

CHARACTERIZATION OF DIPEPTIDYL
AMINOPEPTIDASE I (CATHEPSIN C)
FROM ATLANTIC SHORT-FINNED SQUID

CENTRE FOR NEWFOUNDLAND STUDIES

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KHWAJA SHAHID HAMEED

**CHARACTERIZATION OF DIPEPTIDYL AMINOPEPTIDASE I
(CATHEPSIN C) FROM SHORT-FINNED SQUID**

by



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**A thesis submitted in partial fulfilment
of the requirements for the
degree of Master of Science**

**Department of Biochemistry
Memorial University of Newfoundland**

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TO MOM

Abstract

Dipeptidyl aminopeptidase I (cathepsin C, E.C.3.14.3.1) was isolated from the hepatopancreas of the Atlantic short finned squid (*Illex illecebrosus*) and partially purified by the successive steps of autolysis of an acid extract, ammonium sulfate precipitation, heat treatment, ultrafiltration and affinity chromatography using p-CMB-Sepharose column. On SDS-polyacrylamide gel electrophoresis, the enzyme gave one major and one minor band corresponding to a molecular weight of about 25 and 62 kdaltons, respectively. The identification of cathepsin C is based on (1) its ability to catalyze a transferase reaction with Gly-Phe-NH₂ and a hydrolase reaction with Gly-Phe-NA or Ser-Tyr-NA at pH optima of 7 and 6, respectively; (2) the Cl⁻ ion and sulfhydryl activation of the enzyme; (3) its inhibition by known inhibitors of cathepsin C; and (4) the apparent existence of a 25 kdaltons subunit, which forms associated complexes of 50, 100 and 200 kdaltons. The dissociation and reassociation of cathepsin C in relation to its catalytic functions as a transferase and a hydrolase were studied by examining the ultrafiltration behavior of the enzyme following heat treatment or exposure to detergent or urea.

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List of Abbreviations

Ala-Ala-NA	: alanyl-alanine 2-naphthylamide
Ala-Ala-NH ₂	: alanyl-alanine amide
Bz-Arg-NH ₂	: benzoyl-arginine* amide
Ala-Phe-NH ₂	: alanyl-phenylalanine amide
BANA	: benzoyl-arginine 2-naphthylamide
CBZ-Glu-Tyr	: carbobenzoxy-glutamyl-tyrosine
CM	: carboxy methyl
D-Ala-Tyr-OMe	: D-alanyl-tyrosine methyl ester
α D-Asp	: α D-aspartate
DEAE	: diethyl amino ethyl
DFF	: diisopropylfluorophosphate
DTE	: dithiothreitol
D-Ser-Lys-	: D-seryl-lysyl
EDTA	: ethylene diaminetetraacetic acid
Glu-His	: glutamyl-histidine
Gly-Arg-MNA	: glycyl-arginine 4-methoxy 2-naphthylamide
Gly-Arg-NA	: glycyl-arginine 2-naphthylamide
Gly-Phe-MNA	: glycyl-phenylalanine 4-methoxy 2-naphthylamide
Gly-Phe-NA	: glycyl-phenylalanine 2-naphthylamide
Gly-Phe-NH ₂	: glycyl-phenylalanine amide

Gly-Phe-OMe	:	glycyl-phenylalanine methyl ester
Gly-Lys-OMe	:	glycyl-lysine methyl ester
Glutaryl-Phe-pNA	:	glutaryl-phenylalanine p-nitroanilide
Gly-Trp-NH ₂	:	glycyl-tryptophan amide
ME	:	2-mercaptoethanol
NA	:	naphthylamine
p-CMB	:	p-chloromercuribenzoate
Phe-Arg	:	phenylalanyl-arginine
PMSF	:	phenylmethanesulfonylfluoride
Ser-Met	:	seryl-methionine
Ser-Tyr-NA	:	seryl-tyrosine 2-naphthylamide
TEMED	:	N,N,N',N'-tetramethylethylenediamine

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Chapter 1

INTRODUCTION

1.1. Historical background

It has long been known that aqueous extracts of various animal tissues exhibit proteinase activity, and therefore, the term cathepsin (to digest) was introduced to define this activity (Willstatter and Bamann, 1929). The multiplicity of proteolytic activities associated with cathepsin was recognized largely because of their differing action on synthetic peptide substrates. In 1941 a cathepsin classification was formulated, and it was later revised in 1952 (Tallen et al., 1952). By the use of the synthetic substrates CBZ-Glu-Tyr, BZ-Arg-NH₂ and Gly-Phe-NH₂ evidence was obtained for the existence of three separate enzymes, namely cathepsin A, B, and C. The specificities of these enzymes appeared to be similar to those of pepsin, trypsin, and chymotrypsin, respectively (Tallen et al., 1952). Since 1952, ten additional cathepsins have been classified (Goll et al., 1983).

1.2. Occurrence

The intracellular exopeptidase, cathepsin C (E.C.3.4.14.1), was first recognized to exist in porcine kidney tissue extract, on the basis of its hydrolytic action on the chymotrypsin substrate, Gly-Phe-NH₂ (Gutmann and Fruton, 1948). Because of its apparent lack of activity on protein substrate, and its action on synthetic substrate such as Gly-Phe-NH₂ as a transferase, the name dipeptidyl transferase was proposed (Metrione et al., 1966). Based on its ability to hydrolyze peptides, the cathepsin C was renamed as dipeptidyl aminopeptidase I (McDonald

et al., 1971), the name which was later accepted by the International Enzyme Commission. The lysosomal localization of cathepsin C was demonstrated in rat liver (Bouma and Gruber, 1966) and bovine pituitary (McDonald et al., 1966a). The enzyme has been found in tissues from cow, rat, chicken and human (McDonald et al., 1974). A survey of tissues showed liver to be the richest source of cathepsin C in the rat (McDonald et al., 1971; Bouma and Gruber, 1966). In human tissues, cathepsin C was about five times more abundant in spleen than in liver (Vanha-Perttula and Kalliomaki, 1973). Wang and Frieden (1973) reported that activity of cathepsin C in the tail tissues of the tadpole increased at least 100 fold during tail resorption. The existence of cathepsin C was reported in carp (*Cyprinus carpio*) muscle (Ikeda and Makinodan, 1971), Pacific whiting (*Merluccius productus*), true cod (*Gadus macrocephalus*) (Anglemier et al., 1983) and chicken (Iodice et al., 1972).

1.3. Purification

Although cathepsin C was originally detected in extracts of porcine kidney, bovine spleen became the popular source of enzyme for studies of purification and characterization (Tallen et al., 1952; De la Haba et al., 1959; Planta and Gruber, 1964; Mettrione et al., 1966, 1970, Mettrione and MacGeorge 1975; Mettrione, 1978; McDonald et al., 1972a).

The preparation of bovine cathepsin C is described by Mettrione et al. (1966). The purification method involves successive steps of acid extraction (or autolysis) at pH 3.5 and 37°C, ammonium sulphate precipitation between 40% and 70% saturation, heat treatment at 65°C for 40 min, and column chromatography on Sephadex G-200, DEAE-cellulose, and CM-cellulose. The resulting preparation of cathepsin C appears to be homogeneous as judged by ultracentrifugation analysis. When cathepsin C was prepared for the purpose of sequencing peptides, the last two purification steps were omitted (Callahan et al., 1972). These preparations were later shown to be contaminated with peptide hydrolases that were termed Ser-Met-dipeptidase and catheptic carboxypeptidase

C (McDonald et al., 1972b). The peak of activity recovered following gel filtration on Sephadex G-200 was treated with EDTA and DFP to inhibit the contaminating peptide hydrolases (McDonald et al., 1972a). To remove the small amount of contaminating protein, Metrione (1978) used affinity adsorbant material in combination with gel filtration and ion exchange chromatography.

1.4. Assay methods for cathepsin C

Hydrolytic and transferase reactions with specific substrates have been utilized to distinguish cathepsin C from other enzymes. With Gly-Phe-NH₂ as substrate at pH 6.0, a deamidation reaction occurs and the liberated ammonia can be measured by a diffusion method (Seligson and Seligson, 1951). At pH 6.8, a transferase reaction occurs in which the substrate Gly-Phe- is transferred to hydroxylamine, and the resulting Gly-Phe-hydroxamic acid can be measured spectrophotometrically (Jones et al., 1952). The esterolytic activity of cathepsin C has been measured on Gly-Phe-OMe substrate by automatic titration at pH 5.0 (Tesser et al., 1964). At pH 6.0 arylamidase activity of cathepsin C with Gly-Phe-pNA has been measured spectrophotometrically (Planta and Gruber, 1963) and with Gly-Phe-NA spectrofluorometrically (McDonald et al., 1968a).

1.5. Physical properties

1.5.1. Size

The molecular weight of bovine spleen cathepsin C has been determined by sedimentation equilibrium and it was found to be 235,000 (De la Haba et al., 1959), and 210,000 (Planta and Gruber, 1964; Metrione et al., 1966). On the basis of sedimentation analysis and molecular exclusion chromatography Metrione et al. (1970) subsequently reported that bovine spleen cathepsin C has a molecular weight of 197,000.

Cathepsin C isolated from rat liver has a molecular weight of 200,000 as estimated by exclusion chromatography on Sephadex G-200 (McDonald et al., 1968a).

1.5.2. Isoelectric point

On the basis of electrophoretic mobilities on analytical polyacrylamide gel at three different pH values, it has been reported that rat liver enzyme has an isoelectric point of about 5.0 as compared to about 6.0 for bovine spleen cathepsin C (McDonald et al., 1969a).

1.5.3. Subunit nature

The subunit nature of bovine spleen cathepsin C was recognized when it was shown that the sedimentation behavior of the enzyme differed in the presence of 1mM p-mercuribenzoate or 1% SDS (Metrione et al., 1966). On the basis of amino terminal amino acid analysis, Metrione et al. (1970) obtained evidence for the presence of eight subunits, possibly of two different types, when cathepsin C is in 6M guanidinium chloride, 8M urea or at pH values below 3.

The subunit nature of the rat liver enzyme was shown by electrophoresis in polyacrylamide gels containing 1% SDS (McDonald et al., 1969a). Rat liver cathepsin C is apparently composed of 2 types of subunits, one of which is three times larger than the other as judged by migration in polyacrylamide gels containing SDS. The possibility that the larger material represented undissociated or partially dissociated enzyme was apparently not ruled out (McDonald et al., 1969a).

Evidence for the allosteric nature of bovine spleen cathepsin C has been reported by Gorter and Gruber (1970) wherein cooperativity was observed at non-saturating concentrations of halide activators when hydrolase activity was determined with Gly-Phe-pNA.

1.6. Catalytic properties

1.6.1. pH optimum

Hydrolysis of dipeptidyl derivatives at the susceptible amide or ester linkages predominates when the pH of the enzymatic reaction is between pH 5-6. At pH values between 6.5 and 8, replacement of amide or ester moiety by an acceptor nucleophilic compound (hydroxylamine or free amino acids or excess dipeptides) prevails and a polymerization (transferase) reaction occurs (Jones et al., 1952; Fruton et al., 1953). The transferase assay of cathepsin C is usually performed at pH 6.8, where the reaction rate is optimal.

1.6.2. Substrate specificity

Cathepsin C catalyzes the transfer of a dipeptidyl group of a suitable substrate to nucleophilic amines, such as hydroxylamine, free amino acids, or excess dipeptide amide substrate to form polymers. In the absence of other acceptors, water serves as an acceptor causing hydrolysis (Wurz et al., 1962; Nilsson and Fruton, 1964).

Early studies on the specificity of bovine spleen cathepsin C led to the belief that the enzyme was narrowly restricted in both its hydrolytic and transferase activity. That is, suitable substrates are amide and ester derivatives of α -amino acid dipeptides, provided that the N-terminal residue possesses an unsubstituted α -amino group (or α -imino in a prolyl peptide) and a short side chain and the penultimate residue has the L-configuration and a hydrophobic side chain (Jones et al., 1952; Fruton et al., 1953; Wiggans et al., 1954; Planta et al., 1964).

Early work with less than pure preparations of bovine spleen cathepsin C revealed no proteinase activity toward hemoglobin, serum albumin, fibrinogen, or ribonuclease (Planta and Gruber, 1961, 1964). Confirming earlier reports, a pure preparation of bovine spleen cathepsin C was found to contain negligible proteinase activity toward native proteins such as bovine hemoglobin, bovine

serum albumin, egg albumin, ribonuclease and insulin (Metrione et al., 1966). However, rat liver cathepsin C, purified to homogeneity was shown to catalyze the hydrolysis of several polypeptide hormones. These include glucagon and secretin (McDonald et al., 1969b), the B chain and the des-Phe B chain of oxidized bovine insulin (Callahan et al., 1969), the A chain of oxidized bovine insulin (McDonald et al., 1972a), angiotensin II amide (McDonald et al., 1972b), and analogues of human angiotensin II (McDonald et al., 1974).

In accordance with earlier studies of bovine spleen cathepsin C, the rat liver enzyme also showed no activity on peptides having a penultimate residue with the D-configuration (McDonald et al., 1974). However, the configuration of the N-terminal residue does not appear to be important as shown by the hydrolysis of D-Ala-Tyr-OMe by bovine spleen (Izumiya and Fruton, 1956), and hydrolysis of D-Ser-Lys- β corticotropinamide (McDonald et al., 1969b), and α D-Asp-angiotensin II by rat liver enzyme (McDonald et al., 1974). Consistent with earlier studies conducted with bovine spleen cathepsin C, the requirement of a free amino terminal has been shown for rat liver enzyme. For example, no enzyme activity was detected on N-acetyl blocked peptides (McDonald et al., 1969b).

The specificity of rat liver cathepsin C was studied on a wide variety of naphthylamide dipeptide derivatives. Dipeptides with penultimate basic residues were especially susceptible to attack by the enzyme, and most notably that Gly-Arg-NA was hydrolyzed about 18 times faster than Gly-Phe-NA. Furthermore, naphthylamide derivatives of Ser-Met, Glu-His, and Phe-Arg were also hydrolyzed faster than Gly-Phe-NA (McDonald et al., 1969a). A comparison of rates of hydrolysis of dipeptide esters by rat liver cathepsin C showed that Gly-Lys-OMe was hydrolyzed several times faster than Gly-Phe-OMe (McDonald et al., 1969a).

There are few reports available on the specificity of the polymerase or transferase activity of the enzyme. The polymerase activity of cathepsin C has been demonstrated with hydrophobic dipeptide amides such as Ala-Phe-NH₂ and Gly-Trp-NH₂ (Nilsson and Fruton, 1964). However, the polymerase action of the

enzyme was found to be rapid on a hydrophilic substrate such as Gly-Arg-NA (McDonald et al., 1969a).

The K_m values for hydrolysis of a series of dipeptidyl substrates were determined with partially pure bovine cathepsin C (Fruton and Mycek, 1956). The hydrolysis of substrates by purified rat liver cathepsin indicates derivatives of dipeptides have K_m values ranging from 0.1 to 0.2mM; whereas the values for esters and amides of the same dipeptides are 10 and 100 times higher, respectively (McDonald et al., 1969a). For bovine cathepsin C comparable values of K_m to the rat liver enzyme have been reported (Fruton and Mycek, 1956; Voynick and Fruton, 1968).

1.6.3. Activation

The activation of bovine spleen cathepsin C by cysteine was recognized during early studies of this enzyme (Gutmann and Fruton, 1948; Tallen et al., 1952). There is also a marked stimulation of hydrolytic activity by other sulfhydryl compounds, for example, 2-mercaptoethylamine, ME, thioglycolic acid, and glutathione (Fruton and Mycek, 1956).

In addition to a sulfhydryl requirement, cathepsin C has an absolute halide requirement. This requirement was not detected for many years because Cl^- and Br^- ions were carried into the usual assay mixture by either substrate (e.g., Gly-Phe-NH₂·HCl) or by the sulfhydryl activator (e.g., cysteine HCl). The halide requirement was first reported for cathepsin C obtained from bovine pituitary (McDonald et al., 1966a) which was then believed to be a newly discovered "dipeptidyl arylamidase" (an enzyme which catalyzes the hydrolysis of arylamide bond). The pituitary enzyme exhibits maximal activation by 5mM NaCl or HBr (McDonald et al., 1966a). Moreover, about 60% of the activation observed with Cl^- was achieved with I^- , F^- and also with NO_3^- . Subsequently, an absolute halide requirement was noted for the hydrolytic reaction catalyzed by cathepsin C from bovine spleen and from rat liver (McDonald et al., 1966b, 1969a). Kakiuchi and

Tomizawa (1964) isolated a halide dependent glucagon degrading enzyme from rat liver. An absolute halide requirement was also demonstrated for Ala-Ala-NA activity from porcine kidney (Hopsu-Havu and Rintola, 1968). McDonald (1969a) concluded that all of the above halide dependent activities are probably attributable to a common enzyme, that is cathepsin C.

1.6.4. Inhibition

Catalysis of hydrolytic reactions by bovine cathepsin C was inhibited by 1mM iodoacetate or 50mM formaldehyde, and was unaffected by 1mM diisopropylphosphorfluoridate or by 2,3-dinitrophenol. (Fruton and Mycek, 1956). The lack of inhibition with DFP was also observed with purified rat liver cathepsin C (McDonald et al., 1969a). Mycek (1970) reported that purified bovine spleen cathepsin C was unaffected by p-CMB; on the other hand, the porcine kidney enzyme was inhibited by the same reagent under similar concentration (Vanha-Perttula et al., 1965). The lack of inhibition of the hydrolytic reaction with bovine spleen enzyme is unexpected in view of the complete inhibition of hydrolytic activity observed for bovine pituitary, and rat liver enzyme with 0.01mM 4-chloromercuriphenylsulphonate (McDonald et al., 1966a, 1972b).

Several phenylalanine derivatives, in particular (L)phenylalaninamide, were reported to be competitive inhibitors of the hydrolytic reaction catalyzed by bovine spleen cathepsin C (Fruton and Mycek, 1956). Mettrione and MacGeorge (1975) have conducted a more detailed investigation into the mechanism of inhibition of bovine spleen cathepsin C by amino acids and peptide amides. Of the compounds studied, derivatives of hydrophobic amino acids were found to be the strongest inhibitors, while basic amino acids and short chain amino acids were somewhat less effective. Moreover, primary amines, for example butylamine, have also been shown to inhibit the hydrolytic activity of the enzyme. Bovine spleen cathepsin C was inhibited by papain inhibitor from chicken egg white, and this was the first naturally occurring inhibitor for cathepsin C (Keilova and Tomasek, 1975). Recently this inhibitor has been characterized and it was found to be a

polypeptide which was named cystatin (Barrett, 1981). A specific inhibitor of cathepsin C (Gly-Phe-diazomethylketone) has been reported by Green and Shaw (1981). Partially purified (lysosomal fraction) cathepsin C from rat liver has been reported to be inhibited by zinc (Misaka and Tappel, 1971).

1.7. Purpose of study

Previous studies have demonstrated the presence of cathepsin B in the hepatopancreas of other species of squid (Inaba et al., 1976) and cathepsins E and D in the muscle of *Illex illecebrosus* and *Loligo pealei* Leseur (Leblanc and Gill, 1982). However, there have been no reports of cathepsin C in squid or other invertebrate organisms. Preliminary studies in our laboratory have indicated the presence of an enzyme in the muscle and hepatopancreas of the Atlantic short-finned squid capable of catalyzing the transferase reaction with Gly-Phe-NH₂. On the basis of pH optima, activity against Gly-Phe-NH₂, and chloride activation, it was suggested that the active component was cathepsin C and a crude preparation of the enzyme from squid hepatopancreas was shown to promote taste development in brined squid (Lee et al., 1982).

The relationship between the subunit structure and the catalysis of polymerase and hydrolase reactions by cathepsin C is not clear at the present time. Urea (2M) was shown to dissociate subunits and simultaneously inhibit the polymerase reaction of bovine spleen cathepsin C and this observation led to the suggestion that the action of the enzyme as a catalyst of polymerization and hydrolytic reactions may be related to specific association and dissociation of subunits (Heinrich and Fruton, 1988).

It was hypothesized that the hydrolytic and polymerase reactions noted previously for an ammonium sulfate fraction from the hepatopancreas of squid were catalyzed by cathepsin C (dipeptidyl aminopeptidase I, E.C.3.4.14.1), and that association of subunits is required for polymerase reaction and the dissociated form of the enzyme catalyzes the hydrolytic reaction.

However, there is no definitive information regarding association and dissociation of this enzyme in relation to its catalytic properties as a polymerase and as a hydrolase. In view of the finding that a crude preparation of cathepsin C from squid potentiated the development of a delicious taste in brined squid, it

was of interest to characterize this enzyme further and to determine which factors control the hydrolytic and transferase reactions.

Chapter 2

MATERIALS AND METHODS

2.1. Biological material

Fresh squid (*Illex illecebrosus*) used in this study were purchased from a local supplier. The hepatopancreas was removed, vacuum packed, and immediately frozen in liquid nitrogen and stored at -70°C until use. Unless otherwise mentioned, all steps of purification were performed at 4°C .

2.2. Chemicals

The following chemicals used were purchased from Sigma Chemical Company, St. Louis, Mo.: Acrylamide, Ammonium sulfate, Ammonium persulfate, BANA, Coomassie brilliant blue (R-250), Copper sulfate pentahydrate, Ferric chloride, Gly-Phe- NH_2 , Gly-Phe-NA, Glutaryl-Phe-pNA, Hydroxylamine hydrochloride, ME, 2-Mercaptoethylamine, Phenylalanine hydroxamic acid, Ser-Tyr-NA, TEMED, Sodium phosphate monobasic, Sodium phosphate dibasic, Sodium EDTA, Trichloroacetic acid, Tris-(hydroxymethyl)-amino methane, Urea.

The chemicals listed below were purchased from J.T. Baker Chemical Company, Ltd., : Acetic acid (glacial), Hydrochloric acid, Sulfuric acid.

p-CMB-Sepharose was purchased from Pierce Chemical Company, Rockford, Il.

Bio-Rad reagent for protein determination, protein standards for SDS-polyacrylamide gel electrophoresis and DEAE-biogel were purchased from BioRad Laboratories Limited, Canada.

2.3. Methods used in this study

2.3.1. Buffer preparation

All buffers used for the purification and assays of the enzyme were prepared as described by Gomori (1955).

2.3.2. Extraction

About 500 g frozen hepatopancreas was thawed overnight at 4°C and homogenized in a Waring blender at full speed for 2 min with 2 parts(w/v) of 1.78mM disodium EDTA. The homogenate was further dispersed by a Polytron tissue homogenizer at high speed for 60 sec. The pH of the homogenate was adjusted to 3.5 with 6N sulfuric acid and it was incubated at 38°C for 18 h to allow autolysis. The autolysate was centrifuged at 10,000 X g for 1 h. The lipid layer which separated as a result of centrifugation was removed by suction and the remaining turbid supernatant was collected and filtered through two layers of cheese cloth. The clear brown filtrate thus obtained was called "acid extract".

2.3.3. Ammonium sulfate fractionation

The acid extract was adjusted to pH 5.0 with 6N NaOH and brought to 40% saturation with ammonium sulfate at 4°C. The mixture was held at 4°C for 2 h before the precipitate was pelleted by centrifugation at 10,000 X g for 1 h. The supernatant obtained was adjusted to 70% saturation with solid ammonium sulfate. The suspension was allowed to stand overnight, and the resulting precipitate was collected after centrifugation at 10,000 X g for 1 h. The precipitate was suspended in 50ml of 154mM NaCl solution and extensively dialyzed against approximately 9L of the same solution. The dialyzed solution was adjusted to pH 5.0 and centrifuged at 20,000 X g for 15 min. The clear solution obtained was collected and referred to as the "40-70% AS fraction".

2.3.4. Heat treatment

The protein concentration of the "40-70% AS fraction" was adjusted to 1.5mg/ml with 154mM NaCl and the pH of the solution was adjusted to 5. The "40-70% AS fraction" was divided into 12 ml portions in conical glass centrifuge tubes, and heated to 65°C. After 40 min at 65°C the enzyme preparation was chilled in an ice bath, and the precipitated protein was pelleted by centrifugation. The supernatants were collected and dialyzed against 6L of 154mM NaCl containing 4mM ME. The dialyzed solution was referred to as the "heated fraction".

2.3.5. Ultrafiltration

The "heated fraction" was concentrated approximately 10 fold by means of ultrafiltration under nitrogen at 40 psi with a 65ml Diaflo ultrafiltration assembly fitted with a UM10 Diaflo membrane. This membrane excludes most components having molecular weights in excess of 10 kdaltons. The concentrate (5ml) was dialyzed against 6L of 154mM NaCl containing 4mM ME. The dialyzed solution was referred to as the "heated fraction concentrate", and was stored at 0°C prior to assay or affinity chromatography.

2.3.6. Affinity chromatography

A 2ml portion of the "heated fraction concentrate" was dialyzed against 6L of 50mM sodium phosphate buffer, pH 7.0 and applied to a p-CMB-Sepharose column (0.5X8cm) which was pre-equilibrated with the same buffer. After sample application, the column was washed with phosphate buffer until protein was no longer eluted. Some of the bound protein was then eluted from the column with 50mM sodium acetate buffer, pH 4.5. A fraction containing cathepsin C was then eluted with 50mM sodium phosphate buffer containing 20mM ME pH 6.8. The eluted fractions were concentrated approximately 5 fold by ultrafiltration using a UM10 membrane and dialyzed against 6L of 154mM NaCl containing 4mM ME and referred to as the "affinity chromatography fraction".

2.3.7. DEAE-Biogel column chromatography

A 3ml portion of the "heated fraction concentrate" was dialyzed against 6L of 100mM sodium phosphate containing 4mM ME pH 7.0. The dialyzed concentrate, which contained 31mg of protein, was applied to a DEAE-Biogel column (1.5X30cm) pre-equilibrated with 100mM sodium phosphate buffer containing 4mM ME, pH 7.0. The protein was eluted with a linear 0-500mM NaCl gradient in the same buffer. Fractions of 7.0ml were collected with a flow rate of 30ml/h. The fractions rich in transferase activity were pooled, and concentrated approximately 4 fold by ultrafiltration using a UM10 membrane. The concentrated solution was referred to as the "DEAE fraction".

2.3.8. Gel permeation chromatography

A 4ml portion of the "heated fraction concentrate" was dialyzed against 100mM sodium acetate buffer, pH 4.5 and 3ml were applied to a Sephacryl S-300 (0.64X72cm) column pre-equilibrated with 100mM sodium acetate buffer containing 154mM NaCl and 4mM ME, pH 4.5. Elution was performed with the same buffer and fractions (2.4ml) were collected at a flow rate of 24ml/h. The column was previously calibrated with Blue dextran, catalase, ovalbumin and ribonuclease.

The fraction collected corresponding to the elution volume of protein of 50 kdaltons from the above run was rechromatographed under the conditions as described above without any further treatment.

2.3.9. SDS-polyacrylamide gel electrophoresis

The "affinity chromatography fraction" was electrophoresed in the presence of SDS and ME using the method of Laemmli (1970).

2.4. Determination of enzyme activity

Cathepsin C was assayed spectrophotometrically using Gly-Phe-NH₂ and Gly-Phe-NA as substrates (Vanha-Perttula et al., 1965; Metrione et al., 1966).

2.4.1. Transferase activity

The transferase activity of cathepsin C was determined with Gly-Phe-NH₂ as described by Metrione et al.(1966). The assay medium was prepared by combining 0.1ml of 1.9M hydroxylamine (prepared fresh from 4.0M hydroxylamine hydrochloride by adjusting to pH 7.0 with 4.0M NaOH), 0.1ml of 0.125M 2-mercaptoethylamine hydrochloride (prepared fresh and adjusted to pH 7.0 with 0.1N NaOH), 0.1ml of 0.25M Gly-Phe-NH₂ (adjusted to pH 7.0 with 0.1N NaOH), and 0.1ml water. The mixture was maintained at 30°C for 3 min, and the reaction was initiated by adding 0.1ml of enzyme preparation (suitably diluted with deionized water). The reaction tubes were incubated at 30°C for 15 min and the reaction was stopped by the addition of 0.5ml 20% TCA, followed by 0.5ml of 5% ferric chloride in 0.1N HCl. The zero time value was obtained by adding the TCA prior to the enzyme solution. The volume was adjusted to 2.0ml with distilled water and the absorbance at 510nm against a water blank was read in a Beckman DU-8 spectrophotometer within 10 min after the reaction was stopped. A standard curve was prepared with phenylalanine hydroxamate and the amount of dipeptide hydroxamate formed was calculated from the standard curve.

One unit of enzyme activity is defined as the amount of the enzyme that catalyzes the formation of 1 nanomole of dipeptide hydroxamate per min under the conditions of assay.

2.4.2. Hydrolase activity

The hydrolase activity of cathepsin C was determined with Gly-Phe-NA (Vanha-Perttula et al., 1985). In a 3.0ml quartz cuvette, 1.88ml of 0.1M sodium phosphate buffer, pH 6.0, containing 20mM 2-mercaptoethylamine.HCl, 0.1ml of suitably diluted enzyme preparation were placed and the cuvette was equilibrated at 30°C for 5 min in the cell compartment. The reaction was initiated by addition of 0.02ml (0.8 μ mole) of substrate. The rate of hydrolysis of substrate was determined by measuring the rate of change of absorbance at 340nm. The amount of 2-naphthylamine formed was calculated from its molar extinction coefficient (Lee et al., 1971).

One hydrolase unit of enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1 nanomole of 2-naphthylamine per min under the conditions of assay.

2.4.3. Protein determination

Protein concentration was estimated from the absorbance at 280nm of fractions obtained by column chromatography. The protein content of fractions obtained after each purification step was determined using Bio-Rad reagent and bovine serum albumin as a standard (Bradford, 1976).

2.5. The pH study

2.5.1. Effect of pH on transferase activity

The influence of pH on the transferase activity was determined using a modified form of the procedure by Jones et al. (1952). The "affinity chromatography fraction" was assayed for transferase activity over the pH range 5.5-7.5 and for this study 150mM citrate-phosphate buffer was used to buffer the pH. The reaction mixture contained 150mM citrate phosphate buffer, 50mM Gly-Phe-NH₂, 400mM hydroxylamine, and 25mM 2-mercaptoethylamine.HCl in a

final volume of 1.0ml. Each assay was performed in quadruplicate at 30°C with 10 μ g enzyme protein and stopped after 0, 5, 10 and 15 min, respectively, by the addition of 1.0ml of 20% trichloroacetic acid followed by 1.0ml of 5% ferric chloride in 0.1N HCl. The volume was then adjusted to 4.0ml with distilled water and the absorbance at 510nm was read within 10 min after the reaction was stopped. The amount of dipeptide hydroxamate formed was calculated from the standard curve prepared with phenylalanine hydroxamate.

2.5.2. Effect of pH on hydrolase activity

The "affinity chromatography fraction" was assayed for Gly-Phe-NA or Ser-Tyr-NA hydrolysis over the pH range 3-7. The reaction mixture contained 150mM citrate-phosphate buffer, 20mM 2-mercaptoethylamine.HCl and 400mM dipeptide NA substrate in a total volume of 2.0ml. Each assay was performed in triplicate with 5 μ g enzyme protein. The reaction tubes were incubated at 30°C for 10 min and the reaction was stopped by the addition of 2.0ml of 0.23N HCl in ethanol followed by 2.0ml of 0.06% p-dimethylaminocinnamaldehyde in ethanol. The absorbance at 540nm was read in a Beckman DU-8 spectrophotometer (Matsutani et al., 1967). A standard curve was obtained using 2-naphthylamine, and the amount of 2-naphthylamine formed during reaction was calculated from the standard curve.

2.5.3. Stability of cathepsin C

The "affinity chromatography fraction" was diluted with buffers of different pH and stored at 30°C for 30 min. The buffers used were 100mM sodium acetate pH 4.0, 4.5, 5.0, and 100mM sodium phosphate pH 5.5, 6.0, 6.5, and 7.0. At the end of the incubation time the enzyme pH was readjusted to the assay pH and transferase activity was measured under the conditions described in 2.4.1.

2.6. Kinetic study

2.6.1. Effect of substrate concentration on transferase activity

The effect of Gly-Phe-NH₂ concentration on transferase activity of squid cathepsin C was determined with the "affinity chromatography fraction". For the transferase reaction 10 μ g of protein was assayed at each concentration of substrate (25-400mM), and the assays were carried out under the conditions described in 2.4.1. The apparent Michaelis-Menten constant (K_m) and V_{max} were determined by using Lineweaver-Burk analysis and by the least square method of Johansen and Lumry (1961) using a computer program based on equations presneted by Cornish-Bowden (1979).

2.6.2. Effect of substrate concentration on hydrolase activity

The effect of either Gly-Phe-NA or Ser-Tyr-NA concentration on hydrolase activity was studied using the "affinity chromatography fraction". The assays were performed with 5 μ g protein under the conditions described in 2.4.2., except the concentration of substrate varied (0.025-0.4mM). The apparent Michaelis-Menten constant (K_m) and V_{max} were determined by using Lineweaver-Burk analysis and by the least square method of Johansen and Lumry (1961) using a computer program based on equations presneted by Cornish-Bowden (1979).

2.7. Halide activation

2.7.1. Effect of Cl⁻ ions on the transferase activity

A 4ml portion of the "affinity chromatography fraction" was dialyzed against 6L of deionized water at 5°C for 48 h. Assays were performed with 12 μ g of protein under the conditions described in 2.4.1., except different concentrations of NaCl or KCl were used. The 2-mercaptoethylamine was replaced with 25mM ME to satisfy the thiol requirement of the enzyme.

2.7.2. Effect of Cl⁻ ions on hydrolase activity

A 4ml portion of the "affinity chromatography fraction" was dialyzed against 6L of 50mM sodium phosphate buffer pH 6.0, at 5°C for 48 h. Assays were performed with 5 μ g of protein under the conditions described in 2.4.2., except different concentrations of NaCl were used and 2-mercaptoethylamine was replaced with 20mM ME.

2.8. Sulfhydryl activation

2.8.1. Effect of thiol compounds on the transferase activity

The "affinity chromatography fraction" was used to study the effect of thiol compounds on the transferase activity of the enzyme. Assays were performed with 10 μ g of protein under the conditions described in 2.4.1. Sodium chloride was included in all assay mixtures at a final concentration of 25mM, except in reaction mixtures containing 2-mercaptoethylamine.HCl.

2.8.2. Effect of ME on hydrolase activity

The "affinity chromatography fraction" was used to test the effect of ME on hydrolase activity of the enzyme. Assays were performed with 5 μ g of protein and the reaction conditions were the same as described in 2.4.2., except NaCl was included in the assay mixture at a final concentration of 20mM.

2.9. Inhibition study

2.9.1. Effects of inhibitors on enzyme activity

The "affinity chromatography fraction" of the enzyme preparation was used to test the effect of inhibitors. Stock solutions of EDTA, iodoacetate and puromycin were prepared in deionized water. Pepstatin A and p-CMB were dissolved separately in DMSO. PMSF was solubilized in 10% (v/v) 2-propanol. Each inhibitor was included in assay buffer at final concentration (preincubation)

of 1.0mM, except mercuric chloride and EDTA which were used at a final concentrations of 0.1mM and 4.0mM, respectively. The enzyme (5 μ g protein) was incubated at 30°C with assay buffer containing each inhibitor. After 30 min, 0.02ml of Gly-Phe-NA (400 μ M) was mixed in to start the reaction, and amount of 2-naphthylamine formed was determined as described in 2.4.2.

2.10. Effect of Triton X-100 treatment

2.10.1. Effect of Triton X-100 treatment on transferase and hydrolase activity of squid cathepsin C

The "heated fraction" of squid cathepsin C was used to study the influence of Triton X-100 on activity. The enzyme solution was diluted to 6.0mg protein/ml with 154mM NaCl. To 25ml sufficient Triton X-100 was added to get a final concentration of 1% and the solution was incubated at 0°C for 1 h. Transferase and hydrolase assays were performed under the conditions described in 2.4.1 and 2.4.2., respectively.

2.10.2. Effect of Triton X-100 treatment on transferase and hydrolase activity of bovine cathepsin C

Bovine cathepsin C was diluted to 1.5mg protein/100ml with 154mM NaCl and dialyzed against 6L volume of 154mM NaCl containing 4mM ME prior to Triton X-100 treatment. Sufficient Triton X-100 was added to the dialyzed enzyme solution to achieve a final concentration of 1% (v/v). The mixture was incubated at 0°C for 1 h and it was then subjected to ultrafiltration using a XM50A membrane. This membrane excludes most components having a molecular weight in excess of 50,000. The enzyme solution (25ml) was concentrated to about 3.5ml, and assays were performed under the conditions described in 2.4.1. and 2.4.2. Since the enzyme solution was not concentrated to complete dryness, the values for the enzyme units reported in the results section were corrected accordingly. To obtain a corrected value, the following expression was used:

$$U_f \times V_i / V_f = U_{fc}$$

Where as V_i = Initial volume of the sample.

V_f = Volume of the filtrate.

V_r = Volume of retentate (3.5ml)

U_f = Units of activity in filtrate (uncorrected).

U_{fc} = Units of activity in filtrate (corrected).

U_r = Total units of activity recovered in retentate
(without correction).

$U_{rc} = U_r - (U_f / \text{ml} \times V_r) = \text{Units recovered}$
in retentate (corrected).

2.11. Effect of urea treatment

2.11.1. Effect of urea treatment on the ultrafiltration behavior of squid cathepsin C

The "heated fraction" of squid cathepsin C was used to study the effect of urea treatment on the ultrafiltration behavior of the enzyme. The enzyme solution was diluted to 6.0mg protein/100ml as described in 2.10.1. To 25ml of the diluted solution, solid urea was added to achieve a final concentration of 2M. The solution was incubated at 0°C for 4 h, and it was subjected to ultrafiltration using a XM50A membrane. The solution was concentrated to about 3.5ml. All assays were performed under the conditions described in 2.4.1. and 2.4.2. Since the enzyme solution was not concentrated to complete dryness, the values for the enzyme units reported in the results section were corrected as in section 2.10.2.

2.11.2. Effect of urea treatment on the ultrafiltration behavior of the bovine cathepsin C

Bovine cathepsin C was also used to study the effect of urea treatment on the ultrafiltration behavior of this enzyme. The enzyme solution was adjusted to 1.5mg protein/100ml using a sodium chloride (154mM) solution, and dialyzed under the conditions described in 2.10.2. Solid urea was added to achieve a final concentration of 2M. The solution was incubated at 0°C for 4 h, and it was subjected to ultrafiltration using a XM50A membrane. The solution was concentrated to about 3.5ml. Since the enzyme solution was not concentrated to complete dryness, the values for the enzyme units reported in the results section were corrected as in section 2.10.2.

Chapter 3

RESULTS AND DISCUSSION

3.1. Partial purification of cathepsin C from squid hepatopancreas

Table 3-1 summarizes the recovery of the enzyme under different conditions of pH, temperature and time during autolysis of the tissue. It is apparent from Table 3-1, that the maximum release of enzyme was found after 18h at pH 3.5, 38° C.

Table 3-2 summarizes the recovery of the enzyme in different fractions obtained by ammonium sulfate precipitation. It is evident from Table 3-2, that a salt saturation of 0-40% or 70-80% did not precipitate the protein exhibiting either of the catalytic activities. It is also evident that the maximum recovery of active enzyme was obtained in the protein-fraction which precipitated at a concentration between 40-70% saturation. The data also show that ammonium sulfate fractionation increases the total units of enzyme. Therefore, it appears that this step removes an inhibitor of cathepsin C or otherwise activates the enzyme.

Cathepsin C obtained from the hepatopancreas was partially purified as described under 2.3. The results of cathepsin C purification are summarized in Table 3-3. The transferase activity was increased 36 fold and the hydrolase activity was increased 51 fold as a result of purification of the acid extract by the indicated steps ending with affinity chromatography. The specific activity of the fraction purified from a heated sample by ion exchange chromatography was

similar to that obtained by affinity chromatography. The elution profile for affinity chromatography of the "heated fraction" of squid cathepsin C preparation on a p-CMB-Sepharose column is shown in Fig 3-1. The enzyme activity peak was closely paralleled the second smaller protein peak, while the larger preceding protein peaks were devoid of activity.

The elution profile for DEAE-Biogel column chromatography of the "heated fraction" is shown in Fig 3-2. The transferase activity eluted in four fractions corresponding to NaCl concentrations of 0.29, 0.31, 0.34 and 0.40M. It is possible that these four fractions correspond to different states of association of the same subunits (see section 3.8.). SDS-polyacrylamide gel electrophoresis of the enzyme preparation after different stages of purification was carried out by the method described by Laemmli (1970), and the results obtained are presented in Fig 3-3.

The R_f values of standard proteins were also estimated and used to plot a graph of the logarithm of molecular weight of the proteins versus mobility (R_f). A summary of the description of the proteins, their R_f values and molecular weights are presented in Table 3-4. It is apparent from Fig 3-3, that the procedure employed for the purification of squid cathepsin C was effective in eliminating a large number of proteins. The main component of the partially purified squid cathepsin C (affinity chromatography fraction) was about 25 kdaltons, corresponding to the expected mass of the subunit of cathepsin C. However, a second minor band, corresponding to 62 kdaltons was also present.

General discussion: Purification of cathepsin C from squid.

The initial steps in the purification of bovine cathepsin C involved the preparation of an acid extract obtained by incubating the homogenate, adjusted to pH 3.5, at 38°C for 22 h, and separation of the acid extract from the autolysate by centrifugation (Metrione et al., 1966). The acid extract was concentrated by ammonium sulfate precipitation between 40-70% saturation.

Table 3-1: Autolysis of tissue homogenate under various conditions and recovery of enzyme in acid extract¹

Autolysis condition			Recovery of the enzyme ² (% Relative activity)	
Time (h)	Temperature (°C)	pH	Transferase	Hydrolase
1	4	3.5	30	34
4	4	3.5	33	30
1	38	3.5	40	39
4	38	3.5	50	51
18	38	3.5	100	100
20	38	3.5	70	76
22	38	3.5	63	67
18	38	7.0	47	41

¹Average of two separate experiments; 500 g hepatopancreas was used.

²Transferase and hydrolase assays were performed under the conditions described in 2.4.1. and 2.4.2., respectively, and are based on total units recovered. 100% of transferase activity represents 4900 units; 100% of hydrolase activity represents 5100 units.

Table 3-2: Recovery of cathepsin C under varying saturations of ammonium sulfate¹

Fraction	Recovery of the enzyme ² (% Relative activity)	
	Transferase	Hydrolase
Acid extract	100	100
Ammonium sulfate		
(0-40%)	0	0
(40-70%)	234	338
(40-80%)	206	202
(70-80%)	0	0

¹Average of two separate experiments; 500 g of hepatopancreas was used.

²Transferase and hydrolase assays were performed under the condition described in 2.4.1, and 2.4.2. respectively, and are based on total units recovered. 100% of transferase activity represents 4600 units; 100% of hydrolase activity represents 4800 units.

**Table 3-3: Summary of purification of cathepsin C
from 500 g squid hepatopancreas¹**

Fraction	Total protein (mg)	Total transferase (Units)	Specific activity (Units/mg)	Total hydrolase (Units)	Specific Activity (Units/mg)
Acid extract	165	4700	28	5000	30
Ammonium sulfate (40-70%)	65	11000	169	16900	260
Heat treated fraction	32	8200	256	16500	515
UM10 filtration	28	7520	268	15300	540
Affinity Chromatography	8	6100	1016	9300	1550
DEAE-Biogel Chromatography ²	4	3200	800	7050	1762

¹Average of two separate experiments.

²The "heated fraction" of the enzyme preparation was used; about 31 mg protein (7500 transferase units) were applied to the column.

³Transferase activity of the enzyme was determined with Gly-Phe-NH₂, methods section 2.4.1.

⁴Hydrolase activity of the enzyme was determined with Gly-Phe-NA₂, methods section 2.4.2.

Fig 3-1: Affinity chromatography of squid cathepsin C on a p-CMB-Sepharose column.

The column (0.5 x 8 cm) was preequilibrated with 50mM sodium phosphate buffer, pH 7.0. At "a" the buffer was changed to 50mM sodium acetate, pH 4.5. Cathepsin C was eluted with the starting buffer containing 20mM ME at "b". Fractions of 5.0ml were collected. The assay for enzyme activity was performed with Gly-Phe-NH₂ as described under 2.4.1. Fractions number 23-27 were pooled, concentrated and dialyzed as described in 2.3.6. The dialyzed enzyme solution was referred to as the "affinity chromatography fraction".

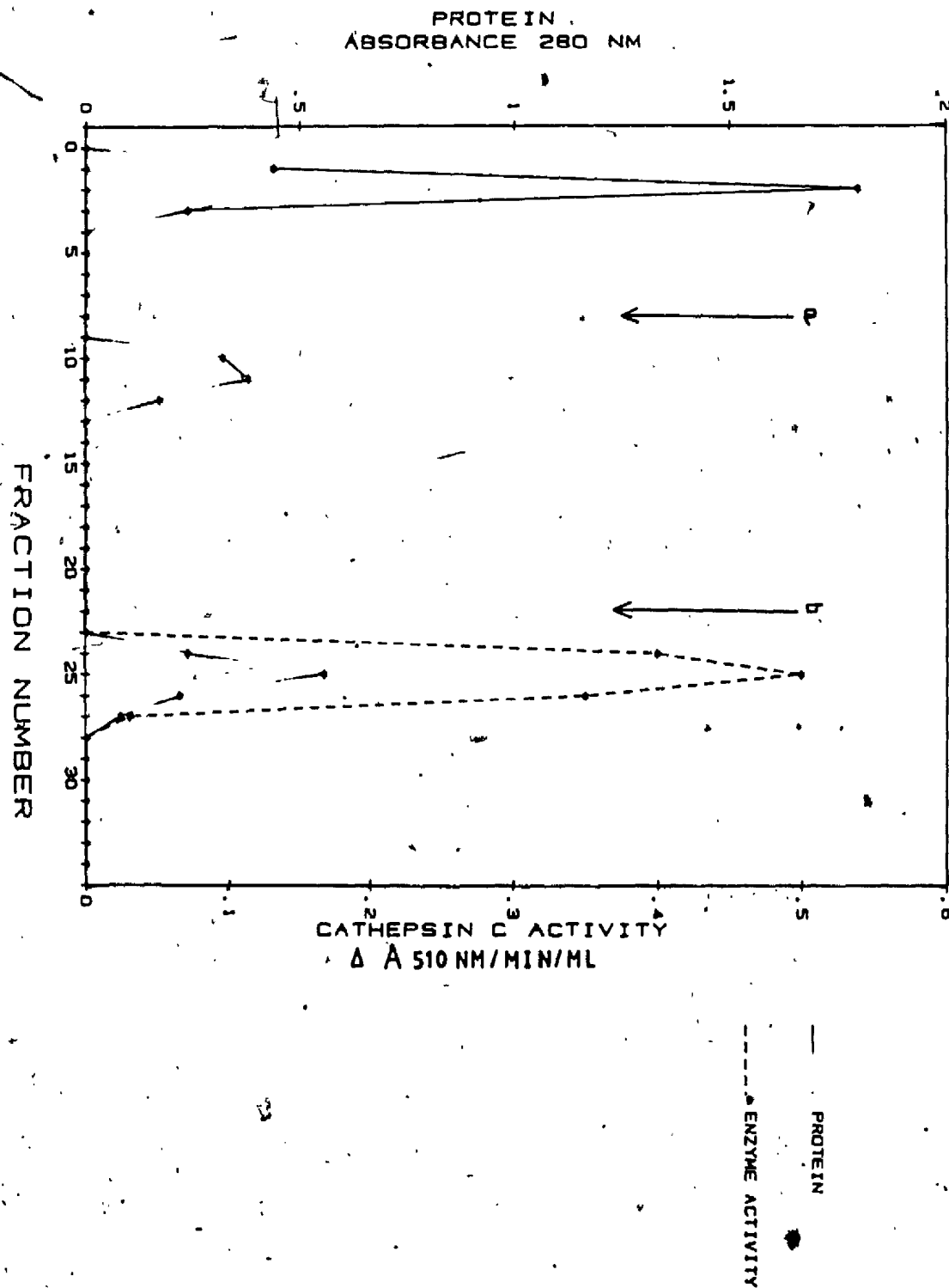


Fig 3-2:DEAE-biogel column chromatography of squid cathepsin C.

The column (1.5 x 30 cm) was preequilibrated with 100mM sodium phosphate buffer containing 4mM ME. The cathepsin C was eluted with a linear gradient of 0-0.5M NaCl in same buffer. Fractions of 7.0ml were collected with a flow rate of 30ml/h. The assay for enzyme activity was performed with Gly-Phe-NH₂ as described under 2.4.1. Fractions number 50-55, 56-57, 58-60 and 65-67 were pooled and concentrated as described under 2.3.7. The concentrated enzyme solutions were referred to as the "DEAE fraction". Data are for one experiment and are representative of two other experiments.

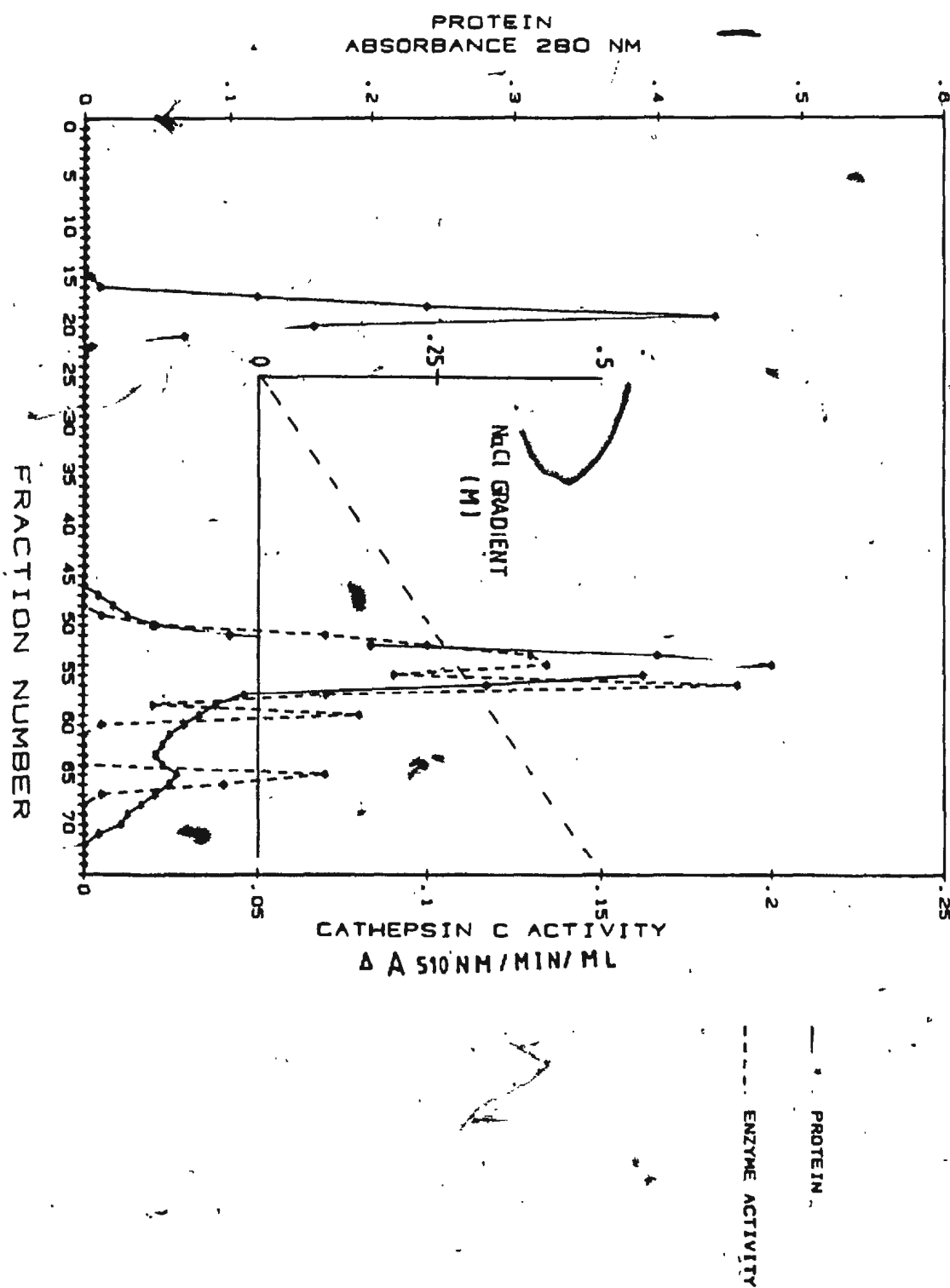


Fig 3-3: SDS-polyacrylamide gel electrophoresis of samples obtained in the partial purification of squid cathepsin C.

1) 40-70% AS fraction (60 μ g); 2) heated fraction (60 μ g); 3) standard proteins; [a] β -galactosidase, [b] Phosphorylase A, [c] Bovine serum albumin, [d] Ovalbumin, [e] Chymotrypsinogen and [f] Ribonuclease A, 40 μ g); 4) standard proteins (20 μ g); 5) affinity chromatography fraction (10 μ g); 6) affinity chromatography fraction (20 μ g). The samples and marker proteins were mixed with marker dye (bromophenol blue) and were electrophoresed under the conditions described by Laemmli (1970). The protein was stained with 1% Coomassie blue in 7% acetic acid.

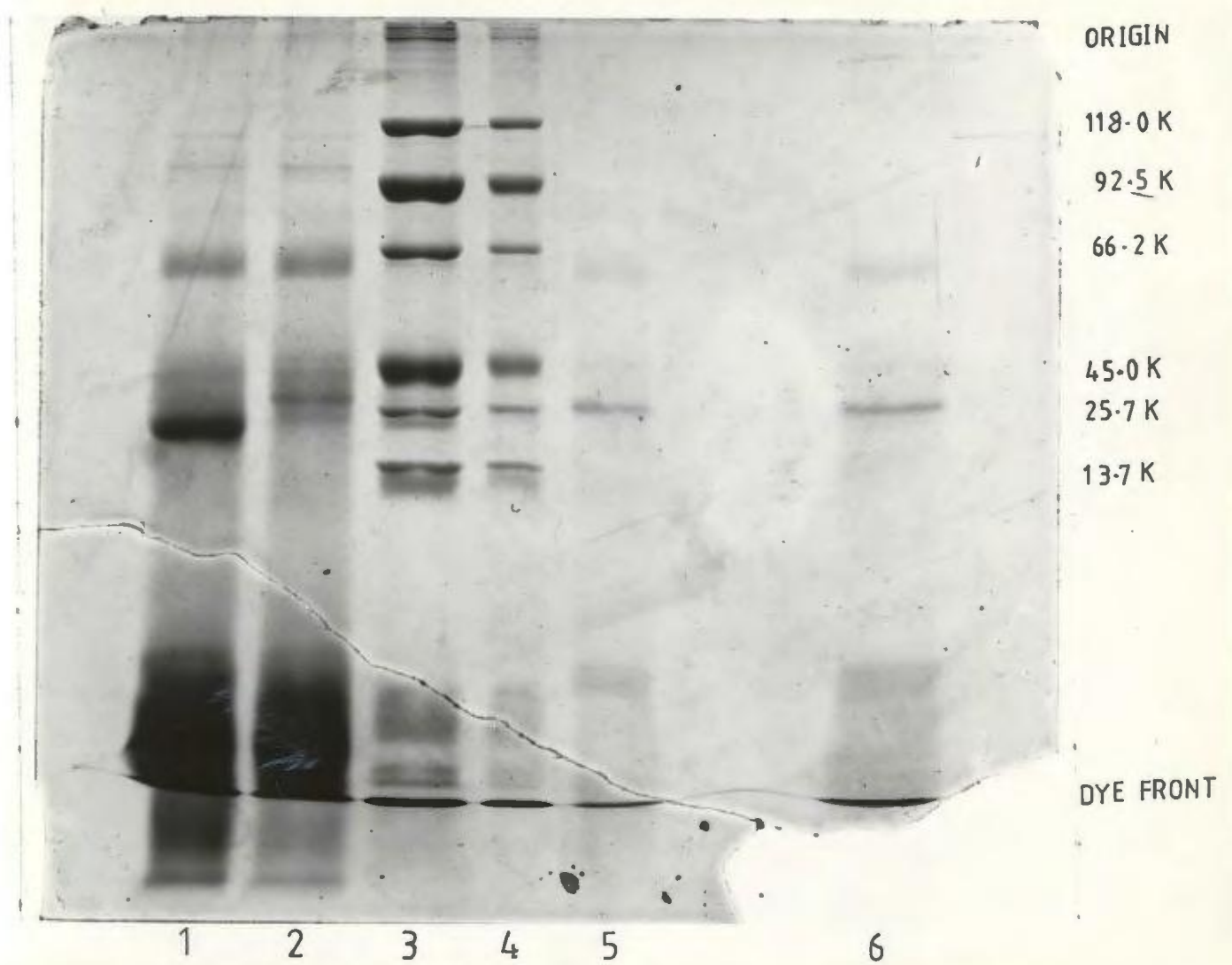


Table 3-4: Mobility of proteins on SDS-polyacrylamide gel electrophoresis

Protein	Rf	Mol.weight(kdaltons)
β -Galctosidase	0.13	118.0
Phosphorylase a	0.21	92.5
Bovine serum albumin	0.30	66.2
Ovalbumin	0.44	45.0
Chymotrypsinogen	0.50	25.7
Ribonuclease A	0.58	13.7
Squid cathepsin C		
(Major band)	0.33	62.0
(Minor band)	0.50	25.7

In the purification of cathepsin C from squid hepatopancreas, the above two steps were successfully applied with an adjustment in the time of autolysis to 18 h. If a longer time of autolysis was allowed, low recovery of enzyme was obtained.

In the initial stages of the present investigation, experiments were conducted in an attempt to optimize the conditions for ammonium sulfate precipitation by bringing the acid extract to 0-40%, 40-70%, 70-80%, and 40-80% saturation with ammonium sulfate. On the basis of data obtained by this study a saturation of 40-70% was used for subsequent experiments. With this operation a 6-9 fold purification was achieved, and more activity was recovered in the "40-70% AS fraction" than was present in the "acid extract" indicating that this step removed inhibitors present in the acid extract or otherwise activated cathepsin C. The increase in specific activity of squid cathepsin C obtained from acid extract to 40-70% AS fraction is comparable to that reported for the bovine enzyme (Metrione et al., 1966).

Purification of proteins by conventional procedure is frequently laborious and incomplete, and the yields are often low. Enzyme isolation based on a highly specific biological property such as strong reversible association with a specific substrate or inhibitors offers a good method for purification. Para-chloromercuribenzoate-Sepharose is known to bind a number of proteins, including bovine cathepsin C, that contain sulphydryl groups (Metrione, 1978). In this study this material was used to purify squid cathepsin C. This material was found to be very effective in binding the enzyme, which was then eluted in a single peak with buffer containing ME. The active fraction eluted from an affinity column was further resolved into 2 bands, corresponding to molecular weights of about 25 and 62 kdaltons, respectively, by SDS-polyacrylamide gel electrophoresis. The major band on Commassie blue staining, corresponds to a protein of 25 kdaltons on SDS-polyacrylamide gel electrophoresis of the affinity chromatography fraction. This protein appears to be a subunit of squid cathepsin

C (refer to section 3.8.3.). The other minor band corresponding to 82 kdaltons would appear to be incompletely dissociated subunits or a contaminant of the preparation.

The use of ion exchange chromatography on different anion and cation exchangers in the purification of cathepsin C was reported by various workers (Planta and Gruber, 1964; Mettrione et al., 1966; McDonald et al., 1966a, 1969a). In an attempt to purify the squid cathepsin C further, an anion exchange material (DEAE-biogel) was used in this study. Chromatography of the heated fraction of the enzyme preparation revealed the following information: i) At a pH of approximately 7.0, squid cathepsin C had a net negative charge, therefore, all the activity loaded on the column was adsorbed. ii) The use of a gradient of increasing ionic strength of NaCl permitted desorption of the enzyme activity in four peaks, which were all eluted between 0-0.50M NaCl. With this operation, a 3-4 fold increase in transferase activity was achieved over the sample applied. The recovery of transferase and hydrolase activities in this fraction was about 43-46%. Further purification of squid cathepsin C by combining ion exchange with affinity chromatography did not improve the specific activity at all.

The specific activity of the affinity chromatography fraction with Gly-Phe-NH₂ as substrate was about 1016 nanomoles of hydroxamate produced per mg of protein per min and was a 36 fold increase over the activity in the acid extract.

The specific activity of the affinity chromatography fraction with Gly-Phe-NA as substrate was 1550 nanomoles of NA formed per mg of protein per min. This was a 51 fold increase over the activity in the acid extract.

3.2. Characterization of catalytic properties

3.2.1. Cathepsin C assay

The time course for transferase and hydrolase reaction was determined with the "affinity chromatography fraction" of squid cathepsin C under the conditions described in 2.4.1. and 2.4.2., respectively. The results obtained are presented in Fig 3-4 and 3-5. The rate of absorbance change with "affinity fraction" was constant for at least 30 min for both transferase as well as hydrolase activity. Incubation of reaction mixtures without enzyme did not cause any change in absorbance, and thus eliminated the possibility of non-enzymic reactions such as hydrolysis or hydroxylaminolysis.

The initial rate for both activities were proportional to the enzyme concentration tested, at least up to 300 μ g protein of crude preparation. The enzyme preparations used in this study were appropriately diluted to fit this range of activity.

General discussion: Cathepsin C assays.

The substrate Gly-Phe-NA was adopted for the hydrolase assay because of the availability of the substrate and previous reports that it is specific for cathepsin C. The sensitivity of the assay could have been increased at least ten to fifteen fold through the use of Gly-Arg-MNA, recently made available by Sigma Chemical Co. The crude preparation of squid cathepsin C hydrolyzed this substrate at a rate which was at least ten times faster than for Gly-Phe-NA. The possibility that some other enzyme active on this substrate was not ruled out. With the acid extract of the squid enzyme preparation the reaction was found to terminate prior to the anticipated completion of the reaction (Fig 3-5). The possibility that substrate depletion or enzyme inactivation was responsible for this was ruled out. The addition of more substrate or enzyme after the reaction started caused the reaction to resume at the initial rate. The exact reason for the

Fig 3-4: Time course for transferase activity of squid cathepsin C.

Either 10 μ g of squid cathepsin C (affinity chromatography fraction) or 285 μ g of "acid extract" was incubated per assay mixture at 30°C, and the reaction was stopped at 5, 10, 15, 20, 25 and 30 min interval. The absorbance at 510nm was measured as described under 2.4.1. Gly-Phe-NH₂ was used as substrate.

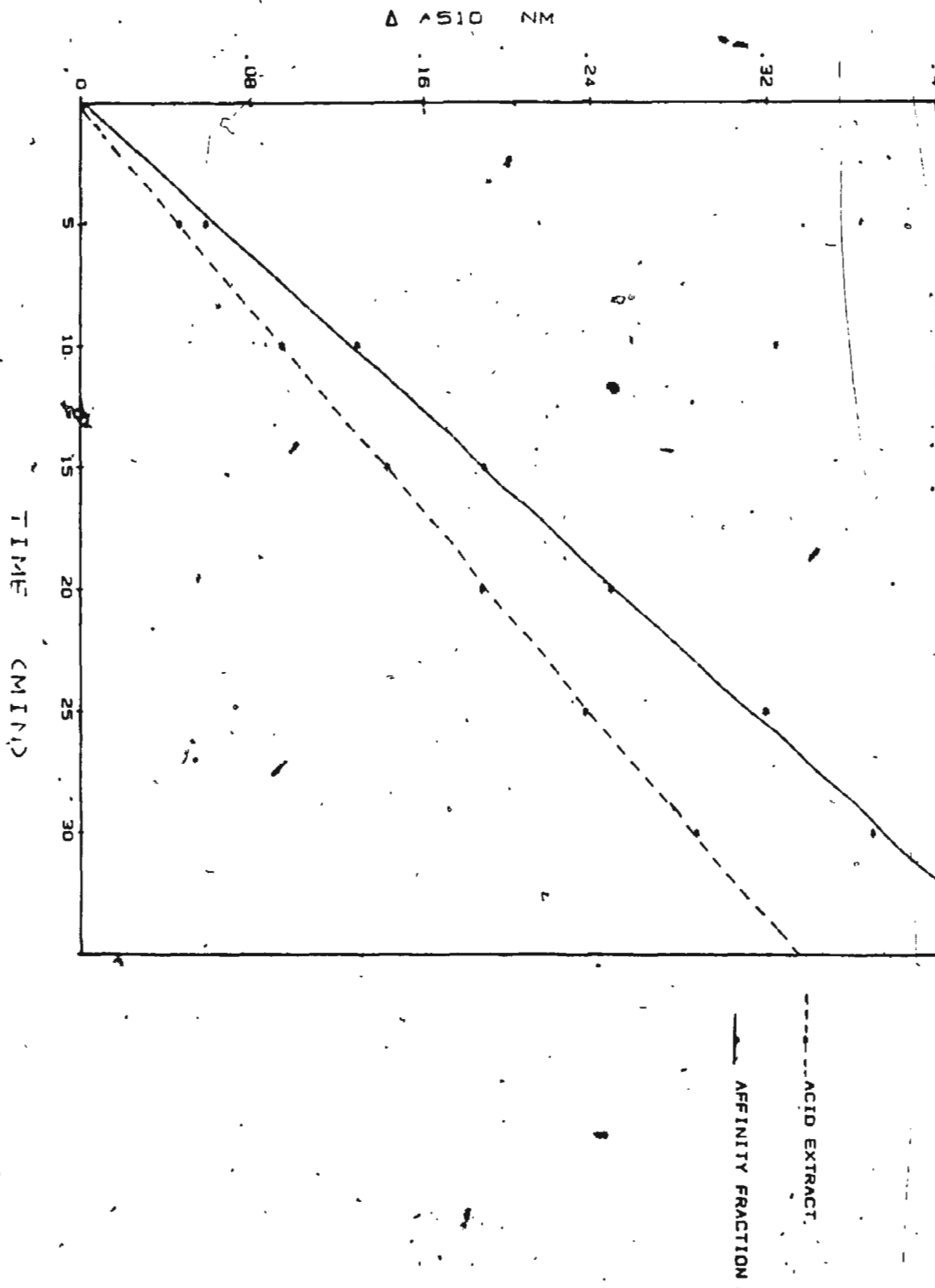
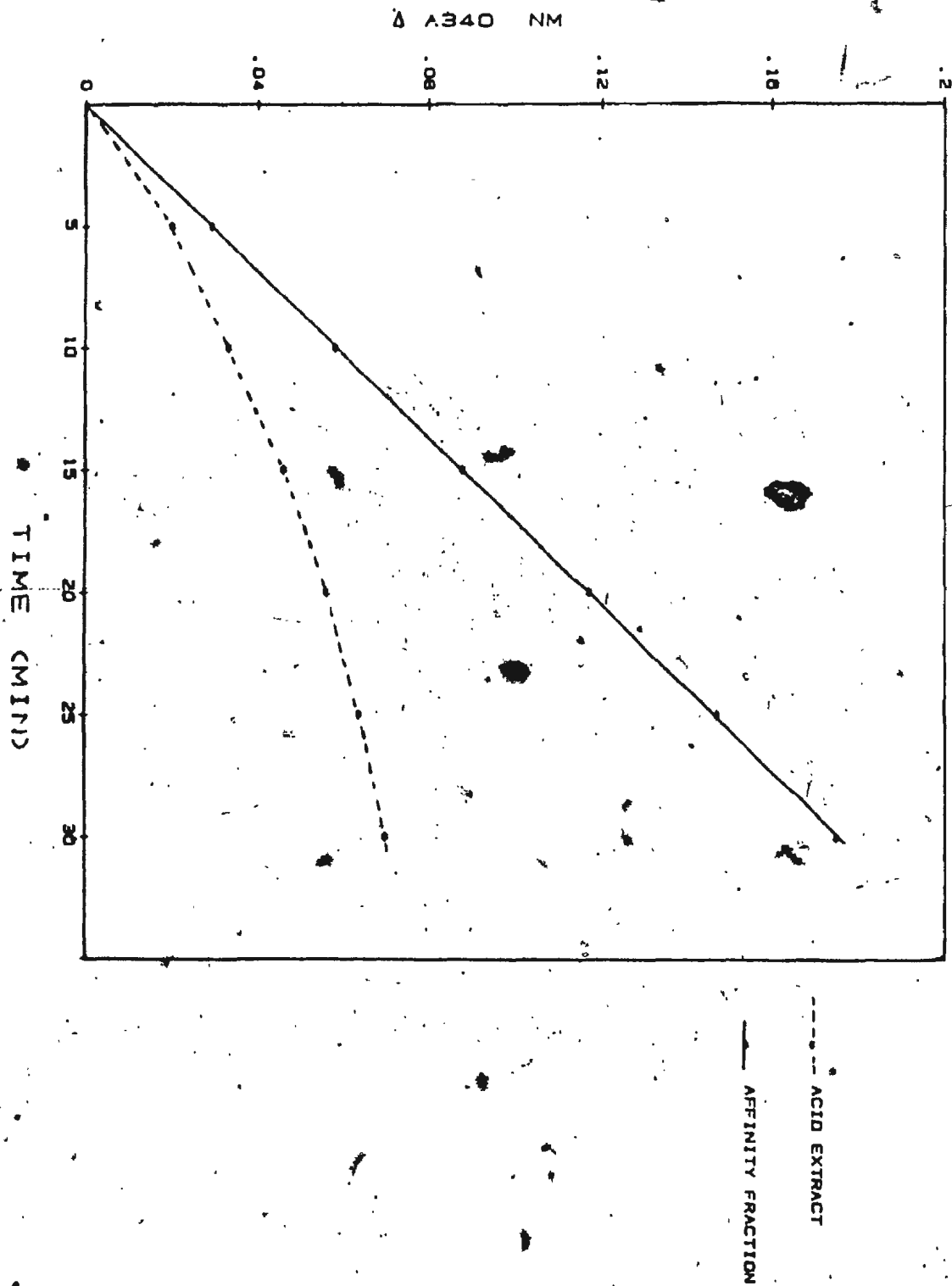


Fig 3-5: Time course for hydrolase activity of squid cathepsin C.

For acid extract 200 μ g protein/assay mixture was used. For "affinity chromatography fraction" 5 μ g protein/assay mixture was used.

The assay mixtures were incubated at 30°C, and the rate of change of absorbance at 340nm was measured at 1 min intervals in the DU-8 spectrophotometer, as described under 2.4.2. Gly-Phe-NA was used as substrate.



non-linearity is not understood. In contrast to the hydrolase activity, the transferase activity was constant with time of assay, even with the acid extract (Fig 3-4).

3.3. Effect of pH on cathepsin C catalyzed reactions

The effect of pH on the transferase activity of squid cathepsin C with Gly-Phe-NH₂ was determined as described under 2.5.1. The results obtained are presented in Fig 3-6. It is apparent from Fig 3-6 that squid cathepsin C showed a narrow pH optima (7.0) for the transferase reaction.

The effect of pH on the hydrolase activity of squid cathepsin C with either Gly-Phe-NA or Ser-Tyr-NA was determined as described under 2.5.2. The results obtained are presented in Fig 3-7. The pH activity profiles of squid cathepsin C for Gly-Phe-NA and Ser-Tyr-NA substrate were similar in so far as both substrates were hydrolyzed more at moderately acidic pH 5-6, and another region of high activity was also recognized at p. 4. Similar results were obtained with the acid extract preparation of the squid enzyme.

The pH stability of the squid cathepsin C was determined with the affinity chromatography fraction of the enzyme as described under 2.5.3. and the results obtained are presented in Fig 3-8. The enzyme was stable at a pH value of 7, but at pH values less than 4.5, more than 20% of the transferase activity was lost.

General discussion: Effect of pH on cathepsin C catalyzed reactions

Cathepsin C catalyzes the transfer of a dipeptidyl unit of a suitable substrate to nucleophilic acceptors, such as hydroxylamine, free amino acids, excess dipeptide amide substrate or water (Wurz et al., 1962; Nilsson and Fruton, 1964).

The relative rate at which the activated dipeptidyl units react with water or with the amino group of another substrate depends upon the pK_as of the

Fig 3-8: Effect of pH on transferase activity of the squid cathepsin C toward Gly-Phe-NH₂.

The assays were performed with the "affinity chromatography fraction" of the enzyme preparation (10 μ g protein) under the conditions described in 2.5.1. at 30°C. Citrate-phosphate buffer (150mM) was used to cover the pH range of 5.5-7.5.

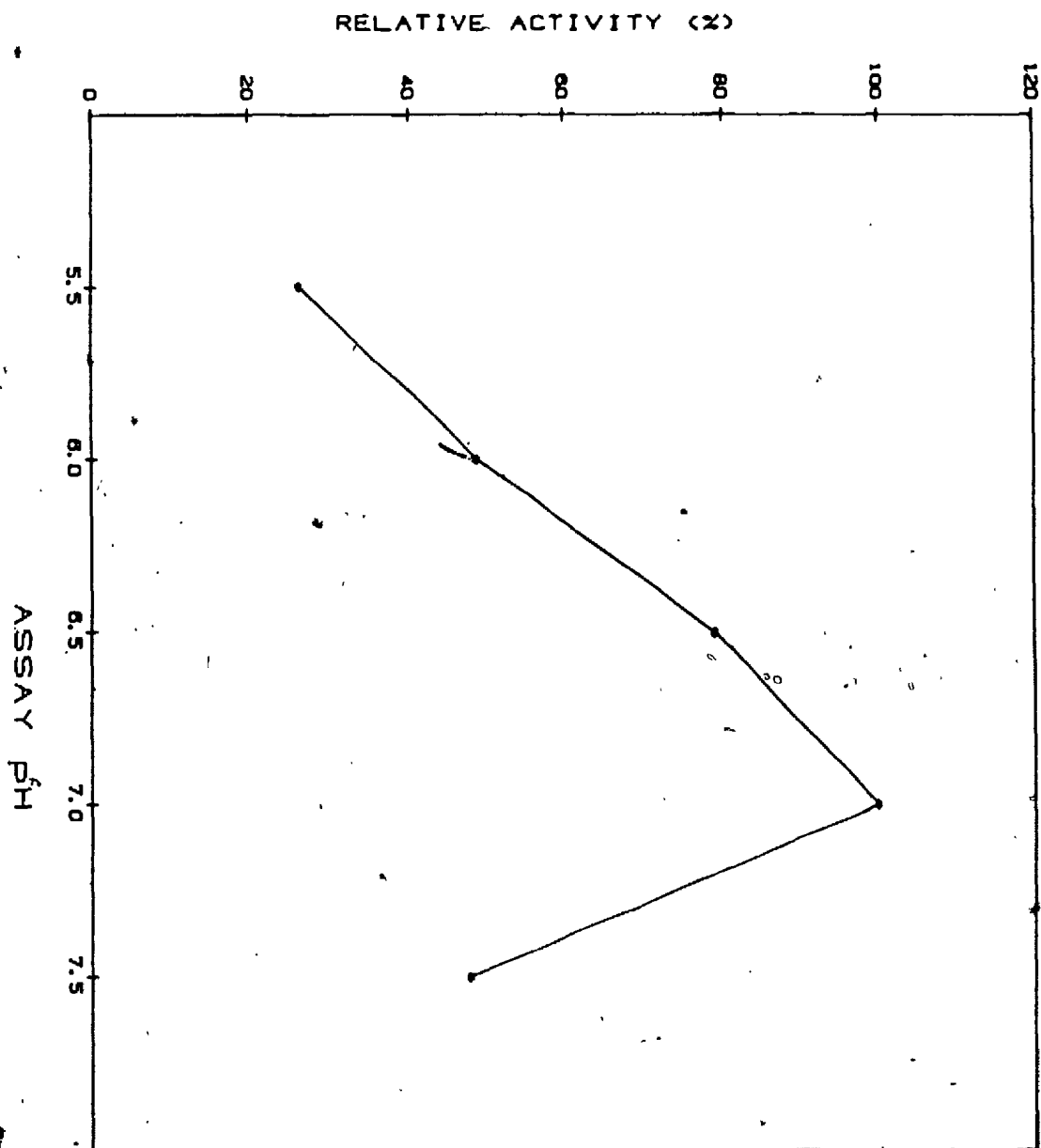


Fig 3-7: Effect of pH on hydrolase activity of the squid cathepsin C.

The assays were performed with the "affinity chromatography fraction" of the enzyme preparation (5 μ g protein) under the conditions described in 2.5.2. at 30°C. Citrate-phosphate buffer (150mM) was used to cover the pH range of 3.0-7.0.

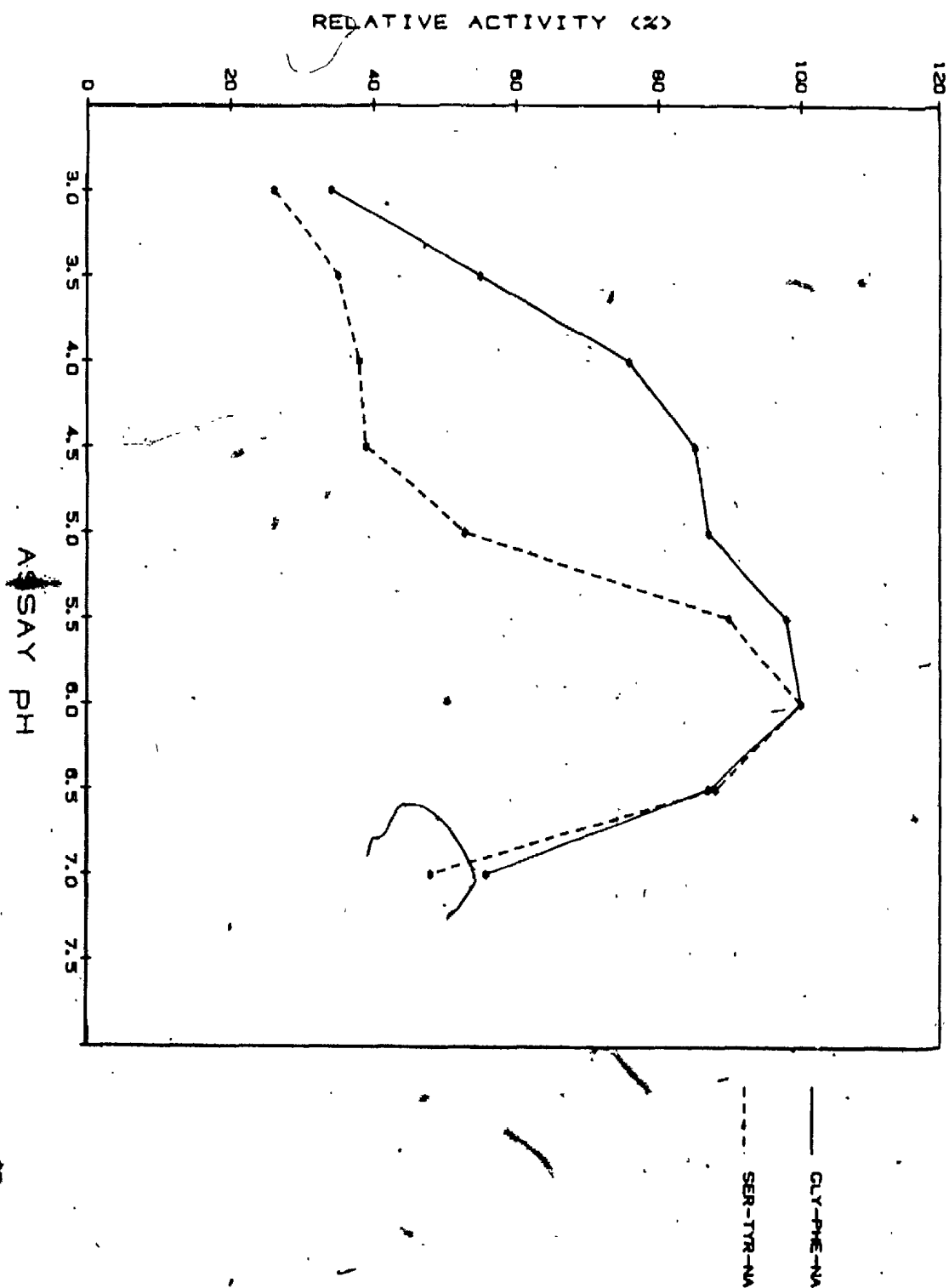
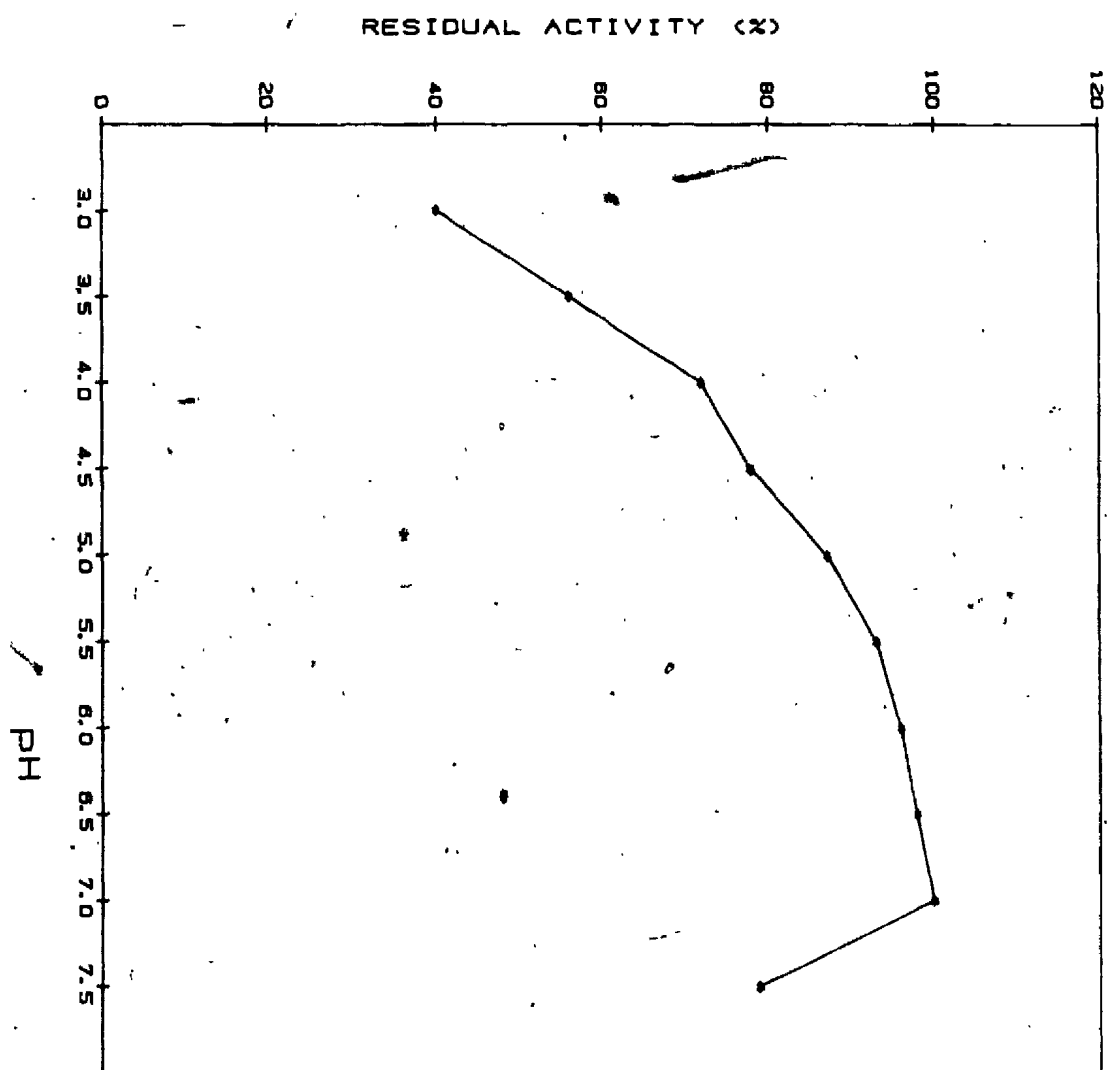


Fig 3-8: pH stability of squid cathepsin C.

The "affinity chromatography fraction" was diluted 1:4 with buffers of different pH and stored at 30°C for 30 min. The buffers used were 100mM sodium acetate pH 4.0, 4.5, 5.0, and 100mM sodium phosphate pH 5.5, 6.0, 6.5 and 7.5. At the end of incubation time the pH was readjusted to the assay pH and transferase activity was measured with Gly-Phe-NH₂ under the standard assay conditions described in 2.4.1.



corresponding ammonium group and upon the pH of the solution. At pH values below 6, where the NH_2 terminal group of the substrate is largely protonated, the hydrolytic reaction is strongly favored. On the other hand, when the pH is above 6 and thus approaches the pK_a of the substrate (pH 7-8), then the predominant reaction is polymerization (Voynick and Fruton, 1968).

When cathepsin C acts on Gly-Phe- NH_2 in the presence of hydroxylamine at pH values near 7, four reactions proceed concurrently.

- i) The substrate is hydrolyzed to Gly-Phe and ammonia.
- ii) Hydroxamic acid is formed by a transamidation or transferase reaction.
- iii) The newly formed hydroxamic acid is subjected to enzymatic hydrolysis.
- iv) The polymer derived from Gly-Phe- NH_2 is formed (Jones et al., 1952).

An estimate may be made of the relative extent of hydrolysis (Gly-Phe- NH_2 and hydroxamic acid) and of transamidation (formation of hydroxamic acid and of the polymer) by analytical determination of the rate of liberation of carboxylate ions and ammonia (Jones et al., 1952).

It should be noted that the transferase assay used in this study will give only the amount of hydroxamic acid present during the assay time at different pH values. As noted earlier, the newly formed hydroxamic acid can also act as a substrate for cathepsin C. Therefore, this method will not reveal the extent of the formation of hydroxamic acid.

Although the hydrolysis of hydroxamic acid is not likely under the conditions in which assays were performed, none the less, the data on pH optima for a transferase assay should be interpreted with caution. The complicated interpretation of pH optima studies with this enzyme might be clarified in future work employing a parallel determination of carboxylate ions, polymerization products and ammonia in addition to hydroxamic acid.

The results obtained with the squid enzyme showing a pH optima of 7 and 6 for the transferase and hydrolase reactions, respectively, are consistent with other reports (Fruton and Mycek, 1956; McDonald et al., 1969a). Besides an optimum pH of 6.0 for hydrolase activity, partially purified squid cathepsin C also showed high activity at pH 4.0 for the same reaction. The possibility that the "affinity chromatography fraction" contains a contaminant active at this pH against Gly-Phe-NA and Ser-Tyr-NA was not ruled out in this study. However, a similar type of pH activity curve for hydrolase reaction by rat liver cathepsin C was reported by McDonald et al. (1969a).

3.4. Kinetic study

The apparent K_m and V_{max} of the squid cathepsin C (affinity chromatography fraction) were estimated by measuring the initial rates for the hydrolysis of either Gly-Phe-NA, Ser-Tyr-NA and transferase of Gly-Phe-NH₂ at different concentrations as described under 2.6.1 and 2.6.2. The apparent K_m and V_{max} were determined by the analysis of Lineweaver-Burk plots and by the least squares method of Johansen and Lumry (1961). The results obtained are summarized in Table 3-5. The apparent K_m and V_{max} estimated for the squid cathepsin C by the Lineweaver-Burk plot were similar to that obtained by the least squares method. Table 3-5 also show that the correlation coefficients between $1/S$ and $1/V$ for each substrate approach unity.

Table 3-6 summarizes the specific hydrolytic activities of squid cathepsin C on different synthetic substrates. It is apparent that Gly-Arg-MNA was hydrolyzed at a rate ten times faster than was Gly-Phe-NA. The rate of hydrolysis of Ser-Tyr-NA was twice that of Gly-Phe-NA.

General discussion: Kinetic study.

Most of the kinetic data available for cathepsin C are for the hydrolytic reaction and there are very few reports concerning the transferase reaction. The

Table 3-5: Squid cathepsin C hydrolysis of Gly-Phe-NA, Ser-Tyr-NA and transferase of Gly-Phe-NH₂

Substrate ¹	Range (mM)	K _m (mM) ²		V _{max} ³		r ⁴
		A	B	A	B	
Gly-Phe-NA	0.025-0.40	0.060	0.055	1593	1553	0.998
Ser-Tyr-NA	0.025-0.40	0.061	0.055	7692	7850	0.997
Gly-Phe-NH ₂	25-400	5.71	5.41	1112	1127	0.991

¹With Gly-Phe-NA or Ser-Tyr-NA, 5μg enzyme protein (affinity chromatography fraction) was used.

All assays were performed under standard assay conditions described in 2.4.1. and 2.4.2.

²"A" calculated from Lineweaver Burk plots; "B" calculated by least squares analysis and for this purpose a computer program was used.

³Nanomoles of product/mg of protein/min.

⁴Correlation coefficient for linear regression analysis of Lineweaver Burk plot.

Table 3-6: Specific hydrolytic activities of squid cathepsin C on different synthetic substrate¹

Substrate	Specific Activity (Units/mg)	Relative (%)
Gly-Phe-NA	492	10
Ser-Tyr-NA	1033	21
Gly-Arg-MNA	4920	100
Glutaryl-Phe-pNA ²	0	0
BANA	0	0

¹The "heated fraction" of the enzyme preparation (65 μ g protein/assay mixture) was used.

²All assays were performed at 30°C as described under 2.4.2., except for Glutaryl-Phe-pNA, change in absorbance was recorded at 410nm.

reported values available on the kinetic of the transferase reaction with Gly-Phe-OMe as substrate, and not with Gly-Phe-NH₂, the substrate which was used in this study.

The K_m values reported for rat liver cathepsin C on several dipeptide-NAs ranged from 0.1 to 0.2mM (McDonald, et al., 1969a). A K_m of 0.17mM was reported for the hydrolysis of Gly-Phe-NA by the rat enzyme at pH 6.0 and at 37°C, whereas, Hopsu-Havu and Rintola (1968) reported a K_m of 2.0mM for porcine kidney cathepsin C for the same substrate at pH 5.6 and at 30°C.

In this present study the hydrolysis of Gly-Phe-NA at pH 6.0 and a temperature 30°C showed a K_m value of 0.055mM which was lower than the values reported for rat liver and porcine kidney enzyme (McDonald et al., 1969a; Hopsu-Havu and Rintola, 1968). Although a K_m of 0.02mM was reported for the hydrolysis of Ser-Tyr-NA by bovine pituitary enzyme at pH 4.0 and 37°C (McDonald et al., 1968a), squid cathepsin C showed a higher K_m for this substrate at pH 6.0 and at 30°C. Squid cathepsin C shows a low rate for hydrolyzing Gly-Phe-NA as compared to Ser-Tyr-NA. Whereas the V_{max} values of Gly-Phe-NA and Ser-Tyr-NA are different, the K_m values are about the same.

Preliminary studies were also carried out with other substrates including Glutaryl-Phe-pNA, BANa and Gly-Arg-MNA. Of these, the heated fraction was inactive with all except Gly-Arg-MNA. The rate of hydrolysis of Gly-Arg-MNA by the squid enzyme was ten fold greater than that observed for Gly-Phe-NA.

Earlier studies by McDonald et al. (1969a) showed that Gly-Arg-NA is a better substrate for rat liver cathepsin C than was Gly-Phe-NA. Since Gly-Arg-MNA is now commercially available (Sigma Chem.Co., St.Louis, Mo), further studies of squid cathepsin C should employ this substrate.

3.5. Halide requirement

The effect of Cl^- ion on the transferase activity of the squid cathepsin C was investigated as described under 2.7.1, and results obtained are presented in Fig 3-9.

As illustrated, squid cathepsin C catalysis of the transferase reaction was stimulated by NaCl. Maximum stimulation was observed at about 20mM. A similar activation curve was obtained with KCl, indicating that the Cl^- ion rather than Na^+ is responsible for the activation.

The effect of Cl^- ions on the hydrolase activity of the squid cathepsin C was investigated as described under 2.7.2, and results obtained are presented in Fig 3-10. Squid cathepsin C showed very low activity in the absence of Cl^- ions. The enzyme exhibited an almost absolute requirement for Cl^- ions and responded to a small amount of this ion. It is also apparent from Fig 3.10, that only 2.5mM NaCl was required for half activation of the enzyme, but maximum activity was obtained at a concentration of 20mM.

General discussion: Halide activation.

In agreement with early reports on halide activation of bovine spleen and rat liver cathepsin C (McDonald et al., 1968a, 1969a; Tappel and Huang, 1972), squid cathepsin C also exhibited an apparent absolute requirement of Cl^- for both transferase and hydrolase activities. The Na^+ and K^+ salts of Cl^- were found to be equally effective in satisfying Cl^- requirement of the squid enzyme. The activation of both transferase and hydrolase activities were found to be almost instantaneous. It should be mentioned here that the dialysis of the enzyme preparation against Cl^- free dialysing medium (deionized water or 50mM phosphate buffer, pH 6.0) results in a decrease in enzyme activity (data not shown), but on addition of Cl^- ions, the enzyme activity was stimulated several fold. This may suggest that there is a reversible binding site for Cl^- ions in squid cathepsin C.

Fig 3-9: Effect of Cl^- ions on transferase activity of squid cathepsin C.

The "affinity chromatography fraction" (4ml) of squid cathepsin C was dialyzed against 6L of deionized water at 5° for 48 h. Each assay was performed in duplicate and $12\mu\text{g}$ enzyme protein was used. The conditions of assay were the same as in 2.4.1 except 2-mercaptoethylamine.HCl was replaced with 25mM ME to satisfy the SH requirement of the enzyme.

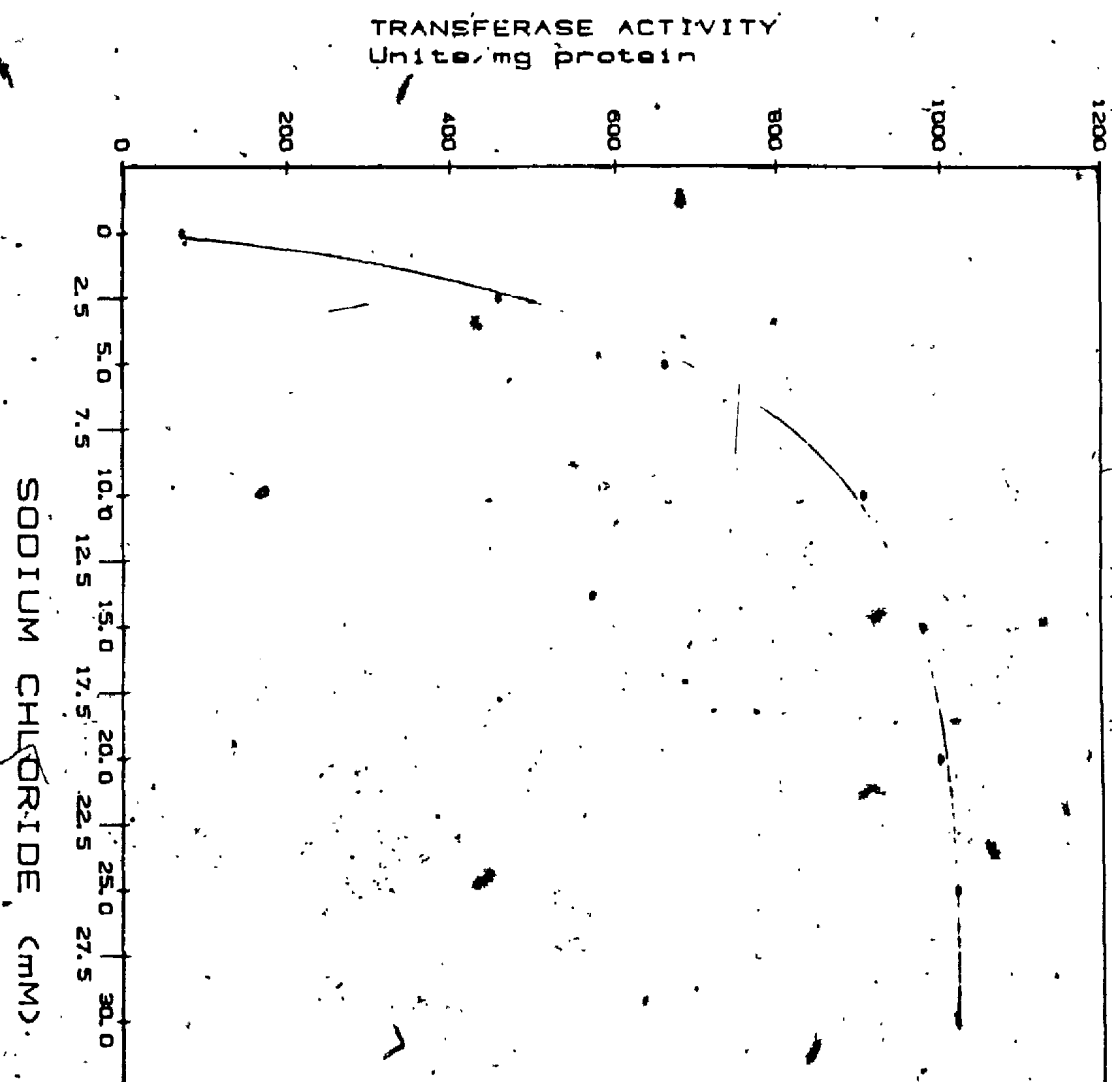
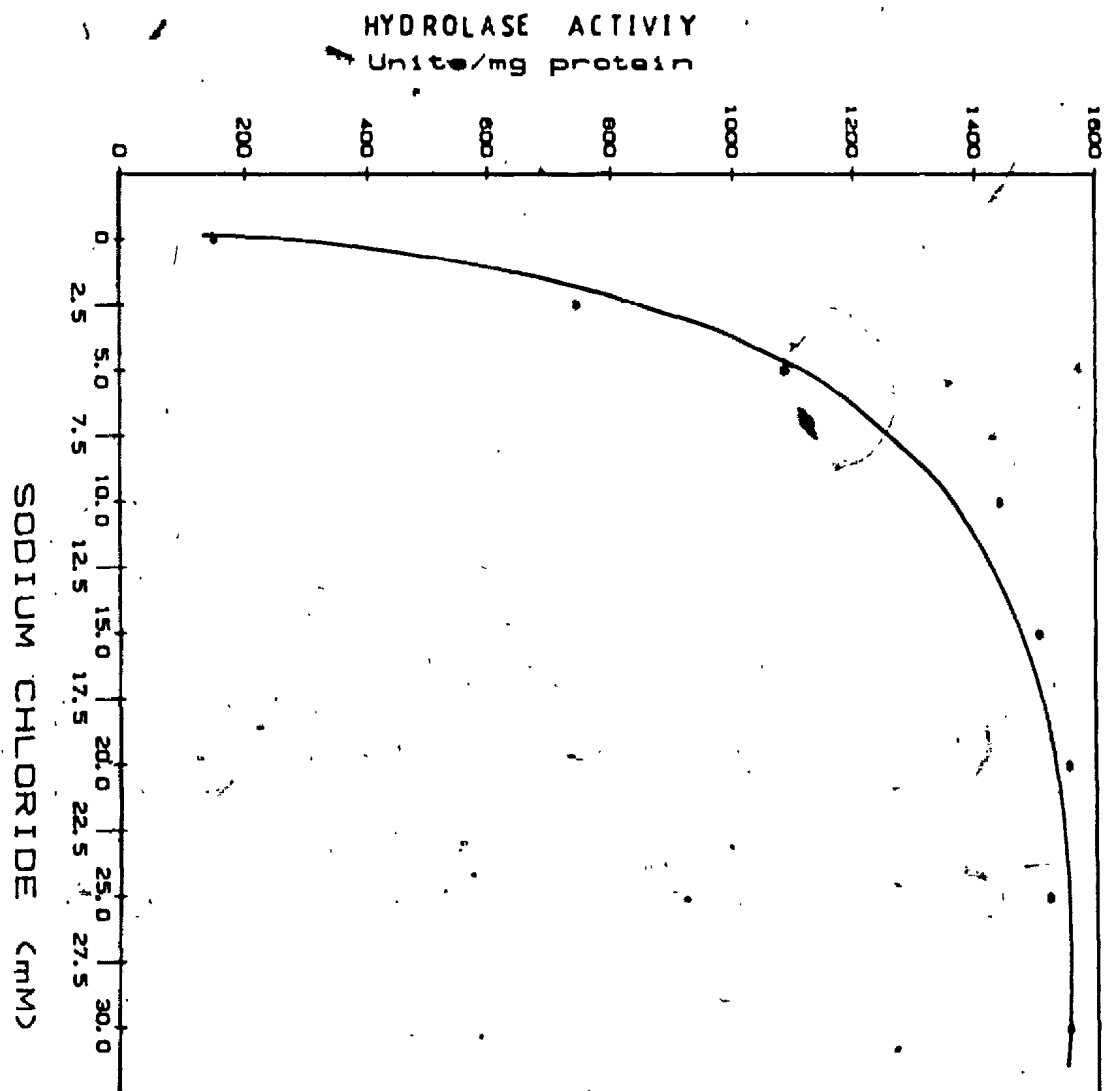


Fig 3-10: Effect of Cl^- ions on hydrolase activity of squid cathepsin C.

The "affinity chromatography fraction" (4ml) of squid cathepsin C was dialyzed against 6L of 50mM sodium phosphate buffer, pH 6.0 at 5°C for 48 h. Each assay was performed in duplicate and $5\mu\text{g}$ enzyme protein was used. The conditions of assay were the same as in 2.4.2 except 2-mercaptoethylamine.HCl was replaced with 25mM ME to satisfy the SH requirement of the enzyme.



The finding that the transferase and hydrolase activities of squid cathepsin C responded in the same way to chloride is further evidence that the two reactions can be attributed to the same enzyme and indicates that it is unlikely that the affinity chromatography fraction contains additional peptidases which act on Gly-Phe-NA.

3.6. Thiol requirement

The effect of thiol compounds on the transferase activity of the squid cathepsin C was determined as described under 2.8.1. The results obtained are shown in Fig 3-11 and Table 3-7. As shown in Fig 3-11, the enzyme showed very low activity in the absence of ME. Maximum activation of the enzyme was achieved with a concentration of 25mM ME.

The effect of ME on the hydrolase activity of the squid cathepsin C was determined as described under 2.8.2. The results obtained are shown in Fig 3-12, with enzyme exhibiting very low activity in the absence of ME. Maximum activation of hydrolase was achieved with a concentration of 20mM ME.

General discussion: Thiol requirement.

Cathepsin C is known to be a sulfhydryl containing enzyme. Compounds such as L-cysteine, ME, 2-mercaptoethylamine and DTE have been used as enzyme activators (Fruton and Mycek, 1956; Tappel and Huang, 1972).

The stimulation of transferase and hydrolase activity of squid cathepsin C by thiol compounds is consistent with these reports. Several other thiol compounds were also tested to determine their relative effects on transferase activity of squid enzyme (Table 3-7). The sulfhydryl dependency of squid cathepsin C coupled with inhibition by p-CMB, mercuric chloride and iodoacetate (section 3.7) suggests the presence of essential cysteine residue(s) in the enzyme. Among those compounds tested, L-cysteine was the most efficient activator. Why a concentration of 25mM sulfhydryl activator was required for maximum

Fig 3-11: Effect of ME on transferase activity of squid cathepsin C.

Each assay was performed in duplicate with 10 μ g enzyme protein ("affinity chromatography fraction"), method 2.8.1. The assay conditions were the same as in 2.4.1. except NaCl was included in the assay mixture at a final concentration of 25mM.

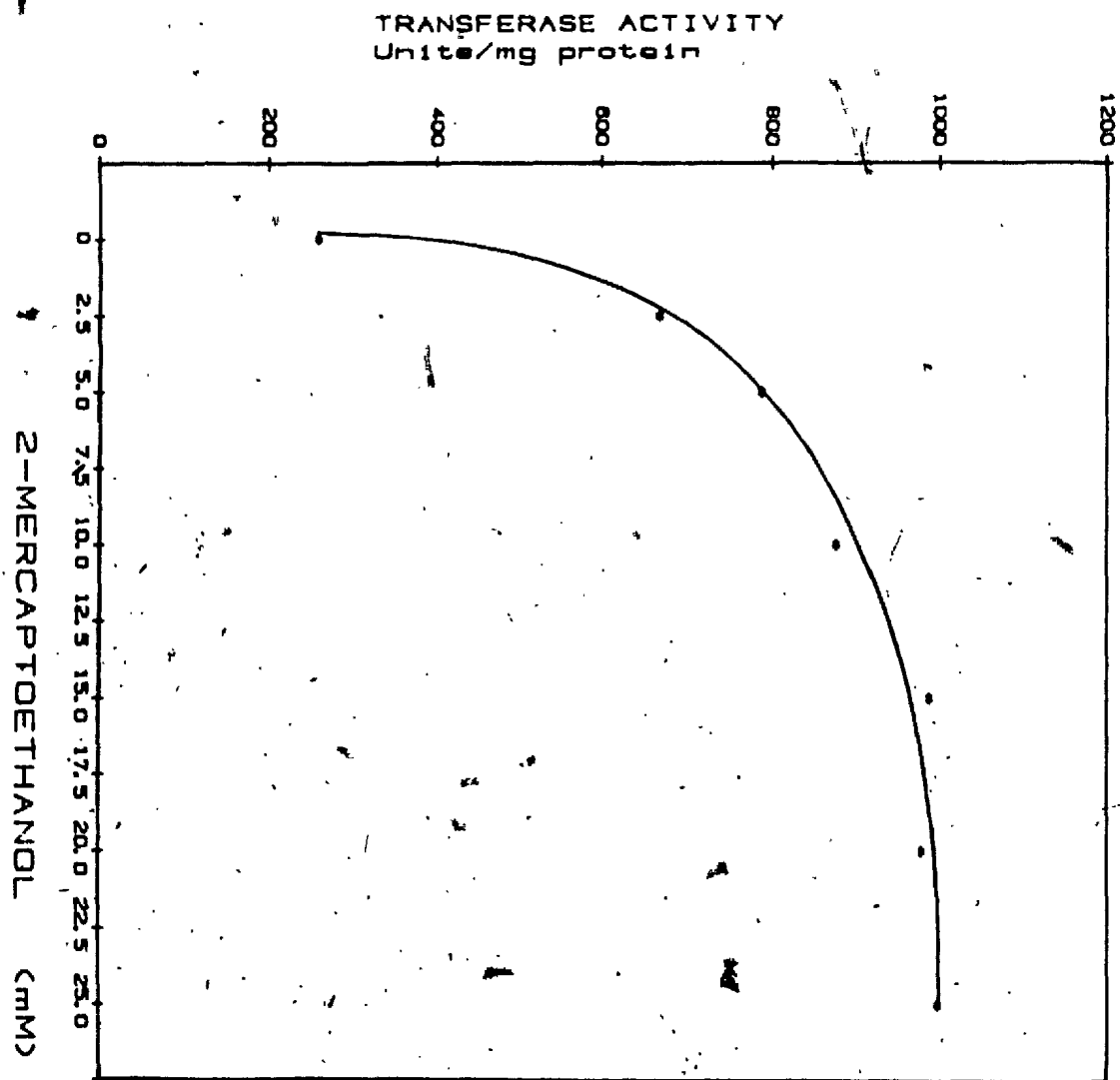


Table 3-7: Effect of thiol compounds on the transferase activity of squid cathepsin C

Activator ¹	Activity ² Units/mg protein
Control	252
ME	1010
2-Mercaptoethylamine.HCl ³	1112
DTE	1020
L-Cysteine	1313

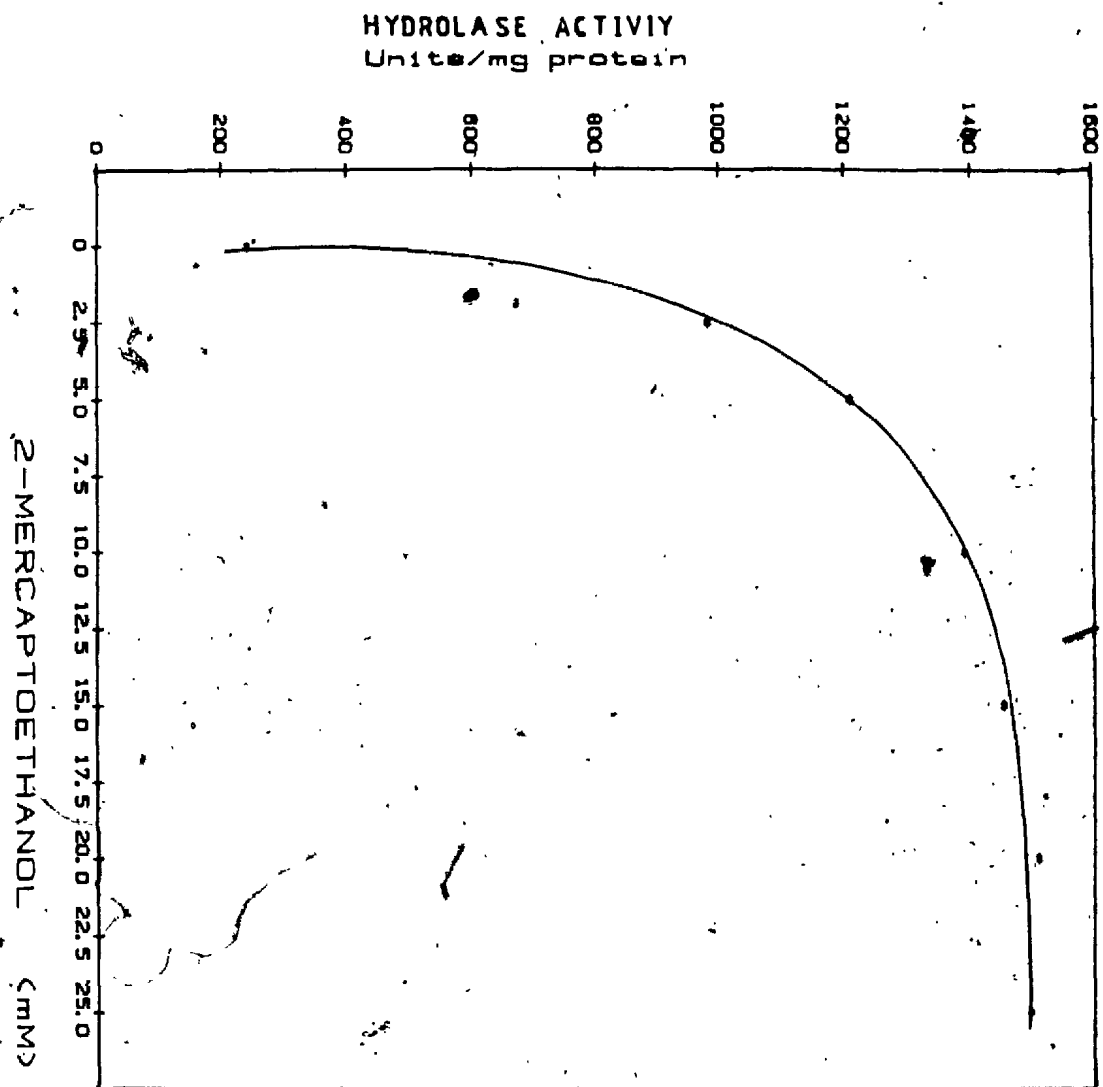
¹All activators were used at 25mM final concentration and were freshly prepared; methods 2.8.1.

²All assays were performed as under the conditions mentioned in 2.4.1.; average of two separate experiments.

³NaCl was omitted from reaction mixture.

Fig 3-12: Effect of ME on hydrolase activity of squid cathepsin C.

Each assay was performed in duplicate and $5\mu\text{g}$ enzyme protein ("affinity chromatography fraction") was used, method 2.8.2. The assay conditions were the same as in 2.4.2. except NaCl was included in the assay mixture at a final concentration of 20mM.



activation is unknown. Such a high concentration of sulfhydryl activator inside the cell would be difficult to maintain; but when the intracellular reducing environment is considered, the in vivo requirement for sulfhydryl activator may be quite different from the in vitro requirement. In vivo protection of sulfhydryl enzymes by reduced glutathione may be afforded through a thiol-disulfide exchange.

3.7. Inhibitor study

The effect of various inhibitors on squid cathepsin C activity was investigated as described under 2.9.1. The results obtained are reported in Table 3-8. The hydrolase activity was completely inhibited by 1mM mercuric chloride and iodoacetate, and only 8% activity remained in the presence of p-CMB. No inhibition was obtained with either EDTA, PMSF, pepstatin-A or puromycin.

General discussion: Inhibitor study.

The squid cathepsin C was not inhibited by EDTA which inhibits metalloproteases, or by PMSF which inhibits serine proteases, or by pepstatin, which inhibits carboxyl or acid proteases, e.g. pepsin and cathepsin D.

The very strong inhibition by iodoacetate and mercuric chloride suggests that the squid enzyme, like the bovine spleen and rat liver cathepsin C, is also inactivated by reagents which interact with sulfhydryl groups.

Puromycin is known to be an inhibitor of aminopeptidase (Ellis and Perry, 1967), and the possibility of a stepwise (aminopeptidase) hydrolysis of Gly-Phe-NA was considered less likely because puromycin did not effect the rate of formation of 2-naphthylamine by squid cathepsin C.

Table 3-8: Effect of inhibitors on hydrolase activity¹

Inhibitor	Final concentration (mM)	Hydrolase activity (% of original activity)
EDTA	4.0	107
Iodoacetate	1.0	0
PMSF	1.0	109
Mercuric chloride	0.1	0
p-CMB	1.0	8
Pepstatin A	1.0	103
Puromycin	1.0	102

¹The "affinity chromatography fraction" of the enzyme preparation was used. The enzyme (5 μ g protein) was added in assay buffer, containing various reagents in the final concentrations indicated, and was preincubated at 30°C for 30 min; method 2.9.1. After preincubation, the residual hydrolase activity was assayed according to the method described in 2.4.2.

3.8. Characterization of the subunit nature of cathpesin C and its relationship to catalytic activity

3.8.1. Heat treatment.

Table 3-9 summarizes the regeneration of transferase activity following heat treatment of the "40-70% AS fraction" at 65°C (for 40 min). Heat treatment caused a complete loss of transferase activity, and a stimulation of hydrolase activity. However, the transferase activity was partially regenerated after 48 h at 4°C.

The regeneration of transferase activity was greater when the "heat-treated fraction" sample was dialyzed against 154mM NaCl and 4mM ME. The regeneration of transferase activity was associated with a concomitant decrease in hydrolase activity (Table 3-9).

General discussion: Heat treatment.

Bovine spleen and rat liver cathpesin C are heat stable enzymes (McDonald et al., 1969a) and this property is exploited in their purification since most of the other cathpesins (A, B, D, E, and carboxypeptidase) are destroyed by heat treatment (McDonald et al., 1972a). When the "40-70% AS fraction" from squid hepatopancreas was heated at 65° C for 40 min, the transferase activity of the enzyme preparation completely disappeared and the hydrolase activity increased by 1.2 fold.

Dialysis of the "heat-treated fraction" against NaCl and ME resulted in optimal regeneration of transferase activity. Of the activities present prior to heat treatment, about 74% of transferase and 93% of hydrolase activities were recovered. The regeneration of transferase activity during dialysis of the "heat-treated fraction" may be due to removal of substances formed during heat treatment which are inhibitory to the transferase reaction, or the regeneration of activity may be related to reversible heat denaturation of a single polypeptide

Table 3-9: Regeneration of the transferase activity following heat treatment

Treatment	Time at 4 °C after heating (h)	Recovery of the enzyme activity (Total units)	
		Transferase	Hydrolase
None ²		10121	15686
65°C, 40 min	0	0	17568
65°C, 40 min	48	3369	12235
Dialysis after 65°C, 40 min			
Deionized water	48	6126	15372
1% NaCl, 4mM ME	48	7658	14588
1% NaCl	48	6841	16313
4mM ME	48	7045	16000

¹Transferase and hydrolase assays were performed as described under 2.4.1. and 2.4.2. respectively.

²Total activity present in the "40-70% AS fraction" before heat treatment.

chain or to association of subunits into a form capable of catalyzing transferase activity.

Since some regeneration of transferase occurred without dialysis it appears that the disappearance and re-appearance of transferase activity is at least partly due to reversible heat denaturation of cathepsin C.

3.8.2. Membrane permeability of squid cathepsin C

The results reported in Table 3-10 show the evidence for the membrane permeability of the "heated fraction" of squid cathepsin C preparation. It is apparent from Table 3-10 that a marked decrease in hydrolase activity was observed when XM100A or PM30 membrane was used. However, about 92-93% activity was retained by UM10 membrane.

General discussion: Membrane permeability of squid cathepsin C.

Ultrafiltration of the "heated fraction" of squid cathepsin C resulted in permeation of part of the hydrolase and transferase activities through various membranes. A greater percentage of hydrolase activity as compared to transferase activity was lost in the filtrate. This would indicate that smaller subunits are more active in catalyzing the hydrolytic reaction. These smaller subunits appear to reassociate to form tetramers and octomers, refer to section 3.8.3.

3.8.3. Gel permeation chromatography

The elution profile for protein and enzyme activity of the "heated fraction concentrate" of squid cathepsin C preparation on a Sephadex S-600 column is shown in Fig 3-13 and the specific activities obtained in different fractions are reported in Table 3-11. The transferase activities obtained in these fractions were associated with proteins corresponding to 200, 100, 50 and 25 kdaltons.

Table 3-10: Retention of squid cathepsin C with various membranes¹

Membrane	Recovery of the enzyme ² (Total units)	
	Transferase	Hydrolase
XM100A		
Retentate	3256	4372
Filtrate	744	3628
PM30		
Retentate	3535	5302
Filtrate	465	2698
UM10		
Retentate	3680	7440
Filtrate	0	0
None (Control) ³	4000	8000

¹ "Heated fraction" (25ml) was concentrated to about 3.5ml in an Amicon filtration assembly using, XM100 (cut-off level 100,000), PM30 (cut-off level 30,000) and UM10 (cut-off level 10,000) membranes, respectively.

It should be noted here that the cut-off level refers to the molecular weight (of a globular solute) at which the membrane exhibits 90% rejection.

² Transferase and hydrolase assays were performed under the conditions mentioned in 2.4.1. and 2.4.2. respectively.

³ Without filtration.

Fig 3-13: Gel filtration of "heated fraction concentrate" of squid cathepsin C preparation on a Sephacryl S-300 column.

The column (0.64 x 72cm) was preequilibrated with 100mM sodium acetate buffer containing 4mM ME, pH 4.5. Elution of protein was performed with the same buffer, and fraction of about 2.4ml were collected with a flow rate of 24ml/h. The standards eluted at the following volumes. Blue dextran (48.0ml), Catalase (76.8ml), Ovalbumin (98.4ml), Ribonuclease A (112.8ml). The assay of enzyme activity was performed with Gly-Phe-NH₂ as described under 2.4.1. Data shown are for one experiment and are representative of results obtained with three other experiments.

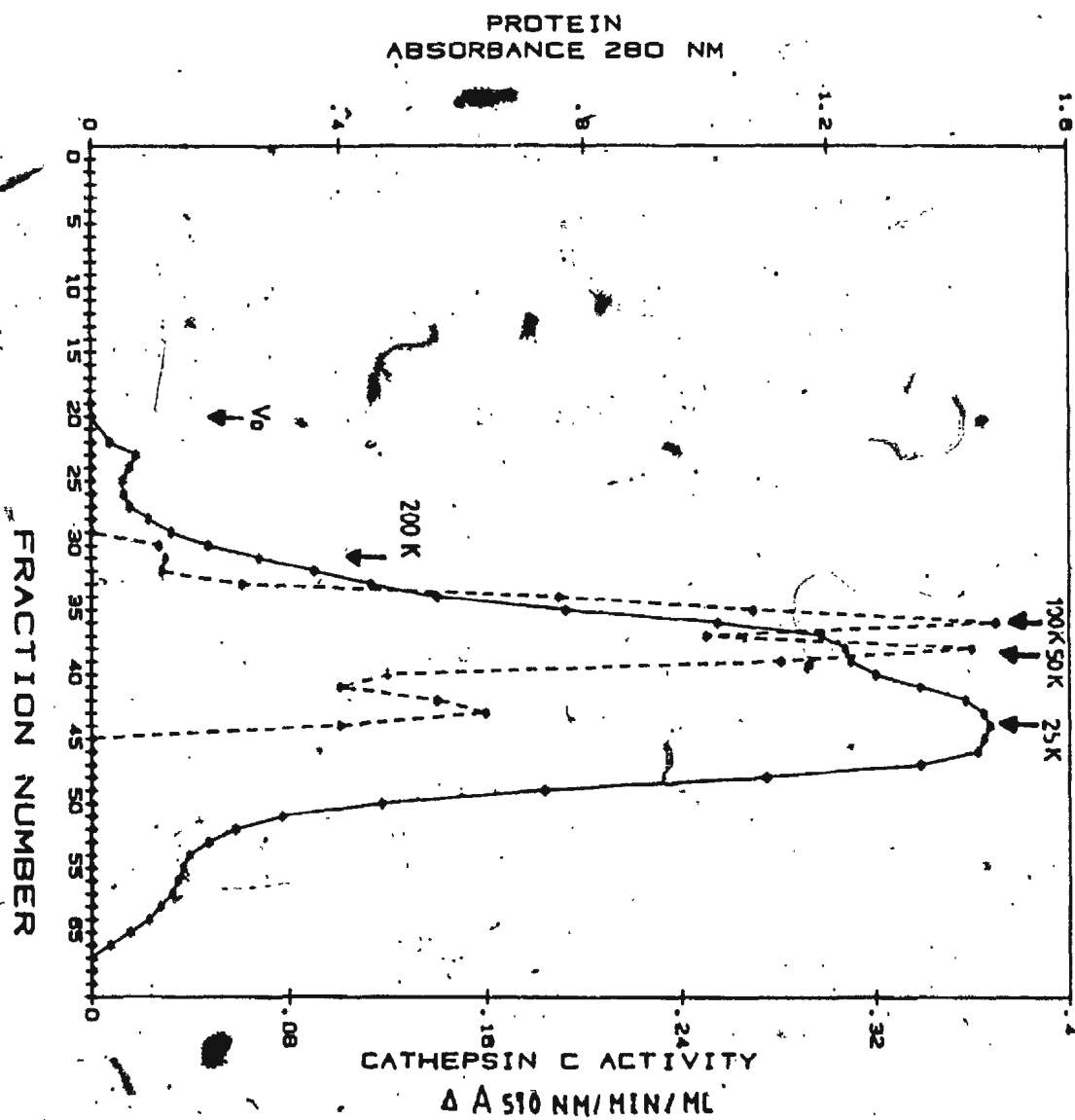


Table 3-11: Recovery of transferase activity in various fractions from Sephacryl S-300 chromatography ¹

Fraction Mol.weight (kdaltons)	Units/Fraction ²	Protein/Fraction (mg)	Specific Activity	%Total Units
200	52	1.2	43	5
100	365	0.4	913	31
50	480	3.1	155	41
25	265	6.4	42	23

¹The "heated fraction concentrate" was chromatographed as described in 2.3.8.

²Transferase activity was determined as described in 2.4.1.

Fig 3-14: Re-chromatography of the "S-50 fraction" on a Sephacryl S-300 column.

Fractions collected at elution an volume of the proteins corresponding to molecular weight a of 50 kdaltons from the first run were re-chromatographed under similar conditions mentioned in legend to Fig 3-13. Data shown are for one experiment and are representative of results obtained with two other experiments.

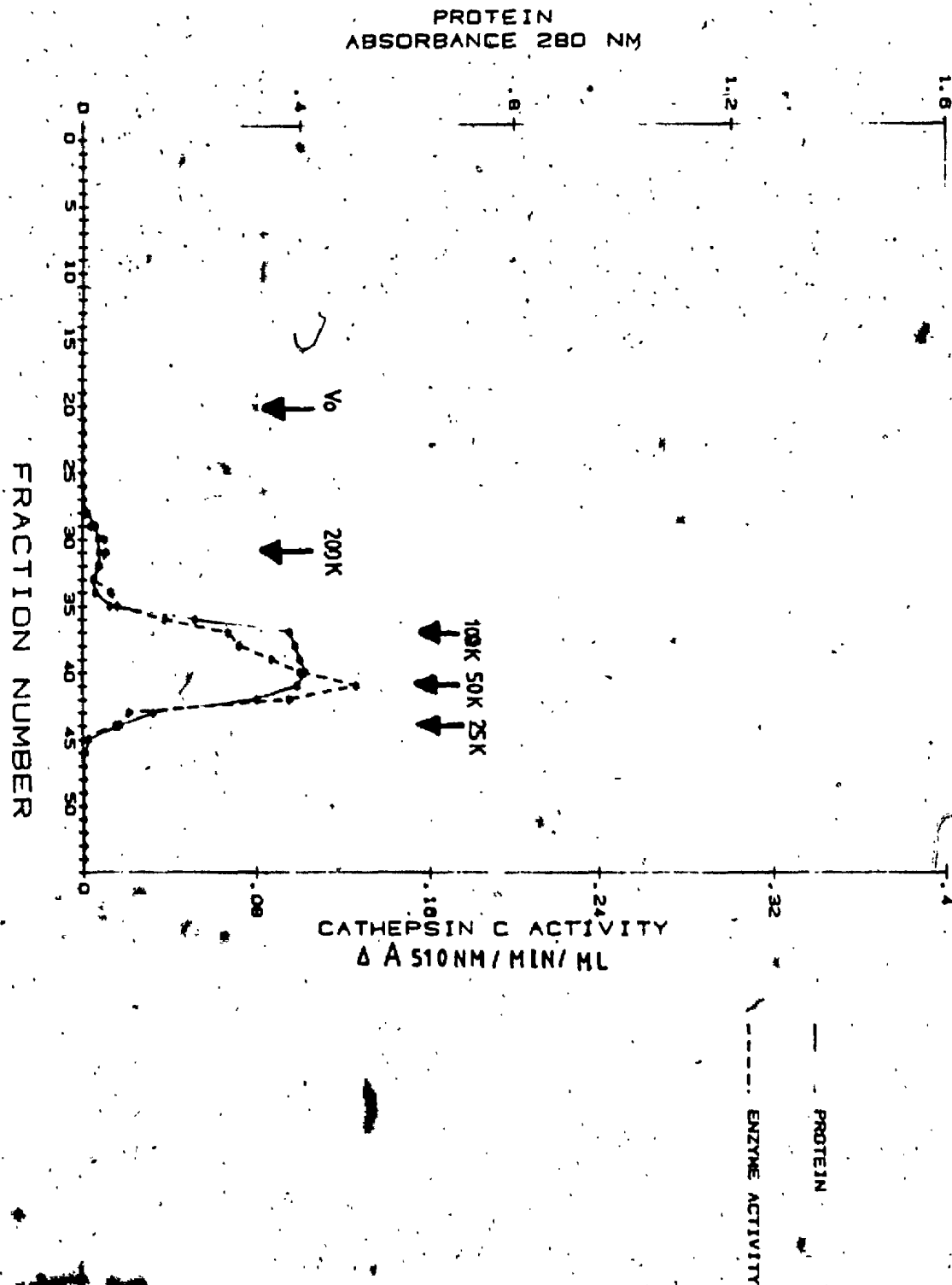


Table 3-12: Recovery of transferase activity in various fractions from Sephacryl S-300 chromatography ¹

Fraction Mol. weight (kdaltons)	Units/Fraction ²	Protein/Fraction (mg)	Specific Activity	% Total Units
200	21	0.2	105	5
100	138	0.3	460	31
50	242	1.2	201	55
25	40	0.6	66	9

¹The fraction corresponding to 50 kdaltons recovered from the first Sephacryl S-300 chromatography step was used.

²Transferase activity was determined as described in 2.4.1.

The elution profile for protein and enzyme activity on re-chromatography of the fraction corresponding to 50 kdaltons recovered from the first Sephacryl S-300 chromatography step is shown in Fig 3-14. The specific activities obtained in different fractions are reported in Table 3-12. It is apparent from Fig 3-14 and Table 3-12, that a redistribution of protein and enzyme activity between 25-200 kdaltons has occurred.

General discussion: Distribution of transferase activity by gel permeation chromatography.

The molecular weight of rat liver cathepsin C was determined by gel filtration and reported to be around 200 kdaltons (McDonald et al., 1969a). bovine spleen enzyme showed a molecular weight of 197,000 by ultracentrifugation analysis (Metrione et al., 1970).

In this study the molecular weight for squid cathepsin C was determined by gel permeation chromatography. The "heated-fraction" of the squid enzyme preparation gave four peaks of enzyme activity between 25-200 kdaltons. The finding that the transferase activity of squid extract is distributed in fractions corresponding to 200, 100, 50 and 25 kdaltons is consistent with its occurrence as a subunit enzyme in various states of dissociation. However, in this study bovine cathepsin C (unheated) gave a single peak of enzyme activity corresponding to 200 kdaltons.

If an equilibrium exists between the monomer and polymer form of squid cathepsin C, and if a substantial amount of monomer exists at all times, exclusion chromatography might reveal different size fractions of the enzyme. Any particular fraction of active material collected from this column should again give the original size distribution of active units. This experiment was conducted on a Sephacryl S-300 column using the "heated fraction" of the enzyme preparation. Four peaks of activity were eluted at volumes corresponding to the elution volumes of proteins of molecular weights 200, 100, 50 and 25 kdaltons.

Alternative interpretations of these data include:

i) That, the molecular weight of the enzyme is 25 kdaltons and under the conditions of gel permeation chromatography, this enzyme may be associated with various proteins and thus fractionate as a higher molecular weight species.

ii) That, under the conditions of gel permeation chromatography, the enzyme with a molecular weight of 200 kdaltons may be degraded to smaller active molecular weight species.

iii) That, there are several distinct enzymes with similar activities but of different molecular weights.

On reloading the 50 kdaltons protein fraction with cathepsin C activity, several peaks of activity corresponding to the molecular weights 25, 50, 100 and 200 kdaltons were obtained. The enzyme activity peaks were always distinctly associated with protein fractions that correspond to 25 kdaltons or a simple multiple thereof. This would argue against the alternate possibilities given above.

Upon re-chromatography of the 50 kdaltons peak on the same column, a redistribution of activity in all four fractions was observed. The data thus suggests that the 50 kdaltons fraction can associate to form tetramers and octomers, and dissociate to form monomers. Further studies employing other experimental techniques would be necessary to prove this.

The suggestion that the protein in the "heated fraction" is largely composed of a 25 kdaltons component is supported by SDS-gel electrophoresis (Fig 3-3). The other major component of this fraction had a molecular weight of 62,000 and would appear to be a contaminant of the cathepsin C preparation.

3.8.4. Effect of Triton X-100 treatment

The effect of Triton X-100 treatment on the "heated fraction" of squid cathepsin C preparation was investigated as described under 2.10.1 and the results are reported in Table 3-13. It is apparent from Table 3-13, that treatment with Triton X-100 resulted in about a 47% decrease in transferase activity and a 58% increase in hydrolase activity. It is also apparent that transferase/hydrolase ratio decreased from 0.54 to 0.19 as a result of detergent treatment.

The effect of Triton X-100 treatment on bovine spleen cathepsin C was studied as described under 2.10.2 and the results obtained are reported in Table 3-14 and 3-15. It is apparent from Table 3-14, that Triton X-100 treatment of bovine cathepsin C resulted in a decrease in transferase activity and a concomitant increase in hydrolase activity. This resulted in a change in the transferase/hydrolase ratio from 0.68 to 0.31.

As shown in Table 3-15, approximately 50% of the activity associated with bovine cathepsin C permeated through a XM50A membrane in the presence of 1% Triton X-100, whereas none of the activity permeated the membrane without Triton treatment. These results indicate that Triton X-100 treatment caused dissociation of the native protein into subunits of 50 kdaltons or less.

General discussion: Effect of Triton treatment.

Oligomeric enzymes are usually dissociated into subunits by treatment with denaturing agent such as extremes of pH, temperature, organic solvents, detergents and chaotropic agents such as guanidine hydrochloride and urea (Tanford, 1968, 1970). The forces involved in the association of the subunits are thus of the weak, non-covalent type, which are involved in the folded structure of a polypeptide chain; that is, hydrogen bonds, electrostatic forces, Van der Waals forces, and hydrophobic forces (Price and Stevens, 1982).

Negatively charged detergents such as SDS frequently cause dissociation of

Table 3-13: Effect of Triton X-100 treatment on
transferase and hydrolase activity of squid
cathepsin C¹

Samples	Transferase (Total units) ²	Hydrolase	Transferase/Hydrolase (Ratio)
Control	313	580	0.54
Triton X-100 treated(1%)	170	915	0.19

¹The "heated fraction" of the enzyme preparation was used, methods 2.10.1.

²All assays were performed as described in 2.4.1. and 2.4.2.; average of
two analyses. For experimental details refer to 2.10.1.

Table 3-14: Effect of Triton X-100 treatment on transferase and hydrolase activity of bovine cathepsin C¹

Samples	Transferase (Total units) ²	Hydrolase	Transferase/Hydrolase (Ratio)
Control	736	1083	0.68
Triton X-100 treated(1%)	544	1758	0.31

¹For experimental details refer to 2.10.2.

²All assays were performed as described in 2.4.1. and 2.4.2.; average of two analyses.

Table 3-15: Ultrafiltration of bovine cathepsin C before and after treatment with Triton X-100¹

Samples	Transferase (Total units) ²	Hydrolase	Transferase/Hydrolase (Ratio)
Control			
(without Triton)	736	1083	0.68
Retentate	640	964	0.66
Filtrate	0	0	0
Treated			
(1% Triton)	544	1758	0.31
Retentate	221	980	0.23
Filtrate	159	724	0.23

¹For experimental details refer to 2.10;2.

²All assays were performed as described in 2.4.1. and 2.4.2.; average of two analyses.

proteins into subunits and denaturation of the individual polypeptide chains. Positively charged detergents (e.g. cetyl trimethyl ammonium bromide) and neutral detergents (e.g. Triton X-100; iso-octylphenoxypolyethoxy ethanol) have also been used to disrupt proteins, and to solubilize proteins from membrane and other structural components.

The "heated fraction" of squid cathepsin C was used to study the influence of Triton X-100 on enzyme activity. The crude preparation was used in this study due to the non-availability of large quantities of purified preparation. The enzyme preparation was incubated with 1% Triton X-100 for 1 h at 0°C. Addition of Triton X-100 to the enzyme preparation reduced the transferase activity, whereas the hydrolase activity was increased. Three alternative explanations for this response are: i) Triton X-100 selectively inhibited transferase activity and activated the hydrolase activity of cathepsin C independent of subunit dissociation, or ii) Triton X-100 treatment resulted in the dissociation of the enzyme into subunits which are more active in catalyzing the hydrolytic reaction and less active in catalyzing the transferase reaction than the associated enzyme, iii) the transferase and the hydrolase activity of the squid enzyme preparation are catalyzed by different enzymes which respond differently to Triton X-100.

To distinguish between alternatives i, ii and iii a similar study was also carried out with pure bovine spleen cathepsin C. Interestingly, a similar effect of Triton X-100 treatment on the bovine enzyme was observed. This is consistent with an interpretation such as i or ii, which assumes that only one enzyme is being effected by Triton X-100. Moreover, part of the Triton X-100 treated bovine cathepsin C penetrated a XM50A membrane indicating that Triton X-100 treatment resulted in a partial dissociation of the enzyme. This observation supports the explanation ii, although the experimental design is such that it is not possible to conclude that Triton X-100 inhibition of transferase activity and activation of hydrolase activity is a direct result of subunit dissociation.

3.8.5. Effect of urea treatment

The effect of urea treatment on the "heated fraction" of squid cathepsin C was investigated as described under 2.11.1 and results are reported in Table 3-16. It is apparent from Table 3-16, that pretreatment of the enzyme with 2M urea resulted in a decrease in both transferase and hydrolase activities. The loss of transferase activity was greater than that of hydrolase and this resulted in a decreased transferase/hydrolase ratio. The ratio of activities after urea treatment was similar to that observed for the detergent-treated enzyme. It was also observed that 2M urea caused a decrease in the size of the fraction containing enzyme activity, as shown by the recovery of all the hydrolase activity in the filtrate of an XM50A membrane. The filtrate, containing dissociated cathepsin C, had a much lower transferase/hydrolase ratio than did the retentate.

The effect of urea treatment on bovine spleen cathepsin C was studied as described under 2.11.2 and the results obtained are reported in Table 3-17. Urea treatment of bovine cathepsin C resulted in similar decreases in transferase and hydrolase activities as was observed for the squid enzyme. It is also apparent from Table 3-17, that about a 50% decrease in the transferase/hydrolase ratio was observed. As shown in Table 3-17, ultrafiltration of the urea treated sample through a XM50A membrane resulted in recovery of most of the hydrolase activity in the filtrate and about two thirds of the transferase activity in the retentate.

General discussion: Effect of urea treatment.

Urea has been used extensively as a denaturing agent for proteins and it is known that high concentrations of the reagent cause unfolding of proteins. Proteins of multiple subunits are likely to be separated into their constituent polypeptide chains (Tanford, 1968, 1970).

It has been suggested that the polymerase activity of cathepsin C may

Table 3-16: Effect of urea treatment on squid cathepsin C¹

Samples	Transferase (Total units) ²	Hydrolase	Transferase/Hydrolase (Ratio)
Control	337	516	0.65
Urea treated	112	375	0.29
<u>Ultrafiltration</u>			
Urea treated filtrate	31	1093	0.03
Urea treated retentate	47	0	∞

¹For experimental details refer to 2.11.1.

²All assays were performed as described in 2.4.1. and 2.4.2.; average of two analyses.

Table 3-17: Effect of urea treatment on bovine cathepsin C¹

Samples	Transferase (Total units) ²	Hydrolase	Transferase/Hydrolase (Ratio)
Control	736	1083	0.68
Urea treated	267	785	0.34
<u>Ultrafiltration</u>			
Urea treated filtrate	121	2295	0.05
Urea treated retentate	221	0	∞

¹For experimental details refer to 2.11.2.

²All assays were performed as described in 2.4.1. and 2.4.2.; average of two analyses.

involve the cooperative interaction of catalytic centers on adjacent subunits of the native enzyme (Wurz et al., 1962; Mettrione et al., 1966). Heinrich and Fruton (1968) reported that the rate of hydrolysis of Ala-Ala-NH₂ by bovine spleen cathepsin C was increased, when 2M urea was present in the reaction mixture.

On the other hand the percentage of reacted substrate which was polymerized was decreased. Therefore, these results led the authors to suggest that an individual subunit may be responsible for catalyzing the hydrolysis of substrate while a cooperative action of more than one subunit may be required for polymerase activity.

Previous studies demonstrated that in the presence of 2M urea, bovine spleen cathepsin C dissociates rapidly into a form having a sedimentation coefficient of 5.6 S corresponding to a molecular weight of about 100,000 (Mettrione et al., 1970). However, the data on the relative formation of the products of hydrolysis and polymerization under that condition were not given.

In the present study experiments were performed to analyse the effect of 2M urea on the ultrafiltration behavior of cathepsin C. The data obtained for squid cathepsin C are generally consistent with those which were obtained for bovine enzyme.

A possible interpretation of experimental results obtained by this study is as follows:

Treatment of the enzyme with urea resulted in dissociation of an octomer into tetramers and this was accompanied by some loss of enzyme activity. Ultrafiltration of the enzyme in the presence of 2M urea at 30 psi resulted in the further dissociation of tetramers to dimers and monomers. The dissociated forms of the enzyme were more active in hydrolytic reactions, and therefore a large decrease in transferase/hydrolase ratio was observed in the filtrate. On the other hand, the retentate obtained after urea treatment showed an increase in the transferase/hydrolase ratio suggesting that this represents undissociated enzyme.

Metrione et al. (1970) studied the dissociation of bovine spleen cathepsin C. In the presence of a low concentration of guanidinium chloride, the enzyme rapidly dissociated into tetramers of molecular weight near 100 kdaltons. Further dissociation occurred with time or with a higher concentration of guanidinium chloride, suggesting that the binding which holds the tetramer together is relatively strong. A similar concentration-dependent dissociation of bovine cathepsin C was observed when the enzyme was treated with different concentrations of urea (Metrione et al., 1970).

3.8.6. Interpretation of studies relating subunit association and dissociation to hydrolase and transferase reactions

The studies described in section 3.8.1-3.8.5 indicate that heat treatment, detergent and urea each cause dissociation of cathepsin C and a corresponding decrease in the ratio of rates of the transferase and hydrolase reactions with the substrate and reaction conditions employed. It is possible that the observed results are a reflection of the particular reaction conditions chosen for this study and further studies employing other substrates and reaction conditions will be necessary to determine whether the hydrolytic and transferase reactions are generally affected by dissociation of the enzyme.

Chapter 4

CONCLUSION AND SUGGESTIONS

The present study clearly demonstrates the existence of a dipeptidyl aminopeptidase I (cathepsin C) in the hepatopancreas of squid, and the similarity of the enzyme from squid to that from mammalian sources. The identification of cathepsin C is based on : (1) its ability to catalyze a transferase reaction with Gly-Phe-NH₂ and a hydrolase reaction with Gly-Phe-NA or Ser-Tyr-NA at pH optima of 7 and 6, respectively; (2) the Cl⁻ ion and sulphhydryl activation of the enzyme; (3) its inhibition by known inhibitors of cathepsin C; and (4) the apparent existence of a 25 kdaltons subunit which forms associated complexes of 50, 100 and 200 kdaltons.

The behavior of squid cathepsin C and bovine cathepsin C on gel filtration columns differed. Whereas bovine spleen cathepsin C gave a single peak for protein corresponding to the elution peak of a protein of 200 kdaltons and transferase activity, multiple peaks for enzyme activity were obtained in the range of 200 to 25 kdaltons, when a "heat-treated" preparation of squid cathepsin C was chromatographed. It is possible that heat treatment caused dissociation of the squid enzyme.

Various experimental approaches can be used to follow the dissociation and reassociation behavior of an oligomeric subunit protein. These include hydrodynamic methods such as viscosity and sedimentation coefficient determination; spectroscopic methods (absorption and fluorescence); circular dichroism; optical rotation and NMR (Jaenicke, 1979).

In the present study the dissociation and reassociation of cathepsin C in relation to its catalytic function as a transferase and hydrolase were studied by the ultrafiltration behavior of the enzyme following heat treatment or exposure to detergent or urea. Although the penetration of enzyme through a XM50A membrane is good indicator of dissociation of the protein into subunits, it is possible that reassociation occurred during assay, and this poses limitations on the interpretation of the results obtained in this study.

It will be necessary to obtain more definitive experimental data on the kinetics of dissociation and reassociation of the pure enzyme, in order to verify the conclusion that there is a relationship between the structure and function of the enzyme.

Other experimental approaches can be taken to resolve this enigma. For example, immobilization of cathepsin C subunits to an insoluble matrix can be used to study the relationship between the enzyme activity and states of dissociation and reassociation.

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