Further characterization of the major cysteine protease of Artemia franciscana cysts, and the isolation of a cDNA encoding the small subunit of the protease.

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FURTHER CHARACTERIZATION OF THE MAJOR CYSTEINE PROTEASE OF ARTEMIA FRANCISCANA CYSTS, AND THE ISOLATION OF A cDNA ENCODING THE SMALL SUBUNIT OF THE PROTEASE

By:

Andrea Lynn Aiton

A Thesis
Submitted to the Faculty of Graduate Studies & Research through the Department of Biological Sciences in Partial Fulfillment of the Requirements for the Degree of Master of Science at the University of Windsor.

Windsor, Ontario, Canada
1997
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ABSTRACT

Dormant cysts (embryos) of Artemia franciscana contain large amounts of a cysteine protease which was previously believed to be cathepsin B-like, but has now been re-classified as cathepsin L-like based on the studies detailed in this thesis. The Artemia cyst cysteine protease was purified to apparent homogeneity using a protocol involving gel filtration, anion exchange, and affinity chromatography. Fast protein liquid chromatography resolved five isoforms of the protease whose subunits (28.5 kDa and 31.5 kDa) were separated using reverse phase high performance liquid chromatography.

Carbohydrate moieties were found associated with both subunits of all cyst protease isoforms. Only the large subunit, however, was sensitive to endoglycosidase F, suggesting that the carbohydrate group associated with it is likely to be an N-linked high mannose, oligosaccharide, or complex biantennary carbohydrate. Assay of the enzyme’s proteolytic activity toward synthetic peptide substrates showed that the enzyme is cathepsin L-like in this respect.

A combination of enzymatic treatments and (reverse phase) high performance liquid chromatography were used to isolate a putative carboxyl-terminal peptide fragment of the small subunit of isoform #3 of the Artemia protease. The amino acid sequence information from this peptide was used to design a primer for use in polymerase chain reactions. A second primer was designed based on amino acid sequence information obtained (previously) from the amino terminus of the protein. These primers were used
in a polymerase chain reaction which amplified a 435 base pair product from a bacteriophage cDNA library. This product was subsequently cloned into the pCR 2.1 bacterial vector. *E. coli* cells were transformed with this vector, and two clones which hybridized with a $^{32}$P-labelled lobster cysteine protease cDNA were selected for further analysis.

These studies have allowed further characterization of a potentially unique cysteine protease. The enzyme’s isoform and subunit composition, carbohydrate content, and preference for synthetic peptide substrates is described in this thesis, and the isolation of a clone containing a cDNA encoding the small subunit of this protein will facilitate further studies of this enzyme at the molecular level.
To Andy,

for your unconditional support and encouragement
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I. INTRODUCTION

A. The History of *Artemia franciscana*

*Artemia franciscana* of North America (previously identified as *Artemia salina*) is a popular organism for the study of many cellular, developmental, and biochemical processes. In recent years, this brine shrimp has received a great deal of attention for several reasons. Encysted *Artemia* embryos (early gastrula stage) are readily available commercially, and are easily cultured in the laboratory. The culture of *Artemia* cysts gives synchronously developing populations with a short life cycle, making them relatively easy and convenient to work with. As well, when not in use, (desiccated) dormant *Artemia* cysts can be stored (at -20°C) for an extended period of time (several years) without loss of viability.

A wide array of studies have been conducted on *Artemia*, in areas ranging from biochemical and metabolic studies, to developmental, cellular, and molecular biology (Bagshaw & Acey, 1979; Warner et al., 1979; Bagshaw et al., 1989; Freeman, 1989; Sastre et al., 1989; Sillero et al., 1989; Warner, 1989; Spooner et al., 1994; Valverde et al., 1994). One interesting aspect of *Artemia* has been the presence of an abundant cysteine protease, and endogenous cysteine protease inhibitors associated with it in dormant cysts. This cysteine protease is believed to be required for the initiation of
developmental events (Perona & Vallejo, 1985; Warner, 1987; 1989; Warner et al., 1995). The presence of endogenous cysteine protease inhibitors whose levels vary with respect to *Artemia* development is thought to regulate the cysteine protease activity, and in turn, participate in the regulation of developmental events in *Artemia* embryos (Warner 1987; 1989). While much work has been done to characterize the major cysteine protease found in *Artemia* cysts, a number of questions remain unanswered. The one major area of investigation which has been pursued to a limited degree only, is the molecular characterization of the cyst protease gene (Lu, 1991), and this will be discussed at the end of this chapter.

B. Proteases

Of the many types of proteins required for the routine functioning and maintenance of any given organism, enzymes play key roles in all metabolic reactions. The presence of many types of enzymes is critical to the survival of all organisms as enzymes catalyze important chemical reactions in all living organisms. As an important group of enzymes, the proteases are of considerable interest and have been found almost ubiquitously in organisms ranging from viruses and bacteria to higher eukaryotes and humans. Based on the classification system of the Nomenclature Committee of the International Union of Biochemistry (1984), proteases are a subclass of the class of enzymes known as peptide hydrolases. They can be divided into two types or subclasses called peptidases (exopeptidases) or proteinases (endoproteinases). The
peptidases or exopeptidases are enzymes which remove an amino acid from the amino or carboxyl-end of a peptide or protein. Also, some peptidases remove a dipeptide from one or both ends of their substrate. These are often referred to as dipeptidases. The peptidases will not be discussed further, however they have been reviewed by Polgár (1989).

The proteinases are composed of four major groups: serine, cysteine, aspartic, and metalloproteinases. These groups are classified based on the composition of their active sites, and their sensitivity to protease inhibitors known to affect each group. It should be noted that the term protease refers to both peptidases and proteinases, however throughout this thesis, proteases will be used interchangeably with the term proteinases. These enzymes are critical for many biological processes, and when they become improperly regulated, or function incorrectly, pathological conditions are often the result. In order to convey the scope of physiological processes involving proteases, a few examples for each type of protease will be given. The main focus, however, will be on the cysteine proteases.

1. **Metalloproteases**

The metalloproteases are a group of mainly extracellular enzymes which range in size from approximately 17 kDa to 800 kDa. This group of enzymes is composed of the matrix metalloproteases, which include the collagenases, gelatinases, stromolysins, and matrilysins, as well as the meprins and brush border membrane enzymes of the intestines and kidneys (Woessner, 1991; Nagase et al., 1992; Twining, 1994). These enzymes are mainly responsible for bone resorption (Everts et al., 1992), and degradation of
extracellular matrix components in vertebrates (Woessner, 1991). They also function in the regeneration of (amphibian) appendages (Groell et al., 1993).

2. **Serine Proteases**

Most serine proteases tend to be extracellular and active at alkaline pH. There are two major families of serine proteases. They are the (eukaryotic) chymotrypsin family, and the (microbial) subtilisin family (Polgár, 1989). The serine proteases of the chymotrypsin family are responsible for many physiological processes. Dietary proteins are broken down in the duodenum of mammals by chymotrypsin, trypsin, and elastin after these digestive enzymes are secreted from the pancreas where they are manufactured (Hirschi et al., 1994). As well, the coagulation, fibrinolysis, and complement systems of mammals all require serine proteases (Mann et al., 1988; Twining, 1994). Finally, when one considers that serine proteases are also involved in embryogenesis, ovulation, neuronal growth, wound healing, vascular injury, inflammatory injury, smooth muscle cell proliferation, tumour growth and tumour cell invasion, the potential for disease associated with these enzymes can be appreciated (Twining, 1994).

3. **Aspartic Proteases**

The aspartic proteases are another important group. These enzymes participate in both normal and abnormal physiological processes. As well, the protease required by the HIV virus for processing of viral polyprotein precursors during assembly of new viral particles is an aspartic protease (Darke & Huff, 1994). The best known members of the aspartic proteases are renin, pepsin, gastracin, cathepsin D, cathepsin E (Twining, 1994),
and the HIV protease (Darke & Huff, 1994). Pepsin and gastracin are digestive enzymes which are active in the stomach of mammals, while the chief function of renin is to cleave angiotensinogen I to angiotensinogen II (Twining, 1994). Cathepsin E functions in the gastric parietal cells of mammals (Saku et al., 1990) while cathepsin D is a lysosomal enzyme which has been linked with the invasion and metastasis of breast cancer cells (Cardiff, 1994).

4. Cysteine Proteases

The cysteine proteases (often referred to as thiol proteases) are divided into several groups or families (Polgár, 1989). These families have recently been expanded, revised, and grouped into clans (Rawlings & Barrett, 1994). The clan of interest in relation to this work is designated CA and contains the families C1, C2, and C10 which correspond to the Papain, Calpain, and Streptopain Families, respectively. The cysteine proteases are generally intracellular enzymes; they are found in the lysosomes and cytosol (Bond & Butler, 1987), and have predominantly acidic or neutral pH optima. The amino acid residues which make up the active site of the cysteine proteases are cysteine (cysteine 25 in papain) and histidine (histidine 159 in papain) (Rawlings & Barrett, 1994). As well, glutamine (glutamine 19 in papain) and asparagine (asparagine 175 in papain) are also important to the formation and conformation of the active site (Rawlings & Barrett, 1994). In vitro, the cysteine proteases are generally inhibited by alkylation agents such as iodoacetamide and iodoacetate, n-ethylmaleimide, epoxide compounds (E-64 and E-475), and ρ-chloromercuribenzoate.
Under normal physiological conditions, proteases can serve to create biologically active molecules and degrade or turn over biologically active proteins or peptides (Bond & Butler, 1987). This is true of both mammalian and non-mammalian cysteine proteases. Mammalian cysteine proteases participate in protein degradation and turnover (Brocklehurst et al., 1987; Bohley & Seglen, 1992), as well as the processing of inactive biological molecules, such as the conversion of the interleukin 1β precursor to its active form (Thornberry et al., 1992), and the conversion of pro-enkephalin to (met)enkephalin (Krieger & Hook, 1991; Krieger et al., 1992; Azaryan & Hook, 1994). As well, cysteine proteases can be secreted by macrophages and fibroblasts in an inactive form, which can be activated extracellularly under the appropriate conditions (Kirschke et al., 1995).

Physiologically, the activity of cysteine proteases is controlled by many factors. The major physiological inhibitors of cysteine proteases are endogenous proteins known as cysteine protease inhibitors. These include the cystatin family of inhibitors, specific for most cysteine proteases (Katunuma & Kominami, 1985; Barrett et al., 1986; Turk & Bode, 1991; Bobek & Levine, 1992), the calpastatins, specific for the calpains (Pontremolli et al., 1991), and the α2-macroglobulin, which is a non-specific inhibitor for all four classes of endoproteinases (Borth, 1992). As well, many other physiological factors are involved in regulating proteolytic activity. These factors, reviewed by Bond & Butler (1987), and Twining (1994), include post-translational modifications, compartmentalization/localization/sequestration, formation of complexes, and the presence of metabolites.

When the balance between cysteine proteases and the in vivo factors which
regulate them is upset, abnormal or pathological conditions often result. Notably, cysteine proteases have been found to be involved in Alzheimer's disease (Marks et al., 1994), cancer (Sloane et al., 1987; Sloane, 1990; Sloane et al., 1990; Keppler et al., 1993; Ogloblina & Aref'eva, 1993; Solovyeva et al., 1995), arthritis (Twining, 1994), and periodontal disease (Lah et al., 1993; Ciborowski et al., 1994; Kadowaki et al., 1994; Takahisa et al., 1994; Kuniaki et al., 1995; Okamoto et al., 1995). Cysteine proteases have also been found to be involved in apoptosis (Miura et al., 1993; Vaux et al., 1994; Wilson et al., 1994; Patel et al., 1996).

a) Viral and Bacterial Cysteine Proteases

In cells infected with viruses, the main role of viral proteases is the cleavage of viral polyprotein precursors, not protein degradation or turnover (Ohkawa et al., 1994). Recently, however, a cysteine protease in the *Bombyx mori* nuclear polyhedrosis virus was discovered which is believed to play a role in the degradation of infected host tissue, to facilitate the spread of viral infection (Ohkawa et al., 1994). This protease was shown to have 35% homology with a *Trypanosoma brucei* cysteine protease precursor (Ohkawa et al., 1994).

There are several cysteine proteases of bacterial origin. Notable examples are porphypain-1 and porphypain-2 (Ciborowski et al., 1994), and argingipain (Kadowaki et al., 1994; Okamoto et al., 1995), produced by *Porphyromonas gingivalis*, which participate in the destruction of periodontal tissue contributing to periodontal disease. Streptopain (Liu & Elliot, 1971), is produced and released by *Streptococcus pyogenes*, contributing to the virulence of this organism (Ohara-Nemoto et al., 1994). As well,
endoproteinase C from *Staphylococcus aureus* strain V8 is believed to be one of the many staphylococcal toxins and enzymes used in virulence (Drapeau et al., 1972). This enzyme also has a very specific substrate specificity (Houmard & Drapeau, 1972) and is very tolerant to protein denaturants (Johnson & Delk, 1994), making it useful *in vitro* in the analysis of peptides and proteins.

b) **Plant Cysteine Proteases**

To date, many plant cysteine proteases have been characterized and described, with the most well known being papain. The plant cysteine proteases will not be discussed in great detail, as they have been reviewed quite extensively elsewhere (Glazer & Smith, 1971; Brocklehurst et al., 1981; Brocklehurst et al., 1987; Polgár, 1989). In general, plant cysteine proteases are usually found associated with the fruit or seeds of a plant. Many of the cysteine proteases found in seeds have been shown to break down or degrade plant storage proteins (Baumgartner & Chrispeels, 1977; Shutov & Vaintraub, 1987; Tanaka et al., 1993; and Yamouchi et al., 1992). Currently, many seed cysteine proteases are being studied at the molecular level to elucidate their exact roles in embryogenesis (Becker et al., 1994; Domoto et al., 1995; Nong et al., 1995). Of particular interest is the timing of mRNA synthesis and expression of seed cysteine proteases, and the regulation of this expression (Becker et al., 1994).

The cysteine proteases of many fruits have been found to be useful in a variety of applications. Ananain and comosain from pineapple stem have been used to remove (debride) necrotic tissue from burn wounds and ulcers (Silverstein et al., 1987), and chymopapain has been used as a chemonucleolytic agent in treating herniated vertebral
discs (Smith, 1991). As well, the latex of papaya (which contains many cysteine proteases) is used commercially in meat tenderizing preparations, and as a chill-proofing agent in beer undergoing cold storage (Polgár, 1989).

c) Insect and Parasitic Cysteine Proteases

Cysteine proteases of several agricultural pests are currently being studied in the hope that cysteine protease inhibitors could be used in agriculture to replace pesticides (McGhie et al., 1995). The cysteine proteases of several species of Coleoptera, or beetles (Terra & Cristofoletti, 1996) including the Colorado potato beetle, *Leptinotarsa decemlineata* Say (Michaud et al., 1995) are currently being studied in this respect. As well, the cysteine proteases of three white grub species, *Lepidiota noxia*, *Lepidiota negatoria*, and *Antitrogus consanguineus*, are also being studied in an effort to find a way of controlling the destruction of sugar cane crops by white grubs (McGhie et al., 1995).

As well, in *Drosophila melanogaster*, a putative cysteine protease cDNA has been isolated which is expressed in the embryonic and larval midgut and is believed to have a digestive function(s) (Matsumoto et al., 1995). These studies were initiated in the hope that *Drosophila* could be used as a model to study the interaction of phytocystatins (plant cysteine protease inhibitors) with insect cysteine proteases, since phytocystatins probably aid plants in defending themselves against attack by insect pests (Matsumoto et al., 1995).

Cysteine proteases are also a major virulence factor used by many parasites to invade human and animal host tissues. These parasites cause a significant level of morbidity and mortality in human hosts. The parasitic proteases have been selected as
potential targets for drug therapies because they are involved in the replication, metabolism, survival, and pathology of many parasites (McKerrow et al., 1993). Notably, members of *Trypansoma, Schistosoma, Leishmania, and Plasmodium* all contain cathepsin B-like or L-like (or both) cysteine proteases.

Three main cysteine proteases from *Trypansoma* have been characterized to date. These enzymes, cruzipain, trypanopain, and congopain, play a role in the pathogenesis of *Trypansoma cruzi, Trypansoma brucei brucei*, and *Trypansoma congolense*, respectively. *T. cruzi* is the causative agent of Chagas’ disease (or trypanosomiasis) and infects more than 24 million people in South and Central America (McGrath et al., 1995). In Africa, *T. brucei brucei* causes nagana in cattle and sleeping sickness in humans (Troéberg et al., 1996), while *T. congolense* causes anemia, weight loss, and overall poor productivity in cattle (Authié, 1994).

It has been estimated that approximately 250 million people suffer from Schistosomiasis in tropical countries (Dalton et al., 1996). *Schistosoma mansoni* and *Schistosoma japonicum* also contain cysteine proteases needed for the survival and infectivity of these parasites (Dalton et al., 1996; Day et al., 1995). The enzymes of *S. mansoni* have been shown to be expressed at many developmental stages, aiding in the penetration of tissues and digestion of host hemoglobin (Smith et al., 1994). Other examples of parasitic cysteine proteases involved in human pathological conditions include Lpcys1 and Lpcys2 of *Leishmania*, the causative agent of Leishmaniasis (Traub-Cesko et al., 1993), and falcipain of *Plasmodium falciparum*, which degrades hemoglobin in the process of causing malaria (Rosenthal et al., 1988).
Finally, there is a considerable amount of evidence to suggest that some insect cysteine proteases are involved in the degradation of insect egg yolk proteins (Kageyama & Takahashi, 1990; Takahashi et al., 1992; Yamamoto et al., 1994; Zhao et al., 1996). Interestingly, two cysteine proteases have been isolated from silkmoth eggs which show some similarity to the *Artemia* cyst cysteine protease (Kageyama & Takahashi, 1990; Zhao et al., 1996). These will be discussed in greater detail later in this chapter.

d) Other Invertebrate Cysteine Proteases

It is noteworthy that the study of cysteine proteases extends to many other organisms. Multiple cysteine proteases have been described in the slime mold, *Dictyostelium discoideum* (Williams et al., 1985; Pears et al., 1985; Presse et al., 1986; Datta & Firtel, 1987; North & Whyte, 1984; North et al., 1988), and recent studies have shown that some of these proteases are activated by treatments with acid during zymography (North et al., 1996). However, North et al. (1988) have suggested that the cysteine proteases of *Dictyostelium discoideum* are more important for nutrition than in development.

Cysteine proteases have also been examined in the American lobster, *Homarus americanus* (Laycock et al., 1991) and the Norway lobster, *Nephrops norvegicus* (Le Bouley et al., 1995). These studies have characterized cathepsin L-like enzymes from both lobster species, and the enzymes from the two lobsters are in fact quite similar (Le Bouley et al., 1995). Because of the limited information about invertebrate cysteine proteases at the time during which their studies were undertaken, Le Bouley et al. (1995) hoped that studies of lobster cysteine proteases could be used to study the evolutionary
relationship among different crustacean cysteine proteases, as well as contribute to the understanding of the evolutionary relationship among cysteine proteases in general.

C. *Artemia* Cysteine Protease

Some time ago, Urbani et al. (1952) and Bellini (1957) observed acid protease activity associated with dormant (encysted) *Artemia* embryos. This work was furthered by Nagainis & Warner (1979), who described an abundant acid protease from *Artemia* cysts in greater detail, followed by reports by Warner & Shridhar (1980; 1985) who found the major cysteine protease of *Artemia* embryos predominantly in the cytosolic fraction of cyst homogenates. This enzyme is composed of two subunits of relative molecular weights of approximately 28.5 and 31.5 kDa as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Initially, Warner and Shridhar (1980) characterized the enzyme as follows. Using bovine serum albumin as a substrate, the enzyme was found to have a pH optimum of pH 3.6-3.8, and a temperature optimum of 45°C. Incubation above pH 8.0 inactivated the enzyme irreversibly, as did incubation at 60°C (in the presence or absence of substrate). As well, the enzyme was not inhibited by phenylmethyl sulfonylfluoride, pepstatin, n-ethylmaleimide or p-chloromercuribenzoate. The enzyme was found to be strongly inhibited by Cu$^{2+}$, Hg$^{2+}$, iodoacetate, and leupeptin. Further studies by Warner & Shridhar (1985), revealed that the enzyme was also inhibited by antipain, chymostatin and Ep-475. The enzyme was found to be stimulated by heavy metal chelators and thiol
reagents, and it hydrolysed many protein substrates including bovine serum albumin, hemoglobin, protamine, *Artemia* embryo soluble proteins, and *Artemia* lipovitelline (at pH 3.5-4.0). At pH 6.0-6.5, the enzyme was found to be highly specific for *Artemia* elongation factor 2 and *Artemia* lipovitelline α1.

There are other (sometimes conflicting) reports of the characteristics of the *Artemia* cyst cysteine protease in the literature. Around the same time that Warner & Shridhar (1980; 1985) were studying the cyst enzyme, Garesse et al. (1980) also reported proteolytic activity found in *Artemia* cysts. They described an enzyme present in an inhibited state (a high molecular weight complex). Then Perona & Vallejo (1982) further described the enzyme. It should be noted that there is some discrepancy as to whether the enzyme described above is the same enzyme described in these reports. This report, in contrast to the work described above, stated that the cyst enzyme was composed of a single polypeptide of 68 kDa, and based on their observations and those of Perona et al. (1991), Perona & Vallejo (1982) characterized the enzyme as a cathepsin B-like lysosomal enzyme, found chiefly in the particulate fraction of cyst homogenates. Minimal proteolytic activity was detected associated with the cytosolic fraction of cysts. This is where the two groups of researchers differ in opinion. Several factors are thought to contribute to these discrepancies. They will be mentioned here briefly, as they have been described in detail elsewhere (Warner, 1987).

It is believed that the "California" cysts used by the Spanish researchers were probably the parthenogenetic strain from China. The enzyme from these cysts has different physical characteristics (molecular weight, subunit composition) and it is
possible that this is not the same enzyme described from "Utah" cysts (A. Warner, personal communications). It is also believed that treatment with hydroxychlorite to dechorionize and sterilize cysts has affected the measurement of proteolytic activity in the cytosolic fraction of cyst homogenates, causing the enzyme activity to decrease markedly (Warner, 1979). Also, if the endogenous protease inhibitors found in cysts are not removed before performing activity measurements, this will greatly affect the amount of proteolytic activity detected. It should be noted however, that both groups agree that the enzyme is a glycoprotein (Perona & Vallejo, 1982; Warner, 1987).

D. Endogenous Artemia Cysteine Protease Inhibitors

The story of the major cysteine protease of Artemia cysts would not be complete without mentioning the endogenous cysteine protease inhibitors found in Artemia cysts. Dormant Artemia cysts contain two types of cysteine protease inhibitors, one which can be removed from cyst extracts by dialysis, and one which can not (Nagainis & Warner, 1979). The dialysable cysteine protease inhibitor has not been characterized to date, but the non-dialysable inhibitor was initially described by Nagainis & Warner (1979), who found that it could be separated from the major cyst protease using ion exchange chromatography. Nagainis & Warner (1979), also purified the non-dialysable inhibitor and demonstrated that it inhibited the cyst protease, and that there were multiple forms of it in cysts. They also measured the effects of calcium and EDTA on Artemia protease-inhibitor interactions.
During early *Artemia* development, the measurable level of the non-dialysable protease inhibitor peaks around six to nine hours of development at 30°C, then drops continuously to extremely low levels by 36 hours of development (Warner, 1987). In 1992, Warner and Sonnenfeld-Karcz further characterized the non-dialysable cysteine protease inhibitors from *Artemia* cysts. Using a purification protocol involving ion exchange and gel filtration chromatography, and high performance liquid chromatography (HPLC), three inhibitors were resolved and named TPI-1, TPI-2, and TPI-3 (thiol protease inhibitor-1, -2, and -3). TPI-1 was found to be a heterogeneous (inseparable to date) mixture of two proteins of relative molecular weights of 11.8 kDa and 13.6 kDa, while TPI-2 and TPI-3 were homogenous proteins of 12.5 kDa each.

The TPI-1(b) peak collected from HPLC purification, TPI-2, and TPI-3 were subjected to tryptic digestion and these digest products were analyzed by HPLC (Warner & Sonnenfeld-Karcz, 1992). Upon comparison of the elution profiles, it was noted that among the different inhibitor isoforms, some peptide peaks were common to all three inhibitors, while some were not. Based on this information, Warner and Sonnenfeld-Karcz (1992) suggested that the different inhibitor isoforms may have evolved from a single protein. During early development, the TPI’s are believed to participate in the regulation of proteolytic activity in *Artemia* embryos along with factors such as ionic environment, intracellular pH, and compartmentalization (Warner, 1987) to prevent unwanted proteolysis.
E. Putative Roles of the *Artemia* Cyst Cysteine Protease

At present, the role of the *Artemia* major cyst cysteine protease has not been determined unequivocally. Upon their initial characterization of this cysteine protease, Nagainis and Warner (1979) suggested that it may be involved in yolk protein utilization. Then in 1985, Warner & Shridhar showed that the enzyme specifically catabolized lipovitelline α1, a major *Artemia* yolk platelet protein *in vitro*, in a pattern that was similar to the *in vivo* degradation of lipovitelline α1 which is observed as *Artemia* embryos develop to the nauplius stage (De Chaffoy de Courcelles & Kondo, 1980; Vallejo & Marco, 1985). Recent studies by Warner *et al.* (1995) have also supported a role for the enzyme in yolk protein utilization, as well as in the hatching of pre-nauplius larvae.

The idea that this enzyme participates in yolk protein degradation *in vivo* is supported by the work of Kageyama & Takahashi (1990), Takahashi *et al.* (1992), Yamamoto *et al.* (1994), and Zhao *et al.* (1996) which examined cysteine proteases in silkmoth eggs. To date, both serine and cysteine proteases have been isolated and purified from insect eggs. The serine proteases will not be discussed here, but have been reviewed by Izumi *et al.* (1994). Two cysteine proteases have been isolated from silkmoth eggs which show some similarity to the *Artemia* cyst cysteine protease. A cysteine protease from the eggs of the silkmoth, *Bombyx mori* was originally described by Kageyama & Takahashi (1990). This enzyme is synthesized as a 47 kDa precursor which is converted to a 39 kDa active form under acidic conditions (Takahashi *et al.*, 1992).
This enzyme was found to be synthesized in the follicle cells of the ovaries of *Bombyx mori* (Yamamoto *et al.*, 1994). When egg homogenates of *Bombyx mori* were incubated at acidic pH, the preferential degradation of the heavy chain of vitellin, a *Bombyx mori* yolk protein was observed (Kageyama & Takahashi, 1990). This pattern of degradation was also observed *in vitro* when purified silkmoth egg protease was incubated with purified yolk proteins such as egg specific protein (Yamamoto, 1994).

A cysteine protease has also been isolated from the eggs of another silkworm, *Antheraea pernyi* (Zhao *et al.*, 1996). This enzyme is not as well characterized as the cysteine protease in *Bombyx mori* eggs, however, it was implicated in yolk protein degradation in *Antheraea pernyi* based on the degradation of yolk proteins observed when egg extracts were incubated at 37°C (Zhao *et al.*, 1996). Also, this degradation was inhibited when cysteine protease inhibitors were added to the extracts during incubation (Zhao *et al.*, 1996).

F. **Research Objectives and Contributions to This Field of Research**

There were two main objectives of this research. The first objective was to further characterize the *Artemia* cyst cysteine protease at the protein level using a variety of methods. The multiple isoforms of the enzyme and the subunits of the major isoform were analyzed using fast protein liquid chromatography and high performance liquid chromatography, respectively. As well, the carbohydrate moiety associated with the enzyme, the preference of the enzyme for synthetic peptide substrates, and carboxyl-
terminal amino acid sequence of the small subunit of the major isoform of the enzyme were examined. Characterization of this enzyme at the protein level will contribute to the knowledge of a potentially novel class of cysteine proteases.

The second, and most important objective of this research was to clone a cDNA encoding the small subunit of the most abundant isoform of the major cysteine protease found in Artemia embryos. The cloning of this cDNA will begin the process of characterizing this enzyme at the molecular level. Sequencing of this cDNA will allow comparisons to be made between the nucleic acid sequence of this protease and others of the Papain Family. Also, this information should help to better understand the function of the small subunit of the enzyme. Amino terminal amino acid sequence of the small subunit obtained thus far (Lu, 1991) has indicated that it may share a high degree of homology with papain. This suggests that the active site of the enzyme may lie in this subunit, and this can be confirmed by sequencing this cDNA. As well, this cDNA may be isotopically labelled, and used to estimate the number of Artemia cysteine protease genes, (by probing a Southern blot of total Artemia genomic DNA), and it may be used to isolate the complete protease gene from an Artemia genomic DNA library.

Finally, future studies could also include measuring the level of expression (mRNA) of the protease at different developmental stages. This information would be of great value to those investigating the function and control of cysteine proteases in areas of research such as the development of anti-parasite drug therapies and protection of agricultural crops against insect pests, as well as those investigating various aspects of developmental biology.
II. MATERIALS & METHODS

A. Materials

The *Artemia* cysts used in all experiments were from the Sanders Brine Shrimp Company (Ogden, UT), lot # 12715. G-25 Sephadex, DEAE Sephadex A-50, Concanavalin A Sepharose, G-150 Sephadex, and the Mono Q column (0.5 x 5.0 cm) were purchased from Pharmacia (Uppsala, Sweden). Acetonitrile (high performance liquid chromatography grade) was purchased from EM Science (Gibbstown, NJ). Acrylamide and bis-acrylamide were purchased from Bio Rad Laboratories (Hercules, CA). Agarose, chloroform, and ethidium bromide (molecular biology grade) were purchased from International Biotechnologies, Inc. (New Haven, CT). Peptone, yeast extract, and agar were purchased from Difco Laboratories (Detroit, MI). Phenol (molecular biology grade) was purchased from Gibco BRL (Grand Island, NY). All membranes were dried under vacuum on a Gel Dryer (Model 583) from Bio-Rad Laboratories (Mississauga, ON). The shaking water bath used in hybridizations was purchased from Precision Science Company (Chicago, IL). [\(\beta^{32}\text{P}\)]dCTP was purchased from NEN Research Products (Boston, MA). Cheesecloth, glass wool, and Carnation dry milk were purchased locally. Unless otherwise specified, other chemicals were from Sigma Chemical Company (St. Louis, MO), and were of reagent grade or better.
B. Methods

1. Assays for Cysteine Protease Activity

a) Trinitrobenzene Sulfonic Acid Assay Method

The majority of cysteine protease assays were conducted using the trinitrobenzene sulfonic acid (TNBS) method developed by Nagainis and Warner (1979) with some modifications. Typically, assays were conducted at pH 4.0 and 40°C, or pH 5.0 and 30°C in a 200 µl reaction volume containing 1 mM ethylenediaminetetraacetic acid (EDTA), 2.5 mg/ml protamine sulfate, 0.1 M sodium acetate (pH 4.0 or 5.0), and an appropriate volume of the sample to be assayed. The reaction was started by the addition of substrate. At the desired incubation times at 40°C (or 30°C), 50 µl were removed and added to 950 µl of stop buffer #1 (53 mM NaOH, 53 mM Na₂B₄O₇). Once all reaction aliquots had been collected in this manner, they were mixed with 25 µl of 220 mM TNBS and incubated for 15 minutes at ambient temperature. Amino groups liberated through proteolysis will combine with TNBS creating a yellow coloured product which absorbs light at 420 nm (Nagainis, 1976). Colour development was stopped after 15 minutes with the addition of 2.0 ml of stop buffer #2 (0.1 M NaH₂PO₄, 1.5 mM Na₂SO₄). Samples were vortexed immediately for 10 seconds and after incubating at ambient temperature for 15 minutes, the absorbance of each sample was read at 420 nm in a Beckman DU-64 spectrophotometer (Mississauga, ON).

b) Standardization of the TNBS Assay

A standard curve for the TNBS assay was constructed in which the substrate
(protamine sulfate) was omitted and known quantities of alanine were used as a source of amino groups. This reaction was conducted in essentially the same manner as protease assays, except that no incubation period (for proteolysis) was necessary. Colour development was measured as in an actual assay. A standard curve derived from the absorbance at 420 nm was used to calculate the free amino groups released in protease catalyzed reactions. One milli-enzyme unit (mEU) corresponds to the release of one nanomole amino groups per minute (Warner et al., 1997).

c) Assay of Artemia Cysteine Protease Activity Using Synthetic Peptide Substrates

To further characterize the Artemia cyst cysteine protease, assays were conducted using synthetic peptide substrates coupled to β-naphthyamide. Assays were conducted based on the method of Barrett & Kirschke (1981) with some modifications. All reactions contained 40 mEU G-150 Sephadex purified Artemia cyst protease (as measured by the TNBS assay at 30°C, pH 5.0), and 0.2 mM substrate (dissolved in 10% dimethyl sulfoxide, giving a final concentration of approximately 0.3% in reaction vessels) in a final volume of 320 µl. Assays were conducted in the presence of 88 mM KH₂PO₄, 12 mM Na₂HPO₄, and 1.33 mM EDTA at pH 5.0 and 30°C. Immediately prior to assays, 2.5 mM cysteine was added to this incubation buffer. All reactions were initiated by the addition of substrate (Nα-Cbz-Arg-Arg 4-methoxy-β-naphthyamide, N-Cbz-Phe-Arg 4-methoxy-β-naphthyamide, or L-Leucine β-naphthyamide). At the desired incubation times, 50 µl were removed from the incubation mixture and added to 50 µl ice cold coupling reagent (5 mM mersalyl acid, 0.03N NaOH, 2% Brij 35, 0.81 mM EDTA, adjusted to a final pH of 4.0 with 1 M HCl). Once all reaction aliquots had been
collected and terminated in this manner, they were mixed with 50 µl of additional coupling reagent containing 0.5 mg/ml Fast Garnet. Colour development was allowed to proceed for 15 minutes at ambient temperature, then was terminated by extracting each sample with 1.0 ml of n-butanol (Fisher Scientific, Fairlawn, NJ). Samples were vortexed thoroughly and centrifuged for five minutes in a bench top clinical centrifuge (Precision Scientific Co.) at 2,500 rpm. The absorbance of the (upper) butanol layer of each sample was read at 520 nm in a Beckman DU-64 spectrophotometer.

d) **Standardization of the Cysteine Protease Assay Using Synthetic Peptide Substrates**

A standard curve for the above assay was conducted in which the substrate was omitted from the assay and known quantities of β-naphthylamine (LAP calibration reagent) were included. Incubation at 30°C was omitted, but otherwise colour development was as described for the protease assays. A standard curve derived from the absorbance at 520 nm was used to calculate the β-naphthylamine groups released in protease catalyzed reactions.

2. **Protein Assays**

Protein measurements were conducted using three different methods. The protein in fractions that eluted from G-25 Sephadex, DEAE Sephadex A-50, G-150 Sephadex and Conconavalin A Sepharose columns was determined by measuring the absorbance of column fractions at 280 nm in a Beckman DU-64 spectrophotometer. For all other protein determinations, the Bio-Rad protein micro-assay (based on the method of Bradford, 1976), or the Pierce bicinchoninic acid (BCA) Assay (Rockford, IL, based on the method of Smith *et al.*, 1985) was used. In the latter two methods, bovine serum
albumin (BSA) was used as a protein standard, and the assays were conducted according to the manufacturers' instructions.

3. **Protein Purification**

a) **Isolation of the Major Cysteine Protease from *Artemia franciscana* Cysts**

The major cysteine protease of *Artemia franciscana* cysts was isolated using a method modified from that of Warner & Shridhar (1985). It should be noted that all steps in the isolation of *Artemia* cyst cysteine protease were performed at 4°C, unless indicated otherwise. Forty grams (dry weight) of *Artemia* cysts were hydrated for a minimum of four hours in 0.25 M NaCl. Floating cysts were removed by aspiration and the remaining liquid above the cysts was decanted. Cysts were washed in cold (0-4°C) distilled water three times. During each wash the cysts were stirred, allowed to settle, and the floaters were aspirated. Next, cysts were collected on a sintered glass filter and washed with cold distilled water and then cold homogenization buffer (buffer A (50 mM Tris-HCl pH 7.3, 5 mM KCl, 1 mM dithiothreitol, 10 mM MgCl₂)). Cysts were ground in 25 g (wet weight) batches for 15 minutes using 7.0 ml of homogenization buffer in an automated mortar and pestle (Torsion, Montreal, PQ). After the initial homogenization, 115 ml of additional homogenization buffer was added to the thick paste and the suspension was stirred slowly for 15 minutes. The homogenate was centrifuged at 10,400g for 20 minutes in 250 ml polycarbonate centrifuge tubes. The supernatant from this centrifugation was filtered through a layer of glass wool "sandwiched" between two layers of cheesecloth. The filtered supernatant was centrifuged at 150,000g in a Beckman L5-65 ultracentrifuge for two and one half hours using a 60Ti rotor. The
resulting supernatant was filtered as above, and designated as the S-150 fraction.

The S-150 fraction was adjusted to 40% ammonium sulfate, stirred for 30 minutes, and proteins were allowed to precipitate overnight. The precipitate was then centrifuged at 12,100g for 30 minutes. The supernatant from this centrifugation was adjusted to 75% ammonium sulfate and protein was allowed to precipitate as described above. Again, the protein was collected by centrifugation as described above. The supernatant from this step was discarded and the ammonium sulfate insoluble protein was saved. This precipitate contained most of the cytoplasmic cysteine protease activity from the cysts (Warner & Shridhar, 1985). This procedure was repeated several times until sufficient crude enzyme was obtained to proceed with further purification steps.

The 40-75% ammonium sulfate precipitates were combined and suspended in a minimal volume of column buffer #1 (15 mM potassium phosphate, pH 6.8, 25 mM KCl, 10% glycerol). Any insoluble material was removed by centrifugation at 12,000g for 30 minutes, and re-suspended in column buffer #1. The centrifugation step was repeated and the supernatants were combined.

b) Fractionation of *Artemia* Cysteine Protease on G-25 Sephadex and DEAE Sephadex A-50

It should be noted that all chromatography of the protease was carried out at 4°C except where indicated otherwise. To desalt the solubilized 40-75% ammonium sulfate fraction, the sample was loaded onto a G-25 Sephadex (medium) column (3.7 x 40.0 cm) that had been equilibrated with column buffer #1. The orange band representing the soluble proteins in the preparation could be visualized as it passed through the column and was collected in one large fraction as it eluted from the column. This fraction was
loaded directly onto a DEAE Sephadex A-50 column (4.2 x 40.0 cm) which had been equilibrated with column buffer #1. The column was washed with column buffer #1 until the column effluent had an absorbance of less than 0.020 at 280 nm. Next, cysteine protease activity was eluted from the column with a 25 to 750 mM KCl gradient in one litre (total volume) of column buffer #1 which was pumped through the column at a rate of 0.5-1.0 ml/minute. Fractions of 10 ml or less were collected and assayed for protein content and cysteine protease activity as described above. Column fractions containing the majority of the cysteine protease activity were pooled.

c) Fractionation of Artemia Cysteine Protease on Concanavalin A Sepharose

The cysteine protease rich fraction from the DEAE Sephadex A-50 column was loaded onto a Concanavalin A Sepharose (Con A) column (1.7 x 10.0 cm) which had been equilibrated with column buffer #2 (15 mM potassium phosphate, pH 6.8, 200 mM KCl). The column was washed with this buffer until the column effluent had an absorbance of less than 0.020 at 280 nm. Protein was eluted with column buffer #3 (15 mM potassium phosphate, pH 6.8, 200 mM KCl, 1.0 M glucose). Fractions of approximately 3.0 ml were collected and assayed for protein content and cysteine protease activity. The fractions containing the majority of the cysteine protease activity were pooled and concentrated by pressure filtration on a YM-10 membrane (Amicon, Oakville, ON) to less than 5.0 ml using 30 psi nitrogen. The sample was then concentrated to less than 2.0 ml using a Centricon-10 concentration unit according to the manufacturer’s directions (Amicon, Oakville, ON).
d) Fractionation of *Artemia* Cysteine Protease on G-150 Sephadex

A sample of the concentrated Con A Sepharose bound protein fraction was loaded onto a G-150 Sephadex (super fine) column (1.5 x 42.0 cm) which had been equilibrated with column buffer #1. Fractions of approximately 1.0 ml were collected and assayed for protein content and cysteine protease activity. Fractions containing the majority of the cysteine protease activity were pooled and concentrated by pressure filtration to approximately 1.0 ml using a YM-10 membrane and a Centricon-10 unit.

e) Fractionation of *Artemia* Cysteine Protease Isoforms Using Fast Protein Liquid Chromatography

A Mono Q column (0.5 x 5.0 cm) was used to separate the major isoforms of the protease by fast protein liquid chromatography (FPLC) at room temperature. The FPLC column was driven by a Beckman 421 Controller system. The column was equilibrated with column buffer #1, and approximately two milligrams of protein were loaded per FPLC run. Protein was eluted from the column with a 25 to 275 mM KCl gradient in column buffer #1 at a flow rate of 1.0 ml/minute over the course of 20 minutes. Fractions of 250-333 µl were collected from the column. Protein eluting from the column was monitored at 280 nm and recorded on a Gilson Holochrome recorder (Middleton, WI). Protease activity in each fraction was measured using the TNBS assay. The fractions containing the various isoforms were identified and pooled separately. The pooled samples were concentrated to a final volume of approximately 250 µl using Centricon-10 units and were re-equilibrated with column buffer #1. The partially resolved isoforms were then re-run on the Mono Q column to enhance purification of each protease isoform.
Separation of the Large and Small Subunits of the *Artemia* Cysteine Protease By High Performance Liquid Chromatography

High performance liquid chromatography (HPLC) was conducted at room temperature on a Chemcosorb C-18 reverse phase column (4.6 x 250 mm) purchased from Phenomenex (Torrence, CA). The HPLC column was driven by a Gilson Model 704 HPLC System Manager. Initially, the column was equilibrated with starting buffer (12% acetonitrile containing 0.1% trifluoroacetic acid) and up to 100 μg of protease were loaded on the column for each chromatography run. Proteins were eluted from the column using a 12-60% acetonitrile gradient containing 0.1% trifluoroacetic acid (TFA) at a flow rate of 1.0 ml/minute and protein elution was monitored at 214 nm and recorded on a Gilson Model 620 Data Master recording system. This procedure served to separate the two sub-units of the protease (A. Warner, unpublished observations). Column fractions of 250-500 μl were collected and those of interest were lyophilized in sterile Eppendorf tubes (1.5 ml) as follows. The open Eppendorf tube containing the liquid sample was covered with two layers of parafilm which was pierced twice with a 26 gauge needle. The tube was frozen at -70°F, then transferred to a lyophilizer (Refrigeration for Science, Inc., Chicago, IL) and freeze-dried overnight. Dried samples were reconstituted in sterile distilled water and assayed for protein content.

4. Detection of Carbohydrate Associated with the *Artemia* Cysteine Protease

a) Electrophoresis on Sodium Dodecyl Sulfate Polyacrylamide Gel

Electrophoresis of proteins was conducted using a 7-18% gradient polyacrylamide gel in a Bio-Rad Mini-Protean electrophoresis unit, but otherwise according to the procedure of Laemmli (1970). Prior to gel loading, the samples and standards were
denatured by heating at 95°C for five minutes in the presence of 1X loading buffer. Gels were run at 125 volts and 40 mA for 1-1.5 hours. The molecular weight standards used were purchased pre-stained from Bio-Rad Laboratories. Following electrophoresis, the protein bands on the gel were visualized by staining with 0.1% Coomassie blue R-250 (Bio-Rad) in 40% methanol and 10% acetic acid for 60 minutes, with gentle shaking. Gels were destained for several hours in mild destain solution (5.0% methanol, 7.5% acetic acid) with gentle shaking.

b) Staining for Carbohydrate Associated with *Artemia* Proteins

Cysteine protease subunits separated by electrophoresis (see above) were tested for the presence of carbohydrate groups using a procedure derived from a modification of previously published protocols (Köiw & Grönwall, 1952; Keyser, 1964; Clarke, 1964; Zacharias et al., 1969). Following electrophoresis, the gel was placed in approximately 50 ml of 20% trichloroacetic acid (TCA) for 30 minutes. The TCA was decanted and the gel was washed with several changes of distilled water over a period of 20 minutes. Next, the gel was placed in 50 ml of fresh 1% periodic acid for 30 minutes. The gel was then transferred to a dish with approximately 50 ml of a solution containing one part 1% periodic acid, and five parts Schiff base (Fisher Scientific, Nepean, ON). The gel was left in this solution for 10 minutes, then the periodic acid and Schiff base was decanted and replaced with fresh Schiff base (alone) until the glycoproteins could be clearly visualized as pink bands on the gel. All treatments of the gel during this procedure were performed at room temperature with gentle shaking.
c) Deglycosylation of the Artemia Cysteine Protease

Deglycosylation of proteins was conducted according to Haselbeck & Hösel (1988) with some modifications. In all experiments, 5.0 μg of protein were used. The proteins were denatured by boiling for two minutes in the presence of 1.0% SDS. The protein samples were adjusted to contain 50 mM NaOAc, pH 5.5, 25 mM EDTA, pH 8.0, 1% β-mercaptoethanol, and 1% Nonidet P-40 in a final volume of 23.0 μl. Samples were boiled again for two minutes, cooled to 37°C and 0.1 unit endoglycosidase F (endo F, E.C. # 3.2.1.96, Calbiochem, San Diego, CA) was added. The reaction was incubated overnight at 37°C, and the products of the reaction were analyzed by electrophoresis on a 7-18% (gradient) polyacrylamide gel. Gels were stained for proteins with Coomassie blue as described above.

5. Isolation of a Carboxyl-Terminal Peptide Fragment of the Small Subunit of the Artemia Cysteine Protease Isoform #3

A combination of enzymatic digests and reverse phase HPLC was used to identify a carboxyl-terminal peptide fragment of the small subunit of Artemia cyst cysteine protease isoform #3 (CP-3). This procedure was developed by Ann Marie Butler in our laboratory. Initially, the small subunit of the protein was incubated with carboxypeptidase P (E.C. # 3.4.16.1, Boehringer-Mannheim, Laval, PQ) in a 1:50 enzyme to substrate (w/w) ratio in 50 mM sodium citrate buffer, pH 5.0, for six hours at 35°C (as per the manufacturer’s instructions). The reaction was terminated by heating the reaction mix to 75°C for 30 minutes. Next, the carboxypeptidase P treated sample was incubated with trypsin, in an enzyme to substrate ratio of 1:50 (w/w), for six hours at 37°C in 25 mM Tris-Cl, pH 8.7 (Fullmer & Wasserman, 1979). The digested samples were then
adjusted to contain 12% acetonitrile and 0.1% TFA, and the peptide fragments were separated on the C-18 (reverse phase) HPLC column (4.6 x 250 mm) using a 12-60% acetonitrile gradient in 0.1% TFA over 45 minutes, at a flow rate of 1.0 ml/minute. The elution profiles were analyzed for differences among peptides in order to identify the peptide of interest (carboxyl-terminal peptide fragment).

6. Identification of the Amino Acid Sequence of the Carboxyl-Terminal Peptide Fragment of the Small Subunit of Artemia CP-3

The amino acid sequence of the carboxyl-terminal peptide was analyzed by technicians at the Macromolecular Core Facility, Department of Biochemistry and Molecular Biology, Wayne State University School of Medicine, (Detroit, MI) under the direction of Michal Ram.

7. Oligonucleotide Primer Construction

Two (mixed) degenerate oligonucleotide primers were constructed at the Macromolecular Core Facility, Wayne State University, under the direction of Michal Ram. An amino-terminal primer was constructed based on amino acid sequence obtained previously (Lu, 1991). The carboxyl-terminal primer was constructed based on the amino acid sequence obtained from the carboxyl-terminal peptide isolated as described above. The two primers constructed were named CP3-n (amino-terminal primer), and CP3-c (carboxyl-terminal primer). Their sequences are: 5’ GGT ACC GAA AAA/G GGI GCT/C ATT/C AC 3’ and 5’ GGA TCC ICC T/CTT T/AGG A/GTA A/GAA/T T/CTG 3’, respectively.
8. *Artemia* Embryo cDNA Library in *lambda* ZAP II

An encysted embryo cDNA library was constructed using the *lambda* ZAP II phage vector and *Artemia* cyst cDNA in the laboratory of Leandro Sastre, Instituto de Investigaciones Biomedicas, Madrid, Spain. A sample of this library was generously donated to Dr. Tom MacRae, Dalhousie University, who in turn provided a sample (with permission) for our use.

a) Growth and Culture Conditions for the *lambda* ZAPII cDNA Library Clones

*Lambda* ZAPII phage containing the *Artemia* cyst cDNA library were propagated according to the methods of Sambrook *et al.* (1989). The phage were grown using *E. coli* strain JM109 as a plating bacterium. *E. coli* cells (100 μl) were infected with phage (1.4 x 10⁵ plaque forming units) for 20 minutes at room temperature, then mixed with 2.5 ml of LB broth (10 g/l peptone, 10 g/l NaCl, 5 g/l yeast extract) containing 7.0 g/l agarose which had been equilibrated to 47-50°C. The mixture was spread on 15 x 100 mm LB agar (15.0 g/l) plates which had been equilibrated to room temperature. The plates were incubated (inverted) overnight at 37°C.

b) Harvest of *lambda* ZAPII Phage and Isolation of Phage DNA

*Lambda* ZAPII phage and its DNA were prepared according to Sambrook *et al.* (1989) with some modifications (Chatterjee, 1991). Five millilitres of storage medium or SM (10 mM NaCl, 10 mM MgSO₄·7H₂O, 50 mM Tris-Cl, pH 7.5, 0.01% gelatin) were added to each of 10 confluently lysed plates (15 x 100 mm) of *lambda* ZAPII phage. The plates were agitated gently at 4°C for four hours. The SM containing the phage was collected and centrifuged at 8,000g (4°C) for 10 minutes. The supernatant was
decanted and treated with 1 μg/ml each of RNase A and DNase I for 30 minutes at 37°C. Next, solid NaCl was added to a final concentration of 1M. The sample was incubated on ice for 60 minutes then centrifuged at 11,000g (4°C) for 10 minutes. Solid polyethylene glycol (PEG) 8000 was stirred slowly into the supernatant at room temperature to give a final concentration of 10% (w/v). The mixture was again incubated on ice for 60 minutes, followed by a 20 minute centrifugation at 10,000g (4°C). The supernatant was discarded and the pellet was drained of excess fluid. The pellet was re-suspended in 0.5 ml TE buffer (10 mM Tris-Cl, pH 8.0, 1 mM EDTA, pH 8.0) to which 5.0 μl each of 10% SDS and 0.5 M EDTA, pH 8.0 had been added. The sample was incubated at 68°C for five minutes. DNA was extracted with phenol, phenol-chloroform-isoamyl alcohol (25:24:1 ratio) and then chloroform alone according to Sambrook et al. (1989). The aqueous phase from the final extraction was adjusted to contain 0.3 M sodium acetate, pH 5.2 and the DNA was precipitated with 2.5 volumes of ice cold 95% ethanol at -20°C overnight (Sambrook et al., 1989). DNA was recovered from the ethanol by centrifuging for 45 minutes in a Beckman microcentrifuge (4°C). The DNA pellet was washed twice with 0.5 ml ice cold 70% ethanol centrifuging for 15 minutes after each wash. The 70% alcohol was removed carefully with a pipette, and the DNA was dried at room temperature, or with the aid of a DNA 10 Speed Vac (Savant, Farmingdale, NY). The dried DNA was reconstituted in TE buffer (pH 8.0), and the concentration and purity of the DNA were determined spectrophotometrically (Sambrook et al., 1989).
9. **Analysis of Plasmid and Bacteriophage DNA by Electrophoresis**

Electrophoresis of DNA on agarose gels was conducted according to Sambrook *et al.* (1989) using 0.5X TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH 8.0) at 30 volts and 10 mA for three to four hours in a "Minnie" Horizontal Submarine Unit (Hoefer Scientific Instruments, San Francisco, CA). Prior to gel loading, the DNA samples were mixed with 6X loading buffer (0.25% bromophenol blue, 40% sucrose, w/v). Following electrophoresis, DNA in the gels was stained with ethidium bromide (0.5 μg/ml) for 30 minutes with gentle shaking. A 1kb ladder of DNA size standards (Gibco BRL, Burlington, ON) was used for reference markers.

10. **Purification of DNA From Agarose Gels Using the Glass Wool Method**

When it was necessary to recover DNA from agarose gels following electrophoresis, the DNA band of interest was excised using a fresh scalpel blade. The DNA was eluted from the agarose gel slices as follows (A. Chatterjee, personal communications). A hole was made at the bottom of a sterile 0.5 ml Eppendorf tube using a 22.5 gauge needle. A small amount of siliconized glass wool was packed in the bottom of the tube with a micro pipette tip. A slice of agarose (alone) containing DNA was placed in the tube and this was placed inside a sterile 1.5 ml Eppendorf tube. The cap was cut off the larger tube and the tubes were centrifuged in this arrangement at 5,000g (4°C) for 10 minutes. The liquid that collected at the bottom of the larger Eppendorf tube was transferred to a fresh 1.5 ml Eppendorf tube, and the centrifugation was repeated to collect additional liquid which was pooled with the first aliquot.
11. **RNase A Treatment of Plasmid and Bacteriophage DNA Preparations**

When deemed necessary, the plasmid and bacteriophage DNA samples were treated with (DNase free) RNase A (20 µg/ml) for 30 minutes at 37°C. Samples were then extracted with phenol, phenol-chloroform-isoamyl alcohol, then chloroform alone. DNA was collected by precipitation with ethanol and recovered by centrifugation as described previously.

12. **Amplification of a cDNA Sequence Encoding a Putative Artemia Cyst Cysteine Protease from the lambda ZAP II cDNA Library Using the Polymerase Chain Reaction Methodology**

Polymerase chain reactions (PCR) were conducted in a Thermolyne thermocycler (Barnstead, Dubuque, IA) according to the established procedures of Saiki et al. (1985), Mullis et al. (1986), and Mullis & Faloona (1987). In all experiments, the reaction volume was 50.0 µl and all reactions were overlaid with 25 µl molecular grade mineral oil. A series of polymerase chain reactions were conducted to generate a PCR product of approximately 435 bp which reacted with a 32P-labelled lobster cysteine protease cDNA probe. This 435 bp cDNA was then cloned as described below. Unless otherwise specified, all PCR reagents were from Bio-Can Scientific (Mississauga, ON).

a) **Polymerase Chain Reactions Using lambda ZAP II Library DNA and the CP3-n and T7 Primers**

Initially 10-100 ng of the lambda ZAPII library DNA was used as template for PCR in the presence of 1X PCR buffer (50 mM KCl, 10 mM Tris-Cl, pH 9.0, 0.1% Triton X-100), 1.5 mM MgCl₂, 0.2 mM deoxynucleoside triphosphates (dNTP’s) and 10 picomoles each of the CP3-n primer (described previously) and T7 primer (Promega, Madison, WI). Initially, all reaction vessels (minus the Taq polymerase) were subjected to a three minute
denaturation step or hot start at 94°C (Saiki, 1990). The reactions were started by adding one unit of Taq polymerase, and 35 cycles of the following program were run: denaturation at 94°C for 45 seconds, annealing at 47°C for 45 seconds, and extension at 72°C for 90 seconds. Following the final cycle, the products were incubated at 72°C for 10 minutes to complete extension of any partial PCR products generated in the reaction.

b) Re-amplification of PCR Products Generated from the lambda ZAPII Library Using the CP3-n and CP3-c Primers

One microlitre of $10^{-2}$, $10^{-3}$, and $10^{-4}$ dilutions of the above PCR products was amplified with 100 picomoles each of CP3-n and CP3-c primers (described previously) in the presence of 1X PCR buffer, 2.5 mM MgCl$_2$ and 0.2 mM dNTP’s. A hot start was used as before, then one unit of Taq polymerase was added to each reaction vessel. Initially, five cycles of the following program were run: denaturation at 94°C for 45 seconds, annealing at 43°C for 30 seconds, and extension at 72°C for 60 seconds. This was followed by 20 cycles of the same program except that the annealing temperature was increased to 58°C. Following the final cycle, the reactions were incubated at 72°C for 10 minutes.

The PCR products from this reaction were visualized by electrophoresis on a 1.2% agarose gel, stained with ethidium bromide (0.5 μg/μl), Southern blotted, and probed with a $^{32}$P-labelled lobster cysteine protease cDNA probe (see below). One PCR product which reacted with this probe was selected for cloning in a bacterial system. DNA was purified from the agarose gel and re-amplified using PCR as follows. The DNA band of interest was excised with a new scalpel from a lane of the gel which had not been
subjected to ethidium bromide staining. The gel slice was subjected to three cycles of freezing (-70°F) and thawing. This served to generate enough liquid (containing DNA) from the gel slice to act as template DNA for PCR (A. Butler, personal communications). The last PCR (described above) was repeated on dilutions \(10^2, 10^3, \text{ and } 10^4\) of this template. The only difference between this reaction and the one described above, was that the final incubation of PCR products at 72°C was extended from 10 minutes to 60 minutes to improve cloning efficiency (Li & Guy, 1996). This entire process yielded a single PCR product which could be ligated directly into a vector and cloned (see below).

13. Immobilization of DNA on Various Membranes

a) Blotting of Nucleic Acids to Zeta Probe Membranes

All supplies for slot blots were from Bio-Rad and blotting was performed according to the manufacturer’s instructions. The Bio-Dot SF Slot Blot apparatus was used with Bio-Dot SF filter paper and Zeta Probe membrane. Five microlitres of various PCR products to be probed were loaded per slot. The nucleic acids were denatured and fixed on the membrane according to the manufacturer’s instructions. Typically, membranes containing DNA were air dried for 30 minutes, then baked under vacuum at 80°C for 30 minutes.

b) Southern Blotting of Nucleic Acids

All transfers of PCR products and plasmid DNA from agarose gels to membranes were performed based on the method of Southern (1975), with various modifications. In all cases, DNA was electrophoresed on an agarose gel \(1.0-1.4\%), stained with ethidium bromide \(0.5 \mu\text{g/ml}\), and transferred to a membrane for 20 hours. Typically, 10 \(\mu\text{l}\) of
PCR products were applied per lane of the agarose gel. When Zeta Probe membranes were used, the DNA was transferred using the alkali transfer method specified in the manufacturer’s instructions. When Biotrace HP membranes (Gelman Sciences, Montréal, PQ) were used, the alkali transfer protocol of Sambrook et al. (1989) or the capillary transfer (to nitrocellulose membrane) protocol of Southern (1975) as modified by Sambrook et al. (1989) was used. In the latter method, DNA was transferred to the membrane using 15x SSC (2.25 M NaCl, 2.25 M citric acid) as a transfer solution. Following transfer of the DNA, all membranes were rinsed in 2x SSC (0.3 M NaCl, 0.03 M citric acid), air dried for a minimum of 30 minutes, and baked under vacuum at 80°C for 30 minutes (Zeta Probe membrane) or 60 minutes (Biotrace HP membrane).

c) Colony Lifts

After selecting for transformed cells on LB agar plates (15 x 100 mm) containing 50 μg/ml ampicillin or kanamycin, bacterial colonies were lifted using Biotrace NT precut membrane discs (Gelman Sciences) according to the manufacturer’s protocol. After lifting, the membranes were treated with solutions to lyse the bacterial cells, denature the bacterial DNA, then neutralize the DNA, according to the manufacturer’s protocol. Membranes were allowed to air dry for 30 minutes, then dried under vacuum at 80°C for 60 minutes.

14. Production of an [α²P]dCTP Labelled Lobster Cysteine Protease cDNA Probe

a) Transformation of E. coli with a cDNA Encoding a Lobster Cysteine Protease

In order to construct a probe from a lobster cysteine protease cDNA, E. coli strain JM109 cells were transformed with a plasmid containing a lobster cysteine protease
cDNA (LCP2 plasmid) which was a gift from Dr. Ron MacKay of the National Research Council of Canada. It consists of the pUC18 plasmid vector with an 1173 base pair cDNA ligated into the EcoR1 site of the polycloning site of the vector. The cDNA encodes a lobster cysteine protease also known as LCP2 (Laycock et al., 1991). A single colony from a fresh overnight culture of *E. coli* JM109 was used to inoculate 100 ml of LB broth in a 250 ml flask for the production of competent cells. The calcium chloride method of Cohen et al. (1972) with modifications by Sambrook et al. (1989) was used to make the cells competent. The competent cells (200 μl) were transformed with 50 ng of plasmid DNA and were selected on 15 x 100 mm SOB agar plates (20 g/l peptone, 1 mM NaCl, 5 g/l yeast extract, 2.5 mM KCl, 10 mM MgCl₂, 15 g/l agar) that contained 50 μg/ml ampicillin.

**b) Isolation of LCP2 Plasmid DNA**

LCP2 plasmid DNA was isolated using the large scale alkali lysis method (R. Treisman, unpublished observations; Birnboim & Doly, 1979; Ish-Horowicz & Burke, 1981) with modifications by Sambrook et al. (1989). The plasmid preparation was treated with RNase A, extracted with phenol, phenol-chloroform-isoamyl alcohol, then chloroform alone, precipitated with ethanol, and recovered by centrifugation as described previously. In order to excise the LCP2 cDNA insert, the plasmid DNA was digested with EcoR1 (1 U/μg DNA) at 37°C overnight in 1X React 3 buffer (10 mM MgCl₂, 100 mM NaCl, 50 mM Tris-Cl, pH 8.0). EcoR1 and React 3 buffer were from Gibco BRL. The digested DNA was electrophoresed on a 1.2% agarose gel, then stained with ethidium bromide as described previously. The 1173 bp cDNA insert was excised
from several lanes of the gel with a new scalpel, and the DNA was eluted from the agarose gel slices using the glass wool purification technique described previously. The pooled supernatants from this procedure were precipitated with ethanol and recovered by centrifugation as described previously, with the exception that 10 μg of yeast tRNA (donated by Aurobindo Chatterjee of this laboratory) was added to aid in precipitation of the nucleic acids. The nucleic acid precipitate was dissolved in 10 μl of TE buffer (pH 8.0) and electrophoresed (in one lane) on a 1.2% agarose gel. The DNA was excised from the gel with a clean scalpel and collected using the glass wool isolation procedure.

c) Random Labelling of LCP2 DNA with \([α^{32}P]dCTP\)

Approximately 100 ng of denatured LCP2 cDNA were used to make a randomly labelled probe with the Pharmacia Oligolabelling Kit (Baie d’Urfé, PQ) using \([α^{32}P]dCTP\) according to the manufacturer’s instructions. The \(^{32}\)P-labelled cDNA was purified on a G-50 Sephadex (super fine) column (8 x 70 mm) using an elution buffer composed of 50 mM NaCl, 1 mM EDTA, pH 8.0, and 10 mM Tris-Cl, pH 8.0 (Sambrook et al., 1989).

15. Hybridizations of the \(^{32}\)P-Labelled LCP2 DNA Probe with PCR Products and Plasmid DNA

In all hybridization reactions, 2.0 x 10^6 dpm of heat denatured \(^{32}\)P-labelled LCP2 DNA was used as probe. The probe was denatured by heating at 95°C for five minutes, then transferring to an ice bath for at least two minutes. All membranes containing DNA to be probed with the \(^{32}\)P-labelled LCP2 cDNA were pre-hybridized in 10 ml of the appropriate hybridization buffer (described below) in sealed plastic bags in a shaking water bath for at least 30 minutes at 52°C. Following this step, the buffer was replaced with 10 ml of fresh hybridization buffer containing 1-2 x 10^6 dpm of the denatured
probe. The composition of hybridization and membrane wash buffers was according to
the particular membrane manufacturer’s instructions, and any modifications are detailed
below. After the final wash, all membranes were wrapped in Saran Wrap and exposed
to X-OMAT AR X-ray film (Kodak, Rochester, NY) in a HI-PLUS Cronex film cassette with
an intensifier screen (DuPont, Mississauga, ON). Typically, X-rays were exposed to
membranes for 12-48 hours, depending on the specific activity of the $^{32}$P-labelled probe.
Film developing chemicals were purchased from Christie Group, Ltd. (St. Eustache, PQ),
and were used according to the manufacturer’s directions.

a) Hybridizations Performed With Zeta Probe Membranes

Zeta Probe hybridization buffer was composed of 1 mM EDTA, pH 8.0, 0.5 M
$\text{NaPO}_4$, pH 7.2, and 7% SDS. Following hybridizations, membranes were washed once
with Zeta Probe wash buffer I (1 mM EDTA, pH 8.0, 40 mM $\text{NaPO}_4$, pH 7.2, 5% SDS),
for 15 minutes at room temperature, and then twice for 15 minutes at 52°C. On
occasion, this was followed by one 15 minute wash at room temperature and two 15
minute washes at 52°C in Zeta Probe wash buffer II (1 mM EDTA, pH 8.0, 40 mM
$\text{NaPO}_4$, pH 7.2, 1% SDS).

b) Hybridizations Performed with Biotrace HP Membranes

Biotrace HP hybridization buffer was composed of 1 mM EDTA, pH 8.0, 0.5 M
$\text{NaPO}_4$, pH 7.2, 7% SDS, and 0.5% Carnation dry milk powder. Following
hybridization, membranes were washed with (modified) Biotrace wash buffer I (1 mM
EDTA, pH 8.0, 250 mM $\text{NaPO}_4$, pH 7.2, 5% SDS, 0.5% Carnation dry milk) for 10
minutes at room temperature, and then for 10 minutes at 52°C. These washes were
followed by one 10 minute wash at room temperature and two 10 minute washes at 52°C using a modified Biotrace wash buffer I containing 100 mM NaPO₄, pH 7.2. Finally, membranes were washed for 10 minutes at room temperature with (modified) Biotrace wash buffer I containing 40 mM NaPO₄, pH 7.2.

c) Hybridizations Performed With Biotrace NT Membranes

Biotrace NT membranes from colony lifts were hybridized with the Biotrace hybridization buffer described above. Membranes were washed with (standard) Biotrace wash buffer I (containing 40 mM NaPO₄, pH 7.2) for 10 minutes at room temperature, and then twice for 15 minutes each at 52°C.

16. Cloning of PCR Products

The TA Cloning Kit (Invitrogen San Diego, CA) was used to ligate the PCR product of interest into the pCR 2.1 vector (supplied with the TA Cloning Kit) according to the manufacturer’s instructions. The products of the ligation were subsequently used to transform *E. coli* strain INVF’ cells (supplied with the TA Cloning Kit) according to the manufacturer’s instructions. The transformed cells were selected on LB agar plates (15 x 100 mm) containing 50 µg/ml kanamycin and 25 µl of 40 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal, Gibco BRL).

17. Screening of Transformed Bacterial Cells

Transformed *E. coli* INVF’ cells were spread on LB agar plates (15 x 100 mm) containing kanamycin and X-gal (see above) and incubated (inverted) overnight at 37°C. Colonies were lifted using pre-cut Biotrace NT membrane discs, and membranes were processed, pre-hybridized, hybridized, washed, and exposed to X-ray film (and
developed) as described above.

18. **Isolation of Plasmid DNA From Putative Positive *Artemia* Cysteine Protease Clones**

Putative positive colonies were picked and grown for 20 hours in 4 ml of LB or SOC (20 g/l peptone, 1 mM NaCl, 5 g/l yeast extract, 2.5 mM KCl, 10 mM MgCl₂, 20 mM glucose) broth at 37°C with moderate shaking (250 rpm). Plasmid was isolated from 3 ml of culture using the Wizard Miniprep Kit (Promega) according to the manufacturer’s directions. In order to excise the cDNA ligated into the pCR 2.1 vector, plasmid preparations were digested overnight with Eco R1 (1 U/μg DNA) in 1X React 3 buffer, and electrophoresed on a 1.4% agarose gel as previously described. Following staining, the DNA was blotted to a Biotrace HP membrane, and the membrane was pre-hybridized then hybridized as usual, with the ³²P-labelled LCP2 probe. After washing, the size of the *Artemia* cDNA sequence(s) cloned into the bacterial cells was identified by exposing the membrane to X-ray film (and developing) as previously described.
III. RESULTS

A. Protein Purification

1. Fractionation of Artemia Cysteine Protease Isoforms Using Fast Protein Liquid Chromatography

The major cysteine protease (CP) of Artemia cysts was purified as described in the Materials and Methods section. The isolation and purification process was repeated several times to generate sufficient quantities of protease for additional analysis. Several hundred grams of dry cysts yielded approximately 800 µg of pure protease. Figure 1 shows the initial fractionation of the major isoforms of the cyst cysteine protease on the Mono Q column (0.5 x 5.0 cm). The first peak of enzymatic activity to elute from the column contains isoforms #1 and #2 (CP-1 and CP-2), the middle peak contains isoform #3 (CP-3), and isoforms #4 and #5 (CP-4 and CP-5) can be seen as smaller, overlapping peaks. It should be noted that re-fractionation of pooled samples on the Mono Q column enhanced the separation of the isoforms (data not shown), especially for CP-1 and CP-2. Peaks of protease activity (representing the different cysteine protease isoforms) were pooled and re-fractionated on the Mono Q column; this yielded five distinct isoforms. Due to its abundance in relation to the other isoforms, and the fact that studies have been conducted on this isoform previously (Lu, 1991), CP-3 was selected for further analysis.
Figure 1. Fractionation of *Artemia* cysteine protease isoforms using FPLC.

Partially purified *Artemia* cysteine protease (1.67 mg) from a G-150 Sephadex column was fractionated on a Mono Q column (0.5 x 5.0 cm) equilibrated with column buffer #1. The protease was eluted with a 25-275 mM KCl gradient in column buffer #1 at a flow rate of 1 ml/minute over 20 minutes. Protein eluting from the column was measured by monitoring absorbance at 280 nm (—). The protease activity ($A_{420}$) in each column fraction was determined using the TNBS assay. The numbers above the protease activity peaks eluting from the column indicate the various isoforms.
2. **Separation of the Large and Small Subunits of *Artemia* CP-3 By High Performance Liquid Chromatography**

In previous studies, all of the *Artemia* cysteine protease isoforms fractionated on the Mono Q column were found to contain two subunits (28.5 kDa and 31.5 kDa) as shown by SDS-PAGE (Warner & Shridhar, 1980) or HPLC (A. Warner, unpublished observations). It should be noted that previous experiments have shown that the two subunits of the protease are not separated by gel filtration, and polyacrylamide gel electrophoresis under non-denaturing conditions (A. Warner, unpublished observations). Since the experiments described in this thesis focussed mainly on the small (28.5 kDa) subunit of CP-3, it was necessary to obtain this protease subunit using HPLC. As shown in Figure 2, the large and small subunits of CP-3 were separated on a Chemcosorb C-18 reverse phase HPLC column (4.6 x 250 mm). The first peak to elute from the column (at approximately 19 minutes) represents the small subunit (SSU) of the protease, whereas the second peak (at approximately 26.5 minutes) represents the large subunit (LSU) (A. Warner, unpublished observations). It should be noted that all CP isoforms show the same pattern of subunit separation on the C-18 reverse phase HPLC column (data not shown).

B. **Analysis of Carbohydrate Group(s) Associated with the *Artemia* Cysteine Protease**

In an effort to further characterize the *Artemia* CP, all isoforms of the CP were tested for the presence of carbohydrate groups associated with the protease subunits. Ten micrograms each of FPLC (Mono Q column) purified CP-1 and 2 (together), CP-3,
Figure 2. Separation of the large and small subunits of the *Artemia* cysteine protease using reverse phase HPLC.

The subunits of FPLC purified *Artemia* CP-3 (75 μg) were separated on a Chemcosorb C-18 reverse phase HPLC column (4.6 x 250 mm) equilibrated with 12% acetonitrile containing 0.1% TFA. The polypeptide subunits were eluted with a 12-60% gradient of acetonitrile in 0.1% TFA at a flow rate of 1 ml/minute over 30 minutes. Absorbance was monitored at 214 nm, (—). SSU and LSU represent the small and large subunits of the protease, respectively.
CP-4, and CP-5 were electrophoresed on a 7-18% (gradient) polyacrylamide gel (in duplicate). Coomassie blue staining of the gel (Figure 3, panel A) shows the large and small subunits of the cyst cysteine protease isoforms with relative molecular weights of 31.5 kDa and 28.5 kDa, respectively. Schiff base staining of the duplicate gel (Figure 3, panel B) detected carbohydrate group(s) associated with both the large and small subunits of all isoforms of the CP tested.

To further characterize the carbohydrate group(s) associated with the protease, CP-3 was subjected to treatment with endo F (see Materials and Methods). As well, samples of CP-3 were treated with endo F under different incubation conditions, since Plummer et al. (1984) have shown that many commercial preparations of endo F are contaminated with peptide:N-glycosidase F (PNGase F) activity. The PNGase F activity (if present) is another carbohydrate cleaving enzyme. Therefore, samples of CP-3 were incubated with endo F under conditions that varied with respect to buffer composition and pH. In experiments designed to demonstrate digestion by endo F, 50 mM sodium acetate, pH 5.5 was used in the incubation buffer, and 50 mM sodium phosphate, pH 7.2 was used in experiments designed to demonstrate PNGase F digestion.

The products of all digestions were electrophoresed on a 7-18% (gradient) polyacrylamide gel and stained with Coomassie blue. Treatment of CP-3 with endo F resulted in a 1.0 kDa shift in the relative molecular weight of the large subunit of the protease (see Figure 4), while the small subunit was insensitive to this treatment. There was no apparent difference between samples digested under conditions appropriate for endo F activity versus conditions appropriate for PNGase F activity. Bovine serum
Figure 3. Detection of carbohydrate group(s) associated with *Artemia* cysteine protease isoforms.

A. Samples of FPLC purified CP-1&2, 3, 4, and 5 along with a sample of crude *Artemia* cytoplasmic proteins were electrophoresed on a 7-18% gradient polyacrylamide gel and stained with Coomassie blue as described in Materials and Methods.

B. Duplicate gel of that described in (A) stained with Schiff base reagent (see Materials and Methods) to detect carbohydrate groups associated with proteins.

Lane 1: Pre-stained standard molecular weight markers: phosphorylase B (107 kDa), bovine serum albumin (76 kDa), ovalbumin (52 kDa), carbonic anhydrase (36.8 kDa), soybean trypsin inhibitor (27.2 kDa), and lysozyme (19 kDa).

Lane 2: Crude *Artemia* cytoplasmic proteins (25-30 μg).

Lane 3: *Artemia* CP-1&2 (10 μg).

Lane 4: *Artemia* CP-3 (10 μg).

Lane 5: *Artemia* CP-4 (10 μg).

Lane 6: *Artemia* CP-5 (10 μg).
Figure 4. Deglycosylation of *Artemia* CP-3 with endo F.

To examine the type of carbohydrate group(s) associated with *Artemia* CP-3, a sample of FPLC purified CP-3 was treated with endo F overnight (see Materials and Methods). As well, bovine serum albumin and alkaline phosphatase were treated in the same manner as negative and positive controls, respectively. The digestion products of all the reactions were analyzed on a 7-18% (gradient) polyacrylamide gel, then stained with Coomassie blue as described in Materials and Methods.

Lane 1: Pre-stained standard molecular weight markers: phosphorylase B (107 kDa), bovine serum albumin (76 kDa), ovalbumin (52 kDa), carbonic anhydrase (36.8 kDa), soybean trypsin inhibitor (27.2 kDa), and lysozyme (19 kDa).

Lane 2: CP-3 (2.5 µg), untreated.

Lane 3: CP-3 (2.5 µg) incubated with 50 mM sodium acetate buffer, pH 5.5, no endo F.

Lane 4: CP-3 (2.5 µg) incubated with 50 mM sodium acetate buffer, pH 5.5, and 0.05 unit endo F.

Lane 5: CP-3 (2.5 µg) incubated with 50 mM sodium phosphate buffer, pH 7.2, no endo F.

Lane 6: CP-3 (2.5 µg) incubated with 50 mM sodium phosphate buffer, pH 7.2, and 0.05 unit endo F.

Lanes 7-10: Same as lanes 3-6, except that the substrate protein is bovine serum albumin (2.5 µg).

Lanes 11-14: Same as lanes 3-6, except that the substrate protein is alkaline phosphatase (2.5 µg).
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albumin and alkaline phosphatase were also treated in the same manner as the CP-3 to serve as negative and positive controls, respectively, for both endo F and PNGase F activity.

C. Activity of Artemia Cysteine Protease Toward Synthetic Peptide Substrates

Previous studies on the Artemia cyst CP have characterized it as a cysteine protease based on its inhibition by several cysteine protease inhibitors (Warner & Shridhar, 1980; 1985). More specifically, the protease has been described as cathepsin B-like, based on its affinity for Nα-Cbz-Arg-Arg 4-methoxy-β-naphthylamide (Warner & Shridhar, 1989), and Cbz-Arg-Arg 4-methoxy-β-naphthylamide dihydrochloride (Perona & Vallejo, 1982). As well, inhibition of this enzyme by leupeptin and antipain is indicative of a cathepsin B-like enzyme (Perona & Vallejo, 1982). It has been shown, however, that cathepsin B does not bind to Con A Sepharose (Barrett & Kirschke, 1981), while this is not the case for the Artemia CP. Therefore, to learn more about the enzyme’s affinity for specific synthetic peptide substrates, Artemia CP activity was measured using synthetic peptide substrates known to be specific for cathepsins B, H and L. Assays were conducted as described in Materials and Methods in a reaction volume of 320 μl containing 0.2 mM of various peptide substrates. Because a single, pure enzyme was being tested, activity toward Nα-Cbz-Arg-Arg 4-methoxy-β-naphthylamide, N-Cbz-Phe-Arg 4-methoxy-β-naphthylamide, or L-Leucine β-naphthylamide indicated cathepsin B-like, L-like, or H-like activity, respectively (Barrett & Kirschke, 1981). The results in Figure 5
Figure 5. Assay of *Artemia* CP using synthetic peptides as potential substrates.

Assays were conducted as described in Materials and Methods, with each reaction vessel containing 40 mEU of purified CP from a G-150 Sephadex column (as measured by the TNBS assay at 30°C, pH 5.0) and 0.2 mM of the appropriate synthetic peptide substrate. At the times indicated, aliquots of the reaction mixtures were terminated and the β-naphthylamine produced by proteolytic cleavage was extracted with n-butanol (see Materials and Methods). The absorbance of the butanol layer from each reaction aliquot was read at 520 nm. Cathepsin B activity was measured as activity toward Na-Cbz-Arg-Arg 4-methoxy-β-naphthylamide ( ■- ), cathepsin L activity was measured as activity toward N-Cbz-Phe-Arg 4-methoxy-β-naphthylamide ( ◆- ), and cathepsin H activity was measured as activity toward L-leucine-β-naphthylamide ( ▲- ).
show that the *Artemia* CP exhibited negligible activity toward the cathepsin H substrate, low activity toward the cathepsin B substrate, and high enzymatic activity when incubated with the cathepsin L substrate.

**D. Isolation of a Carboxyl-Terminal Peptide of the Small Subunit of *Artemia* CP-3**

Originally, the main objective of this work was to isolate a cDNA clone encoding the small subunit of CP-3 using PCR amplification of a cDNA library. Therefore, amino acid sequence information from two areas (preferably the amino- and carboxyl-termini) of the small subunit of the protein was required to construct oligonucleotide primers to be used in PCR. Sequence information from the amino terminus of the small subunit of CP-3 was obtained previously (Lu, 1991), however carboxyl-terminal sequence was required for construction of a second primer. Because conventional amino acid sequencing reactions proceed from the amino to carboxyl-end of a peptide/protein, a carboxyl-terminal peptide of the small subunit of CP-3 was required, which could then be sequenced beginning at the amino end of the peptide.

Using a combination of enzymatic treatments and HPLC (A. Butler, personal communications), a putative carboxyl-terminal peptide was isolated. A sample (28 μg) of the small subunit of CP-3 was treated with carboxypeptidase P as described in Materials and Methods. As a control, an identical sample was incubated in buffer alone, then both samples were treated with trypsin (see Materials and Methods). The products of each digest were separated on a Chemcosorb C-18 reverse phase HPLC column (4.6 x 250
mm) as described in Materials and Methods to locate the peak (peptide) corresponding to the carboxyl-terminus. The elution profile of the products of trypsin digestion of the small subunit of CP-3 are shown in Figure 6 (panel A). When this profile was compared to the profile in panel B (same digest as in panel A except that the small subunit of CP-3 was treated with carboxypeptidase P before trypsin), a difference in the peak eluting from the column at approximately 7.5 minutes was seen. Based on previous experimentation, this peak is believed to be the carboxyl-terminal peptide of the small subunit of CP-3. This peptide was collected as it eluted from the HPLC column, lyophilized, and submitted for sequencing as described in Materials and Methods. The first eight amino acids of the peptide were analyzed and the sequence obtained is shown in Figure 7. When this sequence, along with amino-terminal sequence obtained previously was analyzed, the results in Figure 8 were obtained.

E. Production of $^{32}$P-Labelled Lobster Cysteine Protease cDNA for use as a Probe

*E. coli* JM109 cells were successfully transformed with a plasmid containing a lobster cysteine protease (LCP2) cDNA (Laycock *et al.*, 1991) as described in Materials and Methods. The transformed cells were propagated in broth, and plasmid DNA was isolated and digested with Eco R1. The digested DNA was electrophoresed on a 1.2% agarose gel, and the LCP2 cDNA released by this digestion (see Figure 9) was isolated and labelled with $^{32}$P. The $^{32}$P-labelled LCP2 DNA was hybridized with nucleic acids (PCR products or plasmid DNA) after their immobilization on membranes.
Figure 6. Identification of a carboxyl-terminal peptide of the small subunit of Artemia CP-3.

A combination of enzymatic treatments and reverse phase HPLC on a Chemcosorb C-18 reverse phase column was used to identify the carboxyl-terminal peptide of the small subunit of CP-3 (see Materials and Methods).

A. Twenty eight micrograms of the small subunit of CP-3 were incubated with trypsin as described in Materials and Methods, and the digestion products were separated on a C-18 reverse phase HPLC column (4.6 x 250 mm) equilibrated with 12% acetonitrile in 0.1% TFA. Elution of the peptides was achieved using a 12-60% acetonitrile gradient in 0.1% TFA at a flow rate of 1 ml/minute over 45 minutes. Absorbance was monitored at 214 nm, (——).

B. Same as in (A), except that the sample was treated with carboxypeptidase P (see Materials and Methods) before trypsin digestion.
NH₂........A F Q E¹ Y W⁰ K G........COOH
 I P

Figure 7: Amino acid sequence of the carboxyl-terminal peptide of the small subunit of *Artemia* CP-3.

The peptide eluting from the reverse phase C-18 HPLC column at a position of 7.5 minutes was sequenced as described in Materials and Methods. The numbers above the sequence refer to the position of the amino acids beginning at the amino terminus.

¹ The sample contained equal amounts of F and I at position 4.

² The sample contained 40% W and 60% P at position 6.
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Figure 8. Comparison of amino- and carboxyl-terminal peptide sequences obtained from the small subunit of CP-3.

Amino-terminal amino acid sequence information obtained previously and carboxyl-terminal amino acid sequence information obtained through these studies (see Materials and Methods) was compared with sequences of other known cysteine proteases. The numbers above the sequences refer to the position of the amino acids beginning at the amino terminus. PAP, LCP2, RCL, and ACP3 refer to papain (Cohen et al., 1986), lobster cysteine protease 2 (Laycock et al., 1991), rat cathepsin L (Ishidoh et al., 1987), and Artemia cysteine protease 3 (CP-3), respectively.
Figure 9. Isolation of the LCP2 cDNA for construction of a $^{32}$P-labelled DNA probe.

Six aliquots (10 µg each) of purified pUC18 plasmid containing the LCP2 cDNA insert were digested with 20 units Eco R1 in the presence of 1X React 3 buffer overnight at 37°C. The digestion products were electrophoresed on a 1.2% agarose gel, and the gel was stained with ethidium bromide (0.5 µg/ml). The 1173 bp LCP2 cDNA was excised from lanes 3-8 of the gel with a clean scalpel, and DNA was collected from the agarose using the glass wool isolation technique (see section 14-b, Materials and Methods).

Lane 1: 1 kb DNA ladder size standards (as indicated).
Lane 2: 10 µg plasmid, undigested.
Lane 3-8: 10 µg plasmid, digested with 20 units Eco R1
F. PCR Products Generated from the lambda ZAP II cDNA Library DNA

In order to avoid the conventional and labour intensive exercise of screening an Artemia (encysted embryo) cDNA library (constructed in lambda ZAP II) using an imperfect DNA probe, I decided to amplify the sequences of interest by PCR before proceeding further. This approach avoided the need to plate the phage, lift individual plates with membranes, and then screen each membrane. In order to amplify a sequence encoding the small subunit of CP-3 from the Artemia cyst cDNA library, the phage containing the cDNA library were first grown on agar plates, and their DNA was harvested and purified (see Materials and Methods). This DNA was used as template in PCR, along with CP3-n and CP3-c as primers. Initially, all attempts to amplify this particular cDNA were unsuccessful at producing a PCR product that hybridized with the $^{32}$P-labelled LCP2 probe (data not shown). Therefore, a slightly different strategy was employed. PCR was conducted using the Artemia cyst cDNA library DNA as template, and the CP3-n primer and a commercially available T7 primer were used. The lambda ZAP II vector contains a T7 promoter site near the polycloning site of the vector. Therefore, the T7 primer was tested as a replacement for the CP3-c primer in the initial PCR. This PCR yielded many products ranging in size from approximately 400 bp to approximately 1500 bp after electrophoresis on a 1.2% agarose gel (see Figure 10). Some of the products were difficult to visualize individually. This is attributed to using the T7 primer in PCR, as it will amplify all sequences inserted into the vector, creating a smear of PCR products on the gel. The intensity of ethidium bromide staining of the
Figure 10. Electrophoresis of PCR products amplified from the lambda ZAP II cDNA library DNA using CP3-n and T7 primers.

Increasing amounts (10-100 ng) of lambda ZAP II cDNA library DNA were amplified (using PCR) with 10 picomoles each of CP3-n and T7 primers, in the presence of 1X PCR buffer, 1.5 mM MgCl₂, and 0.2 mM dNTP’s. The reaction mixture was treated with a three minute hot start at 94°C, then one unit of Taq polymerase was added. Thirty five cycles of the following program were run: denaturation at 94°C for 45 seconds, annealing at 47°C for 45 seconds, and extension at 72°C for 90 seconds. The final cycle was followed by a 10 minute incubation at 72°C. The PCR products (10 µl) were separated on a 1.2% agarose gel and stained with ethidium bromide (0.5 µg/ml).

Lane 1: 1 kb DNA ladder of size standards (as indicated).
Lane 2: Negative control PCR product (no DNA template added).
Lane 3: PCR product from 10 ng lambda ZAP II cDNA library DNA template.
Lane 4: PCR product from 25 ng lambda ZAP II cDNA library DNA template.
Lane 5: PCR product from 50 ng lambda ZAP II cDNA library DNA template.
Lane 6: PCR product from 100 ng lambda ZAP II cDNA library DNA template.
PCR products was seen to increase as the amount of DNA template was increased (see Figure 10).

The gel containing the PCR products was blotted to a Biotrace HP membrane and probed with the $^{32}$P-labelled LCP2 DNA. The membrane was washed and exposed to X-ray film. Details of the probing, washing, and exposure of membranes to X-ray film can be found in Materials and Methods. No hybridization signal resulted from this experiment. Therefore, this experiment was repeated using an alternative method of immobilizing nucleic acids on solid supports. The same PCR products were blotted to a Zeta Probe membrane using a Slot Blot apparatus (see Materials and Methods), and probed with the $^{32}$P-labelled LCP2 DNA probe. The results in Figure 11 show the autoradiogram of this experiment. The PCR products generated from the cDNA library DNA template using the CP3-n and T7 primers generated a hybridization signal on the slot blot when probed with the $^{32}$P-labelled LCP2 DNA.

Based on the results obtained in the slot blotting experiment described above, it was decided that a sample of the same PCR products that were probed on the slot blot would be re-amplified (with PCR) using the CP3-n and CP3-c primers. The PCR products obtained using 100 ng of template DNA (in the experiment described above) were diluted 100 and 1000 fold, and 1 µl of each dilution was amplified as described in Materials and Methods. The results in Figure 12 (panel A), show the products of this PCR separated on a 1.4% agarose gel. This amplification reaction yielded two major bands; one of approximately 435 bp, and one of approximately 300 bp. The gel was stained with ethidium bromide, then blotted to a Zeta Probe membrane (see Materials
Figure 11. Autoradiogram of PCR products amplified from lambda ZAP II library DNA with CP3-n and T7 primers.

Five microlitres each of the PCR products seen in Figure 10 were applied to a Zeta Probe membrane using a Slot Blot apparatus (see Materials and Methods). The membrane was probed with the \(^{32}\)P-labelled LCP2 DNA at 52°C overnight, washed, and exposed to X-ray film.

Position A1: Negative control PCR product (no DNA template added).

Position A2: PCR product from 10 ng lambda ZAP II cDNA library DNA template.

Position A3: PCR product from 25 ng lambda ZAP II cDNA library DNA template.

Position A4: PCR product from 50 ng lambda ZAP II cDNA library DNA template.

Position A5: Negative control PCR product (no DNA template added).

Position A6: PCR product from 100 ng lambda ZAP II cDNA library DNA template.

Position B1-B6: Empty wells.

Position C6: 125 ng LCP2 cDNA (positive control).
Figure 12. Electrophoresis, blotting, and autoradiography of PCR products re-amplified using CP3-n and CP3-c primers.

The PCR products obtained by amplifying 100 ng of lambda ZAP II library DNA with 10 picomoles each of CP3-n and T7 primers were diluted 100 and 1000 fold. One microlitre of each of these dilutions was re-amplified with 100 picomoles each of CP3-n and CP3-c in the presence of 1X PCR buffer, 2.5 mM MgCl₂, and 0.2 mM dNTP's. The reaction mixture was treated with a three minute hot start at 94°C, then one unit of Taq polymerase was added. Initially, five cycles of the following program were run: denaturation at 94°C for 45 seconds, annealing at 43°C for 30 seconds, and extension at 72°C for 60 seconds. This was followed by 20 cycles of the same program except that the annealing temperature was increased to 58°C. Following the final cycle, the reactions were incubated at 72°C for 10 minutes. Ten microlitres of the products of the reaction were separated on a 1.4% agarose gel, stained with ethidium bromide (0.5 µg/ml) and blotted to a Zeta Probe membrane. The membrane was probed with the ⁵²P-labelled LCP2 DNA overnight at 52°C, washed, and exposed to X-ray film.

A. 1.4% agarose gel of the above PCR products. Note that the stained material at the bottom of lanes 2, 3, and 4 is believed to be "primer-dimers".

B. Autoradiogram of Southern blot of the gel seen in (A) after three hours exposure.

C. Same autoradiogram as seen in (B), except after 24 hours exposure.

Lane 1: 1 kb ladder of DNA size standards (as indicated).

Lane 2: Negative control PCR product (no DNA template added).

Lane 3: PCR product from amplifying 1 µl, 100 fold dilution of template DNA.

Lane 4: PCR product from amplifying 1 µl, 1000 fold dilution of template DNA.
and Methods). The membrane was probed with the $^{32}$P-labelled LCP2 DNA, washed, and exposed to X-ray film (Figure 12, panels B and C). The 435 bp PCR product generated in this reaction showed a hybridization signal on the autoradiogram. Some non-specific binding between the DNA size standards and the LCP2 DNA probe was also seen, however during autoradiography, it was noted that the signal generated by hybridization with the PCR product could be seen after exposing the probed membrane to X-ray film for as little as three hours. The non-specific signal required 24 hours exposure to appear.

G. Cloning of the 435 base pair PCR Product

Since the main objective of this work was to isolate a cDNA clone encoding all (or part) of the small subunit of the Artemia CP-3, I chose to clone the 435 bp cDNA which reacted with the LCP2 probe into a stable (bacterial) system. The 435 bp PCR product was ligated into the pCR 2.1 vector supplied with the TA Cloning Kit, and the products of the ligation reaction were used to transform E. coli INVaF' cells (also supplied with the kit) as described in Materials and Methods. Transformed cells were selected on LB plates (15 x 100 mm) containing 50 $\mu$g/ml kanamycin and 25 $\mu$l of 40 mg/ml X-gal. After overnight incubation at 37°C, plates were lifted with Biotrace NT pre-cut membrane discs. Membranes were processed, probed with the $^{32}$P-labelled LCP2 DNA, washed, and exposed to X-ray film as described in Materials and Methods (see Figure 13).
Figure 13. Autoradiogram of first screening of *E. coli* cells transformed with the 435 base pair PCR product.

The 435 bp PCR product generated from a combination of PCR's (described previously) was ligated into the pCR 2.1 vector, and this ligation mix was used to transform *E. coli INVaF*’ cells (see Materials and Methods). The transformed cells were selected on LB plates (15 x 100 mm) containing 50 µg/ml ampicillin. After overnight incubation at 37°C, cells were lifted with Biotrace NT pre-cut membrane discs. Membranes were processed, probed with the ³²P-labelled LCP2 DNA, washed, and exposed to X-ray film (as described in Materials and Methods). Colonies were grown on the plate in duplicate. Colonies that were picked for further analysis are underlined and positive controls (*E. coli* cells transformed with the pUC18 vector containing the LCP2 cDNA) are marked with arrows. It should be noted that several positive colonies grew on this plate, however only those picked for the experiments detailed in this thesis are underlined.
Several colonies on the membrane generated positive hybridization signals when they were hybridized with the $^{32}$P-labelled LCP2 DNA. Two of the colonies showing positive hybridization signals (see Figure 13) were selected for further analysis. Each colony was picked and grown overnight in LB broth containing 50 μg/ml kanamycin, then plasmid DNA was isolated from each culture using the Wizard Miniprep Kit (see Materials and Methods). The plasmid DNA was digested overnight with Eco R1 and the digestion products were separated on a 1.4% agarose gel. The gel was stained with ethidium bromide, and the DNA was blotted to a Biotrace HP membrane. The membrane was probed with the $^{32}$P-labelled LCP2 DNA, washed, and exposed to X-ray film. Digestion with Eco R1 cut an insert of 435 bp from the plasmid (see Figure 14) which hybridized with the $^{32}$P-labelled LCP2 probe.

To ensure that this cDNA was amplified with both of the primers employed in the PCR, a final PCR was performed in which the entire pCR 2.1 plasmid containing the 435 bp cDNA from one of the positive clones was used as template. The cloned cDNA insert was amplified with CP3-n and CP3-c under the same conditions in which it was amplified originally from the cDNA library. A 435 bp PCR product which hybridized with the $^{32}$P-labelled LCP2 probe was the observed result of this reaction and no products were produced when either of the primers were omitted from the reaction (see Figure 15, panel A). The products of the reactions were separated on a 1.4% agarose gel, stained with ethidium bromide, and blotted to a Biotrace HP membrane. The membrane was then probed with the $^{32}$P-labelled LCP2 DNA, washed, and exposed to X-ray film. A hybridization signal corresponding with the position of the 435 bp PCR
product can be seen on the autoradiogram in Figure 15 (panel B).
Figure 14. Electrophoresis and autoradiography of Eco R1 digested plasmid DNA from clones containing the 435 bp PCR product.

Two clones which generated a hybridization signal when probed with the $^{32}$P-labelled LCP2 DNA (see Figure 13) were picked and grown overnight at 37°C in LB broth containing 50 μg/ml kanamycin. Plasmid DNA was isolated from these cells using the Wizard Miniprep Kit. Approximately 12 μg plasmid DNA was digested with 6 units Eco R1 in the presence of 1X React 3 buffer overnight at 37°C. The products of the digest were separated on a 1.4% agarose gel and the gel was stained with ethidium bromide (0.5 μg/ml). The gel was blotted to a Biotrace HP membrane, probed with the $^{32}$P-labelled LCP2 DNA, washed, and exposed to X-ray film.

A. 1.4% agarose gel of the Eco R1 digest products.

B. Autoradiogram of the gel seen in (A) after blotting and probing with the $^{32}$P-labelled LCP2 DNA. Note that the non-specific hybridization between the DNA probe and the pCR 2.1 vector is believed to have resulted from incomplete Eco R1 digestion of some plasmid molecules. This issue is addressed in the Discussion.

Lane 1: 1kb ladder of DNA size standards (as indicated).

Lane 2: Self ligated pCR 2.1 plasmid (3 μg).

Lane 3: Plasmid DNA from clone #1 undigested.

Lane 4: Plasmid DNA from clone #1 digested with Eco R1.

Lane 5: Plasmid DNA from clone #2 undigested.

Lane 6: Plasmid DNA from clone #2 digested with Eco R1.

Lane 7: Empty.

Lane 8: Approximately 50 ng LCP2 cDNA.
Figure 15. Electrophoresis and autoradiography of the PCR product generated from clone #2 plasmid DNA with CP3-n and CP3-c primers.

A sample (49 ng) of the plasmid DNA isolated from clone #2 was amplified (using PCR) with 100 picomoles each of CP3-n and CP3-c in the presence of 1X PCR buffer, 2.5 mM MgCl₂, and 0.2 mM dNTP's. The reaction mixture was treated with a three minute hot start at 94°C, then one unit of Taq polymerase was added. Initially, five cycles of the following program were run: denaturation at 94°C for 45 seconds, annealing at 43°C for 30 seconds, and extension at 72°C for 60 seconds. This was followed by 20 cycles of the same program, except that the annealing temperature was increased to 58°C. Following the final cycle, the reactions were incubated at 72°C for 10 minutes. The products of the PCR were separated on a 1.4% agarose gel. The gel was stained with ethidium bromide (0.5 μg/ml), and blotted to a Biotrace HP membrane. The membrane was probed with the ³²P-labelled LCP2 DNA, washed and exposed to X-ray film as described in Materials and Methods.

A. 1.4% agarose gel of PCR products from plasmid DNA isolated from clone #2.

B. Autoradiogram of a Southern blot of the gel seen in (A), probed with the ³²P-labelled LCP2 DNA.

Lane 1: 1 kb ladder of DNA size standards (as indicated).

Lane 2: Negative control PCR (no DNA template added).

Lane 3: Plasmid DNA from clone #2 amplified with CP3-n and CP3-c primers.

Lane 4: Plasmid DNA from clone #2 amplified with CP3-n primer alone.

Lane 5: Plasmid DNA from clone #2 amplified with CP3-c primer alone.
IV. DISCUSSION

Although the major cysteine protease of Artemia franciscana cysts has been investigated previously, many unanswered questions still remained. In an attempt to further characterize the physical aspects of this enzyme, studies were conducted in which the preference of the enzyme for specific synthetic peptide substrates was assessed, and the glycosylation of this enzyme was investigated. As well, a cDNA encoding the small subunit of CP-3 (or part of it) was isolated from a cDNA library, and cloned into a bacterial system to facilitate further studies of the protease at the molecular level.

The purification alone of the Artemia CP allows one to see its uniqueness as a cysteine protease. The fact that the Artemia CP binds to Con A Sepharose suggests that it is a glycosylated enzyme. The major lysosomal cysteine proteases, cathepsin B, H, L, and S have also been found to be glycosylated to varying degrees (Kirschke et al., 1995), however it is of interest that cathepsin L and H are known to bind Con A Sepharose, while cathepsin B does not (Barrett & Kirschke, 1981). As well, other non-mammalian examples of glycosylated cysteine proteases exist, such as CC-III, isolated from the latex of the mountain papaya (Carica candarcensis Hook) by Jaziri et al. (1994) melain, isolated from the sarcocarp of the bead tree fruit (Melia azedarach) by Kaneda et al. (1994), and comosain and bromelain from pineapple stem, described by Napper et al. (1994). One final noteworthy comparison is that previous studies have determined that
the Artemia cyst enzyme is not bound by a Gly-Phe-glycinal semicarbazone column (A. Warner & M. Ryan, unpublished observations) which is known to bind cathepsin B (Rich et al., 1986).

Also of interest was the elution profile obtained when the isoforms of the Artemia enzyme were chromatographed on an FPLC (Mono Q) column (see Figure 1). Previous studies using iso-electric focussing have determined that the isoforms differ with respect to their iso-electric points (A. Warner, manuscript in preparation). The basis for the separation of these isoforms has not yet been fully determined, although it has been speculated that post-translational modifications of the enzyme may be responsible. Indeed, experiments described in this thesis involving specific staining techniques (Schiff base stain for carbohydrate detection), and digestion of the Artemia CP with enco F, a carbohydrate cleaving enzyme, confirmed that this protease is in fact glycosylated (see Figures 3 and 4). These experiments will be discussed in greater detail later.

It is noteworthy that multiple isoforms of other cysteine proteases have been documented. While conducting studies aimed at separating and characterizing the mixture of enzymes in pineapple stem, Napper et al. (1994) found that one of the enzymes in crude pineapple stem preparations, ananain, can be separated into five isoforms (three active, two inactive) using Mono S chromatography after treatment with thiol modifying agents. Their work concluded that the basis of the multiple enzyme forms was post-translational modifications, however it should be noted that ananain is not glycosylated. Although the effect of purification (ie. the creation of artifacts) on the Artemia protease or any other enzyme must be considered, Napper et al. (1994) state
that they obtained the same FPLC profiles over an extended period of time (two years), while chromatographing numerous pineapple stem preparations. This is consistent with observations made during purification of the *Artemia* CP. Thus it is believed that the FPLC elution profiles of the *Artemia* CP are not the result of purification induced artifacts. Alternatively, the multiple isoforms of the *Artemia* CP may be the result of multiple genes encoding different forms of the enzyme, or alternative splicing of *Artemia* mRNA, however, there is no experimental evidence to date to support this assumption.

The subunit composition of the isoforms of the *Artemia* CP is also somewhat unique. All isoforms of the *Artemia* cyst CP are composed of two subunits which can be separated by HPLC (see Figure 2) or SDS-PAGE (see Figures 3 and 4). The two subunits have been found to have relative molecular weights of 28.5 kDa and 31.5 kDa. It should be noted that the order of elution of the subunits of the CP from the HPLC column was determined by previous studies (A. Warner, unpublished observations), by collecting the subunits after HPLC separation and analyzing their molecular weights using SDS-PAGE. This subunit composition is in contrast to other well known cysteine proteases. The mature forms of mammalian cathepsin B, H, and L, all have relative molecular weights of 30 kDa or less. These enzymes are composed of a heavy chain and a light chain, however the light chain for all three enzymes is only about 5 kDa. The plant cysteine proteases (papain, etc.), also tend to have relative molecular weights of 30 kDa or less. As well, these enzymes are generally composed of only one polypeptide.

The structural characteristics of the *Artemia* CP do not allow it to be classified
easily into any of the established cysteine protease families, however, it seems to share some characteristics with the calpains, papain, and cathepsin L. Both the Artemia CP and the calpains are composed of heterodimers, however the two enzymes differ in overall size as the Artemia CP is approximately 60 kDa, while the calpains are approximately 110 kDa (Melloni et al., 1992). Although the Artemia enzyme does not require calcium for activity, the presence of low levels of calcium in the reaction mixture during the TNBS assay has been found to increase Artemia CP activity slightly (Nagainis & Warner, 1979). The main point on which the Artemia CP and the calpains differ however, is in their sensitivity to the typical cysteine protease inhibitors. The calpains are sensitive to the specific calpastatins only, while the Artemia enzyme is sensitive to the traditional cysteine protease inhibitors and its own endogenous cysteine protease inhibitors (Nagainis & Warner, 1979; Warner & Shridhar, 1980; 1985; Warner, 1987; 1989). The similarities between the Artemia CP, papain, and cathepsin L will be discussed later.

It has been suspected for some time that the Artemia CP is glycosylated based on its affinity for Con A Sepharose. Therefore, in order to pursue this idea, the isoforms of the Artemia CP were stained with a Schiff base reagent (see Figure 3) which detects glycoproteins. Interestingly, both subunits of all of the isoforms tested stained for carbohydrate. To further analyze the type of carbohydrate groups associated with the enzyme, a sample of CP-3 was treated with endo F, a carbohydrate cleaving enzyme which hydrolyses many, but not all (N-linked) high mannose, oligosaccharide, and complex biantennary carbohydrate groups (Tarentino et al., 1985). The large subunit of
CP-3 was sensitive to this treatment (see Figure 4), undergoing a shift in relative molecular weight of approximately 1.0 kDa. This suggested that this subunit has an N-linked glycan associated with it. The small subunit was not affected by this treatment, suggesting that the carbohydrate moiety associated with it was most likely an O-linked, rather than an N-linked glycan.

Because it has been noted by Plummer et al. (1984) that commercial preparations of endo F are often contaminated with PNGase F activity, the incubation conditions of the experiment (buffer and pH) were altered in an attempt to take advantage of any contaminating PNGase F activity (if present). Tarentino et al. (1985), have found that PNGase F has the ability to hydrolyse all classes of N-linked glycans. The results of the treatment which attempted to demonstrate the effect of PNGase F activity on CP-3 were not appreciably different than the result of endo F treatment (see Figure 4). Therefore, it is believed that there was not enough contaminating PNGase F activity present in the endo F preparation to be active on CP-3. This conclusion is supported by the behaviour of the alkaline phosphatase used in the experiment as a positive control (see Figure 4). PNGase F is capable of removing a second N-linked carbohydrate group from alkaline phosphatase which is not sensitive to endo F cleavage (Haselbeck & Hösel, 1988). Therefore, the alkaline phosphatase should have experienced a further shift in mobility on the gel if PNGase F activity was present (Haselbeck & Hösel, 1988).

As another way of characterizing the Artemia CP, its activity toward synthetic peptide substrates was tested (see Figure 5). Of the substrates tested, the Artemia CP exhibited the most activity toward N-Cbz-Phe-Arg 4-methoxy-ß-naphthylamide (cathepsin
L substrate). Although this substrate is also sensitive to cathepsin B activity, the *Artemia* activity was classified as cathepsin L-like because the enzyme showed very low activity toward *Nα*-Cbz-Arg-Arg 4-methoxy-β-naphthylamide (cathepsin B substrate). These results contradict the results of Warner (1987) who suggested that the enzyme is cathepsin B-like based on its ability to hydrolyse BANA (*Nα*-benzoyl-DL-Arg-β-naphthylamide) and *Nα*-Cbz-Arg-Arg 4-methoxy-β-naphthylamide. At that time, however activity toward the cathepsin L substrate had not been assessed. These data indicate that the *Artemia* CP is a cathepsin L-like protease with regards to its preference of (synthetic peptide) substrates.

Using a novel method developed in our laboratory (A. Butler, unpublished observations), a carboxyl-terminal peptide fragment of the small subunit of the *Artemia* CP-3 was isolated. This method was initially developed in conjunction with research being conducted on the major non-dialysable cysteine protease inhibitor (CPI) found associated with the CP in *Artemia* cysts. It was devised in an attempt to find an alternative strategy to the conventional method of screening DNA libraries when adequate probes are not available. The objective of this method was to obtain amino acid sequence information from the two termini of the protein of interest (in this case, the small subunit of CP-3). Primers for PCR could then be designed based on this sequence, and used to screen a cDNA or genomic DNA library using PCR, hopefully amplifying a complete cDNA or gene sequence, respectively.

There are many examples in the literature of using PCR to screen DNA libraries using primers designed based on conserved regions of cysteine proteases (Tanaka et al.,
1994; Mallinson et al., 1994; Harrop et al., 1995; Martinez et al., 1995; Nong et al., 1995), however these experiments normally yield incomplete cDNA's or gene sequences. The strategy described here could theoretically amplify a complete sequence from a cDNA or genomic DNA library, provided that the functional limits of the Taq polymerase used in the PCR were not exceeded (this might occur when amplifying an entire gene from a genomic library).

In order to isolate the carboxyl-terminal peptide of the Artemia CP-3, a combination of enzymatic manipulations and HPLC (previously described) was used. The major difference observed between the HPLC column elution profiles seen in Figure 6 (panel A and B), is the peak which elutes from the column at approximately 7.5 minutes. The difference in the size of these two peaks is attributed to digestion by carboxypeptidase P (panel B) before trypsin digestion. The sensitivity of this peptide to carboxypeptidase P identifies it as the carboxyl-terminus of the protein. The amino acid sequence obtained from this peptide necessitated designing a mixed primer containing a relatively high level of degeneracy (approximately 50-fold more than the amino-terminal primer) and this may have contributed to difficulties experienced with initial PCR reactions. This will be discussed later on in this chapter. The amino acid sequence obtained from the carboxyl-terminal peptide (and the amino-terminal sequence obtained previously) was compared to that of other cysteine proteases. There is a high degree of sequence homology seen between the Artemia CP-3 and papain, LCP2, and rat cathepsin L at the amino terminus of these proteins. It was more difficult to assess homology at the carboxyl-end of the proteins because of limited sequence information (only eight cycles
of amino acid sequencing were run); however, two conserved amino acids (tyrosine and proline) were seen in the *Artemia* sequence (see Figure 8).

In order to analyze the PCR products generated from subsequent experiments described in this thesis, it was decided that a cDNA encoding a lobster cysteine protease, or LCP2 (Laycock *et al.*, 1991) would be labelled isotopically (\(^{32}P\)) and used to probe various PCR products and nucleic acids of interest, after their immobilization on membranes. The strategy of using a cDNA sequence from one organism to identify a similar cDNA in another organism has been used successfully in the past, while screening cDNA or genomic DNA libraries for cysteine protease genes (Domoto *et al.*, 1995; Matsumoto *et al.*, 1995). Because the amino-terminal region of the *Artemia* CP (first 20 amino acids) shares a high degree of homology (approximately 65%) with LCP2, this cDNA was chosen for use as a probe. As well, it was found that the CP3-n and CP3-c primers reacted readily during PCR with the LCP2 cDNA (data not shown), adding further support to the decision to use LCP2 cDNA as a probe.

Initial attempts to amplify a cDNA from the lambda ZAP II library DNA (containing the *Artemia* cDNA's) using the CP3-n and CP3-c primers were unsuccessful at producing a PCR product that hybridized with the LCP2 DNA probe. It is believed that a combination of factors contributed to this result. Although a number of manipulations of PCR conditions were tried, it is possible that conditions were still not completely optimal. Less than optimal PCR conditions may have produced such a low amplification of the targeted DNA that insufficient DNA transferred to membranes when blotted, and therefore could not be detected by the LCP2 probe. As well, it is possible
that the degree of degeneracy of the CP3-c primer interfered with the amplification of the targeted cDNA. When initial PCR’s failed to generate a PCR product which reacted with the LCP2 DNA probe, it was decided that PCR would be conducted again, using a T7 primer in place of the CP3-c primer. This would result in several PCR products, as a T7 promoter site is present near the polycloning site of the lambda ZAP II vector, and theoretically, this should result in at least one copy of every cDNA in the library. The annealing temperature of the initial five cycles of this reaction was dropped to 43°C to allow annealing between the T7 primer and promoter. Subsequent cycles were conducted with an annealing temperature of 58°C in an effort to selectively amplify the cDNA encoding the small subunit of CP-3. This experiment generated a smear of PCR products ranging in size from approximately 300 bp to 2000 bp, however no predominant DNA bands were observed (see Figure 10). When these PCR products were blotted to a Biotrace HP membrane, and probed with the 32P-labelled LCP2 DNA probe, no hybridization signal was detected (data not shown).

When the PCR products from this experiment were immobilized on Zeta Probe membrane in the form of a slot blot however (see Figure 11), a hybridization signal was evident after probing with the 32P-labelled LCP2 DNA probe. This suggested that the targeted cDNA was in fact amplified through PCR. According to the manufacturer, when used in alkaline blotting protocols, Zeta Probe membrane (used in the slot blot procedure) has a higher sensitivity and resolution than other membranes used in traditional Southern blots. Therefore, it is possible that the amount of PCR product produced in the reaction described above was so low that it was not detectable using the
Biotrace HP membrane. Also, if very small amounts of the PCR product of interest were made, it may have resulted in poor transfer to the membrane.

Because the hybridization signal was seen on the slot blot (described above), it was decided that these PCR products would be used as template for PCR with the CP3-n and CP3-c primers. This PCR strategy was successful in amplifying a cDNA sequence that reacted with the LCP2 probe (see Figure 12). This PCR generated two major products (as visualized by agarose gel electrophoresis) in Figure 12 (panel A) of which only one band of approximately 435 bp reacted with the probe (see Figure 12, panels B and C). This size is smaller than expected, as the calculated size based on the relative molecular weight of the small subunit of CP-3 is approximately 260 amino acids (approximately 28.5 kDa), which should result in a cDNA of approximately 780 bp. The size of the carbohydrate moiety associated with the small subunit has not yet been determined, and this may account for some discrepancies in size between the calculated size of the cDNA encoding the small subunit of CP-3 and the actual 435 bp cDNA obtained.

Once an Artemia cDNA which hybridized with the LCP2 probe was amplified from the cDNA library, it was cloned into the bacterial vector, pCR 2.1. The initial screening of the colonies transformed with this plasmid (see Figure 13) yielded several potentially positive clones. Therefore, several clones were selected for analysis, and all contained inserts. Two of the clones containing inserts were selected for further analysis. Their plasmid DNA was isolated and digested with Eco RI to cut out their cDNA insert, and the digestion products were analyzed by agarose gel electrophoresis (see Figure 14).
The cloned inserts were of the expected size of approximately 435 bp, and when the above gel was blotted to a Biotrace HP membrane and probed with the LCP2 probe, the cDNA inserts showed a hybridization signal (see Figure 14). However, a hybridization signal was also seen from the pCR 2.1 vector even after the cDNA insert was cut out with Eco RI. This result was attributed to digesting a relatively large amount of plasmid DNA with Eco RI in this experiment. In previous experiments, difficulty was experienced in transferring the small (435 bp) insert to the membrane. It has been shown by Sambrook et al. (1989), that small (less than 500 base pairs) pieces of DNA transfer much less efficiently from agarose gels to membranes during blotting procedures than do large pieces. Therefore, the amount of plasmid DNA in the digest was increased, while keeping the amount of Eco RI constant so that the concentration of glycerol (included in the Eco RI storage buffer) did not exceed 5%, thereby interfering with Eco RI activity. This most likely resulted in less than complete digestion of the plasmid DNA leaving some pCR 2.1 plasmid molecules linearized but not free of their cDNA inserts, causing a hybridization signal when probed. It should be noted that the intensity of the hybridization signal associated with the 435 bp cDNA insert is quite strong considering the amount of DNA in the cDNA insert band when compared to the signal generated by the large amount of cut vector. If completely non-specific binding between the vector and the probe was occurring, the hybridization signal given by the vector should be much more intense. Finally, it should be noted that the self-ligated pCR 2.1 vector (containing no cDNA insert) did not generate any hybridization signal, as expected.
To verify the putative CP clones further, plasmid DNA from clone #2 was used as DNA template in PCR using the same primers and reaction conditions that generated this cDNA initially. Also, to ensure that both primers were needed to amplify the cDNA, control reactions were run containing only one primer each. These reactions generated a PCR product only when both primers were used, and the product hybridized with the LCP2 probe. This confirmed that a specific cDNA encoding all (or part) of the small subunit of the CP-3 of Artemia has been obtained (and cloned) by screening a bacteriophage cDNA library using PCR employing primers designed based on amino acid sequence information from the amino- and carboxyl-termini of the small subunit of the protein.

Sequencing of this cDNA will resolve some unanswered questions which exist at this time, such as the size discrepancy between this cDNA and the predicted size of the protein. Also, sequencing this cDNA will allow for comparisons to be made between the Artemia CP and related proteases allowing further characterization of this enzyme at the molecular level. This characterization could include the elucidation of molecular control mechanisms acting on this gene, characterization of the intron/exon structure of this gene, as well as provide information about the pro-region of this enzyme, which has been found to be important to the targeting and processing of many cysteine proteases (Kirschke et al., 1995). Finally, this information will contribute to the knowledge of a cysteine protease which may be a member of a unique group of cysteine proteases.
ADDENDUM

Since the writing of this thesis, the cDNA encoding the small subunit of the
Artemia CP-3 has been sequenced by A. H. Warner (unpublished results). The cDNA
was found to represent approximately 70% of the small subunit of the protein, based on
comparisons made with a Drosophila melanogaster cysteine protease sequence
(Matsumoto et al., 1995). The Artemia sequence was also found to have 73% and 60%
homology with the Drosophila sequence and a lobster cysteine protease sequence (LCP2)
(Laycock et al., 1991), respectively. The Artemia CP-3 (small subunit) sequence and the
comparison of it with the Drosophila and lobster sequences are as follows.

Comparison of the Artemia CP-3 SSU with Drosophila and Lobster CP Sequences

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<td>Drosophila</td>
<td>DIPQGEKKMPEAVAVGPVSA IDASHESFQFYSEG VYNEPO QCDAQNLHGVLV VVGFTDE</td>
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**Artemia** CP-3 SSU cDNA Clone Sequence

```
1---10---20---30---40---50
GGTACCGAAAAAGGGGGCTATTACTCTCTGTAAGAAGCCAAGGACAGTGTTG
E K G A I T P V K D Q G Q C G

60---70---80---90---100
TTGCTGCTGGGCTTTTCATCTACTGCTGTGCCTGGGTAAAAGTTCA
S C W A F S S T G A L E G R T F

110---120---130---140---150
GAAAAACAGGGAGCTCGTTTTCTTTGAGTGCAAGAAGTTGAGTATTGT
R K T G K L V S L S E Q K L I D C

160---170---180---190---200
TCTGGAAAAATATGGAATGAAGGATGCAATGGAGGATTAATGGACCAAGC
S G K Y G N E C N G L M D Q A

210---220---230---240---250
TTTCCAGTATATCAAGGATAAACAAGGCAATTGACACTGAAAATACTGACC
F Q Y I K D N K A I D T E N T Y

260---270---280---290---300
CTTATGAAAGCTGAAAGCGATGTCTGTCCTTTATCTACTCCAGGAAACCGAGGT
P Y E A E D D V C R Y N P R N R G

310---320---330---340---350
GCAGTTGGACCCGTTGCTCGACATATCCATCTGGGAAAGAAGATAAGCCT
A V D R G E V D I P S G E E D K L

360---370---380---390---400
TAAGGCAGCTGTTGCAAGGGGCTTGTGATCTGTTGGCATTCATGTAGGCCT
K A A V A T V G P V S V A I D A

410---420---430---440---450
CTCATGAAAGATTCCAAATCTACCTAAGGGGCGGATCC
S H E S E Q I Y P K G
```

- Hexanucleotide sequences added to primers, to facilitate future directional cloning using Kpn I and Bam HI.

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