AN ABSTRACT OF THE THESIS OF

CHAO-YUN TING (Name) for the M.S. (Degree) in FOOD SCIENCE (Major)

Date thesis is presented March 15, 1965

Title PARTIAL PURIFICATION OF CATHEPSINS FROM SALMON MUSCLE

Abstract approved _____ (Major professor)

Cathepsins are intracellular proteinases that hydrolyze the peptide bonds of proteins. These enzyme have been implicated in the tenderization of aging beef, with the deterioration of radiation-stabilized meats on storage, and in the spoilage of fish prior to processing. Hence, the cathepsins of edible muscles are of concern to the food scientist.

The purpose of the research reported herein was to develop procedures for the purification of the cathepsin from salmon muscle. The availability of a purified preparation of salmon muscle cathepsins should stimulate interest and research in the characterization of these enzymes and lead to better means for the control of catheptic activity in fish muscle.

Results from these investigations indicate that salmon muscle cathepsins exhibit pH optima at 3.7, 6.9, and 8.5 when Folin's

reagent was used to determine the products of protein hydrolysis; whereas, pH optima at 3.7 and 7.3 were obtained when the products of protein hydrolysis were determined by absorption at 280 mμ. Possible reasons for the differences in pH optima are discussed. It was decided to attempt the purification of the cathepsin optimally active at pH 3.7. The stability of this enzyme was found to be maximal at pH 6.5.

The purification was accomplished by extracting the salmon muscle cathepsin with two parts 0.2 N KCl. The pH of this crude extract was adjusted to 5.5 and the precipitated proteins were removed by centrifugation before the pH of the supernatant was readjusted to the original pH of the extract. Upon dialysis of this fraction against 0.005 M phosphate (pH 6.5) a precipitate formed and was removed by centrifugation. The catheptic activity was precipitated from the supernatant at 0.50 saturation with $(NH_4)_2SO_4$. The precipitate was recovered by centrifugation, dissolved in 0.005 M phosphate (pH 6.5), and dialyzed against the same buffer. A precipitate formed and was removed by centrifugation. This fraction was placed on a 2.5 x 35 cm column of DEAE-cellulose equilibrated with the starting buffer (0.005 M phosphate at pH 6.5). A concave concentration gradient was used to elute the proteins from the ion-exchange resin. Final buffer was 0.005 M phosphate (pH 6.5) containing 0.5 N NaCl. The absorbance (280 mμ) of the column effluent

was continuously recorded and 10 ml fractions of the effluent were collected. Fractions comprising the various protein peaks were combined and concentrated by lyophilization. Two catheptic enzymes appeared to be separated by this procedure with maximum purification of 117 fold with 6.8 per cent recovery.

During the development of the procedure, it was found that the fractions obtained from column chromatography could not be successfully concentrated by ultrafiltration, pervaporation, or "Aquacide #2" and that the presence of cysteine in the eluting buffer was not beneficial.

PARTIAL PURIFICATION OF CATHEPSINS FROM SALMON MUSCLE

by

CHAO-YUN TING

A THESIS

submitted to

OREGON STATE UNIVERSITY

in partial fulfillment of
the requirements for the
degree of

MASTER OF SCIENCE

June 1965

APPROVED:

Assistant Professor of Food Science and Technology
In Charge of Major

Head of Department of Food Science and Technology

Dean of Graduate School

Date thesis is presented March 15, 1965

Typed by Luanne Bayless

ACKNOWLEDGEMENT

The author wishes to express sincere thanks to Dr. Morris W. Montgomery for suggesting the subject of this thesis and for his understanding, encouragement and guidance in the course of the work as well as during the preparation of the manuscript.

Gratitude is also expressed to Dr. Allen F. Anglemier for his help and suggestions for the manuscript.

TABLE OF CONTENTS

INTRODUCTION 1

LITERATURE REVIEW 3
Location of Cathepsins 3
Classification of Cathepsins 4
Catheptic Enzymes in Muscle 7
Purification of Cathepsins 11

EXPERIMENTAL AND RESULTS 14
General Methods 14
Determination of pH Optimum 17
Extraction of Cathepsin from Salmon Muscle 18
pH Stability of Salmon Muscle Cathepsin 20
Acid Treatment 23
Dialysis 24
Ammonium Sulfate Fractionation 24
Column Chromatography 26
Over-all Purification Procedure 41

DISCUSSION 42

SUMMARY 45

BIBLIOGRAPHY 47

LIST OF FIGURES

Figure

1 pH activity curve for salmon muscle cathepsins..... 19

2 Chromatography of fraction C..... 32

3 Chromatography of fraction D (trial 1)..... 34

4 Chromatography of fraction D (trial 2)..... 37

5 Chromatography of fraction D (trial 3)..... 39

LIST OF TABLES

Table

1	Extraction of salmon muscle cathepsin with various solutions	21
2	Effect of KCl concentration on extraction of salmon muscle cathepsin	21
3	Stability of cathepsin at various pH values	22
4	Effect of acid treatment on catheptic activity	23
5	Effect of dialysis of fraction B on catheptic activity	24
6	Ammonium sulfate precipitation of catheptic activity from fraction C	26
7	Chromatography of fraction D (trial 1)	35
8	Chromatography of fraction D (trial 2)	38
9	Chromatography of fraction D (trial 3)	40

PARTIAL PURIFICATION OF CATHEPSINS FROM SALMON MUSCLE

INTRODUCTION

Cathepsins are intracellular proteinases that hydrolyze the peptide bonds of proteins. This is not the only reaction that is catalyzed by these enzymes. Several of the cathepsins have been shown to hydrolyze simple peptides and amides and esters of amino acids and peptides. One of the cathepsins (cathepsin C) has been reported to catalyze transpeptidation reactions involving small peptides leading to the formation of polypeptides (Fruton and Mycek, 1956). This has caused speculation that the cathepsins are involved in protein synthesis within cells; however, the physiological role of the cathepsins is not understood at this time.

Catheptic enzymes are important in several areas of food science. These enzymes have been implicated in the tenderization of aging beef; however, the exact nature of the changes brought about by these enzymes in beef is not well defined. Catheptic activity has been correlated with the deterioration of radiation-stabilized meats on storage and the application of heat is the only known practical method for control of the proteolytic activity in irradiated meat products (Chiambalero, Johnson, and Drake, 1959). The cathepsins of fish muscle have been reported to play a role in the spoilage of fish prior to processing (Siebert, 1962). Heat treatment (300 sec at 150°F or 15 sec at 160°F) is required prior to irradiation for

inactivation of the enzymes that cause release of amino nitrogen in cod muscle (Sinnhuber and Landers, 1964).

The purpose of the investigation reported in this thesis was to purify the catheptic enzyme from salmon muscle. It is anticipated that a purified enzyme preparation will lead to investigations on the basic characteristics and function of the enzyme and, possibly, to provide better means for the control of the catheptic activity of fish muscle.

LITERATURE REVIEW

The bulk of the work published on the catheptic enzymes of tissues stems from investigations of these enzymes in spleen, kidney, and liver; whereas, the catheptic enzymes of muscle have received comparatively little attention. This situation is probably due to the relatively low activity levels found in muscle as compared to organs. Zender et al. (1958) reported that the proteolytic activity of psoas muscle cathepsin was only 1/40 that of the lung. In fish muscle the situation appears to be more favorable, since Siebert (1958) reported that the catheptic activity of fish muscle was about ten-fold greater than the activity of mammalian muscle.

Location of Cathepsins

de Duve (1959) found that several hydrolytic enzymes, including the cathepsins, were associated with a subcellular particle, which he called the lysosome. The investigations of Bouma and Gruber (1964) on the intracellular distribution of enzymes in rat tissues indicated cathepsins B, C, and D are located in the lysosomes. Studies on the distribution of cathepsins D and E in different types of rabbit leucocytes have shown that cathepsin D is present in polymorphonuclear cells, macrophages, and lymphocytes (Lapresle and Webb, 1962; Stefanoric, Webb and Lapresle, 1962).

Cathepsin E is present in larger amounts in polymorphonuclear cells, in smaller amounts in macrophages and only traces in lymphocytes. Terlizzi and Mitolo (1955) observed that in the brain of the cow, horse, or pig the amount of cathepsin is more abundant in the gray matter than in the white.

Classification of Cathepsins

Hartley (1960) proposes that proteolytic enzymes might be classified into four groups based on the enzymatic properties. These groups are serine, thiol, acid, and metal proteinases. However, this author indicates that most catheptic preparations are not of sufficient purity and their properties are not sufficiently defined to be placed into this classification.

Fruton and co-workers described the properties of cathepsins A, B, and C which have been obtained from both spleen and kidney (Tallan, Jones and Fruton, 1952; Fruton and Mycek, 1956; Greenbaum and Fruton, 1957; Fruton, 1960; Lichtenstein and Fruton, 1960; Stein and Fruton, 1960). These enzymes are distinguished from one another by their substrate specificities, optimun pH values, and activation and inhibition characteristics.

Cathepsin A

This enzyme is characterized by its optimal action on

carbobenzoxy-L-glutamyl-L-tyrosine (a substrate for pepsin) near pH 5.7, and the absence of a requirement for activation by cysteine or of inhibition by iodoacetic acid (Lichtenstein and Fruton, 1960).

Cathepsin B

The cathepsin which exhibits trypsin-like activity has been designated cathepsin B by Greenbaum and Fruton (1957). This enzyme acts optimally between pH 4.2 and 5.8 toward benzoyl-L-argininamide. The specificity of cathepsin B appears to be primarily directed toward the hydrolysis of amide (or ester) bonds involving α-N-acylated-L-arginine or L-lysine. Cathepsin B is activated by sulfydryl compounds and inhibited by reagents that combine with SH-groups. The rate of enzyme action increases with increasing cysteine concentration.

Cathepsin C

Cathepsin C has been shown to be specific for the catalysis of the hydrolysis at the terminal CO group in the compound $NH_2(CHR)CO\text{-}NH(CHR')CO\text{-}X$, where X is NH_2 (as in an amide) or OC_2H_5 (as in an ethyl ester), of which the glycyl -L-tyrosine amide or ethyl ester is a typical substrate (Fruton and Mycek, 1956). Under certain conditions (pH 7 to 8) cathepsin C catalyzes the

polymerization of these amide or ester substrates leading to the formation of insoluble polypeptides. For this reason, the determination of the pH-dependence curve of hydrolysis by cathepsin C has been limited to pH values below 6.5, and studies on the specificity of the enzyme are conducted near pH 5, where the extent of transamidation is negligible.

Cathepsin D

Press, Porter and Cebra (1960) reported that the optimum pH for cathepsin D is at pH 3.0 with hemoglobin as substrate and at pH 4.2 with serum albumin as substrate. The hydrolysis of serum albumin is only about one-fourth of that of hemoglobin. This proteinase does not hydrolyse the synthetic substrates described by Fruton for cathepsin A, B, and C (Tallan, Jones and Fruton, 1952). Cathepsin D is heat-labile, all activity being lost after heating at neutral pH to 60°C for 40 min, while cathepsin C is stable under these conditions. Cathepsin D has been found in beef, rabbit, and human spleen (Press, Porter and Cebra, 1960; Lapresle and Webb, 1962; McMaster and Webb, 1963). In beef spleen there are ten different molecular forms of cathepsin D, which have been separated by chromatography on carboxymethyl-cellulose (CM-cellulose) and starch-gel electrophoresis. No difference in specificity could be detected between the various forms of this enzyme. The

proteolytic activity of cathepsin D in beef and rabbit spleen is not affected by cysteine, iodoacetamide, p-chloromercuribenzoate, ethylenediaminetetra-acetate or diisopropyl phosphofluoridate. Since human spleen cathepsin D is activated by cysteine, it is different from rabbit and beef spleen cathepsin D.

Cathepsin E

Cathepsin E, extracted from rabbit bone marrow, degrades human serum albumin in the pH range 1.5 to 4.5 with the maximum activity at pH 2.5 (Lapresle and Webb, 1962). The presence of cysteine does not affect the activity of cathepsin E at pH 2.5, however, there is a slight increase in the pH range 3.5 to 4.5. The synthetic substrates for cathepsins A, B, and C are not hydrolysed by cathepsin E. This enzyme is completely inactivated after heating at 80°C for 10 min and is not inhibited by iodoacetate or diisopropyl phosphofluoridate. Cathepsin D and E differ greatly in their electrophoretic mobility at pH 8.2, which facilitates their separation.

Catheptic Enzymes in Muscle

Snoke and Neurath (1950) have shown that the optimum pH of the proteinase from striated rabbit muscle is 4.0. Maximum activation was obtained at low concentration of Fe^{++} (0.02 M) and

cysteine produces no change in the activity when added to the extract. At least two proteolytic enzymes are present in chicken breast muscle (Bandack-Yuri and Rose, 1961). One fraction showed maximum activity at pH 4 (on hemoglobin), the other at pH 7 (on casein). No report was made on the effect of cysteine or metal ions on the activity of these enzymes.

Proteolytic enzymes in rat skeletal muscle have been reported to attack the endogenous muscle proteins optimally at pH 3.5 to 4.0 and 8.5 to 9.0 (Koszalka and Miller, 1960a). The latter enzyme has been purified by Koszalka and Miller (1960b) and is able to hydrolyze serum albumin, casein, and hemoglobin. The cathepsin is activated by cysteine and glutathione and inhibited by p-chloromercuribenzoate but not by iodoacetate. At concentrations of 0.001 M or greater, the proteolytic reaction is inhibited by the presence of Mg^{++}, Mn^{++}, Ca^{++}, Zn^{++} and is enhanced by Fe^{++}.

Investigations on the proteolytic activity of beef muscle extracts, in which the extracts serve both as enzyme and substrate, have indicated that three pH optima exist at 4 to 5, 8 to 9 and 10 (Sliwinski, Doty, and Landmann, 1959; Sliwinski, et al., 1961). Activities with synthetic substrates indicated that these enzymes are similar to, but not identical with, cathepsin B and C, and do not require the presence of cysteine, which is necessary for full activity of cathepsin B. The activity of the beef muscle enzyme against

hemoglobin was found to be much higher than that against synthetic substrates.

Siebert (1962) reported that the attack of fish muscle cathepsins on proteins of the fish muscle is regarded as one of the factors which cause the rapid spoilage of fish after death. This investigator found that fish muscle cathepsins are able to split 10 mg of protein per g of fresh muscle tissue which is approximately ten times the rate found in mammalian muscle. These cathepsins are of true muscular origin and occur in an active state. Siebert (1962) was able to purify a cathepsin from cod muscle about 90 fold and studied some of the properties of the enzyme. The pH optimum is near 4.3. This cathepsin has about the same resistance against heat inactivation as mammalian enzymes and is inhibited by 10^{-5} M Co^{++}, but only slightly by 10^{-3} M Hg^{++}. No activation was found by any metal ion; however, cyanide does activate, and several sulfhydryl reagents are inhibitory (p-chloromercuribenzoate, iodobenzoate, iodoacetamide). None of the substrates known to be hydrolyzed by mammaliam cathepsins A, B, and C are split by cod muscle cathepsin.

The experiments of Groninger (1964) showed a proteinase to be present in the muscle of albacore. The enzyme exhibits a pH optimum at 2.4 to 2.5 with very little activity below pH 1.5 or above 4. Hemoglobin gave the greatest activity of all the substrates tested. There was much less activity against casein and

the proteinase was inactive against bovine serum albumin, lysozyme, and albacore muscle extract. The proteolytic activity with hemoglobin as substrate was not affected by 1.4 mM cysteine at pH 2.5; however, there was approximately a 10 per cent increase in activity at pH 3.5. The optimum temperature of the enzyme with hemoglobin as substrate was 42°C. The enzyme did not appear to lose activity after holding for short periods at ambient temperature, or by a single freezing and thawing treatment, but was inactivated by heating at 70°C for 10 min. Groninger (1964) reported that the albacore muscle proteinase has properties considerably different from cathepsins A, B, and C. Albacore muscle proteinase differs from cathepsin A by being inactive toward carbobenzoxy-L-glutamyl-L-tyrosine, from cathepsin B by being inactive toward benzoyl-L-arginine amide and does not require activation by sulfhydryls, and from cathepsin C in being able to attack hemoglobin while being inactive toward glycyl-L-tyrosine amide. A comparison of the properties of the albacore muscle proteinase with those of the cathepsin from cod muscle (Siebert, 1962), cathepsin D (Press, Porter and Cebra, 1960) and E (Lapresle and Webb, 1962) shows some similarities to these three enzymes. Albacore muscle proteinase differs from cod muscle cathepsin in having a much lower pH optimum and somewhat different inhibition characteristics and from cathepsin D in pH optimum and electrophoretic characteristics.

The properties of albacore muscle proteinase are similar to those of cathepsin E with the exception of the slight inhibition of the albacore muscle proteinase by iodoacetamide.

Purification of Cathepsins

Cathepsins from various sources have different methods for purification. Greenbaum and Fruton (1957) developed a method for purification of cathepsin B from beef spleen, which consists of extraction at pH 2.6, fractional precipitation by means of ammonium sulfate, treatment with the cation exchange resin IRC-50-XE64, and fractional precipitation with $HgCl_2$ in ethanol. De la Haba, Cammarata, and Timasheff (1959) have purified cathepsin C about 250 fold from beef spleen by ammonium sulfate precipitation, heat treatment, and acetone or ethanol-zinc precipitation.

The chromatographic technique has been widely and successfully applied to the separation of these enzymes. In the last five years, chromatography on diethylaminoethyl-cellulose (DEAE-cellulose), carboxylmethyl-cellulose (CM-cellulose), and Sephadex columns have been the most useful methods for purification of cathepsins from different animal sources. The purification of cathepsins have been the subject of a number of publications (Evtikhina and Chernikov, 1962; Levyant, 1962; McMaster and Webb, 1963; Firfarova, Morozkin, and Orekhorick, 1964;

Groninger, 1964; Lishko, 1964).

Press, Porter and Cebra (1960) reported that at least ten forms of cathepsin D can be isolated from beef spleen by chromatography on DEAE-cellulose, CM-cellulose, and sulphomethyl-cellulose (SM-cellulose), and by electrophoresis on cellulose and starch-gel. There seems no doubt that cathepsin D is the principal proteolytic enzyme of beef spleen as judged by the hemoglobin assay. More than 90 per cent of the activity of the spleen was extracted and about 2/3 of this activity was characterized as cathepsin D. Lapresle and Webb (1962) used DEAE-cellulose, CM-cellulose, Sephadex G-75, CM-Sephadex G-50, and gel-filtration to purify cathepsin D and E from rabbit bone marrow. From their behavior on Sephadex G-75, cathepsin E shows a higher molecular weight than cathepsin D.

Recently Planta and Gruber (1964) used chromatography for the purification of beef spleen cathepsin C. DEAE-cellulose at or near pH 7 adsorbed all the cathepsin C activity and about 80 per cent of the protein. The enzyme then could be eluted by lowering the pH and simultaneously increasing the ionic strength of the eluent. With CM-Sephadex, 60 per cent of the enzymic activity and 8 per cent of the protein were eluted in a fairly narrow peak at pH 5.5.

Groninger (1964) purified a proteinase from water extract of albacore muscle by using acid treatment at pH 3.5, fractionation

at 0.3 to 0.6 saturated ammonium sulfate, and chromatography on DEAE-cellulose. A 260 fold purification of the enzyme with 2.3 per cent recovery of the activity was obtained. Some of the properties of the enzyme were determined with this purified preparation.

EXPERIMENTAL AND RESULTS

General Methods

Collection and Treatment of Samples

One year old Chinook salmon (Oncorhynchus tshawytscha) were obtained from the Food Toxicology and Nutrition Laboratory in the Department of Food Science and Technology. These fish were grown on a purified diet of casein, dextrin, corn oil, salts and vitamins. After sacrificing, the fish were eviscerated and stored at -28°C until required. The thawed, minced muscle tissue, excised from skin and bone, was homogenized with two parts water or solution for 3 min. in a Virtis "45" homogenizer at a setting of 75. All operations, except the assay for catheptic activity, were performed in the cold room (4°C).

Assay for Catheptic Activity

Preparation of Substrate

A 2.5 per cent solution of urea denatured hemoglobin (Nutritional Biochemicals Corporation) was used as the substrate throughout this work for the determination of catheptic activity. Hemoglobin powder (12.5 g) was weighed into a 50 ml beaker,

dissolved in distilled water, and transferred to a dialysis bag. Dialysis was carried out for two days against four changes of 100 volumes of distilled water. After dialysis, the hemoglobin solution was removed from the dialysis bag and 12.5 mg of Thimerosal, N. F. (sodium ethylmercurithiosalicylate) were added as a preservative. The volume was then made to 500 ml with distilled water and the resulting solution was stored in the refrigerator until used.

Preparation of Folin's Reagent

Folin's reagent was prepared by the method of Folin and Ciocalteu (1927). Into a 2 liter flask was introduced 100 g of sodium tungstate ($Na_2WO_4 \cdot 2H_2O$), 25 g of sodium molybdate ($Na_2MoO_4 \cdot 2H_2O$), 700 ml of water, 50 ml of 80 per cent phosphoric acid and 100 ml of concentrated hydrochloric acid. After refluxing for 10 hr, 150 g of lithium sulfate, 50 ml water and few drops of bromine were added. To remove the excess bromine the mixture was boiled for 15 min without condenser. The solution was cooled and diluted to one liter and filtered. The reagent was protected from dust and diluted with an equal volume of water before used.

Determination of Catheptic Activity

The following procedure is a modification of the method of Tallan, Jones, and Fruton (1952). Into 25 ml Erlemeyer flasks

were pipetted: 1 ml of 0.4 M acetate buffer (pH 3.2), 0.25 ml of 0.5 N HCl, 0.75 ml of water, and 4 ml hemoglobin solution. The flasks were placed in a shaker water bath at 37°C. Throughout the incubation period the flasks were shaken 120 times per min through an oscillation of 1 1/2 in. After temperature equlibration, 1 ml of the enzyme solution was added. The pH of the assay mixture was 3.7. Exactly 1 hr later, 9 ml of 0.3 M trichloroacetic acid (TCA) were added to stop the reaction; the flasks were shaken and replaced in the water bath for 20 min. The precipitated protein was removed by filtration through Whatman No. 1 filter paper. An enzyme blank was prepared as above, except that the enzyme solution was added immediately after the TCA. Absorbance at 280 mμ of the clear filtrate was determined in the Beckman DU spectrophotometer standardized with the filtrate of the enzyme blank. All determinations were made in duplicate. Activity is expressed in absorbance units of the filtrate.

In studies on the effect of pH on catheptic activity of salmon muscle, the presence of tyrosine and tryptophan in the above filtrates were determined with Folin's reagent by the method of Anson (1938). Three ml of filtrate were placed in a test tube with 6 ml of 0.5 N NaOH. After mixing, 2 ml of diluted Folin's reagent were added. This solution was immediately mixed and the absorbance at 650 mμ was read exactly 8 min later in the Beckman

Model B spectrophotometer, standardized with water. The absorbance of the blank was subtracted from the absorbance of the determination and the activity was expressed as the difference in absorbance units.

Determination of Protein Concentration

The protein concentration of the solutions was determined by measuring the absorbance at 280 mμ. It was assumed that in a 1 cm cell a solution containing 1 mg of average protein per ml has an absorbance of 1 at 280 mμ (Dixon and Webb, 1964).

Determination of pH Optimum

In the review of literature, it was noted that several investigators, working with a variety of tissues, have found cathepsins with pH optima ranging from pH 2.5 to 10. Therefore, before undertaking the purification of the cathepsin from salmon muscle and to determine the pH to be used in the assay mixture, it was necessary to undertake a study of the effect of pH on catheptic activity. Using muscle homogenate as a source of enzyme, the pH of the assay mixture was varied from pH 1 to 11. Below pH 6, 0.4 M acetate was used as the buffer; above pH 6, 0.4 M phosphate was used as the buffer. The pH of the assay mixture was adjusted by the addition of various amounts of 0.5 N HCl and 0.5 N NaOH.

Water was added to maintain a constant volume in all flasks. The pH was determined on the individual reaction mixtures during the incubation period with a Beckman pH meter, Model G. In some cases, the pH of the reaction mixtures was far removed from the maximum buffering capacity of the buffers; however, no significant change in pH was noted between the initial and the final portion of the incubation period. Presence of tyrosine and tryptophan in the TCA filtrates was determined with the use of Folin's reagent described earlier, as well as by absorption at 280 mμ.

Figure 1 shows the pH-activity curve of the catheptic activity of salmon muscle as determined by the above method. Both curves show a pH optimum between 3.6 and 3.8. The curve determined by absorption at 280 mμ shows a high activity peak with an optimum at pH 7.3, while the curve determined by Folin's reagent shows two relatively minor peaks at pH 6.9 and 8.5. Both curves tend to show an increase in proteolysis above pH 10. Consequently, it was decided to attempt the purification of the enzyme, which exhibits a pH optimum at 3.6 to 3.8.

Extraction of Cathepsin from Salmon Muscle

To purify the cathepsin from salmon muscle, the enzyme must first be extracted from the tissue. The muscle tissue was homogenized with various salt and buffer solutions and the homogenates

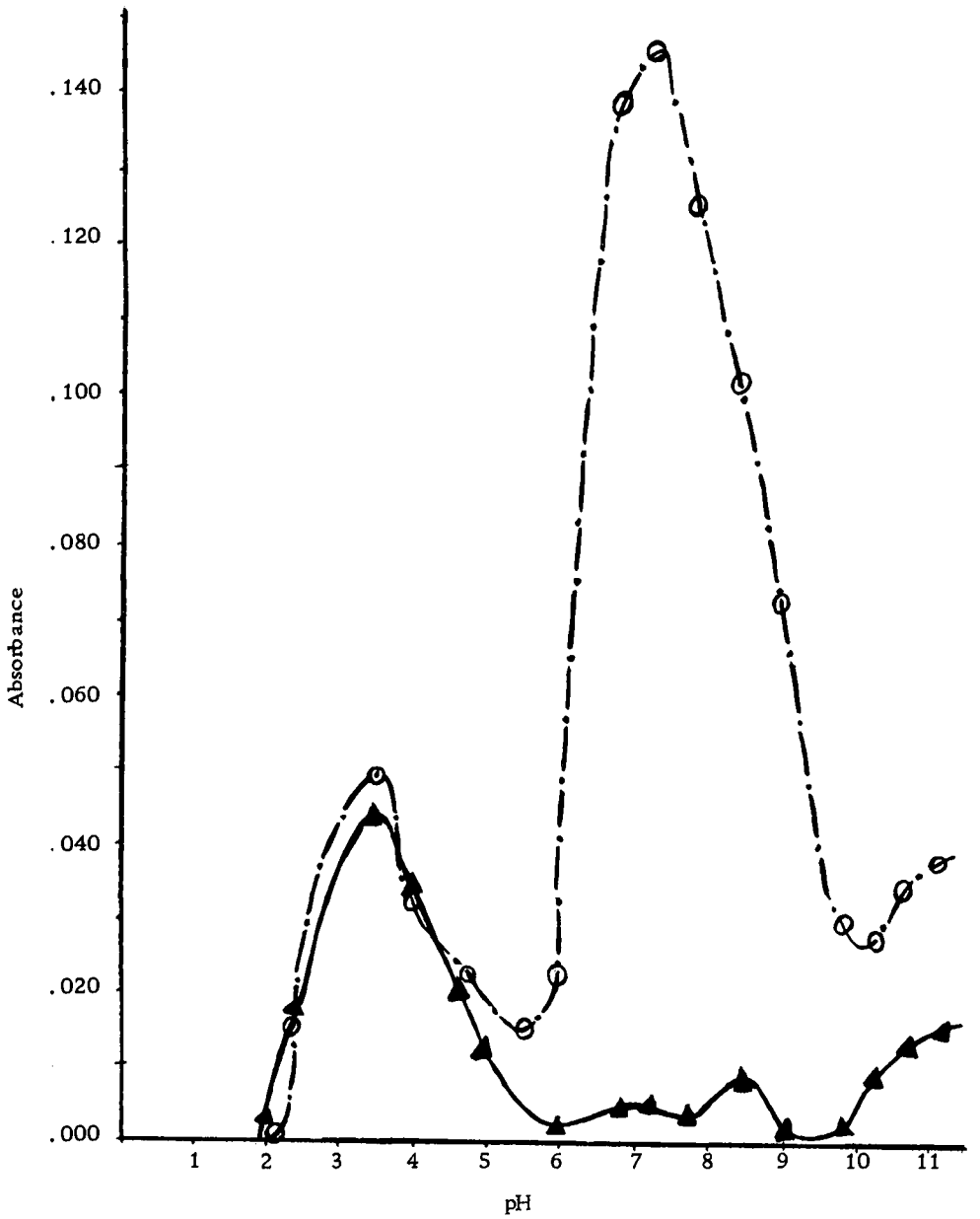


Figure 1. pH-activity curve for salmon muscle cathepsins.

— · — · — · — Salmon muscle catheptic activity measured at 280 mμ.

——————— Salmon muscle catheptic activity measured with Folin's reagent at 650 mμ.

were centrifuged at 17,000 rpm (34,800 x G) for 20 min. Catheptic activity of the clear supernatants were determined and a summary of the data is presented in Table 1. Maximum specific activity was obtained by extracting with 0.15 M acetate buffer (pH 4.5) containing 0.1 N KCl, however, the recovery was low. Solutions of KCl appeared to show the greatest promise as extracting solutions. Homogenates prepared with 0.15 M phosphate (pH 7.5) and 0.15 M phosphate (pH 7.5) containing 0.1 N KCl formed gels and could not be separated at this centrifugal force.

Further investigations were made to determine the optimum concentration of KCl for extracting the catheptic activity from salmon muscle. These data are presented in Table 2. Extracts with water yielded the maximum specific activity although recovery was low. Extracts obtained with 0.2 N KCl yielded high specific activity consistent with high recovery. Hence, 0.2 N KCl was used as the extracting solution in the following investigations. This crude extract was called fraction A.

pH Stability of Salmon Muscle Cathepsin

In ion-exchange chromatography the proteins are eluted from the resin by changing the pH and/or the ionic strength of the eluting buffer. Therefore, to determine the pH of the buffers to be used, it is necessary to know the stability of the cathepsin at various pH

Table 1. Extraction of salmon muscle cathepsin with various solutions.

solution	activity (absorbance)	protein concentration (mg/ml)	specific activity (activity / mg protein)	percentage recovery
homogenate	0.090			
water	0.054	31.2	0.00173	60
0.1 N KCl	0.078	34.4	0.00227	87
0.6 N KCl	0.060	36.0	0.00167	67
0.1 N LiCl	0.033	35.2	0.00094	37
acetate (pH4.5, o.15M)	0.033	13.4	0.00246	37
acetate (pH 4.5, O.15M)+O.1N KCl	0.041	15.2	0.00270	46

Table 2. Effect of KCl concentration on extraction of salmon muscle cathepsin.

solution	activity (absorbance)	protein concentration (mg/ml)	specific activity (activity / mg protein)	percentage recovery
homogenate	0.097			
water	0.067	30.1	0.00223	69
0.05 N KCl	0.074	37.8	0.00196	76
0.1 N KCl	0.086	46.2	0.00186	89
0.2 N KCl	0.092	46.6	0.00197	95
0.3 N KCl	0.102	54.2	0.00188	105

levels. To fraction A various quantities of 0.5 N HCl and 0.5 N NaOH were added to decrease and increase the pH, respectively. Sufficient water was added to the samples to maintain the volumes constant. These samples were stored in the cold room for 24 hr. The pH was readjusted to the original pH of fraction A before activity was determined.

The results presented in Table 3 show that as the pH was increased or decreased, catheptic activity was lost. The pH levels in the acid region did not appear as harmful as those in the basic region. From these data it was concluded that eluting buffers in the region of pH 6.5 were necessary to prevent loss of catheptic activity.

Table 3. Stability of cathepsin at various pH values

basic region		acid region	
pH	activity (absorbance)	pH	activity (absorbance)
[a]6.45(control)	0.154	[a]6.54(control)	0.059
6.91	0.125	5.59	0.058
8.06	0.082	4.44	0.048
9.76	0.014	3.41	0.044
		2.58	0.032

[a] Extracts were from different muscles.

Acid Treatment

In the preceeding experiment precipitates formed when the pH was reduced to 5.5 and 4.5. It was of interest to determine if the catheptic activity was precipitated or if inactive protein could be removed by this process. The pH of fraction A was reduced by the careful addition of 0.5 N HCl. The samples were allowed to stand for 10 min before centrifugation at 1000 x G for 20 min. The pH of the clear supernatant was readjusted to the original pH by the addition of 0.5 N NaOH. Sufficient water was added to maintain the volume in all samples constant. The data are present in Table 4.

Specific activity of the cathepsin was increased 2.5 fold by reduction of the pH to 5.5. Hence, acid treatment was used as the first step in the purification of catheptic activity from salmon muscle. The fraction resulting from this procedure was called fraction B.

Table 4. Effect of acid treatment on catheptic activity

pH	activity (absorbance)	protein concentration (mg/ml)	specific activity (activity/mg protein)	percentage recovery
6.49 (control)	0.055	76.8	0.00072	
5.49	0.047	25.5	0.00184	86
4.45	0.047	33.0	0.00145	86

Dialysis

In preparation for column chromatography, fraction B was dialyzed against 0.005 M phosphate buffer (pH 6.5) for 12 hr. A precipitate formed during dialysis was removed by centrifugation at 1,000 x G for 20 min. Catheptic activity of clear supernatants was determined and the data are presented in Table 5. The specific activity increased approximately two fold with very high recovery of catheptic activity after dialysis. This fraction was called fraction C. Fractionation of fraction C was attempted by column chromatography, however, the effluent was not of sufficient concentration to permit accurate activity determinations. The column chromatography procedure will be described in a later section.

Table 5. Effect of dialysis of fraction B on catheptic activity

treatment	activity (absorbance)	protein concentration (mg/ml)	specific activity (activity/mg protein)	percentage recovery
before dialysis	0.060	22.3	0.00269	
after dialysis	0.054	10.0	0.00545	98.1

Ammonium Sulfate Fractionation

Since the catheptic activity of fraction C was not sufficiently concentrated to obtain significant quantities from column chromatography, fractionation by $(NH_4)_2SO_4$ was attempted to concentrate

the activity prior to column chromatography. Solid $(NH_4)_2SO_4$ was added to portions of fraction C to give various concentrations. The precipitates were removed by centrifugation at 34, 800 x G for 10 min, dissolved in minimum amounts of 0. 005 M phosphate at pH 6. 5, and dialyzed against the same buffer for 12 hr. Precipitates formed on dialysis were removed by centrifugation at 34, 800 x G for 10 min. Activity was determined on the supernatants. Preliminary results showed that most of the proteolytic activity was present in the 0. 25 to 0. 50 saturated fraction. This range of concentration was investigated in detail and the data are presented in Table 6.

The major portion of the catheptic activity of fraction C was precipitated between 0. 25 and 0. 50 saturation of $(NH_4)_2SO_4$ with an increase in specific activity. Hence, this fraction was used for column chromatography and was designated fraction D.

Table 6. Ammonium sulfate precipitation of catheptic activity from fraction C.

fraction	activity (absorbance)	protein concentration (mg/ml)	specific activity (activity/mg protein)	percentage recovery
A	0.062	46.9	0.00132	
B	0.060	17.8	0.00337	91
C	0.041	7.6	0.00540	65
0.25[a] to 0.40[b]	0.136	14.4	0.00944	23
0.40 to 0.50[b]	0.114	18.9	0.00603	22
0.50 to 0.60[b]	0.026	8.7	0.00299	4.5
0.60 to 1.00[b]	0.006	11.6	0.00517	17

[a]no precipitation appeared at 0.25 saturation

[b]saturation of ammonium sulfate

Column Chromatography

Cellulose ion-exchange resins have been widely used for the chromatographic fractionation of protein and enzyme mixtures. Recently, Rampton (1965) reported the chromatographic fractionation of bovine sacroplasmic proteins on diethylaminoethyl-cellulose (DEAE-cellulose). Hence, an investigation was undertaken to determine if catheptic activity from salmon muscle could be fractionated by chromatography on DEAE-cellulose.

Packing the column

A water suspension of 30 g of DEAE-cellulose, an anion exchange resin, was prepared in a 1 liter graduated cylinder. After standing for 30 min the fines were removed by decantation. The resin was conditioned by treatment with 500 ml 1 N NaOH followed by washing on a Buchner funnel with 1 liter of water, resuspended in 500 ml 1 N HCl, washed with 1 liter of water on a Buchner funnel, resuspended in 500 ml of 1 N NaOH, washed with 3 liter of water on a Buchner funnel and finally resuspended in 500 ml of 0.1 N NaOH. The resin was deaerated by placing under reduced pressure at the water aspirator for a few min. The column (2.5 x 50 cm) was packed by siphoning the agitated slurry into a column filled with 0.1 N NaOH. The stopcock was partially opened and the resin was allowed to settle by gravity flow. A disc of filter paper was placed on top of the column to preserve the flat surface. A column of 35 cm in height was obtained.

Washing and Regeneration of Resin

After the DEAE-cellulose column was packed, the column was placed in a cold room and washed successively with water until the pH of the effluent was neutral, 100 ml of 0.1 M phosphate buffer (pH 6.5), and finally, starting buffer (0.005 M phosphate, pH 6.5)

until the pH of the effluent was 6.5.

After the chromatographic run, the resin was regenerated in the column by successive treatments of 100 ml of 0.1 N NaOH (to remove any protein material), distilled water until the effluent pH was neutral, 750 ml of 0.1 M Na_3PO_4-0.2 per cent Triton X-100 (Peterson and Chiazze, 1962), distilled water until the pH of the effluent was neutral, 100 ml 0.1 M phosphate buffer (pH 6.5), and finally, starting buffer until the pH of the effluent was 6.5.

Chromatography of Protein

The protein solution was placed on top of the column and allowed to flow into the resin by gravity. When all of the sample had entered the resin, the column was rinsed with several small portions (usually 5 ml) of starting buffer, each being allowed to flow into the column before the next was added. Approximately 20 ml of starting buffer were placed on the resin before the column was attached to a constant flow micropump adjusted to 80 ml per hr. Effluent from the column was conducted to a Beckman DB recording spectrophotometer equipped with a flow-through cell and the absorbance at 280 mμ was recorded continuously throughout the chromatographic run. The effluent was collected by a fraction collector equipped with a 10 ml volumetric siphon.

After the first protein fraction, which was not retained by the

resin, had been eluted, gradient elution was started. The gradient was obtained with the aid of a nine chambered Varigrad (Peterson and Sober, 1959). The starting buffer was 0.005 M phosphate at pH 6.5 and the limit or final buffer was 0.005 M phosphate at pH 6.5 containing 0.5 N NaCl. The volume percentages of limit buffer in chambers one through nine was 0, 0, 4, 0, 15, 0, 25, 40, and 100, respectively. This resulted in a concave gradient, where the change in salt concentration of the eluting buffer was small initially and increased gradually throughout the elution procedure. A total of 2250 ml of buffer was used in the Varigrad taking approximately 40 hr to complete a single chromatographic run.

Chromatography of Fraction C

Three trials were made in an attempt to purify the catheptic activity from fraction C by chromatography on DEAE-cellulose. Figure 2 shows a typical chromatogram from one of these trials. The major portion of the protein was not retained on the resin and was eluted in the first peak. The remainder of the protein was chromatographed and appeared to be well separated. Catheptic activity determinations were made on the fractions containing the highest protein concentration. The levels of activity were low and no confidence could be placed in the activity measurements. The fractions comprising one protein peak were combined and attempts

were made to concentrate the proteins.

Ultrafiltration was the first method used to concentrate the protein solutions. The protein solution was placed in a short cylinder and a plastic cage with dialysis tube stretched over the outside was placed in the solution. Vacuum from a water aspirator was used to remove the water through the dialysis tubing. A safety trap was installed between the dialysis tubing and the aspirator. The water removed from the protein solution was collected in the safety trap. Approximately 24 hr were required to remove 200 ml of water. Catheptic activity and protein concentration were determined on the solution remaining in the cylinder and the solution in the safety trap. The results of several trials of this method showed that the levels of catheptic activity and protein of the two solutions were almost identical. Hence, this method was not concentrating the protein.

Pervaporation was the next method investigated. The combined fractions were transferred to dialysis bags and placed in the air stream from an electric fan at room temperature. The rate of evaporation was sufficient to keep the solution cool. Catheptic activity and protein concentration were determined on the residual solutions. One protein fraction (peak 7 of Figure 2) showed an activity of 0.048 absorbance unit with an increase in specific activity of approximately four fold over fraction C with 52 per cent

recovery. Since the recovery was low, it was decided to abandon this method of concentration.

The third method investigated to concentrate the protein fractions was the use of "Aquacide #2" (Calbiochem). "Aquacide #2" is a water soluble modified cellulose preparation of very high molecular weight containing a minimum of enzyme poisons. The protein fractions were transferred to dialysis bags and placed in glass trays. The dialysis bags were covered with the dry "Aquacide #2" powder and the trays were covered with "Saran Wrap". After 24 hr in the cold room the volume of the fractions was reduced approximately 95 per cent. From activity and protein concentration data, one protein fraction in the region of peak 7 of Figure 2 showed an activity of 0.044 absorbance unit with an increase in specific activity of approximately five fold over fraction C with only 18 per cent recovery. With this very low recovery, it was decided to abandon this method of concentrating the fractions from column chromatography.

Chromatography of Fraction D

Trial 1

Since concentration of the protein fractions after chromatography did not appear to be very successful, ammonium sulfate fractionation of the catheptic activity in fraction C was investigated

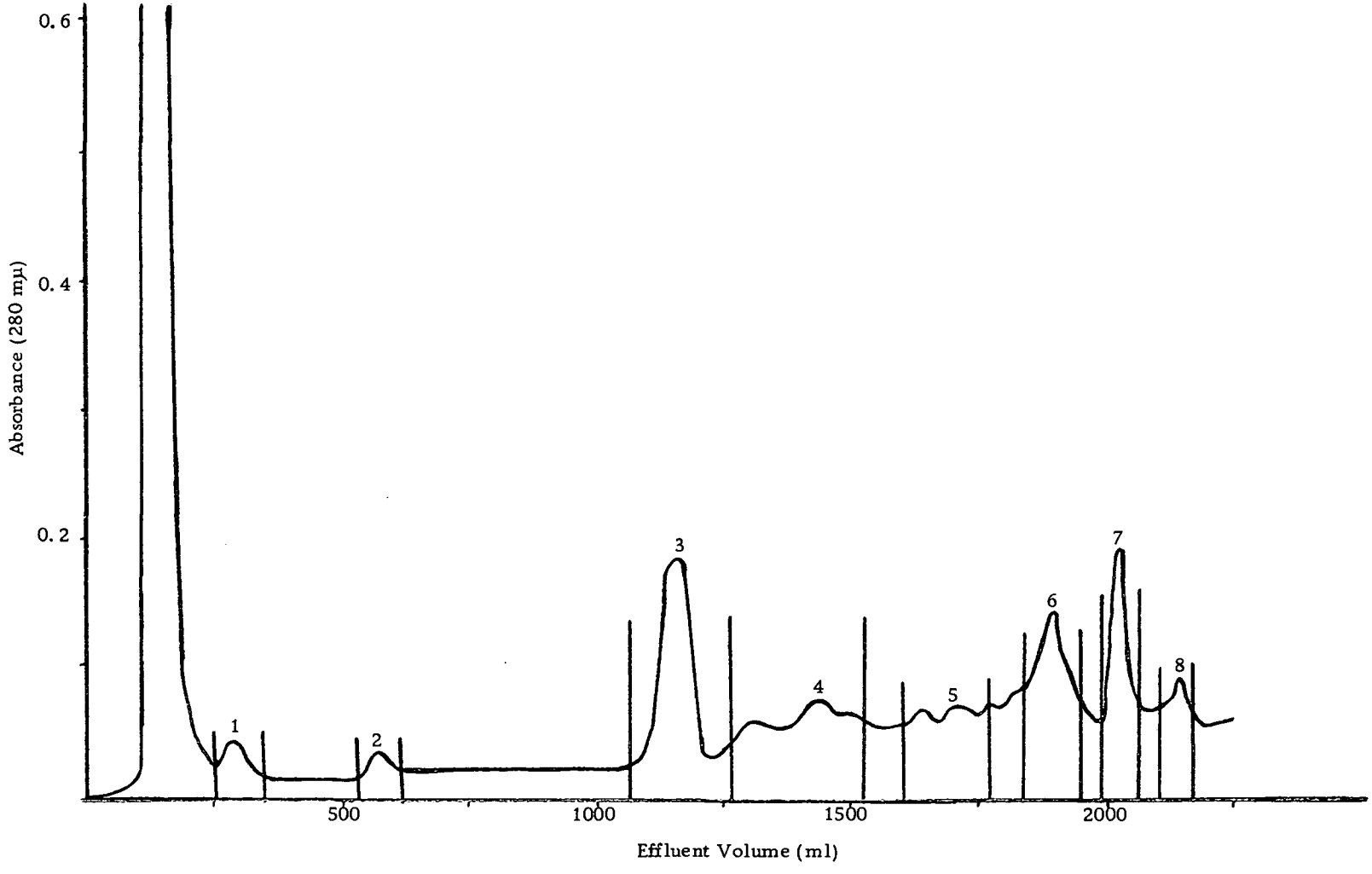


Figure 2. Chromatography of fraction C. Sample size: 27.8 ml (150 mg of protein) with activity of 0.049 absorbance unit/ml. Gradient elution: starting buffer, 0.005 M phosphate (pH 6.5); limit buffer, 0.005 M phosphate-0.5 N NaCl (pH 6.5). Gradient started at 200 ml. The fractions within the vertical lines were combined.

and resulted in fraction D. Using DEAE-cellulose to purify cathepsin C from beef spleen, Planta and Gruber (1964) found that the addition of 0.01 M cysteine to the eluting buffers reduced the tailing and caused the enzyme to be eluted in a narrower band. Therefore, fraction D was chromatographed on DEAE-cellulose with the starting and limit buffers containing 0.01 M cysteine. The chromatogram is presented in Figure 3.

The catheptic activity was determined on the fractions containing the highest protein concentration. Again the activity levels were too low to be meaningful. It was decided to investigate lyophilization as a means of concentrating the proteins. The fractions comprising the protein peaks were combined, transferred to glass trays, covered with "Saran Wrap", and frozen at -28°C. The trays were transferred to the lyophilizer which consists of an 8 cubic foot chamber with two shelves heated by circulating water. A vacuum of 0.6 mm of Hg was maintained on the chamber and the heating water was not allowed to raise above 38°C throughout the 18 to 24 hr. lyophilization period. After lyophilization, the white powder was dissolved in a minimum quantity of distilled water and dialyzed against starting buffer for 24 hr. A precipitate formed which was removed by centrifugation at 1000 x G for 10 min. Catheptic activity and protein concentration were determined on the supernatants. These data are presented in Table 7.

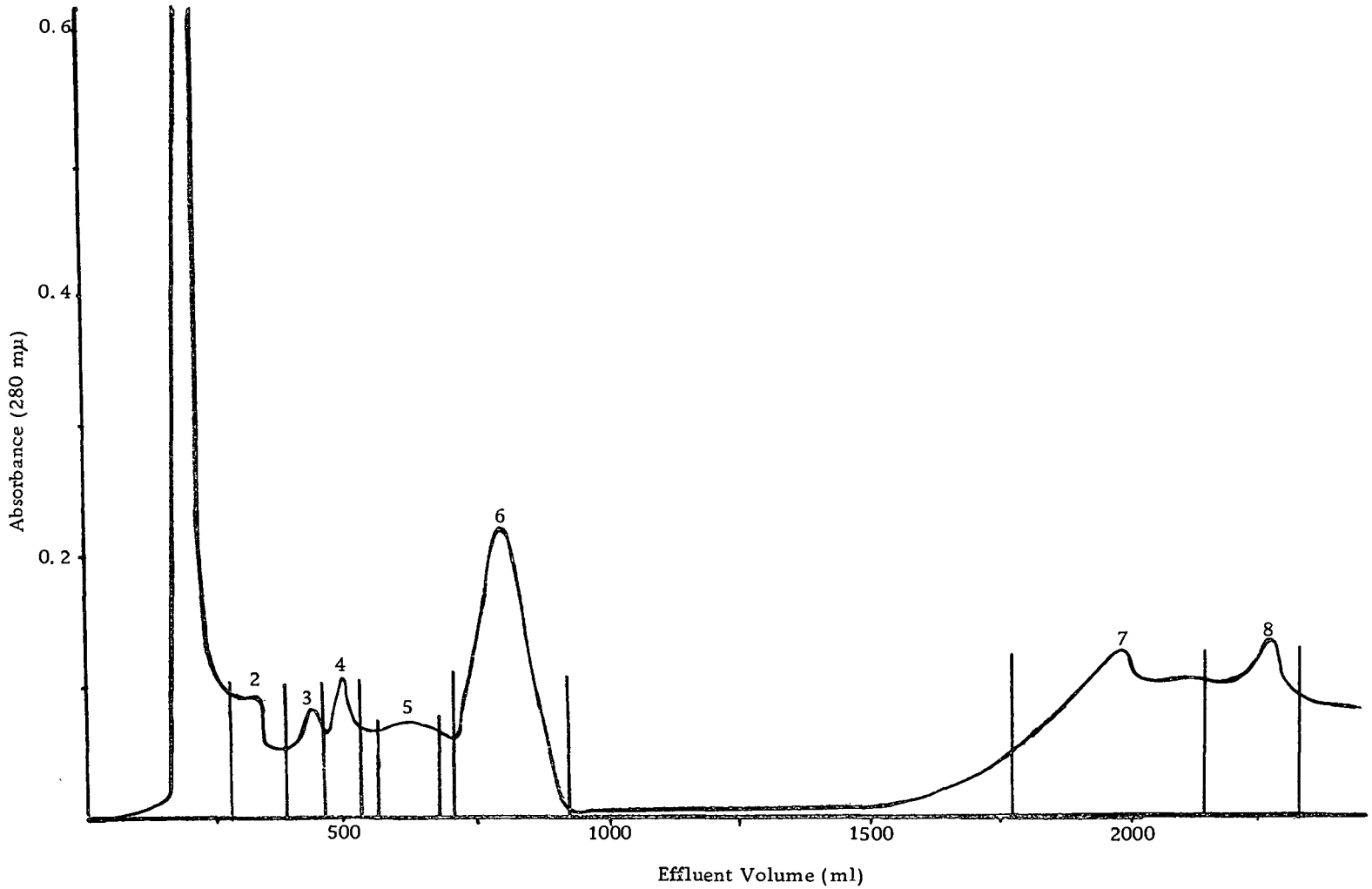


Figure 3. Chromatography of fraction D (trial 1). Sample size: 39.5 ml (995 mg of protein) with activity of 0.380 absorbance unit/ml. Gradient elution: starting buffer, 0.005 M phosphate-0.01 M cysteine (pH 6.5); limit buffer, 0.005 M phosphate-0.5 N NaCl-0.01 M cysteine (pH 6.5). Gradient started at 250 ml. The fractions between the vertical lines were combined.

Table 7. Chromatography of fraction D (trial 1).

fraction	activity (absorbance)	protein concentration (mg/ml)	specific activity (activity/mg protein)	percentage recovery	fold purification
A	0.144	40.2	0.00358		
B	0.116	41.6	0.00279	77	0.78
C	0.092	16.8	0.00548	61	1.53
D	0.380	25.2	0.0151	42	4.21
peak					
2	0.002	0.226	a	a	a
3	0.006	0.242	a	a	a
4	0.008	0.335	a	a	a
5	0.007	0.488	a	a	a
6	0.000	0.448	a	a	a
7	0.104	1.42	0.0732	12	20.4
8	0.009	0.830	a	a	a

[a] Activity too low to be meaningful

Purification was increased approximately five fold over fraction D in peak 7. This is comparable to that obtained with fraction C in the preceeding section. Hence, the presence of cysteine in the buffer does not appear to be beneficial.

Trials 2 and 3

Two chromatographic separations of fraction D were made using the same procedure as in trial 1, except that cysteine was not added to the buffers. The protein fractions were concentrated by lyophilization and catheptic activity and protein concentration were determined on the supernatants. The results of trial 2 are presented in Figure 4 and Table 8.

Purification was increased eight fold in peak 4 and seven fold in peak 7 over fraction D. The data presented in Table 8 show two activity maxima; that is, the activity was high in peak 4, decreased in peak 5, increased in peak 6, and finally decreased in peak 7. This suggests the possible presence of two cathepsins in fraction D.

The results of trial 3 are presented in Figure 5 and Table 9. An increase in purification of 5 and 21 fold over fraction D was noted in peaks 5 and 8, respectively. The maximum purification of 117 fold with respect to fraction A, the crude extract, was obtained in peak 8. The data in Table 9 are similar to those presented in

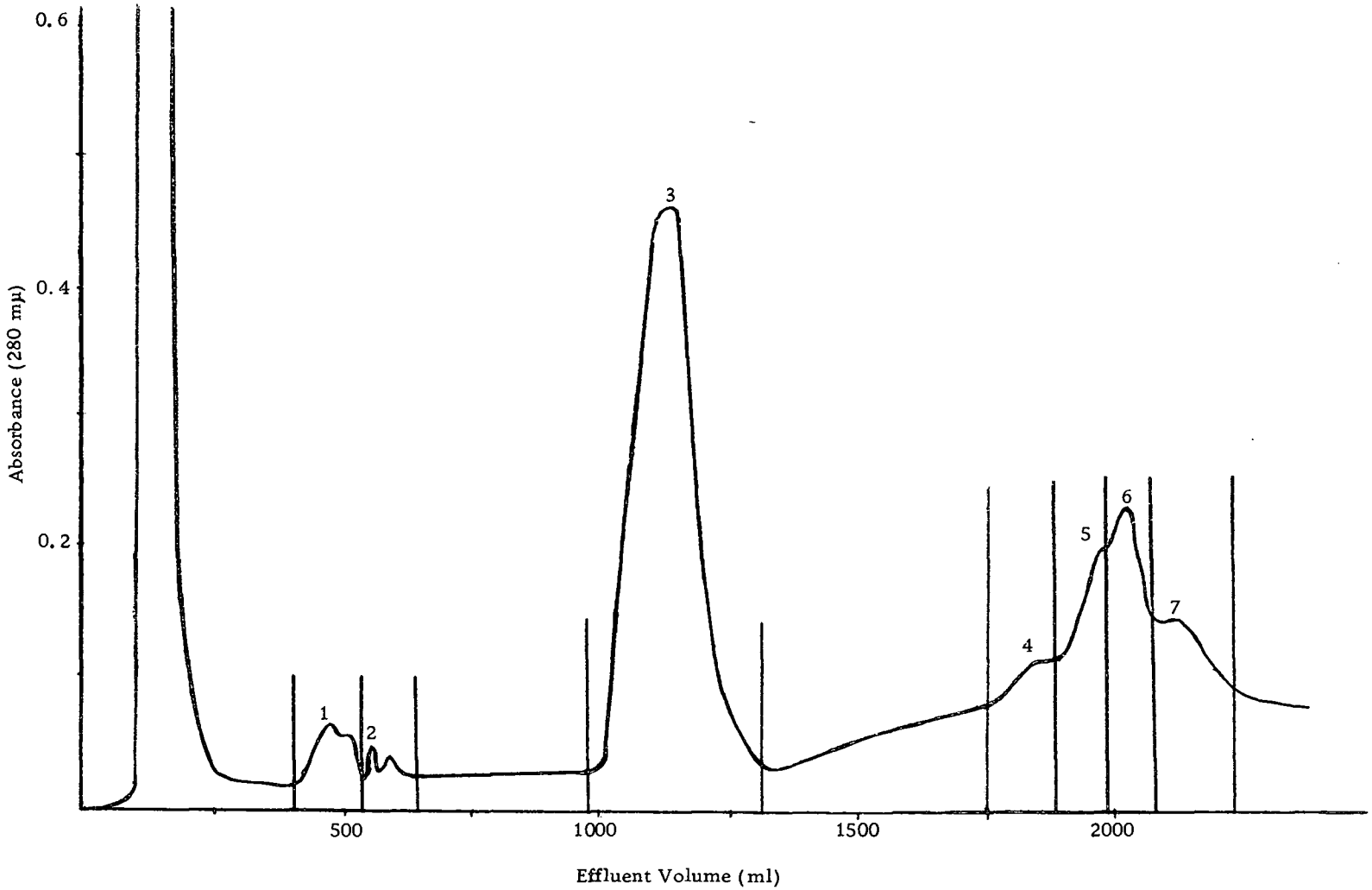


Figure 4. Chromatography of fraction D (trial 2). Sample size: 32 ml (633 mg of protein) with activity of 0.475 absorbance unit/ml. Gradient elution: starting buffer, 0.005 M phosphate (pH 6.5); limit buffer, 0.005 M phosphate-0.5 N NaCl (pH 6.5). Gradient started at 230 ml. The fractions between the vertical lines were combined.

Table 8, in that two activity maxima appear to be present. This lends further evidence to the possible presence of two catheptic enzymes in fraction D.

Table 8. Chromatography of fraction D (trial 2).

fraction	activity (absorbance)	protein concentration (mg/ml)	specific activity (activity/mg protein)	percentage recovery	fold purification
A	0.153	47.3	0.00323		
B	0.124	25.2	0.00492	78	1.5
C	0.108	14.4	0.00750	69	2.3
D	0.475	19.8	0.0240	39	7.4
peak					
1	0.022	0.262	0.0839	0.5	26.0
2	0.016	0.138	[a]	[a]	[a]
3	0.005	1.32	[a]	[a]	[a]
4	0.076	0.417	0.182	3.7	56.4
5	0.048	0.429	0.112	1.8	34.6
6	0.159	1.37	0.116	6.0	35.9
7	0.076	0.492	0.154	6.3	47.8

[a] Activity too low to be meaningful.

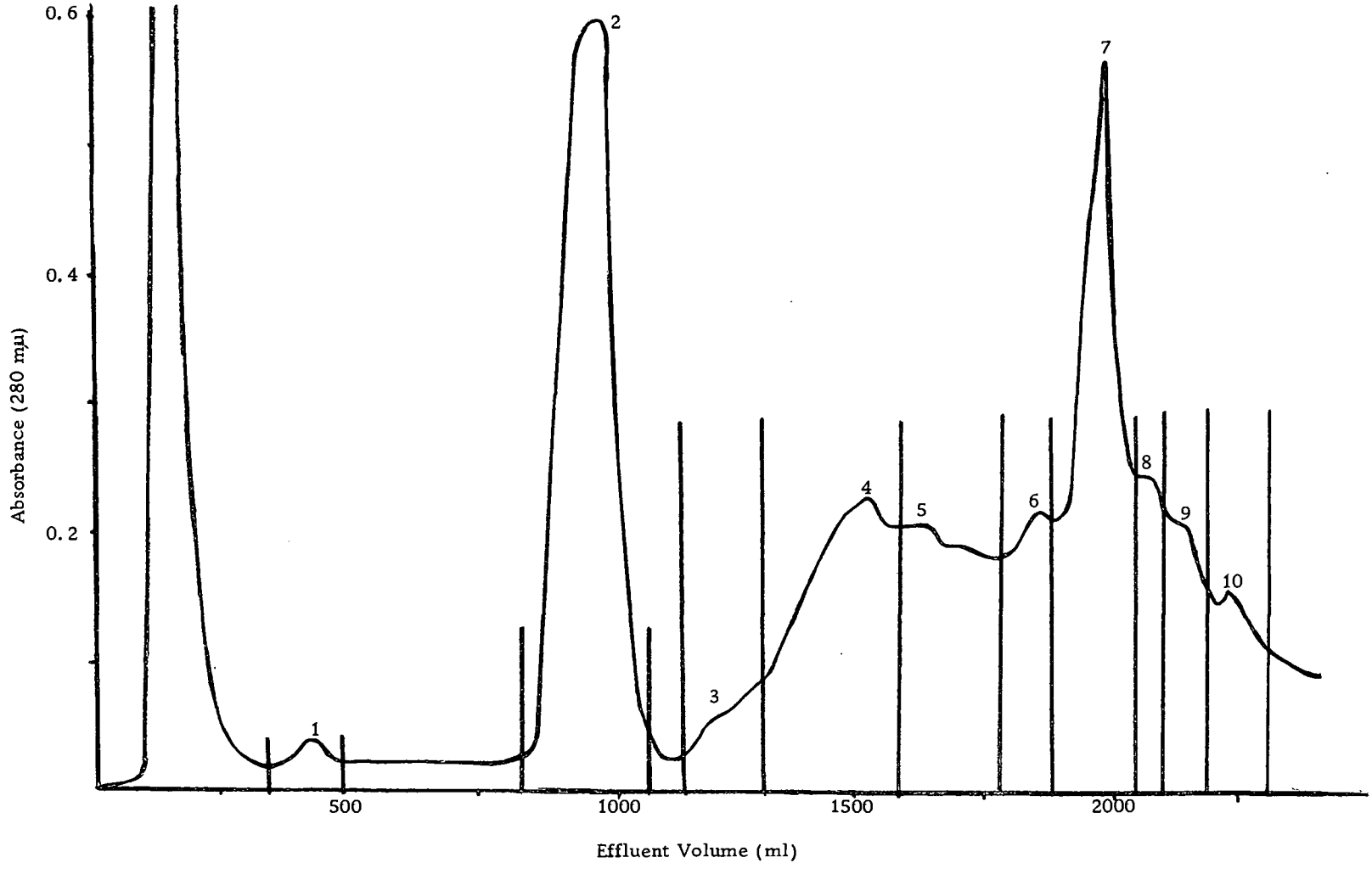


Figure 5. Chromatography of fraction D (trial 3). Sample size: 25 ml (1215 mg of protein) with activity of 0.514 absorbance unit/ml. Gradient elution: starting buffer, 0.005 M phosphate (pH 6.5); limit buffer, 0.005 M phosphate-0.5 N NaCl (pH 6.5). Gradient started at 280 ml. The fractions between the vertical lines were combined.

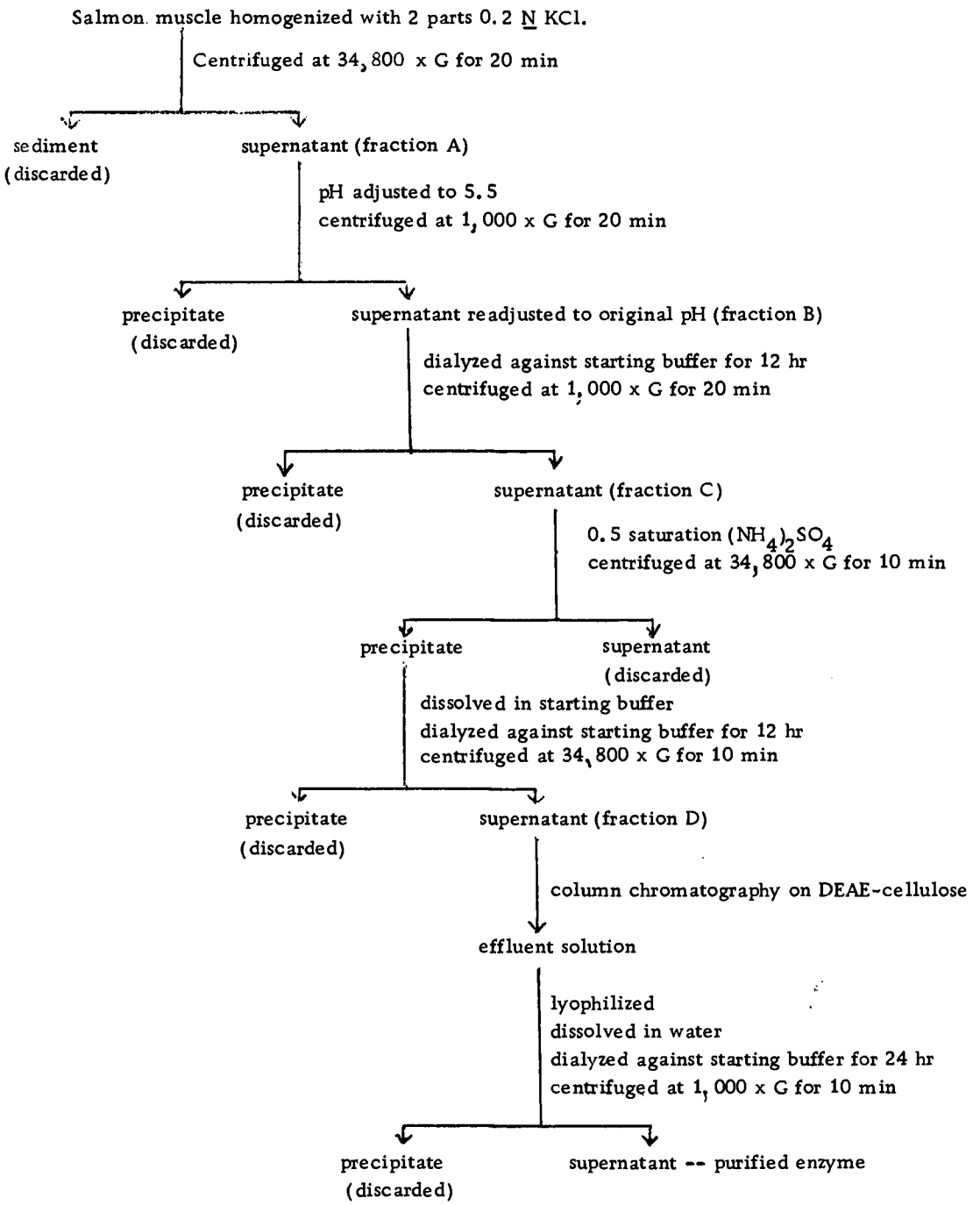
Table 9. Chromatography of fraction D (trial 3).

fraction	activity (absorbance	protein concentration (mg/ml)	specific activity (activity/mg protein)	percentage recovery	fold purification
A	0.110	57.9	0.00189		
B	0.101	29.7	0.00340	88	1.80
C	0.074	19.7	0.00388	74	2.05
D	0.514	48.6	0.00106	56	5.60
peak					
1	0.010	0.448	[a]	[a]	[a]
2	0.002	8.18	[a]	[a]	[a]
3	0.006	0.441	[a]	[a]	[a]
4	0.020	0.662	0.00295	1.2	1.56
5	0.184	3.54	0.00520	17	27.5
6	0.051	1.69	0.00302	1.8	16.0
7	0.140	4.40	0.00320	8.5	16.9
8	0.190	0.860	0.00221	6.8	117
9	0.076	0.950	0.00800	4.6	42.3
10	0.027	0.560	0.00482	1.7	25.5

[a] Activity too low to be meaningful.

Over-all Purification Procedure

The flow scheme for the purification of salmon muscle cathepsin is presented below.



DISCUSSION

The pH activity curve (Figure 1), determined with the use of Folin's reagent, shows three pH optima at pH 3.7, 6.9, and 8.5. Conversely, the pH-activity curve as determined by absorption at 280 mμ indicates two pH optima at pH 3.7 and 7.3. Since Folin's reagent has been reported to be specific for tyrosine and tryptophan (Anson, 1938), the pH optimum observed at pH 7.3 by absorption at 280 mμ might also be due to the presence of substances other than tyrosine and tryptophan that are released from the muscle homogenate at this pH. No evidence is available as to the identity of these substances. However, it is possible that nucleic acids might be responsible, since ribonuclease from animal tissues has a pH optiumn of 7 to 8 (Anfinsen and White, 1961).

Cathepsin purified from albacore muscle has a pH optimum of 2.4 to 2.5 (Groninger, 1964), while cod muscle cathepsin has a pH optimum of 4.3, both values obtained with hemoglobin as a substrate. From the data presented in Figure 1, it would appear that the salmon muscle cathepsin would be similar to the cod muscle cathepsin and differs from the cathepsin purified from albacore muscle on the basis of pH optimum.

The catheptic activity noted at pH 6.9 with Folin's reagent (Figure 1) might be due to an aminopeptidase similar to that

described by Dvorak (1960) in beef muscle. Koszalka and Miller (1960a) reported a proteinase in rat skeletal muscle with a pH optimum at 8.5 to 9.0. A similar enzyme in fish muscle would account for the activity evident at pH 8.5. Since the activities of these enzymes are low in salmon muscle, it should be pointed out that there is some doubt of their existance. The increase in proteolysis above pH 10 might be caused by the hydrolysis of muscle proteins at this high pH. Since the muscle homogenate is not added to the enzyme blank until after the TCA is added, the enzyme blank would not compensate for this proteolysis.

The catheptic activity of salmon muscle was purified approximately 117 fold by the methods used in this report. Chromatography on DEAE-cellulose with concentration of the fractions by lyophilization does appear to be a useful method for the purification of salmon muscle cathepsin. The difficulty encountered in the concentration of the catheptic activity after column chromatography by ultrafiltration, pervaporation, and "Aquacide #2" would indicate that the enzyme is not stable under these conditions. In attempts to purify cathepsin C by ion-exchange chromatography and gel filtration, Planta and Gruber (1964) reported that concentration of the fractions "by all methods tried" caused heavy losses in enzyme activity. The methods used by these authors were not mentioned.

In Figure 4 and 5, it would have been ideal to determine the

catheptic activity on each fraction obtained. Since a laboratory lyophilizer was not available during this work, this was not considered practical. Higher purification may have resulted if each fraction had been analyzed for catheptic activity or if the fractions had been combined in a different manner. Also, in future work it would be advisable to check the protein concentration of the fractions with the micro-Kjeldahl or another suitable method to determine if absorption at 280 mμ is an accurate estimation of the protein concentration.

Further investigations are required to confirm the presence of two cathepsins in fraction D. This could be done by concentrating those fractions showing catheptic activity and attempting further purification by chromatography on DEAE-cellulose or another cellulose ion-exchange resin and by column electrophoresis. The properties of the purified enzymes could be determined, which would lead to the classification and a greater understanding of the salmon muscle cathepsin.

SUMMARY

Catheptic activity of salmon muscle was found to exhibit pH optima at pH 3.7, 6.9 and 8.5 when Folin's reagent was used to determine the products of protein hydrolysis; whereas, pH optima at pH 3.7 and 7.3 were obtained when the products of protein hydrolysis were determined by absorption at 280 mμ. Possible reasons for the differences in pH optima were discussed.

A method was developed for the partial purification of salmon muscle cathepsin, which shows optimal activity at pH 3.7. The stability of this enzyme appeared to be maximal at pH 6.5. Purification was accomplished by extracting the salmon muscle cathepsin with two parts 0.2 N KCl. The pH of this crude extract was adjusted to 5.5 and the precipitated proteins were removed by centrifugation before the pH of the supernatant was readjusted to the original pH of the extract. Upon dialysis of this fraction against 0.005 M phosphate (pH 6.5), a precipitate formed, which was removed by centrifugation. The catheptic activity was precipitated from the supernatant at 0.50 saturation with $(NH_4)_2SO_4$. The precipitate was recovered by centrifugation, dissolved in 0.005 M phosphate (pH 6.5), and dialyzed against the same buffer. A precipitate formed and was removed by centrifugation. This fraction was placed on a 2.5 x 35 cm column of DEAE-cellulose equilibrated

with the starting buffer (0.005 M phosphate at pH 6.5). A concave concentration gradient was used to elute the proteins from the ion-exchange resin. Final buffer was 0.005 M phosphate (pH 6.5) containing 0.5 N NaCl. The absorbance (280 mμ) of the column effluent was continuously recorded and 10 ml fractions of the effluent were collected. Fractions comprising the various protein peaks were combined and concentrated by lyophilization. Two catheptic enzymes appeared to be separated by this procedure with maximum purification of 117 fold with 6.8 per cent recovery.

During the development of the procedure, it was found that the fractions obtained from column chromatography could not be successfully concentrated by ultrafiltration, pervaporation, or "Aquacide #2" and that the presence of cysteine in the eluting buffer was not beneficial.

BIBLIOGRAPHY

1. Anfinsen, B. Christian and Frederick H. White, Jr. The ribonucleases: occurrence, structure, and properties. In: Paul D. Boyer, Henry Lardy, and Karl Myrback's The enzymes. vol. 5. New York, Academic Press, 1961. p. 95-122

2. Anson, M. L. The estimation of pepsin, trypsin, papain, and cathepsin with hemoglobin. Journal of General Physiology 22:79-89. 1938.

3. Bandack-Yuri, S. and Dyson Rose. Proteases of chicken breast muscle. Food Technology 15:186-188. 1961.

4. Bouma, J. M. W. and M. Gruber. The distribution of cathepsin B and C in rat tissues. Biochemica et Biophysica Acta 89:545-547. 1964.

5. Chiambalero, C. J., D. A. Johnson, and M. P. Drake. A time-temperature relationship for heat-enzyme inactivation of radiation-sterilized beef and pork. Journal of Agricultural and Food Chemistry 7:782-784. 1959.

6. de Duve, C. Lysosome, a new group of cytoplasmic particles. In: Teru Hayashi's Subcellulose particles. New York, The Ronald Press, 1959. p. 128-157.

7. De la Haba, G., Peter S. Cammarata, and S. N. Timasheff. Partial purification and some physical properties of cathepsin C from beef spleen. Journal of Biological Chemistry 234:316-319. 1959.

8. Dixon, Malcolm and Edwin C. Webb. Enzymes. 2d ed. New York. Academic Press, 1964. 950 p.

9. Dvorak, Z. Proteolytische Aktivitat im Rindermuskel post mortem. Collection of Czechoslovak Chemical Communications 25: 2059-2069. 1960.

10. Evtikhina, Z. F. and M. P. Chernikov. Purification and some properties of cathepsin from the spleen of cattle. Aktual'nye Voprosy Sovremennoi Biokhimii Akademiya Meditsinskikh Nauk SSSR, Institut Biologicheskoi i

Meditsinskoi Khimii 2:78-84. 1962. (Abstracted in Chemical Abstracts 58:699e. 1963).

11. Firfarova, K. F., A. D. Morozkin, and V. M. Orekhorick. Isolation of proteinase from the brain tissues. Biokhimiya 29:673-679. 1964. (Abstracted in Chemical Abstracts 61:14958h. 1964).

12. Folin, O. and V. Ciocalteu. On tyrosine and tryptophane determinations in proteins. Journal of Biological Chemistry 73:627-650. 1927.

13. Fruton, S. Joseph and Mary J. Mycek. Beef-spleen cathepsin C. Archives of Biochemistry and Biophysics 65:11-20. 1956.

14. Fruton, S. Joseph. Cathepsins. In: Paul D. Boyer, Henry Lardy, and Karl Myrback's The enzymes. vol. 4. New York, Academic Press, 1960. p. 233-240.

15. Greenbaum, M. Lowell and Joseph S. Fruton. Purification and properties of beef-spleen cathepsin B. Journal of Biological Chemistry 226:173-180. 1957.

16. Groninger, Jr., H. S. Partial purification and some properties of a proteinase from albacore (Germo alalunga) muscle. Archives of Biochemistry and Biophysics 108:175-182. 1964.

17. Hartley, B. S. Proteolytic enzymes. Annual Review of Biochemistry 29:45-72. 1960.

18. Koszalka, Thomas R. and Leon L. Miller. Proteolytic activity of rat skeletal muscle. I. Evidence for the existence of an enzyme active optimally at pH 8.5 to 9.0. Journal of Biological Chemistry 235:665-669. 1960a.

19. Koszalka, Thomas R. and Leon L. Miller. Proteolytic activity of rat skeletal muscle. II. Purification and properties of an enzyme active optimally at pH 8.5 to 9.0. Journal of Biological Chemistry 235:669-672. 1960b.

20. Lapresle, C. and R. Webb. The purification and properties of a proteolytic enzyme, rabbit cathepsin E, and further studies on rabbit cathepsin D. Biochemistry Journal 84:455-462. 1962.

21. Levyant, M. I. Purification and properties of cathepsin from swine kidney. Aktual'nye Voprosy Sovreminnoi Biokhimii Akademiya Meditsinskikh Nauk SSSR, Institut Biologicheskoi i Meditsinskoi Khimii 2:85-91. 1962. (Abstracted in Chemical Abstracts 57:17049g. 1962).

22. Lichtenstein, Noah and Joseph S. Fruton. Studies on beef spleen cathepsin A. Proceedings of the National Academy of Science 46:787-791. 1960.

23. Lishko, V. K. Purification and some properties of brain cathepsin. Ukrains'kii Biokhimicheskii Zhurnal 36:565-573. 1964. (Abstracted in Chemical Abstracts 61:14962g. 1964).

24. McMaster, P. B. and T. Webb. Purification and properties of cathepsin D from the human-spleen. Annales de l'Institut Pasteur 104:90-101. 1963. (Abstracted in Chemical Abstracts 58:794e. 1963).

25. Peterson, Elbert A. and Ellen A. Chiazze. Some experimental factors in the gradient chromatography of serum proteins. Archives of Biochemistry and Biophysics 99:136-147. 1962.

26. Peterson, Elbert A. and Herbert A. Sober. A variable gradient device for chromatography. Analytical Chemistry 31:857-862. 1959.

27. Planta, R. J. and M. Gruber. Chromatographic purification of the thiol enzyme cathepsin C. Biochimica et Biophysica Acta 89:503-510. 1964.

28. Press, E. M., R. R. Porter and J. Cebra. The isolation and properties of a proteolytic enzyme, cathepsin D, from bovine spleen. Biochemical Journal 74:501-514. 1960.

29. Rampton, James Henry. Fractionation of bovine sarcoplasmic proteins by DEAE-cellulose chromatography. Master's thesis. Corvallis, Oregon State University, 1965. 40 numb. leaves.

30. Siebert, Gunther. Protein-splitting enzyme activity of fish flesh. Experientia 14:65-66. 1958.

31. Siebert, Gunther. Enzymes of marine fish muscle and their role in fish spoilage. In: Fish in Nutrition, International Congress. Washington D. C. 1961. London, Fishing News (Books), 1962. p. 80-82.

32. Sinnhuber, R. O. and M. K. Landers. Enzyme-inactivation studies on irradiation-sterilized cod fillets. Journal of Food Science 29:190-191. 1964.

33. Sliwinski, R. A., D. M. Doty and W. A. Landmann. Overall assay and partial purification procedure for proteolytic enzymes in beef muscle. Agricultural and Food Chemistry 7:788-791. 1959.

34. Sliwinski, R. A. et al. Proteolytic enzymes in beef muscle tissue. In: Annual report, 1959-1960, Chicago, 1961, p. 20. (American Meat Institute Foundation. Bulletin 45).

35. Snoke, John E. and Hans Neurath. The proteolytic activity of striated rabbit muscle. Journal of Biological Chemistry 187:127-135. 1950.

36. Stefanoric, J., T. Webb and C. Lapresle. Cathepsin D and E in rabbit polymorphonuclear cells, macrophages, and lymphocytes. Annales de l'Institut Pasteur 103:276-284. 1962. (Abstracted in Chemical Abstracts 58:794e. 1963).

37. Stein, Olya and Joseph S. Fruton. Proteinase activity of rat lymph nodes. Yale Journal of Biology and Medicine 33:71-78. 1960. (Abstracted in Chemical Abstracts 59:4338d. 1963).

38. Tallan, H. Harris, Mary Ellen Jones and Joseph S. Fruton. On the proteolytic enzymes of animal tissues. Journal of Biological Chemistry 194:793-805. 1952.

39. Terlizzi, L. and M. Mitolo. The catheptic activity of central nervous substance. Bollettino della Soceita Italiana di Biologia Sperimentale 31:1487-1489. 1955. (Abstracted in Chemical Abstracts 50:12245c. 1956).

40. Zender, R. et al. Aseptic autolysis of muscle: Biochemical and microscopic modifications occurring in rabbit and lamb muscle during aseptic and anaerobic storage. Food Research 23:305-326. 1958.