1976

Characterization of proteases and protease inhibitors in embryos of the brine shrimp Artemia salina.

Peter A. Nagainis
University of Windsor

Follow this and additional works at: https://scholar.uwindsor.ca/etd

Recommended Citation

Nagainis, Peter A., "Characterization of proteases and protease inhibitors in embryos of the brine shrimp Artemia salina." (1976). *Electronic Theses and Dissertations*. 1406.
https://scholar.uwindsor.ca/etd/1406

INFORMATION TO USERS

THIS DISSERTATION HAS BEEN
MICROFILMED EXACTLY AS RECEIVED

This copy was produced from a micro-
fiche copy of the original document.
The quality of the copy is heavily
dependent upon the quality of the
original thesis submitted for
microfilming. Every effort has
been made to ensure the highest
quality of reproduction possible.

PLEASE NOTE: Some pages may have
indistinct print. Filmed as
received.

Canadian Theses Division
Cataloguing Branch
National Library of Canada
Ottawa, Canada   K1A ON4

AVIS AUX USAGERS

LA THESE A ETE MICROFILMEE
TELLE QUE NOUS L'AVONS RECUE

Cette copie a été faite à partir
d'une microfiche du document
original. La qualité de la copie
dépend grandement de la qualité
de la thèse soumise pour le
microfilmage. Nous avons tout
fait pour assurer une qualité
supérieure de reproduction.

NOTA BENE: La qualité d'impression
de certaines pages peut laisser à
désirer. Microfilmée telle que
nous l'avons reçue.

Division des thèses canadiennes
Direction du catalogage
Bibliothèque nationale du Canada
Ottawa, Canada   K1A ON4

CHARACTERIZATION OF PROTEASES AND

PROTEASE INHIBITORS IN EMBRYOS

OF THE BRINE SHRIMP <u>ARTEMIA</u> <u>SALINA</u>

BY

PETER A. NAGAINIS

A Thesis
Submitted to the Faculty of Graduate Studies through the
Department of Biology in Partial Fulfillment
of the Requirements for the Degree of
Master of Science at the
University of Windsor

Windsor, Ontario

1976

ABSTRACT

Proteolytic activity has been implicated in several key processes in early embryonic development. In an attempt to correlate proteolytic activity with developmental events, a protease assay was developed using 2,4,6-trinitrobenzenesulfonic acid to determine the release of amino groups upon protein hydrolysis. The versatility and sensitivity of this method made it possible to detect and characterize the proteolytic activity in small quantities of cysts and developing embryos of the brine shrimp, Artemia salina. A protease with a molecular weight of 84,000, a pH optimum of 3.6 and a temperature optimum of $45^\circ C$ was partially purified from cysts. An inhibitor of this acid protease was also partially purified and found to have a molecualr weight of between 5,000 and 20,000. The inhibitor is inactivated slowly when incubated in the presence of the acid protease. Experiments using developing embryos revealed another protease activity appearing between 20 and 30 hours development (at $30^\circ C$). This activity has a molecular weight of 27,500 and a pH optimum of 8.5. This alkaline protease is inhibited by naturally occurring trypsin inhibitors but noy by the cyst inhibitor. Methods of quantitation of the proteases and inhibitor were developed to monitor their relative levels during early embryonic development in Artemia.

ACKNOWLEDGMENTS

I would like to thank my research advisor Dr. A. H. Warner for his inspiring direction and patient encouragement during the course of this research work.

I would also like to thank Dr. D.E. Schmidt for invaluable advice concerning this project.

Finally I would like to thank the Department of Chemistry for their generous loan of the Bausch & Lomb Spectrophotometer without which this work would not have been possible.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

Proteolytic enzyme activity has been implicated in a number of cell processes essential to early stages of embryonic development, although evidence of specific function(s) of proteases has not been clearly established. The onset of development following fertilization in the sea urchin is characterized by polysome formation and a burst of protein synthesis (1). Of interest is the finding that an inhibitor of protein synthesis associated with the ribosomes can be inactivated by treatment with trypsin to yield ribosomes capable of protein synthesis in vitro (2). In this regard, the observation by Lundblad (3) that a burst of proteolytic activity occurs immediately upon fertilization is of considerable interest. Thus proteolytic activity has been implicated in derepression of protein synthesis at the beginning of development.

It has also been demonstrated that stored yolk protein undergoes a redistribution in the developing embryo probably due to proteolytic activity (4). Proteolytic enzymes are also thought to be important in the activation of certain enzymes (similar to the activation of trypsin and chymotrypsin) present in an inactive form in the undeveloped egg (5).

The study of proteolytic activity in developing systems suffers from the fact that most assay methods have serious drawbacks. The method of Anson (6) which was developed in 1938, and still is in common use today, involves measurement of acid soluble fragments of protein substrates. However, this procedure is of limited value because it may be insensitive to limited proteolysis where large

acid insoluble fragments are produced. A relatively new method
for the measurement of proteolysis uses an insoluble cowhide sub-
strate with a covalently bound azo-dye (7). As the cowhide is
hydrolysed, the azo-dye is released and can be determined spectro-
photometrically. Although this method is sensitive, the reagents
are expensive and the versatility of this method is limited. Other
assays involving synthetic substrates are easy to use but extremely
selective for specific types of proteases and therefore not useful
as a general proteolytic assay.

Previous work on Artemia proteases was described by Bellini (8)
in 1957. In this study, whole homogenates of cysts and embryos
were assayed and protease levels were monitored using synthetic
substrates. Although proteolytic activity was detected in these
embryos, no attempt was made to characterize the protease.

The purpose of this study was to design a general protease
assay in that substrates could be used to detect and characterize
all types of protease activity in cysts and developing embryos of
Artemia salina. In addition, this new assay procedure was used to
elucidate the composition of protease(s) in Artemia cysts and
nauplii and to study some of these proteases in developing embryos.

MATERIALS AND METHODS

Determination of Amino Groups Liberated During Proteolysis

The determination of amino groups was routinely carried out using the method developed by Fields (9) with minor modifications. To 0.05 ml samples of material to be assayed were added 0.95 ml of a solution containing 0.053 M $Na_2B_4O_7$ and 0.053 M NaOH. Next, 0.025 ml of 1.1 M 2,4,6-trinitrobenzenesulfonic acid (TNBS, Sigma Chemical, St. Louis, Mo.) was added and the tubes allowed to stand at room temperature for 15 min. The color development was stopped by the addition of 2.0 ml of 0.1 M NaH_2PO_4 containing approximately 1.5 mM Na_2SO_3 and the intensity of color in each sample determined immediately at 420 nm in a Bausch & Lomb Spectronic 100 read against a reagent blank.

Assay for Proteolytic Activity

Stock substrate solutions containing bovine serum albumin (BSA, fraction V, Worthington Biochemical, Montreal) and buffer were prepared to be diluted when needed. All stock substrate solutions contained 16 mg/ml BSA and either 0.4 M sodium acetate, pH 4.0, 0.4 M sodium phosphate, pH 6.0 or 0.4 M sodium phosphate, pH 8.0. The pH values of all buffer solutions were determined under assay conditions and at 37°C. BSA was denatured prior to use by incubation in the appropriate buffer at 70°C for 30 min. Unless indicated otherwise, all reaction vessels contained 4.0 mg/ml BSA and 0.1 M buffer at the desired pH, and the fraction to be assayed for proteolytic activity in a final volume of 0.4 ml. The reaction /

3

vessels were incubated in a thermostated water bath at $37^{\circ}C$,
and at various times after enzyme addition, 0.05 ml aliquots were
removed for the determination of amino groups as described above.
Since BSA contains free amino groups (all N-terminal moieties and
$\in$-groups on the protein) it reacts with the colorimetric reagents
to produce background absorbance which must be considered when
evaluating the extent of proteolysis..

Assay of Trypsin Activity

Trypsin 2x recrystallized and salt-free (Worthington Bio-
chemical, Montreal) was assayed for activity as described by
Worthington (10) and found to contain 167 TAME units/mg protein in
0.05 M sodium borate containing 0.01 M $CaCl_2$, pH 8.1, at $25^{\circ}C$. Vary-
ing amounts of trypsin in 1 mM HCl were added to a reaction vessel
containing 4 mg/ml BSA, 0.10 M sodium borate and 0.01 M $CaCl_2$, pH 8.1,
in a final volume of 0.4 ml. The reaction mixture was incubated at
$25^{\circ}C$ and aliquots were removed at 10 min intervals for the deter-
mination of liberated amino groups by the TNBS method described
above. The background absorbance at time zero was subtracted from
all other values to give the actual increase in absorbance (ΔA_{420})
due to production of amino groups by the hydrolysis of substrate.

One unit of proteolytic activity was defined as that quantity
of enzyme which gave rise to a ΔA_{420} of 0.01 in 1 min.

Isolation of Proteolytic Enzymes from Artemia Cysts and Nauplii

Encysted Artemia salina embryos (Longlife Aquarium Products,
Harrison, N.J.) were hydrated in distilled water or artificial
sea water (11) then washed free of floating cysts, debris and

sand. All extraction and purification procedures were performed at 0-4°C unless otherwise indicated. Twenty grams (wet weight) of hydrated cysts were homogenized in a glass tissue grinder (Duall type) in 30 ml of 0.1 M KCl containing 0.015 M potassium phosphate, pH 7.0. (It should be noted that all subsequent solutions were buffered with 0.015 M potassium phosphate, pH 7.0.) The homogenate was centrifuged at 50,000 rpm in a 60 Ti rotor (Beckman Instruments Co.) for 2 h and the supernatant fluid removed by syringe avoiding the floating orange lipid layer. This fraction was either dialysed overnight against 2 to 3 changes of buffered 0.025 M KCl (200 volumes/change) or filtered through a G-25 Sephadex column (2.5 x 40 cm) using the same buffer. The dialysed or protein-rich fraction was applied to a DEAE-cellulose column (2.5 x 30 cm) previously equilibrated with buffered 0.025 M KCl and the column washed with an additional 50 ml of buffered 0.025 M KCl. The adsorbed material was eluted using a 1 liter linear gradient of 0.025 M KCl to 0.6 M KCl buffered with 0.015 M potassium phosphate, pH 7.0. Column fractions were assayed for proteolytic activity at pH 4.0, 6.0 and 8.0 as described above. The contents of the active fractions were pooled and concentrated either by pressure dialysis at 40 psi N_2 (Diaflo membrane, Um-10) or by vacuum dialysis to about 2.0 ml. The protease was purified further by gel filtration on a column of Ultrogel AcA34 (2.0 x 52 cm) previously equilibrated with buffered 0.1 M KCl and eluted with the same buffer. The contents of the active fractions were pooled, concentrated by pressure dialysis as above and stored at -15°C.

Swimming nauplii were obtained from 18 grams (wet weight)

of _Artemia_ cysts permitted to develop 40 h at 30°C as previously described (12). The nauplii were homogenized in 30 ml of buffered 0.1 M KCl and the homogenate centrifuged for 2 h at 50,000 rpm in a 60 Ti rotor (Beckman Instruments Corp). The supernatant fraction was processed on ion-exchange and gel filtration columns as described above.

The protein content of _Artemia_ cyst and nauplii preparations was determined by the method of Lowry _et al._ (13) after delipidization according to the procedure of Bligh & Dyer (14).

Isolation and Quantitation of a Protease Inhibitor from Extracts of _Artemia_ Cysts

From kinetic data on acid protease activity in the crude unfractionated enzyme preparation it appeared that cyst extracts contained a substance(s) inhibiting proteolytic activity. Therefore, an attempt was made to separate the inhibitory substance from the enzyme in the cyst homogenate. A crude cyst enzyme preparation was chromatographed on a column of DEAE-cellulose, and column fractions were assayed for inhibitory activity using a reaction mixture containing 4 mg/ml BSA, 0.1 M sodium acetate, pH 4.0, 0.05 ml of partially purified cyst enzyme obtained earlier and 0.25 ml of each column fraction to be tested in a final volume of 0.4 ml. The contents of those fractions displaying inhibitory activity were concentrated by pressure dialysis to about 2.0 ml and purified further by gel filtration on AcA34 as previously described. The protein fractions with inhibitory activity were concentrated by pressure dialysis and stored at -15°C.

When varying amounts of inhibitor were added to a constant

amount of acid protease a reduction of the initial rate of pro-
tease activity occurred; thereafter a gradual loss in inhibition
occurred and complete enzyme activity was restored as the time
of incubation progressed. The latter response appeared to be due
primarily to enzyme inactivation of the inhibitor. Therefore,
the amount of inhibitory activity that was present in the protein
preparation was determined by measuring the time required to
attain a predetermined level of hydrolysis, as determined by the
liberation of amino groups, compared to a control reaction with
enzyme only. The difference in time required to attain the same
level of hydrolysis was proportional to the amount of enzyme added.
Therefore one unit of inhibitory activity was defined as that
quantity of protein when added to one unit of activity of acid
protease caused a lag of one minute under standard incubation
conditions.

The proteases isolated from Artemia embryos were also tested
for sensitivity to naturally occurring inhibitors found in other
systems. Ovomucoid and soy bean trypsin inhibitor (SBTI, both
from Worthington Biochemical) in final concentrations of 0.01,
0.1 and 1.0 mg/ml were used in reaction mixtures containing 4 mg/ml
BSA in 0.1 M sodium acetate, pH 4.0, for the acid protease, and
4 mg/ml BSA in 0.1 M sodium phosphate pH 8.0 for the alkaline
protease, in a final volume of 0.4 ml. The reaction vessels were
incubated at 37°C and at various times aliquots were removed and
assayed for the extent of hydrolysis of the substrate as previously
described.

Determination of pH Optima

Stock substrate solutions containing BSA and buffer similar to those described above were prepared using the following buffers: citric acid-sodium-phosphate for assays between pH 2.5 and 4.0; sodium acetate for pH 4.0 to 5.5; sodium phosphate for pH 6.0 to 8.0; sodium borate for pH 8.0 to 9.0; and sodium borate-NaOH for pH 10.0. The cyst protease activity was measured at various pH values from 2.5 to 8.0 whereas the nauplii protease activity was measured from pH 4.0 to 10.0 using the TNBS method described above.

Determination of Temperature Optimum for Acid Protease Activity

The cyst acid protease was assayed at pH 4.0 using BSA (4 mg/ml) as substrate. Reaction mixtures were incubated at temperatures ranging from $25^{\circ}C$ to $55^{\circ}C$. Aliquots were removed at 15 min intervals and the liberated amino groups determined as before.

Molecular Weight Determinations

Molecular weight determinations were carried out using a gel filtration technique. All standards and embryo fractions were applied to a column of Ultrogel AcA34 (2.0 x 52 cm) and eluted with buffered 0.1 M KCl as described above. The void volume was determined using blue dextran, and the column calibrated using five proteins of known molecular weight (chymotrypsinogen, 25,000; ovalbumin, 45,000; bovine serum albumin 65,000; aldolase 145,000 and globulin 160,000). The relationship between elution volume of the standard proteins was determined, and the molecular weight of the cyst acid protease, the cyst protease inhibitor, and the nauplius alkaline protease determined based upon the

elution position of their biochemical activities.

Protease and Inhibitor Levels During Development of Artemia salina

Dry encysted embryos of Artemia salina were sterilized in
7% antiformin solution for 30 min at $4^{\circ}C$ (15), washed as described
above then hydrated for 3 hours at $0^{\circ}C$ in artificial sea water (11).
One gram cysts (wet weight) was added to a 250 ml Erlenmeyer flask
containing 35 ml artificial sea water (11) fortified with penicillin-
G (1,000 IU/ml) and streptomycin sulfate (100 μg/ml) to retard
bacterial growth. Embryos were incubated at $30^{\circ}C$ with gentle
shaking until needed, then collected on a scintered glass filter
and stored at $-15^{\circ}C$.

For extraction of the proteases and inhibitor, the frozen
embryos were homogenized directly in a glass homogenizer (Duall
type) in 10-12 ml of ice-cold 0.1 M KCl containing 0.015 M potassium
phosphate, pH 7.0, and the homogenate centrifuged at 40,000 rpm
in a SW 41 rotor (Beckman Instruments Inc.) for 2 hours. The
supernatant fluid was collected and passed through a G-25 Sephadex
column (1.1 x 50 cm) as previously described. The protein eluting
in the void volume was collected and 1.0 ml was retained for
protein analysis. From the remainder, 10 ml was applied to a small
DEAE-cellulose column (1.3 x 4 cm) previously equilibrated with
buffered 0.025 M KCl. After application of the protein sample,
the column was washed with 5 ml of additional buffered 0.025 M KCl
(to remove residual inhibitory protein) and the effluent and wash
were combined. This non-adsorbable material was free of protease·
activity but contained all of the protease inhibitor. The protease(s) was
(were) eluted from the column using 10 ml of buffered 0.6 M KCl.

Embryos from various developmental stages were treated in the same manner. The amount of protease activity in the buffered 0.6 M KCl washes was determined at pH 4.0, 6.0 and 8.0 in the presence of 1 mM EDTA as described previously using the TNBS method to determine the liberation of amino groups. The amount of acid protease inhibitor activity in the non-adsorbable DEAE-cellulose fraction was determined as described below. The reaction mixture contained 4 mg/ml BSA in 0.1 M sodium acetate, pH 4.0, 1.71 units of acid protease activity, and 250 μl of the inhibitor-rich DEAE-cellulose fraction in a volume of 0.4 ml. Aliquots were removed at various times for the determination of liberated amino groups by the TNBS method. The units of acid protease inhibitor activity were determined as above and corrected for variations in protein content and/or volume of the G-25 Sephadex protein fraction.

Assay of Amino Groups Liberated by Proteolysis

The use of a sensitive colorimetric determination for the detection of liberated amino groups was employed in the development of a new assay for proteolytic activity. A standard curve using alanine revealed that the relationship between absorbance at 420 nm and the concentration of amino groups in the assay was linear from 0 to 250 nmoles as shown in Figure 1. Therefore, samples with absorption values greater than 1.75 A_{420} were discarded and smaller aliquots were removed from assay mixtures for the determination of amino groups liberated.

The enzymatic hydrolysis of a peptide bond in a protein results in the production of two smaller polypeptides concomitant with the liberation of a new free amino terminus as shown in reaction (1). This amino group will react with trinitrobenzenesulfonic acid (TNBS) under alkaline conditions to produce a colored complex as shown in reaction (2).

$$\ldots NH\text{-}\overset{R}{C}H\text{-}CO\text{-}NH\text{-}\overset{R'}{C}H\text{-}CO\ldots \quad + \quad H_2O$$

$$\downarrow \text{protease} \tag{1}$$

$$\ldots NH\text{-}\overset{R}{C}H\text{-}COO^- \quad + \quad H_3N\text{-}\overset{R'}{C}H\text{-}CO\ldots$$

$$\downarrow \text{pH 9.5}$$

$$H_2\overset{R'}{N}\text{-}CH\text{-}CO\ldots \quad + \quad O_2N\text{-}\underset{NO_2}{\overset{NO_2}{\bigcirc}}\text{-}SO_3^- \tag{2}$$

$$\downarrow \text{pH 9.5}$$

$$O_2N\text{-}\underset{NO_2}{\overset{NO_2}{\bigcirc}}\text{-}NH\text{-}CH\text{-}CO\ldots \quad + \quad SO_3^{-2} + H^+$$
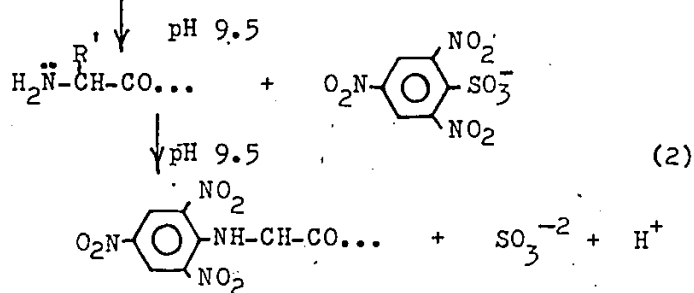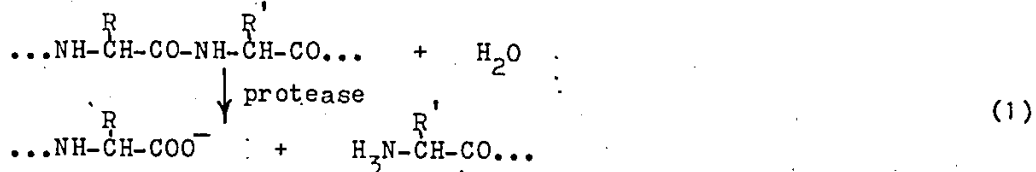
colored complex

FIGURE 1

Standard curve for assay of amino groups using alanine.

To reaction vessels containing 0 to 500 nmoles alanine in
0.5 ml distilled H_2O were added 0.5 ml of 0.1 M $Na_2B_4O_7$
containing 0.1 M NaOH. To this mixture 0.025 ml of 1.1 M
2,4,6-trinitrobenzenesulfonic acid (TNBS) was added and
the reaction vessels allowed to stand at room temperature.
After 15 min 2.0 ml of 0.1 M $Na_2H_2PO_4$ containing approx-
imately 1.5 mM Na_2SO_3 was added and the color intensity
immediately determined at 420 nm against a reagent blank.
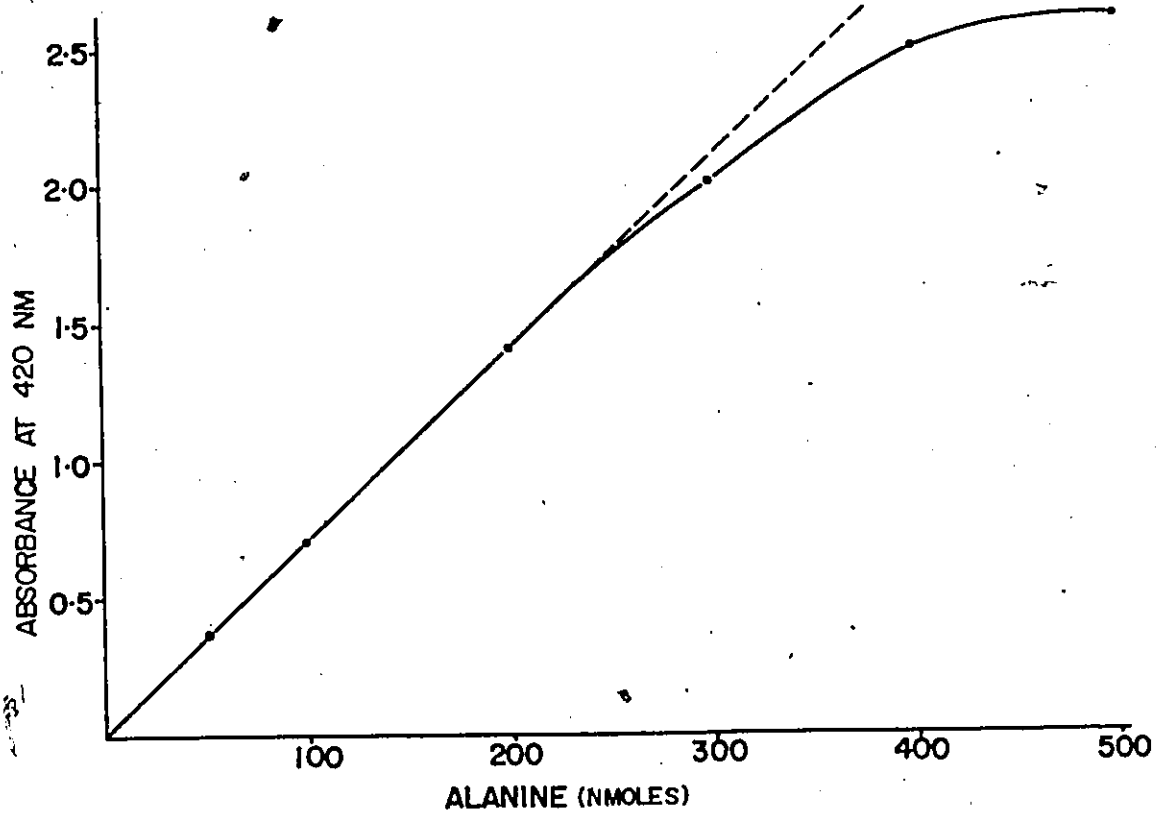
The greater the number of peptide bonds split the greater will
be the number of amino groups available to react with TNBS.
Therefore the reaction should permit us to detect limited levels
of proteolytic activity in addition to extensive hydrolysis of
protein substrates to their amino acid constituents.

Assay of Proteolytic Activity Using Trypsin

A commercially available preparation of trypsin was used to
test the efficacy of this procedure as an assay for proteolytic
activity. The results of an experiment using increasing concen-
trations of trypsin in a reaction mixture containing BSA as substrate
are shown in Figure 2. The increase in absorbance at 420 nm as
a function of time and enzyme concentration was as expected for
trypsin activity and comparable to that reported by Kunitz using
a different assay procedure (16).

Studies of Proteolytic Activity in Artemia Cysts

Subsequent to the development of the TNBS method for the
detection of protease activity, a study was undertaken to determine
the amount and type(s) of proteolytic activity in the undeveloped
cysts. The post-ribosomal supernatant fluid was obtained from
the cyst as described above and assayed at pH 4, 6 and 8. No
activity was observed at pH 6 and 8, however some activity was
detected at pH 4. After dialysis of this fraction, the proteolytic
activity at pH 4 increased by over 300% as shown in Figure 3.
When varying amounts of the dialysed protein fraction were assayed
for proteolytic activity, the data in Figure 4 were obtained.
In each case it should be noted that the initial rate of proteolysis

FIGURE 2

Assay of proteolytic activity using trypsin.

All reaction vessels contained 4 mg/ml BSA, 0.1 M sodium borate, pH 8.1, 0.01 M CaCl$_2$ and 0 to 200 ug trypsin in 0.001 M HCl in a final volume of 0.4 ml. The vessels were incubated at 25°C and at the desired time 0.05 ml aliquots were removed for the determination of amino groups as described in Materials and Methods.
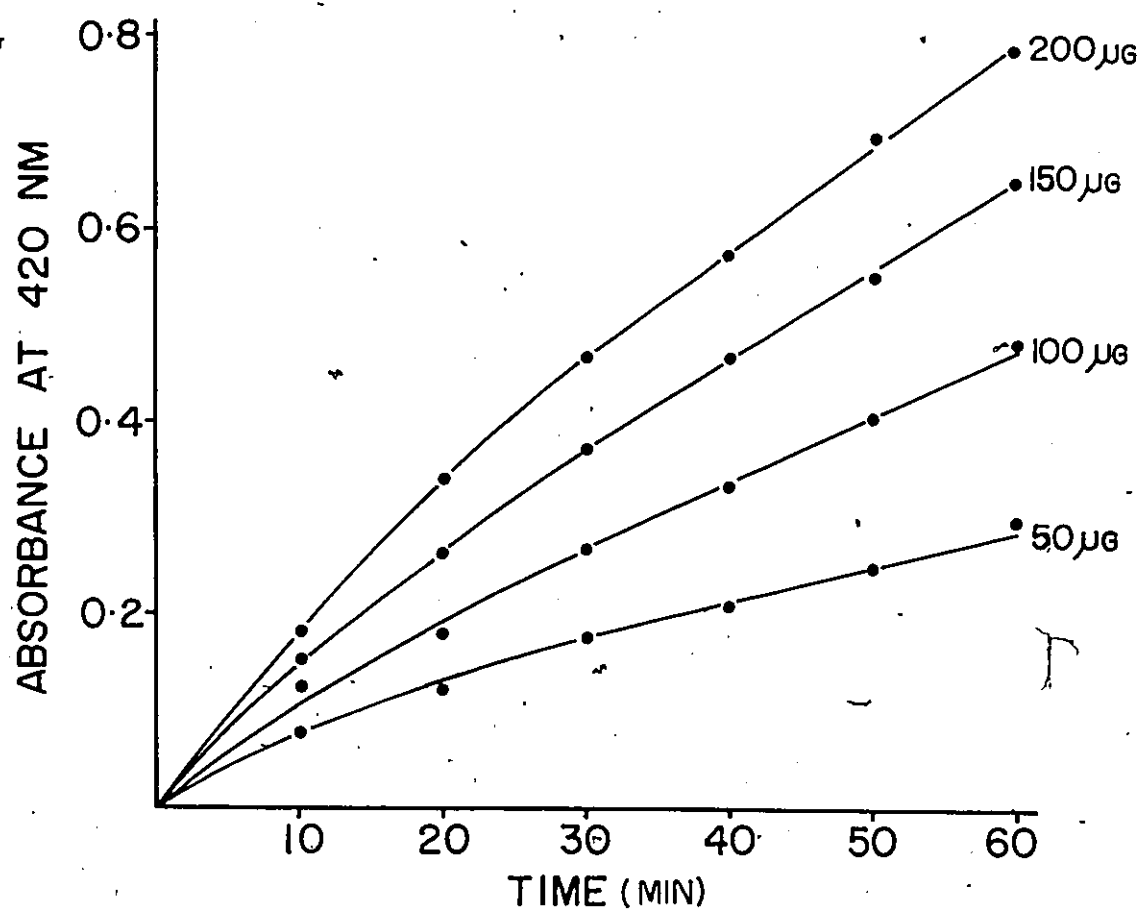
FIGURE 3

Proteolytic activity in dialysed and undialysed extracts of Artemia cysts.

One gram (wet weight) of hydrated cysts were homogenized in 10 ml of 0.1 M KCl buffered with 0.015 M potassium phosphate, and the homogenate centrifuged at 40,000 rpm in a SW 41 rotor. The supernatant was removed and part was dialysed overnight against two changes of 4.0 liters of the homogenization medium. Aliquots (300 μl, 4.2 mg protein) of both the dialysed and undialysed preparations were assayed in standard reactions containing 4 mg/ml BSA and 0.1 M sodium acetate pH 4.0 in a total volume of 0.4 ml. The reaction vessels were incubated at $37^{o}C$ and at the desired times aliquots were removed and assayed for amino groups as described in Materials and Methods.
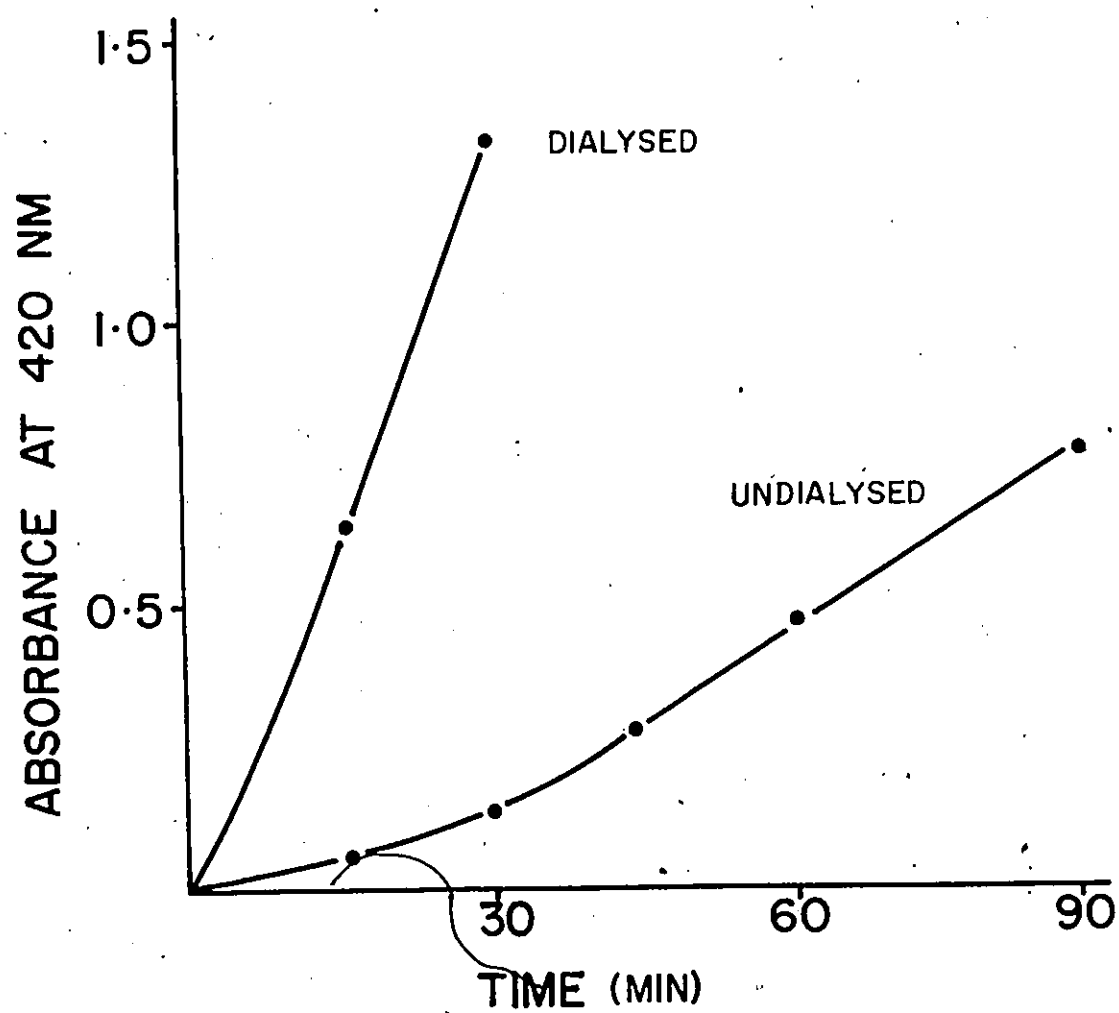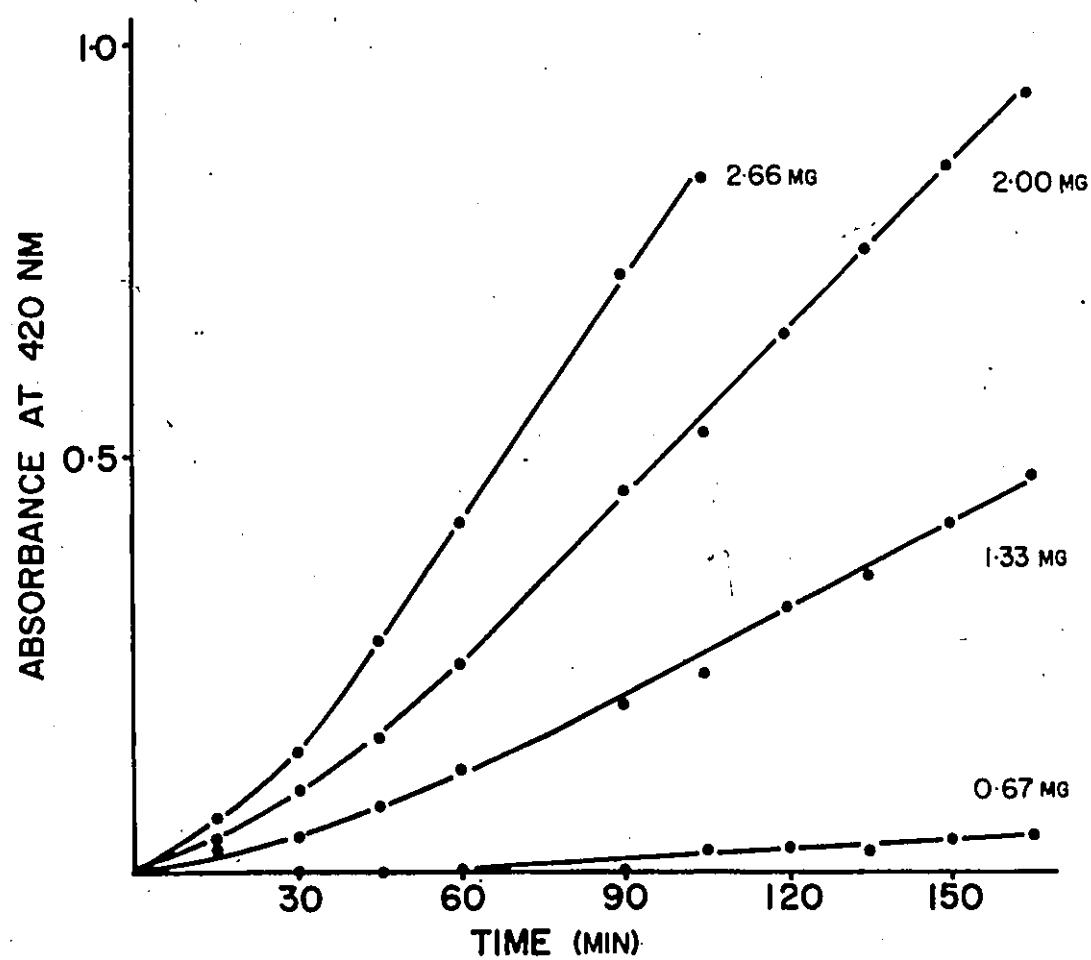
FIGURE 4

Proteolytic activity in dialysed extracts of _Artemia_ cyst.

Dialysed post-ribosomal supernatant fluid from _Artemia_ cysts
was   tested at four different concentrations as indicated
above. The reaction conditions were as described for Fig. 3.

was low, but as the time of incubation increased, a linear rate of hydrolysis was achieved and sustained for the duration of the assay.

Fractionation of the Cyst Protease and Inhibitor on DEAE-Cellulose

In an attempt to purify the proteolytic activity, the dialysed post-ribosomal fraction was chromatographed on a column of DEAE-cellulose and the column fractions analysed at 280 nm in a UV spectrophotometer and assayed for both proteolytic and inhibitor activity. The results of these experiments are shown in Figure 5 and indicate that a single peak of proteolytic activity is present in Artemia cysts, active at pH 4.0, and eluting at about 0.24 M KCl. A study of the kinetics of the hydrolytic reaction indicated that the initial lag in activity observed in the crude dialysed extract had been eliminated by this fractionation procedure. However, when enzymatically active fractions were pooled and assayed again in the presence of the other column fractions, it was observed that the non-adsorbable column fractions essentially restored the lag in activity that was observed with the unfractionated crude enzyme preparation. Therefore, it appears that Artemia cysts contain a non-dialysable protease inhibitor in addition to a dialysable component(s) that is also inhibitory.

Chromatography of the Cyst Acid Protease on Ultrogel AcA34

In order to further purify the acid protease from the DEAE-cellulose column, the combined protease fractions were concentrated by pressure dialysis and chromatographed on a column of Ultrogel AcA34. The elution profile of the column is shown in Figure 6

· FIGURE 5 .

Chromatography of <u>Artemia</u> cyst enzyme preparation on DEAE-
cellulose.

Twenty grams (wet weight) hydrated cysts were homogenized in

30 ml of 0.1 M KCl buffered with 0.015 M potassium phosphate,

pH 7.0, and centrifuged for 2 hours at 50,000 rpm in a 60 Ti

rotor. The supernatant fluid was dialysed overnight against

2 changes (2 liters each) of 0.025 M KCl buffered with 0.015 M

KCl solution. The column was washed with an additional 50 ml

of buffered 0.025 M KCl then eluted using a 1 liter linear

gradient of buffered KCl from 0.025 to 0.6 M KCl. Aliquots

of selected column fractions were assayed for proteolytic

activity at pH 4.0, 6.0 and 8.0 and for inhibitory activity

as described in Materials and Methods. Since no proteolytic

activity was detected at pH 6.0 and 8.0 only the results

obtained at pH 4.0 are shown.

●▬▬▬●, absorbance at 280 nm

○▬▬▬○, proteolytic activity, pH 4.0

●▬▬▬●, inhibitory activity
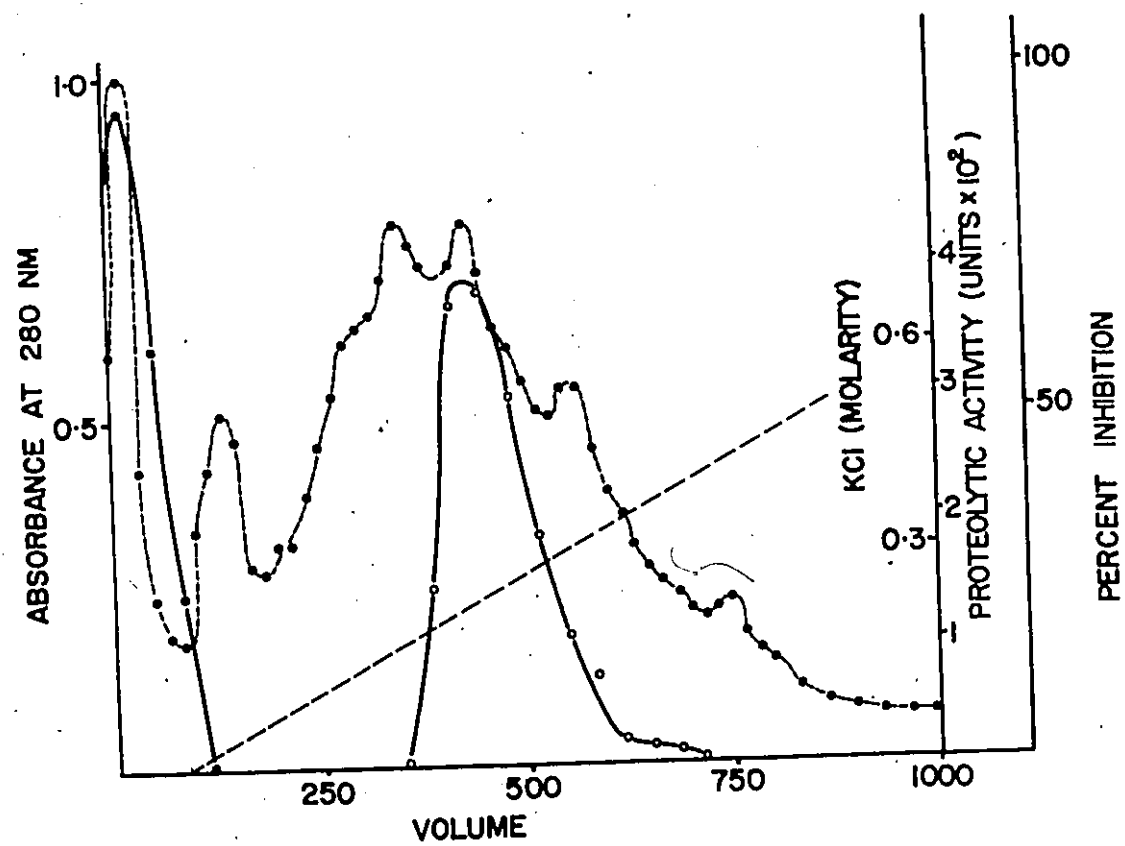
▬ ▬ ▬, KCl concentration

FIGURE 6

Chromatography of the cyst acid protease on Ultrogel AcA34.

The fractions from the DEAE-cellulose column containing
acid protease activity in Fig. 5 were pooled and concentra-
ted by pressure dialysis to a volume of 2.0 ml. The sample
was then applied to a column of Ultrogel AcA34 (2.0 x 52 cm)
previously equilibrated with 0.1 M KCl containing 0.015 M
potassium phosphate, pH 7.0, and eluted with the same
buffer. Aliquots of all column fractions were assayed at
pH 4.0 as described in Materials and Methods.

O————O , absorbance at 280 nm

●————● , proteolytic activity at pH 4.0
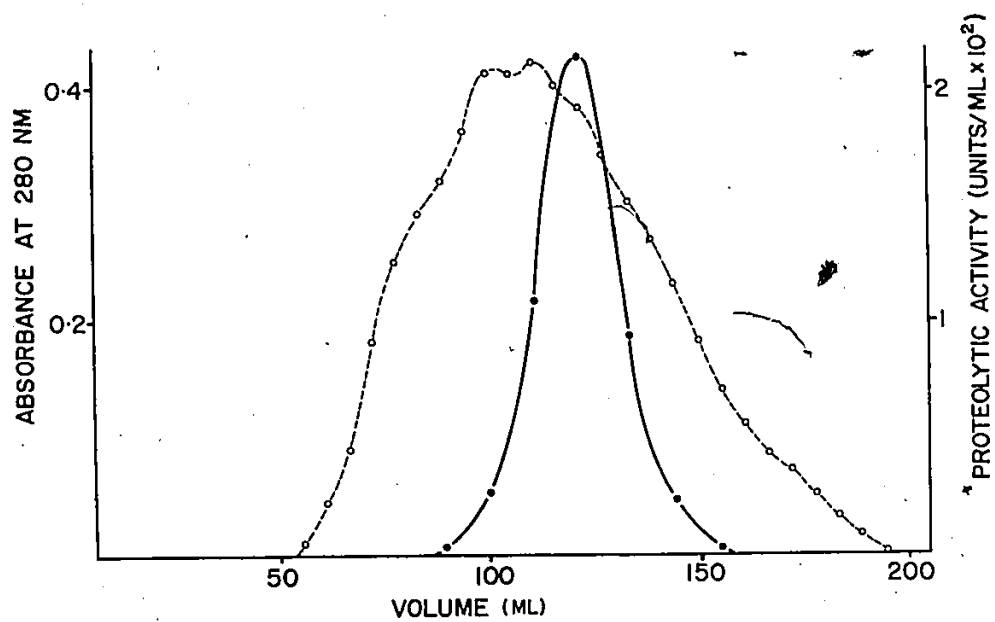
and it is clear that the protease activity elutes as a single peak. The elution position of this protease compared with other proteins of known molecular weight corresponds to a molecular weight of 84,000 (Figure 7).

pH and Temperature Optima of the Cyst Acid Protease

The partially purified acid protease was assayed at various temperatures and pH in order to determine the conditions of optimum hydrolysis. Using BSA as the substrate, the enzyme was found to have a pH optimum of about 3.6 (Figure 8) and a temperature optimum of about $45^{\circ}C$ (Figure 9).

Effect of $CaCl_2$ and EDTA on the Kinetics of Acid Protease Activity

When the partially purified acid protease was assayed at various enzyme concentrations, the initial rate of hydrolysis was found to be linear, but the activity was not proportional to enzyme concentration at low levels of enzyme as shown in Figure 10. The exact cause of this deviation from expected kinetics is not known but an attempt was made to eliminate the problem. When the assays were performed in the presence of 0.01 M $CaCl_2$ the response to enzyme concentration was nearly linear. However, the addition of 1 mM EDTA to the reaction mixture completely eliminated the problem and a linear activity curve was obtained as a function of enzyme concentration. The results of these experiments are shown in Figure 11. Therefore, all subsequent enzyme kinetic assays were carried out in the presence of 1 mM EDTA.

FIGURE 7

Molecular weight estimation of <u>Artemia</u> cyst and nauplii
    proteases.

A column of Ultrogel AcA34 equilibrated with 0.1 M KCl
containing 0.015 M potassium phosphate, pH 7.0, was used.
The reference proteins used were (1) chymotrypsinogen A,
(3) ovalbumin, (4) bovine serum albumin, (6) aldolase,
and (7) gamma globulin. The acid protease eluted at a
K_{av} of 0.84. Arrows indicate molecular weights of 84,000
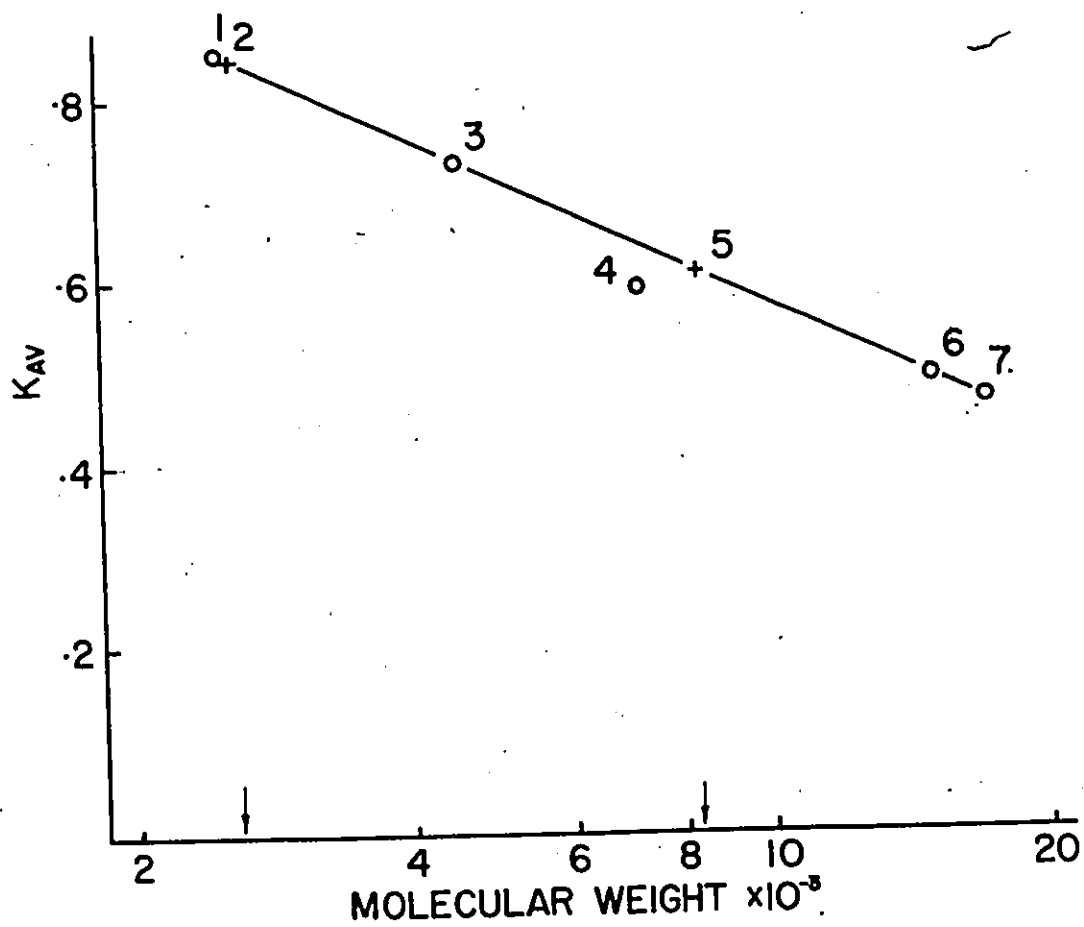and 27,500 for the acid and alkaline proteases, respectively.

FIGURE 8

Determination of pH optimum of the cyst acid protease.

The cyst acid protease used in this assay was partially
purified on DEAE-cellulose and AcA34. The protease activity
in a standard reaction mixture was assayed at varying pH
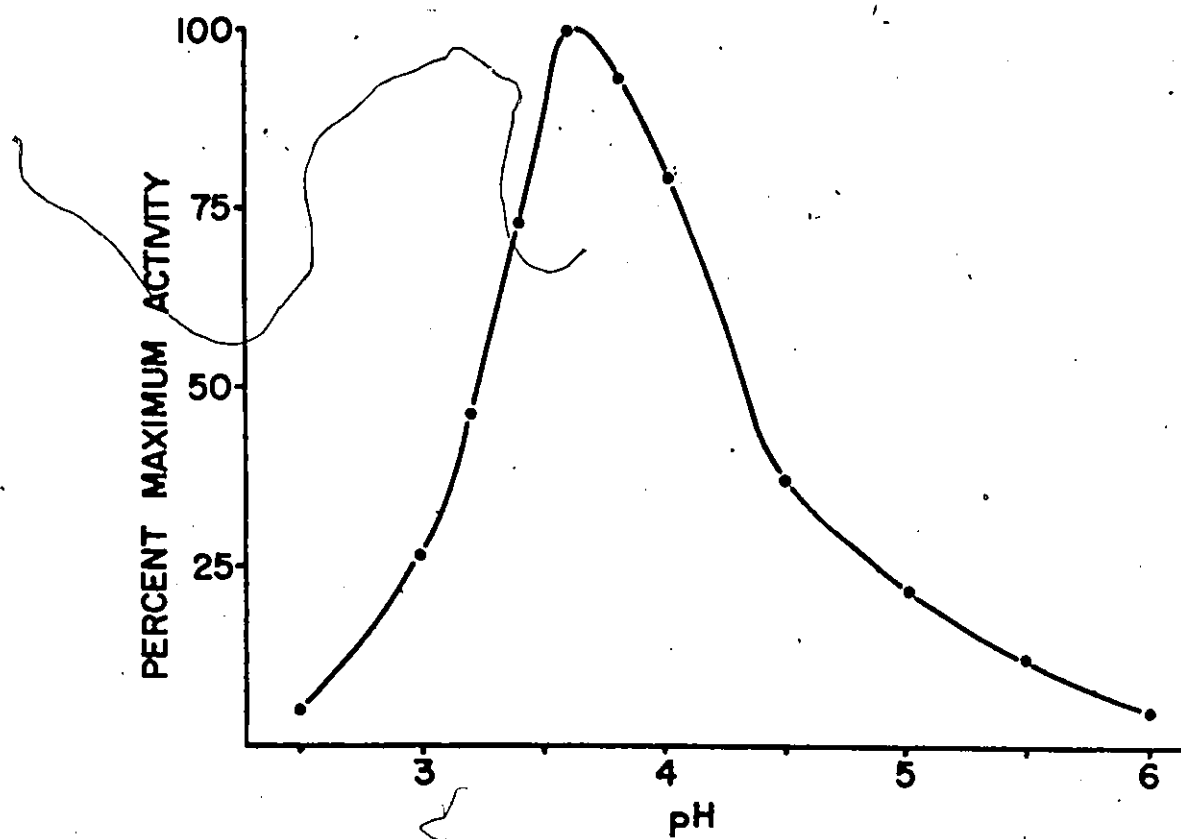from 2.5 to 6.0 at $40^{\circ}C$ as described in Materials and
Methods.

FIGURE 9

Determination of temperature optimum of the cyst acid protease.

The DEAE-cellulose and AcA34 partially purified acid protease
was assayed in a standard reaction mixture at pH 4.0 at
temperatures ranging from $25^{\circ}C$ to $55^{\circ}C$ as described in
Materials and Methods.
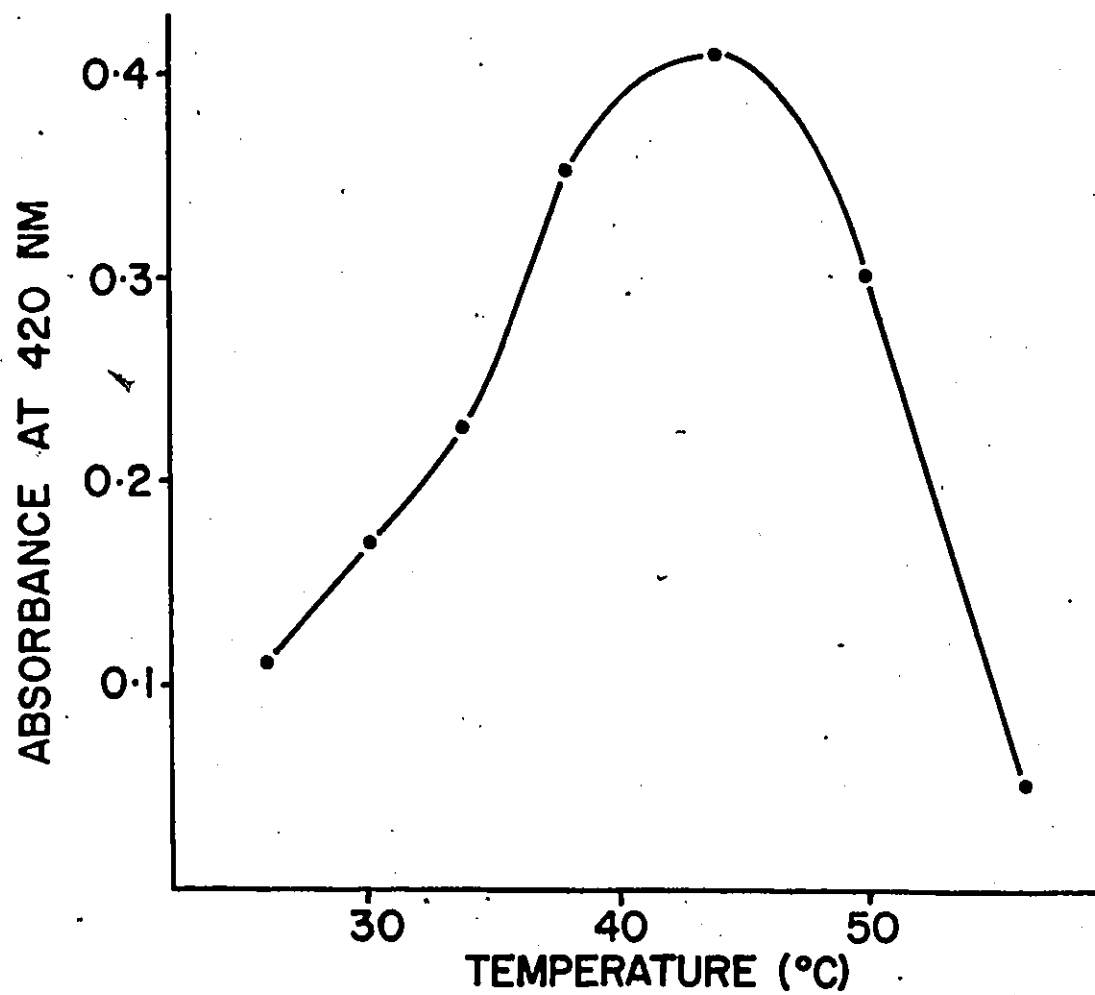
FIGURE 10

The effect of concentration on acid protease activity.

Varying quantities of partially purified acid protease
as described in Fig. 8 were assayed at pH 4.0 as described
in Materials and Methods. The increase in absorbance after
30 min incubation in each case was plotted against enzyme
concentration expressed in milligrams protein.
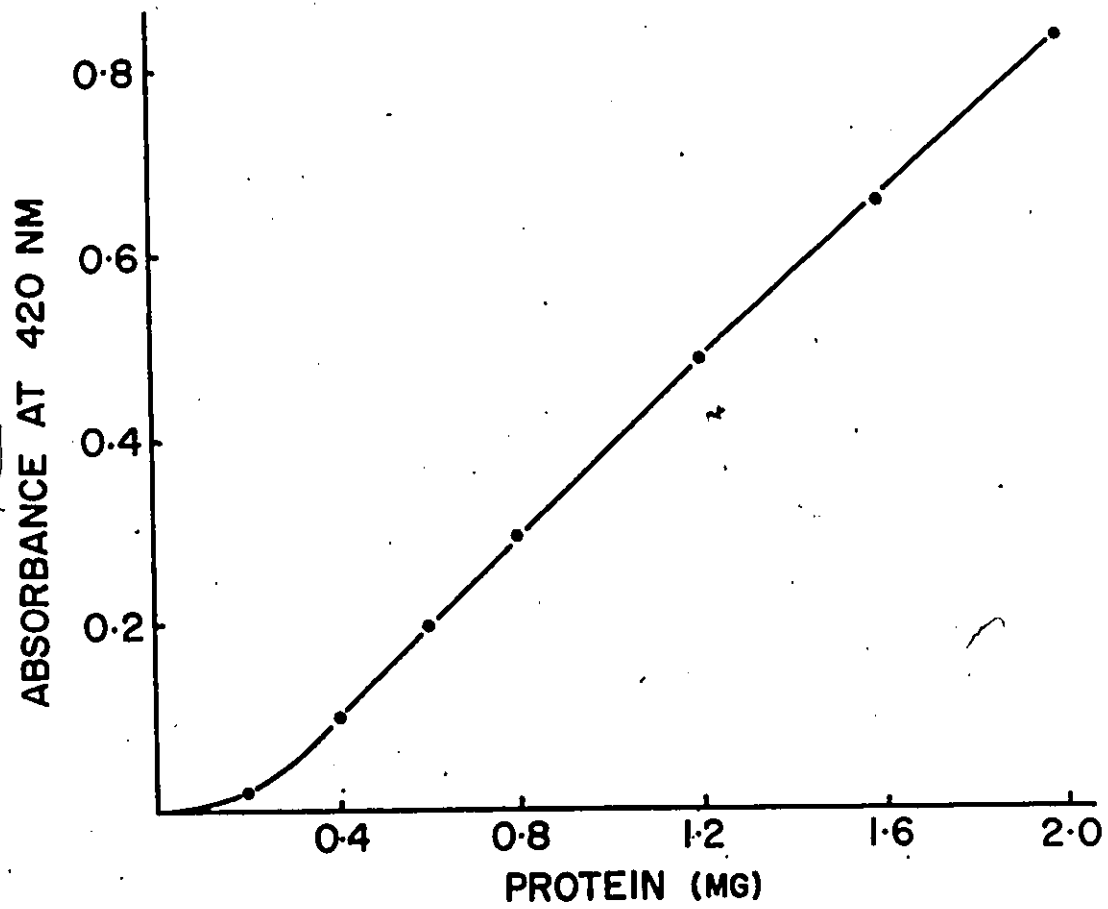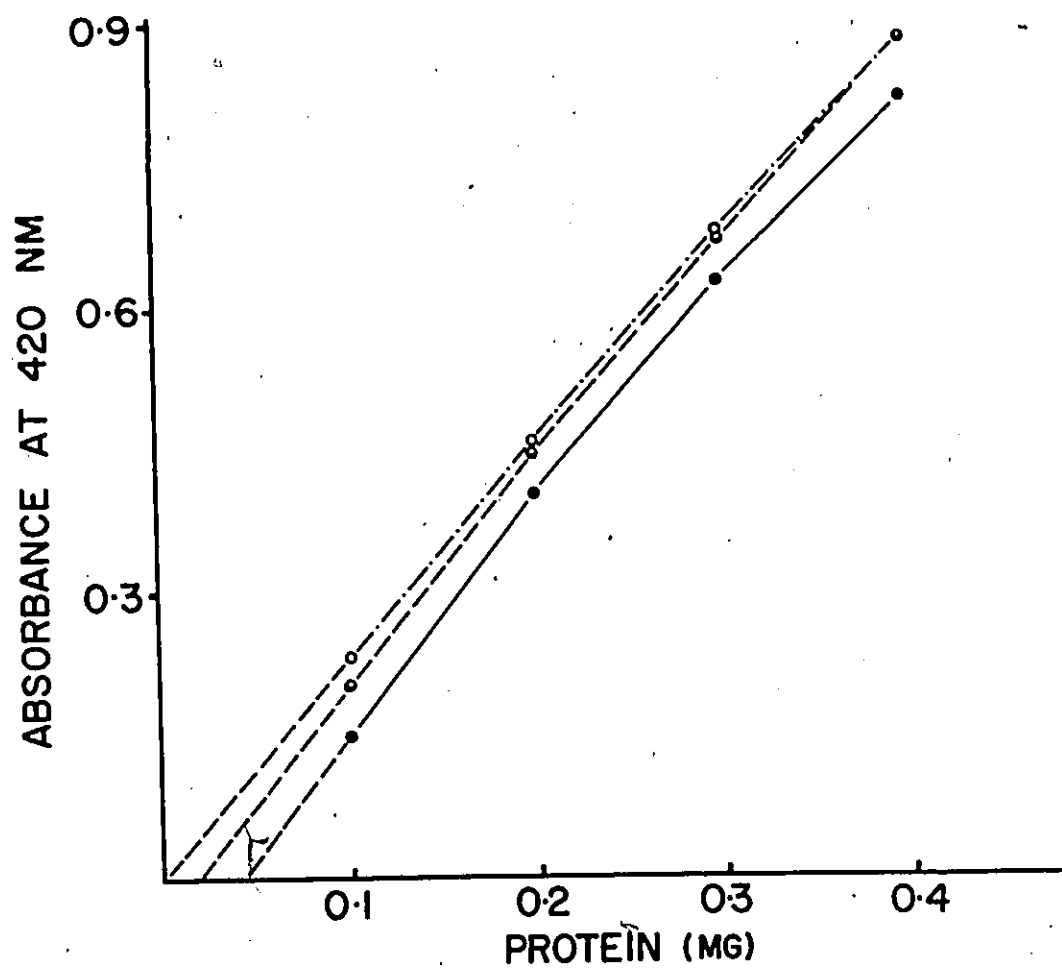
FIGURE 11

Effect of $CaCl_2$ and EDTA on kinetics of acid protease activity.

The partially purified acid protease was assayed as in Fig.

10 but included 0.01 M $CaCl_2$ or 1 mM EDTA in reaction mixtures

as indicated.

●———● ,   control (no addition)

◓—·—◓ ,   plus 0.01 M $CaCl_2$

○—·—○ ,   plus EDTA

Chromatography of the Acid Protease Inhibitor on Ultrogel AcA34

In order to further purify the inhibitor of acid protease activity, the combined inhibitory fractions from DEAE-cellulose were concentrated and chromatographed on a column of Ultrogel AcA34. The elution profile is shown in Figure 12. The inhibitor eluted as a single peak and preliminary data indicate that this component has a molecular weight of less than 20,000.

Protease-Inhibitor Kinetics

As described earlier, incubation of the acid protease and inhibitor together in a standard protease reaction mixture produced considerable inhibition initially, followed by a resumption of activity at the expected rate of hydrolysis. In order to determine whether the inactivation of the inhibitor in the reaction mixture was the result of thermal inactivation during the course of the reaction and not due to the presence of the acid protease, the inhibitor was incubated in a standard reaction mixture, lacking only the protease, for up to two hours prior to the introduction of the protease. The results of this experiment are illustrated in Figure 13 and indicate that very little inhibitory activity was lost by pretreatment of the inhibitor at 30°C in the standard reaction mixture. Therefore, it appears that inhibitor inactivation is due primarily to the presence of the acid protease and not to thermal effects. Consequently it should be possible to study the kinetics between the protease and inhibitor.

When varying amounts of inhibitor were added to a constant amount of acid protease it was observed that the duration of inhibition was directly proportional to the amount of inhibitor

FIGURE 12

Chromatography of the acid protease inhibitor on Ultrogel
   AcA34.

The fractions containing inhibitory activity from a DEAE-
cellulose column (see Fig. 5) were concentrated by pressure
dialysis and chromatographed on a column of Ultrogel AcA34
(2.0 x 52 cm) as described in Fig. 6. The column fractions
were analyzed for protein at 280 nm and for inhibitory
activity as described in Materials and Methods.

O———O , absorbance at 280 nm

●———● , inhibitor activity
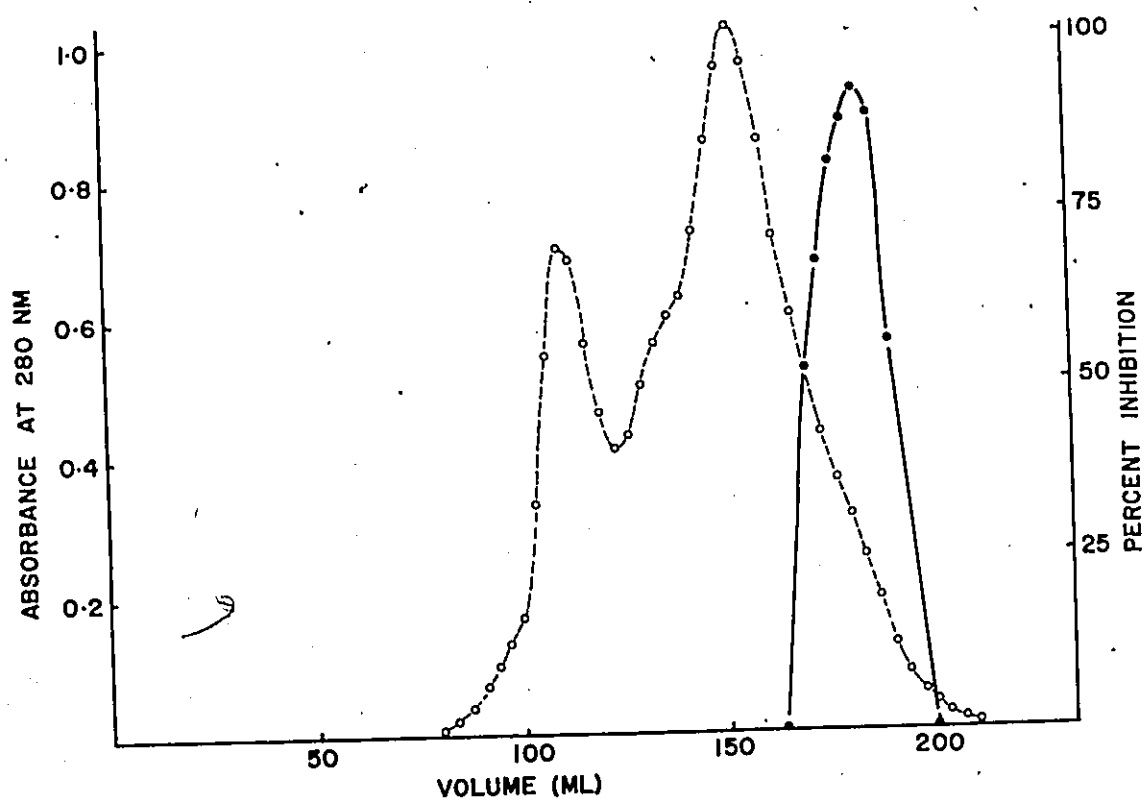
FIGURE 13

Effect of preincubation of inhibitor on its inhibitory
  activity.

Partially purified inhibitor (91 units) was incubated at
30^0C in 5 separate standard acid protease mixtures lacking
only the acid protease. After various times of incubation,
1.5 units of acid protease was added to one of the reaction
vessels and the reactions allowed to proceed for the time
indicated on the abscissa. The liberation of amino groups
from BSA was determined as described in Materials and
Methods.
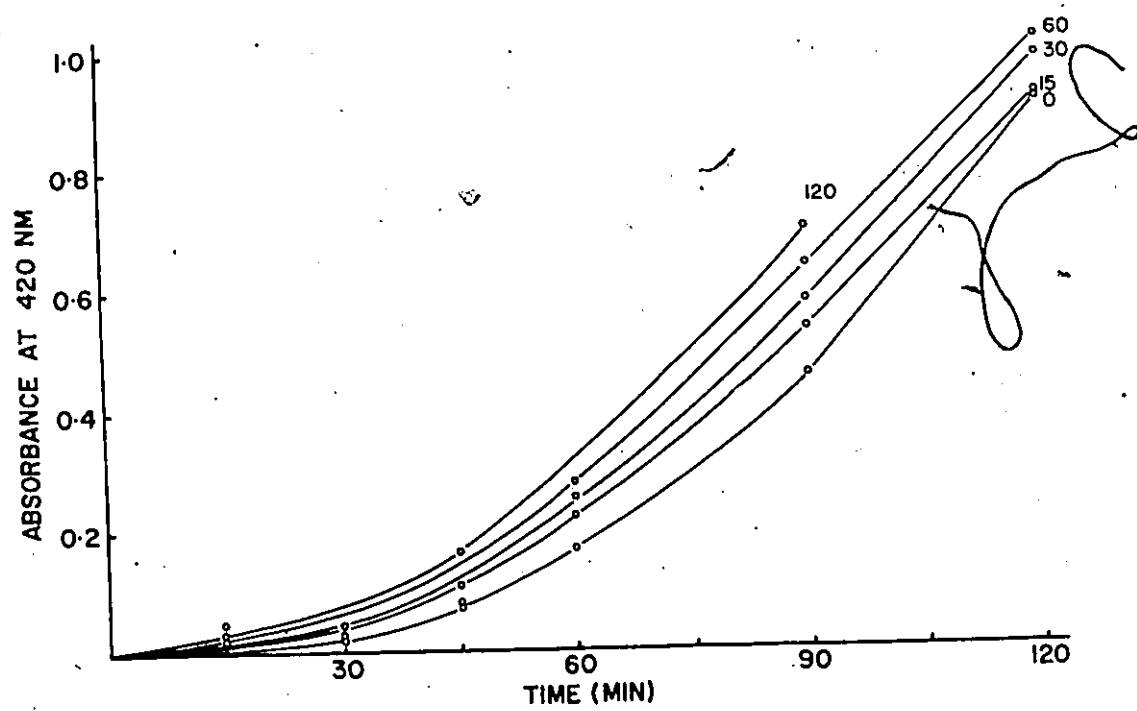
added. These findings are shown in Figure 14. However it should

be noted that at low concentrations of inhibitor this relation-

ship is not linear. The effect of adding EDTA and/or $CaCl_2$ to

an enzyme-inhibitor mixture is shown in Figure 15. When EDTA is

added, the inhibitory period is shortened slightly, whereas in the

presence of $CaCl_2$, the inhibitory period is decreased greatly.

In the presence of both EDTA and $CaCl_2$ still less inhibition

occurs and the response to these reagents appears to be additive.

The results of experiments using various inhibitor concentrations

in the presence of EDTA and/or $CaCl_2$ are shown in Figure 16. In

this illustration the duration of inhibition is plotted against

inhibitor concentration, and it should be noted that as the rate

of inactivation of the inhibitor is increased, such as in the presence

of $CaCl_2$ or EDTA and $CaCl_2$, the slope of the line decreases,

indicating a decrease in the period of inhibition prior to re-

sumption of the expected rate of hydrolysis. Also it should be

noted that the control reaction lacking EDTA or $CaCl_2$ shows a

non-linear response at low inhibitor concentrations but that

linearity could be restored without reduction in inhibitor function

by including EDTA in the reaction. However, $CaCl_2$ alone or in

combination with EDTA increased the rate of inactivation of the

inhibitor by over 200%.

Levels of Protease(s) and Inhibitor in Developing Artemia Embryos

Embryos of Artemia salina were collected at various stage of

development and the post-ribosomal supernatant fraction processed to

separate the inhibitor from the protease fraction. The amount of pro-

tease activity in each sample was measured at pH 4, 6 and 8 and the

FIGURE 14

Effect of increasing inhibitor concentration on acid protease
    activity.

All reaction mixtures contained 5.0 units of acid protease,
4 mg/ml BSA, 0.1 M sodium acetate, pH 4, and increasing
amounts of inhibitor as indicated. The reaction vessels were
incubated at $37^{\circ}C$ and aliquots were removed with time for
determination of amino groups as described in Materials and
Methods.
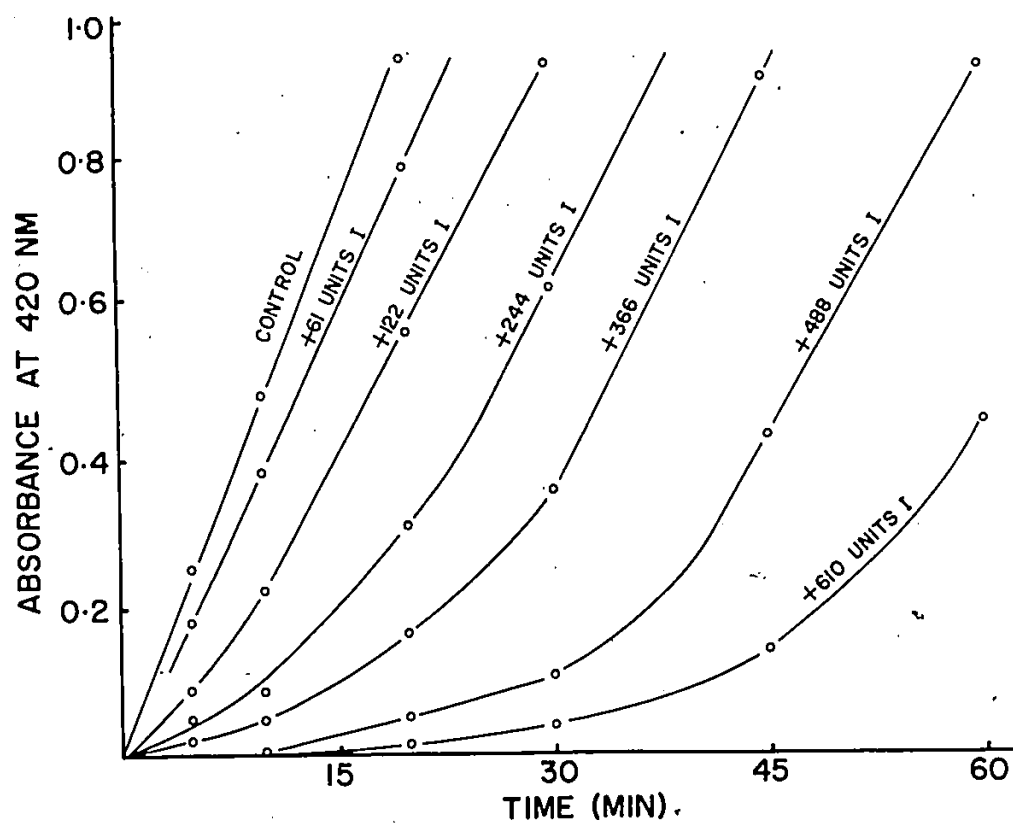
FIGURE 15

Effect of $CaCl_2$ and EDTA on inhibitor inactivation.

All reaction mixtures contained 1.5 units of acid protease, 366 units of inhibitor, 4 mg/ml BSA, 0.1 M sodium acetate, pH 4.0 and either 0.01 M $CaCl_2$ and/or 1 mM EDTA. All vessels were pre-incubated at $37^{O}C$ and at times indicated, aliquots were removed for the determination of amino groups as described in Materials and Methods.

FIGURE 16

A summary of the effect of $CaCl_2$ and EDTA on inhibitor
    inactivation.

The results shown in Fig. 14 (control) and Fig. 15 plus
identical experiments including 0.01 M $CaCl_2$ and/or 1 mM
EDTA in the reaction mixtures are summarized here. The lag
in resumption of protease activity was measured as des-
cribed in Materials and Methods and plotted against
inhibitor concentration.

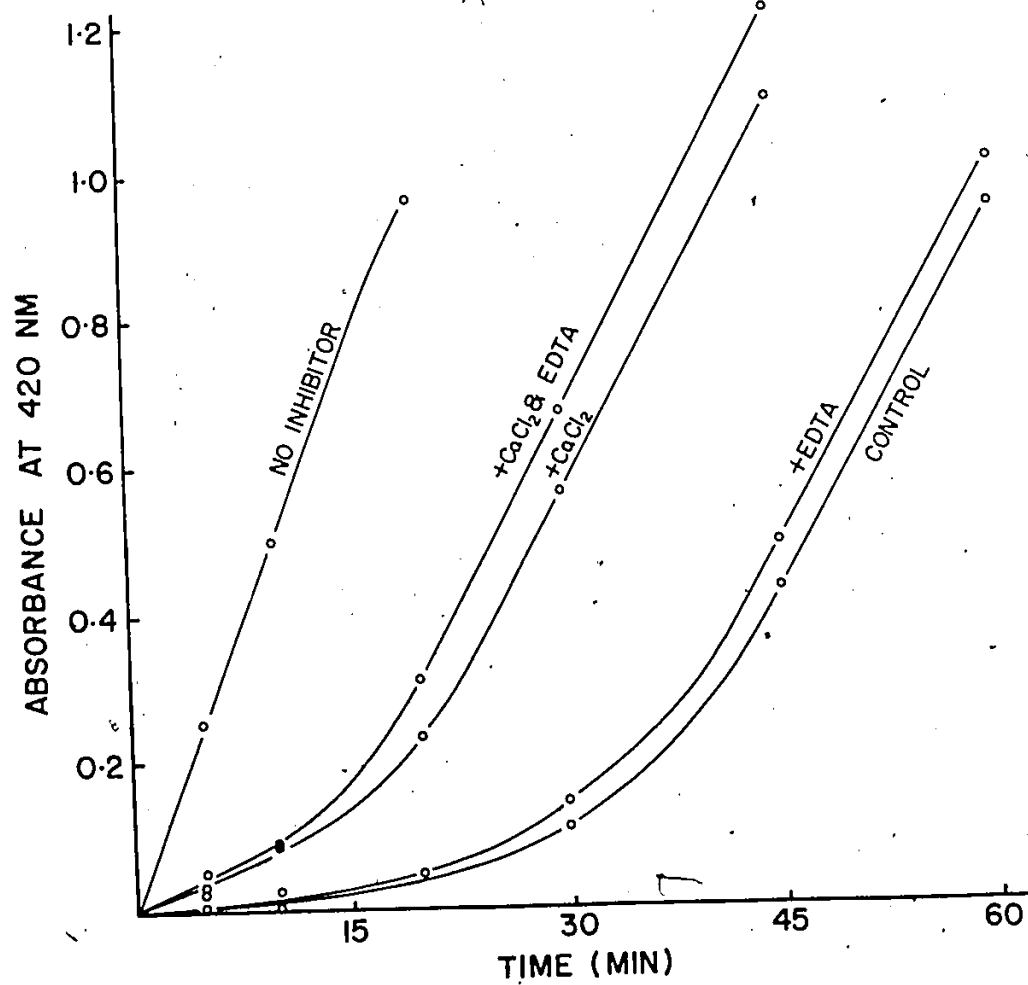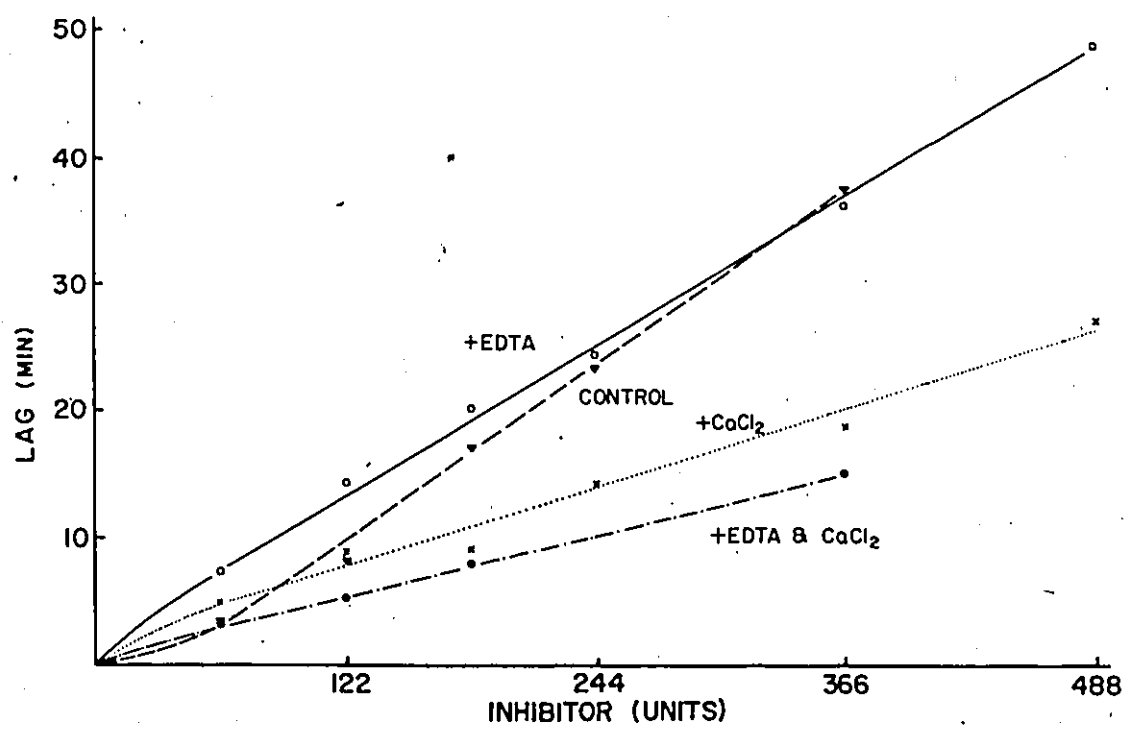.level of protease inhibitor in the DEAE-cellulose column effluent determined as described above. The results of these assays are shown in Figure 17. The acid protease activity remains relatively constant during the first day of development then drops sharply after 25 hours of development to a lower but constant level of activity. In contrast, the level of inhibitor rises markedly during the first 12 hours of development reaching a maximum just before hatching, then declines steadily to an undetectable level by 48 hours. During pre-hatched development, no alkaline protease activity was detectable. However at 21 hours, shortly after the onset of hatching, protease activity at pH 8 was detected. The level of this protease rose sharply during the next 10 hours, and continued to increase gradually as development progressed further.

Partial Purification of Alkaline Protease from Nauplii

In order to study further the late appearing alkaline protease observed in the developmental studies, nauplii were allowed to develop for 40 hours, then collected and processed as described above for the acid protease. When the nauplii protease preparation was chromatographed on a DEAE-cellulose column the elution profile shown in Figure 18 was obtained. The alkaline protease activity eluted as a broad peak at about 0.45 M KCl. In order to further purify the alkaline protease, the combined protease fractions were concentrated and chromatographed on a column of Ultrogel AcA34. The elution profile of this column is shown in Figure 19. The protease activity eluted in a peak corresponding to a molecular weight of 27,500 (Fig. 7). From the shape of the alkaline protease peaks from both DEAE-cellulose and Ultrogel AcA34

FIGURE 17

Upper.   Developmental pattern of Artemia embryos incubated
         at 30°C.

Hydrated cysts were allowed to develop in individual flasks
at 30°C.   At various times aliquots from each flask were
spread on a strip of filter paper and the number of cysts,
prenauplii and nauplii counted.   The data are presented as
percent of emergence (O——O)   and hatching (●——●) as
a function of incubation time.

Lower.   Protease and inhibitor levels in developing Artemia
         embryos.

At the times indicated the total population of embryos
were collected and the post-ribosomal supernatant fluid
fractionated and assayed for protease levels at pH 4, 6 and
8 and inhibitor levels as described in Materials and Methods.
The activities were calculated based on the protein content
of the post-ribosomal supernatant fraction.

O——O , proteolytic activity at pH 4.0

X·········X , proteolytic activity at pH 8.0

●——● , inhibitor activity
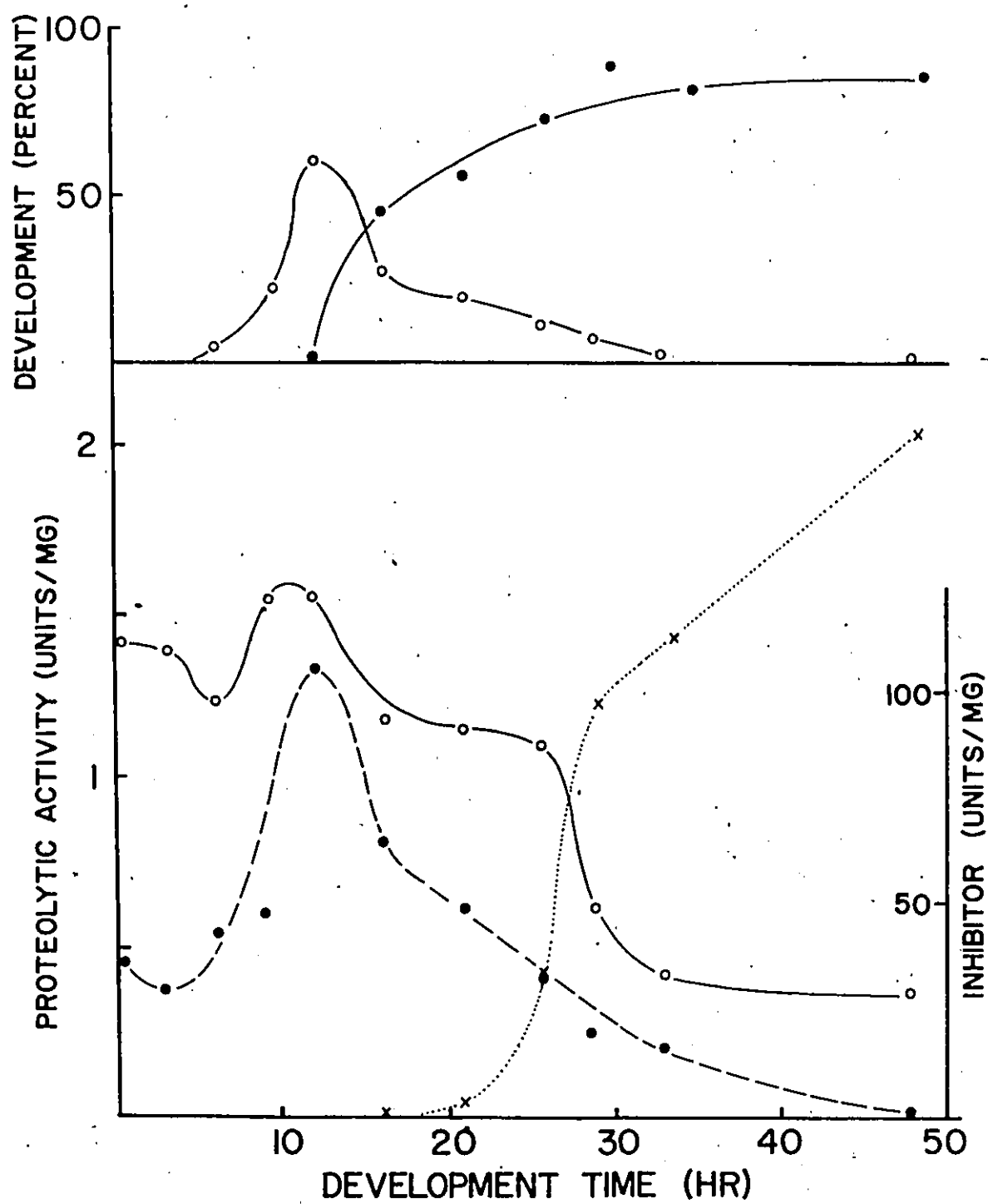
FIGURE 18

Chromatography of 40-hour nauplii post-ribosomal fraction on
    DEAE-cellulose.

The post-ribosomal supernatant was prepared from 40 hour

nauplii obtained from 18 grams (wet weight) hydrated cysts

as previously described and chromatographed on a column of

DEAE-cellulose as described in Fig. 5.   Column fractions

were assayed for proteolytic activity as described in

Materials and Methods.

O----O , absorbance at 280 nm

●——● , proteolytic activity at pH 4.0

X——X , proteolytic activity at pH 8.0
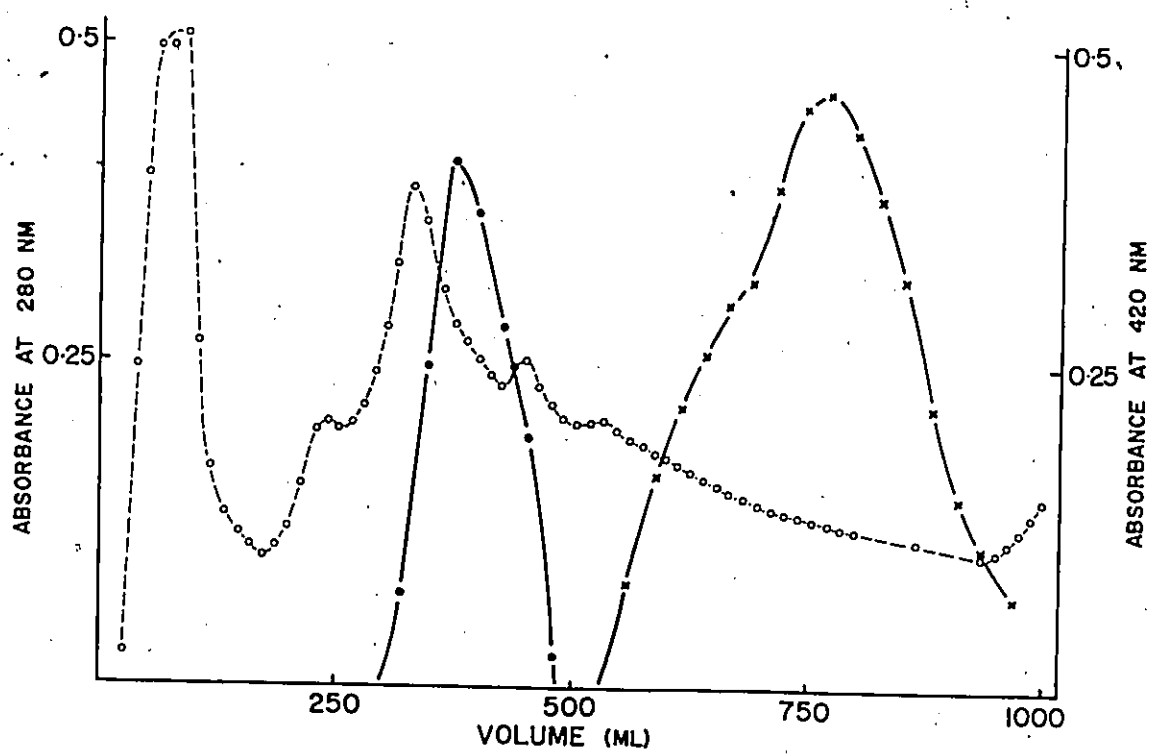
FIGURE 19

Chromatography of 40 hour nauplii protease activity on
    Ultrogel AcA34.

The combined fractions containing alkaline protease activity

from a DEAE-cellulose column (see Fig. 18) were concentrated

by pressure dialysis at 40 psi N_2 and chromatographed on

a column of Ultrogel AcA34 (2.0 x 52 cm) exactly as described

in Fig. 6. The column fractions were analysed for protein

at 280 nm and for proteolytic activity at pH 8.0 as

described in Materials and Methods.

            O-----O ,   absorbance at 280 nm

            ●——● ,   protease activity
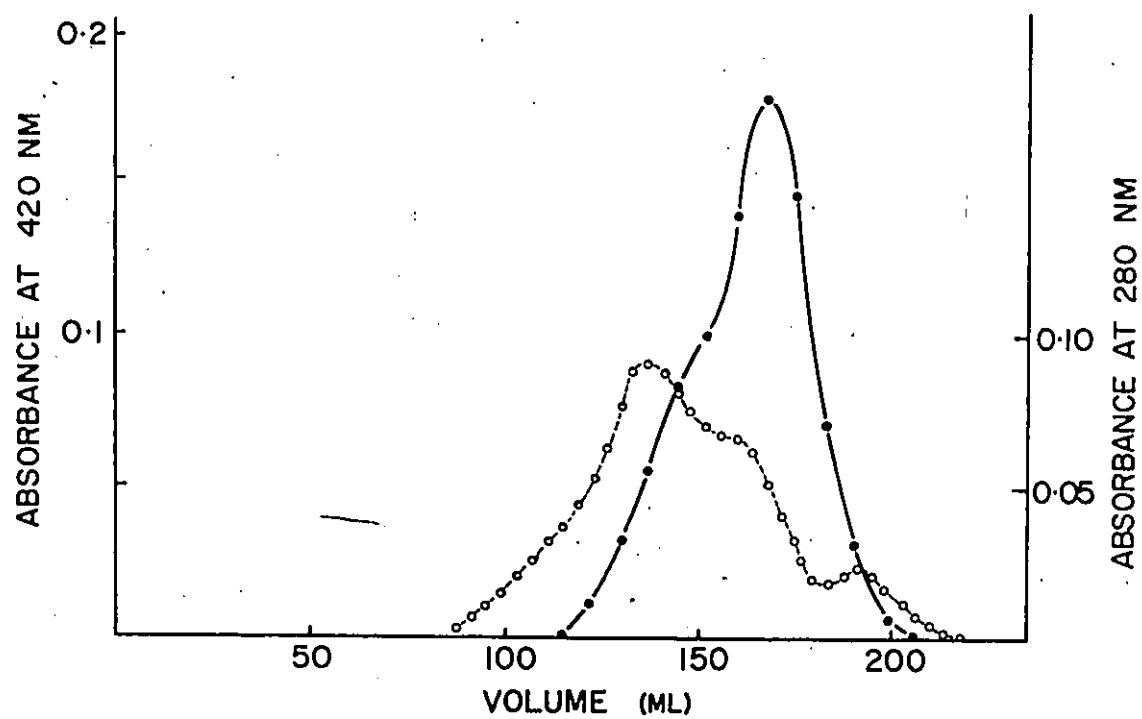
columns it appears that more than one alkaline protease may be present in 40 hour nauplii.

pH Optimum of the Alkaline Protease

When the partially purified alkaline protease from the Ultrogel AcA34 column was assayed at varying pH, it was observed that maximal hydrolysis of BSA occurred at pH 8.5. The result of this experiment is shown in Figure 20.

Sensitivity of Artemia Proteases to Inhibitors

Both the alkaline and acid proteases were tested for sensitivity to naturally occurring trypsin inhibitors and compared to the activity of the brine shrimp cyst protease inhibitor. When soy bean trypsin inhibitor (SBTI), ovomucoid, or cyst protease inhibitor was added to reaction mixtures containing BSA and assayed at the optimum pH, the results shown in Table 1 were obtained. It is clear that the nauplii alkaline protease was inactivated by both SBTI and ovomucoid, even at low concentrations, but unaffected by the Artemia protease inhibitor. In contrast, the cyst acid protease was inactivated by the Artemia protease inhibitor only.

Hydrolysis of Artemia Yolk Platelets and Ribosomes by the Cyst Protease

To determine if the Artemia acid protease is able to utilize yolk as a substrate, yolk platelets from Artemia cysts were prepared and tested as a substrate compared to a standard reaction containing BSA. The results of this experiment are presented in Figure 21 and show that yolk platelets and BSA are almost equally

FIGURE 20

Determination of pH optimum of the alkaline protease.

The 40-hour nauplii alkaline protease used in this assay
was purified on DEAE-cellulose and AcA34. The protease
activity was assayed in a standard reaction mixture at pH
values from 4.0 to 10.0 at 37°C as described in Materials
and Methods.
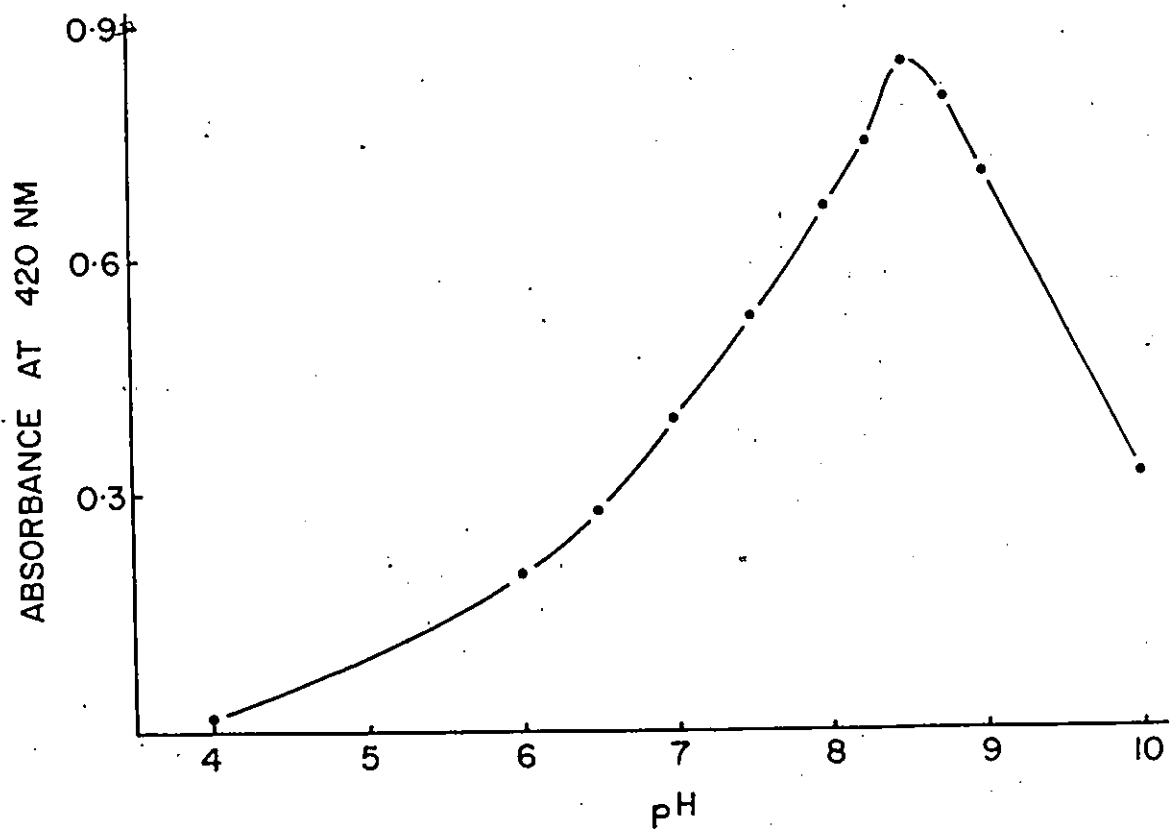
Table 1. Effect of naturally occurring protease inhibitors
on proteases isolated from brine shrimp cysts and nauplii.

Protease Inhibitor	Concentration		Percent of Control	
			Acid Protease	Alkaline Protease
	1.0 mg/ml		98	0
SBTI	0.1 "		102	3.9
	0.01 "		97	6.0
	1.0 mg/ml		97	4.5
Ovomucoid	0.1 "		103	11.7
	0.01 "		103	35.5
Artemia Inhibitor	245 units		11.2	97
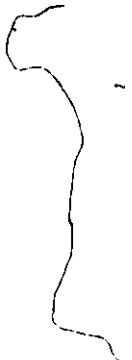
FIGURE 21

Hydrolysis of yolk platelet protein by the cyst acid

   prbtease.

Five units of partially purified byst acid protease was

incubated in reaction mixtures containing either 4 mg/ml

BSA or dried yolk platelets in 0.1 M sodium acetate, pH

4.0, with or without 0.01 M $CaCl_2$. At selected incubation

times aliquots were removed and assayed for amino groups

as described in Materials and Methods.

●——● ,   BSA

●———● ,   BSA + $CaCl_2$

✕——✕ ,   Yolk platelets

✕———✕ ,   Yolk platelets + $CaCl_2$

○——○ , Acid protease only.
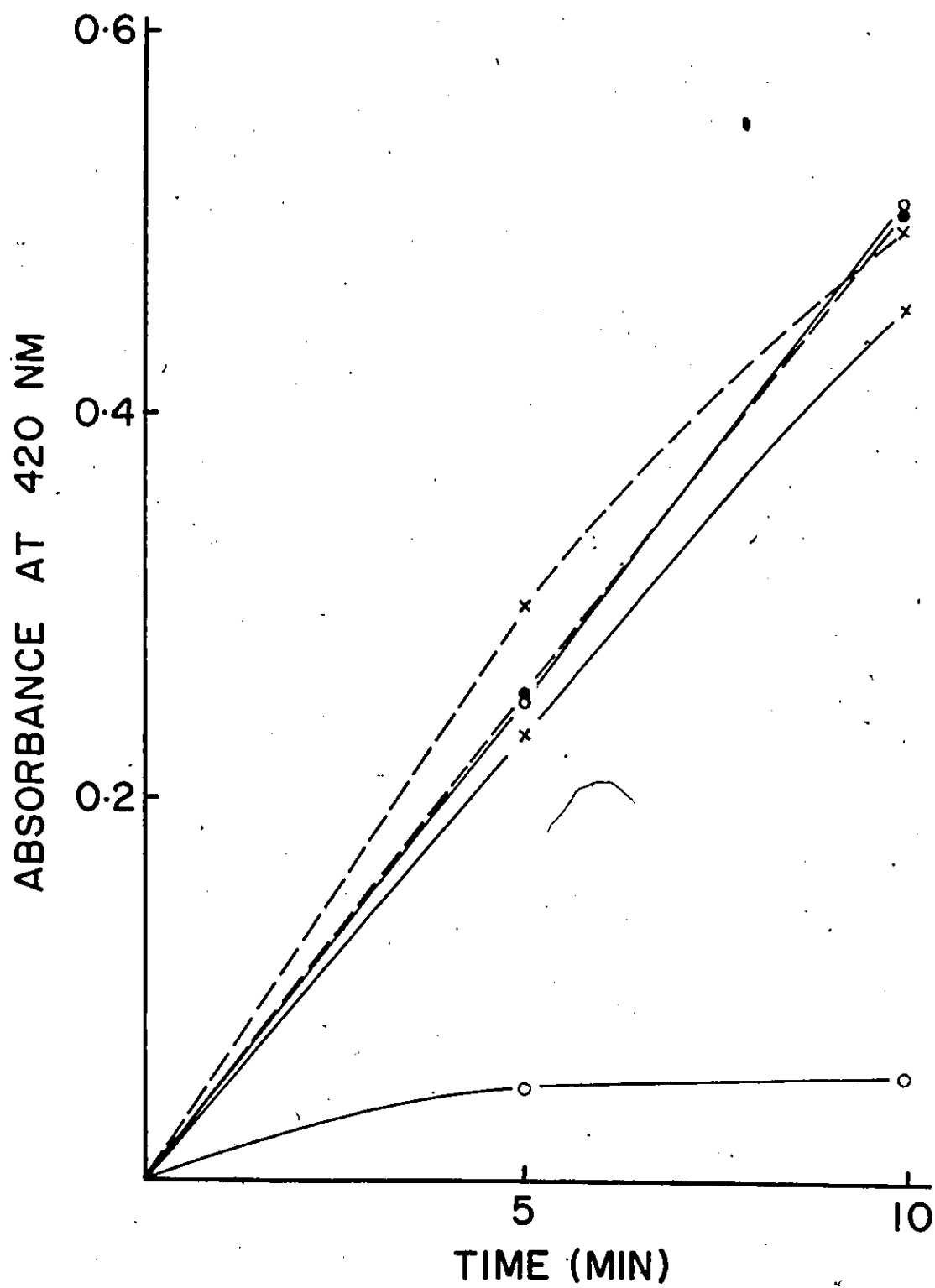
effective as substrates. Moreover, the addition of $CaCl_2$ had

only a slight stimulatory effect on the activity of the acid

protease with either yolk platelets or BSA.

Two preparations of cyst ribosomes were also tested as

substrate, one washed free of loosely bound protein, and the other

containing unwashed ribosomes. As shown in Figure 22, both

ribosome preparations contain protein that serves as substrate

for the acid protease.

FIGURE 22

Ribosomes as substrate for the cyst acid protease.

Two ribosome preparations were tested as substrates for the
acid protease. One reaction vessel contained 0.1 M sodium
acetate, pH 4.0, 1.6 units of acid protease and unwashed
ribosomes containing 1.6 mg protein and 2.25 mg RNA in
a final volume of 0.4 ml (O———O). A second vessel
contained the same amount of buffer and acid protease plus
salt-washed ribosomes containing 2.3 mg protein and 2.5 mg
RNA in a final volume of 0.4 ml (●———●). A third
vessel contained the buffer and acid protease alone (X———X).
Reaction mixtures were incubated at 37°C and aliquots re-
moved for the determination of amino groups as described
in Materials and Methods. No activity was detected in
reactions containing ribosomes alone and therefore these
values are not shown.
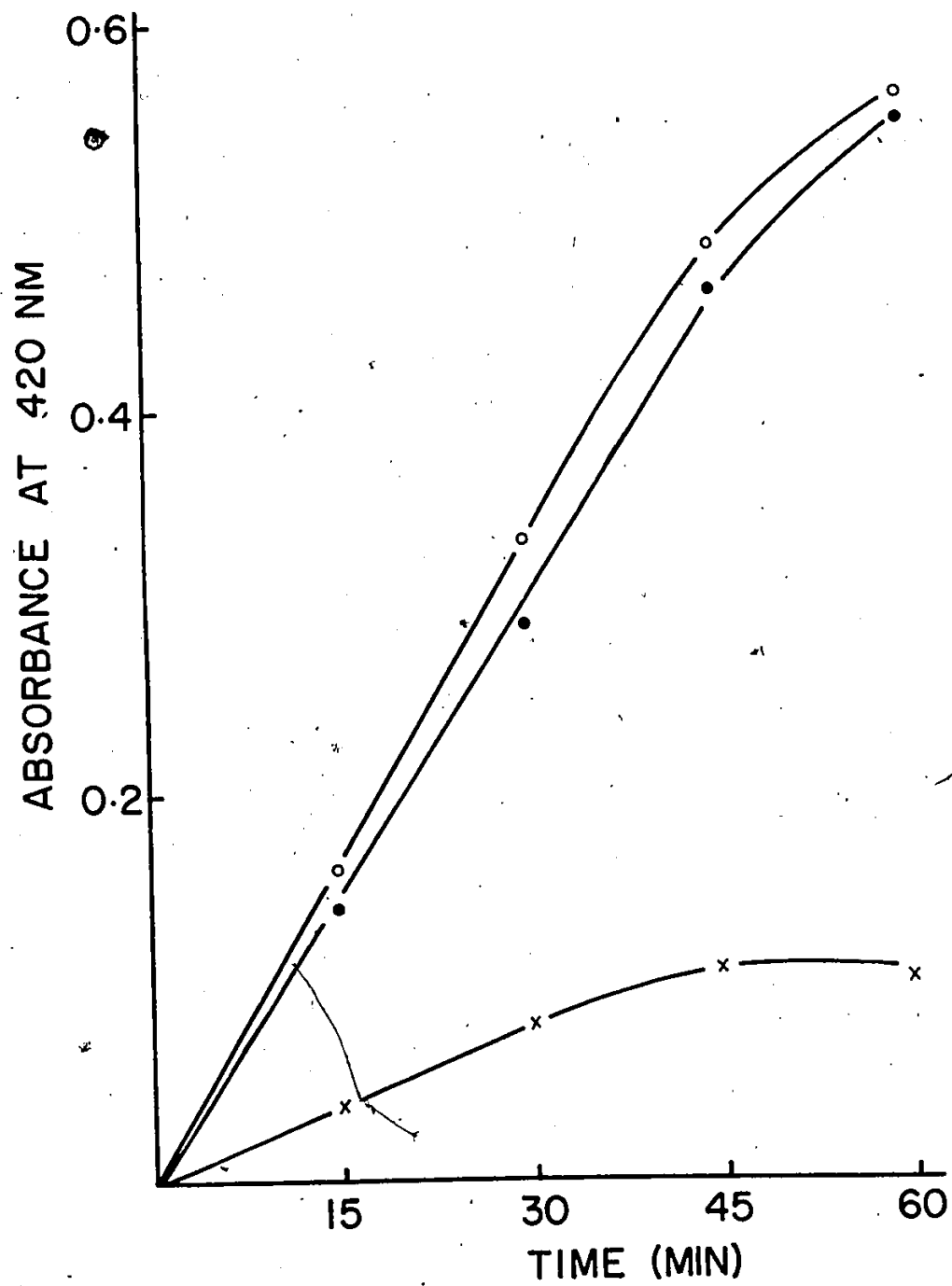
DISCUSSION

Assays for proteolytic activity usually depend on measuring acid soluble products detectable by UV absorption (6) or using insoluble protein substrates which when hydrolysed release a soluble dye (7). These procedures have serious limitations if one is searching for a variety of proteolytic activities including proteases active in a limited way that produce very specific cleavages and few acid-soluble fragments. Therefore, it was necessary to develop an assay system which did not rely upon extensive hydrolysis for the detection of proteolytic activity. In the assay developed for this study, the substrate used is a soluble protein which remains in solution during the assay for peptide bond hydrolysis, thus limited proteolysis of the substrate can be detected by simply measuring the number of amino groups produced during incubation.

The kinetics of hydrolysis of protein substrates by trypsin, an enzyme with known characteristics, using the TNBS method was determined and compared to trypsin data obtained by other methods. The results were very similar to those obtained by Kunitz (16) using casein as a substrate. In both cases, the rate of hydrolysis increased with increasing concentrations of trypsin but the response was not proportional to the quantity of trypsin used (Fig. 2). These results are probably due to the narrow range of specificity of trypsin for peptide bonds involving only arginine and lysine. Preliminary data indicated that the TNBS method used in this study is at least 10 times more sensitive than the acid soluble assay of Anson for measuring proteolysis.

The only protease detectable in undeveloped <u>Artemia</u> cysts was an acid protease. Studies using this enzyme revealed that it has a pH optimum of 3.6 and a temperature optimum of 45°C for the hydrolysis of BSA. In addition, this enzyme was found to have a molecular weight of 84,000 by gel filtration. The partially purified enzyme loses activity readily when stored in dilute solutions at $0-4^\circ$C; however, as a concentrated solution greater than 1 mg/ml it could be stored at 4°C for several weeks or at -15°C for months without significant loss in activity. The enzyme was also found to be sensitive to pressure and loses activity readily when subjected to pressures over 40 psi. The activity of the acid protease was stimulated slightly by addition of EDTA to reaction mixtures; this response may be due to removal of inhibitory heavy metal ions. However the addition of $CaCl_2$ to reaction mixtures produced a slight stimulation in activity. These findings are in general agreement with those of Lundblad <u>et al</u>. (17) who studied proteases with similar properties in extracts of newly fertilized sea urchin eggs.

When the kinetics of hydrolysis of BSA by the acid protease were studied, it was observed that the rate of hydrolysis was linear throughout the reaction in contrast to the kinetic pattern observed when trypsin was tested. These differences suggest that the acid protease, unlike trypsin, is able to hydrolyse a broad spectrum of peptide bonds. Also, in some experiments not reported here, we observed that when the acid protease was assayed using various <u>Artemia</u> cyst protein fractions as substrate (obtained from a DEAE cellulose column fractionation experiment of soluble

cyst proteins as illustrated in Fig. '5), that all Artemia protein
fractions were equally active as substrates. Therefore, it appears
that the acid protease from Artemia cysts has considerably less
specificity for peptide bonds than does trypsin.

During development the acid protease level remains relatively
constant throughout the period of emergence and hatching, and
declines only after about 25 hours of development to a lower but
constant level.

Unfractionated post-ribosomal supernatant fractions from
Artemia contain at least two protease inhibitors. When the cyst
post-ribosomal fraction was assayed for proteolytic activity,
very little was detected. However after dialysis or gel filtration
the crude extract was at least 3 times more active. These data
indicate the presence of a small molecular weight inhibitor in
the cyst extract. When a dialysed or G-25 Sephadex treated
fraction was assayed for proteolytic activity at pH 4, an initial
restriction of activity was observed followed by a gradual increase
in activity until a linear rate of hydrolysis was obtained (Fig. 5).
This restriction in proteolytic activity or lag in initial
activity was shown to be due to the presence of another inhibitor
in the cyst extract which could be separated from the protease by
chromatography on DEAE-cellulose. Therefore, when the DEAE-
cellulose purified acid protease was assayed using BSA as substrate,
no initial lag in activity was observed. The inhibitory fraction
in the cyst extract was easily removed from the protease fraction
since it didn't bind to DEAE-cellulose.

The cyst acid protease inhibitor has been studied to some

extent. It has a molecular weight of between 5,000 and 20,000 and is not stable in dilute solutions when stored at 0-4°C but is stable to freezing as a concentrated solution. The inbibitor-enzyme interaction described above indicates that the acid protease has a high affinity for the inhibitor. This is particularly evident when one considers the fact that a large excess of BSA (or substrate) in the reaction mixtures had no effect on the kinetics of inhibitor inactivation by the protease in the presence of EDTA. Moreover, the rate of inactivation of the inhibitor was increased by over 200% when $CaCl_2$ was added to the reaction mixture, whereas $CaCl_2$ addition had no stimulatory effect on hydrolysis of BSA. These observations support further the conclusion that the protease has a high affinity for the inhibitor The inactivation of the acid protease inhibitor probably occurs due to proteolysis as has been described for the inactivation of the bovine pancreatic trypsin inhibitor (18). However, the mechanism of inhibitor inactivation in the Artemia system is still open to question.

The role of the protease inhibitor in developing Artemia embryos remains to be studied. However, it appears that the acid protease may be maintained in an inactive form during pre-embryonic development.

During the developmental studies it was observed that another protease(s) appears in Artemia embryos after hatching that could not be detected in undeveloped cysts. In fact, the first indication of alkaline protease activity occurs at 21 hours development (at 30°C) shortly after the appearance of nauplius larvae in

the incubation medium. Subsequent to this developmental stage alkaline protease activity in the post-ribosomal supernatant fraction increases markedly. After 30 hours development, the total amount of this protease(s) began to level off (data not shown) although the specific activity continued to increase. The alkaline protease(s) from 40 hr nauplii was (were) partially purified and found to have a pH optimum of 8.5 for the hydrolysis of BSA and a molecular weight of 27,500 by gel filtration. The activity is inhibited strongly by the naturally occurring trypsin inhibitors ovomucoid and SBTI, but not inhibited by the Artemia cyst protease inhibitor. The nauplii protease has not been studied extensively but the elution patterns of this activity from DEAE-cellulose and Ultrogel AcA34 suggest that there may be more than one alkaline protease in 40 hour nauplii. The function of the alkaline protease in developing Artemia embryos is not known but it appears to have properties similar to trypsin and other trypsin-like enzymes found in a wide variety of tissues and organisms (19).

Very little is known about the exact function of acid proteases in developing systems, thus we can only speculate about their actual purpose. The results presented here have demonstrated that the acid protease from Artemia has the ability to utilize yolk as a substrate and therefore it could function in yolk utilization for the release of amino acids required for the synthesis of structural and enzymatic proteins. However yolk utilization is thought to occur primarily after hatching and therefore this protease may not be important for this purpose since it decreases markedly after hatching. What then is the function

of the acid protease? In <u>Artemia</u>, this protease may be instru-
mental in the derepression of cyst ribosomes and thus assist
in the activation of the protein synthesizing apparatus (2).
The observation that the acid protease is able to utilize ribosome
associated proteins as substrate is consistent with this hypothesis.
However, this function seems unlikely unless the pH of the cyto-
plasm is sufficiently low to permit the acid protease to function.

SUMMARY

A new method for the measurement of proteolytic activity was developed using BSA as a substrate and 2,4,6-trinitrobenzene-sulfonic acid (TNBS) to detect amino groups produced as the result of peptide bond hydrolysis. Using this assay a single protease was isolated from *Artemia salina* cysts and studied. After partial purification the enzyme was found to have a pH optimum of 3.6, a temperature optimum of $45^{\circ}C$ and a molecular weight of about 84,000. $CaCl_2$ and EDTA were required for maximal activity. An inhibitor of acid protease activity was also found in *Artemia* cysts. The inhibitor has a molecular weight of between 5,000 and 20,000 and a high affinity for the acid protease. However this protease inhibitor can be inactivated by incubation with the protease at $37^{\circ}C$.

A second protease appeared between 20 and 30 hours of development at $30^{\circ}C$. This protease has a pH optimum of 8.5 and a molecular weight of 27,500. This alkaline protease is inactivated by ovomucoid and soy bean trypsin inhibitor but not by the cyst inhibitor; therefore, it appears to by a trypsin.

The properties of the enzymes and inhibitor were discussed with respect to their possible function in the developing embryo of *Artemia salina*.

LITERATURE CITED

1. Monroy, A. & Tyler, A. (1963) _Arch. Biochem. Biophys._ 103, 431-435.

2. Tyler, A. (1963) _Am. Zoologist._ 3, 109-126.

3. Lundblad, G. (1950) _Nature_ 163, 643.

4. Monroy A. (1957) in _The Beginning of Embryonic Development_ (A. Tyler, R.C. von Borstel, and C. B. Metz, eds.) pp. 169-174. Am. Assoc. Advanc. Sci., Washington, D. C.

5. Monroy A.& Tyler, A. (1967) in _Fertilization_ (C. B. Metz & A. Monroy, eds.) pp. 369-412. Academic Press Inc. New York, N. Y.

6. Anson, M. L. (1938) _J. Gen. Physiol._ 22, 79-89.

7. Rinderknecht, H., Silverman, P., Geokas, M. C., & Haverback, B. J. (1970) _Clin. Chim. Acta_ 28, 239-246.

8. Bellini, L. (1957) _Rend. Acc. Naz. Lincei_ 22, 340-346.

9. Fields, R. (1971) _Biochem. J._ 124, 581-589.

10. _Worthington Enzyme Manual_ (1972) pp. 125-127. Worthington Biochemical Corportation, Freehold, N. J.

11. Warner, A. H. & McClean, D. K. (1968) _Develop. Biol._ 18, 278-293.

12. McClean, D. K. & Warner, A. H. (1971) _Develop. Biol._ 24, 88-105.

13. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) _J. Biol. Chem._ 193, 265-275.

14. Bligh, E. G. & Dyer, W. J. (1959) _Can. J. Biochem._ 37, 911-917.

15. Nakanishi, Y. H., Iwasaki, T., Okigaki, T. & Kato, H. (1962) _Annot. Zool. Jap._ 35, 223-228.

16. Kunitz, M. (1947) _J. Gen. Physiol._ 30, 291-304.

17. Lundblad, G., Lundblad, M., Immers, J. & Schilling, W. (1966) _Arkiv. für. Kemi._ 25, 395-409.

18. Tschesche, H., Klein, H. & Reidel, G. (1970) in _Proc. Int. Conf. on Proteinase Inhibitors,_ Munich. pp. 299-304. Walter de Gruyter, New York, N. Y.

19. Walsh, K. A. (1970) in _Methods in Enzymology_ (Perlman, G. E. & Lorand, L. eds.) 19, pp. 41-63. Academic Press Inc. New York, N. Y.

VITA AUCTORIS

1951:   Born in Sault St. Marie, Ontario, January 5, 1951.

1956-1964:   Attended Cody Public School,
                Sault Ste. Marie, Ontario.

1964-1969:   Attended Bawating Collegiate and Vocational School,
                Sault Ste. Marie, Ontario.

1969-1973:   Attended University of Windsor, Windsor, Ontario
                Graduated in 1973 with degree of Bachelor of Science.

1973:   Accepted into the Factulty of Graduate Studies
                at the University of Windsor, Windsor, Ontario.