Coppin, 2005). This phylum includes parasites of humans including *Plasmodium*, *Toxoplasma*, *Cryptosporidium*, and *Isospora* (Blackman and Bannister, 2001). In addition, apicomplexan parasites include *Theileria*, *Neospora*, *Sarcocystis*, *Eimeria*, and *Babesia*, which are parasites of cattle, poultry, or other animals (Blackman and Bannister, 2001). The majority of apicomplexan parasites store glucose in the form of amylopectin granules related to floridian starch in different life cycle stages (Coppin, 2005). However *Plasmodium*, the agent of malaria, lack these storage polysaccharides (Coppin, 2005). Since *Plasmodium* must go directly from the host to the intermediate host, the mosquito, before infecting a new host, it is thought that it can use the nutrients from the intermediate host and does not need amylopectin to survive in the environment.

The polysaccharide stores synthesized in *Eimeria tenella* were first characterized as glycogen in 1944 using an iodine histochemical stain (Coppin, 2003). However, it was shown by electron microscopy that the structure of the granules differ from that of animal glycogen and led to the proposal of the term “Coccienglykogen” by Scholtyseck (Coppin, 2003). Ryley et al. (1974) showed that the maximal absorbance of the PAS-positive polysaccharides extracted from *E. tenella* sporozoites was measured at 520-537 nm, which is in agreement with waxy maize starch from plants at 530-540 nm but distinct from that of glycogen at 439 nm (Nakai and Ogimoto, 1987, Guerardel et al., 2005). Structural and gas chromatography/mass spectroscopy analysis also demonstrated that granules in *Toxoplasma gondii* are genuine amylopectin, the highly branched component

of starch, composed of α -1,4 linked glucan linear chains with a low proportion of α -1,6 branches, like low amylose waxy maize plant starch (Guerardel et al., 2005). While the unit chain profile was quite different from the single bell shaped peak of the glycogen profile, it also differed slightly from typical amylopectin in its length distribution (Ryley et al., 1974). However, similar to traditional amylopectin, acid hydrolysis of the granules yielded only glucose, while the addition of an excess of salivary α amylase resulted in the formation of glucose and maltose. This confirmed the presence of α 1,4 linked glucose chains and the branch points were characterized as α 1,6 glucosidic linkages by examination of beta amylase attack (Von Brand, 1973, Wang et al., 1975). These results indicate that the reserve polysaccharides are similar to typical amylopectin although the average chain lengths are relatively short at 19-23 carbons (Ryley et al., 1974). Although the unit chain profiles indicate that the reserve polysaccharide differ slightly from typical plant amylopectin, there is a close resemblance to the polysaccharide of the related gregarine *Gregarina blaberae* (Ryley et al., 1974). Thus, ultrastructurally and biochemically, the polysaccharide granules in *T. gondii* and other apicomplexans are more similar to plant-like amylopectin than β -glycogen of animals and this material was subsequently identified as amylopectin (Coppin, 2003). Therefore coccidia have similar energy reserves to rumen ciliates which have been shown chemically to store amylopectin and not “paraglycogen” as was first suggested (Ryley et al., 1969, Von Brand, 1973).

Since it was determined that the amylopectin produced by the parasites is similar to plant starch, it was investigated to determine if the parasites form amylopectin in a similar location to plants, within plastids (Coppin, 2003). Some protozoans such as *T. gondii* have a plastid, the apicoplast, but this does not appear to be associated with

amylopectin formation (Coppin, 2003, Harris et al., 2004). *T. gondii* accumulates storage polysaccharide in a strictly cytoplasmic localization, which differs from plants where the amylopectin granules form within plastids termed amyloplasts and chloroplasts (Coppin, 2003). In addition, plastids have not been detected in all apicomplexa and are apparently absent in *C. parvum* (Coppin, 2003, Harris et al., 2004). The absence of a plastid in *Cryptosporidium* may be related to the recent observation that *Cryptosporidium* is more closely related to gregarines such as *G. blaberae* than to apicomplexans (Huang et al. 2004). Nevertheless, the synthesis and accumulation of amylopectin, a plant-like crystalline storage polysaccharide in the cytoplasm, together with the acquisition of the apicoplast can be considered tracers of the evolutionary origin of apicomplexan parasites to green and red algae (Guerardel et al., 2005).

Amylopectin in Protozoan Life Stages

In addition to the presence of amylopectin in the oocyst cytoplasm, it has been found in other life cycles of these parasites. Within the *Cryptosporidium* oocyst, there exists a residual body or oocyst residuum, a membrane-bound structure containing lipid, polysaccharide/amylopectin, and possibly protein/amino acids as a storage depot of metabolites for the sporozoites (Harris et al., 2004). The *C. parvum* residual body contains a uniform population of large smooth-surface amylopectin granules of somewhat variable shape (Harris et al., 2004). Thin sectioning reveals that the larger amylopectin granules within the residual body and those that have escaped from damaged residual bodies during *in vitro* excystation possess a fine granular structure, with some suggestion of internal organization (Harris et al., 2004). While amylopectin is often present within the mature and immature apicomplexan parasites, it has not always been convincingly

demonstrated within *C. parvum* sporozoites (Tetley et al., 1998). However, some research suggests that sporozoites contain small amylopectin granules (Harris et al., 2004).

Eimeria

Considerable amounts of amylopectin are found in oocysts of *Eimeria* species (30.1-36.7 μ g glucose/10⁶ oocysts) according to Vetterling and Doran (Vetterling and Doran, 1969, Von Brand, 1973). In *Eimeria tenella*, *E. burnetti*, *E. stedai*, and *E. bovis*, amylopectin granules have been shown to be distributed in the middle and anterior part of the sporozoites, oocysts, sporocyst residual bodies, merozoites and in the cytoplasm of the schizont, while in *E. acervulina*, *E. necratix*, and *E. meleagridis* they are found in the anterior globule around the nucleus and behind the posterior refractile globule (Wang et al., 1975, Dubremetz and Elsner, 1979, Nakai and Ogimoto, 1983b, Heise et al., 1999, Jenkins et al., 2000, Coppin, 2005).

Amylopectin is found in almost all stages of the *E. tenella* life-cycle and has been thought to be the parasite's only carbohydrate reserve (Michalski et al., 1992). These granules appear and disappear throughout the life cycle of this coccidia (Heise et al., 1999). Two populations of polysaccharide granules are typically found in endogenous forms of *Eimeria brunetti* (Harris et al., 2004). The larger ones (approx. 500-620 nm by 250-500 nm) were observed in mature merozoites, macrogamonts, and developing oocysts, and a smaller population (15 to 30 nm) was seen at the periphery of the residual cytoplasmic mass of mature microgamonts (Von Brand, 1973, Wang et al., 1975, Beier et al., 1977, Harris et al., 2004). However, other research reports that while the residual body of microgametocytes and zygotes contain large amounts of amylopectin, no polysaccharide was visualized in microgamete bodies or in the microgamonts of *E.*

acervulina nor in *E. burnetti* and was found only in 5% of the mature microgamonts of *E. labeana* (Beier et al., 1977, Mueller et al., 1981). Wagner and Foerster (1967) demonstrated a large number of granules in differentiating merozoites of *E. tenella*, but the granules vanished gradually following maturation and development of the merozoites into trophozoites (Wagner and Foerster, 1967, Wang et al., 1975). Despite the decrease of the amylopectin source during sporulation, the size and shape of the granules did not change; however, the total number of granules did decline (Harris et al., 2004). In dormant sporozoites the association with dense granules and amylopectin is common even before host cell penetration (Dubremetz and Elsner, 1979). These granules were located in front of the anterior refractile globule, around the nucleus, and behind the posterior globule (Vetterling and Doran, 1969).

Periodic acid-Schiff (PAS) staining revealed several PAS-positive granules inside the merozoites at days 3–5 post infection. PAS-positive granules were found inside eimerian sporozoites and oocysts; however, individual species and developmental stages differ from each other in the amount of these granules (Slapeta et al., 2001). Amylopectin granules present in the schizont increased in number with onset of merogenesis (Dubremetz and Elsner, 1979). This appears to be a manifestation of metabolic changes known to take place when the cell metabolism starts supporting the differentiation of organelles and the formation of energetic reserves for the merozoites (Dubremetz and Elsner, 1979). The only difference between merogenesis as seen *in vitro* and *in vivo* conditions are the early appearance of amylopectin and its partial incorporation into the forming merozoites, and the formation of amylopectin granules after the merozoites are pinched off the residuum under the latter conditions (Dubremetz and Elsner, 1979).

Toxoplasma

Amylopectin is found in all stages of *T. gondii*. However, it has been experimentally shown that the conversion of dormant encysted bradyzoites into newly transformed tachyzoites correlates with the disappearance of amylopectin granules (Coppin, 2003). In *T. gondii*, during tissue cyst formation, there is synthesis of numerous (average: 21.8, range: 7-38) amylopectin granules within the bradyzoite at an average size of 358nm (range: 192-630nm) and the presence of specific lectin binding sugars in the cysts well (Von Brand, 1973). As shown by electron microscopy, the bradyzoite form produces an extraordinarily high amount of amylopectin (glucose polymer) while it develops in glucose rich environments such as brain or muscle cells because of the decreased need for nutrients during this inactive period (Tomavo, 2001). This situation raises the question of whether the transport of glucose through the cyst wall is reduced or abolished when encystation progresses (Tomavo, 2001). In contrast, tachyzoites may lack these amylopectin granules or have few small granules with an average size of 135-201nm x 79nm (Nakai and Ogimoto, 1987, Coppin, 2003, Guimaraes et al., 2003).

Amylopectin Structure

Amylopectin granules isolated using a sucrose gradient following French press disruption of sporozoites and residual bodies have been found to be present in a considerable range of sizes (Harris et al., 2004). Although the morphology of populations of large digested amylopectin granules and the population of small possibly sporozoite-derived granules differ, they are considered to be similar in structure (Harris et al., 2004). The large amylopectin granules exhibit a rigid compact coiled ball of string structure to store large amounts of glucose molecules with less effect on terminal osmotic potential

and had dimensions of the order of 0.4µm with a cell membrane thickness of 0.02 µm (Harris et al., 2004). The neatly coiled, smooth-surfaced ‘ball of string’ structure of the larger amylopectin contrasts markedly with the more irregular shape and rod-like particulate composition of the smaller granules (Harris et al., 2004). Smaller granules may represent a residual amylopectin core that has been metabolically degraded from initially larger granules within the sporozoite or a unique population of smaller undegraded amylopectin granules within the sporozoites (Harris et al., 2004).

Amylopectin Functions

The physiological role of amylopectin during the maturation and survival of the *C. parvum* oocysts and utilization of the sporozoites appears to be linked to a population of large amylopectin granules within the residual body and smaller amylopectin granules within the sporozoites (Harris et al., 2004). The present observations suggest that amylopectin plays an important role in establishing infection (Nakai and Ogimoto, 1987). These polysaccharide granules serve as an energy reserve in coccidia and are utilized by the parasite for respiration, excystation, and survival in the environment (Karkhanis et al., 1993). Amylopectin is also believed to allow the parasite to access host cells, allow sporozoites to actively move, persist as a cyst during infection, invade, and subsequently develop in the host cell (Nakai and Ogimoto, 1987, Nakai and Ogimoto, 1988, Karkhanis et al., 1993, Coppin, 2003, Guerardel et al., 2005). It has been concluded that amylopectin as polysaccharide storage could be a key factor for excystation followed by invasion of host cells by coccidian sporozoites (Michalski et al., 1992, Entrala and Mascaro, 1997, Fayer et al., 1998, King et al., 2005). When the amylopectin falls below a critical level, sporozoites lack sufficient energy to move and invade cells resulting in

less infectivity (Michalski et al., 1992, Entrala and Mascaro, 1997, Fayer et al., 1998, King et al., 2005). These facts indicate that sporozoites may invade cells by using energy from the amylopectin reserve (Nakai and Ogimoto, 1987). The failure to convert from one stage to another in the parasite life cycle may also be related to deficiencies in the synthesis or degradation of amylopectin (Coppin, 2003). These investigations should provide a fuller understanding of the intracellular development of Apicomplexan parasites and may identify metabolic targets suitable for future drug intervention (Coppin, 2003).

Infectivity

While water-borne *Cryptosporidium* oocysts appear to be resilient at a wide range of temperatures, increased holding temperatures performed *in vitro* correspond to decreased oocyst infectivity due to finite amylopectin stores (Fayer et al., 1998, King et al., 2005). The amount of the storage polysaccharide amylopectin is generally found to be correlated with infectivity in many apicomplexan parasites (Heise et al., 1999). One hypothesis is that elevated temperatures result in the exhaustion or consumption of oocyst energy reserves in direct response to ambient environmental temperatures, resulting in the inability of sporozoites to initiate infection (King et al., 2005).

Oocyst storage at temperatures above 5°C produced a marked reduction in amylopectin and infectivity over a period of several weeks, whereas at 0°C and 5°C the amylopectin depletion was very slow (Harris et al., 2004). Vetterling and Doran (1969) observed that *E. acervulina* and *E. tenella* oocysts lost amylopectin reserves after storing oocysts for 6 to 8 months at 4°C (Vetterling and Doran, 1969). After storage, the infectivity and ability to invade cultured host cells by oocysts were lowered. When

stored for 6 years at 4°C, infectivity was less than 5% of that in fresh oocysts, and it was concluded that amylopectin was required for energy to successfully penetrate a host cell, because the stored oocysts did not produce a patent infection in chickens (Nakai and Ogimoto, 1987). It has also been observed that decreases in amylopectin levels due to storage above 4°C were associated with marked reduction of oocysts viability and infectivity *in vivo* (Coppin, 2003). Thus amylopectin was required for energy to successfully penetrate a host cell. Sporozoites having almost no amylopectin granules inoculated into chicken embryos showed lower lethality and lower ability of oocyst production (Nakai and Ogimoto, 1987).

All fresh sporozoites contained a large amount of amylopectin granules (PAS positive rate, 100%; PAS index 4.7). Sporozoites which were incubated at 0°C for 16h contained a large amount of amylopectin (PAS index 4.4), and showed a high rate of invasion (5.1%). By incubating sporozoites at a higher temperature, amylopectin contents decreased accordingly with a PAS index of 2.3 at 29°C and 1.0 at 37°C. The invasion rate also decreased to 2.7% at 29°C and 1.0% at 37°C (Nakai and Ogimoto, 1987). The PAS index of sporozoites after 41°C incubation was much lower (0.4) and no sporozoites were observed in the cells. The sporozoites which had been incubated for 16 h at 41°C contained a small amount of amylopectin (PAS positive rate, 5%; PAS index 0.1) and majority of these did not have amylopectin granules (Nakai and Ogimoto, 1987). There was a higher value of the coefficient of correlation between amylopectin content (PAS indices) and invasion of sporozoites into the cells (invasion rates), which suggests that the two factors are directly correlated. The number of viable sporozoites after incubation was 33% of that of fresh sporozoites (Nakai and Ogimoto, 1987). Lower mortality rates were observed in the group of chicken embryos that were inoculated with

Eimeria sporozoites incubated at 41°C. Although 1×10^4 fresh sporozoites killed 7 of 9 embryos, 3.3×10^4 incubated sporozoites killed only 2 of 9 embryos and smaller numbers of oocysts were harvested from embryos. Embryos inoculated with 1×10^3 fresh sporozoites produced a mean number of 3.2×10^4 oocysts. After the sporozoites were incubated at 41°C for 16h, however, the embryo with 3.3×10^4 sporozoites produced only 1×10^4 oocysts (Nakai and Ogimoto, 1987).

In a previous study by Nakai and Ogimoto (1987), it was observed that *E. tenella* sporozoites survived for longer periods of incubation at lower temperature and they consumed the amylopectin reserve at a slower rate. Amylopectin reserve was consumed quicker by incubating sporozoites at a higher temperature, and those sporozoites containing a smaller amylopectin reserve invaded cells at a lower rate (Nakai and Ogimoto, 1987). Oocysts of *E. acervulina* stored for either three months or one year produced patent infections in chicks with a dosage of 5×10^4 oocysts, but only a few were infective. It was determined that a dosage of 2×10^6 of these stored oocysts was necessary to produce a patent infection (Vetterling and Doran, 1969). Oocysts which had been stored for 6 years did not produce a patent infection (Vetterling and Doran, 1969). Thus storage time results in a coincident loss of polysaccharide storage which indicates that polysaccharide is probably necessary for survival during dormancy and is required as a source of energy for the oocysts to excyst and successfully penetrate the host cell (Vetterling and Doran, 1969). Thus when the parasite is incubated at high temperatures and the content of amylopectin falls below a critical level, sporozoites lack sufficient energy to perform the above functions and can neither invade chorioallantoic membrane cells nor develop in host cells (Nakai and Ogimoto, 1987, Karkhanis et al., 1993, Fayer et al., 1998, Guerardel et al., 2005).

Amylopectin and the mRNA encoding amyloglucosidase can serve as viability markers of *C. parvum* oocysts due to the finding that non-infectious *C. parvum* oocysts contain negligible quantity of amylopectin, a known energy store for sporozoites within oocysts (Jenkins et al., 2000, Harris et al., 2004). However, assays measuring amylopectin content or levels of amyloglucosidase mRNA display only marginal agreement with mouse infection and cell culture infection (King et al., 2005).

Survival

When the life cycle of the parasite is examined it may be of significance that the infectious stages which are normally involved in transmission of infection between hosts, the bradyzoite, oocyst, and the sporozoite, contain numerous amylopectin granules. In contrast, the stages involved in transmission between cells within the host, the rapidly replicating tachyzoite and merozoite, lack or have few of these granules (Vetterling and Doran, 1969, Coppin, 2003, Harris et al., 2004). The bradyzoite and sporozoite forms await ingestion by a carnivorous host and herbivorous host, respectively, and are dependent on sufficient energy supply to survive the resting periods and the rigors of infecting a new host (Harris et al., 2004).

Amylopectin found in the residual body may be looked upon as carbohydrate storage granules, utilized during the transmission period of the oocyst stage in the environment whereas the sporozoite amylopectin is likely to be closely involved in the immediate metabolism of the sporozoite once liberated from the oocysts in the intestines of the new host (Harris et al., 2004). Coccidia are notable for their ability to survive as oocysts in the environment, apparently utilizing stored energy sources (Tilley et al., 1997). In this situation, the parasite has no access to nutrients and could survive on its

amylopectin granules, a glucose storage source for the parasite (Coppin, 2003). They are entirely dependent on endogenous sources of energy for sporulation to remain viable (Schmatz, 1997). The energy requirements and biosynthetic substrates of coccidian parasites are mostly dependent on carbohydrate metabolism and prolonged exposure of oocysts to increased environmental temperatures results in the depletion of energy reserves (Fayer et al., 1998, Guimaraes et al., 2003).

Respiration/Excystation

Approximately 50% of the carbohydrate store was consumed during sporulation of *Eimeria acervulina*, and its depletion might be closely associated with respiration (Guerardel et al., 2005). Vetterling and Doran (1969) also showed that two-thirds of the amylopectin in sporozoites of *E. acervulina*, *E. necatrix* and *E. meleagridis* were utilized by respiration during in vitro excystation, with a calculated ratio of about 6 mol O₂ consumed/mol of glucose (Guerardel et al., 2005). Ryley et al. (1969) incubated *E. tenella* sporozoites anaerobically, and correlated the decrease in amylopectin stores of the parasite with production of lactic acid plus lesser amounts of carbon dioxide and glycerol (Ryley et al., 1969, Guerardel et al., 2005).

Depletion of amylopectin and rapid consumption of oxygen during excystation indicates that a large amount of energy is required for dormant sporozoites to excyst (Vetterling and Doran, 1969). When the quantity of amylopectin used during excystation is compared with oxygen consumption during the same period, the factors for carbohydrate metabolism ($\mu\text{l oxygen}/\mu\text{g glucose}$) of *E. acervulina*, *E. necatrix*, and *E. meleagridis* are 0.79, 0.73, and 0.88, respectively while another study found it at 0.8 (Vetterling and Doran, 1969). Vetterling and Dorn observed a disappearance of 21-37 μg

amylopectin from 10^6 oocysts of the three *Eimeria* spp. during excystation of sporozoites (Von Brand, 1973). In all three species of *Eimeria*, 20-23 μ g glucose/ 10^6 oocysts was utilized after three min of excystation (Vetterling and Doran, 1969). The quantity of amylopectin found in dormant sporozoites of all three species was nearly three times greater than in those that had excysted (Vetterling and Doran, 1969). In contrast to rapid utilization by sporozoites is the fact that amylopectin content of dormant oocysts of *Eimeria acervulina* required 6 years to decrease from 33 μ g/ 10^6 specimens to 1.5 μ g (Von Brand, 1973).

Mobility

Amylopectin in *E. tenella* plays an important role in establishing the infection (Slapeta et al., 2001). When sporozoites were incubated for 16h at 4°C, many of them (87%) showed vivid movements such as bending and stretching their bodies or shaking the anterior parts of their bodies (Nakai and Ogimoto, 1987). The merozoites with high amounts of amylopectin are highly motile and capable of penetrating cultured cells (Slapeta et al., 2001). Whereas sporozoites incubated for 16h at 41°C contained a scarce amount of amylopectin, and only 4% were motile indicating that sporozoites may survive and move by consuming the amylopectin reserve as the energy source (Nakai and Ogimoto, 1987). Amylopectin granules may also help the oocysts to reach the appropriate host cells. *E. acervulina* and *E. hagani*, which parasitize favorably in the upper part of the small intestine of chickens, contained very small amounts of amylopectin, and *E. maxima* which parasitize the middle part of the small intestines contains a small to medium amount of amylopectin (Nakai and Ogimoto, 1987). However, *E. tenella* which parasitizes in the cecum contained a large amount of

amylopectin (Nakai and Ogimoto, 1987). These observations suggest that *E. tenella* sporozoites reach the cecum in 1 to 2 h after excystation (Nakai and Ogimoto, 1988) and many of them remain in the lumen of the cecum for several hours before invasion to host cells (Nakai and Ogimoto, 1988). Sporozoites of *E. acervulina* and *E. tenella* have both been observed to excyst from oocysts at the upper or middle part of small intestines (Nakai and Ogimoto, 1987). *Eimeria acervulina* sporozoites may be able to find their host cells within a comparatively short period of time after excystation with low energy consumption, but *E. tenella* sporozoites have to move for a longer period of time to the cecum, actively to passively, by consuming large amounts of amylopectin as the energy source (Nakai and Ogimoto, 1987). There is a possibility, therefore, that the amylopectin content of the sporozoites of chicken *Eimeria* species have been modified by the adaptation of the parasites to the infection site in the intestines (Nakai and Ogimoto, 1987).

Sporozoites were shown to metabolize exogenous sugars as the substitution of amylopectin reserve (Nakai and Ogimoto, 1988). When little amount of sugars or some substrates are available in intestine, sporozoites have to survive by using its own amylopectin reserve as the energy source (Nakai and Ogimoto, 1988). Sporozoites of the passage line may consume amylopectin reserve while moving down and staying in the intestines and cecum, and many of them may lose viability or ability to infect host cells (Nakai and Ogimoto, 1988). Sporozoites of the passage strain consume amylopectin reserves quickly while moving down the intestines and cecum and a small population of sporozoites with enough amylopectin are able to infect (Nakai and Ogimoto, 1988). Sporozoites within the lamina propria that were devoid of amylopectin probably lacked the energy to reach cells at the base of crypts (Vetterling and Doran, 1969). Therefore,

low pathogenicity of the chicken embryo passage line to chickens might be related to the smaller amylopectin reserve in sporozoites (Nakai and Ogimoto, 1988).

ENZYMES

Amylopectin Degradation

Recent findings including a genomic approach has identified several genes encoding for proteins associated with amylopectin synthesis or degradation and glucose metabolism including different isoforms of certain glycolytic enzymes which are stage-specifically expressed (Coppin, 2003). Several genes encoding enzymes involved in polysaccharide metabolism were identified in the genome of *C. parvum* and the corresponding homologies can be classified in two categories: (1) enzymes involved in amylopectin degradation, such as starch debranching enzymes, alpha amylases (Accession No. B88566), starch phosphorylases, amyloglucosidases (Accession Nos. B88444 and AF268073) and alpha glucosidases; (2) enzymes involved in amylopectin synthesis such as: starch branching enzymes, pullulanases, and glucosyltransferases (Coppin, 2003).

Phylogenetic data, together with enzyme assays, establish that apicomplexans *Toxoplasma gondii* and *Cryptosporidium parvum*, use a UDP-glucose pathway to build insoluble crystalline amylopectin and consist of genes used in plants and green algae to breakdown and synthesize semi-crystalline polysaccharides (Coppin, 2005).

Cryptosporidium parvum does not depend upon a functional respiratory chain for viability suggesting that glycolysis is the main mechanism for energy generation (Entrala and Mascaro, 1997). Substrate-level phosphorylation appears to be the major route of energy generation in the sporozoite life-cycle stage also since mitochondria have not been

observed and pyrophosphate-dependent phosphofructokinase (PPi-PFK) activity has been detected (Strong and Nelson, 2000). With the exception of PPi-PFK, all of the classical glycolytic activities were found leading to the formation of phosphoenolpyruvate (PEP) from glucose 6-phosphate (Entrala and Mascaro, 1997).

In *T. gondii*, while both tachyzoites and bradyzoites utilize the glycolytic pathway for energy, tachyzoites utilize the glycolytic pathway with the production of lactate as their major source of energy, but bradyzoites lack a functional TCA cycle and respiratory chain (Weiss and Kim, 2000). Since *T. gondii* modifies its morphology and probably also its metabolism and expression pattern of glycolytic enzymes to adapt to the varying environment during its life cycle, it is possible that the stage-specific isoenzymes may have different characteristics, which could also be required for the parasite to adapt to a particular microenvironment (Dzierszynski, 2001, Coppin, 2003).

Enzymes for metabolism of glycogen, starch, and amylopectin are present in sporozoites, which is consistent with suggestions that amylopectin represents an energy reserve for sporozoites (Xu, 2004). Endogenous carbohydrate can be used, as mentioned previously, for synthesis but serves usually as an energy reserve for anaerobic or aerobic fermentations and oxidations (Von Brand, 1973). In Apicomplexans, there may also be stage and function dependent variations in the expression of amylopectin degradation and synthesis enzymes (Heise et al., 1999). In *T. gondii*, one gene was found for UDP-glucose phosphorylase, none for ADP-glucose pyrophosphorylase, one for starch synthase, two for branching enzymes, one for isoamylase, one for indirect debranching enzyme, one for alpha 1,4 glucantransferase, one for phosphorylase, one for alpha amylase, one for alpha glucosidase, and one for glycogenin (Coppin, 2005). It is noteworthy that transcripts coding for enzymes known to be involved in the catabolic

functions such as the R1 protein (GWD), alpha-glucan phosphorylase, alpha-glucosidase, and alpha-amylase seem to be preferentially expressed in bradyzoites (Coppin, 2005).

Thus strategies to prevent illness and infectivity may include identifying molecules involved in stage conversion and amylopectin utilization as possible targets for chemotherapy and vaccination (Yang and Parmley, 1995).

Enolase and lactate dehydrogenase

Enolase (2-phospho-D-glycerate hydrolase, EC 4.2.1.11) converts 2-phosphate-D-glycerate to phosphoenolpyruvate (Vetterling and Doran, 1969, Yang and Parmley, 1995, Dzierszinski et al., 1999, Dzierszinski, 2001, Tomavo, 2001). However, in drug untreatable encysted bradyzoites of *T. gondii*, enolase genes are overexpressed or exclusively expressed at both transcriptional and protein levels (Dzierszinski et al., 1999). This suggests that the use of enolase to break down amylopectin or another metabolic energy source is related to infectivity (Dzierszinski et al., 1999).

Lactate dehydrogenase (L-lactate: NAD⁺ oxidoreductase, EC 1.1.1.27) catalyses pyruvate to lactate (Vetterling and Doran, 1969, Yang and Parmley, 1995, Dzierszinski et al., 1999, Dzierszinski, 2001, Tomavo, 2001). LDH is a physiologically significant enzyme for metabolism and possibly for regulation of gene expression in a variety of organisms (Yang and Parmley, 1995). LDH plays an indispensable role when glycolysis becomes the only pathway by which the cell can obtain energy (Yang and Parmley, 1995). This may be true when the function of mitochondria is impaired or not present as in the case of *C. parvum* in the presence of inhibitors or under anaerobic conditions (Yang and Parmley, 1995).

The two bradyzoite-specific glycolytic enzymes, enolase and lactate dehydrogenase show considerable homologies to land plant counterparts, which differ from those of animals (Entrala and Mascaro, 1997, Dzierszinski et al., 1999). These glycolytic enzymes are useful to trace the evolutionary origin of Apicomplexa and might offer novel chemotherapeutic targets in diseases caused by these parasites (Entrala and Mascaro, 1997, Dzierszinski et al., 1999). Enolase and lactate dehydrogenase are stage-specific. Expression at the transcript and protein level can supply each parasitic stage with the appropriate enzymes needed for its environmental adaptation and metabolic requirements (Vetterling and Doran, 1969, Yang and Parmley, 1995, Dzierszinski et al., 1999, Dzierszinski, 2001, Tomavo, 2001). They have distinct enzymatic activities in the parasite consistent with the metabolic and environmental differences in lifestyle between bradyzoites and tachyzoites of *T. gondii* (Dzierszinski et al., 1999, Dzierszinski, 2001, Ferguson et al., 2002). The more stable and less active ENO1 and LDH2 are expressed specifically by the bradyzoite with possibly lower energetic requirements whereas the more active and less stable ENO2 and LDH1 is expressed by the virulent tachyzoite stage (Vetterling and Doran, 1969, Dzierszinski, 2001, Tomavo, 2001, Coppin, 2003). LDH1 is replaced by LDH2 during development from tachyzoites to bradyzoites. Although they all catalyze the interconversion between pyruvate and lactate, different LDH isoforms do not have the same kinetics and properties (Yang and Parmley, 1995). Nevertheless, Yang and Parmley (1997) have clearly demonstrated that LDH1 mRNA is equally abundant in both tachyzoites and bradyzoites using RT-PCR while the LDH1 enzyme is only detectable in tachyzoites and is absent in the bradyzoite (Tomavo, 2001). Thus, it is likely that LDH1 is replaced by LDH2 during the interconversion of tachyzoites into bradyzoites (Yang and Parmley, 1997, Tomavo, 2001). However, preliminary studies

reported that recombinant LDH1 and LDH2 enzymes display no significant kinetic differences and there were no biochemical differences (Tomavo, 2001, Coppin, 2003). The two stage-specific enolases (ENO1 and ENO2) of *T. gondii* can also have different sub cellular localization (i.e. cytoplasm or cytoplasm plus nuclear location) and may function in ways additional to its involvement in glycolysis (Ferguson et al., 2002). If enolase is associated with nuclear activity, it is possible that the two isoforms may in some way control stage-specific gene transcription (Ferguson et al., 2002). Even though enolase has been found in other subcellular compartments as described earlier, it is a glycolytic enzyme and consistent with its primary function, it is predominantly localized in the cytoplasm where it can be free or associated with the cytoskeleton with other glycolytic enzymes (Ferguson et al., 2002).

It is not known whether the induction of alternative isoenzymes causes *T. gondii* differentiation and encystment or whether it is a by-product of molecular signals that trigger the differentiation process (Coppin, 2003). The fact that mitochondrial inhibitors can induce the expression of bradyzoite-specific antigens suggests that alterations in parasite mitochondrial functions may be correlated to stage conversion, and that these parasitic forms rely predominantly on anaerobic glycolysis (Dzierszinski, 2001). This is likely to be related to differences in the tricarboxylic acid (TCA) cycle and oxidative phosphorylation pathway (Coppin, 2003). It has been suggested that the induction of bradyzoite development may be associated with stress responses (Coppin, 2003).

However, the presence of stage-specific enzymes being due to metabolic and environmental changes may be an oversimplification (Ferguson et al., 2002). In the early cysts, bradyzoites are extremely “active” undergoing active proliferation (endodyogeny) and development including synthesis of large numbers of polysaccharide granules

(Ferguson et al., 2002). If the isoforms expressed were purely dependent on parasite activity (energy requirements), it would be expected that the early active cysts would express the same isoforms as expressed by the tachyzoites and would only convert to the bradyzoite specific forms when the cyst entered the resting stage (Ferguson et al., 2002).

Alpha amylase and Amyloglucosidase

Alpha amylase (EC 3.2.1.1) an extracellular enzyme specific for alpha 1,4 linkages and is capable of bypassing alpha 1,6 linkages (Kelly and Fogarty, 1983). Amyloglucosidases or glucoamylase (EC 3.2.1.3) is an extracellular enzyme specific for alpha 1,6 linkages (Kelly and Fogarty, 1983). These enzymes successively degrade starch or glycogen into maltose and to free glucose (amyloglucosidase, alpha and beta amylases, sucrose, trehalase, and lactase) and were below detection limits in *Cryptosporidium* (Entrala and Mascaro, 1997). Although clearly speculative, inhibition of these or similar polysaccharide hydrolytic enzymes might well disrupt *C. parvum* energy metabolism by limiting the supply of glucose available to the anaerobic glycolytic pathway, thereby reducing substrate-level phosphorylation (Strong and Nelson, 2000).

The mere demonstration of disintegration of engulfed starch granules allows for deduction concerning the mechanism of starch digestion by parasitic protozoa, since starch can be degraded by phosphorylysis to glucose-1-phosphate or hydrolyzed by the successive action of amylase and maltase to maltose (Von Brand, 1973). A preliminary glycohydrolase study provided evidence for partial degradation of the *C. parvum* amylopectin granules by [alpha]-amylase, but amyloglucosidase had little effect on the overall structure (Harris et al., 2004).

Analysis of the radioactive product after digestion with alpha amylase resulted in the formation of radioactive glucose and maltose (Karkhanis et al., 1993). For the control (i.e., without alpha amylase and beta amylase) all radioactive material remained at the origin. When treated with alpha amylase, 82% of the product was radioactive glucose and maltose (Karkhanis et al., 1993).

Phosphorylase

Amylopectin 5-glucohydrolase (amylopectin phosphorylase) readily attacks protozoan polysaccharides and genuine amylopectin (Von Brand, 1973). Earlier work on *Eimeria tenella*, using surfactant and protease digestion to release amylopectin granules from oocysts, showed that the enzyme amylopectin phosphorylase was present, and likely to be involved in the breakdown of amylopectin (Harris et al., 2004). It has been pointed out that degradation of amylopectin is an assumed biologic function of *E. tenella* based on the close relations between the enzyme activity and rate of disappearance of polysaccharides in sporulating *E. tenella* oocysts (Wang et al., 1975).

However, there may be stage- and function-dependent variations in the expression of molecules related to granule formation and degradation such as amylopectin synthase and amylopectin phosphorylase, respectively (Heise et al., 1999). During sporulation, amylopectin granules are degraded by amylopectin phosphorylase and the absence of detectable amylase activity indicates that amylopectin phosphorylase is probably the major means of amylopectin utilization (Wang et al., 1975). Although Wang et al. detected a small amount of amylopectin phosphorylase in the sporulated oocyst of *E. tenella*, it has previously been observed that amylopectin phosphorylase activity declined at a linear rate during oocyst sporulation with less than 8% of the original amylopectin

phosphorylase remaining in fully sporulated oocysts (Wang et al., 1975, Michalski et al., 1992). Phosphorylase activity also steadily declines in parallel with the oocysts decreasing respiration rate (Wang et al., 1975).

Even though amylopectin reserve was reduced during sporulation, the size and shape of the purified granules did not change (Wang et al., 1975). In electron-microscopy studies, other investigators have also shown that only the number of granules change during sporulation, not the overall form (Wang et al., 1975). This suggests the phosphorylase must attack and completely degrade each granule before moving to the next one (Wang et al., 1975). Though the mechanism by which this is accomplished is not known. The observation that purified granules do not serve as a substrate for phosphorylase suggests that there might be some factor associated with the granules *in vivo* which increases their susceptibility to phosphorylase (Wang et al., 1975). This factor could be responsible for the granules decrease in phosphorylase activity during sporulation due to enzyme mediated inactivation or simple denaturation (Wang et al., 1975). It was suggested that phosphorylase activity during sporulation is probably not regulated by cAMP (Wang et al., 1975). Thus the mechanism by which amylopectin phosphorylase is turned off during sporulation and probably turned on again in the sporozoites following the subsequent encystation remains to be determined (Wang et al., 1975).

An extract of unsporulated oocysts was assayed for amylopectin phosphorylase, and a specific activity of 13 IU/mg protein was recorded which compared favorably with that of potato amylopectin phosphorylase (28.5U/mg protein) (Wang et al., 1975). Apparently the endogenous amylopectin in the extract was sufficient for maximum activity (Wang et al., 1975). The phosphorylase activity had a fairly sharp optimum at

pH 6 and phosphate is an essential cofactor using amylopectin phosphorylase in utilization of amylopectin by the parasite (Wang et al., 1975).

Amylopectin Synthesis

Both ADP-glucose pyrophosphorylase and ADP-glucose utilizing starch synthase could not be found in apicomplexans or in red algae (Coppin, 2005). This suggests that amylopectin is synthesized through a UDP-glucose-based pathway is much simpler than that described for plants (Coppin, 2005). It is also evident that both *C. merolae* and *T. gondii* contain a UDP-glucose utilizing glycogen (starch) synthase-like sequence and glycogenins (Coppin, 2005).

Glycogen Synthase

Rumen ciliates must contain very potent branching systems since their amylopectin does not contain amylose (Von Brand, 1973). A soluble enzyme, amylopectin synthase (UDP-glucose alpha1,4-glucan alpha-4-glucosyltransferase) which transfers glucose from uridine 5'-diphosphate glucose (UDP-glucose) to a primer to form alpha-1,4-glucosyl linkages has been identified in the crude extracts of unsporulated oocysts of *E. tenella* (Karkhanis et al., 1993). Since alpha amylase is an alpha 1,4 glucan 4-glucohydrolase and beta amylase is an alpha 1,4 glucan malthydrolase, their effect on the enzyme activity was critical in determining the linkage formed by amylopectin synthase in transferring glucose from UDPG to glycogen (Karkhanis et al., 1993). Both enzymes inhibited enzyme activity, which presumably prevented the formation of new alpha 1,4 linkages (Karkhanis et al., 1993).

The pH optimum of glycogen synthase is 7.5 and it was fully active at temperature between 22-37°C; at 45°C it lost only 10% of the activity (Karkhanis et al., 1993). This enzyme used UDPG-glucose not ADP-glucose as substrate to transfer glucose to the primer and has been shown to be involved in these processes: a) self glycosylation protein b) glycogen synthase and c) branching enzyme (Karkhanis et al., 1993). The product formed by the reaction was predominantly a glucan containing alpha-1,4 linkages (Karkhanis et al., 1993). Corn amylopectin, rabbit liver glycogen, oyster glycogen, and corn starch served as primers; the latter two were less efficient (Karkhanis et al., 1993). The enzyme exhibited typical Michaelis-Menten kinetics with dependence on both the primer and substrate concentrations (Karkhanis et al., 1993). The Michaelis constants (K_m), with respect to UDP-glucose, was 0.5 mM; and 0.25 mg/ml and 1.25 mg/ml with respect to amylopectin and rabbit liver glycogen (Karkhanis et al., 1993). The specificity of the enzyme suggests that this enzyme is similar to glycogen synthase in eukaryotes and has been designated as amylopectin synthase (UDP-glucose-alpha-1,4-glucosyltransferase EC 2.4.1.11) (Karkhanis et al., 1993).

Transcripts coding for enzymes known to be involved in glycogen or starch synthesis are preferentially expressed in tachyzoites (glycogenin, starch [glycogen] synthase, one branching enzyme isoform) but can also be detected in lesser amounts in bradyzoites (Coppin, 2005). This pattern is consistent with the production of amylopectin during differentiation of tachyzoites into bradyzoites and with the mobilization of the glucose stores during bradyzoite-to-tachyzoite interconversion (Coppin, 2005).

Glucose

When sporozoites were incubated at 37°C in phosphate buffer without glucose, the PAS indices decreased as incubation time increased and they decreased in number gradually (Nakai and Ogimoto, 1983a). However, the decrease of the indices ceased after the addition of 0.05M (final concentration) glucose (Nakai and Ogimoto, 1983a). PAS indices and PAS ratios neither decreased nor increased in the case of the addition of glucose at 8, 16, and 24h incubation (Nakai and Ogimoto, 1983a). Since the number of viable sporozoites was constant for 8h after addition of glucose, it is suggested that the sporozoites which had lost amylopectin granules when stored without glucose possessed the granules again (Nakai and Ogimoto, 1983a). When glucose was added and stored for 16 h of incubation, PAS indices and PAS ratios increased as the incubation time proceeded (Nakai and Ogimoto, 1983a). This suggested that the amount of the amylopectin content in the sporozoites increased (Nakai and Ogimoto, 1983a). This was particularly seen in the sporozoites incubated for 16h where ratios increased by 15% in 2 h after addition of glucose (Nakai and Ogimoto, 1983a). The increase of the indices and the ratios may result from the increase of the amylopectin content in the viable sporozoites, since the number of viable sporozoites was constant after the addition of glucose at one hour incubation (Nakai and Ogimoto, 1983a). The difference from the case of 16h incubation may be attributed to the change of amylopectin synthetic ability, the property of detection methods by PAS stain, or synthesis of amylopectin in sporozoites (Nakai and Ogimoto, 1983a).

When ¹⁴C-glucose was added to fresh sporozoites as soon as they were excysted, 68.69 degradation per minute (dpm) of the ratio activity was counted in the polysaccharide reaction (Nakai and Ogimoto, 1983a). When ¹⁴C-glucose was added to

the sporozoites at 16h of incubation, 101.18 dpm was counted in the fraction (Nakai and Ogimoto, 1983a). These values of dpm were equivalent to 0.16 μ g glucose or 0.29 μ g glucose, respectively (Nakai and Ogimoto, 1983a) These results indicated that the ^{14}C from glucose was incorporated in polysaccharide of the sporozoites (Nakai and Ogimoto, 1983a).

When the ratio activity of polysaccharide extracted from the sporozoites incubated with ^{14}C (radioactive carbon) was determined, it was detected from the polysaccharide fraction in the cases of addition of glucose at 0 and 16h incubation (Nakai and Ogimoto, 1983a). The count of dpm of the sporozoites to which ^{14}C -glucose was added after 16h incubation was higher than that of the fresh sporozoites (Nakai and Ogimoto, 1983a). This fact may suggest that glucose is apt to be catabolized in the presence of larger amount amylopectin reserve in the body, in contrast, it is apt to be synthesized to amylopectin in the condition of smaller amount of the reserve (Nakai and Ogimoto, 1983a). From these results, it is confirmed that the sporozoites can synthesize amylopectin from glucose (Nakai and Ogimoto, 1983a).

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**III. ROLE OF METABOLIC ENZYMES AND AMYLOPECTIN
CONCENTRATION ON INFECTIVITY AND EXCYSTATION OF
CRYPTOSPORIDIUM PARVUM OOCYSTS**

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Key words; *Cryptosporidium parvum*, amylopectin, metabolic enzymes, infectivity, alpha-amylase, amyloglucosidase, enolase, lactate dehydrogenase, phosphorylase, electroporation, excystation

ABSTRACT

Cryptosporidium parvum an Apicomplexan parasite has garnered considerable interest due to outbreaks of gastrointestinal illness (sometime fatal) associated with consumption of contaminated foods and water caused by this parasite. It is hypothesized that *C. parvum* uses amylopectin granules, which are found in many Apicomplexans, for energy to infect hosts and survive in the environment for long periods of time. This study was performed to determine if degrading amylopectin granules with metabolic enzymes: alpha amylase, amyloglucosidase, lactate dehydrogenase, enolase, and phosphorylase could result in decreased infectivity of freshly excreted and stored *C. parvum* oocysts. An excystation assay was also performed to determine if the decrease in infectivity was related to a decreased ability of the parasite to release infectious sporozoites. In freshly excreted oocysts, alpha amylase and amyloglucosidase caused the greatest reduction of infective oocysts with reductions of 99.6% and 99.7%, respectively. Furthermore, amylopectin reduction was correlated with a decrease in infectivity ($R^2=0.69$) and excystation ($R^2=0.72$). This suggests that amylopectin may be an energy source for infectivity of *C. parvum* due to a decreased ability to excyst. In stored oocysts, enzymes did not significantly decrease infectivity, pointing to another energy source that may be present in dormant oocysts.

INTRODUCTION

Cryptosporidium has become a food and waterborne hazard because of its sustainability in the environment and resistance to many chemical disinfectants. The cell wall of *C. parvum* is practically impervious allowing this parasite to survive in the environment and resist commonly used water treatment chemicals. Coccidia are notable for their ability to survive as oocysts in the environment, a trait that has been attributed in part to its ability to utilize stored energy sources (Tilley et al., 1997). In this situation, the parasite has no access to nutrients and could survive on energy from amylopectin granules, a glucose storage source for the parasite (Haas and Aturaliye, 1999, Coppin, 2003). Similar to other parasites in the Apicomplexan phylum, the oocysts of *C. parvum* contain amylopectin granules which may also be present throughout their life cycles and used for energy.

Amylopectin granules may aid survival of the parasites during dormancy, allow latent stages to excyst or release infective stages and allow them to be mobile to access infection sites, invade host cells, and continue conversion to other life stages continuing their life cycle (Karkhanis et al., 1993, Coppin, 2003, Guerardel et al., 2005).

Amylopectin also serves as an energy reserve in coccidia and is utilized by the parasite for respiration, excystation, and survival in the environment (Karkhanis et al., 1993).

Residual body amylopectin may be looked upon as carbohydrate storage granules that are utilized during the transmission period of the oocysts stage in the environment whereas the sporozoite amylopectin are likely to be closely involved in the immediate metabolism of the sporozoite once liberated from the oocysts in the intestines of the new host (Harris et al., 2004). It has been concluded that when the content of amylopectin falls below a

critical level, sporozoites lack sufficient energy to move and invade cells resulting in reduced infectivity (Fayer et al., 1998, King et al., 2005). Harris et al. (2004) showed that enzymes could be used to degrade *Cryptosporidium* amylopectin granules but no work has been performed showing the relation of decreasing amylopectin to change in infectivity of *Cryptosporidium*. While amylopectin has been attributed to modes of survival and infection of other parasites in the phylum Apicomplexa, limited research has been done related to the function of amylopectin in *Cryptosporidium*.

This study seeks to determine if amylopectin is a primary source of energy for the infection process of both freshly excreted and stored oocysts. It will also be determined if amylopectin is an energy source responsible for excystation, allowing for release of the infective sporozoites. Currently there are few effective drugs available and no vaccines for *C. parvum*. This research may help support development of better control methods or may help with research to determine the potential of these enzymes for use as an anti-parasitic drug or for vaccine development.

MATERIALS AND METHODS

Cryptosporidium parvum oocyst preparation

Purified viable *C. parvum* oocysts (bovine Beltsville isolate, Genotype C) were kindly provided by Dr. Ron Fayer, USDA, Beltsville, MD. Both freshly excreted (less than 1 week old) and stored oocysts (6 months old) were used to determine the effect of the metabolic enzymes on amylopectin and infectivity of *C. parvum* oocysts. Freshly excreted oocysts were propagated and purified within 1 week of use to represent highly infectious oocysts that would be recently excreted and would have large amounts of amylopectin. Stored oocysts were passaged through calves and purified approximately 6 months prior to use and stored at 4°C until use. This storage creates a representation of oocysts that would have been found in the environment and would have lower levels of amylopectin due to environmentally stressful conditions. Oocysts were purified from bovine fecal material by washing and sieving through various graded pore sizes down to 45µm, followed by density centrifugation over cesium chloride for purification as previously described by Kilani and Sekla (Kilani and Sekla, 1987). Residual cesium chloride was removed by three wash cycles of centrifugation at 1,000 x g for 10 minutes each followed by aspiration of the supernatant and oocysts were resuspended in distilled water. Oocyst stocks were enumerated using a Reichert hemocytometer and evaluated with an Olympus BX60 microscope. Oocysts were diluted to 10⁵ oocysts/ml in sterile RNase DNase free molecular grade water (Mediatech Cellgro, Herndon, VA) for treatment.

In vitro cell culturing of *C. parvum* and oocyst treatment

Human ileocecal adenocarcinoma (HCT-8; ATCC CCL-244; American Type Culture Collection, Manassas, VA) cells were maintained in RPMI 1640 medium (Mediatech Cellgro, Herndon, VA) supplemented with L-glutamine (300 mg/L; Mediatech Cellgro, Herndon, VA), and HEPES (25mM; Mediatech Cellgro, Herndon, VA). For normal cell maintenance, the medium was supplemented with 2% fetal bovine serum (pH 7.2; Biofluids, Inc., Rockville, MD) and was increased to 10% fetal bovine serum for parasite infection. Cell culture maintenance medium consisted of RPMI 1640 medium with 2% fetal bovine serum (pH 7.2), L-glutamine (2mM), HEPES (20mM), 100,000 U of penicillin G liter⁻¹, 100 mg of streptomycin liter⁻¹ and 100mM sodium pyruvate. Forty-eight hours prior to inoculation, approximately 5 x 10⁶ HCT-8 cells were cultivated in 6 well plates (Corning, Corning, NY) with 22-mm² coverslips for immunohistochemistry and 24 well cell culture microplates for real-time PCR. The cell culture plates were incubated at 37°C in a 5% CO₂ humidified incubator to allow the development of ~85 to 90 % confluency in 10% RPMI media for 48 hours. The cell monolayer in each well of the microplate was considered a single replicate.

Enzyme treatment

For enzyme treatment, the following enzymes were used at 1mg/ml: alpha-amylase (Sigma # A6211, Sigma, St. Louis, MO), amyloglucosidase (Sigma # A7420, Sigma, St. Louis, MO), enolase (Sigma # E6126, Sigma, St. Louis, MO), lactate dehydrogenase (Sigma # L3888, Sigma, St. Louis, MO), and phosphorylase (Sigma # P6685, Sigma, St. Louis, MO). Since the *C. parvum* oocyst wall is highly impermeable,

electroporation (the application of brief, high voltage above 1kV) was used so that pores are formed and the enzymes could gain access to the amylopectin within the residual body and sporozoites (Haas and Aturaliye, 1999). All oocyst samples were diluted to log 5 oocysts/ml in sterile DNase RNase water (Fisher Scientific, Pittsburg, PA) and 700µl was placed into a 4mm gap electroporation vial (Fisher Scientific, Pittsburg, PA). The electroporation vials were placed into an ice bath for 10 minutes prior to electroporation to ensure that temperature fluctuations or warm room temperatures would not allow any pores to form in the cell wall. Each vial was then electroporated at approximately 1.8kV (1.56-1.58kV actually received due to conductivity of media) with two pulses at approximately 1.080-1.089 ms using a BTX600 Bacto Cell Manipulator electroporator (BTX; San Diego, CA). A control that has no enzymes but was electroporated (Control-electroporated) and a control that contains no enzymes and was not electroporated (Control) were also utilized. Since the control was not electroporated, it was taken out of the ice bath, placed at room temperature for 5 seconds and then placed directly back into the ice bath for 10 minutes. Following electroporation, the vials were placed back into the ice bath for 10 minutes to close the cell wall pores formed by electroporation. The vials were then incubated at 37°C for one hour to allow the enzymes to degrade the amylopectin.

Inoculation of monolayers with oocysts

Cryptosporidium oocysts were prepared as previously stated. Prior to oocyst infection of the cell monolayer, the cell maintenance media was removed and the cells were washed with HBSS to remove any cellular debris. Three mls of growth media

containing 0.01% Tween 20 was then added to the six well plates containing seeded coverslips and 1 ml of growth media was added to the 24 well cell culture plates (for real-time PCR) with a confluent monolayer. Since amylopectin may be responsible for excystation which may be affected if amylopectin is decreased, Tween 20 was used to prevent non-specific binding of the oocysts to the cell line (Fayer et al., 1998). Following the above incubation of the oocysts with the enzymes, 100µl of a 1×10^5 oocyst/ml suspension (1×10^4 oocysts total) were added to duplicate wells of both the six well cluster plates for immunohistochemistry and the 24 well culture plates for real-time PCR. The plates were then placed in an incubator at 37°C for 48 hours to allow for infection of the cells.

DNA extraction from oocysts and cell culture monolayers

After incubation, the cell monolayers were washed two times with HBSS to remove unexcysted unattached oocysts. Then 200µl of 1X Tris-EDTA buffer (10mM Tris-HCl, 1 mM EDTA) pH 8.0 was added to each well (Keegan et al., 2003, Di Giovanni and LeChevallier, 2005). The cells were dislodged using a plastic inoculating loop and placed into a separate 1.5 ml microcentrifuge tube and centrifuged at 8,000 x g to form a pellet. The supernatant was aspirated off and 50µl of thoroughly mixed Instagene (Biorad # 732-6030; specially formulated, 6% weight to volume Chelex® resin; Bio-Rad, Hercules, CA) was added to the samples and vortexed. The samples were then incubated in a 56°C water bath for 10 minutes followed by 100°C on a heat block for 20 minutes, and vortexed for 10 seconds. The samples were then centrifuged at 17,000 x

g for 5 minutes to separate out the Instagene matrix. All samples were frozen at -20°C until analysis for real-time PCR quantification.

Real-time PCR Quantification

Real-time PCR quantification was performed with a Bio-Rad iQ cycler (Bio-Rad, Hercules, CA). PCR primers specific for the *C. parvum* bovine (isolate 6) oocyst wall protein (COWP) gene (Accession: AF266273; position 613 to 838) were used from the 985 bp locus. The primer sequences were as follows:

forward primer 5' TGTATGGCACCAGAATCAGC 3'

reverse primer 5' AGGGCAGACAGGTTGAGTTG 3'

SYBR Green was used to measure the PCR products based on a fluorescent signal emitted in relation to the amount of DNA present. Each 25- μ l reaction contained 12.5 μ l of iQ Supermix (Invitrogen #1730-017 Invitrogen Corporation; Carlsbad, CA); 300nM (2.5 μ l of each forward and reverse 10X stock of 300 μ M) 0.25 μ l SYBR Green; 3.25 μ l sterile RNase DNase free water (Fisher; Pittsburg, PA) and 4.0 μ l of DNA template. All reactions were thoroughly mixed and 25- μ l was placed into iQ 96-Well PCR plates ensuring that no air bubbles were present (BioRad, Hercules, CA). The plates were then sealed with optical tape (Biorad # 223-8495; Biorad Hercules, CA) and centrifuged at 5,000 rpm for five minutes using a CL5R ThermoElectron centrifuge (ThermoElectron Waltham, MA). Amplification conditions were as follows: initial denaturation at 95°C for three min, 40 cycles of denaturation at 95°C for 20s, and annealing at 60°C for 20s, followed by a final extension at 72°C for 45s. A SYBR Green melt curve was performed

at 95°C for one minute followed by 80 cycles of 55°C to 95°C with a 0.5°C degree gradient. For real-time PCR, each sample duplicate was performed in triplicate.

For the melt curve and standard curve analysis, purified cell culture DNA that was extracted from log 5 *C. parvum* oocysts/ml was serially diluted from log 5 oocysts/ml down to log 1 oocyst/ml and a negative control with sterile RNase DNase free sterile water replacing template DNA was used as templates to create a standard curve. A *C. parvum* standard curve and equation to determine relative quantitation of cell culture infection was generated on the basis of the serially diluted template C_T values of samples plotted against the log number of oocysts inoculated per cell monolayer.

Immunohistochemistry

Following the 48 hour incubation of the infected monolayer, parasite infection was determined using the ABC immunohistochemistry assay as previously described by Kniel et al. 2003 (Vecatastain ABC Kit, Vector Laboratories, Burlingame, CA) (Kniel, 2003). Prior to immunohistochemistry the media in the six-well plates were removed and the cells were washed with HBSS one time for five minutes to remove non-attached cells. The coverslips were fixed with 100% methanol followed by two 5-minute washes with PBS. Immunohistochemistry was carried out by initially applying R Cp00 (provided by Dr. Ron Fayer), a rabbit anti-*Cryptosporidium* parvum primary antibody that was diluted 1:1,000 in PBS, followed by a biotinylated anti-rabbit secondary antibody (Vecatastain ABC Kit, Vector Laboratories, Burlingame, CA). An avidin biotinylated complex (ABC reagent; Vecatstain ABC Kit, Vector Laboratories, Burlingame, CA) was applied and life stages were visualized using an immunoperoxidase stain assay (to form an enzyme

complex) which contained 7µl hydrogen peroxide (Sigma, St. Louis, MO), and diaminobenzidine tetrahydrochloride (DAB, Sigma, St. Louis, MO). Hematoxylin (Fisher Scientific, Pittsburg, PA) was used as a counter stain and Scott's tap water substitute (1X, Fisher Scientific, Pittsburg, PA) was used to blue the nuclei. The coverslips were dehydrated using 50%, 70%, 95%, and 100% ethanol and affixed to slides using Permount. The immunohistochemistry slides were visualized with an Olympus BX60 microscope at 400X magnification. *Cryptosporidium* life stages appeared brown on the purple colored HCT-8 cell line. All immunohistochemistries were performed in duplicate for each sample replicate. All oocysts on the entire coverslip were counted to determine the amount of infective *C. parvum* oocysts present after enzyme treatment.

Amylopectin Assay

The concentration of amylopectin in *C. parvum* oocysts was determined using the method as described by Fayer et al. (1998). Prior to enzyme treatment, 100µl of each duplicate sample of oocysts was placed into a cryovial and refrigerated (4°C) until use to determine a baseline amylopectin level prior to treatment. Following enzyme treatment and incubation at 37°C for one hour, 100µl of each sample was placed into cryovials for the amylopectin assay. The oocysts were freeze/thawed in a liquid nitrogen tank followed by a 37°C water bath for 5 minutes each for ten cycles to lyse the oocysts and sporozoites. A stock of potato amylopectin (Sigma # A8515, Sigma, St. Louis, MO) was used to prepare standards of 50, 100, 200 and 300 µg/ml in deionized water. Amylopectin standards and negative controls were also measured in triplicate of duplicate samples. A

20 μ l sub-sample of each treatment or standard was pipetted into triplicate wells of a 96 well flat bottom microtiter plate (Costar # 3596, Costar, Cambridge, MA). An amyloglucosidase stock was prepared using 10mg amyloglucosidase in 3.2 M ammonium sulfate solution. Then 10 mls of the amyloglucosidase in the ammonium sulfate solution was put into 90mls 0.1M sodium acetate buffer as a working stock. All wells received 20 μ l of 1 mg/ml amyloglucosidase (Sigma #A7420, Sigma, St. Louis, MO) in sodium acetate buffer prepared as stated above. The plates were sealed and incubated at 37°C for one hour. During the incubation period, reagents were prepared. Reagent A consisted of 20 mls potassium phosphate buffer (pH 7.0) and 0.8 mg type II horseradish peroxidase (Sigma# P8250) and 1 mg glucose oxidase Type X from *Aspergillus niger* (Sigma # G7141). Reagent B consisted of 2.5 mg O-dianisidine (Sigma# D3252) in 0.5 ml deionized water. Following the incubation, reagents A and B were mixed and each well received 100 μ l of the mixture. The plates were resealed and incubated at 37°C for 20 minutes. The reaction was stopped by adding 70 μ l of 50% H₂SO₄. Absorbance for each well was measured using a Versamax tunable microplate reader (Molecular Devices, Sunnyvale, CA) at 530nm using Softmax 3.4 software (Molecular Devices, Sunnyvale, CA). To prevent any inaccuracies due to the amylopectin assay reacting with the enzymes, a baseline reading was performed for each enzyme with no oocysts present. The concentration of amylopectin was calculated based on the total amount of amylopectin present in the sample on the basis of the standard curve generated from the serially diluted standard absorbance values of samples plotted against amylopectin concentration (μ g).

Excystation Assay

Following the one hour incubation of the treated oocysts, approximately 200ul of each sample was incubated in 0.75% taurocholic acid (Sigma, St. Louis, MO) for one hour at 37°C. The excysted samples were observed at 400X and 1,000X magnification using Differential Interference Contrast microscopy (DIC) using an Olympus BX60 microscope. A total of 100 oocysts were counted for each duplicate sample. Oocysts that contained sporozoites were considered to be unexcysted while those containing no sporozoites or whose cell wall had collapsed were considered to be excysted. Excystation rates were determined using the following equation as described by Kniel et al. (2003) where % excystation = $(\text{[oocysts excysted/total oocysts counted]} \times 100)$.

Data analysis and statistics

C_T data for experiments were analyzed and expressed as the percent mean and standard deviation of replicates adjusted to experimental controls. To determine the statistical significance of results, data were analyzed by one-way analysis using JMP statistical software. The effects of enzyme treatment on infectivity, measured through real-time PCR, immunohistochemistry, and % excystation were considered to be significant when $p < 0.05$. All significant ($\alpha = 0.05$) main effects were analyzed using Tukey's HSD to separate treatment means. The data was analyzed using JMP statistical software (Statistical Analysis System, Cary, NC).

RESULTS

Comparison of real-time PCR and Immunohistochemistry

Real-time PCR and immunohistochemistry were compared to measure the infectivity of *C. parvum* oocysts on HCT-8 cells after treatment with metabolic enzymes. Using freshly excreted oocysts, immunohistochemistry had significantly higher ($p < 0.05$) infective oocyst counts compared to real-time PCR results (Figure 1). However, using stored oocysts (Figure 2), there was not a significant difference between the two methods with the exception of lactate dehydrogenase and phosphorylase which had a 23% and an 18.6% difference in mean percent infection between the two methods, respectively. Since it can not be determined whether real-time PCR or immunohistochemistry more accurately determined how many oocysts were present, it is important to average the counts from both methods to get more uniform results as shown for results in Table 1.

Comparison of Enzymes Used to Decrease Amylopectin

When 10^4 oocysts of freshly excreted oocysts were added to HCT-8 cells, about 91.8% were actually able to infect the cells (Table 1). Based on the data, the change in infectivity of the control sample of the fresh oocysts was only 11.8% different compared to the stored oocysts, which is not significantly different ($p > 0.05$). This suggests that the storage at refrigeration temperatures did not cause a significant decrease in infectivity as was expected.

When the oocysts were electroporated, infectivity decreased slightly by 17.3% but was not significantly different ($p > 0.05$) in freshly excreted oocysts and was not

significantly different ($p>0.05$) in stored oocysts. Therefore it is suggested that electroporation has no structural effect that would cause for a loss of infectivity.

When metabolic enzymes were added to decrease amylopectin there was some significant differences in infectivity caused by the enzymes on the fresh oocysts (Table 1). However in the stored oocysts, there were no significant differences between the enzymes and the control or electroporated control ($p>0.05$) (Table 1). Thus the enzymes were more effective in freshly excreted oocysts compared to those that were stored. Since electroporation may have had a slight but not significant effect on infectivity, all enzyme samples were compared to the electroporated control to determine if significant differences exist.

When alpha amylase was used on fresh oocysts (Table 1), the mean percent infectivity decreased to approximately 40.5% which was significantly different from both controls ($p<0.01$). Therefore alpha amylase caused the initial load of oocysts to be reduced by 99.6% and amyloglucosidase decreased the fresh oocysts by 99.7%. Therefore both amylase and amyloglucosidase reductions were statistically significant from the control and electroporated control (Table 1; $p<0.01$). However, amyloglucosidase and alpha amylase were not significantly different from each other. In contrast, when treated with enolase, lactate dehydrogenase and phosphorylase (Table 1), lactate dehydrogenase was not significantly different from both controls but enolase and phosphorylase were significantly different from the control but not significantly different from the electroporated control in fresh oocysts. While there was some effect of using metabolic enzymes on amylopectin utilization and thus on infectivity in fresh oocysts,

overall there was no significant effect of any of the enzymes on the infectivity of the stored oocysts (Table 1).

Table 1 also shows the comparison of mean percent infectivity, amylopectin content, and percent excystation after metabolic enzymes were used to decrease amylopectin in fresh and old oocysts, respectively. As amylopectin content decreased after treatment, the infectivity also decreased, although enolase and amyloglucosidase were somewhat divergent. Since there was strong correlation of infectivity decreasing when the amount of excysted oocysts decreased ($R^2=0.93$), it is likely that one of the mechanisms of decreased infectivity is related to less energy for excystation. In addition there is the trend ($R^2=0.72$) that as amount of amylopectin decreases the percent of oocysts that excysted decreased and also as amylopectin decreased the % infective oocysts also decreased ($R^2=0.69$). In the stored oocysts, the same trend occurred with a decrease in amylopectin relating to a decrease in excystation although there were some discrepancy with lactate dehydrogenase, enolase, and phosphorylase. However, in stored oocysts there was no change in infectivity related to decreasing amylopectin using metabolic enzymes.

DISCUSSION

Limited research had been performed to determine if amylopectin is responsible for infection of *Cryptosporidium* similar to other apicomplexan parasites. This project investigated whether glycolytic enzymes could be used to decrease these amylopectin granules hypothesized to be related to survival in the environment, excystation, invading host cells, among other survival/infectivity routes. In addition it was determined whether a decrease in amylopectin would relate to decreased infectivity in freshly excreted oocysts and stored oocysts and whether this was related to decreased energy for excystation.

Comparison of Immunohistochemistry and real-time PCR

The results showed that immunohistochemistry had significantly higher percent of infection compared to real-time PCR for fresh oocysts. However, there was no significant difference between the two methods, with the exception of lactate dehydrogenase and phosphorylase, for the stored oocysts. Typically real-time PCR is a more sensitive method, thus would yield higher levels of *C. parvum* oocysts. However, real-time PCR may have detected fewer infective oocysts for fresh oocysts because it only detected those oocysts that were infective. Although Tween 20 was added to prevent non-specific binding, it is possible that for immunohistochemistry some oocysts stayed attached but were unable to excyst and become infective. Some investigators have proposed that dead oocysts may be more likely than live oocysts to adhere to each other and to debris, resulting in false positives which may have occurred for immunohistochemistries (Bukhari et al., 1998). Loss of infectivity related to decreased

amylopectin may be due to reduced excystation of the oocysts. So it is possible that the fresh oocysts had enough energy to attach to the cells but not to excyst since it requires a lot of energy whereas the stored oocysts did not have enough energy to attach at all or may have had age-related damage to components of the cell wall.

In addition, counting the immunohistochemistry slides is very time consuming and tedious for the counter, so there is potential for overlapping of counting fields or worker fatigue. There is also a likelihood that some of the oocysts clumped together in larger groups creating variations among wells which could potentially increase the overall counts for one test while lowering counts for another test since only a set 100µl sample was aliquoted per well. In addition, there is a possibility of not all of the oocysts being dislodged from the 24 well plates around the edges of the plates or potentially some attached to the plastic loop causing some losses for the real-time PCR.

Comparison of Metabolic Enzymes to Decrease Amylopectin

In this study, oocysts that were not treated with any enzymes were considered to be a control. However it was thought that large enzymes would not be able to enter the oocyst wall. Therefore, electroporation, which is the application of brief high voltage (>1kV) was used to create temporary pores in the oocyst wall to allow the enzymes to enter and react with the amylopectin. Other research shows that electroporation can be used to affect the lipid membrane component of the outer cell wall so that transient pores formed and disinfectants could enter. Haas and Aturliye (1999) showed that disinfectants that typically can not go across the *C. parvum* cell wall caused significant decreases in viable cells when combined with electroporation (Haas and Aturaliye, 1999). A single

50 μ s pulse of 5-15kV cm⁻¹ resulted in significant uptake of a 70kDa dye that is normally excluded by yeast and red blood cells. This suggests that the electroporation level in this study, 1.8kV for 1.078 ms, was significant enough to allow for *C. parvum* to uptake enzymes because the enzymes had a size range of 5-80kDa.

This study showed that electroporation did slightly decrease infectivity but was not significantly different ($p < 0.05$) in fresh oocysts and had no effect on stored oocysts. In addition, a separate electroporation study showed that it did increase excystation of the oocysts slightly, from 9% to 15% (data not shown) but did not cause a significant decrease in infectivity. This was similar to results shown by Haas and Aturaliye (1999) where electroporation alone appears to little to no effect on the viability of *Cryptosporidium* oocysts (Haas and Aturaliye, 1999).

Both fresh oocysts and stored oocysts were used to determine if amylopectin was related to infectivity of *Cryptosporidium parvum*. The fresh oocysts represent oocysts with higher amylopectin levels because they have recently been excreted. This would represent a worse case scenario because it is likely that these oocysts would be more infective and found at higher levels if they contaminated water or food. The stored oocysts represent those oocysts that would have been excreted into the environment and had survived under the environmental conditions for 6 months. When the controls were examined for stored oocysts compared to fresh oocysts it was noted that the mean percent infectivity was only 11.8% different and not significantly different from the freshly excreted oocysts. Since amylopectin typically degrades with increased storage time, it was hypothesized that the infectivity of the stored oocysts would be significantly lower than the fresh oocysts since they would have less energy stores available to infect the

HCT-8 cells. This lack of significant difference could be related to increased variation of mean percent infection by the two infectivity methods for the fresh oocysts. In addition, the oocysts were refrigerated prior to use, so there may have been less decrease in amylopectin compared to other studies. Vetterling and Doran (1969) showed that low temperatures (0-4°C) resulted in a metabolic rate that was two times lower for dormant sporozoites compared to storage at 30°C, making the ability to infect to still remain high (Vetterling and Doran, 1969). Another study showed that oocyst storage at temperatures above 5°C produced a marked reduction in amylopectin and infectivity over a period of several weeks, whereas at 0°C and 5°C the amylopectin depletion was very slow (Harris et al., 2004). When high densities of *Cryptosporidium* oocysts are stored in deionized water some excysted sporozoites may be able to complete their life cycle using the available energy sources of amylopectin, lipid, and protein from broken and excysted oocysts, increasing the apparent length of time that oocysts retained their infectivity (King et al., 2005). However, it was found that *E. meleagridis* sporozoites did not survive at 0-4°C longer than 34 weeks (8.5 months) without considerable loss of sporozoites and that any sporozoites from stored cultures that penetrated cells died or failed to develop into schizonts (Vetterling and Doran, 1969). Therefore, it is possible that the lessened storage time of 6 months still allowed some oocysts to be infective, but based on the *Eimeria* research, it is expected that the levels of infective stored oocysts would be lower.

When metabolic enzymes were used to decrease amylopectin, there was no significant effect on infectivity of stored oocysts ($p>0.05$). However in fresh oocysts, lactate dehydrogenase was not significant from either control while enolase and

phosphorylase was significantly different from the control ($p < 0.05$) but not from the electroporated control ($p > 0.05$). However, amylopectin and amyloglucosidase had the greatest decrease in mean percent infectivity but were not significantly different from each other, which was somewhat different than what Harris et al. (2004) reported. Harris showed that incubation with amyloglucosidase caused aggregation of the granules and release of sticky oligosaccharides while alpha amylase caused some breakdown of the amylopectin granules (Harris et al., 2004). This breakdown of amylopectin by amylase suggests that there would be more reduction in amylopectin content and thus infectivity compared to amyloglucosidase. However, it is possible that any breakdown of amylopectin may cause a decrease in infectivity because there would be less amylopectin available to the oocysts to form free glucose as an energy source. Therefore since the Harris study showed that both enzymes caused some change in amylopectin structure, it is possible that both enzymes caused similar decreases in infectivity. However, studies with transmission electron microscopy would be better able to relate whether loss of structure both corresponds to lower amylopectin levels and less infectivity.

While the decreases caused by both enzymes may not seem very significant biologically, parasites are unable to grow outside of the host. Therefore this level of decrease may relate to lessened symptoms or decreased duration of illness. Also the amount of infective oocysts decreased by approximately 99.6-99.7%, so it is possible that no symptomatic illness would occur unless the individual is severely immunocompromised. This result suggests that alpha amylase and amyloglucosidase should be researched further to determine if they have potential to be used as a control

method or target for anti-parasitic drugs especially since the enzymes are more closely relates to plant enzymes than human enzymes.

Phosphorylase was selected to decrease infectivity because amylopectin phosphorylase is considered to be the rate limiting step for amylopectin degradation in *Eimeria*, but has not been proven to be the primary means of amylopectin utilization in *C. parvum*. Phosphorylase decreased the mean percent infectivity by approximately 50% compared to the control but was not significantly different from the electroporated control (p.0.05). It is possible that phosphorylase was not significantly different because there may not have been enough phosphorylase present, although approximately 20 IU/mg protein were used, which is above the 13 IU amylopectin phosphorylase/mg protein found in an extract of unsporulated *Eimeria* oocysts (Wang et al., 1975). However, if the total amount of enzyme was not transported into the cell, the utilization of amylopectin or glycogen may have been decreased. The phosphorylase used typically forms 1.0 μ mole of glucose-1-phosphate from glycogen and orthophosphate in the presence of 5' AMP per minute at pH 6.8 at 30°C. Therefore, the oocysts may not have had a lot of energy in the form of AMP available to use for this reaction. However, the most likely reason that phosphorylase was not significantly different was because amylopectin phosphorylase was not available and phosphorylase from rabbit muscle may not have had the same active sites. Although it has been stated that the amylopectin found in these oocysts are slightly different from typical amylopectin it may not be similar enough to animal glycogen to allow the enzyme to react properly to form maltose or glucose.

Lactate dehydrogenase may have been ineffective in fresh oocysts because it had such a high conductance resulting in an electroporation pulse that was only 0.05-0.967ms, so the pulse may not have created a high enough number of pores or large enough pores to let the same level of enzyme to enter the oocyst or may have produced enough heat to affect oocyst infectivity. It is also thought that alpha amylase and amyloglucosidase most likely had more of an effect because they work directly on amylopectin whereas enolase and lactate dehydrogenase are farther down in glycolysis so the parasites can possibly use feedback inhibition to regulate the enzymes and product formation to prevent amylopectin degradation, although this is unknown. In addition, adding precursors, such as UDPG and NADP, to the reactions may have increased the amount of amylopectin that was reduced. However, further research is needed in this area and would be needed to determine which enzyme was most effective on an mg/ml basis.

In stored oocysts, the enzymes decreased amylopectin and excystation but did not decrease infectivity which suggests that the decrease in amylopectin may also relate to other sources of energy besides just excystation. However, it is possible that oocysts are capable of infecting mice and cell cultures even though amylopectin concentrations are extremely low. In addition, Jenkins et al (2003) stated that amylopectin levels may underestimate the risk of infection because an assumption is being made that amylopectin is the principal energy source utilized by sporozoites (King et al., 2005). Quantification of amylopectin or targeting enzymatic reactions acting upon this substrate may therefore underestimate energy reserves available for *Cryptosporidium* oocysts during long periods of storage (King et al., 2005). Therefore it is proposed that another potential source of

energy other than amylopectin especially in dormant oocysts may relate to the decreased impact on infectivity after enzyme treatments, as shown in Table 1.

From previous literature, it is possible that two other major potential energy sources: lipids or a mannitol cycle exists and may be used for energy by the oocysts. Within the oocyst residuum, a large lipid body, lipid droplets, and a crystalline protein body reside alongside amylopectin granules which may contribute as a source of metabolites in addition to amylopectin or be involved with amylopectin metabolism for energy production during long storage periods (King et al., 2005). Researchers concluded that carbohydrate provided energy for the early stages of sporulation while oxidation of lipids was used during the latter stages and during dormancy (Vetterling and Doran, 1969). In addition, Vetterling and Doran (1969) showed that oocysts that were 48 hours old were catabolizing lipid and that carbohydrates were being stored (Vetterling and Doran, 1969). Since glycolysis is thought to be the principal source of energy in coccidian, the breakdown of lipid may enter glycolysis via the conversion of glycerol to dihydroxyacetone phosphate (King et al., 2005).

In addition to the potential use of lipids to provide energy, sporozoites are capable of converting exogenous glucose into mannitol suggesting that mannitol may be present in stored oocysts allowing them to remain infective even after the amylopectin levels have decreased dramatically (Michalski et al., 1992). According to Schmatz (1997), the mannitol cycle is present in the 6 major species of *Eimeria* which infect chickens and is also present in *Toxoplasma gondii* and *Cryptosporidium parvum* oocysts (Schmatz, 1997). However, Entrala and Mascaró (1997) stated that mannitol may be unsuitable for energy generation in *C. parvum* sporozoites, since fructose generated from mannitol

could not be phosphorylated and reintroduced into the glycolytic pathway (Entrala and Mascaro, 1997). Therefore it is unknown whether a mannitol cycle is being used or not in dormant or older oocysts.

Mannitol has been detected in another Apicomplexan parasite, *Eimeria tenella*, so the possibility exists that *Cryptosporidium* also has a mannitol cycle. There is no evidence that mannitol is being used by the host, suggesting that mannitol is an exclusive energy source for the parasite and a rapid decrease in amylopectin and free glucose increased mannitol suggesting mannitol was synthesized in oocysts from glucose released from amylopectin (Michalski et al., 1992). Thus the presence of a high glucose concentration would result in the synthesis of mannitol suggesting that mannitol and amylopectin metabolism are interrelated (Michalski et al., 1992).

According to Michalski et al. (1992) freshly prepared oocysts contained large quantities of amylopectin and a relatively small amount of mannitol, suggesting the difference in infectivity between the fresh and the stored oocysts even when the same enzymes were used (Michalski et al., 1992). When enzymes were used on fresh oocysts it would have reduced the level of amylopectin and thus the energy reserve, however in stored oocysts there would primarily be more mannitol in comparison to amylopectin which these enzymes would not be effective against.

Inhibiting the mannitol cycle works exclusively on the sexual stage of the life cycle which would have significant effects on pathogens like *Isospora* and *Cryptosporidium* where oocyst cycling is responsible for the continued transmission of the infection between hosts (Schmatz, 1997). Therefore, the mannitol cycle in coccidian parasites is an attractive drug target since higher eukaryotic hosts do not biosynthesize or

catabolize mannitol (Schmatz, 1997). Therefore further research should be performed to determine if a mannitol cycle truly exists in *C. parvum* and whether it acts simultaneously with amylopectin as an energy source for infectivity.

This study showed that the change in amount of excysted oocysts that were stored were less dramatic compared to the fresh oocysts suggesting that the decrease in amylopectin decreased excystation but apparently there was enough energy for these oocyst to remain infective. Therefore, the use of amylopectin activity as a surrogate marker for oocyst infectivity may be problematic (King et al., 2005). Total amylopectin includes the amylopectin content of broken oocysts and oocysts already inactivated (King et al., 2005). Thus the amylopectin content found in stored oocysts may overestimate the amount truly present.

Since there was strong correlation of infectivity decreasing when the amount of excysted oocysts decreased ($R^2=0.93$), it is likely that one of the mechanisms of decreased infectivity is related to less energy for excystation. In addition there is the trend ($R^2=0.72$) that as the amount of amylopectin decreases the percent of oocysts that excysted decreased suggesting that the loss of amylopectin is related to decreased energy available for the oocysts to excyst relating to decreased infectivity. Initially there was 54.5 μg and 44.7 μg amylopectin per 10^4 oocysts amylopectin in fresh and stored, respectively. In their study for sporulation, Wilson and Fairbain (1961) reported a value of 50 μg alkalai-stable carbohydrate/ 10^6 oocysts (Wilson, 1961, Vetterling and Doran, 1969). This value is higher than the values obtained in this study for polysaccharide in dormant sporulated oocysts although differences may be related to the number of oocysts and methods used (Vetterling and Doran, 1969). When the oocysts were stored at 4°C

for 162 days, the amylopectin level decreased to 65% of the original level (Augustine, 1980). In contrast to this rapid utilization by an active oocyst state is the fact that amylopectin content of dormant oocysts of *Eimeria acervulina* required 6 years to decrease from 33 $\mu\text{g}/10^6$ oocysts to 1.5 μg (Vetterling and Doran, 1969, Von Brand, 1973). Therefore the amount of amylopectin appears to decrease with oocyst age (Jenkins et al., 2003). A significant ($p < 0.05$) two-fold decrease in amylopectin content was observed each month at 1-3 months storage (Jenkins et al., 2003). The amount of amylopectin/oocyst remained at a steady-state level of 0.2-0.8 pg/oocyst over 3-9 months storage, showing a significant ($p < 0.05$) decrease relative to 1-2 months storage (Jenkins et al., 2003). A slight elevation at 8-9 months may have been due to breakdown of oocysts and release of cellular material that interfered with the absorbance readings (Jenkins et al., 2003).

Overall this study showed that amylopectin may be related to infectivity of *C. parvum* especially to the energy used for excystation. Although other mechanisms such as such as: sporozoite motility, survival in the environment, and stage conversion should also be analyzed to determine if amylopectin may supply energy for these functions also. Amylopectin and amyloglucosidase were the most effective at decreasing infective oocysts. Thus further research is needed in this area to further explain the role of amylopectin in *C. parvum* and to determine if metabolic enzymes such as alpha amylase and amyloglucosidase could be used as a control method or an anti-parasitic drug target.

Since limited research has been done in this area, there are many other avenues that this research could take. It should be determined whether oocysts can uptake glucose and other sugars from the host through their cell wall before excretion to allow for

survival in the environment. Also studies should be done to determine if *Cryptosporidium* has a mannitol cycle as another metabolic route present in the oocysts and whether amylopectin synthase would have a significant effect on infectivity since it is the major rate limiting step of amylopectin degradation for *Eimeria*. To determine if amylopectin is the primary source of metabolism, knockout genes for amylopectin synthesis enzyme or mutant with excessive amylopectin degradation enzymes should be used. Finally, TEM could be used to detect amylopectin granule degradation while also looking at radio labeled or immunolabeled enzymes to see if they only attack the amylopectin in the residual body or also in the sporozoites.

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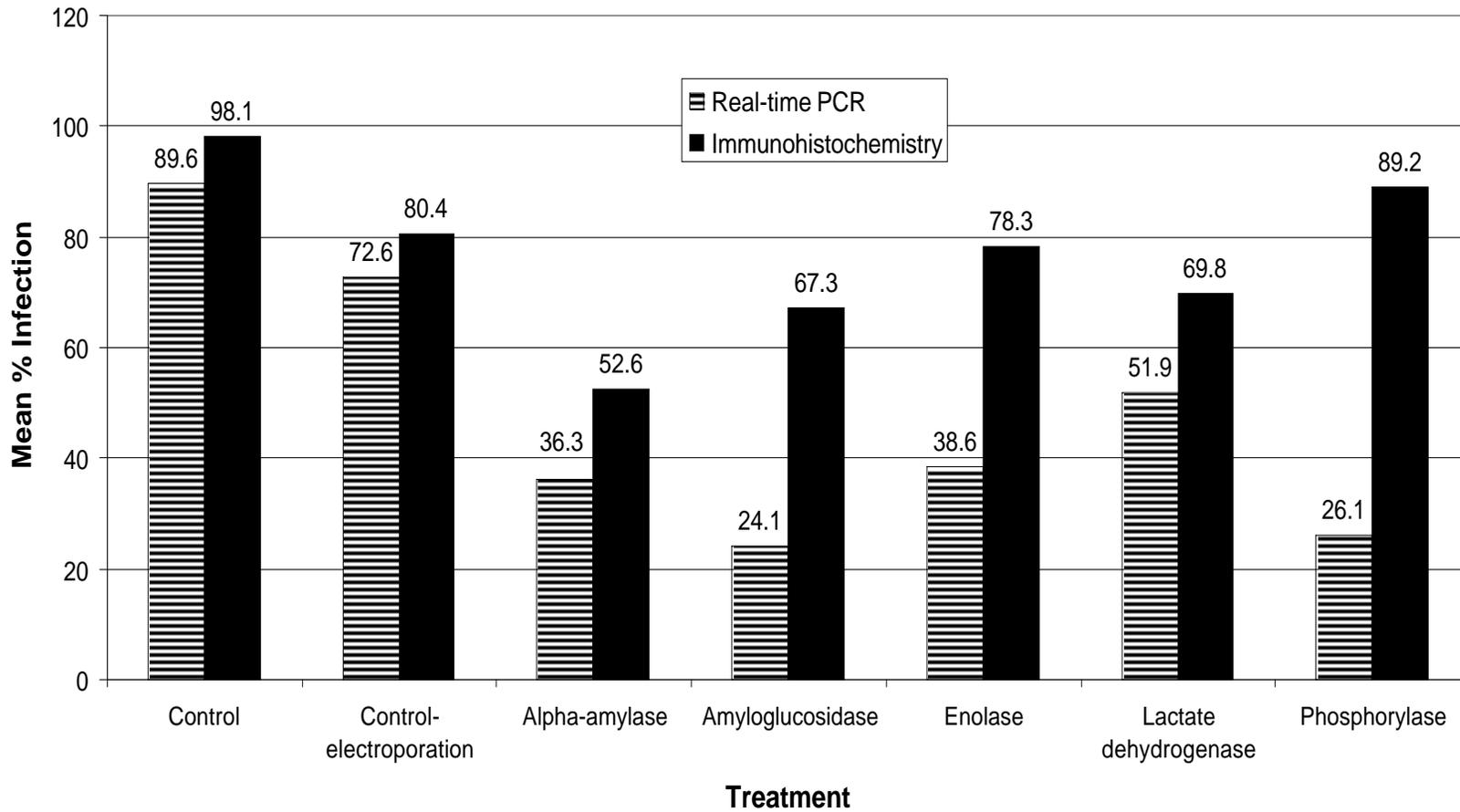


FIGURE 3.1: Comparison of real-time PCR and immunohistochemistry to measure mean percent infection of HCT-8 cells after enzyme treatment of freshly excreted *C. parvum* oocysts to decrease amylopectin (n=3).

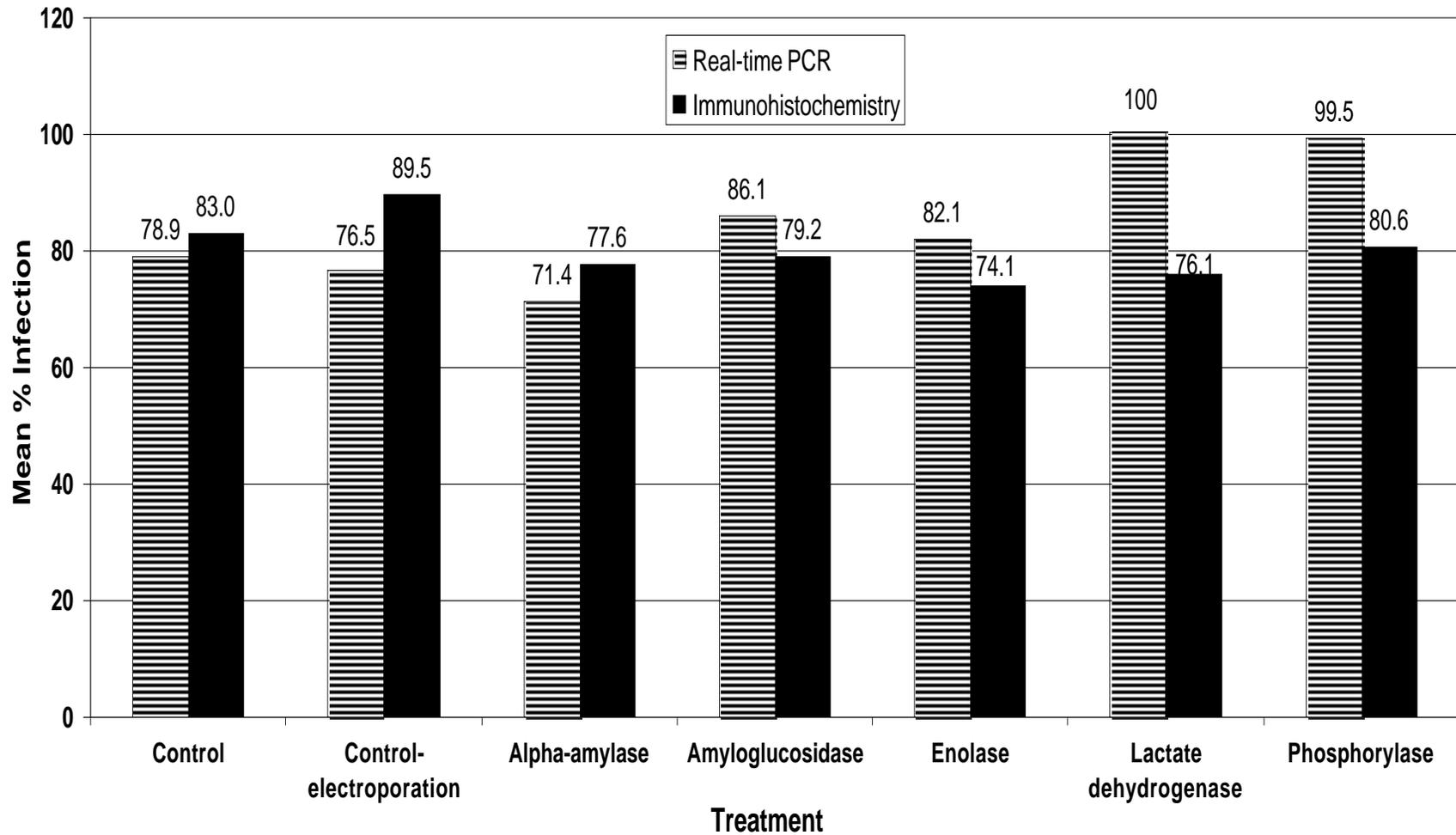


FIGURE 3.2: Comparison of real-time PCR and immunohistochemistry to measure mean percent infection of HCT-8 cells after enzyme treatment of stored *C. parvum* oocysts to decrease amylopectin (n=3).

TABLE 3.1: Comparison of mean percent infection, amylopectin content, and percent excystation of fresh and stored *C. parvum* oocysts after treatment with metabolic enzymes (n=3).

Treatment	Fresh Oocysts ¹			Stored Oocysts ¹		
	% Infection of HCT-8 cells (SD) ^{2,3}	Amylopectin Content (µg)	% Excysted ⁴	% Infection of HCT-8 cells (SD) ^{2,3}	Amylopectin Content (µg)	% Excysted ⁴
Control	91.8 (0.7)a	54.5a	92.66a	80.0 (2.1)a	44.7b	67.66a
Control-Electroporation	74.5 (3.3)ab	48.6a	84.66a	82.2 (3.0)a	39.9bc	66.33a
Lactate dehydrogenase	60.8 (3.4)abc	41.9a	56.66b	91.8 (1.2)a	82.4a	48.66a
Enolase	46.5 (3.6)bc	48.9a	42.66bc	87.5 (6.7)a	38.1bc	47.00ab
Phosphorylase	41.8 (5.3)bc	38.2a	47.00b	92.0 (2.3)a	63.6ab	34.33b
Alpha Amylase	40.5 (3.5)c	31.5b	27.00c	74.5 (2.6)a	18.5d	26.33b
Amyloglucosidase	35.0 (3.3)c	34.3b	29.33c	84.3 (4.0)a	22.7cd	26.00b

¹Values followed by different letters in the same column are significantly different (p>0.05)

² % Infection of HCT-8 cells is expressed as: I/T

I: average infective oocysts measured by immunohistochemistry and real-time PCR

T: total amount of oocysts in each sample (10⁴ oocysts)

³ SD stands for standard deviation

⁴ % Excysted = ([oocysts excysted/total oocysts counted] x 100).

**IV. EFFECT OF GLYCOLYTIC ENZYMES AND SUBSTRATES
ON THE INFECTIVITY AND EXCYSTATION OF
CRYPTOSPORIDIUM PARVUM OOCYSTS**

Angela D. Hartman

Key words; *Cryptosporidium parvum*, amylopectin, metabolic enzymes, infectivity, glucose, glucose-1-phosphate, glycogen synthase, electroporation, excystation

ABSTRACT

Cryptosporidium parvum has recently been the cause of several major outbreaks in food. However, detection limits in food are particularly low because certain isolation and concentration methods used for food may cause a loss of infectivity. Research studies with these parasites are restricted due to loss of infectivity with long-term storage and infectivity of *C. parvum* is linked to polysaccharide storage in the form of amylopectin granules that decrease with increased storage. Metabolic enzymes and substrates: glucose, glucose-1-phosphate, and glycogen synthase were used to increase amylopectin of the parasite to determine the effect on parasite infectivity. Excystation was measured to determine if the increase in infectivity was related to increased release of sporozoites. Glucose and glucose-1-phosphate increased the amount of infection of HCT-8 cells to 98.6% and 97.6%, respectively compared to the 81% infection for the control, while glycogen synthase caused no significant increase. In addition, adding the substrates to increase amylopectin content was related to increased infectivity ($R^2=0.52$) and increased excystation ($R^2=0.82$). Since incubation with these substrates for one hour created slight increases, there is a need to determine if longer incubation time with these substrates could lead to a greater increase in infectivity. This study suggests that incubating the oocysts with metabolic substrates such as glucose or glucose-1-phosphate may increase infectivity. Further research is needed to determine if adding them to the growth medium may lead to increased infectivity to aid in better detection methods or to increase the amount of storage time for oocysts used for research.

INTRODUCTION

Parasites belonging to the genus *Cryptosporidium* are an important and widespread cause of enteric disease in humans and in many other vertebrates (Balabat, 1996, Wiedenmann, 1998, Caccio, 2003). *Cryptosporidium parvum* is an intracellular protozoan parasite responsible for an acute gastrointestinal, and less frequently, respiratory infection in humans that is self-limiting in immunocompetent people. In contrast, immunocompromised people generally experience prolonged and potentially life-threatening diarrhea for which little effective treatment exists (Bonnin et al., 1996). Transmission of the parasite occurs through the fecal-oral route, by person-to-person contact, and exposure to contaminated water and food (Hallier-Soulier, 2000, Caccio, 2003). *Cryptosporidium parvum* is a parasite which already causes significant public health problems in the water industry and is now emerging as a potential food contaminant. The infectious form of *C. parvum*, environmentally resistant oocysts, can be found in many surface waters (Vesey et al., 1998). Although their concentrations may be low, oocysts are capable of surviving for weeks in surface waters, and the infective dose for humans is low at 30 oocysts (Rodgers, 1995). The low infective dose, monoxenous (single host) life cycle, resistance to chlorination, and prolonged survival of the transmissible (oocyst) form of the parasite in the environment favors transmission via potable water (Patel et al., 1999).

Although most cases of cryptosporidiosis are attributed to oocyst-contaminated drinking or recreational water, cryptosporidiosis associated with contaminated foodstuffs has been occasionally reported (Deng and Cliver, 2001). *Cryptosporidium* has demonstrated the potential of causing large numbers of human cases via a single

introduction/contamination event of water or food, and secondary transmission frequently associated with primary infections (Millar et al., 2001). The parasite is difficult to detect in the laboratory in comparison with bacterial foodborne pathogens. This is made even more difficult as most primary diagnostic food and clinical laboratories do not normally have the money or capability in terms of necessary consumable reagents or equipment (IMS, PCR, sequencing facilities, etc.) in order to reliably detect this agent from potentially contaminated foodstuffs or water (Millar et al., 2001). It is also postulated that as oocysts age, the oocyst wall degrades and this may explain why aged oocysts have lower excystation rates and potential lower detection rates (Kniel, 2003).

In a survey of published methods, LeChevallier et al. (1995) reported that most methods had recorded recovery efficiencies of less than 50% for *Cryptosporidium* oocysts from water samples (LeChevallier et al., 1995, Toze, 1999). The limitations of this procedure include loss of cysts or oocysts and/or decreased infectivity (killing them) during the various stages of selective isolation, resulting in reported recovery efficiencies ranging from 80% to 90% to less than 1% for *Cryptosporidium* (Rochelle et al., 1997, Robertson, 2001). An evaluation of commercial laboratories demonstrated average recovery efficiency of 3% for *Cryptosporidium* oocysts (Rochelle et al., 1997). Detecting oocysts washed from foods is considered difficult, with one study reporting that only 1% of oocysts experimentally added to fruit and vegetables were recovered (McEvoy, 2003). Trials and comparison of these methods by LeChevallier et al. (1995) determined that *Cryptosporidium* oocyst losses could be as high as 30% for each centrifugation step (LeChevallier et al., 1995, Toze, 1999). These reported losses during the concentration and/or recovery procedures could greatly decrease the detection sensitivity of the PCR

method (Toze, 1999). Considerable effort has been expended in evaluating different methods of oocyst isolation, yielding a wide variety of recovery efficiencies, mostly below 40% for *Cryptosporidium* (Rochelle et al., 1997). For example, large numbers of oocysts ($>10^4$ oocysts) have been reported to yield recoveries between 20 and 74%, and the recoveries depend on both the numbers of oocysts originally present in the sample and the viability of the oocysts (Bukhari et al., 1998, McCuin et al., 2001). Using a detergent washing procedure and sonication followed by IMS on strawberries the recovery was 27-54%, using detergent washing procedure and sonication followed by IMS on bean sprouts resulted in recoveries of 15-43% (Duffy and Moriarty, 2003). Due to the loss of oocysts, there is potential of not detecting contaminated food.

Nakai and Ogimoto (1983) showed that after addition of 0.05M glucose (final concentration) sporozoites that adding 0.05M of glucose to sporozoites stabilized decreasing amylopectin granules after 8h. When glucose was added at 16h of incubation, the amylopectin content of these sporozoites increased by 15% suggesting that sporozoites can synthesize amylopectin when provided with glucose substrates (Nakai and Ogimoto, 1983a). The purpose of this study was to determine if metabolic substrates could increase amylopectin and subsequently infectivity. The use metabolic substrates to increase amylopectin and infectivity may potentially be used increase detection of *Cryptosporidium* in food.

MATERIALS AND METHODS

Oocysts

Purified viable *C. parvum* oocysts (bovine Beltsville isolate, Genotype C) were kindly provided by Dr. Ron Fayer, USDA, Beltsville, MD. Stored oocysts were used to determine the effect of the metabolic enzymes on increasing amylopectin and consequently infectivity of *C. parvum* oocysts. Oocysts were purified from bovine fecal material by washing and sieving through various graded pore sizes down to 45µm, followed by density centrifugation over cesium chloride for purification as described by Kilani and Sekla (1987). Residual cesium chloride was removed by three wash cycles of centrifugation at 1,000 x g for 10 minutes each followed by aspiration of the supernatant and oocysts were resuspended in distilled water. Freshly excreted oocysts were propagated and purified within 1 week of use to represent highly infectious oocysts that would be recently excreted and would have large amounts of amylopectin. Stored oocysts were passaged through calves and purified approximately 6 months prior to use and stored refrigerated at 4°C until use. Oocyst stocks were enumerated using a Reichert hemocytometer and evaluated with an Olympus BX60 microscope. Oocysts were diluted to 10⁵ oocysts/ml in sterile RNase DNase free molecular grade water for treatment.

In vitro cell culturing of *C. parvum* and oocyst treatment

Human ileocecal adenocarcinoma (HCT-8; ATCC CCL-244; American Type Culture Collection, Manassas, VA) cells were maintained in RPMI 1640 medium (Mediatech Cellgro, Herndon, VA) supplemented with L-glutamine (300 mg/L; Mediatech Cellgro), and HEPES (25mM; Mediatech Cellgro). For normal cell

maintenance, media was supplemented with 2% fetal bovine serum (pH 7.2; Biofluids, Inc., Rockville, MD) and increased to 10% fetal bovine serum for parasite infection. Cell culture maintenance medium consisted of RPMI 1640 medium with 2% fetal bovine serum (pH 7.2), L-glutamine (2mM), HEPES (20mM), 100,000 U of penicillin G liter⁻¹, 100 mg of streptomycin liter⁻¹ and 100mM sodium pyruvate. Forty-eight hours prior to inoculation, approximately 5 x 10⁶ HCT-8 cells were cultivated in 6 well plates (Corning, Corning, NY) with 22-mm² coverslips for immunohistochemistry and 24 well cell culture plates for real-time PCR. The cell culture plates were incubated at 37°C in a 5% CO₂ humidified incubator to allow the development of ~85 to 90 % confluency in 10% RPMI media for 48 hours. The cell monolayer in each well of the plates was considered a single replicate.

Enzyme Treatment

For enzyme and substrate treatments, 0.05M glucose (Sigma # G7528, Sigma, St. Louis, MO), 1mg/ml glucose-1-phosphate (Sigma # G6750, Sigma, St. Louis, MO), and 1U/ml glycogen synthase (Sigma # G13989, Sigma, St. Louis, MO) were used only on stored oocysts to increase amylopectin. Since the *C. parvum* oocyst wall is highly impermeable, electroporation was used to form pores in the oocyst cell wall so that the enzymes could gain access to the amylopectin within the residual body and sporozoites. Electroporation is the application of a brief, high voltage (> 1 kV) pulse with the aim of creating pores in the cell membranes of a suspension of organisms (Haas and Aturaliye, 1999). All oocyst samples were diluted to 1 x 10⁵ oocysts/ml in sterile DNase RNase water (Fisher Scientific, Pittsburg, PA) and 700µl was placed into a 4mm gap

electroporation vial (Fisher Scientific, Pittsburg, PA). The electroporation vials were placed into an ice bath for 10 minutes prior to electroporation to ensure that room temperature would not cause any pores to form in the cell wall. Each vial was then electroporated at approximately 1.8kV (samples actually received 1.56-1.58kV due to conductivity of media) with two pulses at approximately 1.080-1.089 ms each using a BTX600 electroporator (BTX; San Diego, CA). Following electroporation, the vials were placed back into the ice bath for 10 minutes to close the cell wall pores formed by electroporation. A control that has no enzymes but was electroporated (Control-electroporated) and a control that contains no enzymes and was not electroporated (Control) were also utilized. For the control that had no enzymes and also was not electroporated (Control) was taken out of the ice bath and placed at room temperature for 5 seconds and then placed directly back into the ice bath for 10 minutes instead of electroporating. The vials were then incubated at 37°C for 1 hour to allow the enzymes to degrade the amylopectin. Each sample was performed in duplicate.

Inoculation of monolayers with oocysts

Cryptosporidium oocysts were prepared as previously stated. Prior to oocyst infection of the cell monolayer, the cell maintenance media was removed and the cells were washed with HBSS to remove any cellular debris. Three mls of growth media containing 0.01% Tween 20 was then added to the six well plates containing seeded coverslips and 1 ml of growth media was added to the 24 well cell culture plates for real-time PCR with a confluent monolayer. Since amylopectin may be responsible for excystation which may be affected if amylopectin is decreased, Tween 20 was used to

prevent non-specific binding of the oocysts to the cell line (Fayer et al., 1998). Following the above incubation of the oocysts with the enzymes, 100µl of a 1×10^5 oocyst/ml suspension (1×10^4 oocysts total) were added to duplicate wells of both the six well cluster plates and the 24 well culture plates. The plates were then placed in an incubator at 37°C for 48 hours to allow for infection of the cells.

DNA extraction from oocysts and cell culture monolayers

After incubation, the cell monolayers were washed two times with HBSS to remove unexcysted unattached oocysts. Then 200µl of 1X Tris-EDTA buffer (10mM Tris-HCl, 1 mM EDTA) pH was added to each well (DiGiovanni and Knepper, 1994, Keegan et al., 2003). The cells were dislodged using a plastic inoculating loop and placed into a separate 1.5 ml microcentrifuge tube and centrifuged at 8,000 x g to form a pellet. The supernatant was aspirated off and 50 µl of thoroughly mixed Instagene (Biorad # 732-6030; specially formulated, 6% weight to volume Chelex® resin; Bio-Rad, Hercules, CA) was added to the samples and vortexed. The tubes were then incubated in a 56°C water bath for 10 minutes followed by 100°C on a heat block for 20 minutes and vortexed for 10 seconds. The samples were then centrifuged at 17,000 x g for five minutes to separate out the Instagene matrix. All samples were frozen at -20°C until used for real-time PCR quantification.

Real-time PCR Quantification

Real-time PCR quantification was performed with a Bio-Rad iQ cycler (Bio-Rad, Hercules, CA). PCR primers specific for the *C. parvum* bovine isolate 6 oocyst wall

protein (COWP) gene (Accession: AF266273; position 613 to 838) were used from the 985 bp locus. The primer sequences were as follows:

forward primer 5' TGTATGGCACCAGAATCAGC 3'

reverse primer 5' AGGGCAGACAGGTTGAGTTG 3'

SYBR Green fluorescence was used to measure the PCR products. Each 25- μ l reaction contained 12.5 μ l of IQ Supermix (Invitrogen #1730-017 Invitrogen Corporation; Carlsbad, CA); 300nm (2.5 μ l of each forward and reverse 10X stock of 300 μ m) 0.25 μ l SYBR Green; 3.25 μ l sterile RNase DNase free water (Fisher; Pittsburg, PA) and 4.0 μ l of DNA template. All reactions were thoroughly mixed and 25- μ l was placed into wells of an iQ 96-Well PCR plates ensuring that no air bubbles were present (BioRad, Hercules, CA). The plates were then sealed with optical tape (Biorad # 223-8495; Biorad Hercules, CA) and centrifuged at 5,000 rpm for five minutes for five minutes using a CL5R ThermoElectron centrifuge (ThermoElectron Waltham, MA). Amplification conditions were as follows: initial denaturation at 95°C for three min and 40 cycles of denaturation at 95°C for 20s and annealing at 60°C for 20s, followed by a final extension at 72°C for 45s. A SYBR Green melt curve was performed at 95°C for one minute followed by 80 cycles of 55°C to 95°C with a 0.5°C degree gradient. For real-time PCR each sample duplicate was performed in triplicate.

For the melt curve and standard curve analysis, purified cell culture DNA that was extracted from 10⁵ *C. parvum* oocysts/ml was serially diluted from 10⁵ oocysts/ml down to 10¹ oocyst/ml and a negative control with sterile RNase DNase free sterile water replacing template DNA was used as standards to create a standard curve. A *C. parvum* standard curve and equation to determine relative quantitation of cell culture infection

was generated on the basis of the serially diluted template C_T values of samples plotted against the log number of oocysts inoculated per cell monolayer.

Immunohistochemistry

Following the 48 hour incubation of the infected monolayer, the parasite infection was determined using the ABC immunohistochemistry assay (Vectastain ABC Kit, Vector Laboratories, Burlingame, CA) as described by Kniel et al. (2003). Prior to immunohistochemistry the media in the 6 well plates were removed and the cells were washed with HBSS one time for five minutes to remove non attached cells. The coverslips were fixed with 100% methanol followed by two 5-minute washes with PBS. Immunohistochemistry was carried out by initially applying R Cp00 (provided by Dr. Ron Fayer) a rabbit anti-*Cryptosporidium* parvum primary antibody that was diluted 1:1,000 in PBS followed by a biotinylated anti-rabbit secondary antibody. An avidin biotinylated complex (ABC reagent; Vectastain ABC Kit, Vector Laboratories, Burlingame, CA) was applied and life stages were visualized using an immunoperoxidase stain assay to form an enzyme complex including 7 μ l hydrogen peroxide (Sigma, St. Louis, MO), and diaminobenzidine tetrahydrochloride (DAB, Sigma, St. Louis, MO). Hematoxylin (Fisher Scientific, Pittsburg, PA) was used as a counter stain and Scott's tap water substitute (1X, Fisher Scientific; Pittsburg, PA) was used to blue the nuclei. The coverslips were dehydrated using 50%, 70%, 95%, and 100% ethanol and affixed to slides using Permount. The immunohistochemistry slides were visualized with an Olympus BX60 microscope at 400X magnification. *Cryptosporidium* life stages appeared brown on the purple colored HCT-8 cell line. All

immunohistochemistries were performed in duplicate for each sample replicate. All oocysts and/or life stages on the entire coverslip were counted to determine the amount of infective *C. parvum* oocysts present after enzyme treatment.

Amylopectin Assay

The concentration of amylopectin in *C. parvum* oocysts was determined using the method as described by Fayer et al. 1998. Prior to enzyme treatment, 100µl of each duplicate sample of oocysts were placed into a cryovial and refrigerated until use so a baseline amylopectin level could be determined prior to treatment. Following enzyme treatment and incubation at 37°C for 1 hour, 100µl of each sample was placed into cryovials for the amylopectin assay. The oocysts were freeze/thawed for 5 minutes ten times in a liquid nitrogen tank followed by a 37°C water bath to lyse the oocysts and sporozoites. A stock of potato amylopectin (Sigma # A8515, Sigma, St. Louis, MO) was used to prepare standards of 50, 100, 200 and 300 µg/ml in deionized water. Amylopectin standards and negative controls were also measured in triplicate of duplicate samples. A 20 µl subsample of each treatment or standard was pipetted into triplicate wells of a 96 well flat bottom microtiter plate (Costar # 3596, Costar, Cambridge, MA). An amyloglucosidase stock was prepared using 10mg amyloglucosidase in 3.2 M ammonium sulfate solution. Then 10 mls of the amyloglucosidase in the ammonium sulfate solution was put into 90mls 0.1M sodium acetate buffer as a working stock. All wells received 20 µl of 1 mg/ ml amyloglucosidase (Sigma #A7420, Sigma, St. Louis, MO) in sodium acetate buffer prepared as stated above. The plates were sealed and incubated at 37°C for one hour. During the incubation period, reagents were prepared. Reagent A consisted of

20 mls potassium phosphate buffer (pH 7.0) and 0.8 mg type II horseradish peroxidase (Sigma# P8250) and 1 mg glucose oxidase Type X form *Aspergillus niger* (Sigma # G7141). Reagent B consisted of 2.5 mg O-dianisidine (Sigma# D3252) in 0.5 ml deionized water. Following the incubation, reagents A and B were mixed and each well received 100µl of the mixture. The plates were resealed and incubated at 37°C for 20 minutes and the reaction was stopped by adding 70µl of 50% H₂SO₄. Absorbance for each well was measured using a Versamax tunable microplate reader (Molecular Devices, Sunnyvale, CA) at 530nm using Softmax 3.4 software (Molecular Devices, Sunnyvale, CA). To prevent any inaccuracies due to the amylopectin assay reacting with the enzymes a baseline reading was performed for each enzyme with no oocysts present. The concentration of amylopectin was calculated based on the total amount of amylopectin present in the sample on the basis of the standard curve generated from the serially diluted standard absorbance values of samples plotted against amylopectin concentration (µg).

Excystation Assay

Following the one hour incubation of the treated oocysts, approximately 200ul of the each sample was incubated in 0.75% taurocholic acid (Sigma, St. Louis, MO) for 1 hour at 37°C. The excysted samples were observed at 400X and 1,000X magnification using Differential Interference Contrast microscopy (DIC) using an Olympus BX60 microscope. A total of 100 oocysts were counted for each duplicate sample. Oocysts that contained sporozoites were considered to be unexcysted while those containing no sporozoites or whose cell wall had collapsed were considered to be excysted. Excystation

rates were determined using the equation previously described by Kniel et al. 2003 where excystation = ([oocysts excysted/total oocysts counted] x 100) (Kniel, 2003).

Data analysis and statistics

C_T data for experiments were analyzed and expressed as the percent mean and standard deviation of replicates adjusted to experimental controls. To determine the statistical significance of results, data were analyzed by one-way analysis using JMP statistical software (Statistical Analysis System, Cary, NC). The enzymatic effects on infectivity measured through real-time PCR, immunohistochemistry, and % excystation was considered to be significant when $p < 0.05$. All significant ($\alpha=0.05$) main effects were analyzed using Tukey's HSD to separate treatment means.

RESULTS

Both immunohistochemistry and real-time PCR were compared as methods to measure infectivity of *C. parvum* after enzyme treatment. Figure 1 shows the comparison of real-time PCR and immunohistochemistry to measure infectivity of *C. parvum* oocysts after adding enzymes to increase amylopectin. There was no significant difference between the counts for immunohistochemistry and real-time PCR (Figure 1). The counts for both methods were averaged to determine changes in infectivity.

Comparison of Metabolic Enzymes and Substrates to Increase Amylopectin

The mean percent infection was determined following the use of metabolic enzymes and substrates added to *C. parvum* to increase infectivity (Figure 2). There was no significant difference ($p>0.05$) between the control and electroporated samples. When glucose was added to increase amylopectin, the percent infectivity increased to approximately 98.6% with a 17% increase in infection compared to the control and electroporated control, respectively. When glucose-1-phosphate was used there was a mean increase of infectivity of 14.6-16.6% compared to the controls. Both glucose and glucose-1-phosphate increased the percent infection significantly ($p<0.01$) compared to the control samples. However glycogen synthase had no increase and actually had a slight decrease in infectivity.

Table 1 shows the relationship of the change in amylopectin to excystation and infectivity of *C. parvum* after treatment with metabolic enzymes and substrates used to increase amylopectin. This table shows that electroporation caused a slight decrease in amylopectin content and excystation but there was no significant difference ($p>0.05$) in

infectivity. This was most likely due to normal variances rather than a real change in amylopectin content. Glycogen synthase had an increase of 37.1 μg amylopectin per 1×10^4 oocysts compared to the control. However, the excystation only slightly increased to 76.3% but was not significantly different ($p > 0.05$) from the controls (66.3% - 67.7%) and infectivity actually decreased slightly, thus was not significantly different ($p > 0.05$). Glucose-1-phosphate and glucose had an increase of approximately 45.31 μg and 76.67 μg amylopectin, respectively which was significantly different ($p < 0.001$) from both the control and electroporated control but not significantly different from each other. These two substrates also had an increase in excystation and infectivity in addition to the increase in amylopectin.

DISCUSSION

Research studies have related amylopectin granules to survival in the environment, excystation, and the infection process of several apicomplexan parasites. However, limited research has been performed relating amylopectin to infectivity of *Cryptosporidium parvum*. This study investigated whether glycolytic enzymes and substrates could be used to increase amylopectin content of *Cryptosporidium* and whether increased amylopectin is related to increased excystation and infectivity.

Comparison of Enzymes Used to Increase Amylopectin

Although glucose may be able to pass through the oocyst wall it would be unlikely that glycogen synthase would be able to pass through. Therefore electroporation, which is the brief application of high voltage (>1kV) to form pores, was used to allow the enzymes to enter the oocyst and react with the amylopectin. The results showed that electroporation had no significant effect on infectivity ($p>0.05$). This was similar to the results shown by Haas and Aturaliye (1999) where oocysts were electroporated alone and showed little if any effect on the viability of *Giardia* and *Cryptosporidium* oocysts. However it was shown that electroporation could be used to affect the lipid membrane component of the outer cell wall to form pores so disinfectants could enter. When disinfectants were used alone, there was no significant decrease of viable cells. However, when combined with electroporation, the disinfectants: combined chlorine, hydrogen peroxide and potassium permanganate created significant decreases in viable cells (Haas and Aturaliye, 1999). Weaver (1994) found that a 70kDa dye could transport across the membranes of yeast and red blood cells with a single 50 μ s pulse of 5-

15kV cm⁻¹ (Weaver, 1994). This suggests that the electroporation level in this study, 1.8kV for 1.078 ms, was significant enough to allow for *C. parvum* to uptake enzymes since the size range used was 5-80kDa. An electroporation study was performed (data not shown) and there was no significant change in infectivity but percent excystation actually increased by about 6%.

When comparing the enzymes, it was found that glucose and glucose-1-phosphate caused an increase of infectivity of approximately 17.5% and 18.5%, respectively compared to the control. Even this amount of increase may have a significant effect for detection since the infectious dose is so low and detection rates in some foods right now are as low as 1-50% so a 15-20% increase may help this rate of detection to become higher. In addition this study only incubated the oocysts for one hour before being placed onto the cell line, so there may not have been enough time for the amylopectin levels to increase to high enough levels to dramatically increase the infectivity. Therefore, longer incubation times of adding these substrates to storage or growth media is suggested and would most likely be in agreement with other research in regard to increased infectivity, although further research is needed.

When the sporozoites of *Eimeria* were incubated without glucose, the PAS indices decreased as incubation time increased (Nakai and Ogimoto, 1983a). The decrease of the indices, however, ceased by the addition of glucose (Nakai and Ogimoto, 1983a). Nakai and Ogimoto (1983) showed that after addition of 0.05M glucose (final concentration) to the sporozoite suspension at 8, 16, or 24h without glucose seemed to survive at least 8 h by the incubation with glucose (Nakai and Ogimoto, 1983a). Since the number of viable sporozoites was constant for 8h after addition of glucose, it is suggested

that the sporozoites which had lost amylopectin granules once possessed the granules again (Nakai and Ogimoto, 1983a). When glucose was added after 16h of incubation, the PAS indices of these sporozoites increased by 15% in 2h with further incubation (Nakai and Ogimoto, 1983a). This suggested that the amount of the amylopectin content in the sporozoites increased although the ratios did not increase in the sporozoites samples to which glucose was added at 8 or 24 h of incubation (Nakai and Ogimoto, 1983a). It is thought that since glucose and glucose-1-phosphate substrates are direct substrates for amylopectin synthesis, there was more increase in infectivity do to the ability to form more amylopectin.

In contrast to glucose and glucose-1-phosphate, glycogen synthase actually decreased infectivity slightly compared the controls, although it was not significantly different ($p>0.05$) from the controls. It is thought that glycogen synthase did not increase infectivity because the glycogen synthase would need glucose initially to form glycogen which was probably in low amounts already in stored oocysts. Although the amylopectin is somewhat similar to glycogen, even if glycogen was formed the parasite may not have the enzymes to break it back down for energy. However, it was shown that both *C. merolae* and *T. gondii* contain a UDP-glucose utilizing glycogen (starch) synthase-like sequence and glycogenins, so there is potential that there was not enough glycogen synthase present to form glycogen or starch (Coppin, 2005). Although amylopectin synthase was not available for use, Karkahanis et al. (1993) stated that the enzyme transfers glucose from UDPG to glycogen and is similar to glycogen synthase in eukaryotes (Karkhanis et al., 1993).

When comparing the amylopectin content to excystation and infection level it was determined that increase in amylopectin may only be slightly correlated with infectivity ($R^2=0.52$) in this study. However amylopectin content was highly correlated with excystation ($R^2=0.89$). It is thought that the one hour incubation time may not have been long enough to synthesize enough amylopectin to be used for energy or that the increased glucose or glucose-1-phosphate put a strain on the oocysts due to increased osmotic pressure. In addition, it should be noted that the large increase in amylopectin content from glucose did not result in increased infectivity or excystation compared to glucose-1-phosphate. It is possible that some of the glucose remained as excess glucose instead of being built up in to amylopectin which may not have resulted in any increase of infectivity after a certain threshold was present. It is also possible that glucose was able to continue to move throughout the cell wall but not enough in abundance at the same time to increase infectivity. It is possible that the glucose remained free instead of crystallizing into amylopectin granules relating to an excess of glucose that can not be used for energy. Isoamylase has been proposed to be responsible for the crystallization of amylopectin and should also be tested to determine if it can be used in combination with one of the substrates to increase infectivity (Coppin, 2005). However overall, as the amount of amylopectin increased the amount of excystation increased with increased infectivity.

In conclusion, amylopectin may be an important source of energy for excystation to release infective sporozoites and cause infection. Glucose and glucose-1-phosphate were the most effective in increasing infection. Therefore these substrates may have potential to be added as a pre-enrichment step to prevent loss of infectivity from isolation and concentration methods prior to detection of oocysts. This research may aid in

increasing detection levels in food. In addition, these substances may be added to storage media to increase the amount of time that oocysts can be stored for use in research.

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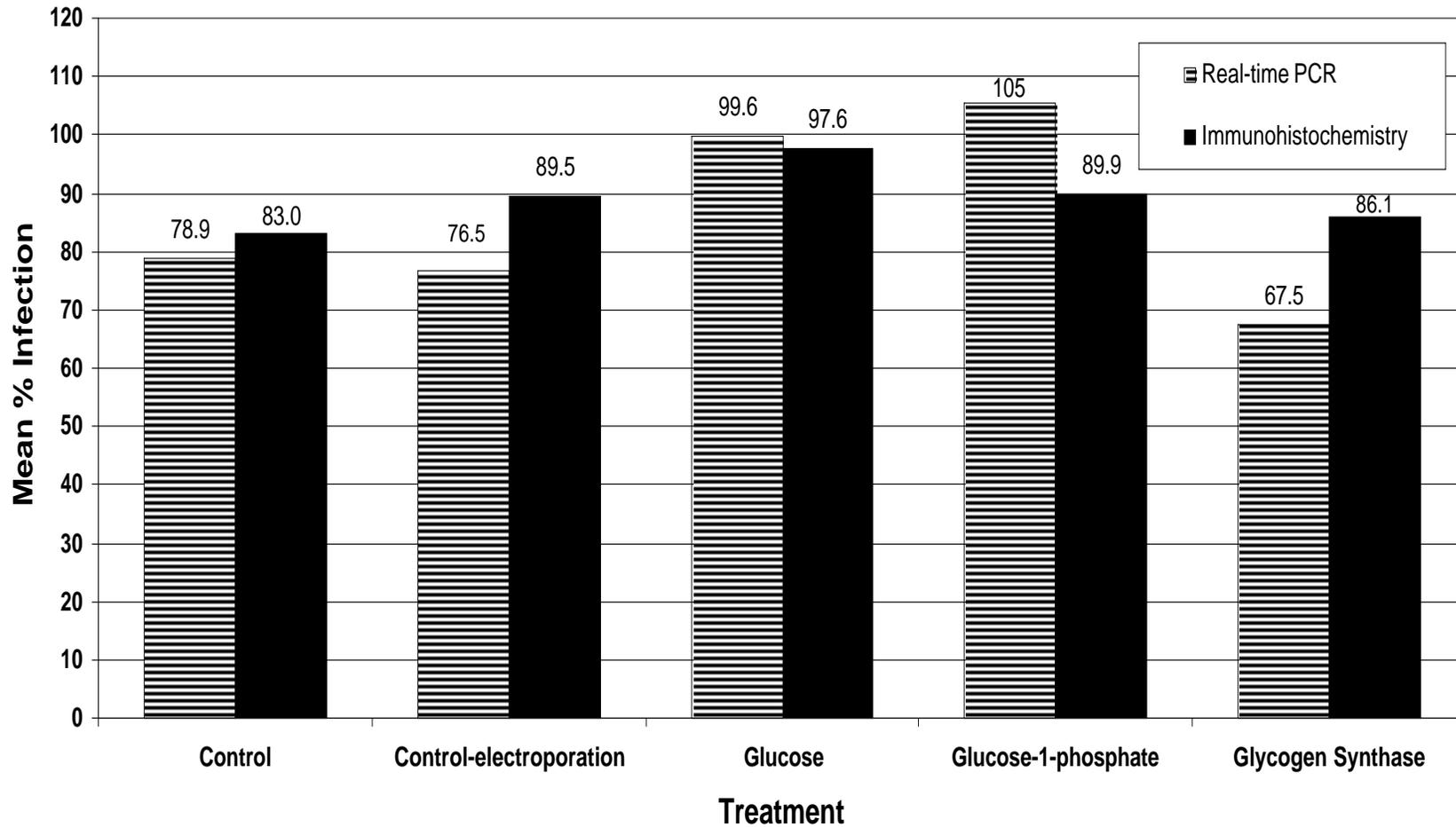


FIGURE 4.1: Comparison of real-time PCR and immunohistochemistry to measure mean percent infection of HCT-8 cells after substrate and enzyme treatment of stored *C. parvum* oocysts to increase amylopectin (n=3).

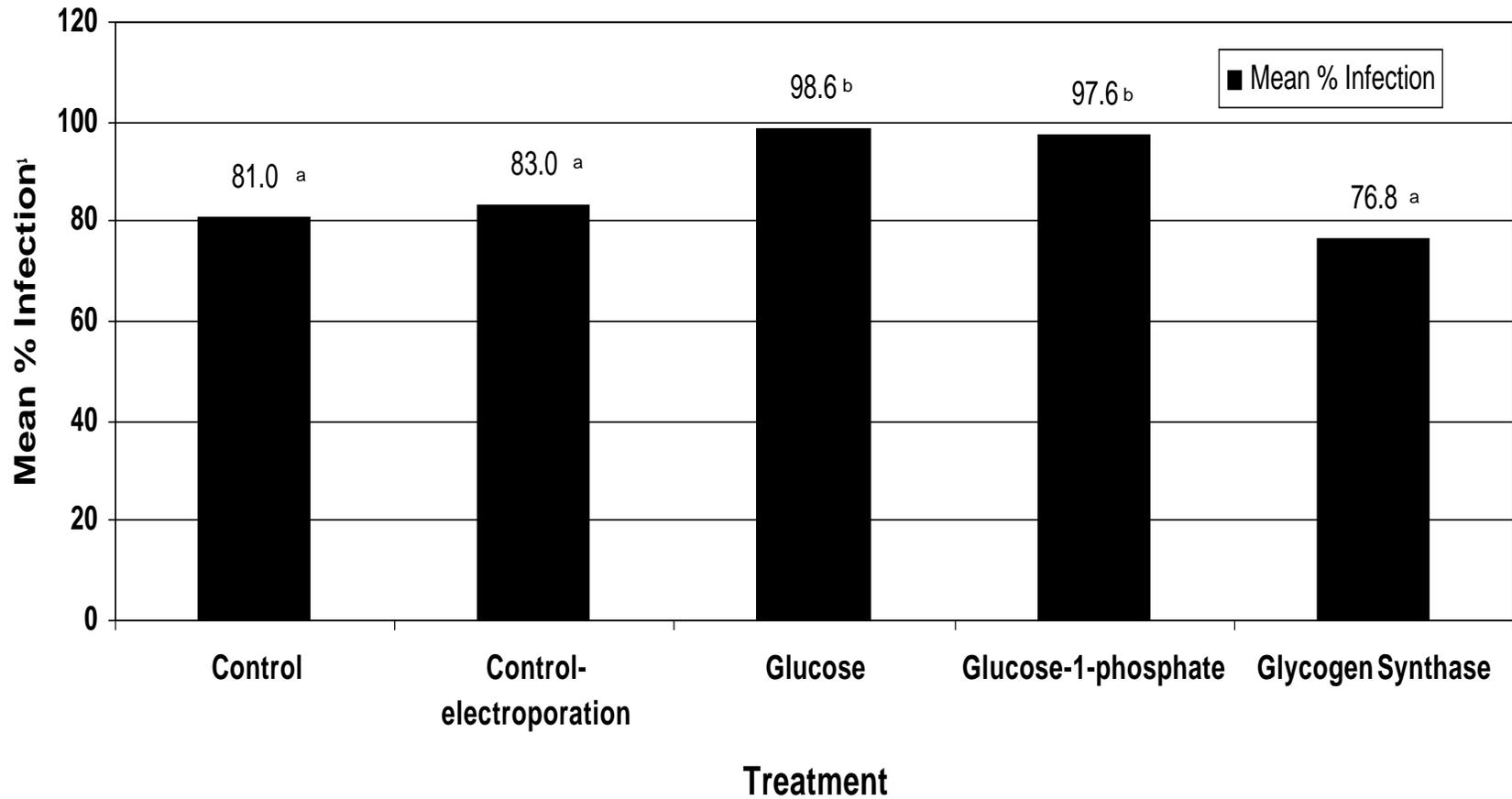


FIGURE 4.2: Comparison of metabolic enzymes to increase amylopectin on log oocysts/ml of stored *C. parvum* oocysts (n=3).

¹Values followed by different letters are significantly different (p<0.05)

TABLE 4.1: Comparison of log oocysts/ml, change in amylopectin content, and percent excystation of stored *C. parvum* oocysts after treatment with substrates and metabolic enzymes (n=3).

Treatment	Mean % Infection ¹	Amylopectin (μg) ¹	% Excysted ¹
Control	80.0a	45.1a	67.7a
Control - electroporation	79.8a	40.0a	66.3a
Glycogen synthase	72.0a	82.2b	76.3ab
Glucose	98.5b	121b	90.7b
Glucose-1-phosphate	97.5b	90.4b	92.3b

¹Values followed by different letters in the same column are significantly different (p>0.05)

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- Virginia Commonwealth Award, 1997 - 1998
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MEMBERSHIPS

- Southeastern Society of Parasitologists, 2005 - present
- Central Atlantic States Association of Food and Drug Officials, 2004 - present
- Gamma Sigma Delta, Honor Society of Agriculture 2004 - present
- International Association for Food Protection, 2001 - present
- Institute of Food Technologists Student Association, Virginia Tech Chapter (Virginia Tech Food Science Club), 1998 - present
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PUBLICATIONS

1. Hartman, A.D., Williams, R.C., Sumner, S.S., and B.W. Zoecklein. 2006. The efficacy of antimicrobials for the control of *Alicyclobacillus acidoterrestris* in tomato juice. (in internal review).
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PAPERS PRESENTED AT PROFESSIONAL MEETINGS

Hartman, A.D., R.C. Williams, S.S. Sumner, and B.W. Zoecklein. 2003. The efficacy of antimicrobial treatments for the inhibition of *Alicyclobacillus acidoterrestris* in apple and tomato juices. Annual Meeting of International Association for Food Protection; New Orleans, LA, August 10 - 13. Abstract is published in the annual meeting program.

Hartman, A.D., R.C. Williams, A.C. Rosypal, and D.S. Lindsay. 2006. Efficacy of the FTA filter-based method to detect DNA of *Cryptosporidium parvum*, *Toxoplasma gondii*, and *Encephalitozoon cuniculi*. Southeastern Society of Parasitologists Annual Meeting, Gatlinburg, TN, March 29 - April 1.

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ACTIVITIES

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- Virginia Tech Chapter, IFTSA College Bowl Team, 2002 and 2006
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