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Characterization of major cysteine protease isoforms in embryos and larvae of Artemia franciscana.

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Characterization of Major Cysteine Protease Isoforms in Embryos and Larvae of *Artemia franciscana*

By

Bo Liu

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Abstract

The brine shrimp *Artemia franciscana* has been used as an important organism in the study of the biochemistry of development in recent years. Among the numerous proteins of importance in developing embryos, proteases are attracting more attention because of their potential role in activation reactions in development. Therefore, an understanding of protease activity at different stages of development is necessary to assess the importance of protease-induced changes in the regulation of development.

Cysteine proteases have been found in different subcellular fractions at different stages of *Artemia franciscana* development, and data suggest that these enzymes may function in different ways during the developmental processes. The different sites of localization of cysteine proteases in first and second instar larvae, and their potentially different functions may be due to different isoforms of the enzyme.

Results in this thesis showed that at least 5 isoforms of a cysteine protease can be resolved by isoelectric focusing and that of these proteases, CP-1 and CP-2 are dominant in the cytoplasm of embryos while CP-3, CP-4 and CP-5 are dominant in the cytoplasm of first (0-h) and second (26-h) instar larvae of *Artemia franciscana*. A preliminary analysis of the cysteine protease isoform pattern in the mitochondria/lysosome fraction showed a profile similar to that found in the cytoplasmic fraction. Results of isoelectric focusing of proteins purified by fast protein liquid chromatography on Mono Q showed

that the cytoplasm of 0-h embryos contains six isoforms of the cysteine protease ranging from a pI 4.3 to 6.8, whereas 1st and 2nd instar larvae contain only three to four isoforms of CP with pIs of 4.5 to 5.5.

These studies demonstrate that the composition of the cysteine proteases changes during development and that the different isoforms may have different functions during development. Structural studies of each CP isoform will be required to ascertain whether differences exist in the amino acid composition of each CP isoform or whether post-translational modifications have occured to the CP for the purpose of localization in specific regions of the larvae.

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Introduction

1. General Properties of *Artemia*

The brine shrimp *Artemia* is found in saline environments on six continents (Browne and Bowen, 1990). Collectively these animals have been classified as 5 species: *Artemia franciscana* (native to North America), *Artemia persimilis* (native to Argentina), *Artemia tunisiana* (native to Mediterranean region), *Artemia urmiana* (native to Iran) and *Artemia parthenogenetica* (native to Asia, Australia and Europe). The encysted embryos of *Artemia* have several remarkable features which make them attractive as research material in molecular biology: they are available in large amounts and can be preserved under desiccated conditions until needed without special attention; the encysted embryos are in a state of dormancy, a physiological situation not observed in mammalian cells and tissues; after rehydration of *Artemia* cysts in the presence of oxygen, the embryos resume their metabolic activities, undergo primary differentiation without cell division, and develop into swimming larvae (nauplii). For these reasons, *Artemia* embryos have been an important organism in the study of the biochemistry of development in recent years. The abundance of *Artemia* dormant cysts and the ease with which these embryos can be cultured into larval forms make this organism ideally suited for studies of biochemistry of invertebrate development.

Most biochemical studies have been conducted using encysted embryos believed to be *Artemia franciscana*. This species has become increasingly important as a useful model system for biochemical studies of development, including the study of enzymes in embryo and larval stages of this organism (Clegg and Conte, 1980; Slegers, 1991; Warner, 1989).

2. General Properties of Proteases

Among several enzymes that have been studied in *Artemia*, considerable research has been conducted on proteases because of their potential role in several developmental processes, including activation reactions, regulatory reactions, and degradative reactions (Slegers, 1991). At the cellular, tissue and systemic levels, proteases function in a variety of biological processes such as protein turnover, control of steady-state concentrations of proteins, metabolic activation reactions, yolk utilization in early embryos and development in general (Warner, 1987, 1989). Proteases have been found in lysosomes, mitochondria, the nucleus, plasma membrane, secretory granules, endoplasmic reticulum, ribosomes, peroxisomes, and the cytoplasm of a variety of cells and tissues (Twining, 1994). These proteases have a variety of functions such as degradation of proteins for cellular adaptation to starvation and differentiation, limited proteolysis during secretion and transport of proteins across membranes, limited proteolysis in the control of blood coagulation, fibrinolysis, blood pressure and fertilization, and in defense reactions involving the complement system (Holzer and Tschesche, 1979). Some of these processes are carried out in lysosomes, while others are carried out in the cytoplasm such as ubiquitin-mediated intracellular protein turnover (Woessner, 1991), and other protease-

mediated processes occur in the extracellular matrix (Stracke and Liotta, 1992). Collectively these processes regulate the lifetime of critical molecules responsible for cellular and extracellular functions. However, regulation of proteolysis in cells and tissues often requires control of multiple proteases that may be involved in a given degradative process. The irreversible modifications that proteases effect, from the cleavage of a peptide bond, to the determination of physiological processes, have profound consequences in biological systems. Numerous papers have been published on the characterization of proteases from different developmental stages using *Artemia franciscana* as an invertebrate model. An understanding of overall protease activity and protease regulation at different stages of development is necessary to assess the importance of protease - induced changes in the regulation of development.

Toward this objective, four major classes of proteases have been identified in eukaryotes based on the mechanism of catalysis: they are the serine, cysteine, aspartic and metallo-protease classes (Twining, 1994).

A. Serine proteases

These enzymes are characterized by the presence of a uniquely reactive serine side chain at the active center, and the catalytic mechanism of these proteases involves the covalent binding of substrates to this serine residues. In addition to serine, the active site contains histidine and aspartic acid in a catalytic triad with serine (Lolis and Petsko, 1990). Two major types of serine endoproteases are involved in a wide range of processes (Hirschi *et al.*, 1994). The first type includes digestive, lysosomal, coagulation,

fibrinolytic, and immune cell secretory enzymes; the second type of serine proteases are homologous to the bacterial protease subtilisin. These proteases evolved by a convergent mechanism relative to the chymotrypsin-serine protease family (Chen *et al.*, 1992). The structures of the two types of serine proteases are different and the catalytic residues are ordered differently. In the subtilisin type the order of the catalytic residues is Asp-His-Ser, whereas in other serine proteases it is His-Ser-Asp (Chen *et al.*, 1992).

B. Cysteine proteases

These proteases contain an essential cysteine residue that is involved in a covalent thioester intermediate complex with substrates (Dunn, 1989). The mechanism of peptide hydrolysis by cysteine proteases is similar to that of the serine proteases. A histidine residue in the active site acts as both a general acid and a general base. In contrast to serine proteases where the residue in the P_1 site of the enzyme is a major contributor to substrate specificity, in cysteine proteases the residue at the P_2 site is the major residue for the determination of substrate specificity (Hasnain *et al.*, 1992). The major protease in *Artemia* embryos was identified as a member of the cysteine group of proteases based on its inhibition by leupeptin, antipain, chymostatin, EP-475, and several other cysteine protease inhibitors (Bird and Roisen, 1986; Twining, 1994). The cysteine proteases from prokaryotes and eukaryotes characterized thus far fall into several evolutionarily related families. Three of these are represented by calpain from *Streptococcus*, clostripain from *Clostridium histolyticum* and papain from *Carica papaya*. The papain superfamily seems to be the predominant family in eukaryotes. Papain is a

23,400-dalton single polypeptide, and the structure and kinetics of the enzyme have been investigated extensively (Drenth *et al.*, 1976). The mammalian cysteine proteases known as cathepsins B, H, L, and S have a high degree of amino acid sequence homology with papain, making it likely that they all evolved from a common ancestor (Barrett and Fritz, 1986). The amino acid residues around the active-site cysteine (Cys-25 in papain) and essential histidine (His-159 in papain) have been highly conserved in the lysosomal cysteine proteases (Bond and Butler, 1987). In mammals, cathepsins B, H, L and S are all localized in lysosomes, while other cysteine proteases known as calpains A and B are found in the aqueous cytoplasm. Their levels of activity vary significantly from one tissue to another, from one cell type to another, and between cells within a tissue (Twining, 1994). Calpains are a special group of cysteine proteases that require calcium for activity (Goll *et al.*, 1992). Most mammalian cells contain two forms of this enzyme, calpain A (μ-calpain), which requires micromolar levels of calcium for activity, and calpain B (m-calpain), which requires millimolar levels of calcium. The lysosomal cathepsins are present in all mammalian cell types, with the exception of nucleated red blood cells. The name cathepsin was derived from a Greek term meaning 'to digest' (Bond and Butler, 1987). The numbers of lysosomes, and hence the protease content, varies in different cells and tissues and is particularly high in liver, spleen, kidney, and macrophages. The properties of the lysosomal cathepsins are very similar in different species and cell types. In general, these proteases are small (20,000-40,000 daltons), optimally active at acidic pH, and unstable at neutral and alkaline pH. Most of these enzymes are glycoproteins, and they are active against a wide range of peptides and

proteins as substrates (Katunuma, 1990). The best-characterized of these proteases are cathepsins B, L, H and D. Cathepsin B is also active against synthetic substrates containing arginine in the P_1 position, and has been thought of as a 'trypsin-like' enzyme with respect to substrate specificity (Bond and Butler, 1987). Cathepsin B acts as a peptidyldipeptidase, cleaving dipeptides from the C-terminus of protein and shows no endoproteolytic specificity for basic residues (Bond and Butler, 1987). The fact that cathepsin B displays both endopeptidase and exopeptidase activity depending on the substrate is not unique. Cathepsin L is considered to be one of the most powerful lysosomal proteases when assayed against protein substrates. The action of cathepsin L against insulin B chain indicates the enzyme has a preference for substrates with hydrophobic residues in the P_2 and P_3 positions (Barrett and Kirschke, 1981).

Cysteine proteases have been found in the gut of many insects and also in the digestive gland of invertebrate larvae and adults including the American lobster (Laycock *et al.*, 1989). The intense staining of a cysteine protease in the lumen of the midgut of second instar larvae of *Artemia franciscana* suggests that a cysteine protease may function in *Artemia* in a way similar to the lobster cysteine proteases (Warner *et al.*, 1995).

C. Aspartic proteases

These enzymes contain two aspartic residues at their active centers that are involved in catalysis (Szecsi, 1992). It is thought that general acid-base catalysis rather than the formation of covalent enzyme-substrate intermediates is operative in the

mechanism of these enzymes. Aspartic proteases have not been identified in prokaryotes but they are present in most eukaryotes. The major aspartic proteases in mammals are cathepsins D and E, renin, and the digestive enzymes pepsin and gastrin (Bond and Butler, 1987). Cathepsin D is found in the lysosomes of most cells, while cathepsin E is a non-lysosomal enzyme found in specific cells in different locations such as canaliculi of gastric parietal cells, renal proximal tubule cells, and bile canaliculi (Yonezawa *et al.*, 1993). Cathepsin D is a glycoprotein that resolves into several forms of similar molecular weight and different isoelectric points upon purification (Huang *et al.*, 1980). It has been proposed that cathepsin D plays a role in the pathological degradation of central nervous system proteins such as myelin basic protein. The inhibition of lysosomal proteolytic activity *in vivo* by pepstatin can be largely attributed to inhibition of cathepsin D activity (Dean and Barrett, 1976).

D. Metalloproteases

The last protease in this group, the metalloproteases contain a metal ion (usually zinc) at the active center. The metal ions are an integral part of the metalloprotease structure, and likely enhance the nucleophilicity of H_2O and polarize the peptide bond to be cleaved prior to nucleophilic attack (Woessner, 1991). The metalloproteases include the matrix metalloproteases and the astacin-like proteases (Twining, 1994). The matrix metalloprotease (MMP) family includes intestinal collagenase (MMP-1), neutrophil collagenase (MMP-8), gelatinase A (MMP-2, 72kDa), gelatinase B (MMP- 9, 92kDa), stromelysin-2 (MMP-10), and matrilysin (MMP-7, PUMP). The mammalian astasin-like proteases include meprin (rodents) and PABA-peptide hydrolyses (human). They are

found on the brush border membranes of the intestine and kidney cells (Werb *et al.*, 1992). Some proteases of unknown catalytic mechanism have been assigned to a new temporary subclass (Rawlings and Barrett, 1993). This subclass accommodates enzymes that either have not been sufficiently purified to allow assignment to one of the mechanistic classes or clearly do not fit one of the four classic groups described above. The class of proteases is usually determined according to the effects of protease inhibitors on enzyme activity (Barrett and Fritz, 1986). For example, all serine proteases are inhibited by diisopropyl fluorophosphate (DFP, DIFP, or diisopropyl phosphofluoridate, DIPF), and most by phenylmethanesulfonyl fluoride (PMSF). Cysteine proteases are inhibited by low concentrations of p-hydroxymercuribenzoate (pHMB, the hydrolysis product of p-chloromercuribenzoate, pCMB) and alkylating reagents such as iodoacetate, iodoacetamide, and N-ethyl-maleimide (NEM) (Bond and Butler, 1987). Aspartic proteases are inhibited specifically by pepstatins (acylated pentapeptides isolated from actinomycetes) (Bond and Butler, 1987). Diazoacetyl compounds, such as diazoacetyl-L-phe-methyl ester, also inhibit aspartyl proteases but will react with other proteases as well. Metalloproteases are inhibited by chelating agents such as EDTA (ethylenediamine-tetraacetic acid) and 1, 10-phenanthroline; some are inhibited by phosphoramidon (rhamnosephosphate-leu-trp) (Komiyama *et al.*, 1975).

3. Proteases in Diseases

Cellular proteases play a major role in diseases such as muscular dystrophy, diabetes, cachexia, cancer and multiple sclerosis (Bond and Butler, 1987; Twining, 1994).

Abnormal conditions can result in elevated levels of cysteine proteases. For example, the synovial cells that are attached to cartilage and bone sites affected by rheumatoid joint erosion display an enhanced transcription of cathepsin B when compared with fibroblasts (Trabandt *et al.*, 1991). Cysteine proteases are released from cells into the extracellular matrix in pathological situations, such as in periodontal diseases (Saito and Sinohara, 1993), and during tumor cells invasion of other tissues as in cancer (Stetler-Stevenson *et al.*, 1993).

The importance of the control mechanisms of proteases is realized when an imbalance occurs between inhibitor and activator level. Examples of conditions that are associated with excessive amounts of proteolytic activity include emphysema (Blank and Brantly, 1994), arthritis (Levine *et al.*, 1993), malignancy (Blasi, 1993), pemphigus vulgaris (Reinartz *et al.*, 1993) and bullous pemphigoid (Kramer and Reinitz, 1993). Excessive protease inhibitor activity is associated with the formation of amyloid deposits associated with Alzheimer's disease (Tooyama *et al.*, 1993) and Downs syndrome (Miyazaki *et al.*, 1993).

4. Regulation of Protease Activity in Eukaryotic Cells

Regulation of cellular protease activity is affected in many ways such as by compartmentalization, interaction with inhibitors and activators, regulation of protease synthesis and degradation, pHi, structure of potential substrates and regulation by metabolites.

A. Compartmentalization

The action of a protease may be limited by its subcellular localization. For example, the localization of the cathepsins in the lysosome provides them with an acidic environment for activity and stability, while restricting their action to those proteins that enter this compartment (Bond and Butler, 1987). Leakage of the cathepsins from lysosomes could result in their inactivation by the higher pHi and inhibitors in the cytoplasm (Bond and Butler, 1987). Similarly, attachment of proteases to membranes results in a loss of freedom which limits their accessibility to substrates. Membrane association may have the effect of increasing the concentration of specific substrates that interact with membrane components (e.g. receptors) (Bird and Roisen, 1986). The "processing" proteases such as enteropeptidases are strategically localized in membranes or secretory granules, which enable access to substrate proteins in specific conformations. The storage of proteases in secretory vesicles ensures specific interactions between protease and substrate (Dunn, 1989).

B. Synthesis and Degradation of Proteases as Regulatory Events

Since there are many examples of tissue-specific proteases, the regulation of protease activity in different cell types is an important factor. For those proteases that are present in most mammalian cells, large variations in the concentrations of the proteases are found in different tissues. For example, immunohistochemical techniques have confirmed that the concentration of lysosomal proteases and presumably their activity, varies with cell type even within one tissue. Also, as for any protein, the concentration of

cellular proteases may be controlled by the rates of protein synthesis and degradation (Beynon and Bond, 1986).

C. Role of Protease Inhibitors and Activators

It is likely that endogenous intracellular inhibitors are important in the control of cellular proteases. An inhibitor can protect a protease from destruction just like a coenzyme can protect the apoenzyme against proteolysis (Bond and Butler, 1987). Substrates and allosteric activators can 'protect' a protein from proteolytic inactivation, while inhibitors can render a protein insensitive to the action of a protease. Thus, protease activities in cells can fluctuate due to changes in inhibitor concentration rather than protease concentration (Barrett and Fritz, 1986). Two types of polypeptide inhibitors have been discovered in cells: they are the cystatins and stefins which inhibit lysosomal cathepsins, and calpastatin which inhibits the calpains (Bond and Butler, 1987). The cystatin superfamily is comprised of three related groups: 1) the stefin family containing small proteins (11 kilodaltons) that do not contain disulfide bonds; 2) the cystatin family containing slightly larger proteins (13 kilodaltons) that have two disulfide loops; and 3) the kininogen family, which consists of more complex proteins (50-120 kilodaltons) that contain nine disulfide bonds. The kininogens are found extracellularly in mammals and are identical to the alpha-1 cysteine protease inhibitors (Muller and Fritz, 1985). The cystatins and stefins are found intracellularly (in the aqueous cytoplasm) and may function to prevent inappropriate proteolysis in the cytoplasm by lysosomal enzymes (Turk *et al.*, 1985).

Calpastatin is a protein and specific inhibitor of the calpains (Takano *et al.*, 1986). It has been isolated from a variety of mammalian and avian tissues. This protein is equally effective in inhibiting calpain A and B, but it does not inhibit any other type of protease. The molecular weight of the calpastatins range from 24,000 to 400,000 daltons according to the source and the method of extraction. The high molecular weight species are capable of binding several calpain molecules simultaneously (Nakamura *et al.*, 1985). The low molecular weight calpastatins are proteins with inhibitor domains that may have evolved from the larger forms of the inhibitor. Calpastatin binds to the large subunit of calpain in the presence of high concentrations of calcium and is not cleaved by the protease.

A protein activator of calpains has been isolated from brain tissue, this polypeptide (17-20 kilodaltons) can stimulate calpain activity, but it does not act by altering the calcium sensitivity of either form of the protease (DeMartino and Blumenthal, 1982).

D. Role of Metabolites in Protease Regulation

The intracellular concentrations of small metabolites may affect proteases in several ways. For example, calcium ions may stimulate calpain activity as well as increase autolysis of the enzyme and promote binding of the enzyme to calpastatin (Pontremoli and Melloni, 1986). Similarly, intracellular nucleotide concentration, especially of ATP, may affect the activity of ATP-dependent proteases as well as the energy-dependent pumps that allow acidification of endosomes, lysosomes, and other

vesicles (Mellman *et al.*, 1986). Physiological concentrations of fatty acids (<100μm) can activate the proteosomes in skeletal muscle (Dahlmann *et al.*, 1983). This form of regulation may be important in diabetes where the intracellular concentration of fatty acids in skeletal muscle is increased (Rogers *et al.*, 1986).

E. Other Factors

Degradation of intracellular proteins to amino acids and small peptides is at least partly determined by the structural characteristics of the protein substrates, and protease regulation may occur through modulation of potential protein substrate structure by intrinsic and extrinsic factors (Bond and Butler, 1987). The heterogeneity in half-lives observed for different proteins in a cell indicates that some proteins are more susceptible to degradation than are others. The observations of 'abnormal' protein structure is determinative in degradation rates (Bond and Butler, 1987). Substrate cofactor levels and intracellular pH (pHi), can also regulate intracellular protease activity. Localized changes in pHi can result in association and dissociation of proteases and their substrates and/or endogenous inhibitors (Twining, 1994). In addition, covalent modifications of proteins such as ubiquitin-conjugation or oxidation may be important in "targeting" proteins for degradation (Rivett, 1986). Therefore, it is clear that proteolytic activity can be regulated in numerous ways both inside and outside of cells.

5. General Functions of Intracellular Proteases in Development

There is such a diversity of processes in which cellular proteases function that it is impossible to cover this aspect adequately. So far, little is known of the role of

intracellular proteases in developing animal embryos, especially young and old larvae. Proteases have been implicated in a variety of metabolic events during early development including activation of metabolically repressed ribosomes (Monroy *et al.*, 1971) and yolk utilization (Williams, 1967). Specific intracellular proteases cleave the signal peptide found on most proteins and the pro-peptide found on many hormones, cytokines and enzymes (Hazuda *et al.*, 1990). Essentially, proteases function to create biologically active molecules or degrade biologically active proteins and peptides. Cytosolic enzymes such as prolyl endopeptidase, are also proteases. The lysosomal cathepsins and ATP-dependent proteases in the cytoplasm are involved in protein degradation or in targeting of previously active molecules to extensive hydrolysis. These catabolic proteases have a role in the removal of defective (abnormal) or normal polypeptides from cells. They control the concentration of polypeptides and enzymes in cells, and generate amino acids from proteins for the synthesis of new proteins in the constant renewal of cellular contents. These two types of protease functions do not always reflect the multitude of protease dependent processes in the cell. Processes such as cytoskeleton reorganization, myoblast fusion, differentiation, protein synthesis, fertilization, growth, apoptosis and creation of immunologically recognizable molecules all require protease intervention (deDuve, 1983). The irreversible modifications that proteases effect processes from cleavage of a peptide bond to changes in protein conformation have profound consequences in the determination of physiological processes.

Mammalian cells contain two distinct proteolytic pathways that are involved in different aspects of protein breakdown. Proteins that enter the cell from the extracellular

milieu (such as receptor-mediated endocytosed proteins) are degraded in lysosomes (Cierchanover and Schwartz, 1994). Also, lysosomal degradation of intracellular proteins can occur but mostly under stressed conditions (Twining, 1994). Non-lysosomal mechanisms are mainly responsible for the highly selective turnover of intracellular proteins that occurs under basal metabolic conditions (Bond and Butler, 1987).

An important non-lysosomal proteolytic pathway is the ubiquitin-dependent proteosome mechanism in which proteins are degraded by a 26S protease complex following conjugation by multiple molecules of ubiquitin (Cierchanover and Schwartz, 1994). The "catalytic core" of the complex is a 20S protease complex also known as the proteasome. The ubiquitin-proteasome mechanism is involved in complete destruction of its protein substrates (Cierchanover and Schwartz, 1994). Ubiquitin, the major component of this proteolytic mechanism is a small (76 amino acids), heat stable, universally conserved protein which exists in all eukaryotic cells either free or convalently joined to proteins in the cell nucleus, cytoplasm or plasma membrane (Wilkinson, 1988). Ubiquitin has been shown to play a key role in a variety of cellular processes, such as protein degradation, maintenance of chromatin structure, cell surface receptor function and differential regulation of gene expression (Monia *et al.*, 1990). Ubiquitination is the ATP-dependent catalysis of proteins. In this process, ubiquitin moieties are covalently linked to target proteins by isopeptide bonds in an ATP-requiring reaction (Cierchanover and Schwartz, 1994). It is estimated that at least 90% of the short-lived proteins within a cell are degraded by the ubiquitin-dependent process (Gregory *et al.*, 1985). Metabolic instability of a protein is a characteristic that ensures

the regulation of its intracellular concentration through changes in the rate of synthesis and degradation.

6. The Intracellular Proteases in *Artemia* Embryos/Larvae

In 1960 studies were initiated to characterize proteases in dormant embryos of the brine shrimp *Artemia franciscana* by Dr. Dutrieu in France (Dutrieu, 1960). Since that time, very few developing invertebrate systems have been investigated for the presence and function of proteases and protease regulators in development. In this respect, *Artemia* is the most studied invertebrate system. Studies of *Artemia* have revealed that both active and latent proteases are present in embryos and larvae, and that these enzymes may be involved in such diverse functions as yolk metabolism (Perona and Vallejo, 1985), protein synthesis regulation (Twardowski *et al.*, 1976) and RNA synthesis regulation (Osuna *et al.*, 1977). Studies from our laboratory showed that dormant and prehatched embryos of *Artemia* contain a cysteine protease that accounts for about 90% of total protease activity in crude homogenates (Warner and Shridhar, 1985). Subsequently, the properties of *Artemia* embryo cysteine proteases have been studied extensively, and it was determined that about 25% of embryo cysteine protease is active and 75% inactive in crude cytoplasmic preparation (Warner, 1987, 1989). As well, total cysteine protease represents about 0.74% of soluble proteins *Artemia* embryos. A preliminary study indicated that the cysteine protease exists in several isoforms with pI values between pH 4.6 and 6.2 (Warner and Shridhar, 1985). The native enzyme has a molecular weight of about 60 kilodaltons and is composed of subunits of 32 and 28 kilodaltons as determined by SDS-polyacrylamide gel electrophoresis (Warner and

Shridhar, 1980). The enzyme has a high specificity for elongation factor 2 (EF-2), but hydrolyzes other proteins including yolk and soluble proteins of *Artemia*. Embryo cysteine protease can be activated by EDTA, dithiothreitol and mercaptoethanol and inhibited by Cu^{2+}, Hg^{2+}, iodoacetate and E-64. In embryos of *Artemia*, 84% of cysteine protease is found in the cytoplasm: during development, some of the cysteine protease becomes associated with a sedimentable fraction that includes mitochondria (Lu and Warner, 1991; Warner *et al.*, 1995).

A major developmental event that occurs in *Artemia* following resumption of development is yolk platelet utilization. Yolk platelets, the major storage organelles of proteins and lipids, are composed mainly of lipovitellin in *Artemia*. An important property of the encysted embryo cytoplasmic protease is its ability to catalyze the hydrolysis of yolk proteins under physiological conditions. Following resumption of development of the encysted embryo of *Artemia*, 50% of the yolk platelets are utilized within 24 hours concomitant with the degradation of lipovitellin (Warner and Shridhar, 1980). Using lipovitellin and cyteine protease isolated from dormant gastrulae of *Artemia*, Warner and Shridhar observed a pattern of lipovitellin hydrolysis *in vitro* which closely assembles that found in intact gastrulae and nauplii (Warner and Shridhar, 1985). This correlation of *in vitro* lipovitellin degradation with the *in vivo* degradation pattern suggests a role for the cysteine protease in yolk metabolism (Lu and Warner, 1991).

In dormant embryos of *Artemia*, cysteine protease activity is regulated by at least two types of low molecular weight protease inhibitors in the cytoplasm, one dializable and the other non-dializable (Nagainis and Warner, 1979). During the first 6 to 9 hours

after resumption of development, the activity of the non-dializable cysteine protease inhibitors (CPI) increases by approximately 60% and then declines to nearly undetectable levels in 2nd instar larvae. Moreover, as the CPI activity decreases, there is an apparent increase (at least *in vitro*) in cysteine protease activity in crude preparation of cytoplasm. This observation suggests that the cysteine protease inhibitor gene(s) may be developmentally regulated in *Artemia* by stage-specific transcription or post-transcriptional control mechanisms (Nagainis and Warner, 1979).

Shortly after hatching begins, a dramatic increase occurs in "alkaline" protease activity in the cytoplasmic fraction (Osuna *et al.*, 1977). Four "alkaline" proteases have been found in second instar nauplius larvae of *Artemia*. The role of these proteases in protein metabolism during early development is not known, but they may reflect gut development (Warner, 1989) or preparation for molting in *Artemia* (Warner *et al.*, 1995).

Many researchers have attempted to determine whether changes in cysteine protease activity in *Artemia* correlate with changes in the development of their organism. However, due to conflicting reports on activity levels where measurements have been influenced by factors which alter the enzyme activity, an assessment of the functional state of the CP activity in embryos has been debated (Warner, 1989). Factors which affect these measurments include endogenous protease inhibitors, buffer composition, sterilization agents, pH, temperature, ionic environment and even the commercial packing method (Bond and Butler, 1987; Twining, 1994).

An immunocytochemical method has been used to determine the localization and potential role of cysteine proteases in development of young larvae (Warner *et al.*, 1995). Results show that in prenauplius larvae there is intense staining for the protease in the epidermal layer in the posterior region, and diffuse staining for the protease throughout the prenauplius larva. In first instar larvae cysteine-protease staining becomes intense in the area where a reticulum-like pattern emerges in cells with an abundance of yolk-platelets. In second instar larvae, cysteine-protease staining becomes intense in the outer zone of epidermal cells and in the basal and apical zones of cells of the midgut. Analysis for the subcellular localization of the protease in the epidermis and midgut of young larvae using immunogold electron microscopy suggests that most of the protease is located in the cytoplasm and extracellular matrix adjacent to these cells. The fact that cysteine protease is not found within yolk platelets, but is capable of degrading yolk protein (lipovitellin) under physiological conditions (*in vitro*), is consistent with the view that yolk-protein metabolism occurs mainly at the outer surface of platelets (Warner *et al.*, 1995). These observations support the view that yolk protein (lipovitellin) is catabolized during early development in *Artemia franciscana* mainly by a non-lysosomal mechanism. The appearance of high concentrations of cysteine protease in epidermal cells of the prenauplius larva, particularly in the posterior region shortly after emergence of the embryo from its shell, and subsequent disappearance from this region in first instar larvae also suggest a role for the cysteine protease in either emergence or hatching processes. Additional studies are needed on the localization and function of the cysteine proteases in midgut cells of *Artemia*, especially to determine whether specific isoforms of

the cysteine protease are functional in midgut cells and different from those in epidermal cells (Warner *et al.*. 1995).

The purpose of this study is to isolate, and to characterize the major isoforms of cysteine protease in embryos and larvae of *Artemia*, and to determine whether the various isoforms of the cysteine proteases are expressed differentially during development.

Materials and Methods

1. Source of Dormant Cysts of *Artemia*

Dried, dormant cysts of *Artemia franciscana* were used as starting materials. They were obtained from Sanders Brine Shrimps Company (Ogden, Utah, U.S.A., lot number 12715) and keep frozen until needed.

2. Preparation of Embryo Homogenate

Dry *Artemia franciscana* cysts (4.2 grams) were hydrated overnight in 50% sea water at 0^0C to minimize development beyond the cyst stage (Warner, 1989). Floating cysts and other debris were removed with suction, and the cysts which sedimented in the sea water were collected on a sintered glass filter under vacuum and washed as described previously (Warner, 1989). Ten grams (wet weight) or about $1x10^6$ of fully hydrated cysts were used in each experiment. Embryos were collected immediately after hydration (0-h) and at the late gastrula stage (12-h), and homogenized initially with about 10 ml of homogenization buffer using a mortar and pestle, then the volume of the ice-cold homogenization buffer was increased to about 50 ml. The homogenization buffer contained 150mM sorbital, 70mM K-gluconate, 5mM KH_2PO_4 and 35mM HEPES, pH 6.8 (Warner *et al.*, 1997).

3. Preparation of Larval Homogenate

Ten grams of hydrated dormant cysts were incubated in 500 ml of Millipore-filtered (0.45μm) sea water fortified with an antibiotic-antimycotic preparation (lot number: 24k1553) containing 20 units/ml of penicillin G Sodium, 20 μg/ml of streptomycin sulfate and 50 ng/ml amphotericin B as Fungizone for 18 hours at 28-30^{0}C with gentle agitation (Lu and Warner, 1991). Newly hatched young larvae were collected at total incubation time of 18 hours (0-h nauplii, first instar) of incubation as described previously (Warner *et al.*, 1979) and divided into three portions for further incubation. Larvae not taken for the 0-h sample were returned to fresh sea water containing the antibiotic-antimycotic for further incubation. Aliquots of larvae were taken at total incubation time of 44 hours (26-h larvae, second instar) and their concentration determined by counting the total number of larvae in 200 μl aliquots, diluted with distilled water and transferred to strips of filter paper (Warner *et al.*, 1979). The 12-h embryos, 0-h and 26-h larvae were collected on a cloth filter and stored at -20^{0}C until needed. The frozen larvae were placed in a prechilled glass homogenizer and homogenized at 0^{0}C (to minimize autolysis) with the homogenization buffer described above, except this buffer contained soybean trypsin inhibitor (10 μg/ml) and phenylmethylsulfonyl fluoride (0.1mM) to limit serine protease degradation (Lu and Warner, 1991). For comparison, newly hatched nauplius larvae, 0-h (18-h total incubation time) larvae were considered to be first instar larva, while those sampled at 44h total incubation time (26-h larvae) were taken as second instar larvae (Hootman and Conte, 1974).

4. Cysteine Protease Activity Determination

The cysteine protease activity in each sample to be tested was measured using the TNBS method as follows (Nagainis and Warner, 1979). Each reaction vessel contained the following components at the final concentration indicated: 0.1M NaOAc, pH 4.0, 4mg/ml protamine sulfate, 0.001M EDTA, 0.5mM DTT and 50 µl of enzyme solution diluted to give linear response (Warner and Shridhar, 1985). Aliquots were taken over 30 minutes incubation at 40^0C. For column fractions and gel slice analysis, 50 µl were taken from each reaction vessel at 0 and 30 minutes, and the reaction stopped by adding 950 µl of a solution containing 53mM NaOH and 53mM $N_2B_4O_7$ at 0^0C. The amino groups released in the reaction were measured by adding 25 µl of 0.22M trinitrobenzene sulfonic acid (TNBS) to the NaOH/borate solution containing the reaction aliquot. Color development of the TNBS reaction was stopped after 15 minutes incubation at room temperature by adding 2ml of a solution containing 0.1 M NaH_2PO_4 and 15mM Na_2SO_3 to each aliquot with the OH/borate stop reagent. The extent of amino groups liberated in each reaction vessel was determined by measuring the absorption at a wavelength of 420 nm (A_{420}) using an extinction coefficient of 0.00575 A_{420}/nmole, where one milliunit of CP activity (mEU) is defined as the release of one nmol min^{-1} of amino peptide from the substrate (protamine sulfate) at pH 4 and 40^0C. To determine the total milli-units of enzyme activity (mEU) in each preparation, aliquots were taken at four time points (10, 20, 30 and 40 minutes), during the reaction to measure the total mEU of CP activity in each preparation (See Appendix A).

5. Determination of the Protein Content and Specific Activity of Various Cysteine Protease Preparations

The total amount of protein in each preparation was determined using the BCA microassay method (See Appendix B). Several dilutions of an albumin standard containing from 1 to 12μg/ml were used to construct a standard curve each time the assay was performed.

The specific activity of each cysteine protease sample was determined by dividing the mEU of protease activity by the microgram of protein in the protease sample assayed.

6. Isolation and Partial Purification of the Soluble Cysteine Protease from *Artemia* Embryos and Larvae

The homogenate of embryos or larvae (see above) was centrifuged at 1650xg, for 15 minutes at 0-4^{0}C (Sorvall, model RC-2B) to sediment the empty shells, yolk platelets and nuclei. The crude sediment was resuspended in 1/10 volume of homogenization buffer and centrifuged again under the same conditions. The sediments were discarded and the 1500xg supernatants were combined and filtered through cheesecloth to remove excess lipid and other floating debris. The filtrate was then centrifuged at 15000xg for 30 minutes to sediment mitochondria and lysosomes (Lu and Warner, 1991). The resulting sediment was resuspended in 1/10 volume of homogenization buffer and centrifuged again at 15,000 xg for 15 minutes. The 15,000 xg supernatants were combined and retained as the cytoplasmic fraction. The resulting 15,000 xg sediments, containing the mitochondria/lysosome fraction was saved at -20^{0}C. All steps were carried out at 0-4^{0}C

and the samples were handled quantitatively. All fractions were stored at -20^{0}C prior to use. The post-mitochondrial/lysosomal supernatant which contains most of the cysteine protease was collected, concentrated using pressure dialysis through a concentrator fitted with an Amicon YM-10 filter, and centrifuged at 10,000 rpm for 10 minutes to remove any denatured protein. The sample was applied to the surface of a G-150 Sephadex filtration column (SF, 65.5cm x 1.4cm) that had been pre-equilibrated with column buffer containing 25 mM KCl, 15 mM K_2HPO_4 and 10% glycerol, pH 6.8. This step separates the major cysteine protease from most large and small proteins in the cytoplasm including the cysteine protease inhibitors. The cysteine protease was washed through the column with elution (equilibration) buffer at 0-4^{0}C, and fractions of 4ml were collected every 30 minutes. The protein elution profile was determined by measuring the absorbance of each fraction at 280 nm in a Beckman spectrophotometer (Model 25). Column fractions were assayed for cysteine protease activity using the TNBS method (Nagainis and Warner, 1979). Column fractions with the highest cysteine protease activity were pooled and concentrated using an Amicon YM-10 filter at 30 psi nitrogen pressure and 4^{0}C. The concentrated cysteine protease preparation was analyzed for protein content and protease activity then stored at -20^{0}C until needed further.

7. Purification of the Cysteine Protease by Fast Protein Liquid Chromatography (FPLC)

To further purify the total cysteine protease, the concentrated protease fraction from the G-150 Sephadex column was applied to a Mono Q column pre-equilibrated with

buffer containing 10% glycerol, 15mM K_2HPO_4 and 25mM KCl, pH 6.8. The protein was eluted from the column using a linear gradient of KCl in the column buffer, from 25mM to 275mM over 60 minutes at a flow rate of 1 ml/min, and fractions of 0.33ml were collected. Selected column fractions were analyzed for cysteine protease activity as described above. The contents of all fractions with cysteine protease were pooled and concentrated to less than 200µl using Amicon Centricon-10 concentrators.

8. Analysis of Cysteine Protease Isoforms by Isoelectric Focusing

An isoelectrofocusing mini cell apparatus (H.B.I. Model 3) was assembled and connected to a refrigerated bath and circulator (D8-G). Isoelectric focusing of the Mono Q column purified cysteine protease was carried out in glass columns (4 x 100 mm) with 150ml of freshly prepared 40mM NaOH in the cathode compartment and 150 ml of freshly prepared 20mM H_3PO_4 in the anode compartment. The running gels consisted of the following components at the final concentration indicated: 12.6% glycerol, 6% acrylamide, and 0.16% bis acryl, 0.006% TEMED, 3.86% BioRad Biolyte 4/6 and 1.92% 3.5/10 carrier ampholytes and 1.2% ammonium persulfate. In one glass tube, standard proteins with known pI values were run to assess the pI value of the cysteine proteases run in parallel, but separate glass columns. The IEF standard preparation contained amyloglucosidase (pI 3.55) , methyl red (pI 3.75), soybean trypsin inhibitor (pI 4.55), β-lactoglobulin A (pI 5.13), carbonic anhydrase (pI 5.85) and myoglobin (pI 7.16). Prior to isoelectric focusing of the cysteine proteases, each preparation was treated with 1mM 2-PDS (2, 2-dithiodipyridine) to inhibit (reversibly) the cysteine protease activity during the focusing. The isoelectric focusing analyses were run at 350 Volts and 10mA (starting

condition) for 18 hours or until the current reached a constant value. After the run, the gel was removed from the glass tube and its length determined. The positions of the brown (myoglobin) and blue (phycocyanin) bands in the standard (unstained) gel were measured, then the gel was stained as described below to determine the position of the other standard proteins. To determine the position of the cysteine protease in the sample gels, each gel was washed in ice-cold distilled water for 1 hour with at least 2 changes, cut into sections of 2mm each, then incubated overnight at 0^0C in buffer containing 50mM NaCl, 1mM DTT, 10% glycerol, 1mM EDTA and 0.015M KH_2PO_4 at pH 6.8 to elute the protein from the gel slices. Proteins in unsliced gels were stained with Coomassie brilliant blue (R-250) in 7.5% acetic acid and 50% methanol, and the gel destained in a solution containing 7.5% acetic acid and 5% methanol. The assay to determine the enzyme activity in each gel section was described previously. Based on the focusing position of the IEF standards, gel slices containing cysteine protease activity were assigned a pI value.

Results

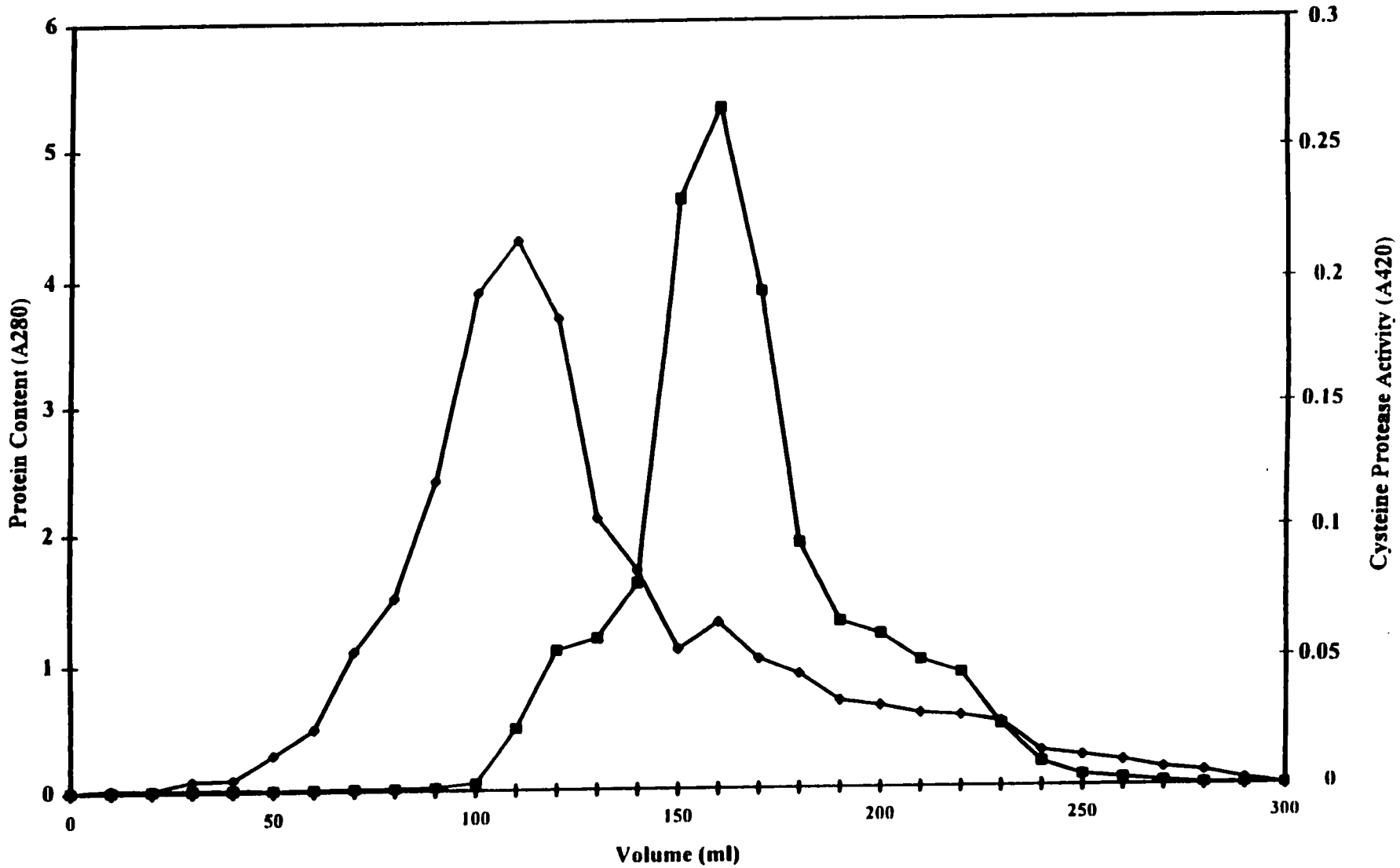
1. Cysteine Protease Purified from G-150 Sephadex Column

Gel filtration is a simple and reliable chromatographic method for separating molecules according to size. In the fractionation of proteins by gel filtration we have selected a gel that gives a good flow rate and the best resolution possible. Generally gel filtration media of large particle size give high flow rates with considerable band spreading, while fine grade media give slower flow rates but higher resolution and less zone spreading. In our experiments, we found that G-150 Sephadex (1.5 x 65cm, superfine) gave optimal separation of the proteins of interest. The results in Figure 1 show the elution profile of the protein and cysteine protease activity in the post-mitochondrial supernate of dormant cysts of *Artemia*. These results show that the bulk of the protein eluted between 80ml and 130ml, while most of the cysteine protease activity eluted between 140ml and 180ml.

When the post-mitochondrial fraction of 12-h embryos, 0-h and 26-h larvae were chromatographed on the Sephadex G-150 SF column, similar results were obtained (data not shown). In all cases those column fractions with highest cysteine protease activity (140-180ml) were pooled and concentrated to less than 1ml by pressure filtration (YM-10 membrane, Amicon Corp.).

Figure 1. Elution Profile of Protein and Cysteine Protease Activity from a Column of G-150 Sephadex

Protein from the post-mitochondrial fraction of 0-h embryos in a volume of 5ml was applied to a column of Sephadex G-150 (1.5 x 65cm, superfine) and eluted with a buffer containing 25mM KCl, 15mM potassium phosphate and 10% glycerol, pH 6.8 at 4^0C. Column fractions were assayed for protein (-◆-) by measuring absorbance at 280 nm, and for cysteine protease activity (-■-) by measuring absorbance at 420 nm as described in the Materials and Methods. The contents of the column fractions between 140 and 180ml were pooled, concentrated using a YM-10 filter, then stored at -10^0C.


Protein Content (A280)
Cysteine Protease Activity (A420)
Volume (ml)
0
1
2
3
4
5
6
0
0.05
0.1
0.15
0.2
0.25
0.3
0
50
100
150
200
250
300

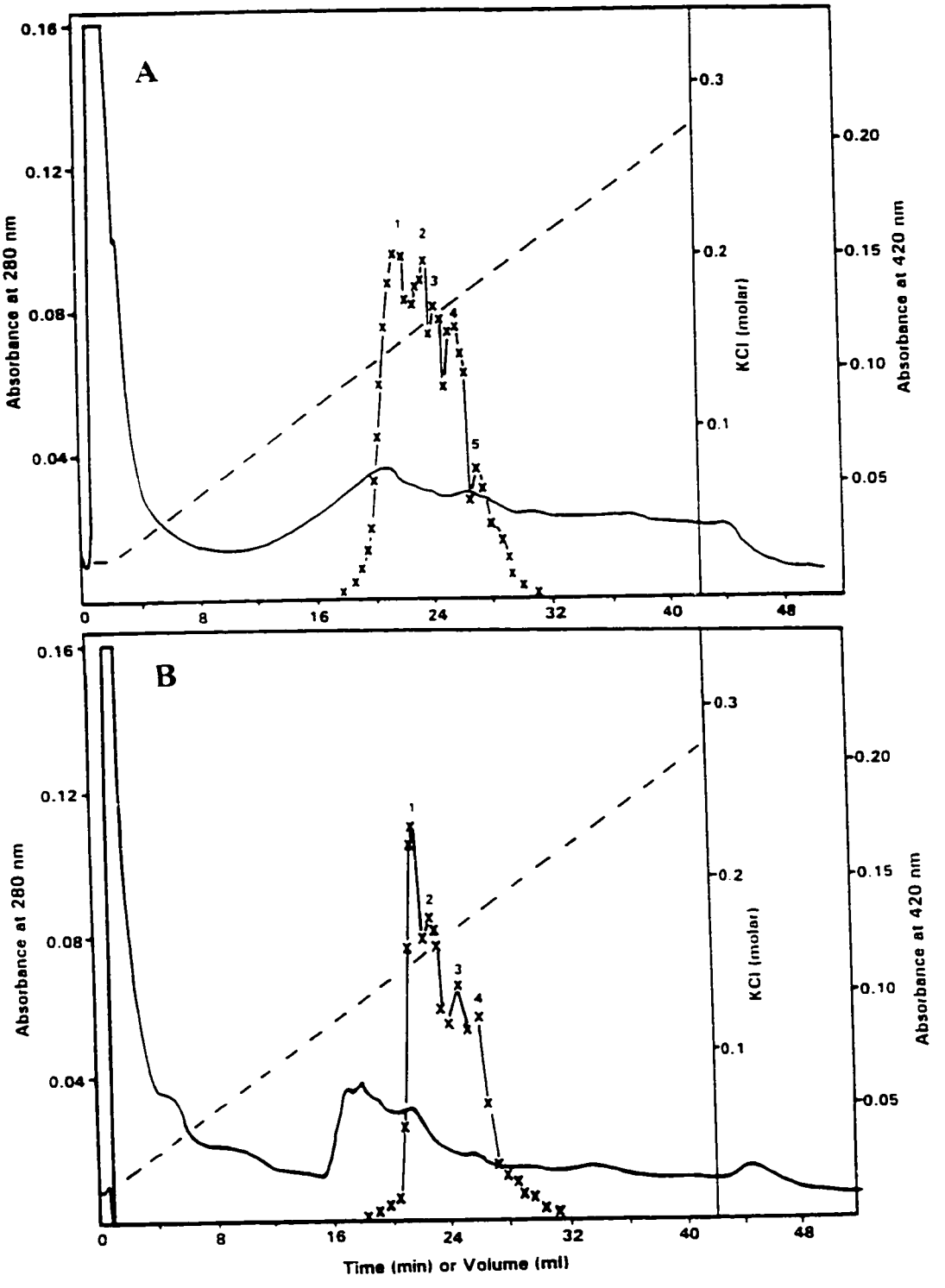
The soluble proteins in the mitochondrial/lysosomal fractions were also fractionated on the Sephadex column (data not shown). These results suggest that the CP preparation may have minor amounts of higher and lower molecular weight forms of the protease, but these fractions were not studied further in this study. In all experiments the protein content of each fraction was estimated by measuring the absorbance at 280 nm, while the cysteine protease activity was measured at 420 nm using the TNBS assay (see Materials and Methods). The enzyme activity was linear over at least 40 minutes incubation (see Appendix A).

2. Fractionation of the Major Cysteine Proteases of *Artemia* Embryos and Larvae by Fast Protein Liquid Chromatography

The partially purified cysteine proteases obtained from the G-150 Sephadex column, from both the cytoplasmic and mitochondria/lysosome fractions of embryos and larvae of *Artemia*, were fractionated further by fast protein liquid chromatography (FPLC) on a Mono Q column as described in Materials and Methods. All samples were run sequentially, and in pairs, with protein from the cytoplasm of 0-h embryos as a control. The results in Figure 2 show that total cysteine proteases from dormant (0-h) and 12-h embryos have a similar isoform pattern with each containing four to five distinct peaks of cysteine protease activity. The cysteine protease isoforms from the 12-h embryos eluted more "sharply" than those from 0-h embryos for reasons which are not clear. Also, the 12-h embryos lack a minor CP isoform (CP-5) found in 0-h embryos. These data suggest that the cysteine protease fraction from early embryos of *Artemia*

Figure 2. Elution Profiles from a Mono Q Column of Cysteine Protease Activity in the Cytoplasm of 0-h and 12-h Embryos of *Artemia*

A. Cysteine protease activity from 0-h embryos. B. Cysteine protease activity from 12-h embryos. In 0-h and 12-h embryos multiple cysteine protease activities were partially resolved using a linear KCl gradient constructed from two buffers: buffer A which contained 15mM KH_2PO_4, 25mM KCl, and 10% glycerol, and buffer B which contained 15mM KH_2PO_4, 275mM KCl, and 10% glycerol. The isoforms were designated as CP-1, CP-2, CP-3, CP-4 and CP-5. The peaks were aligned and analyzed on the basis of their elution position in the KCl concentration gradient of the eluting buffer. For example, CP-1 eluted at a KCl concentration 0.15 M KCl, while CP-2 eluted at 0.16 M KCl. Protein eluting from the column was monitored continuously at 280 nm (——), whereas the CP activity in various column fractions was measured using the TNBS method and assayed at 420 nm (x——x). The KCl concentration gradient (------) is linear in eluting buffer.


A
B
Absorbance at 280 nm
KCl (molar)
Absorbance at 420 nm
Time (min) or Volume (ml)
0.16
0.12
0.08
0.04
0.3
0.2
0.1
0.20
0.15
0.10
0.05
0
8
16
24
32
40
48
1
2
3
4
5

contains at least four, possibly five isoforms which have been designated as CP-1, CP-2, CP-3, CP-4 and CP-5 (Fig. 2, panel A).

The results in Figure 3 show the elution profile of the cysteine protease activity from the cytoplasm of 0-h larvae compared with that of 0-h embryos. In 0-h larvae the CP isoform pattern is different from that in 0-h embryos, and suggests that 0-h larvae lack CP-1 and have reduced amounts of CP-2 compared to 0-h embryos. CP-3 appears to be the dominant isoform in newly hatched 0-h (first instar) larvae.

The results in Figure 4 compare the cysteine protease activity in the cytoplasm of 26-h larvae with that of 0-h embryos. These results show that the CP in the cytoplasm of 26-h larvae have a similar isoform pattern to that of 0-h larvae with each lacking CP-1 and having reduced amounts of CP-2 compared to the cytoplasm of 0-h embryos.

The results in Figure 5 compare the cysteine protease activity in the mitochondria/lysosome fraction of 12-h embryos with the cysteine protease activity in the cytoplasm of 0-h embryos. Inspection of these results show that the dominant CP fractions in the mitochondria/lysosome fraction are CP-3, CP-4, while the dominant CP activities in the cytoplasm of embryos (ie, CP-1 and CP-2) are absent from mitochondria/lysosomes in *Artemia*.

The result in Figure 6 compare the cysteine protease activity in the mitochondria/lysosome fraction of 0-h larvae with the cysteine protease activity from the cytoplasm of 0-h embryos. Inspection of these results show the dominant CP isoforms in

Figure 3. Elution Profiles from a Mono Q Column of Cysteine Protease Activity in the Cytoplasm of 0-h Embryos and 0-h Larvae of *Artemia*

A. Cysteine protease activity from 0-h embryos. B. Cysteine protease activity from 0-h larvae. In 0-h embryos multiple cysteine protease activities were partially resolved using a linear gradient of KCl as described in the legend to Figure 2. The protease isoforms were designated as CP-1, CP-2, CP-3, CP-4 and CP-5. The peaks were aligned and identified on the basis of their elution position in the KCl gradient. In 0-h larvae (Panel B) CP-1 is absent and CP-2 is considerably reduced, while CP-3, the dominant CP isoform eluted at 0.17 M KCl. The protein content, CP activity, and KCl concentration in the buffer are as described in the legend to Figure 2.

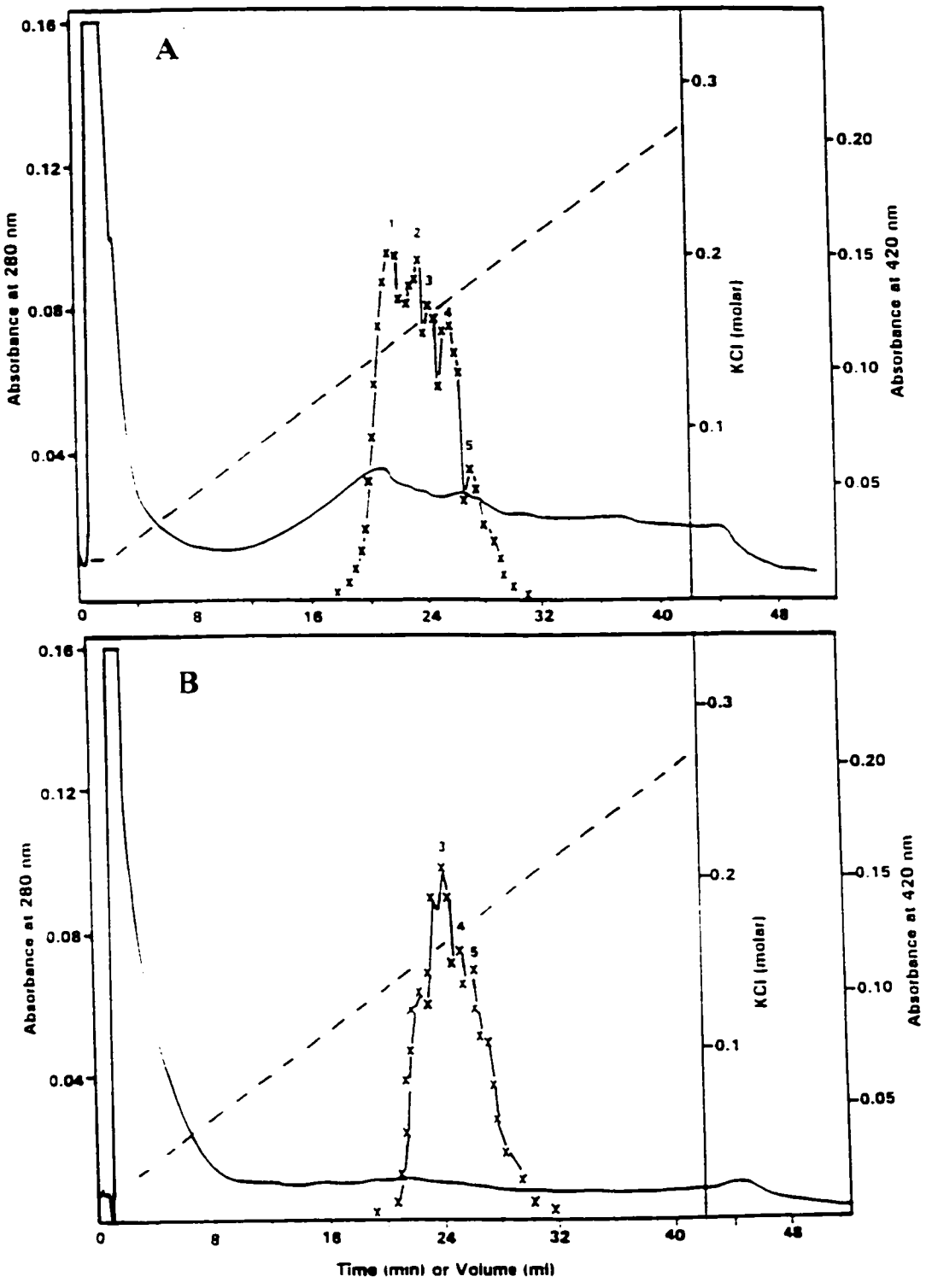

A
B
Absorbance at 280 nm
KCl (molar)
Absorbance at 420 nm
Time (min) or Volume (ml)
0.16
0.12
0.08
0.04
0.3
0.2
0.1
0.20
0.15
0.10
0.05
0
8
16
24
32
40
48
1
2
3
4
5

Figure 4. Elution Profiles from a Mono Q Column of Cysteine Protease Activity in the Cytoplasm of 0-h Embryos and 26-h Larvae of *Artemia*

A. Cysteine protease activity from cytoplasm of 0-h embryos. B. Cysteine protease activity from cytoplasm of 26-h larvae. In 0-h embryos multiple cysteine protease activities were partially resolved using a linear gradient of KCl in a phosphate buffer as described in the legend to Figure 2. The protease isoforms were designated as CP-1, CP-2, CP-3, CP-4 and CP-5. The peaks were aligned and analyzed on the basis of their elution position in the KCl concentration gradient in the eluting buffer. In the cytoplasm of 26-h larvae, CP-1 is absent, CP-2 is reduced, while CP-3 (the major isoform) eluted at 0.17 M KCl in the eluting buffer. The protein content, CP activity, and KCl concentration in the buffer are as described in the legend to Figure 2.

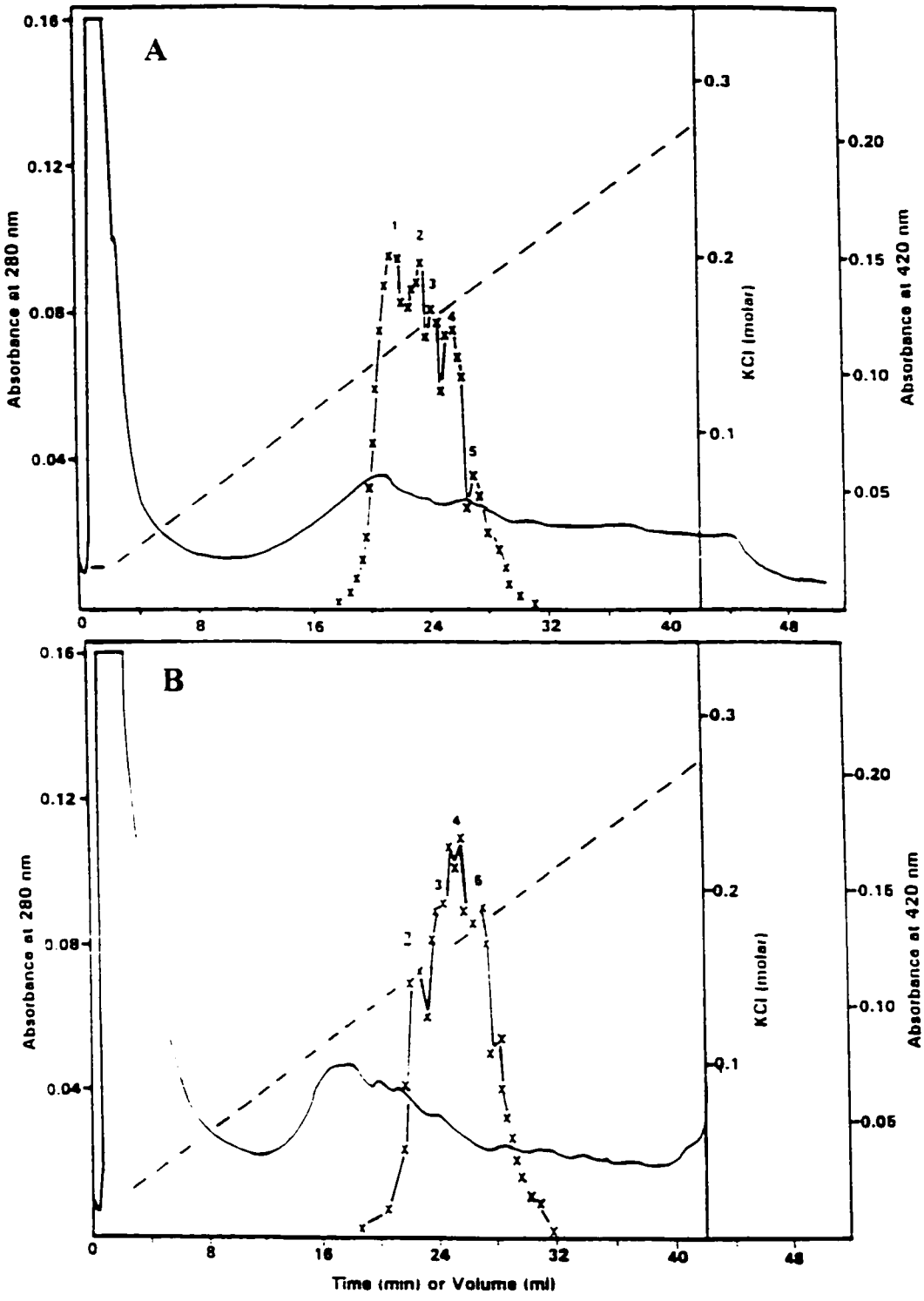

A
B
Absorbance at 280 nm
KCl (molar)
Absorbance at 420 nm
Time (min) or Volume (ml)
0.16
0.12
0.08
0.04
0.3
0.2
0.1
0.20
0.15
0.10
0.05
0
8
16
24
32
40
48

Figure 5. Elution Profiles from a Mono Q Column of Cysteine Protease Activity in the Mitochondria/Lysosome of 12-h Embryos Compared to the Cytoplasm of 0-h Embryos of *Artemia*

A. Cysteine protease activity from cytoplasm of 0-h embryos. B. Cysteine protease activity from the mitochondria/lysosome of 12-h larvae. The multiple cysteine protease activities in 0-h embryos and 12-h larvae were partially resolved by elution using a KCl gradient in phosphate buffer as described in the legend to Figure 2. The protease isoforms were designated as CP-1, CP-2, CP-3, CP-4 and CP-5. The peaks were aligned on the basis of their elution position along the KCl concentration gradient in the eluting buffer. In the mitochondria/lysosome fraction of 12-h embryos, CP-1 and CP-2 are virtually absent, while CP-3 and CP-4 are the major isoforms, eluting at 0.17 and 0.18 M KCl, respectively. The protein content, CP activity, and KCl concentration in the buffer are as described in the legend to Figure 2.

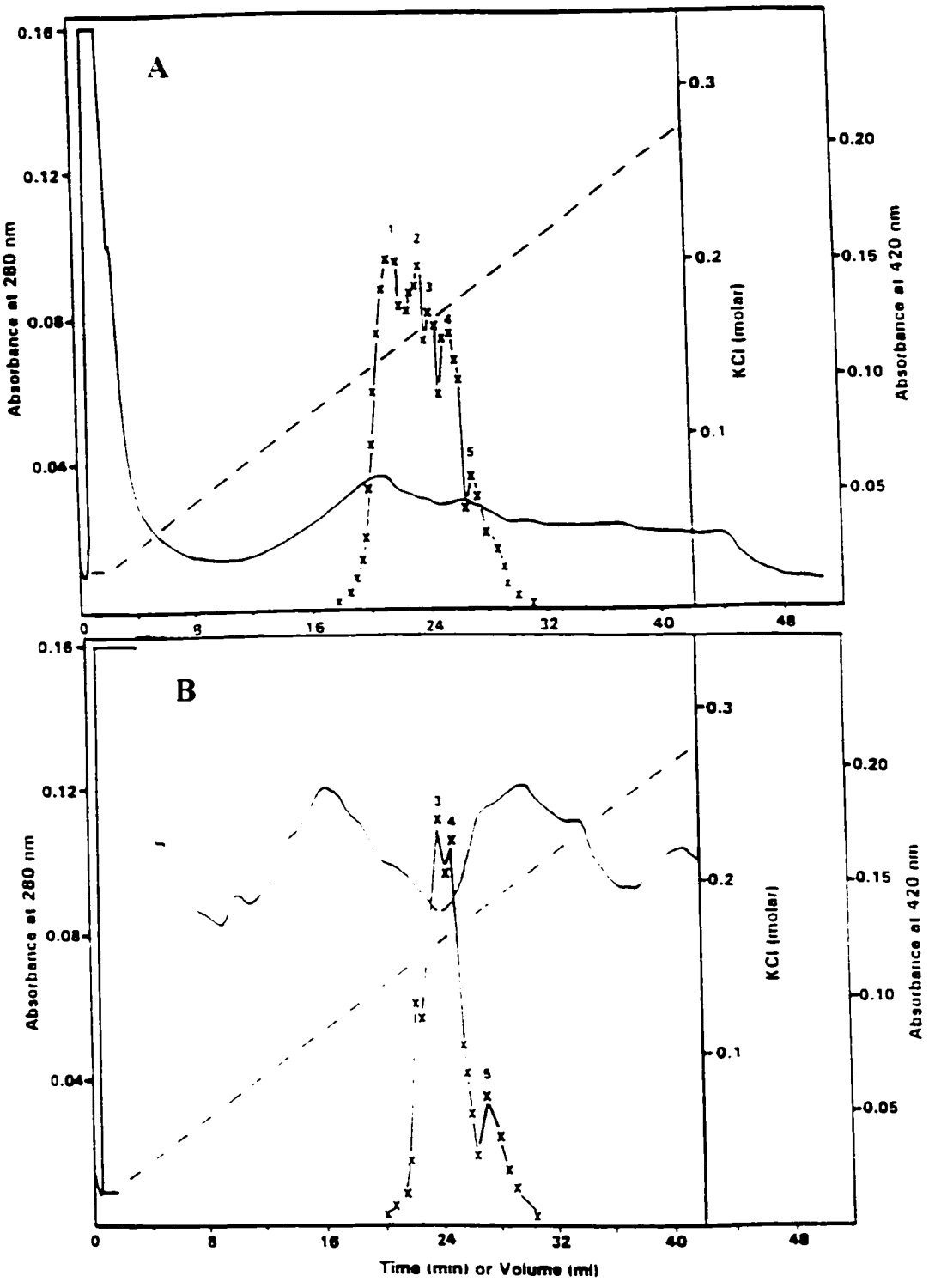
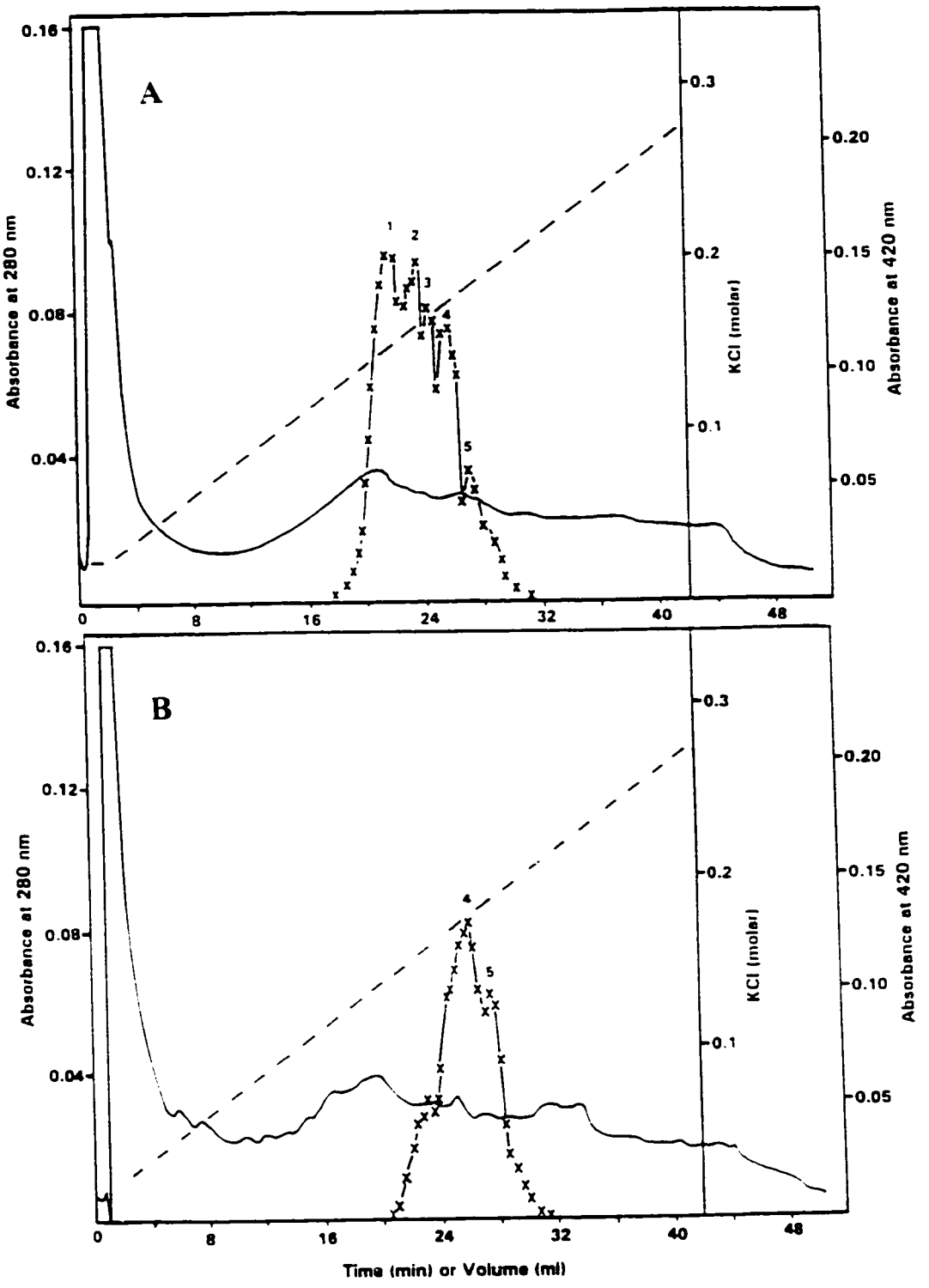

A
B
Absorbance at 280 nm
KCl (molar)
Absorbance at 420 nm
0.16
0.12
0.08
0.04
0.3
0.2
0.1
0.20
0.15
0.10
0.05
0
8
16
24
32
40
48
Time (min) or Volume (ml)

Figure 6. Elution Profiles from a Mono Q Column of Cysteine Protease Activity in the Cytoplasm of 0-h Embryos and in the Mitochondria/Lysosome Fraction of 0-h Larvae of *Artemia*

A. Cysteine protease activity in cytoplasm of 0-h embryos. B. Cysteine protease activity in the mitochondria/lysosome fraction of 0-h larvae. The multiple cysteine protease activities were partially resolved using a linear gradient of KCl in a phosphate buffer as described in the legend to Figure 2. The protease isoforms were designated as CP-1, CP-2, CP-3, CP-4 and CP-5. The peaks were aligned on the basis of their elution position in the KCl concentration gradient. In panel B, CP-4 and CP-5 eluted at a KCl concentration of 0.18M and 0.19M, respectively. The protein content, CP activity, and KCl concentration in the buffer are as described in the legend to Figure 2.


A
B
Absorbance at 280 nm
KCl (molar)
Absorbance at 420 nm
Time (min) or Volume (ml)
0.16
0.12
0.08
0.04
0.3
0.2
0.1
0.20
0.15
0.10
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1
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contains at least four, possibly five isoforms which have been designated as CP-1, CP-2, CP-3, CP-4 and CP-5 (Fig. 2, panel A).

The results in Figure 3 show the elution profile of the cysteine protease activity from the cytoplasm of 0-h larvae compared with that of 0-h embryos. In 0-h larvae the CP isoform pattern is different from that in 0-h embryos, and suggests that 0-h larvae lack CP-1 and have reduced amounts of CP-2 compared to 0-h embryos. CP-3 appears to be the dominant isoform in newly hatched 0-h (first instar) larvae.

The results in Figure 4 compare the cysteine protease activity in the cytoplasm of 26-h larvae with that of 0-h embryos. These results show that the CP in the cytoplasm of 26-h larvae have a similar isoform pattern to that of 0-h larvae with each lacking CP-1 and having reduced amounts of CP-2 compared to the cytoplasm of 0-h embryos.

The results in Figure 5 compare the cysteine protease activity in the mitochondria/lysosome fraction of 12-h embryos with the cysteine protease activity in the cytoplasm of 0-h embryos. Inspection of these results show that the dominant CP fractions in the mitochondria/lysosome fraction are CP-3, CP-4, while the dominant CP activities in the cytoplasm of embryos (ie, CP-1 and CP-2) are absent from mitochondria/lysosomes in *Artemia*.

The result in Figure 6 compare the cysteine protease activity in the mitochondria/lysosome fraction of 0-h larvae with the cysteine protease activity from the cytoplasm of 0-h embryos. Inspection of these results show the dominant CP isoforms in

Table 1. Comparison of the Specific Activity of the Cysteine Proteases from Embryos and Larvae of *Artemia* after Chromatography on Sephadex G-150 and Mono Q

	embryos		larvae	
Specific Activity*	0-h	12-h	0-h	26-h
mEU/mg protein	3835±388	2714±261	5256±710	642±79
mEU/10^4 animals	46±5.7	35±2.9	26±8.1	19±7.6

* Specific activity equals mEU/mg protein or mEU/10^4 animals. All measurements were made at pH 4.0 and 40^0C as described under Materials and Methods. Standard deviations are were carried out on all samples with N=3.

preparation of the protease. This observation/conclusion will be discussed further in the thesis.

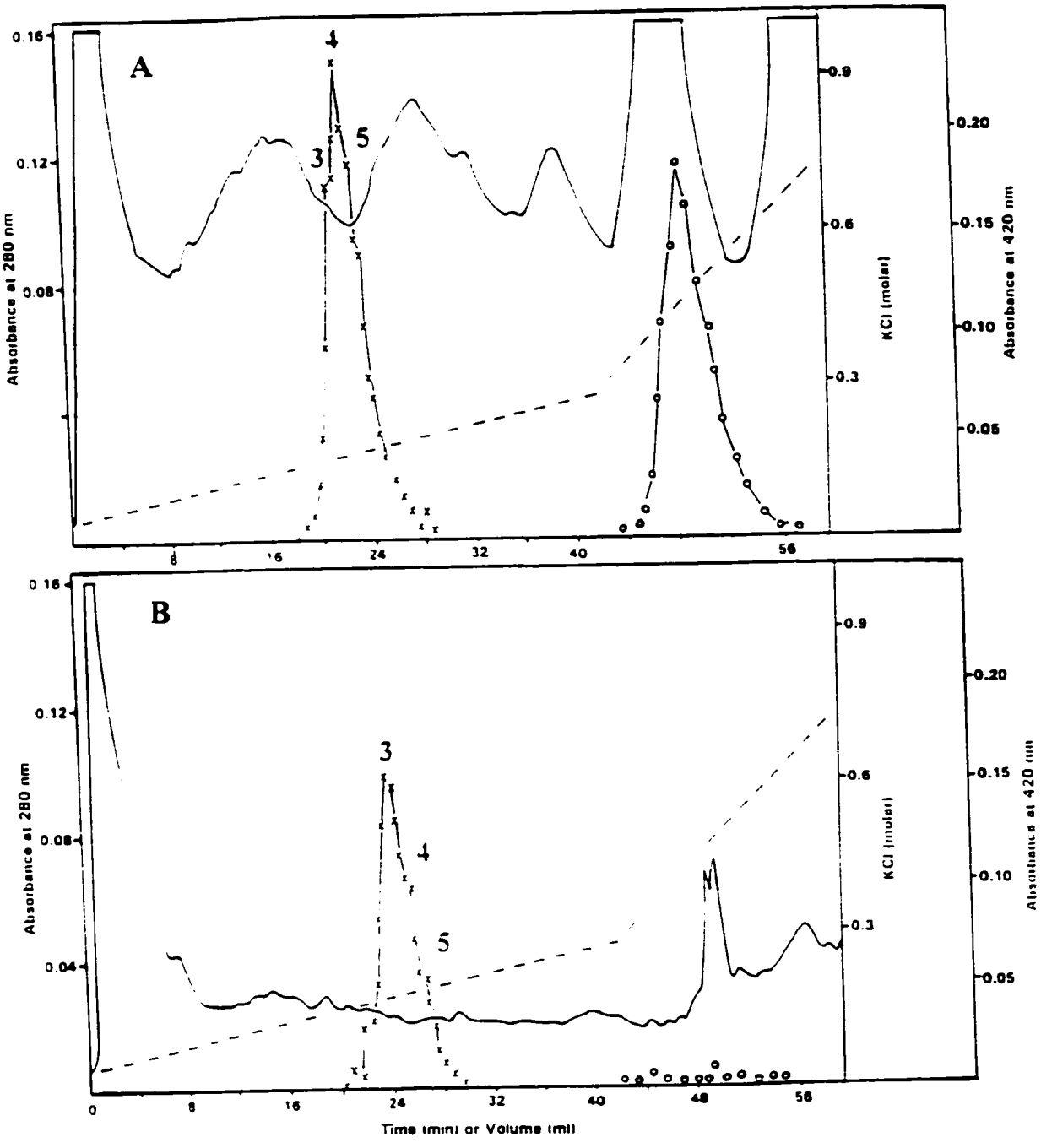
3. Separation of Cysteine Proteases from Alkaline/Neutral Proteases in Extracts of *Artemia* larvae

Shortly after the larvae hatch, large amounts of serine proteases with optimal activity at an alkaline pH are synthesized (Osuna *et al.*, 1977). Thus second instar larvae of *Artemia* contain considerable protease activity with a slightly alkaline pH optimum that interferes with cysteine protease assays of larval extracts (Warner, 1989). To determine whether the major alkaline/neutral proteases in second instar larvae of *Artemia* have similar chromatographic characteristics to that of the CP, partially purified cysteine protease from the cytoplasm and mitochondria/lysosome fractions of second instar larvae (26-h), were chromatographed on a Mono Q column after G-150 Sephadex and assayed for both types of proteases as described in Materials and Methods.

Elution of the proteases from the Mono Q column was carried out with a linear, two-step concentration gradient of KCl in regular column buffer from 25-300mM and 300-750mM KCl, respectively over 60 minutes. The results of this experiment are shown in Figure 7. Under these elution conditions, the CP isoforms of both cytoplasm and mitochondria/lysosome origins are poorly resolved, but their elution characteristics are clearly different from the alkaline/neutral proteases, suggesting that the CP fractions after chromatography on Mono Q are free of contaminating alkaline/neutral proteases. As well, no significant alkaline protease activity was found in the mitochondria/lysosome

Figure 7. Elution Profile of Total Protease Activity in the Cytoplasm and Mitochondria/Lysosome Fraction from 26-h Larvae of *Artemia*

A. Protease activity in the cytoplasm. B. Protease activity in the mitochondria/lysosome fraction. The cytoplasm and mitochondria/lysosome fraction preparations were first chromatographed on a G-150 Sephadex column, then applied to a Mono Q column. In both Panel A and Panel B, the CP isoforms are poorly resolved for unknown reason (The initial KCl concentration gradient was as before then increased to 0.75 M and time increased to 60 minutes). The alkaline/neutral protease activity was assayed at pH 7.4 (o-o), while the cysteine protease activity (x-x) was measured at pH 4.0 using the TNBS assay at 420 nm. The peak of CP activity eluted at 0.15 M KCl, while the alkaline/neutral protease activity eluted at 0.45 M KCl. No alkaline/neutral protease activity was detected in the mitochondria/lysosome fraction, except for trace activity suspected to come from contaminating cytoplasm during the preparation of the crude mitochondria/lysosome. The protein content, CP activity, and KCl concentration in the buffer (up to 40 minutes elution) are as described in the legend to Figure 2.


A
B
Absorbance at 280 nm
Absorbance at 420 nm
KCl (molar)
Time (min) or Volume (ml)
3
4
5

fraction of of 26-h larvae. These data support the view that the alkaline/neutral proteases are cytoplasmic enzymes and not of mitochondria/lysosome origin in larvae of *Artemia*. The trace amount of alkaline/neutral protease activity in the mitochondria/lysosome fraction (see Figure 7, panel B) is probably due to contamination from the cytoplasmic fraction.

4. Fractionation of Cysteine Protease Isoforms by Isoelectric Focusing on Polyacrylamide Gels

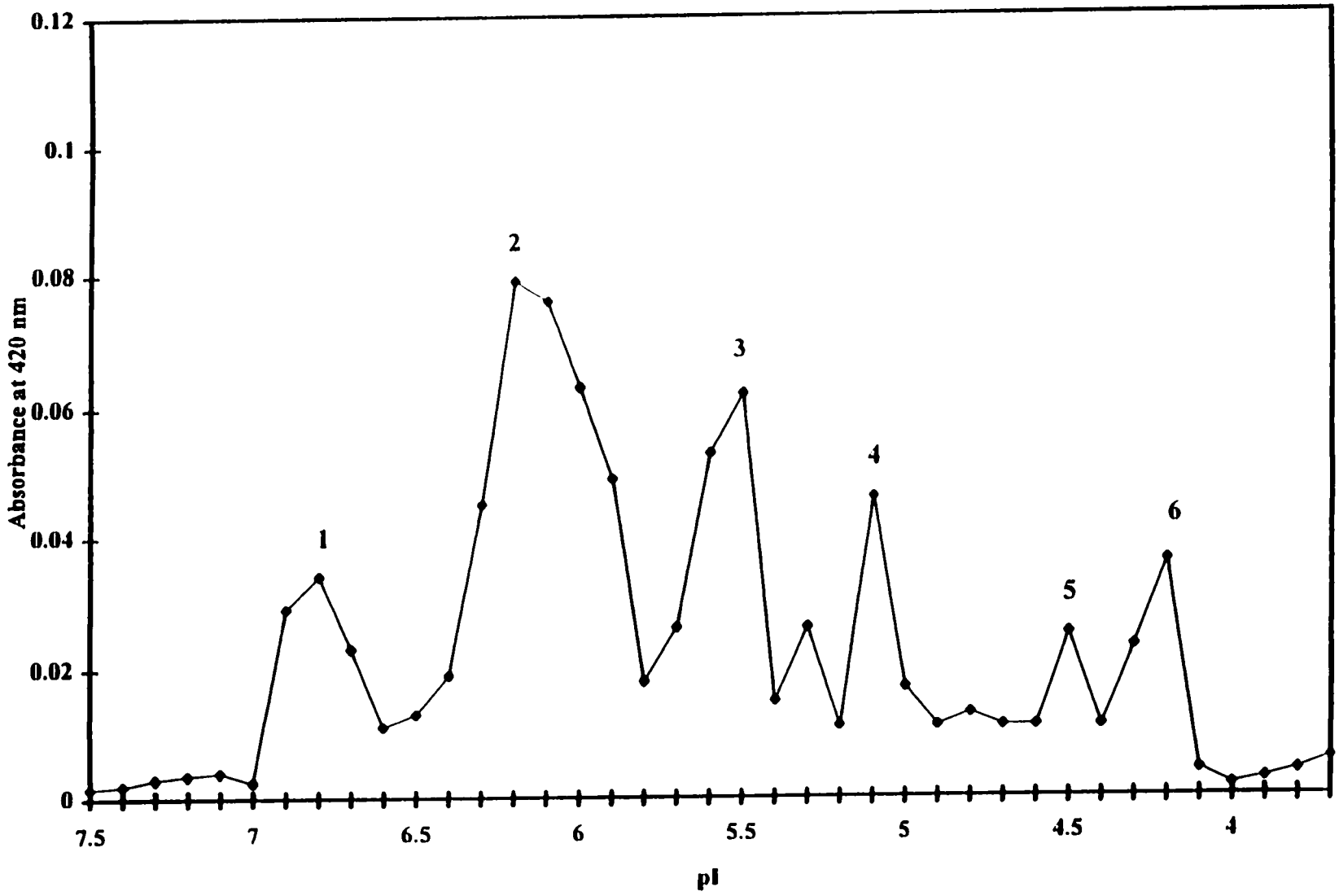
Cysteine protease fractions from 0 and 12-h embryos and 0 and 26-h larvae, which had been purified by FPLC on a Mono Q column, were analyzed further by isoelectric focusing (IEF) in an attempt to resolve the various isoforms of the CP. During each analysis, four IEF gels (in glass columns) were run simultaneously. One gel contained standard proteins as a control, one gel contained total CP from 0-h embryos as a comparator, and two gels contained samples (duplicates) from one other developmental stage.

Isoelectric focusing of the CP from the cytoplasm of 0-h embryos showed six major isoforms of the cysteine protease activity, ranging in pI from pH 4.2-4.3 to 6.7-6.9. The results are shown in Figure 8 and indicate that CP-2 (pI of 6.0 to 6.3) is the dominant CP isoform in 0-h embryos.

The results in Figure 9 compare the CP isoforms in the cytoplasm of 12-h embryos to those in the cytoplasm of 0-h embryos, and show that isoform CP-1 is lacking in 12-h embryos, while 12-h embryos contain a CP at pI 4.8-4.9 (CP-4a) which is lacking

Figure 8. Analysis of the Cysteine Protease Isoforms in the Cytoplasm of 0-h Embryos of *Artemia* by Isoelectric Focusing

Approximately thirty mEU of cysteine protease (CP) activity were separated on an IEF gel as described in Materials and Methods. At the end of the separation, the CP activity was determined in 2 mm gel slices over 30 minutes incubation at pH 4.0 and 40^0C. The CP activity in each gel slice (as absorbance at 420 nm) was plotted against the pI value of each gel slice as determined from a gel with standard proteins run under identical conditions.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Absorbance at 420 nm
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pI

in 0-h embryos. The absence of CP-1 from 12-h embryos as shown on IEF gels is not consistent with the isoform pattern observed on Mono Q (see Figure 2), but at this point we have no evidence to show that CP-1 from Mono Q is the same as CP-1 from the IEF gel.

The results in Figure 10 compare the CP isoforms in the cytoplasm of 0-h larvae to those in 0-h embryos, and show that CP-1, CP-5 and CP-6 are absent from 0-h larvae, while CP-2 is resolved into CP-2a (pI 6.3) and CP-2b (pI 6.1).

The results in Figure 11 compare the CP isoforms in the cytoplasm of 26-h larvae to CP isoforms in 0-h embryos, and show that CP-1, CP-5 and CP-6 are absent or markedly reduced from 26-h larvae, and that all other CP isoforms are reduced in amount, while CP-2 is resolved into CP-2a (pI 6.2) and CP-2b (pI 6.0).

The results in Figure 12 compare the CP isoforms in the cytoplasm and mitochondria/lysosome of 12-h embryos. The mitochondria/lysosome fraction of 12-h embryos appear to contain mainly CP-2, suggesting that the CP isoform pattern seen after Mono Q (Figure 5) may not reflect multiple forms of CP.

The results in Figure 13 compare the CP isoforms in the cytoplasm and mitochondria/lysosome of 0-h larvae. These preparations show a similar CP pattern with CP-2 (a,b) being the dominant isoforms in both the cytoplasm and mitochondria/lysosome of 0-h larvae.

The results in Figure 14 compare the CP isoforms in the cytoplasm and mitochondria/lysosome of 26-h larvae. The two larval fractions show a similar CP

Figure 9. Analysis of Cysteine Protease Isoforms in the Cytoplasm of 12-h Embryos of *Artemia* Compared to Cysteine Protease Isoforms in the Cytoplasm of 0-h Embryos by Isoelectric Focusing

A. Cysteine protease isoforms in cytoplasm of 0-h embryos. B. Cysteine protease isoforms in cytoplasm of 12-h embryos. Approximately thirty mEU of cysteine protease (CP) activity were separated on each IEF gel as described in Materials and Methods. At the end of the separation, the CP activity was determined in 2 mm gel slices over 30 minutes incubation at pH 4.0 and 40^0C. The CP activity (absorbance at 420 nm) in each gel slice was plotted against the pI value of each gel slice as determined from a gel with standard proteins run under identical conditions.

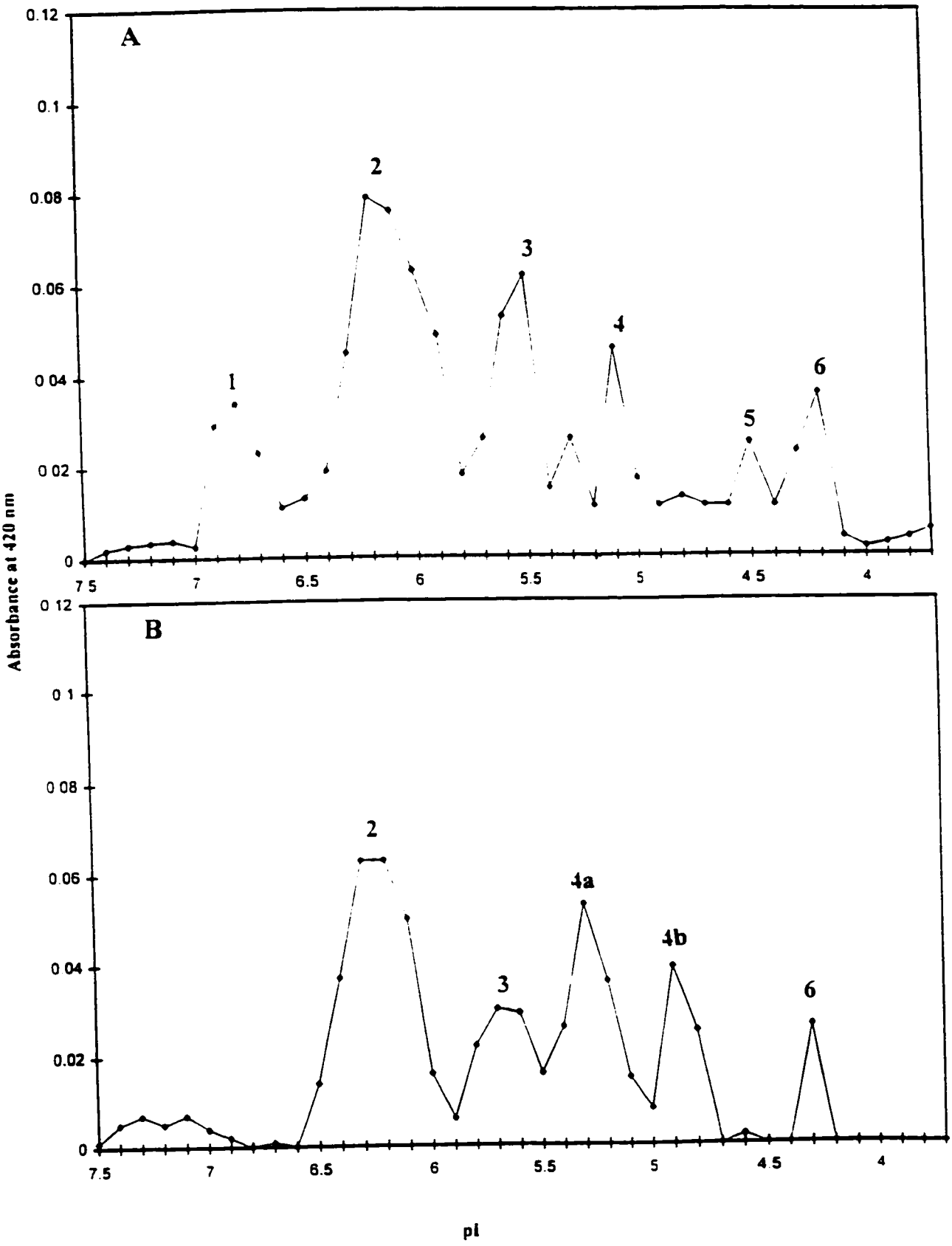
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Figure 10. Analysis of Cysteine Protease Isoforms in the Cytoplasm of 0-h Larvae of *Artemia* Compared to Cysteine Protease Isoforms in the Cytoplasm of 0-h Embryos by Isoelectric Focusing

A. Cysteine protease isoforms in the cytoplasm of 0-h embryos. B. Cysteine protease isoforms in the cytoplasm of 0-h larvae. Approximately thirty mEU of cysteine protease (CP) activity were applied to each IEF gel as described in Materials and Methods. At the end of the focusing, the CP activity was determined in 2 mm gel slices over 30 minutes incubation at pH 4.0 and 40^0C. The CP activity (absorbance at 420 nm) in each gel slice was plotted against the pI value of each gel slice as determined from a gel with standard proteins run under identical conditions.

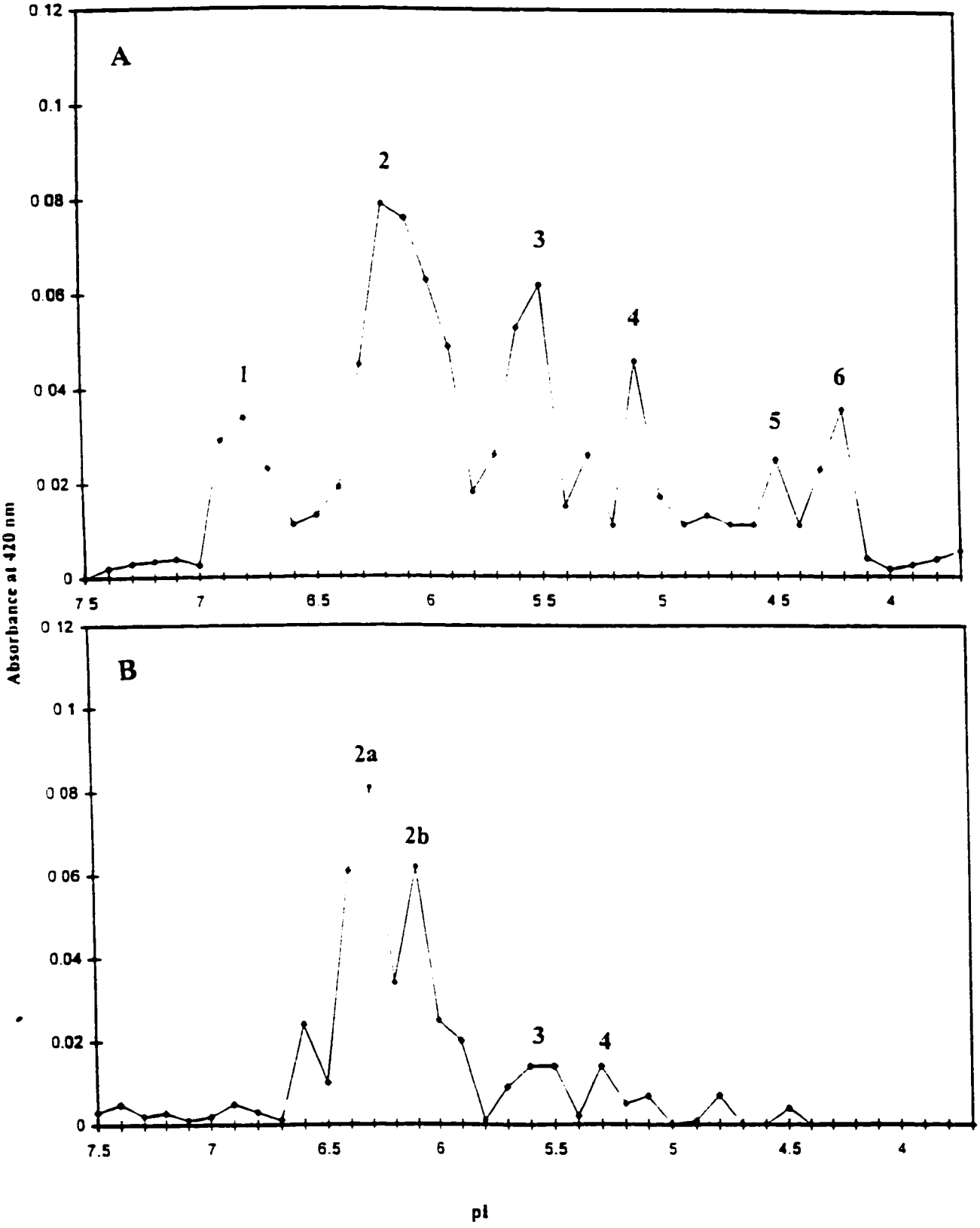
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Figure 11. Analysis of Cysteine Protease Isoforms in the Cytoplasm of 26-h Larvae of *Artemia* Compared to Cysteine Protease Isoforms in the Cytoplasm of 0-h Embryos of *Artemia* by Isoelectric Focusing

A. Cysteine protease isoforms in cytoplasm of 0-h embryos. B. Cysteine protease isoforms in cytoplasm of 26-h larvae. Approximately thirty mEU of cysteine protease (CP) activity were separated on each IEF gel as described in Materials and Methods. At the end of the focusing, the CP activity was determined in 2 mm gel slices over 30 minutes incubation at pH 4.0 and 40^0C. The CP activity (absorbance at 420 nm) in each gel slice was plotted against the pI value of each gel slice as determined from a gel with standard proteins run under identical conditions.

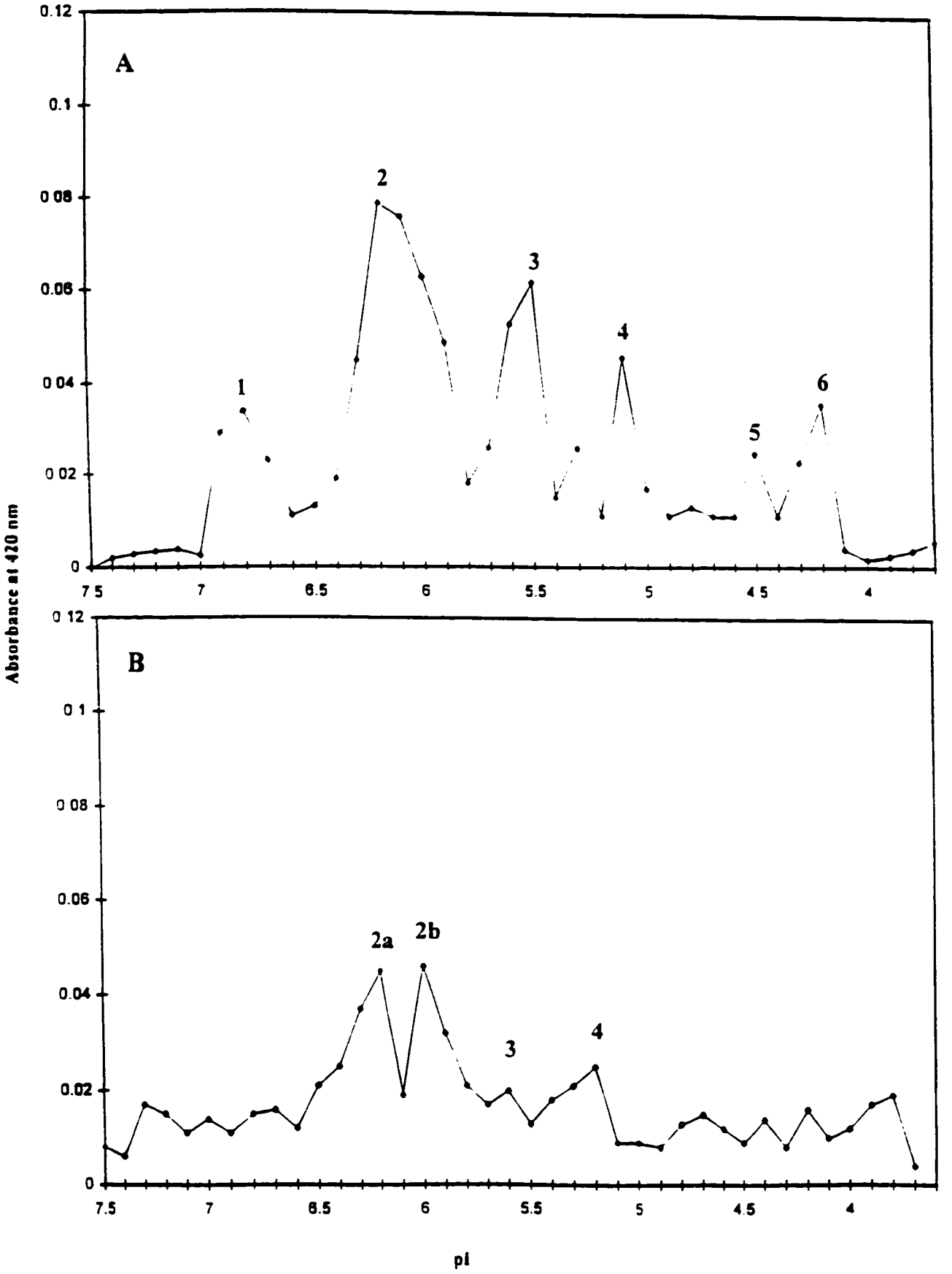
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Figure 12. Analysis of Cysteine Protease Isoforms in the Cytoplasm of 12-h Embryos of *Artemia* Compared to Cysteine Protease Isoforms in the Mitochondria/Lysosome of 12-h Embryos by Isoelectric Focusing

A. Cysteine protease isoforms in the cytoplasm of 12-h embryos. B. mitochondria/lysosome cysteine protease isoforms of 12-h embryos. Approximately thirty mEU of cysteine protease (CP) activity were separated on each IEF gel as described in Materials and Methods. At the end of the separation, the CP activity was determined in 2 mm gel slices over 30 minutes incubation at pH 4.0 and 40^0C. The CP activity (absorbance at 420 nm) in each gel slice was plotted against the pI value of each gel slice as determined from a gel with standard proteins run under identical conditions.

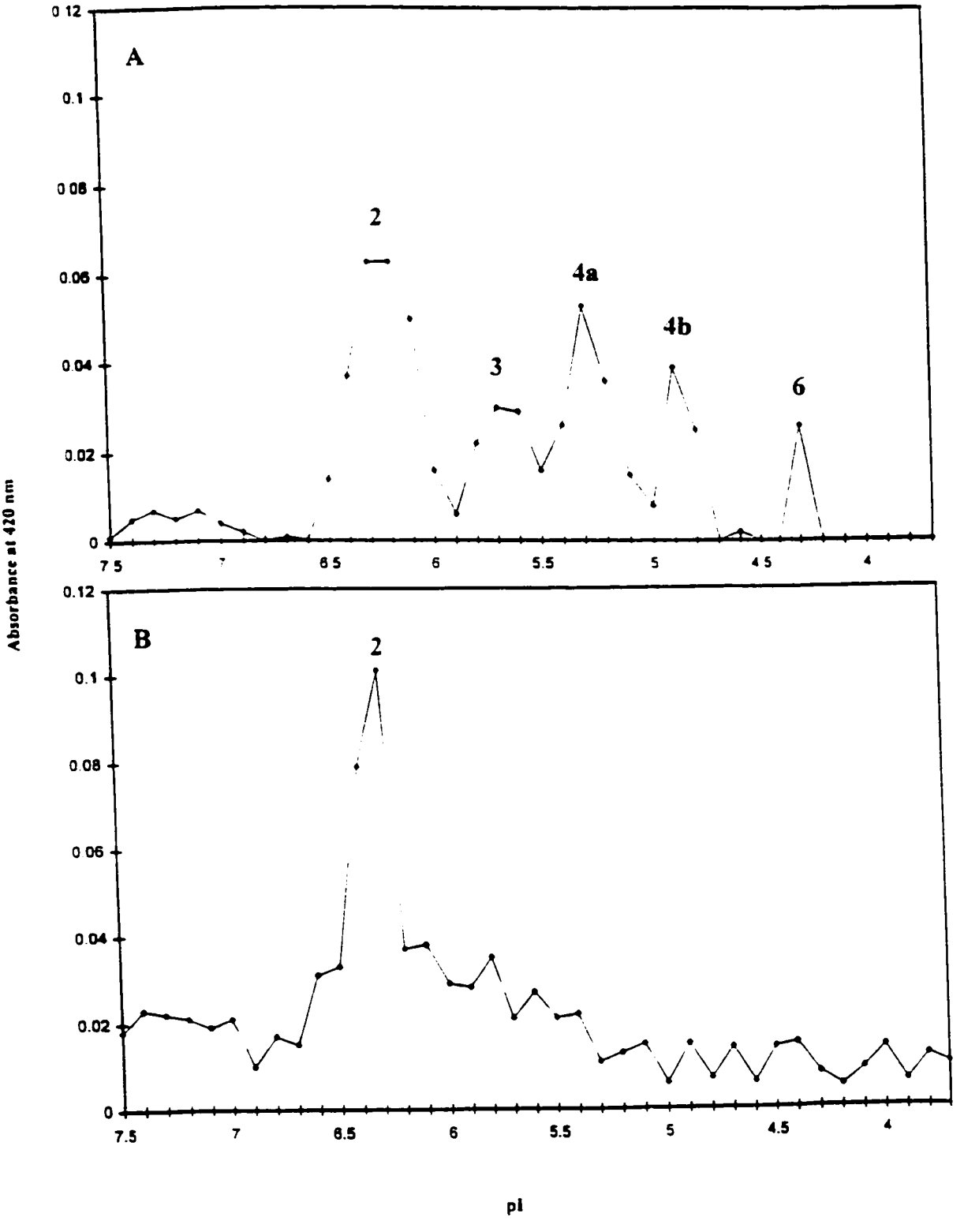
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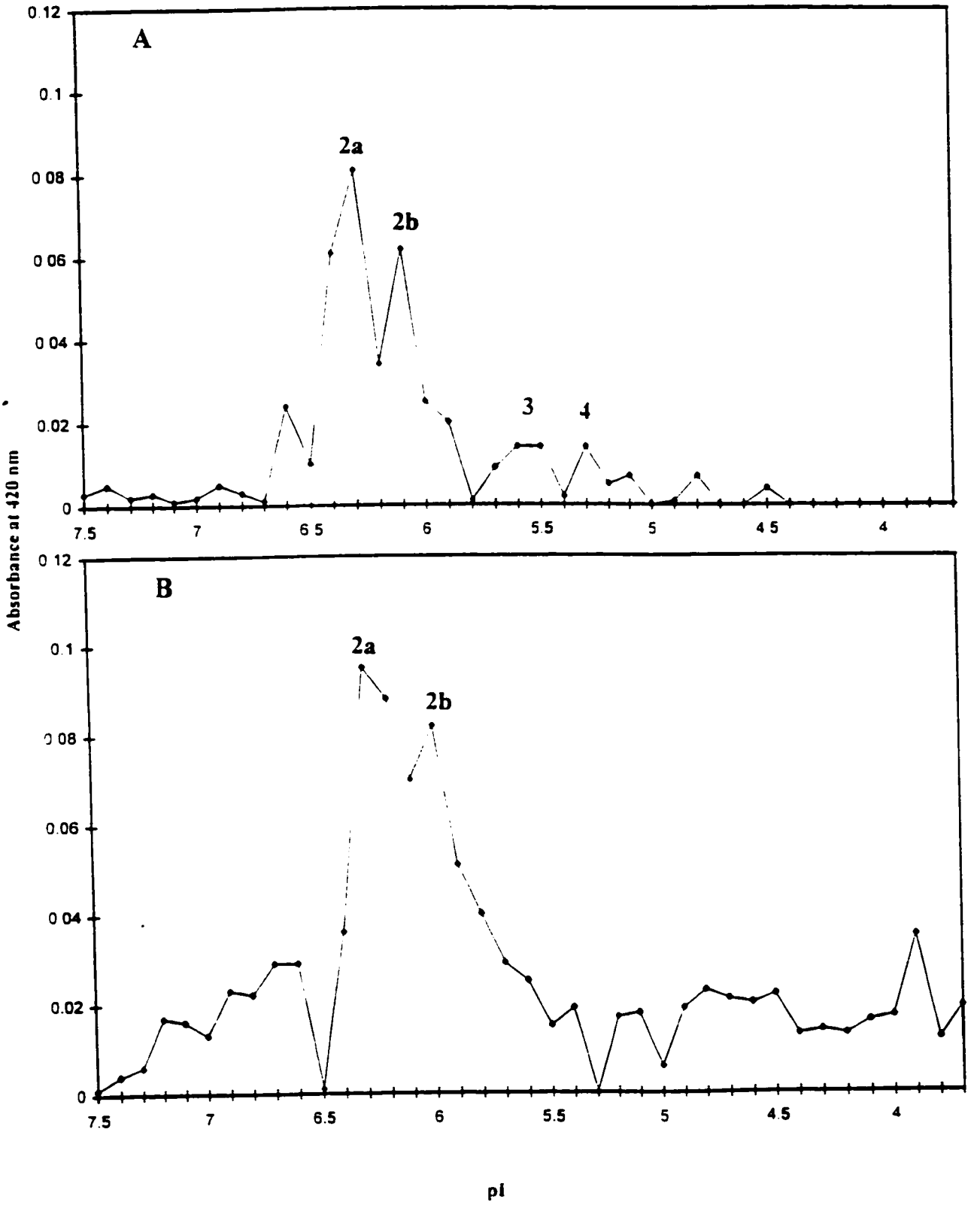
Figure 13. Analysis of Cysteine Protease Isoforms in the Cytoplasm of 0-h Larvae of *Artemia* Compared to Cysteine Protease Isoforms in 0-h Mitochondria/Lysosome of Larvae by Isoelectric Focusing

A. Cysteine protease isoforms in cytoplasm of 0-h larvae. B. Cysteine protease isoforms in mitochondria/lysosome of 0-h larvae. Approximately thirty mEU of cysteine protease (CP) activity were separated on each IEF gel as described in Materials and Methods. At the end of the separation, the CP activity was determined in 2 mm gel slices over 30 minutes incubation at pH 4.0 and 40^{0}C. The CP activity (absorbance at 420 nm) in each gel slice was plotted against the pI value of each gel slice as determined from a gel with standard proteins run under identical conditions.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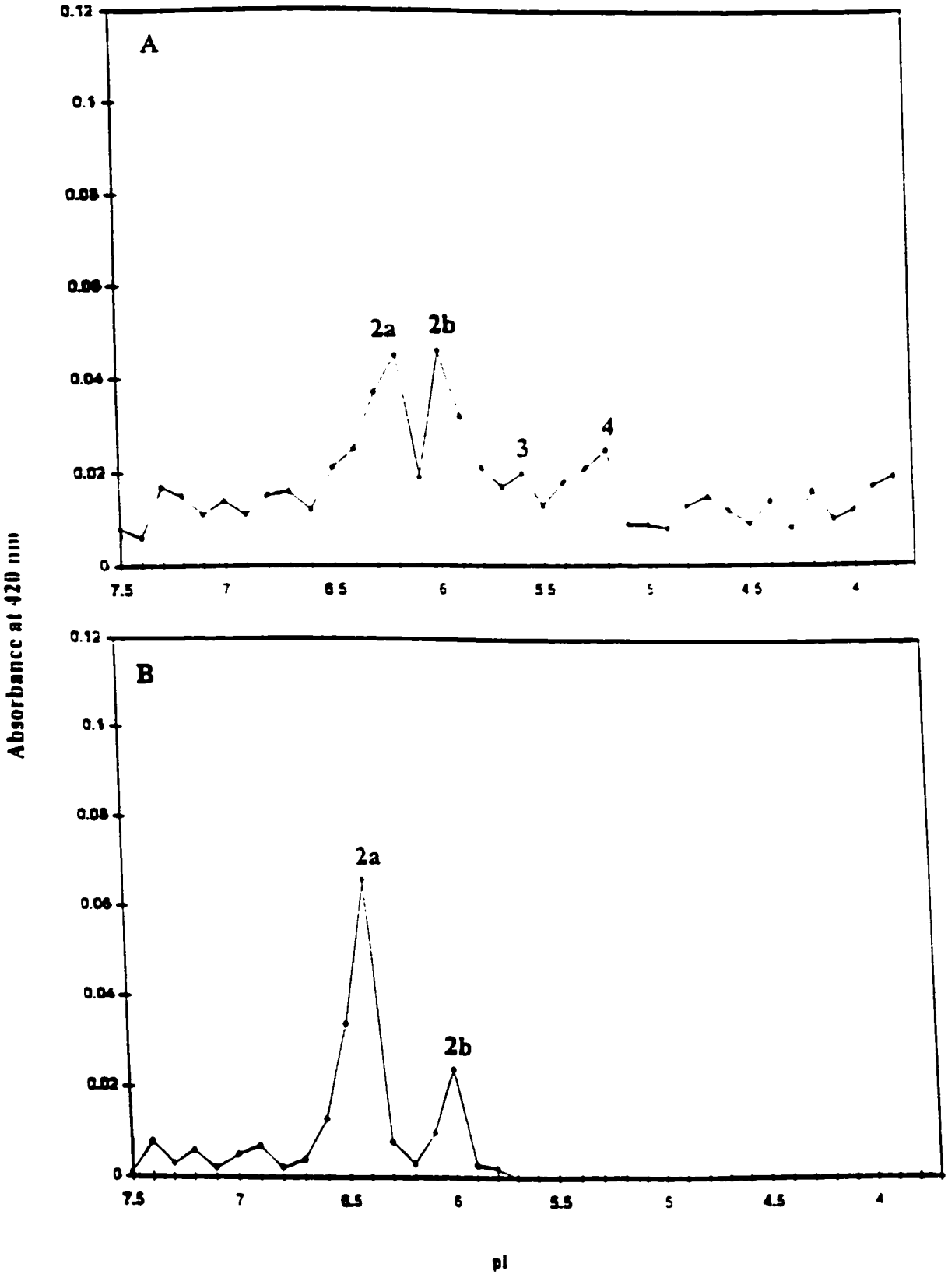
isoform pattern with each containing two distinct peaks of activity as CP-2a and CP-2b, while lacking significant amounts of isoforms found in the embryo stages.

The results of the IEF analyses were repeated three times to confirm our conclusions. The data in Table 2 summarize the CP isoform composition in embryos and larvae of *Artemia* as shown in Figures 9 to 14. The recovery of CP activity from the gel varied from 25 to 36% of that applied and is lower than expected. The implication of this result is discussed below. We found isoelectric focusing to be an excellent method to resolve of the CP isoforms despite the low recovery of activity from the gel. Staining of IEF gels (data not shown) with CP from 0-h embryos, showed that the major protein in the gel has a pI of 6.2, while a second major protein focused as an intense band at pH 5.6. Other bands were clearly resolved at pH 6.8 and pH 5.2, but they did not stain intensely, nor did the bands at pH 4.8 and pH 4.3. The reason for some of the faint bands in the gels is not clear, but it could be due to aggregation of the cysteine protease with ampholytes, or to auto-degradation. The position of the protein bands observed by staining with various stains (Coomassie and Silver) supports the activity measurements, but further work is needed to improve CP band detection by protein staining. It would appear that 30 mEU of total enzyme activity is not enough enzyme to obtain clearly stained bands of some of the minor isoforms of the enzyme.

Figure 14. Analysis of Cysteine Protease Isoforms in the Cytoplasm of 26-h Larvae of *Artemia* Compared to Cysteine Protease Isoforms in 26-h Mitochondria/Lysosome of Larvae by Isoelectric Focusing

A. Cysteine protease isoforms in cytoplasm of 26-h larvae. B. Cysteine protease isoforms in mitochondria/lysosomes of 26-h larvae. Approximately thirty mEU of cysteine protease (CP) activity were separated on each IEF gel as described in Materials and Methods. At the end of the separation, the CP activity was determined in 2 mm gel slices over 30 minutes incubation at pH 4.0 and 40^0C. The CP activity (absorbance at 420 nm) in each gel slice was plotted against the pI value of each gel slice as determined from a gel with standard proteins run under identical conditions.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Absorbance at 420 nm
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Table 2. Summary of CP Isoform Distribution in the Cytoplasm of 0-h and 12-h Embryos and in 0-h and 26-h Larvae of *Artemia franciscana**

		embryos		larvae	
		0-h	12-h	0-h	26-h
Peak #	pI	Percentage of total CP activity applied to gel			
1	6.7-6.9	4.6%	1.1%	2%	3%
2	6.0-6.3	19.1%	17%	21%	16%
3	5.5-5.6	5.2%	9%	2%	2%
4	5.1-5.2	2%	6%	1%	2.7%
5	4.8-4.9	1.6%	2%	0.9%	0.6%
6	4.2-4.3	0.9%	1%	0.1%	0.4%
Totals		33.4%	36.1%	27.0%	24.7%

*In the calculations the activity in CP-2a and 2b were combined as were the activity in CP-4a and 4b.

Discussion

It has now become evident that proteolytic reactions play a key role not only in the regulation of intracellular protein turnover but also in the control of various cellular functions (Bond and Butler, 1987). The role of proteases in animal development appears to be primarily for utilization and/or mobilization of yolk proteins during embryogenesis and to supply amino acids for synthesis of embryonic/larval proteins during differentiation and development. Other potential functions include hatching and digestion (Yamamoto and Takahashi, 1993; Warner *et al.*, 1995).

Yolk platelets are the most abundant inclusions in the cytoplasm of the encysted *Artemia* embryos. It was reported that yolk platelets are composed of 74.1% protein, 8.1% lipid, 4.0% carbohydrate, 4.6% acid-soluble nucleotides, and 0.1% DNA and RNA (Warner *et al.*, 1972). Yolk is the major internal food supply of embryos, and yolk platelets constitute the main storage compartment that supplies the developing embryo with an endogenous source of amino acids, sugars, lipids, phosphate, and ions (Fagotto, 1990). The products of yolk protein hydrolysis are utilized as amino acids for the synthesis of other proteins during embryogenesis. Regulation of yolk utilization is essential to provide nutrients at the right time to the developing tissues, and to support survival of the embryo until it becomes a free-living organism and able to feed (Fagotto, 1995).

Comparison of proteases from divergent species has allowed us to study the function of protease systems. In the soft tick *Ornithodoros moubata*, maximum degradation of vitellin is at pH 3-3.5, whereas no proteolysis is detected at neutral or weakly acidic pHs. Acidic proteolysis is maintained at high level in a compartmentalized way throughout embryonic development, and proteolytic activity has been essentially attributed to a cysteine protease through substrate specificity and inhibitors (Fagotto, 1990). Similar conclusions were drawn in *Artemia*, so the cysteine protease would presumably need to be located in a relatively acidic region of the cell in order to hydrolyze lipovitellin during development (Utterback and Hand, 1987). Also a cysteine protease with properties similar to the mammalian cathepsins has been characterized in eggs of silkmoth (Yamamoto, 1996), suggesting that cysteine proteases are important enzymes in yolk protein degradation during early embryonic development.

Among the proteases found in cysts, developing embryos and larvae of the brine shrimp *Artemia franciscana*, a cysteine protease has been the most studied (Warner *et al.*, 1995). Data have shown that this enzyme represents more than 90% of the total protease activity in extracts of *Artemia* cysts and embryos during embryogenesis (Warner, 1989).

Previous studies from this laboratory have demonstrated that the purified cysteine protease (CP) from encysted embryos (0-h) of *Artemia franciscana* has a molecular weight of about 60 kDa and is composed of two subunits, one of 32 kDa and one of 28 kDa (Warner and Shridhar, 1985; Lu and Warner, 1991). Analysis of the specific activity of the CP of *Artemia* embryos indicated that pure CP has a specific activity of about 18 enzyme units/mg (Eu/mg) protein at pH 4.0 and 40^{0}C (unpublished data). Thus

from previous work with the cysteine proteases of *Artemia*, it would appear that the CP isoforms characterized in my studies, which have a specific activity in a range of 0.64±0.79 to 3.84±0.39 EU/mg protein (see Table 1) are only about 4-21% pure. It should be noted that the CP preparations in this study had not been treated with ConA Sephadex (which improves the purity of the enzyme) so as to avoid the potential loss of CP isoforms that might be lacking carbohydrate and other charged groups. Early studies of *Artemia* suggested the presence of multiple forms of the CP, but only two isoforms could be resolved by isoelectric focusing in an earlier study (Warner and Shridhar, 1985). In the present experiment we used a shallow pH gradient in isoelectric focusing in order to better resolve the cysteine protease fractions which had been purified by FPLC on a Mono Q column. We found that our isoelectric focusing conditions were excellent in resolving the CP isoforms. Using a shallow pH gradient, six isoforms of the cysteine protease were resolved between pH 4.2 and 6.9. Compared to FPLC which gave only partial resolution of the CP isoforms, IEF allowed us to resolve each isoform of the cysteine protease of *Artemia* embryos and larvae, and to determine the pI value of each isoform despite the low recovery of activity from the gel (the recovery level was 15-30%, see Table 2). It still remains to be determined whether the isoenzymes of the cysteine protease resolved on IEF gels have unique amino acid compositions or whether the presence of variable amounts of carbohydrate (and possibly charged groups) in the various isoforms of protease are the reasons for the apparent heterogeneity of the protease on ion-exchange columns and in IEF gels.

In studies by other researchers a different cysteine protease was described in *Artemia* embryos suggesting that the CP is a lysosomal protease consisting of a single polypeptide of 68 kDa (Perona and Vallejo, 1982). These researchers suggested that the protease in the soluble fraction is enzyme which leaks from lysosomes due to manipulations *in vitro* during the isolation procedure. However, our results, including those published previously, demonstrate that most of the CP in embryos and larvae is of cytoplasmic origin and not from lysosomes (Lu and Warner, 1991; Warner *et al.*, 1995).

A recent study on CP localization in *Artemia* by immunocytochemistry demonstrated that CP is found in different tissues in different developmental stages of larvae (Warner *et al.*, 1995). In prenauplius larvae the CP was found mainly on the basal side of the epidermal layer in the posterior region of larvae. In first instar larvae, the cysteine protease appeared to be widely distributed in yolk platelet-rich regions of the larva to form a reticular network around yolk platelets. In second instar larvae CP was found mainly in the outer regions of epidermal cells, in the basal and apical zones of cells of the midgut and in the lumen of the midgut. Analysis for the subcellular localization of the cysteine protease in second instar larvae suggests that most of the enzyme is in the cytoplasm and extracellular matrix adjacent to midgut cells and not in organelles such as lysosomes and mitochondria (Warner *et al.*, 1995).

Does the localization of the cysteine protease change during development, or are different isoforms synthesized in different tissues during development? First, we investigated whether embryos and larvae of *Artemia* at different developmental stages have different isoforms of the cysteine protease, and therefore potentially different roles

at the subcellular level. We used the same purification procedures to isolate and characterize the CP isoforms from both embryos and larvae, and to evaluate the isoform composition in the cytoplasmic fraction and mitochondria/lysosomes in dormant cysts (0-h embryos), 12-h embryo, and first and second instar larvae (0-h and 26-h, respectively) after hatching of *Artemia*.

In general, our results showed that the cysteine protease isoform composition is different in embryos and larvae suggesting that perhaps each isoform of the protease may play different functions. Previous experiments from our laboratory using immunochemical methods to determine the CP localization showed that CP is found around yolk platelets but not within yolk platelets (Warner *et al.*, 1995). These data support the view that yolk-protein metabolism occurs mainly outside of the platelet. The results of my experiments showed that CP-1, CP-5 and CP-6 are absent from first and second instar larvae, while CP-1 to CP-6 are present in embryos. Because of the close association of yolk platelets and CP in embryos, it would appear that during embryonic development CP isoforms function in yolk degradation (Warner *et al.*, 1995). In young larvae, the CP may also function in hatching, and when swimming larvae begin to feed, the CP isoforms (mainly CP-2, CP-3 and CP-4) may function as digestive enzymes in the mid-gut area. There is also evidence that CP may function (indirectly) in the molting process (Warner *et al.*, 1995).

One group of researchers has suggested that yolk proteins (lipovitellins) are metabolized rapidly in *Artemia*, and have proposed that yolk platelet degradation in

Artemia is dependent on a lysosomal cysteine protease (Perona *et al.*, 1985). However, cell fractionation data in our laboratory showed that most of embryo cysteine protease activity (*ie.* 81.5%) is in the cytoplasm and only 4.1% is in the mitochondria/lysosome fraction. The remaining 14% of CP activity was found in the nuclei/yolk platelet fraction (Lu and Warner, 1991). The results of my experiments are consistent with this view. As well, previous immunocytochemical experiments did not show significant staining for CP in either mitochondria or lysosome (Warner *et al.*, 1995). Therefore, it would appear that the cysteine protease in *Artemia* is mainly in the cytoplasm of embryos and larvae of *Artemia*, and functions in the degradation of protein-rich yolk platelets, at least in embryos, and perhaps also in first instar larvae (Warner *et al.*, 1995). Our results support the view that yolk proteins (*i.e.* lipovitellins) must leave the platelets by some non-degradative mechanism before they are degraded by CP (Utterback and Hand, 1987).

As mentioned above, other researchers have claimed that cysteine proteases are present in lysosomes of dormant gastrulae of *Artemia* (Perona and Vallejo, 1982). The "lysosome" associated protease was shown to have an apparent molecular weight of 68,000 and composed of a single polypeptide chain (Perona and Vallejo, 1982). Our CP is clearly not the same as theirs. Previous results in our laboratory using an immunodetection assay on Western blots shows that no protein (band) of 68 kDa reacted with the CP antibodies, and no evidence for limited proteolysis in cyst extracts has been found to suggest that the two subunits found in our experiments originated from a single polypeptide during the processing of extracts for SDS-polyacrylamide gel electrophoresis and Western blotting (Lu and Warner, 1991). The results in my experiment show that

experiment show that cysteine proteases are present mainly in the cytoplasm, but that some is present in the lysosome/mitochondria fraction where CP-2 seems to be the dominant isoform.

Are the CP isoforms in midgut cells of second instar larvae of *Artemia* the same as those in epidermal cells? Further analysis needs to be done to resolve this question. The presence of CP-2, 3 and 4 in the cytoplasm of second instar larvae may reflect the activity of different developmental processes, such as metabolic activation reactions, metamorphosis, organogenesis or tissue remodeling.

One of the great difficulties in obtaining reproducible trials for the soluble cysteine protease in second instar larvae of *Artemia* has been due to contamination of CP preparations by endogenous alkaline/neutral proteases which can not be completely inhibited by phenylmethylsulfonyl fluoride (PMSF), soybean trypsin inhibitor (STI) or any combination of protease inhibitors in the homogenization buffer. These proteases could interfere with CP activity measurement in crude protease preparations, but since they are removed by FPLC, all CP fractions subjected to isoelectric focussing appear to be free from alkaline/neutral proteases.

There is a report in the literature, not consistent with our findings, which show that the activity of the *Artemia* embryo cysteine protease increases markedly at the time of hatching of the swimming larvae (Perona and Vallejo, 1985). We believe that the reason for this apparent increase in activity is due to a decrease in the endogenous

protease inhibitor level at this developmental stage (Warner *et al.*, 1989). In embryos of *Artemia* we believe that the expression of the cytoplasmic cysteine protease is highly regulated. In contrast, results from researchers in our laboratory have shown that the cysteine protease content remains relatively constant over 42 hours incubation during early development in *Artemia*, suggesting that the activity of the cysteine protease gene(s) may be constitutive and not developmentally regulated in *Artemia* embryos (Lu and Warner, 1991). The data from Perona and Vallejo suggesting that CP activity increase at the time of hatching could be due to changes in the level of endogeous inhibitors which influence the activity of the protease *in vitro*. These observations have suggested that cysteine protease inhibitors present in *Artemia* embryos must be important in the regulation of CP activity (Warner, 1989). These regulatory factors have been partially characterized and shown to decrease markedly in newly-hatched larvae and to be present in very low levels in second instar larvae (Warner, 1987). There are still many questions in this area, which can be resolved by comparative studies of protease activity.

Since various isoforms of CP may be cell-type specific, and thus play different roles in cell differentiation, work must be done with isoform specific antibodies in immunocytochemical experiments to determine whether there are cell-type specific CP isoforms in different tissues in *Artemia*. Only then will we be able to ascertain whether specific developmental activities are linked with specific CP isoforms. Experiments in our laboratory demonstrated that as many as 6 isoforms of CP may exist in embryos based on isoelectric focusing experiments, with pI values in the pH range of 4.2-6.9.

However, the relationship between these isoforms, subunit composition, and specific function has not been determined. In future studies we should be able to assign one or more isoforms to specific cells/tissues in *Artemia* embryos and larvae, to help elucidate their function in embryonic/larval cells and tissues.

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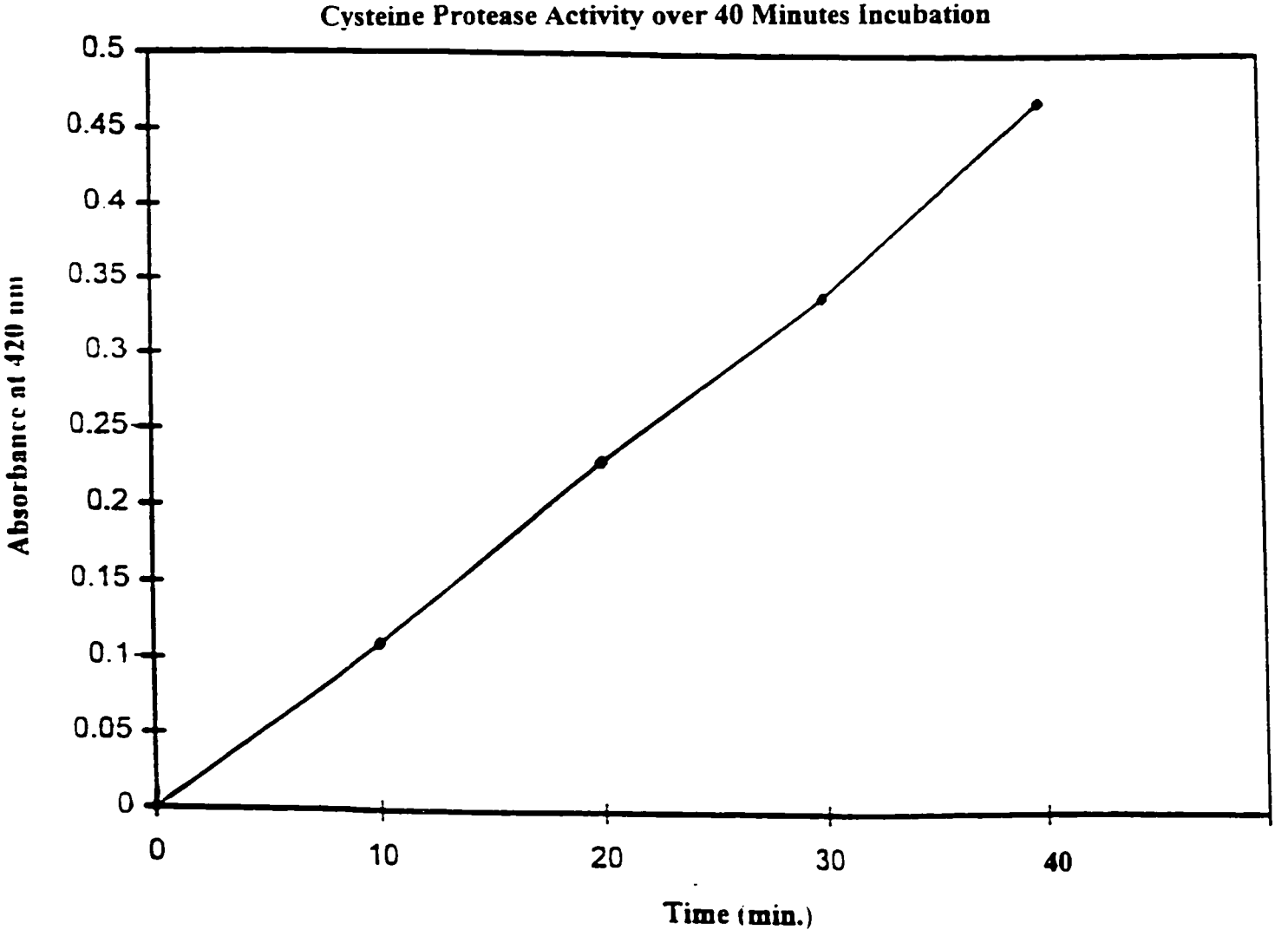
Appendix A



In all experiments, cysteine protease activity was measured at 420 nm using the TNBS assay (see Materials and Methods). The enzyme activity was linear over at least 40 minutes incubation at pH 4.0 and 40^{0}C.

Appendix B

BCA microassay procedure:

1. Place 0.8 ml of standards and appropriately diluted samples in clean, dry test tubes. Place 0.8 ml sample buffer in " blank " test tube.
2. Add 0.2 ml Dye Reagent Concentrate.
3. Vortex (avoid excess foaming) or mix several times by gentle inversion of the test tube.
4. After a period from 5 to 60 minutes, measure the absorbance at 595 nm versus a reagent blank.
5. Plot the absorbance at 595 nm versus concentration of standards then read the unknowns from the standard curve (Technical Assistance, Pierce Chemical Co. USA 1995).

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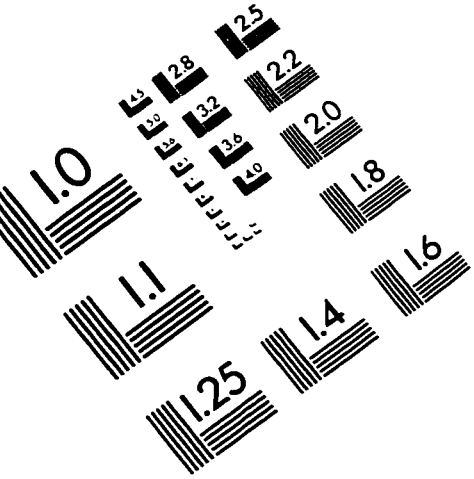
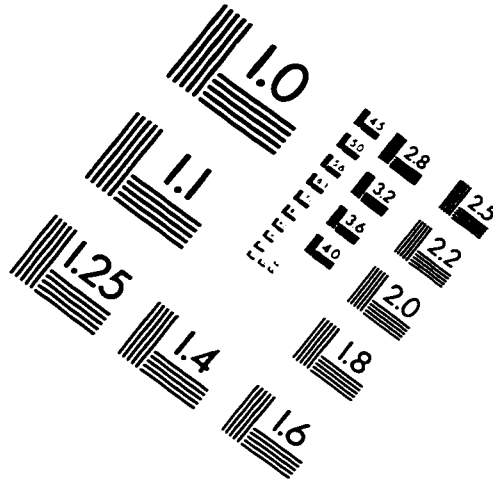
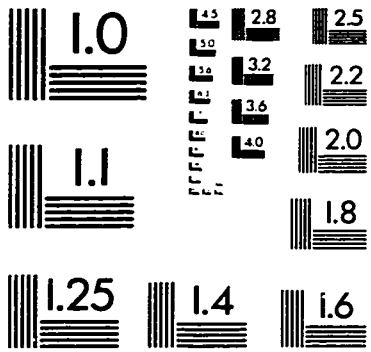
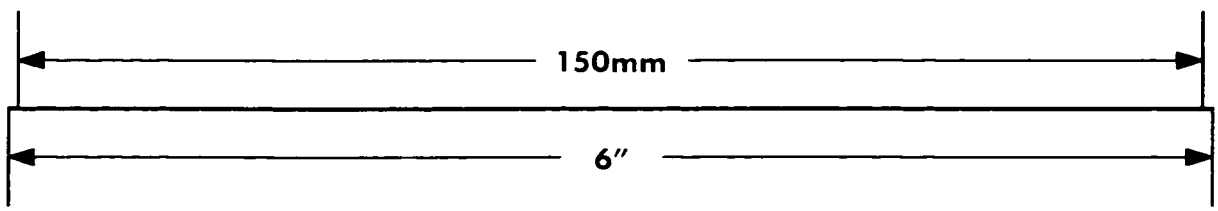
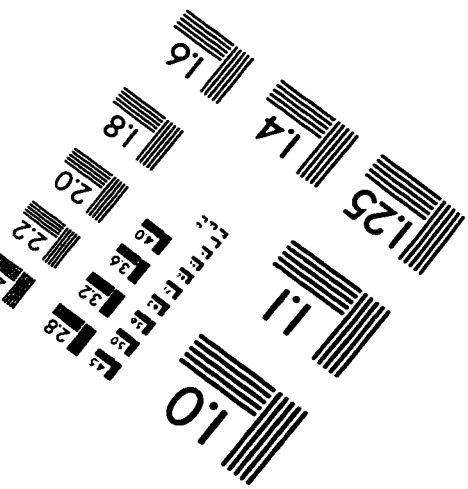
IMAGE EVALUATION TEST TARGET (QA-3)

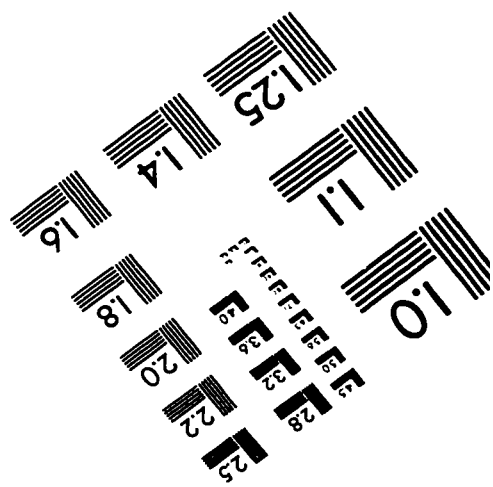












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