ISOLATION AND CHARACTERIZATION OF GASTRIC PROTEASES FROM THE GREENLAND COD (GADUS OGAC)

ELI JAMES SQUIRES

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ISOLATION and CHARACTERIZATION of GASTRIC PROTEASES

from the

GREENLAND COD (*GADUS OGAC*)

A thesis submitted by

Eli James Squires, BSc., MSc.

in partial fulfilment of the requirements for
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ABSTRACT

The Greenland cod *Gadus ogac* is a sub-arctic species that thrives year round in the North Atlantic. It is postulated that the gastric proteases of this fish have several properties in common with the gastric proteases from mammalian species but also have several characteristics unique to fish species. Therefore, the gastric proteases and their zymogens were isolated from the stomach mucosa of the Greenland cod *Gadus ogac* and their properties compared to mammalian gastric proteases and the gastric proteases of other fish. Attempts were also made to purify porcine pepsin A and porcine gastricsin from a crude commercial pepsin preparation. The properties of the purified "gastricsin fraction" obtained differed substantially from the literature data on porcine gastricsin so that direct comparisons of many of the properties of gastricsin and the cod proteases could not be made.

The zymogens of three gastric proteases were separated and purified by Sephadex G100 chromatography at pH 7, chromatofocusing and, after activation of the zymogens, Sephadex G75 chromatography at pH 2.5. The zymogens of protease 1, 2, and 3 had isoelectric points of >7.5, 6.2 and 5.2 respectively. The zymogens of the Greenland cod gastric proteases were activated much more rapidly at low temperature than porcine pepsinogen. All three of the cod proteases had more alkaline pH optima with protein substrates than porcine

pepsin, especially with methylated protein substrates. The pH optima of cod protease 2 and 3 and porcine pepsin with peptide substrates were all near pH 2 while the pH optimum of cod protease 1 with APDT was near pH 3. The specific activities of the individual cod proteases at 26 °C with protein substrates were generally lower than porcine pepsin. However, a mixture of the cod proteases had activity with the protein substrates that was greater than the sum of the activities of the individual proteases. Cod protease 2 and 3 were active on a number of peptide substrates that are good substrates for gastricsin while cod protease 1 was active only with APDT (N-acetyl-phenylalanine-diiodotyrosine) of all the peptide substrates investigated. The milk clotting activities of the cod proteases were much greater than that of porcine pepsin and the cod proteases had CU/PU ratios (the ratio of the clotting activity to hydrolytic activity with hemoglobin) that were 20-50 times higher than porcine pepsin. The individual cod proteases hydrolysed hemoglobin to a greater extent than porcine pepsin, indicating their wider substrate specificity. Porcine pepsin had a high Vmax and low Km' compared to cod protease 1 which had low to moderate Vmax and Km' with all substrates. Cod protease 2 had comparatively high Vmax and Km' with hemoglobin as substrate and moderate to low Vmax and Km' with the other substrates. Cod protease 3 had comparatively low to moderate Vmax and Km' with hemoglobin and casein, high Vmax and Km' with methylated hemoglobin and low Vmax and high Km' with the peptide substrates. No significant differences were found in the activation energy for the hydrolysis of the various substrates by the different protease preparations.

The cod proteases were less stable to heating and retained less activity at

extremes of pH (less than 3 and greater than 6.5) than porcine pepsin. The activities of cod protease 1 and 2 with hemoglobin as the substrate were doubled in the presence of 25 mM NaCl while cod protease 3 and porcine pepsin were not stimulated by salt. The cod proteases did not cross react with antibodies raised against porcine pepsin. Antibodies were also obtained against the purified proteases T16 and T25 from psychrotrophic pseudomonads. Anti-T16 IgG precipitated cod protease 1 and porcine pepsin at similar IgG/enzyme ratios but did not affect cod proteases 2 and 3. Anti-T25 IgG precipitated all the proteases, cod protease 2 and porcine pepsin being precipitated at similar IgG/enzyme ratios. The subunit molecular weights of all the proteases were in the range of 35-37 kdal as estimated by SDS-PAGE and amino acid composition. The amino acid compositions of the cod proteases differed from the mammalian gastric proteases by about the same extent that pepsin, gastricsin and chymosin differ from each other. Cod protease 1 was the most different of the cod proteases from the mammalian proteases, while cod protease 3 was more like chymosin. Cod protease 1 had the lowest hydrophobicity index and chymosin had the highest. The hydrophobicity indices of cod protease 2 and 3 were intermediate between that of porcine pepsin A and chymosin.

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List of Abbreviations

APDT	N-acetylphenylalanine diiodotyrosine
asx	aspartic acid + asparagine
BSA	bovine serum albumin
DAN	diazoacetyl-DL-norleucine methyl ester
DOC	deoxycholate
Ea	activation energy
EPNP	1,2-epoxy-3-(p-nitrophenoxy)propane
glx	glutamic acid + glutamine
HPLC	high performance liquid chromatography
Km'	apparent Michaelis-menton constant
PAGE	polyacrylamide gel electrophoresis
pI	isoelectric point
Rf	relative mobility
SDS	sodium dodecyl sulfate
TCA	trichloroacetic acid
TFA	trifluoroacetic acid
TNBS	trinitrobenzene sulfonic acid
Z-	benzyloxycarbonyl-

Chapter 1
INTRODUCTION

1.1. Historical

Some of the earliest observations on the action of enzymes were with proteolytic enzymes involved with the process of digestion. These early works that will be referred to here can be found in a recent book edited by Friedmann (1981). The first of these papers was written by R. A. deReaumur in 1761 and was entitled "Sur la Digestion des Oiseau, Second Memoire: De la manniere dont elle se fait dans l'estomac des oiseau de proie". In this work, deReaumur takes advantage of the natural ability of buzzards to regurgitate what they cannot digest. He fed these birds meat that was enclosed in a metal tube with wire gauze covering the ends and found that the meat was partially digested even though it was protected from physical grinding. Comparable studies with plant materials showed that they were not digested. This work was extended by A. L. Spallanzani in 1789 in his "Dissertation Relative to the Natural History of Animals and Vegetables" using other animals. He also noted that his own gastric juice *in vitro* could digest meat. He recognized the active principle in gastric juice had the requirement for warm temperatures during the digestion and that the rate of digestion was proportional to the amount of gastric juice. In 1833, W. Beaumont showed that gastric juice contained "some chemical principles" as well as

hydrochloric acid and that the activity was found in gastric mucosa as well as in gastric juice.

The word "pepsin" was first used in T. Schwann's 1836 paper "On the Nature of the Digestive Process". In this work, Schwann stressed the analogy of the action of the unchanged acid in gastric digestion to the action of acid in the hydrolysis of starch. He recognized that digestion by pepsin was another example of the type of activity represented by alcoholic and acetic acid fermentation. He was also the first to suggest a method for the purification of an enzyme that involved a sequence of purification steps. E. W. von Brucke (1861), in his paper "Contribution to the Theory of Digestion, Second Part", was the first to use adsorption methods for enzyme purification. These methods were developed further by R. M. Willstatter in 1926 with his paper "On Progress in Enzyme Isolation". In fact, enzymes were purified to the point that their enzymatic activity was still strong but the protein could not be detected by the methods then available. This supported the widely held view of that period that enzymes were not proteins at all but were catalytic groups somehow associated with protein. The crystallization of urease by J. B. Sumner in 1926 was the first example of a crystallized enzyme and was followed in 1930 by J.H. Northrop when he crystallized pepsin. These works and others that followed on the crystallization of trypsin and chymotrypsin established that enzymes were in fact proteins. J. N. Langley in 1882 and Langley and Edkins in 1886 demonstrated the presence of pepsinogen when they found that a slightly alkaline extract of gastric mucosa which was inactive had peptic activity after acidification. Pepsinogen was first crystallized by R.M. Herriott (1938).

1.2. Properties of Gastric Proteases

1.2.1. General

From the earliest work of Northrop (1930) on the crystallization of porcine pepsin, a considerable heterogeneity was found in pepsin preparations as indicated by measurements of their solubility behavior. With the advent of ion exchange chromatography, Ryle and Porter (1959) separated parapepsins I and II from porcine gastric mucosa. The predominant enzyme from this tissue has since been named pepsin A (EC 3.4.4.1) and is secreted by the fundic area of the stomach. Parapepsins I and II have been renamed pepsin B (EC 3.4.4.2) and pepsin C (EC 3.4.4.3) respectively. Pepsin B was formerly called "gelatinase" by Northrop (1932). Pepsin C was isolated from human gastric juice by Tang *et al.* (1959) and was reisolated from porcine gastric mucosa (Chiang *et al.*, 1967) and called "gastricsin". It is secreted by the pyloric region of the stomach. The zymogen of pepsin B has been studied by Ryle (1965) and the zymogen of pepsin C has been studied by Tang and Tang (1963) and by Ryle and Hamilton (1966). In addition, Lee and Ryle (1967,1967a) reported the existence of pepsin D and its zymogen which are the dephosphorylated forms of pepsin A. Other investigators have separated enzymes from human and porcine stomachs and have used other symbols to denote the various fractions. The early literature in this area has been reviewed by Taylor (1962). Foltmann and Pedersen (1977) have suggested that the gastric proteases can be classified in three groups; (1) the main component, pepsin A, (2) the minor components, pepsin B (gelatinase) and pepsin C (gastricsin) and (3) the fetal or neonatal proteases, which have historically been called chymosin or rennin. Heterogeneity may exist within each of these groups because of multiple

gene products resulting in only a few amino acid substitutions, variable degrees of phosphorylation or carbohydrate content and autolysis of the pepsins, especially during activation and purification.

In addition to the pepsins from pig and man, pepsins have been isolated from cow (Northrop, 1933), horse (Stepanov *et al.*, 1976), sheep (Fox *et al.*, 1977), monkey (Kageyama and Takahashi, 1976), mouse (Esumi *et al.*, 1978), chicken (Donta and Vunakis, 1970), toad (Ward *et al.*, 1978), and various fish species including sardine (Noda and Murakami, 1981), salmon (Norris and Elam, 1940), tuna (Norris and Mathies, 1953), dogfish (Merrett *et al.*, 1969) and bonito (Kubota and Ohnuma, 1970). Trout pepsin has been studied in crude homogenates (Owen and Wiggs, 1971). Partially purified pepsins have also been studied from Atlantic cod *Gadus morhua* (Brewer *et al.*, 1984), Greenland cod *Gadus ogac*, smelt *Osmerus mordax* (Haard *et al.*, 1982) and Arctic cod *Boreogadus saida* (Arunchalam and Haard, 1984).

1.2.2. Properties of Pepsin A

The properties of porcine pepsin have been summarized by Ryle (1970). Pepsin is unstable at pH values above 6 and below 1 while pepsinogen is unstable below pH 5, being activated to pepsin at a more acid pH. Pepsinogen is unstable at elevated temperatures at pH 7 or at room temperature in the pH 8.5-11 range. The amino acid compositions of pepsin A and C and their zymogens are known (Ryle, 1970, Fruton, 1970). Pepsin D is not distinguishable in amino acid composition from pepsin A. Porcine pepsin A consists of a single polypeptide chain cross-linked by three disulfide bridges. The amino terminal amino acid is

leucine for the zymogen and isoleucine for the active enzyme; the carboxyl terminal residue is alanine for both the zymogen and the active enzyme. The complete sequence of porcine pepsin is known (Dayhoff, 1972). The phosphate group of pepsin A is bound to serine 33 but is not required for activity. However, the reduction of the disulfide bridges of pepsinogen results in the loss of the potential enzyme activity. The molecular shape of pepsin A is a prolate elipsoid (roughly egg-shaped) with an a/b axial ratio of 2.5-3.0 (Fruton, 1971). The molecular weight of pepsin is about 35 kdal and the molecular weight of the zymogen is approximately 40 kdal. The isoelectric point (pI) of pepsin is below 1, dephosphorylation shifts the pI to 1.7. However, the isoelectric point of pepsinogen is 2.8, since the 44 residue portion of pepsinogen removed during the activation contains a majority of basic residues.

Pepsin has a rather broad specificity compared to the serine proteases (e.g. trypsin only attacks peptide bonds with a carboxyl arginine or lysine). Generally, activity is greatest when the bond to be hydrolysed is next to bulky nonpolar amino acid residues, particularly tyrosine and phenylalanine. However, even this specificity is not absolute as poly-L-glutamic acid is rapidly hydrolysed to oligopeptides (Neumann *et al.*, 1962). Pepsin A readily hydrolyses N-acetylphenylalanine diiodotyrosine (APDT) while Z-tyr-ala is not hydrolysed. The specificity of pepsin A has been systematically studied using peptide substrates (Fruton, 1971, 1982). With small peptides of the type A-X-Y-B, where X-Y is the susceptible bond, maximal rates of hydrolysis are obtained when X= phenylalanine or *p*-nitro-L-phenylalanine and Y= phenylalanine, tyrosine or tryptophan. If either X or Y is another hydrophobic amino acid such as leucine or

methionine, the bond is hydrolysed, but at a slower rate. However, if X= valine or isoleucine the bond is not cleaved while if Y= valine or isoleucine the bond is broken. Structural modification of the A and B groups of A-X-Y-B is important. If these groups are lengthened to include a number of hydrophobic amino acids, the rate of hydrolysis of the X-Y bond is increased by several orders of magnitude and peptide bonds that were previously not susceptible may be hydrolysed. This increased susceptibility of bonds located in a larger peptide is probably due to very significant secondary enzyme-substrate interactions between the A and B groups and hydrophobic groups on the enzyme. These effects are based on Vmax rather than on Km. Fruton (1976) has suggested that this effect is associated with a flexible, extended active site of pepsin and this has been substantiated by X-ray crystallography studies (Andreeva *et al.*, 1977).

1.2.3. Properties of Minor Pepsins

Porcine pepsins B and C are stable at pH 6.9 at room temperature while pepsin A is unstable above pH 6. Pepsin B is a highly active gelatinase (Northrop, 1932), a poor milk clotting enzyme and has little activity on hemoglobin. It has not received a lot of attention in the literature. The specificities of the minor pepsins have been studied using the β chain of oxidized insulin (Ryle, 1970) and they have been found to be slightly more restricted than pepsin A with this substrate. Pepsin C has little activity with APDT but is very active with protein as the substrate (Ryle *et al.*, 1969). The amino acid composition of porcine pepsin C differs from that of pepsin A (Fruton, 1971), notably a decrease in lysine, serine and isoleucine and an increase in glutamate, alanine and leucine in pepsin C. Good agreement was found between the amino

acid compositions of pepsin C (Ryle and Hamilton, 1966) and gastricsin (Chiang *et al.*, 1967) to support the conclusion that they are the same protein. The substrate specificity of gastricsin with peptide substrates has been determined (Tang, 1970). Peptides that are readily hydrolysed by human gastricsin yet are not suitable substrates for pepsin A include Z-tyr-ala, Z-tyr-thr, Z-trp-ala, Z-tyr-leu and Z-tyr-ser. The substrate specificities of human pepsin and gastricsin have also been investigated with glucagon and oxidized ribonuclease as substrates (Huang and Tang, 1969). There were 23 common hydrolytic sites, 2 pepsin specific and 4 gastricsin specific sites with the two protein substrates. Human and porcine gastricsin and pepsin have similar specific activities with the milk clotting assay. The pH optimum of human and porcine gastricsin with hemoglobin as substrate is pH 3 compared to the pH 2 optimum for pepsin (Richmond *et al.*, 1958). Gastricsins from both human and porcine have slightly lower molecular weights than their corresponding pepsins (Mills and Tang, 1967). The amino terminal residue of human gastricsin is serine. The C-terminal sequence of human gastricsin is known (Tang, 1970) and it is very homologous to the C-terminal sequence of human pepsin. The zymogen of gastricsin has been isolated from humans (Foltmann and Jensen, 1982). Bovine gastricsin has been isolated (Martin *et al.*, 1982). In this species, the heterogeneity of the gastricsin preparations appear to be due to varying degrees of phosphorylation. These authors have pointed out that in the amino acid compositions of the minor pepsins the ratios of isoleucine/leucine and asx/glx are in the range of 0.5-0.7, while for the pepsin A group these ratios are higher (1.3-1.5).

1.2.4. Properties of the Fetal Proteases

Fetal proteases or their zymogens have been found in humans (Hirsch-Marie et al, 1976), rats (Kotts and Jenness, 1976), rabbits (Henschel, 1973), lambs (Alais *et al.*, 1962), calves (Foltmann, 1966) and juvenile and adult harp seals (Shamsuzzaman and Haard, 1984). The importance for this group of enzymes is their extraordinary ability to clot milk and calf chymosin has been used in cheese production for centuries. Chymosin has been purified from calf mucosa by salt precipitation and ion exchange chromatography (Foltmann, 1970). The zymogen form of the enzyme, prochymosin, is stable from pH 5.5 to 9 and is activated to chymosin at lower pH values. The amino acid compositions of the enzyme and zymogen are known. The N-terminal amino acid of prochymosin is alanine and the N-terminal amino acid residue of chymosin is glycine. The molecular weight of the zymogen is 40.8 kdal and the molecular weight of the enzyme is 35.7 kdal. Several fractions (A, B, C) of chymosin have been separated by an ion exchange chromatography and are separable due to minor changes in amino acid composition (eg. position 244 may be glycine or aspartate). Bovine chymosin has a high degree of sequence homology with pepsin A and it moves the slowest on agar gel electrophoresis at pH 8.2 of all of the pepsin-like enzymes. Solutions of chymosin have optimal stability in the pH 5.3-6.3 range but in salt free solutions the enzyme is stable at pH 2. Salt also increases the rate of activation of the zymogen. The concentration of salt also greatly affects the solubility of the enzyme, chymosin being very soluble in 1M NaCl at pH 5.5. The isoelectric point of bovine chymosin is 4.6.

Chymosin was first found in the stomach of young ruminants but later chymosin-like enzymes have been found in the young of some simple-stomached mammals including human, rat, pig and seal. It is probable that chymosin is found in all young mammals that receive maternal antibodies in the milk. Foltmann (1980) has suggested that the function of chymosin is to clot the milk by selective proteolysis of casein. In this manner, the rate of passage of the milk through the digestive tract is slowed down so that digestion can occur in the intestine. Meanwhile very little destruction of the antibodies from the mothers milk occurs in the stomach, allowing them to be absorbed by the young. Harp seal appears to be unique since it produces significant amounts of chymosin-like enzyme after maturity (Shamsuzzaman and Haard, 1984).

1.2.5. Properties of the Gastric Proteases from Other Mammalian Species

As mentioned above, gastric proteases have been isolated from a large number of different species. Although the human, porcine and bovine proteases have been classified (Barrett and McDonald, 1980), no such compendium of the gastric proteases from other species exists. This is probably due to the somewhat sketchy data available to date. In the discussion below, the properties of the gastric proteases from a wide variety of species will be compared to the properties of the porcine gastric proteases.

The gastric proteases from the Japanese monkey *Macca fuscata fuscata* have been characterized in a series of papers by Kageyama and Takahashi (1976, 1976a, 1980, 1982). Five enzyme fractions were isolated by gel filtration and

DEAE cellulose chromatography and designated as I, II, III-1, III-2 and III-3. All five fractions produced active enzymes on exposure to acid. The molecular weights of pepsin I and II were 48 kdal and 43 kdal respectively and because of almost identical properties the authors felt that they represented the same enzyme with different contents of carbohydrate. Pepsinogens III-1, III-2 and III-3 all had molecular weights of 40 kdal and very similar amino acid compositions, sensitivity to inhibitors, and stability characteristics. Pepsin III-3 was thought to correspond to the type A pepsin since it accounted for more than half of the total pepsin content. In their later work, these authors further purified fraction III-2 and isolated a protease that was similar to pepsin C (gastricsin). Pepsin III-1 was found to be dephosphorylated pepsin A.

Shaw and Wright (1976) have isolated the pepsinogens from the cat gastric mucosa. They have found at least eight different zymogens which produce discrete zones of proteolytic activity on agar gels. Only one of these proteases was active on APDT. An electrophoretically slow moving zymogen was identified by Jensen *et al.* (1982) as prochymosin. The active chymosin showed partial immunochemical identity with calf chymosin. Comparison of the N-terminal 27 residues of both cat prochymosin and chymosin to the corresponding sequence of the calf prochymosin and chymosin gave only 13 differences out of the 54 residues. Chymosin from the cat had a molecular weight of 36 kdal. It was present in new born kittens before any of the other proteases.

Pepsin has been isolated from sheep by Fox *et al.* (1977) using pH fractionation, chromatography on a Sepharose 4B-poly-L-lysine column and gel

filtration. These authors did not identify more than one sheep gastric protease using these techniques. The molecular weight of sheep pepsin was 34 kdal with N-terminal valine and C-terminal alanine. The pH optimum was 1.8 using both methylated hemoglobin and methylated casein as substrates. The apparent Km (Km') for both methylated substrates was 50 μM while the kcat (defined as the Vmax/[enzyme] ratio) for methylated casein was 420 min^{-1} and the kcat for methylated hemoglobin was 1100 min^{-1}. The amino acid composition of sheep pepsin was similar to that of porcine pepsin.

Six different proteases were isolated from the gastric juice of the horse (Stepanov *et al.*, 1976) using Sepharose-6B-Gramacidin-S-adsorption chromatography. The proteases all had pH optima with hemoglobin at 1.8 and were more stable at alkaline pH than porcine pepsin. The different proteases had isoelectric points at pH 2.1, 2.3, 2.6, 2.8, 3.2 and 3.6 with the pI 3.2 and 3.6 enzymes present in the greatest amounts. The amino acid composition of the pI 2.3 protease has been reported by these authors. Proteases with pI 2.1 and 2.3 contained 2 phosphorous atoms per molecule of enzyme, all other proteases contained one. The authors have sequenced the amino terminal portion of each of the proteases. They have found that the amino acid sequences of the various horse proteases are more similar to each other than comparable sequence comparisons between porcine pepsin and gastricsin. The sequence of the pI 3.2 protease differs to the greatest extent from the other horse proteases. The authors have found that the relative yields of the various proteases differed between batches of gastric juice.

Esumi *et al.* (1978) have purified two pepsinogens from mouse gastric mucosa using Sepharose 4B-pepstatin affinity chromatography, gel filtration and Sepharose CL6B chromatography. Three other minor bands of activity were found on polyacrylamide gels but these accounted for only 10% of the total gastric protease activity. The molecular weight of the major pepsinogens was 41 kdal. Each pepsinogen gave rise to two separate pepsins after initial exposure to acid. The molecular weights of these pepsins were 38 kdal and 36 kdal. When the activation was complete, only the 36 kdal pepsins remained. Thus, it is apparent that the activation of mouse pepsinogen can occur in more than one step. Mouse pepsins are active with hemoglobin as substrate and in the milk clotting assay.

The presence of chymosin and pepsin was inferred in rats *Rattus norvegicus* at different stages of development by Kotts and Jenness (1976). These authors used crude stomach homogenates and compared the activity at various pH values to that of crystalline calf chymosin and porcine pepsin. They found that in one day old rats the pH profile of the stomach homogenate was more like chymosin than pepsin with a pH optimum at 3.5-4.0. However in 28 day old rats the main activity was at pH 2.2 which corresponded to the pH optimum of porcine pepsin. The authors therefore speculate that the main gastric protease in the young rat is chymosin-like and that this protease is gradually replaced by a pepsin-like protease after weaning.

The mammalian gastric proteases can be classified as pepsins, gastricsins and chymosins according to their different properties as listed in Table 1-1.

Table 1-1: Summary of the Properties of Mammalian Pepsins, Gastricsins and Chymosins

Property	Pepsin	Gastricsin	Chymosin
pH Optimum with Hemoglobin	2.0	3.0	3-4
Hydrolysis of APDT	v. active	inactive	inactive
Milk Clotting Activity	moderate	moderate	v. high
Elution from Amberlite Column	pH 2.1	pH 4.6	
Molecular Weight	35 kdal	32 kdal	35.7 kdal
Ratios ile/leu, asx/glx	1.3-1.5	0.5-0.7	
Site of Production	fundus	pylorus	fetal stomach
Immunology	distinct	distinct	distinct

1.2.6. Properties of Non-Mammalian Gastric Proteases

The various pepsins and their zymogens have been studied in the chicken *Gallus gallus* by two groups; Bohak (1970) and Donta and Van Vunakis (1970). The first group found that chicken pepsinogen eluted from a DEAE cellulose column as a single peak. The activated pepsin was stable between pH 1 and 8 and had a free cysteine in addition to the three disulfide bridges present in all the pepsins discussed so far. There were also 2 glucosamines and 6-7 hexoses per molecule of chicken pepsin. In contrast, Donta and Van Vunakis have described 4 separate pepsinogens from the chicken. The three major pepsinogens, A, C and D have high activity with hemoglobin after activation and are present in a ratio of 3:1:2 respectively. Pepsinogen B was present in very small amounts. Pepsin B was

inactive in the milk clotting assay and with hemoglobin as substrate but was active with Z-glutyr at pH 4. Pepsins A and D have similar amino acid compositions and immunological reactivities that were different from those of pepsin C. They have confirmed the greater pH stability of the chicken pepsins compared to porcine pepsin and suggest that this may be due to the higher number of basic residues in chicken pepsin. The major chicken pepsins were less active than porcine pepsin in the milk clotting assay. These authors also confirm the presence of a free cysteine in chicken pepsin A.

Two zymogens have been isolated from the gastric mucosa of the toad *Caudiverbera caudiverbera* by Ward *et al.* (1978) by gel filtration and an ion exchange chromatography. Zymogen I (Z1) gave a gastricsin-like enzyme after activation that eluted from an Amberlite IRC-50 column at pH 4.6, had optimal activity with hemoglobin as the substrate at pH 3, and had virtually no activity on APDT. Zymogen II (Z2) produced a pepsin-like enzyme after activation that was only 13% as active with APDT as porcine pepsin. These authors have noted the increased stability of the toad proteases to alkali compared to porcine gastric proteases. They have speculated that there may be an inverse correlation between the position of the species on the evolutionary scale and the alkali stability of its gastric proteases.

1.2.7. Properties of the Gastric Proteases of Fish

The gastric proteases from a number of fish species have been isolated and characterized. The first of the fish pepsins to be studied was that of the Pacific salmon *Oncorhynchus tschawytscha* by Norris and Elam (1940). In this early work pepsin was purified by crystallization and the purity of the preparations was estimated by solubility curves. These authors found that salmon pepsin contained twice as much cysteine as porcine pepsin. The pH optimum with hemoglobin as the substrate was pH 2 for both salmon and porcine pepsin prepared by acid extraction of the gastric mucosa, except that salmon pepsin still retained 50% of its activity at pH 4.5 while porcine pepsin was completely inactivated. However, when salmon pepsin was prepared by alkaline extraction of the gastric mucosa followed by acidification of the zymogen to form active pepsin, a pH curve with a plateau from pH 2-3.5 was obtained. The rate of hemoglobin hydrolysis by salmon pepsin was tripled in the presence of 0.1M NaCl. Salmon pepsin was unable to hydrolyse small peptides of glutamate and tyrosine which were good substrates for porcine pepsin (Fruton and Bergman, 1940).

Pepsin was also crystallized from three species of tuna by Norris and Mathies (1953). These pepsins had greater activity towards hemoglobin than any other pepsin isolated to that time. Tuna pepsin was less stable to alkali than salmon pepsin but was more stable than porcine pepsin. In the absence of salt, tuna pepsin displayed double pH optima with hemoglobin as the substrate. When buffer salts were present, the activity was increased and a single pH optimum at pH 2.5 was obtained. At pH 2 tuna pepsin was 50% inactivated by heating to 56

°C while porcine pepsin was 50% inactivated at 63 °C. The isoelectric point of tuna pepsin determined as the point of minimum solubility was 3.8.

The first report of the isolation of different pepsinogen isoenzymes from a fish was that of Merrett *et al.* (1969) and Bar-Eli and Merrett (1970). These authors separated four pepsinogens from the gastric mucosa of the smooth dogfish *Mustelus canis* by DEAE cellulose chromatography. Pepsin B was active with Z-glu-tyr but not with hemoglobin while pepsins A, C and D were active with protein substrates. Pepsins A and D were similar in chromatographic behavior, immunological reactivity and amino acid composition, and were different from pepsin C. Pepsin B had a higher molecular weight (45 kdal) compared to the other dogfish pepsins (34-36 kdal). The dogfish pepsins were 3-5 times more active than porcine pepsin with hemoglobin as substrate but had only 5% of the milk clotting activity of porcine pepsin. Dogfish pepsin A displayed a broader pH optimum with hemoglobin as the substrate than porcine pepsin A.

Two gastric proteases have been isolated from the sardine by Noda and Murakami (1981). These workers used ammonium sulfate fractionation, CM cellulose and gel filtration chromatography in their purification scheme. The amino acid compositions of the two proteases were similar except that enzyme I was higher in threonine, serine, and proline than enzyme II. Enzyme I was reported to contain 9 cysteine residues while enzyme II contained 7. Both enzymes were inhibited by pepstatin to almost the same extent as porcine pepsin. Enzyme I had a pH optimum with hemoglobin at pH 4.0 while the optimum pH for enzyme II was 2.0. The enzymes were unstable above pH 7, enzyme I being less stable

than enzyme II. Sardine gastric proteases hydrolysed-APDT at only 10% of the rate of porcine pepsin. In strong contrast to other fish gastric proteases, the sardine gastric proteases were strongly inhibited by NaCl with casein or hemoglobin as the substrate. This inhibition by salt was somewhat lessened with sardine muscle proteins as the substrate. These proteins were hydrolysed at a slower rate than hemoglobin in the absence of salt.

Pepsin has also been partially purified from the bonito *Sarda sarda* (a relative of the mackerels and tunas) by Kubota and Ohnuma (1970) by ammonium sulfate fractionation of a distilled water extract of gastric mucosa. No attempt was made by these authors to separate pepsin isoenzymes. The pH optimum of the bonito pepsin preparation was near pH 2 but more activity remained at higher pH compared to porcine pepsin. Bonito pepsin was twice as active with hemoglobin but only 5% as active with APDT as porcine pepsin. Bonito pepsin had a lower temperature optimum, decreased thermal stability, and increased stability to alkali compared to porcine pepsin.

Gildberg and Raa (1983) have separated two gastric proteases from the capelin *Mallotus villosus* by ammonium sulfate fractionation, DEAE cellulose chromatography and gel filtration. The pH optimum of protease I, the major protease, was 3.7 and the pH optimum of protease II was 2.8 with hemoglobin as the substrate. These enzymes had lower molecular weights and higher isoelectric points than porcine pepsin; protease I (pI= 6.6, molecular weight= 23 kdal), protease II (pI= 3.5, molecular weight= 27 kdal). The optimum temperature for hemoglobin hydrolysis was 38 °C for protease I and slightly higher for protease II.

The amino acid compositions of the capelin gastric proteases were reported to be similar to the sardine gastric proteases except that the capelin gastric proteases have higher serine and lower cysteine contents.

The gastric proteases have been isolated by Sanchez-Chiang and Ponce (1981, 1981a, 1982) from the hake *Merluccius gayi* (a relative of the cod). In their early paper, two proteases were separated using DEAE cellulose and DEAE Sephadex A-50 chromatography. The pH optima of the proteases was pH 3 with hemoglobin as substrate and the proteases did not hydrolyse APDT. These authors have classified these proteases as gastricsins because of these properties. However, the proteases were also inactive with Z-trp-ala and Z-tyr-ala which were reported to be good substrates for human and porcine gastricsin (Huang and Tang, 1969). The proteases also eluted from a column of Amberlite IRC-50 at higher pH values than gastricsins. The molecular weights of the fish proteases were lower than for porcine gastric proteases. The fish gastric proteases were stable up to pH 10, more active than porcine pepsin with hemoglobin as the substrate and less active in the milk clotting assay than porcine pepsin. In a later paper these authors report that the hydrolysis of hemoglobin by fish proteases was stimulated dramatically by NaCl, as has been reported for a number of other fish species.

The gastric proteases of the cod *Gadus callarias* have been studied in crude stomach homogenates and gastric juice by Labarre *et al.* (1951). They measured proteolytic activity using skim milk powder incorporated into agar plates. The assay was based on the clarification of the agar due to proteolytic action. In this

assay, the size of the clear zone around the well containing enzyme was a function of the concentration of enzyme in the well. These workers found that the pH optimum for both cod mucosal extract and gastric juice was pH 5 while the optimum pH for porcine pepsin was pH 2. Gastric juice also contained an enzyme with activity at pH 9. The temperature optimum for the hydrolysis of milk protein by gastric juice was 30 °C, while the temperature optimum for mucosal extract was 45 °C. This compared to an observed temperature optimum for porcine pepsin of 52 °C.

The gastric protease activity of the brook trout *Salvelinus fontinalis* was investigated in crude stomach homogenates by Owen and Wiggs (1971). They found that the pH optimum with methylated hemoglobin was at pH 3. Arrhenius plots were discontinuous at 13.3 °C. Below this temperature, the activation energy was 15.2 kcal/mole and above this temperature the activation energy was 9.4 kcal/mole. The kinetic characteristics of the extract obtained from fish held at 5 °C were similar to those held at 15 °C. However, increased amounts of activity were present in the fish held at the colder temperature.

Two pepsins (A and B) have been isolated from the Arctic cod *Boreogadus saida* by Arunchalam and Haard (1984). Both enzymes had pH optima at 2.0, temperature optima of 37 °C and activation energies of approximately 3 kcal/mol with hemoglobin as the substrate. The Km' with hemoblobin as the substrate was 0.06 mM for pepsin A and 1.33 mM for pepsin B, but the physiological efficiencies (Vmax/Km') of both enzymes were similar. Arctic cod pepsin had a temperature coefficient (Q_{10}) of 1.2-1.4 in the milk clotting assay compared to 2-2.2 for

mammalian pepsins. A partially purified preparation of pepsin from the Atlantic cod *Gadus morhua* has been studied by Brewer *et.al.*, (1984). The preparation consisted of a mixture of 4 pepsinogens of subunit molecular weight 41 kdal as determined by SDS-PAGE. The pH optimum was at pH 3 at 30 °C and broadened to pH 2-3 at 5 °C. The enzyme preparation was not very active with APDT and had an Ea for the hydrolysis of hemoglobin of 7.3 kcal/mol at pH 1.9.

It is apparent from the discussion above that the gastric proteases of fish and other lower vertebrates have unique properties that are different from the mammalian gastric proteases. These properties are summarized in Table 1-2.

Table 1-2: Properties of the Gastric Proteases of Fish that are Different from Those of Mammalian Gastric Proteases

generally stimulated by salt
increased stability to alkali
decreased thermal stability
lower temperature optimum
active at higher pH
higher isoelectric points

1.3. Biological Diversity of Acid Proteases

Acid proteases are defined as those having two catalytically important aspartic acid residues in the active site and are also known as aspartate or acid proteases. They are inhibited by compounds that bind to these aspartate residues, namely 1,2-epoxy-3-(p-nitrophenoxy)propane (EPNP) and diazoacetyl-DL-norleucine methyl ester (DAN). In addition, all acid proteases are inhibited by pepstatin, which is a hexapeptide isolated from culture broths of *Streptomyces* bacteria and contains two residues of the unusual amino acid statine (4-amino-3-hydroxy-6-methylheptanoic acid). This residue is thought to be transition state analog of the acid proteases for the hydrolysis of peptide bonds (Marciniszyn *et al.*, 1976).

Acid proteases are found inside the cells of a number of animal tissues as well as serving a purely digestive role extracellularly. These have been summarized by Barrett and Mcdonald (1980). An important group of these intracellular acid proteases are the cathepsins which are localized in the lysosomes (Mycek, 1970). Five cathepsins have been identified (A-E). Cathepsins A and B have pH optima with proteins in the pH 4-5 range while cathepsins D and E are active at pH 2.5-3.5. Cathepsin C is not active on protein substrates and is sometimes referred to as dipeptidyl transferase and also catalyses a dipeptidyl hydrolase reaction. Cathepsin A is characterized by its ability to hydrolyse Z-glu-tyr while cathepsin B requires a sulfhydryl activator such as 2-mercaptoethanol for activity *in vitro*. Cathepsins are found in a wide variety of tissues and are usually isolated from tissues that are rich in lysosomes such as spleen or liver. It is

thought that they are responsible for tissue turnover and that they may play an important role in certain diseased states such as the degradation of cartilage in arthritis (Barrett, 1977). Cathepsin D shows some structural homology to the gastric proteases but does not have a zymogen form. The molecular weight of cathepsin D is larger than pepsin due to the presence of a 100 residue hydrophobic tail at its C-terminal end. It has been suggested (Tang, 1979) that the gastric proteases and cathepsin D may have evolved from the same ancestral protease.

A pepsin-like enzyme has also been isolated from human urine (Zoller *et al.*, 1976). It is thought to originate from either the kidney or the plasma and has been named uropepsin. It has not been well characterized to date but it appears to be related to the major pepsin A group of acid proteases. The kidney produces the acid protease renin which plays a regulatory role in converting angiotensinogen to active angiotensin I. The activated hormone produced by this limited proteolysis is involved in the maintainance of blood pressure. Renin has also been found in the mouse submandibular salivary glands and a high molecular weight precursor for the enzyme has been identified. An acid protease has also been isolated in zymogen form from the seminal plasma of human and guinea pig. The zymogen is thought to become activated in the slightly acidic environment of the vaginal discharge and work to allow the sperm to penetrate the cervical mucus and fertilize the egg. The enzyme appears to have gastricsin-like properties (Chiang *et al.*, 1981).

Pepsin-like acid proteases are produced by a number of yeasts and molds including *Aspergillus, Penicillium, Paecilomyces, Rhizopus* and *Trametes* (Sodek

and Hofmann, 1970). The amino acid compositions and structural and kinetic properties of the various microbial proteases have been reviewed (Matsubara and Feder, 1971). These enzymes are extracellular and have been isolated from the culture broths; they do not exist in zymogen form. One of the most studied of these is penicillopepsin, isolated from the mold *Penicillium janthinellum* (see Hsu *et al.*, 1977 for crystal structure). This enzyme has a very similar specificity and catalytic mechanism to porcine pepsin (Chu and Nakagawa, 1982). A related group of acid proteases, the microbial rennins, are produced by *Endothetica parasitica*, *Mucor pusillus*, *Mucor mehei* and related organisms (Arima *et al.*, 1970). These enzymes have been used in commercial cheese manufacturing in place of calf rennin. An evolutionary scheme for the carboxyl proteases has been suggested (Tang, 1979).

1.4. Structure and Catalytic Mechanism of Acid Proteases

A wide variety of acid proteases have been discovered that differ in their functional locations, biological roles and enzymatic properties. However, all of these enzymes share common structural features and similar catalytic mechanism (Tang, 1979). The structural and catalytic similarities between the acid proteases were confirmed following the elucidation of the complete crystal structure of pepsin (Andreeva *et al.*, 1977) and penicillopepsin (Hsu *et al.*, 1977). As well, the crystal structures of two other fungal acid proteases have been worked out and the complete amino acid sequences of pepsin, chymosin and penicillopepsin are known. The catalytically important residues in the active site of the acid proteases were identified using the two affinity labelling reagents previously mentioned; DAN (diazoacetyl-DL-norleucine methyl ester) esterified to aspartate

215 and EPNP (1,2-epoxy-3-(p-nitrophenoxy)propane) reacted with aspartate 32 of the pepsin sequence. These residues, as well as serine 35, have been conserved in all acid proteases.

The three dimensional structure of pepsin A consists mainly of β sheet structure with only three short segments of α-helix. An apparent substrate binding cleft sufficient to accomodate 7-8 amino acid residues runs the whole length of the molecule. The active site aspartate 32 and aspartate 215 are located in the center of the binding cleft and are hydrogen bonded to each other. Aspartate 32 is also hydrogen bonded to serine 35. The pKa of the carboxyl group of aspartate 32 is estimated to be 2.8 while the pKa of the carboxyl group of aspartate 215 is estimated at 5 due to difference in hydrogen bonding of the two residues. At the acid pH optimum of pepsin, the carboxyl group of aspartate 32 is therefore ionized and the carbonyl carbon of the peptide bond to be hydrolysed undergoes nucleophillic attack by this ionized group. Aspartate 215 has been proposed to be a proton donor to the carbonyl oxygen or amide nitrogen; tyrosine 75 may also be the proton donor to the amide nitrogen. The catalytic mechanism of pepsin has been studied by fitting substrates to the active site of the enzyme in models (Blundell *et al.*, 1980, Foltmann, 1981).

1.5. Mechanism of Pepsinogen Activation

The mechanism of pepsinogen activation has been extensively studied (Marciniszyn *et al.*, 1976a) and involves the removal of 44 amino acid residues from the N terminal end of the zymogen to generate the active enzyme. The activation segment is not released as one intact polypeptide (Kay and Dykes,

1977) and it is now known that leu16-ile17 bond is first hydrolysed in the activation process at pH 2.5. The segments removed during the activation process are composed of a majority of basic residues and contain an inhibition peptide of 29 amino acid residues. This inhibitor binds to pepsin at pH 5 but is cleaved at lower pH values (Kumar *et al.*, 1977). A heterogeneity of pepsin molecules has been found under some activation conditions, notably at higher pH, and Rajagopalan *et al.* (1966) have recommended that pepsinogen be activated at pH 2 in order to produce a homogenous pepsin preparation. The heterogeneity of pepsin is thought to be due to the removal of different number of amino acid residues from pepsinogen during the activation process.

The process of pepsinogen activation can occur by either intramolecular or intermolecular mechanisms. The intramolecular mechanism predominates at low concentrations of protein and below pH 3. The activation is first order with respect to zymogen concentration and is inhibited by pepstatin and the purified globin portion of hemoglobin which is a good substrate for pepsin. The proposed mechanism of intramolecular pepsinogen activation is as follows. At neutral pH, the zymogens are stabilized in an inactive conformation by the interaction of the basic amino acid residues in the N-terminal portion of the zymogen with the carboxyl groups in the catalytic portion of the molecule. At low pH, the carboxyl groups are protonated and the interaction with the basic amino acids in the N-terminal region is decreased. The zymogen then undergoes a conformational change to become active and splits the leucine 18-isoleucine 19 bond to remove the activation peptide. This mechanism has been further supported by the finding that pepsinogen will bind to a pepstatin-Sepharose affinity column at slightly acid

pH and is recovered as the native zymogen by elution with pH 8 buffer. Above pH 3, the activation of pepsinogen involves the intermolecular activity of active pepsin on the zymogen to form even more pepsin. Because of the broad specificity of active pepsin, the zymogen is attacked at a number of sites which generates a number of slightly different pepsin species and results in heterogeneity in the activated pepsin.

1.6. Immunological Comparisons of Acid Proteases

A large number of workers have used various immunological techniques to determine the structural relatedness of the acid proteases. The technique of immunochemical titration has been used to compare vertebrate lactate dehydrogenase isoenzymes (Holmes and Scopes, 1974) and aspartate aminotransferase in different species (Porter *et al.*, 1981). This method involves the incubation of the enzyme with antiserum and, after a suitable incubation period, the antigen-antibody complexes that have formed are separated by centrifugation. The amount of antiserum (titer) required to inhibit or precipitate the enzyme is then determined by measuring the enzymatic activity or protein remaining in the supernatant. Enzymes that are precipitated or inhibited by similar titers of antiboby are considered to be immunologically similar. Foltmann (1980), using rocket immunoelectrophoresis, found that anti-human pepsin A antibody reacted with mucosal extracts from cow, pig, goat, zebra, horse, dog, cat and seal while rat, hare, porcupine, kangaroo and hen pepsin gave no reaction. Meanwhile, anti-calf chymosin would precipitate the same extracts from goat, piglets, seal, dog, cat, young rat, porcupine, horse, zebra and kangaroo indicating that these species contained a chymosin-like enzyme. These studies have shown

that there is a strong correlation between sequence homology and the extent of antigenic similarity. With this in mind, it is interesting to note that while the pepsins and chymosins from different species show immunological similarity, no cross reaction has been found among the pepsin, gastricsin and chymosin of the same species. The various techniques that are useful in detecting the antibody-antigen reactions have been summarized (Reichlin, 1977).

1.7. Objectives of This Study

The Greenland cod (Rock cod) is an inshore sub-arctic species which thrives in near freezing water temperatures of the North Atlantic (Liem and Scott, 1966). It occupies a consistently low temperature environment while the Atlantic cod can occupy different thermal strata in the offshore water column. It was felt therefore that the gastric proteases from this species may have adopted some unique properties that may have useful commercial applications. These properties may include lower thermal stability and temperature optimum with a higher molecular activity at lower temperature. These properties have been reported for trypsins isolated from this species (Simpson, 1983). In order to evaluate the possible usefulness of the gastric proteases of this species to the food processing industry, the enzymes must first be isolated and characterized.

It is apparent from the literature review above that the gastric proteases of many different species have many characteristics in common. The properties of the mammalian pepsins, gastricsins and chymosins are summarized in Table 1. As has been discussed in section 1.2.7, the gastric proteases of several fish species have been isolated and these enzymes have shown several unique properties while

still retaining some of the basic properties of the mammalian gastric proteases (Table 2). Some of these characteristics, which enable the animal to survive in its environment, have been successfully exploited in the food processing industry. In this regard, the gastric proteases from the seal *Pagophilus groenlandicus* (Shamsuzzaman and Haard, 1984) and Atlantic cod (Brewer *et al.*, 1984] have been successfully used in cheese making. Other food processes that utilize proteolytic enzymes have been discussed by Haard *et al.*, 1982.

It is therefore hypothesized that the gastric proteases of the Greenland cod have several catalytic and structural features in common with the mammalian gastric proteases but also may possess properties unique to this species. The approach taken in this thesis is to compare and contrast the properties of the Greenland cod gastric proteases with the porcine gastric proteases and to answer the following questions:

1. What are the common characteristics of the mammalian gastric proteases? Do the gastric proteases of the Greenland cod share any of these characterictics?

2. What are the differences between the Greenland cod gastric proteases and the mammalian gastric proteases? Are these differences also seen in the gastric proteases from other fish species.

In this study the focus will be more on those characteristics that are useful in deciding whether the Greenland cod gastric proteases may have commercial applications rather than on structural properties of the enzymes. These properties include substrate specificity and pH optima as well as Vmax and Km with different substrates and assay techniques. Thermal and pH stability of the Greenland cod gastric proteases will be compared to porcine pepsin. Finally,

structural similarities between porcine pepsin and the Greenland cod gastric proteases will be investigated by amino acid composition measurements and immunological studies.

Chapter 2

MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. Animals

Specimens of Greenland Cod *(Gadus ogac)* were caught at Lake Melville, Labrador, by fishermen using trawl lines. Initial samples were taken in February, 1980. Further samples were obtained from local fishermen in April, 1983 and May, 1983 and in June, 1983. These fish were caught in 3 to 8 meters of water, one quarter mile south of Green Island (53° 33" 05' N, 64° 00" 00' W). A further sample was obtained by the Fisheries and Oceans Canada vessel "Gadus" in November, 1982 off the Labrador coast (54° 53" N, 55° 33" W) in ICNAF Division 2J.

Stomachs were removed from the fish and immediately frozen for transportation to the labratory. Once there, the stomachs were partially thawed and the inner mucosal linings peeled away from the outer muscular layer. Special care was taken to remove any parasites that were present, since these may contribute proteolytic enzymes of their own. The presence of Trypanosome worms has been reported in Atlantic Cod *Gadus morhua* by Khan and Kiceniuk (1983). The inner linings were cleaned (mucus has been reported to affect the activity of

gastric proteases by Mikuni-Takagaki and Hotta, 1979) in distilled water, frozen in liquid nitrogen, ground up with a mortar and pestle and stored at -70 C until used.

New Zealand white rabbits used for the generation of antibodies were obtained from the Animal Care Facility, Memorial University.

2.1.2. Chemicals

Bovine hemoglobin (type 2), gelatin (type 3, from calf skin, 225 bloom) and APDT (N-Acetylphenylalaninediiodotyrosine) were obtained from Sigma Chemical Co., St. Louis, Mo. Soluble casein was purchased from BDH. All protein substrates were dialysed extensively (molecular weight cutoff of dialysis tubing was 6,000-8,000) against pH 2 or 3 HCl before use in order to remove low molecular weight contaminants and lower the blank values of the assays. Peptide substrates Z-trp-ala (benzyloxycarbonyl tryptophanylalanine), Z-tyr-ala (benzyloxycarbonyl tyrosylalanine), Z-tyr-leu (benzyloxycarbonyl tyrosylleucine), Z-tyr-ser (benzyloxycarbonyl tyrosylserine) and Z-tyr-thr (benzyloxycarbonyl tyrosylthreonine) were obtained from Chemical Dynamics Corp., South Plainfield, N.J. Porcine pepsin A (1:10,000 and 1:60,000), porcine pepsinogen (grade 1) and Nessler's Reagent were obtained from Sigma. The reagents for gel electrophoresis were obtained from Bio-Rad (Mississauga, Ontario). The reagents for isoelectric focusing and chromatofocusing were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Coomassie Blue G250, Nin-Sol (ninhydrin reagent) and mercaptoethanesulfonic acid were the products of Pierce Chemical Co., Rockford, Illinois. Freund Adjuvant was obtained from Difco Laboratories, Detroit,

Michigan. All laboratory chemicals used were of analytical grade or equivalent and were obtained from major suppliers (Fisher Scientific, Canlab, BDH or Sigma)

2.2. METHODS

2.2.1. Isolation of Greenland Cod Proteases

2.2.1.1. Preparation of Homogenates

Inner stomach linings that had been previously frozen in liquid nitrogen and ground up with a mortar and pestle were homogenized using a Polytron homogenizer (Brinkmann Instruments, Rexdale, Ontario) in two volumes of 50 mM Tris buffer adjusted to pH 7.4 with phosphoric acid. Short periods of homogenization at setting 7 were used with the mixture cooled over ice. The homogenate was centrifuged for 10 min at 17,000 rpm (34,000 x g) using a Sorval RC-5B centrifuge (DuPont Instruments). The pellet obtained was homogenized again with two volumes of buffer, centrifuged and the supernatants were combined.

For certain preparations of Greenland Cod pepsinogens, ammonium sulfate precipitation was used as an initial purification step. Solid ammonium sulfate was slowly added with constant stirring to the cleared homogenate to 23% saturation at 5 °C. Sufficient 1N NaOH was added to maintain a neutral pH during the addition of the ammonium sulfate. The mixture was centrifuged at 34,000 x g for 10 min. and the pellet was discarded. The ammonium sulfate concentration was then adjusted to 63% saturation and the mixture was centrifuged as before. The pellet obtained was then used in further purification steps. For some experiments, the ammonium sulfate fractionation step was ommitted and the "supernatant" fraction from the homogenate was used for further purification.

2.2.1.2. Sephadex G100 Chromatography

Aliquots of Greenland cod stomach homogenate (10 ml) or 63% ammonium sulfate pellet were applied to a 2.5 x 32 cm column of Sephadex G100 and eluted with 50mM Tris-phosphate pH 7.0. The column effluent was monitored continuously at 280 nm and collected into 3.2 ml fractions. Alternate fractions were assayed for proteolytic activity by the method described in section 2.2.4.1 and active fractions were pooled, concentrated by freeze-drying and rechromatographed on Sephadex G100. This purified material gave a single band on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) but multiple bands on Ornstein-Davis gel electrophoresis.

2.2.1.3. Chromatofocusing

The different proteolytic enzymes present in the mucosa from Greenland cod stomach were resolved by chromatofocusing. Enzyme preparation was applied to a 1.5 x 30 cm column of Polybuffer exchanger 94 (PBE 94) equilibrated with 25 mM imidazole-HCl buffer, pH 7.4. The column was eluted with Polybuffer 74, diluted 1:8 and adjusted to pH 4 with HCl. The column effluent was monitored continuously at 280 nm, approximately 3 ml fractions were collected and the pH of alternate fractions was measured using a Photovolt model 112 pH meter equipped with a semi-micro calomel combination electrode (Corning Glass Works, Corning, N.Y.). The proteolytic activity of alternate fractions was determined by the method of section 2.2.4.1 and fractions containing activity were pooled and lyophilized. In certain experiments, enzyme fractions from chromatofocusing were concentrated using a continuous-feed ultrafiltration system (Amicon Corp., Danvers, Mass.) with YM10 filters at 50 psi nitrogen pressure. However,

approximately 50% of the enzyme activity was lost by this procedure and it was subsequently abandoned. Polybuffer was removed from the enzyme preparations by chromatography on a 1.5 x 80 cm column of Sephadex G75 (40-120 μ dry bead diameter) equilibrated and eluted with 50 mM Tris-phosphate, pH 7.0. Active enzyme was prepared by incubating the zymogen at pH 2, 5 °C for 30 minutes followed by chromatography on Sephadex G75 equilibrated and eluted with .003N HCl. In this way, the peptides released by the activation of the zymogen were removed, since it has been reported (Bustin and Conway-Jacobs, 1971) that they may inhibit the enzyme activity. In earlier studies, the various proteolytic activities were separated by preparative isoelectric focusing. A flat bed system (FBE 3000) was used with Sephadex IEF and Pharmalyte 3-10. However, chromatofocusing was found to give superior resolution of the pepsinogens and was more convenient than isoelectric focusing.

2.2.2. Purification of Porcine Pepsin and Gastricsin

Porcine pepsin and gastricsin were purified as described by Chiang *et al.* (1967). Crude porcine pepsin preparation (1:10,000), 2g, was dissolved in 0.2M citrate buffer adjusted to pH 2.1 with NaOH and applied to a 1.5 x 30 cm column of Amberlite CG-50 resin (hydrogen form, 200-400 mesh). The resin was first cycled once through the sodium form by treatment with NaOH, water, and then HCl as described by Hirs *et al.* (1953). The column was eluted succesively with 0.2M citrate buffer adjusted with NaOH to pH 2.1, 3.8, 4.2 and 4.6. Fractions eluted as peaks of protein and containing proteolytic activity were pooled, lyophylised and applied to a 1.5 x 87 cm column of Sephadex G75 equilibrated and eluted with .003N HCl. The enzyme preparations were pure as judged by polyacrylamide gel electrophoresis as described in section 2.2.3.1.

2.2.3. Estimation of Purity of Enzyme Preparations

2.2.3.1. Polyacrylamide Gel Electrophoresis

The purity of the enzyme preparations was examined by electrophoresis on 7.5% polyacrylamide gels (Davis, 1964). Electrophoresis was carried out in glass tubes (0.5 x 13 cm) with a gel length of 10 cm using a model 155 gel electrophoresis cell (Bio-Rad). Cold tap water was circulated through the apparatus to maintain a low temperature and a current of 2mA per gel was applied using a Gelman power supply (Gelman Instruments, Ann Arbour, Mich.). Bromophenol blue dye was used to estimate the leading edge of the electrophoresis pattern.

Electrophoresis was also carried out in the presence of sodium dodecyl sulfate (Laemmli and Faire, 1973) and used to separate proteins of different molecular weights. To estimate the molecular weight of a protein with this method, a calibration mixture containing phosphorylase B, bovine albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and lysozyme was included. A graph of the logarithm of the molecular weight of these standards versus the Rf was then constructed and used to estimate the molecular weight of a test protein once its Rf had been determined. The preparation of the various reagents and the details of the procedure for pouring the polyacrylamide gels are described in Appendix 1.

The electrophoresis was judged to be complete when the bromophenol blue dye was 1 cm from the bottom of the tube. The gels were removed immediately from the glass tubes using a syringe filled with distilled water fitted with a 21

guage needle and were fixed in 14 ml of 12% TCA. Approximately 3 ml of 0.25% Coomassie Blue G250 was then added and the gels allowed to stain. A sufficient staining time was easily estimated since the background takes up very little stain (Diezel *et al.*,1972). The gels were destained overnight in 7.5% acetic acid with 5% methanol in a diffusion destainer containing activated charcoal (Bio-Rad).

2.2.3.2. Use of High Performance Liquid Chromatography

Numerous attempts were made to utilize high performance liquid chromatography (HPLC) to demonstrate the purity of the enzyme preparations. Several types of reverse phase columns were used, including octyl (Altex Ultrosphere C_8) and octadecyl (Waters Scientific μBondapak C_{18}) columns. Solvent systems used included acetonitrile/water with 0.1% trifluoroacetic acid (TFA), isopropanol/water with 0.1% TFA and 0.1M formate and phosphate buffers, with and without various concentrations of sodium chloride and at pH values from 2 to 8. However, with all the various combinations of columns and solvent systems, no satisfactory method was found to elute protein of either porcine pepsin or Greenland cod proteases from the reverse phase column. Samples of bovine trypsin, however, were chromatographed using a known method (linear gradient from water with .1% TFA to 45% acetonitrile with .1% TFA in 30 min). It was surmised that the interaction of the acid proteaeses with the reverse phase column must be unusually strong.

2.2.3.3. Estimation of Protein Concentration

Three methods were used to estimate the protein concentration of solutions. These were (1) biuret method (Gornall *et al.*, 1949) (2) Coomassie Blue G250 method (Sedmak and Grossberg, 1977) (3) and the determination of total nitrogen by the micro-Kjeldahl method (Lang, 1958). The biuret method was used with protein solutions of 10 mg/ml and greater while the Coomassie Blue G250 method was used with protein solutions in the 50 μg/ml range. The micro-Kjeldahl method is sensitive in the 100 μg/ml range but was rather tedious. It was used with solutions of extremely pure enzymes since it was felt that this method would be less subject to artifacts and would provide a more reliable estimate of the true protein concentration.

The biuret method involves the formation of a purple colored complex between copper and the peptide bonds in a protein, and is interferred with by salts such as ammonium sulfate. The Coomassie Blue G250 method is based on the formation of a complex between the dye and the protein which causes a shift in absorbance maximum from 465 nm for the unbound dye to 620 nm for the protein-dye complex. The micro-Kjeldahl method involves the digestion of the protein with strong acid and the determination of the total nitrogen by a colorimetric reaction with Nessler's Reagent. It is useful only when the protein solution is free of other nitrogen-containing substances. The details of the biuret, Coomassie Blue and micro-Kjeldahl methods are given in Appendix 2.

2.2.4. Assay of Proteolytic Activity

A number of methods were used to assay the hydrolytic activity of the enzyme preparations. All assays, except those done to determine the elution profile of a column, were performed in triplicate and duplicate blanks were included. The reaction rate versus time was determined for all assay methods and appropriate incubation times were chosen to keep the reaction in the linear range. Appropriate concentrations of enzyme were used in all assays to keep the reaction rate in the linear range.

2.2.4.1. Determination of the Increase in Absorbance of TCA Soluble Material

This method is based on the traditional procedure described by Anson (1938) and involved incubating the enzyme preparation with a protein substrate at a suitable pH and, after an appropriate time, stopping the reaction by adding one volume of 12% trichloroacetic acid (TCA). The mixture was stored in the cold for at least 2 h to allow complete precipitation of protein to occur and then centrifuged at 3000 x g for 15 min. The absorbance at 280 nm of the supernatant was measured using a Gilford Model 250 Spectrophotometer and used as a measure of proteolytic activity. Standard conditions for the assay were; 1.5 or 2.5 ml of 2% protein at pH 2 to 3 incubated at 27 °C for 10-60 min. The method is convenient, sensitive and linear over a wide range of absorbance values. However, it gives no indication of the number or type of peptide bonds hydrolysed since only the absorbance at 280 nm of the TCA soluble peptides is measured. Also, only that fraction of the products that absorbs at 280 nm is measured. The concentration of TCA used to stop the reaction was increased from 6% in the

initial studies to 12% when it was found that some of the cod proteases were not completely inactivated at a final TCA concentration of 3%.

2.2.4.2. Use of Methylated Protein Substrates

The use of methylated protein substrates in the assay of proteolytic enzymes allows the rate of hydrolysis of peptide bonds to be measured. The procedure involves the use of a protein substrate in which all free amino groups are methylated so that the formation of free amino groups by hydrolysis of peptide bonds can be detected by reaction with trinitrobenzenesulfonic acid (TNBS). The preparation of methylated substrates was decsribed by Lin *et al.* (1969) and is outlined in Appendix 3. Methylated hemoglobin, methylated casein and methylated gelatin were prepared as described and the concentration of protein was determined by the biuret method.

Proteolytic activity with the methylated substrates was determined by the method of Fox *et al.*, (1977). Enzyme was incubated with 0.4% methylated protein substrate (0.5 ml) and 0.2M citrate buffer (.05 ml) adjusted with NaOH to an appropriate pH. The reaction was stopped by the addition of 0.1M sodium borate (0.5 ml) pH 9.5. No further hydrolysis was noted in the reaction mixtures stored overnight in the refrigerator after the addition of borate. Color was developed by the addition of .025ml of 1.1M TNBS. The mixture was incubated at 27 °C for 10 min and 2 ml of a solution of 1.5 mM sodium sulfite in 98.5 mM sodium dihydrogen phosphate was added. The absorbance at 420 nm was then measured using a Gilford Model 250 Spectrophotomer. The number of peptide bonds hydrolysed was calculated using an experimentally determined micromolar extinction coefficient at 420 nm for alanine of 6.057.

2.2.4.3. Determination of Milk Clotting Activity

The time required for an enzyme to clot a 1.0 ml solution of 12% milk solids containing 10 mM $CaCl_2$, pH 6.2, was determined. The reaction was carried out in 13 x 100 mm test tubes and the milk solution was examined by rolling it along the sides of the tube. The clot time was judged to be the time when the initial flocculation occured in the milk solution. The clotting activity was calculated from the clot times using a clotting unit defined as the amount of enzyme that will clot 10 ml of milk in 100 sec at 27 °C.

2.2.4.4. Use of Peptide Substrates

The hydrolytic activity of the enzyme preparations against a number of peptide derivatives (Z-tyr-ala, Z-trp-ala, Z-tyr-ser, Z-tyr-thr, Z-tyr-leu and APDT) was determined. Peptide solutions (2mM) were made by adding sufficient NaOH to distilled water to bring the pH to 7. Peptide solution (.5 ml except .25 ml of APDT), .05 ml of .2M citrate buffer adjusted to pH 2 or 3 with NaOH and sufficient water and enzyme to 1.0 ml were incubated together. The reaction was stopped by adding 0.5 ml of ninhydrin reagent and the mixture heated at 90 °C for 15 min. After cooling, 5 ml of 60% ethanol was added and the absorbance at 570 nm was measured using a Gilford Model 250 Spectrophotometer. The extinction coefficients at 570 nm of the C-terminal amino acids of the peptides were determined following treatment with ninhydrin and used to calculate the rate of peptide hydrolysis.

Attempts were made to use sulfite esters as a kinetic method to determine pepsin activity (Reid and Fahrney,1967). However, these esters proved to be

extremely insoluble and no activity could be detected using porcine pepsin with these compounds as substrates.

2.2.4.5. Use of the pH Stat

The pH stat method was used to determine the extent of hydrolysis of a 2% hemoglobin solution by each of the proteases. A comparison of the total extent of hydrolysis of substrate by each of the proteases gives an indication of the percentage of the total peptide bonds in hemoglobin that are hydrolysed by each of the enzymes. The equipment used in this study was a Metrohm pH titrator (Brinkmann Instruments, Rexdale, Ont.) In this procedure, 5 ml of 2% hemoglobin pH 3.0 was equilibrated at 25 °C and, once a flat baseline was obtained for 20-30 min, 25-50 µl of enzyme (equivalent to 25-100 µg) was added. The acid uptake was recorded for 24-48 h with the titrator set at pH 3.0 and standardized 0.05M HCl in the burette. The endpoint of the reaction was noted when there was no further uptake of acid and the number of µmoles of HCl taken up was calculated. This figure, which represents the amount of HCl used to titrate the free amino groups formed by the peptide bond hydrolysis, was then divided by 0.83 to calculate the number of bonds hydrolysed (Bohak, 1970). The degree of hydrolysis (DH) of the hemoglobin substrate was then calculated assuming a value of 8.0 meq of peptide bonds/g of hemoglobin (h_{tot}) using the following equation;

$$DH = h/h_{tot}$$

where h is the hydrolysis equivalent defined as the milliequivalents of peptide bonds cleaved per gram of protein.

2.2.5. Activation of Zymogens

The activation of the zymogens of porcine pepsin and the three Greenland Cod proteases was measured as described by Christensen *et al.* (1977). Zymogen (.05 ml) was mixed rapidly with .07M HCl (.05 ml) solution to give a pH of 2-2.5 and the mixture was incubated at various temperatures from 0-35 °C. The activation process was stopped by the addition of 0.1M citrate (.4 ml) adjusted to pH 6.2 with NaOH. Aliquots of this mixture were then assayed in triplicate for milk clotting activity as described in section 2.2.4.3. An increase in milk clotting activity following exposure to acid was taken as a measure of activation of the zymogen. Similar concentrations of the zymogens were used and the percentage of activation was calculated using the clotting activity of totally activated zymogen. Non-activated zymogens from Greenland cod mucosa failed to clot milk in experiments left overnight.

2.2.6. Immunological Comparisons of the Enzyme Preparations

2.2.6.1. Preparation of the Antisera

Antisera to purified porcine pepsin and porcine gastricsin were prepared in randomly bred New Zealand white rabbits. Approximately 100 μg of enzyme preparation in Freund adjuvant (Freund, 1956) was injected subcutaneously at 2 week intervals. Blood samples were withdrawn from an ear vein immediately before injection of the enzyme preparations. The presence of antibody in the rabbit sera was examined by Ouchterlony Double Diffusion tests after the eighth week (Ouchterlony, 1949). Two weeks after the presence of antibody was detected, the blood was collected by heart puncture and the animals were killed. The IgG fraction of the serum was purified by sodium sulfate precipitation and DEAE Cellulose chromotography as described in Appendix 4.

2.2.6.2. Immunodiffusion Tests

Antibody titers were estimated using Ouchterlony Double Diffusion plates (for the preparation of these plates, see Appendix 5). Antibody was placed in the center well and successive dilutions of enzyme were placed in the outer wells. The plates were then developed in a humid atmosphere at 5 °C for 24-48 h. Concentrations of antibody and enzyme that produced sharp precipitin bands were judged to be of equivalent titer. The antigenic relatedness of the various proteases were examined by Ouchterlony Double Diffusion tests using multiple outer wells (Stollar and Levine, 1963). With this technique, the formation of a spur at the junction of the precipitin bands is indicative of the presence of different antigenic determinants on the adjacent proteins.

2.2.6.3. Immunochemical Titration Studies

The inhibition of the activity of the various proteases by antibodies raised to either porcine pepsin, porcine gastricsin or to proteases T16 or T25 isolated from psychrotrophic pseudomonads (Jackman *et al.*, 1983) was measured. Purified IgG fraction was incubated with enzyme in 15 mM sodium phosphate pH 5.5 in proportions ranging from 0-25 mg IgG/mg enzyme. The reaction was carried out in 1.5 ml Eppendorf centrifuge tubes (Brinkman Instruments) with a total reaction volume of 0.4 ml at 5 °C for overnight. The tubes were then centrifuged at 12,000 x g for 20 min at 5 °C and aliquots of the supernatant solution were assayed for proteolytic activity using 2% hemoglobin as substrate. The residual proteolytic activity remaining after antibody treatment was calculated for each enzyme by comparison with the activity of enzymes similarly incubated in the buffer without IgG.

2.2.7. Measurements of Stability of the Enzyme Preparations

2.2.7.1. Thermal Stability

The thermal stabilities of the proteases were determined by incubating enzyme in pH 1.9 or 3 HCl or 50 mM Tris phosphate, pH 6.5, at various temperatures ranging from 20 to 60 °C for 60 min. Aliquots were then assayed under standard conditions (20 min at 27 °C with 2% hemoglobin as substrate). Similar concentrations (30-50 μg) of all proteases were used in these studies and the activity remaining after the heat treatment was expressed as a percentage of the activity present before treatment.

2.2.7.2. pH Stability

The pH stability of the different protease preparations was compared. Aliquots of the proteases (60-70 μg) in pH 2.5 HCl were added to 25 μl of 0.2M citrate buffer adjusted to pH 2-6.5 with NaOH or 0.2M Tris-HCl buffer pH 6.5-8. Sufficient water was added to bring the final volume to 225 μl and the mixtures were stored at 5 °C for 96 h. Samples (40 μl) from each pH treatment were then assayed under standard conditions (30 min at 27 °C with 2% hemoglobin as substrate). Similar concentrations of the different enzymes were used in this study and the activity remaining after treatment was expressed as a percentage of the initial activity present.

2.2.8. Determination of Amino Acid Composition

The amino acid contents after acid hydrolysis of the purified enzyme preparations were determined with a Beckman Model 121MB Amino Acid Analyser. A single column of Beckman AA10 resin was used with a three buffer lithium system as described in Beckman Technical Bulletin 121MB-TB-017. Enzyme preparations were hydrolysed in triplicate with 6N HCl for 24 h, 48 h, and 72 h. The amino acid composition was estimated as follows; (1) for those amino acids with constant values for all hydrolysis times (aspartate, glutamate, proline, alanine, glycine and tyrosine) the arithmetic mean was used, (2) for those which increase with time of hydrolysis (lysine, histidine, arginine, valine, isoleucine, leucine and phenylalanine) the maximum value obtained was taken, (3) for those which decrease with time of hydrolysis (serine and threonine) the decomposition curves were extrapolated to zero time. Cysteine and methionine were determined by performic acid oxidation followed by acid hydrolysis (Bailey, 1967). Performic acid was prepared by adding 1 volume of 30% hydrogen peroxide to 9 volumes of 90% formic acid. After 1 h at room temperature the mixture was cooled to 0 °C and a 2 ml aliquot was added to the protein sample (2-5 mg) and stored at 5 °C overnight. Distilled water (8 ml) was then added, the mixture was freeze dried and then hydrolysed in 6N HCl for 24 h. Cysteine and methionine were then determined by amino acid analysis as cysteic acid and methionine sulfone. Tryptophan was determined after hydrolysis of the protein in 3N mercaptoethanesulfonic acid as described by Penke *et al.*, (1974).

2.2.9. Calculation of Results

The initial velocity data obtained at different substrate concentrations were fitted to a Michaelis-Menton model

$$v = Vmax[S] / Km + [S]$$

using the ROSFIT program (Greco *et al.*,1982) on a Hewlett Packard HP-85 microcomputer. This package fits the data to a nonlinear regression curve and allows weighting to be used to offset the differences in accuracy of velocity measurements made at low and high substrate levels. All data from the initial velocity versus substrate determinations were weighted proportional to $1/v^2$.

Activation energy for the hydrolytic reaction was calculated from the slope of the Arrhenius plots (ln Vmax versus 1/Temperature in ^{o}K) using a least squares program.

Chapter 3

RESULTS

3.1. Purification of Proteases

3.1.1. Greenland Cod Proteases

Homogenates were prepared from the inner lining of the stomach of the Greenland Cod *(Gadus ogac)* as described in section 2.2.1.1. Figure 3-1 shows the profile obtained when the homogenate is chromatographed on Sephadex G100. The protease activity, measured with 2% hemoglobin as substrate, was eluted as a shoulder of the large void volume peak. The active fractions (24-36) were then pooled, concentrated by freeze-drying, and re-chromatographed on Sephadex G100 until a single symmetrical peak of protein was eluted which corresponded with the protease activity. This peak was then applied to a chromatofocusing column (Figure 3-2) and the protein eluted with a pH gradient from 7.5 to 4. With this technique, the proteins are eluted from the column at their respective isoelectric points (pI). Three main peaks, representing the zymogen forms of the proteases, were found with pI's of >7.5, 5.9-6.2 and 5.1-5.4 with a variable amount of activated protease at pH 4.5. The zymogens were numbered 1-3 in order of decreasing isoelectric points for future reference. The zymogen preparations were then concentrated by freeze drying, activated, and chromatographed on Sephadex G75 as described in section 2.2.1.3. The active

proteases were eluted with pH 2.5 HCl solution as symmetrical peaks separated from the Polybuffer (Figure 3-3). A summary of the purification scheme, specific activity and percentage yield is shown in Table 3-1.

The purity of the protease preparations was confirmed by polyacrylamide gel electrophoresis. Single, clean bands were obtained for all proteases on both Ornstein-Davis type gels (Figure 3-4) and gels containing SDS (Figure 3-5). The zymogen for cod protease 1 was the least mobile and the zymogen for cod protease 3 was the most mobile on Ornstein-Davis type gels. The picture of the zymogen for protease 1 before the Polybuffer was removed illustrates the necessity of the Sephadex G75 chromatography step.

3.1.2. Porcine Pepsin and Gastricsin

Porcine pepsin and gastricsin were purified from a crude (1:10,000) pepsin preparation as described in section 2.2.2. The elution profile of this crude preparation from an Amberlite CG-50 column is shown in Figure 3-6. Porcine pepsin A was eluted as a large peak with the initial buffer at pH 2.1. The column was then washed with pH 3.8 and pH 4.2 buffer. The peak of activity that eluted with pH 4.6 buffer was pooled and designated the "gastricsin fraction". Both enzyme fractions were then concentrated by freeze drying and chromatographed on a Sephadex G75 column equilibrated and eluted with pH 2.5 HCl solution (Figure 3-7). The enzyme preparations were judged to be pure by polyacrylamide gel electrophoresis (Figure 3-8). Porcine "gastricsin fraction" was found to run slightly behind pepsin A on both Ornstein Davis and SDS gel systems.

Figure 3-1: Elution Profile of Greenland Cod Gastric Proteases from Sephadex G100

Approximately 10 ml of the "supernatant" fraction of Greenland cod stomach mucosa homogenate was applied to a 2.5 x 32 cm column of Sephadex G100 (40-120 μm dry bead diameter) equilibrated and eluted with 50mM Tris phosphate pH 7. Fraction size was 3.2 ml. (–) absorbance at 280 nm of the column effluent; (●) proteolytic activity with 2% hemoglobin as the substrate.

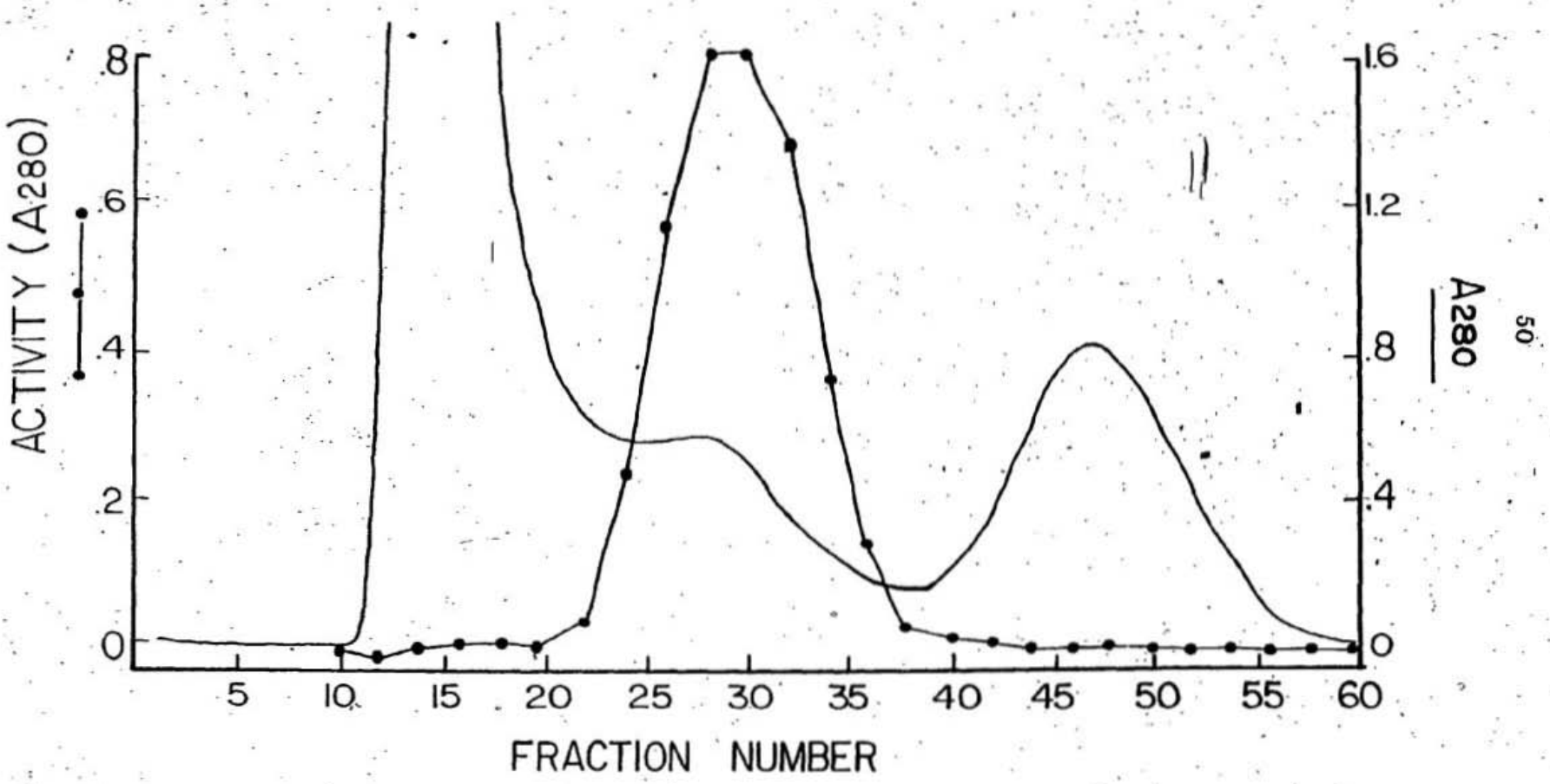


Figure 3-1: Elution Profile of Greenland Cod Gastric Proteases from Sephadex G100

Figure 3-2: Chromatofocusing of the Greenland Cod Gastric Proteases

The fractions containing proteolytic activity from the Sephadex G100 column were concentrated and applied to a 1.5 x 30 cm column of PBE 94 equilibrated to pH 7.4 with 25 mM imidazole-HCl buffer. The column was eluted with Polybuffer 74 diluted 1:8 with distilled water and adjusted to pH 4 with HCl. Fraction size was 3.2 ml. (x) pH measurements made on alternate tubes; (●) proteolytic activity with 2% hemoglobin as the substrate, (–) absorbance at 280 nm of the column effluent.

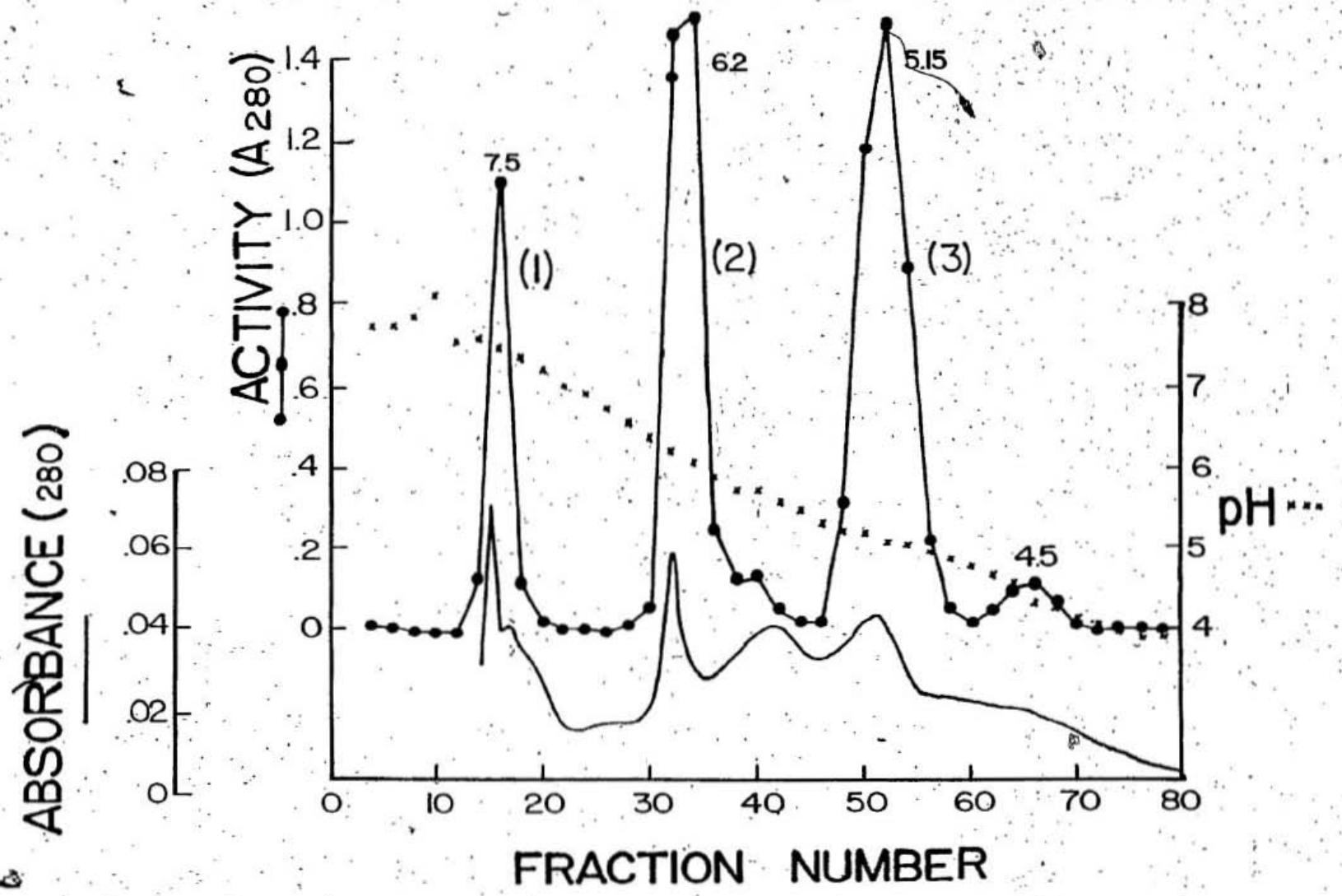


Figure 3-2: Chromatofocusing of the Greenland Cod Gastric Proteases

Figure 3-3: Sephadex G75 Chromatography of Active Greenland Cod Gastric Proteases

Greenland cod zymogen fractions were activated at pH 2, 5°C for 30 min and applied to a 1.5 x 90 cm column of Sephadex G75 (40-120 μm dry bead diameter) equilibrated and eluted with pH 2.5 HCl solution. Fraction size was 3.2 ml; (–) absorbance at 280 nm of the column effluent; (●) proteolytic activity with 2% hemoglobin as the substrate.

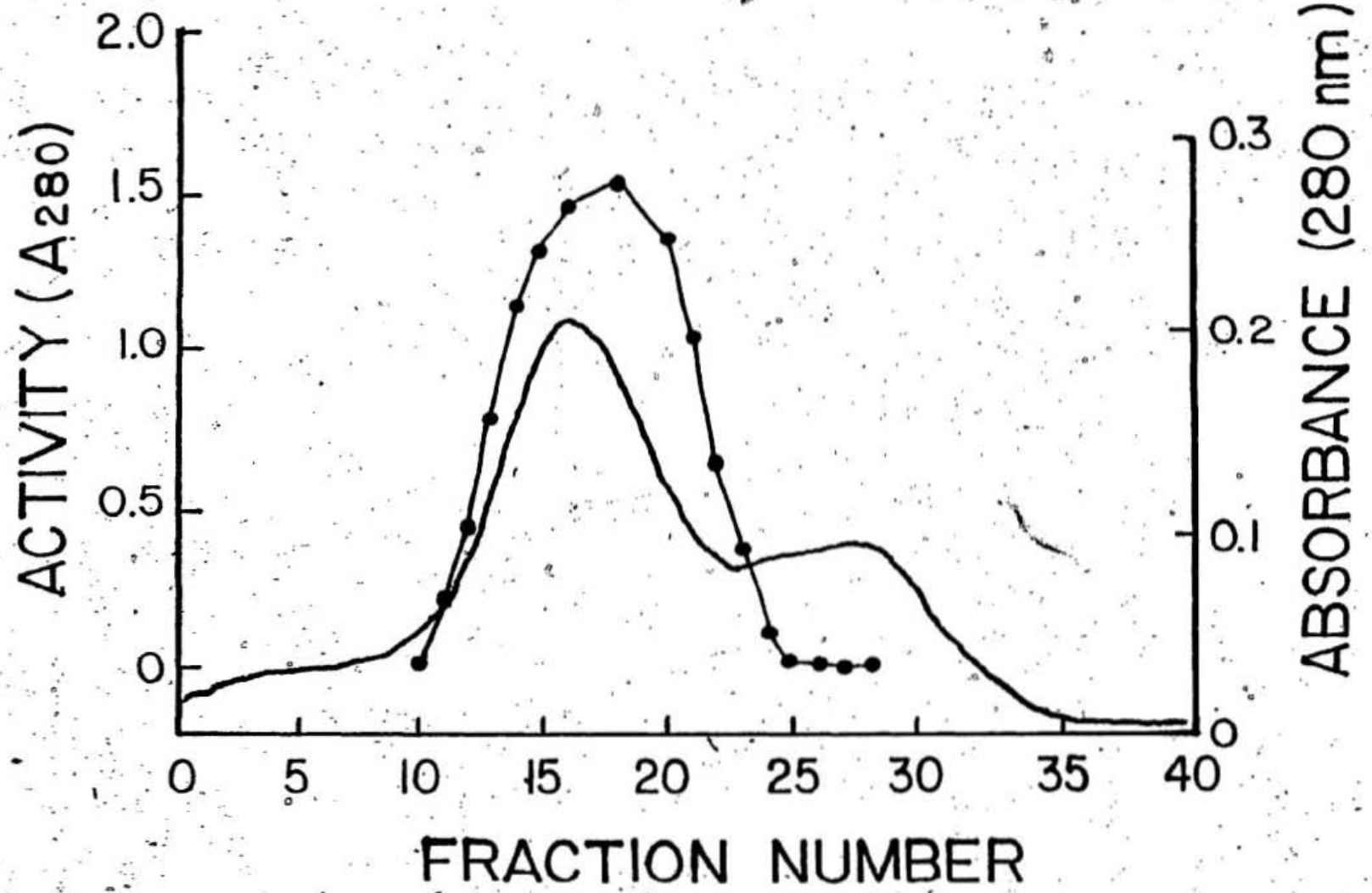


Figure 3-3: Sephadex G75 Chromatography of Active Greenland Cod Gastric Proteases

Table 3-1: Purification of the Greenland Cod Gastric Proteases

Procedures	Total Volume	Total Protein	Total Activity	Specific Activity	Yield (%)
1.Homogenate	147	2,072.6	1.133	5.47	100.0
2.First Sephadex G100	355	134.5	0.534	39.7	113.8
3.Ultrafiltration and Second Sephadex G100	87	166.9	1.220	73.1	63.7
4.Zymogen 1	41.5	165.6	0.450	27.2	11.2
5.Zymogen 2	28	28.0	1.224	437.2	20.6
6.Zymogen 3	50	13.7	0.925	675.2	27.8
Following activation, Sephadex G75 and Ultrafiltration					
7.Protease 1	18.3	116.7	0.398	34.1	4.4
8.Protease 2	18.3	13.5	1.301	963.7	14.3
9.Protease 3	18.3	10.9	1.184	1086.2	13.0

Stomachs used in the above purification were from fish caught on September, 1982. Ultrafiltration was not used in subsequent purification schemes due to low yields from this step. Total activity is reported as ΔA_{280}/20 min/50μl and the specific activity is reported as ΔA_{280}/min/mg protein determined with 2% hemoglobin as the substrate by measuring the TCA reaction products (section 2.2.4.1). Protein concentration is reported in μg/ml.

Figure 3-4: Polyacrylamide Gel Electrophoresis of Greenland Cod Gastric Proteases

Purified Greenland cod gastric proteases (50-100 μg) were run on Ornstein-Davis polyacrylamide gel electrophoresis at pH 8.3 as described in Appendix 1. **A** (left to right) activated protease 1 (P1) after Sephadex G75 chromatography at pH 2.5, zymogen of protease 1 (Z1) (that had been inadvertently activated) after Sephadex G75 chromotography at pH 7 and (Z1A) zymogen of protease 1 from the chromatofocusing column before Sephadex G75 chromatography. **B** (left to right) activated protease 2 (P2) after Sephadex G75 chromatography at pH 2.5, Zymogen of protease 2 (Z2) after Sephadex G75 chromatography at pH 7. **C** (left to right) activated protease 3 (P3) after Sephadex G75 chromatography at pH 2.5, zymogen of protease 3 after Sephadex G75 chromatography at pH 7. The arrow indicates the position of the tracking dye.

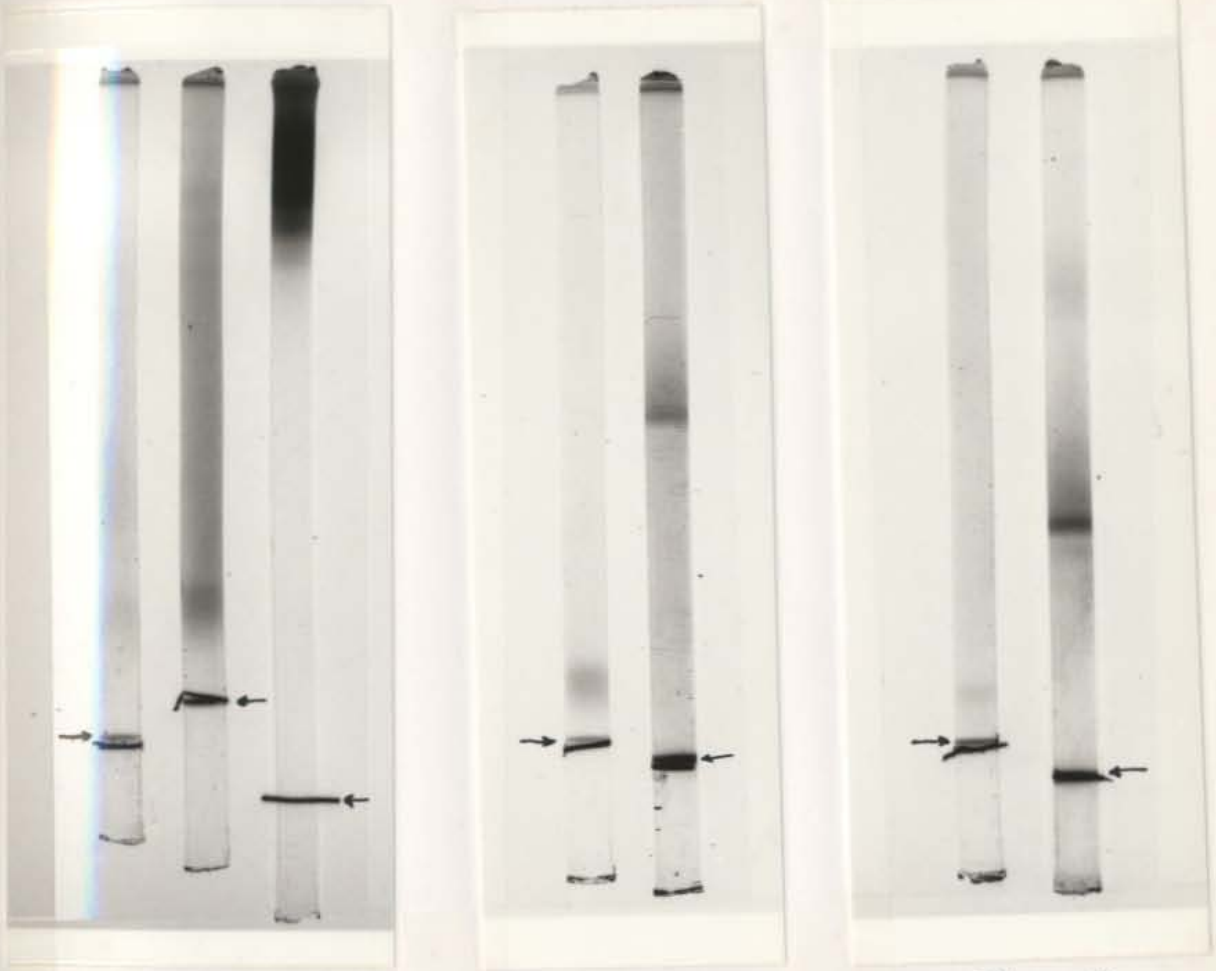

P1 Z1 Z1A
P2 Z2
P3 Z3
A
B
C

Figure 3-5: SDS PAGE of Purified Greenland Cod Gastric Proteases and Zymogens

A (left to right) molecular weight standards; zymogen of protease 1; zymogen of protease 2; zymogen of protease 3. **B** The zymogens were activated at pH 2, 5°C for 30 min and chromatographed on Sephadex G75 pH 2.5 (left to right) molecular weight standards; activated protease 1; activated protease 2; activated protease 3. The arrow indicates the position of the tracking dye.

A

B

Figure 3-6: Chromatography of 1:10,000 Pepsin Preparation on Amberlite CG-50

Crude 1:10,000 porcine pepsin, 2g, was applied to a 1.5 x 30 cm column of Amberlite CG-50 ion exchange resin. The column was eluted successively with 0.2M citrate buffer adjusted with NaOH to pH 2.1, 3.8, 4.2 and 4.6 and collected into 3.2 ml fractions. The active protease eluting with the pH 2.1 buffer was designated the "pepsin fraction" and the active protease eluting with the pH 4.6 buffer was designated the "gastricsin fraction". (▲) proteolytic activity measured with 2% hemoglobin as the substrate.

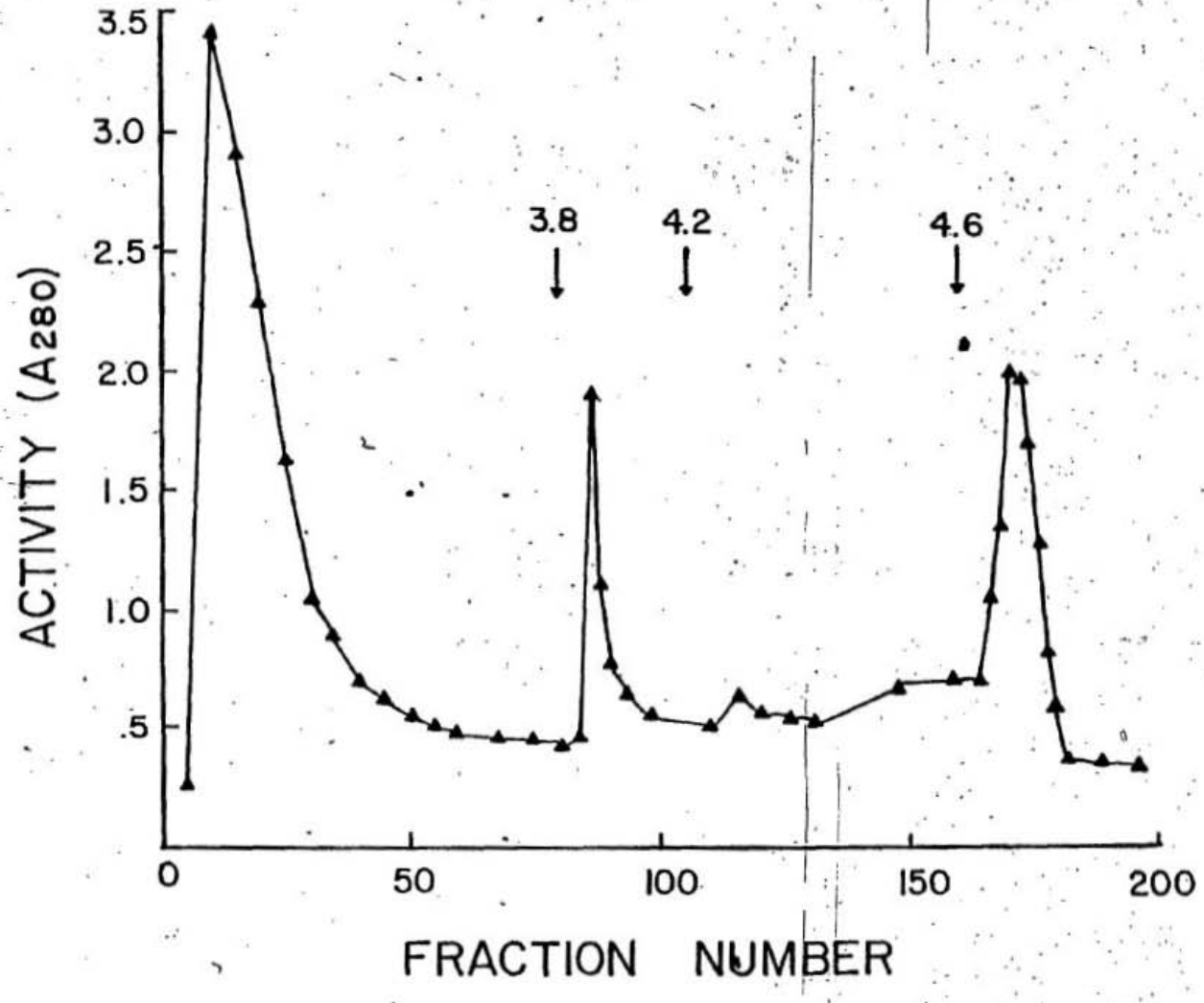


Figure 3-6: Chromatography of 1:10,000 Pepsin Preparation on Amberlite CG-50

Figure 3-7: Sephadex G75 Chromatography of Porcine Gastric Proteases

Fractions from the Amberlite CG-50 ion exchange column were concentrated and applied to a 1.5 x 90 cm column of Sephadex G75 (40-120 μm dry bead diameter) equilibrated and eluted with pH 2.5 HCl. The chromatography of the pepsin fraction is illustrated here. The proteolytic activity eluted as a large initial peak well separated from lower molecular weight contaminants. Where necessary, the active fractions were rechromatographed on Sephadex G75 to obtain a clean symmetrical peak of protein (▲) absorbance at 280 nm.

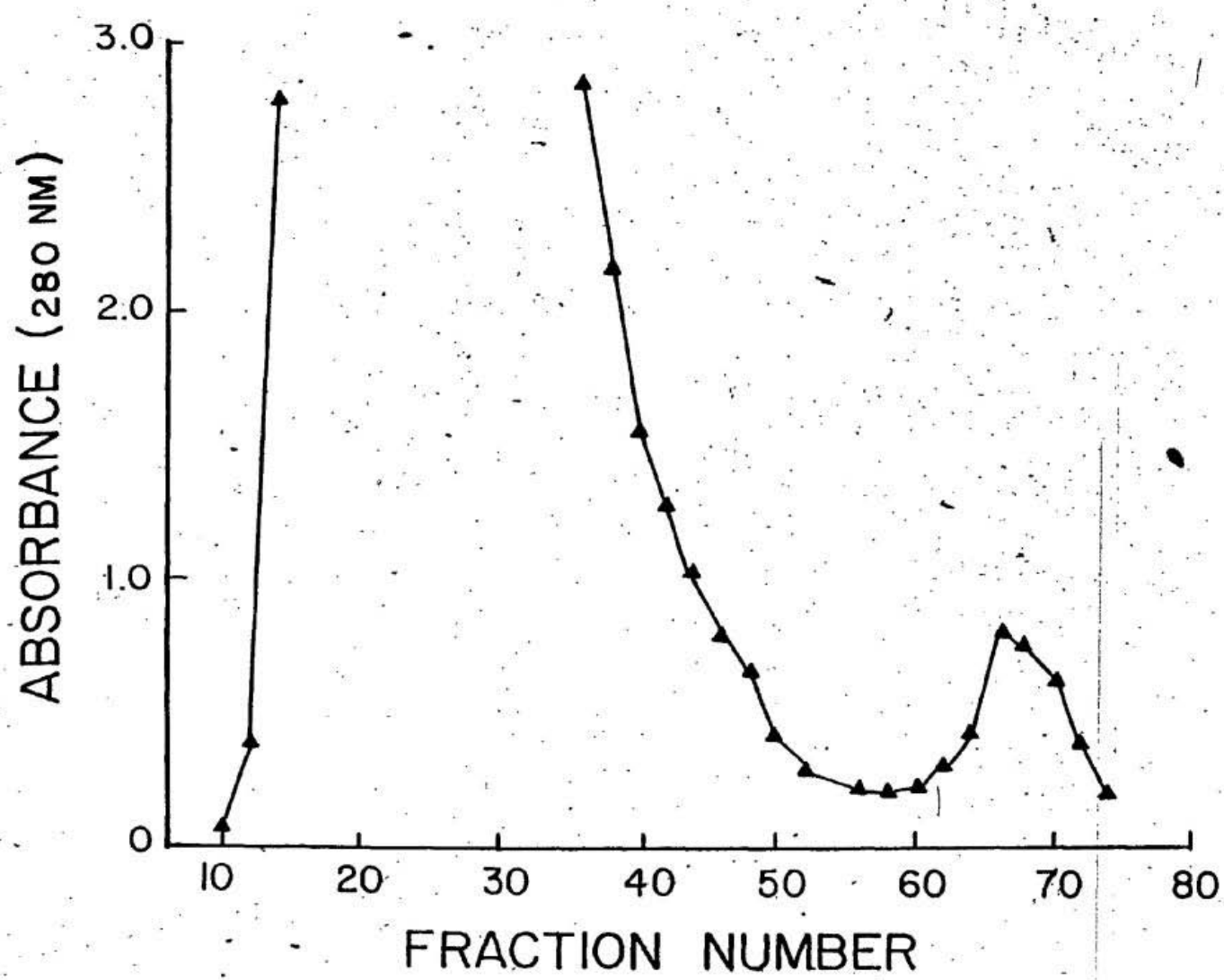
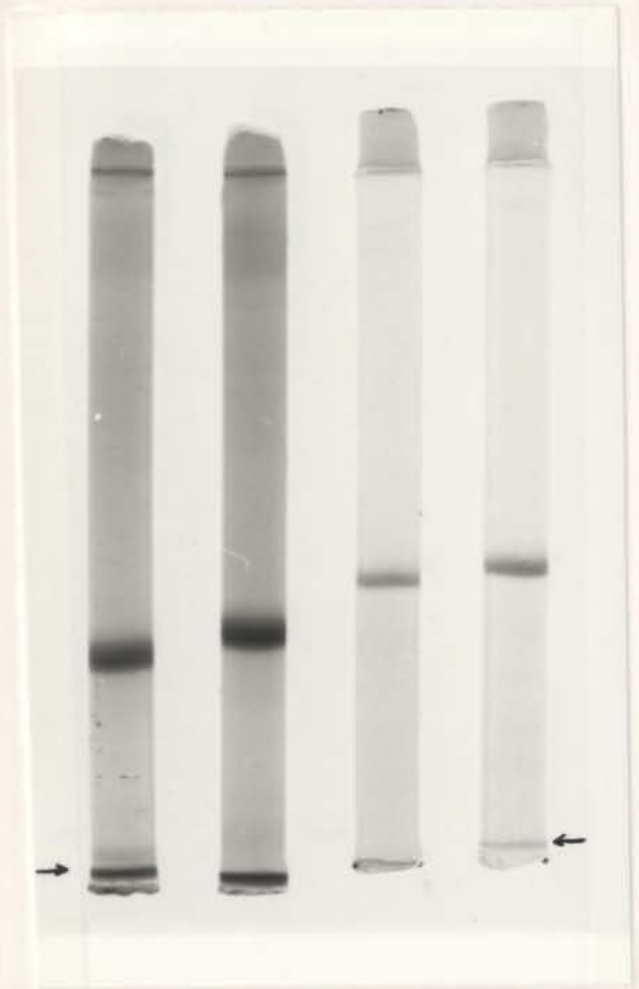


Figure 3-7: Sephadex G75 Chromatography of Porcine Gastric Proteases

Figure 3-8: Polyacrylamide Gel Electrophoresis of Porcine Gastric Proteases

Polyacrylamide gel electrophoresis of purified porcine gastric proteases in the absence **A** and presence **B** of sodium dodecyl sulfate. **A** (left to right) first two gels are purified porcine pepsin A fraction, last two gels are purified porcine "gastricsin fraction" **B** (left to right) purified porcine "gastricsin fraction", purified porcine pepsin A fraction, 1:60,000 porcine pepsin A obtained from Sigma. The arrow indicates the position of the tracking dye.



A

B

3.2. Activation of Zymogens

Preparations of the zymogen forms of the various Greenland cod gastric proteases from the chromatofocusing column were further purified by Sephadex G75 chromatography. The column was eluted with 50 mM Tris-phosphate, pH 7.4, and by this procedure the zymogens were separated from the Polybuffer, which eluted as an inactive peak of A_{280} absorbing material after the enzyme (Figure 3-3). The activation rates of these purified Greenland cod zymogens were measured and compared to the activation rate of porcine pepsinogen as described in section 2.2.5.

The activation of porcine pepsinogen at 0 °C, 5 °C, 10.1 °C, 20.1 °C and 35.1 °C is shown in Figure 3-9. At 35.1 °C, porcine pepsin was approximately 90% activated in 2 min and completely activated in 5 min. However, at 0 °C it took 20 min for the zymogen to become completely activated. Activation rates between these values were obtained at intermediate temperatures. It can be seen that temperature has a dramatic effect on the activation rate of porcine pepsinogen.

The activation of the Greenland Cod zymogens are shown in Figures 3-10 to 3-12. It is immediately obvious that that the effect of temperature on the activation of these zymogens is much less marked than with porcine pepsinogen. Zymogen 1 (Figure 3-10) was the slowest of the Greenland Cod zymogens to activate at 0 °C and was 65% activated in 1 min. At 5 °C it was 86% activated and at 10 °C it was 90% activated in 1 min. The activation of zymogen 2 (Figure 3-11) was relatively independent of temperature and was virtually complete in 1

Figure 3-9: Activation of Porcine Pepsinogen

The activation of porcine pepsinogen at various temperatures was determined as described in section 2.2.5. Pepsinogen (50 µl of a 0.5 mg/ml solution in 50 mM Tris phosphate pH 7.0) in a series of tubes was mixed with 50 µl of HCl of sufficient strength to bring the final pH of the mixture to 2.0. The activation was stopped at various times up to 10 min after the addition of acid by adding 0.4 ml of 0.1M sodium citrate buffer pH 6.2. Triplicate aliquots of each tube (100 µl) were then assayed for milk clotting activity. The average milk clotting activity at various times of activation was expressed as a percentage of the milk clotting activity of the fully activated proteases. (▲) 0° activation temperature, (■) 5° activation temperature, (●) 10.1° activation temperature, (○) 20.1° activation temperature, (△) 35.1° activation temperature.

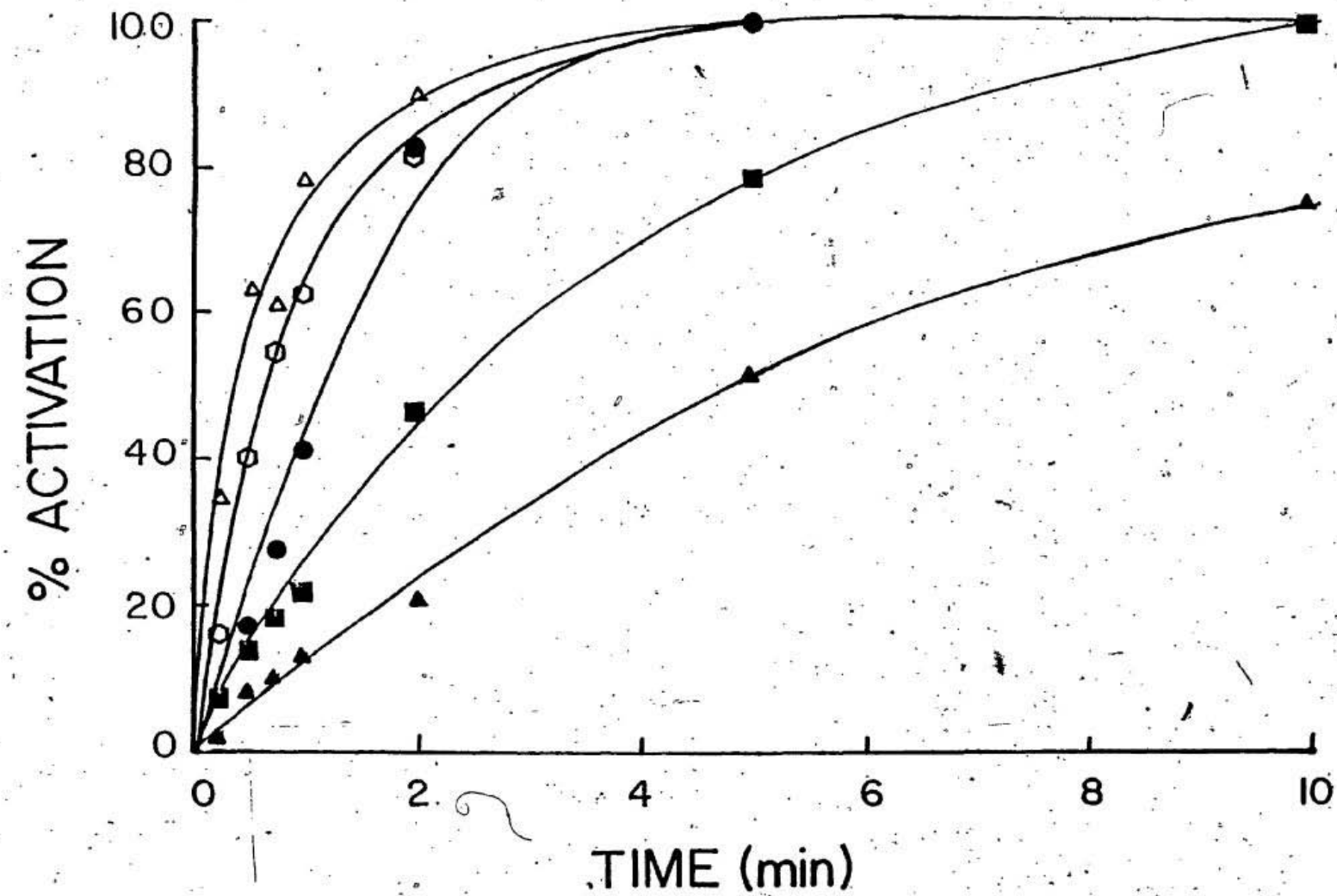


Figure 3-9: Activation of Porcine Pepsinogen

min. Similarly, zymogen 3 (Figure 3-12) was 85-90% activated after 1 min at 0 °C or 5 °C. Thus, zymogen 1 is more like porcine pepsinogen than zymogen 2 or 3 in the temperature dependence of activation.

3.3. Substrate Specificity

The hydrolytic activity of the proteases was measured using the following substrates: (1) protein substrates; hemoglobin, casein and gelatin (2) methylated protein substrates; methylated hemoglobin, methylated casein and methylated gelatin (3) various peptide derivatives; APDT, Z-trp-ala, Z-tyr-ala, Z-tyr-leu, Z-tyr-ser and Z-tyr-thr and (4) 12% milk powder with 10 mM $CaCl_2$ at pH 6.2 (milk clotting activity). The experimental protocol was as follows. For each enzyme, the reaction rate versus time was measured with all substrates. Suitable assay times were then chosen to insure that the linear part of the reaction rate versus time curve was used. The pH profile of each of the proteases with many of the substrates was then measured and further kinetic measurements were made usually at the optimum pH.

3.3.1. Determination of pH Optima

The pH profiles of porcine pepsin with the various substrates are shown in Figure 3-13. The maximum activity was found at pH 2 with both hemoglobin and casein as substrates. However, with methylated substrates, the pH optimum differed between the two substrates. A pH optimum was obtained at pH 1.5 with methylated casein while with methylated hemoglobin a sharp optimum was obtained at pH 2.5. Using the peptide APDT as substrate, a pH optimum was obtained at pH 1.5-2.0.

Figure 3-10: Activation of the Zymogen of Greenland Cod Protease 1

The activation of the zymogen of Greenland Cod protease 1 at various temperatures was determined as described in section 2.2.5. Zymogen in a series of tubes was mixed with 50 μl of HCl of sufficient strength to bring the final pH of the mixture to 2.0. The activation was stopped at various times up to 10 min after the addition of acid by adding 0.4 ml of 0.1M sodium citrate buffer pH 6.2. Triplicate aliquots of each tube (100 μl) were then assayed for milk clotting activity. The average milk clotting activity at various times of activation was expressed as a percentage of the milk clotting activity of the fully activated proteases. Unactivated zymogen failed to clot milk over the course of the day. (▲) 0° activation temperature, (■) 5° activation temperature, (●) 10° activation temperature.

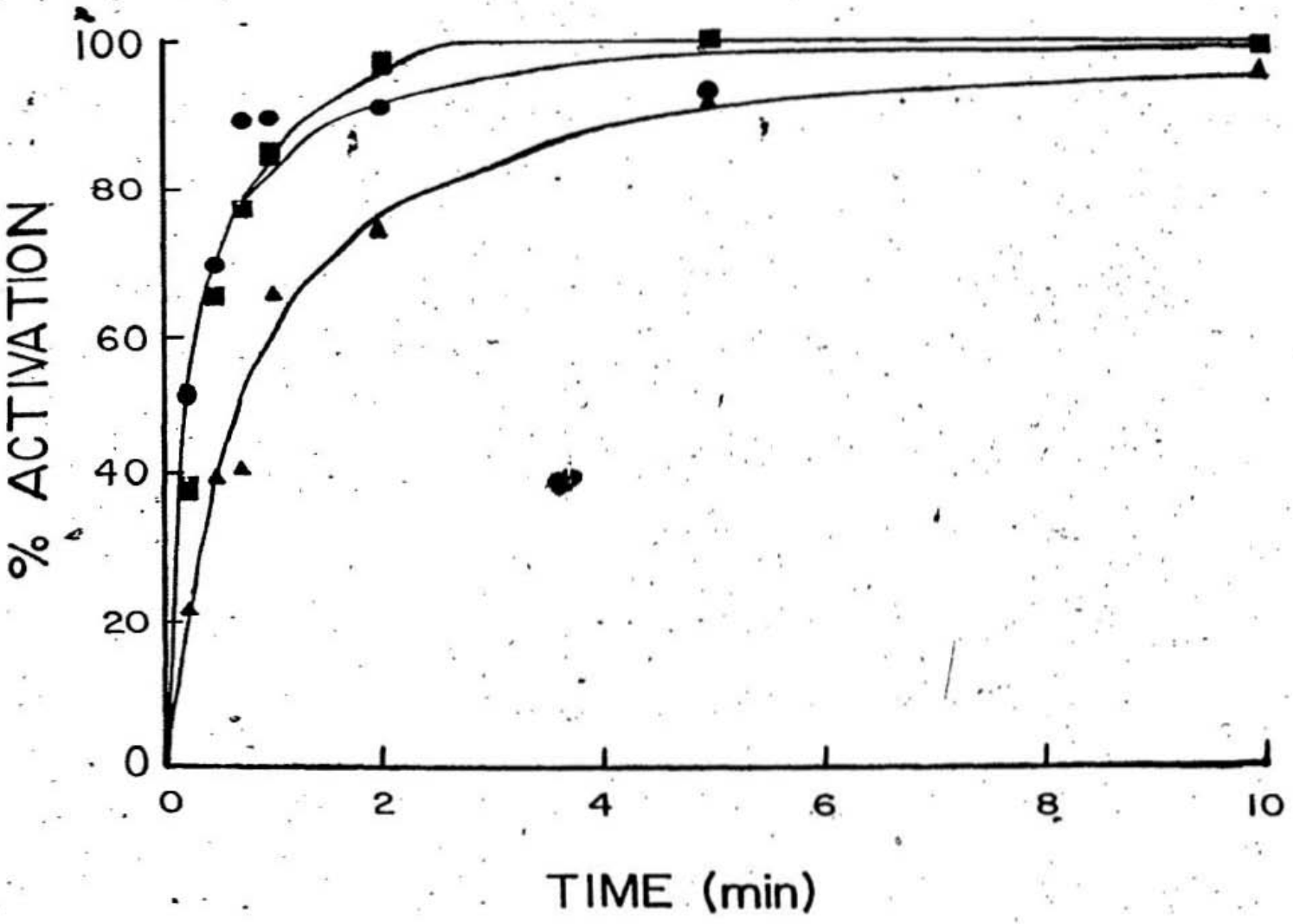


Figure 3-10: Activation of the Zymogen of Greenland Cod Protease 1

Figure 3-11: Activation of the Zymogen of Greenland Cod Protease 2

The activation of the zymogen of Greenland cod protease 2 at various temperatures was determined as described in section 2.2.5. Zymogen in a series of tubes was mixed with 50 μl of HCl of sufficient strength to bring the final pH of the mixture to 2.0. The activation was stopped at various times up to 10 min after the addition of acid by adding 0.4 ml of 0.1M sodium citrate buffer pH 6.2. Triplicate aliquots of each tube (100 μl) were then assayed for milk clotting activity. The average milk clotting activity at various times of activation was expressed as a percentage of the milk clotting activity of the fully activated proteases. Unactivated zymogen failed to clot milk over the course of the day. (▲) 0° activation temperature, (■) 5° activation temperature.

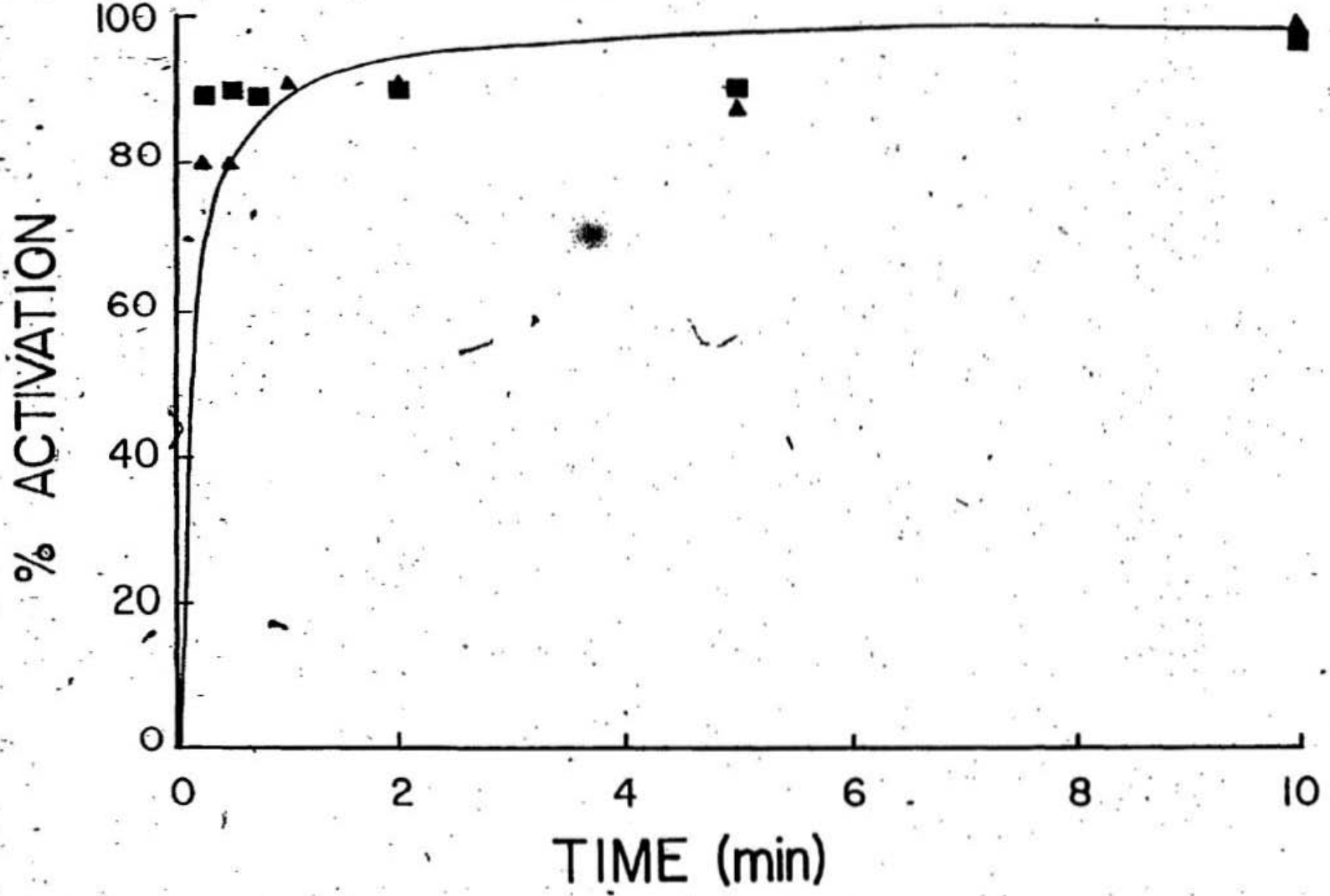


Figure 3-11: Activation of the Zymogen of Greenland Cod Protease 2

Figure 3-12: Activation of the Zymogen of Greenland Cod Protease 3

The activation of the zymogen of Greenland cod protease 3 at various temperatures was determined as described in section 2.2.5. Zymogen in a series of tubes was mixed with 50 μl of HCl of sufficient strength to bring the final pH of the mixture to 2.0. The activation was stopped at various times up to 10 min after the addition of acid by adding 0.4 ml of 0.1M sodium citrate buffer pH 6.2. Triplicate aliquots of each tube (100 μl) were then assayed for milk clotting activity. The average milk clotting activity at various times of activation was expressed as a percentage of the milk clotting activity of the fully activated proteases. Unactivated zymogen failed to clot milk over the course of the day. (▲) 0° activation temperature, (■) 5° activation temperature.

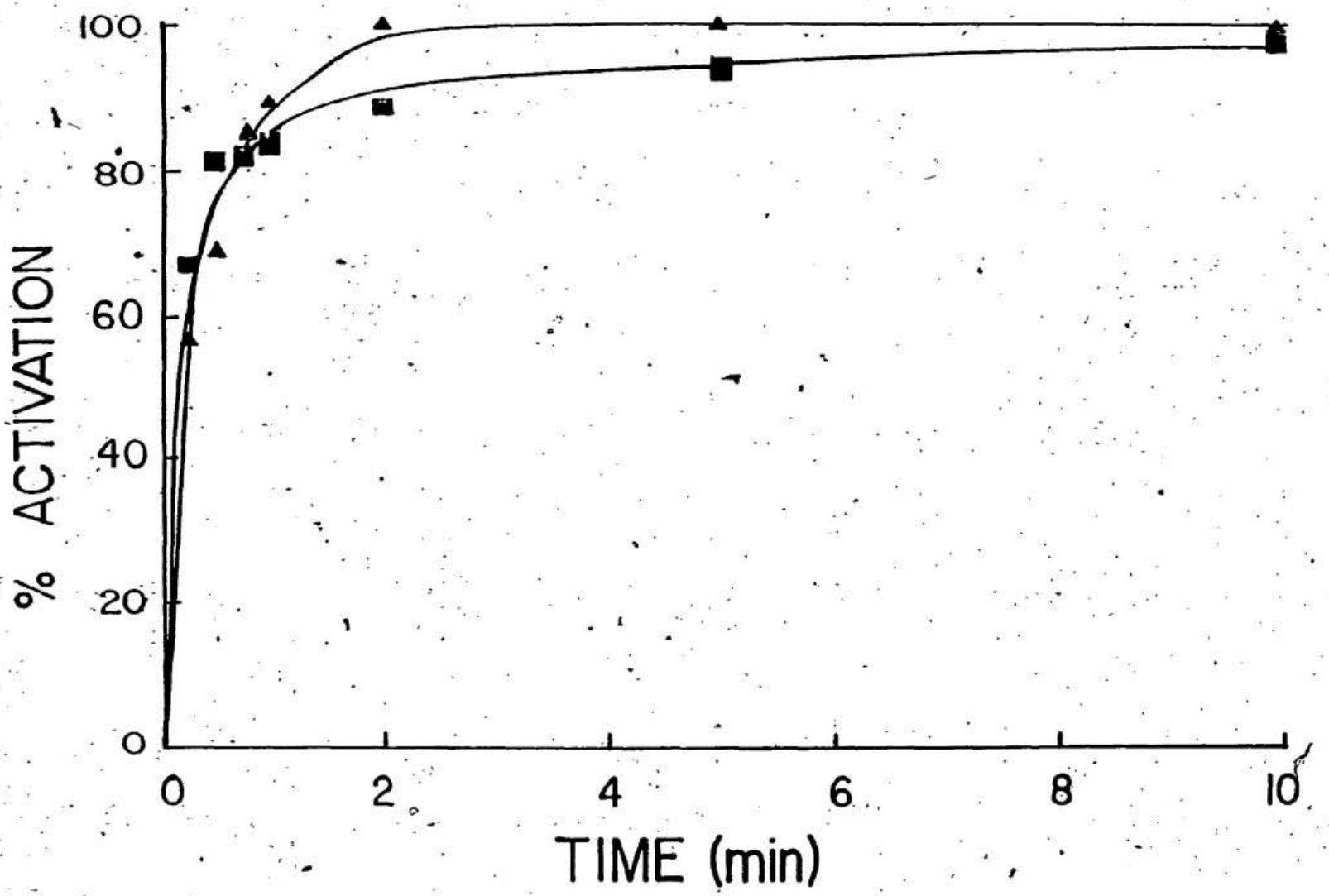


Figure 3-12: Activation of the Zymogen of Greenland Cod Protease 3

Figure 3-13: pH Profiles for Porcine Pepsin A

The activity of porcine pepsin A was measured with different substrates and at different pH at 27° C as described below. **A** (▲) hemoglobin substrate, (●) casein substrate. Triplicate tubes containing 1.5 ml of 2% protein substrate and .075 ml of 0.2M citrate HCl buffer (adjusted to pH values below 2 with HCl and above 2 with NaOH) were incubated with 50-100 μg of enzyme for 20 min. The reaction was stopped by adding 1.5 ml of 12% TCA. Specific activity is expressed as ΔA_{280}/h/mg protein. **B** (■) methylated hemoglobin as substrate, (◆) methylated casein as substrate. Triplicate assay mixtures containing 0.5 ml of 0.3% methylated protein, 0.25 ml of 0.2 M citrate HCl buffer and 10 μg enzyme were incubated at 27 ° C for 10 min. The reaction was stopped by adding 0.5 ml of 0.1M sodium borate and the free amino groups formed by the proteolysis were detected with TNBS as described in section 2.2.4.2. Specific activity is expressed as μmoles of peptide bonds hydrolysed/h/mg protein. **C** (△) Triplicate assays were performed with APDT as the substrate. 2mM APDT (0.25 ml) plus 0.2M citrate HCl buffer (.05 ml) plus 35 μg of enzyme were incubated in a total volume of 1 ml for 2 h. The reaction was stopped by adding 0.5 ml of ninhydrin reagent and the color was developed as described in section 2.2.4.4. Specific activity is expressed as μmoles of peptide bonds hydrolysed/h/mg protein.

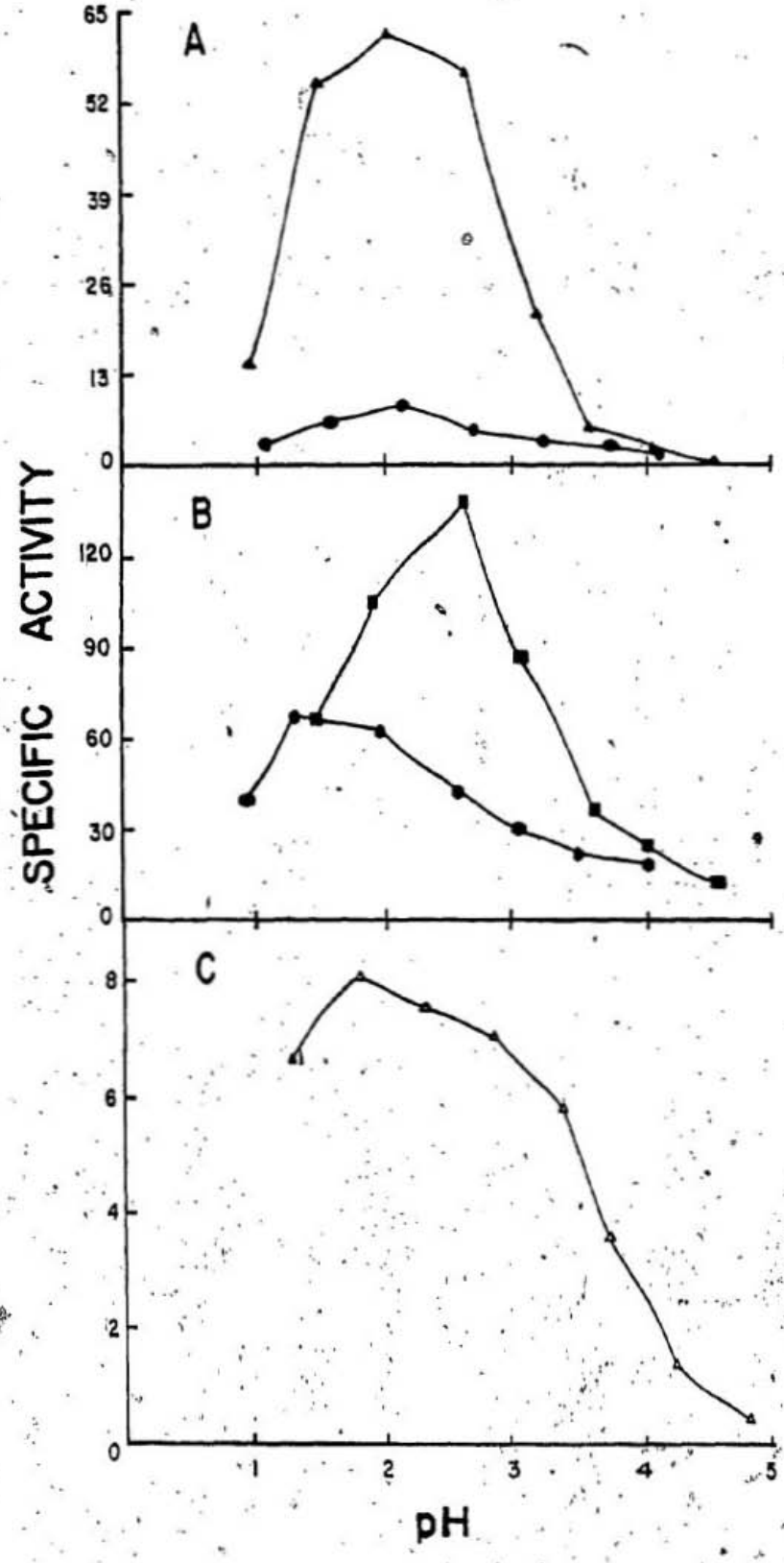


Figure 3-13: pH Profiles for Porcine Pepsin A

The pH optima for porcine "gastricsin fraction" are shown in Figure 3-14. The pH optimum was at pH 2 with either hemoglobin or casein as substrate. However, as found with porcine pepsin A preparation, the pH optimum of the "gastricsin fraction" was different with the two methylated substrates. The pH optimum with methylated casein was at pH 1.5 while the pH optimum with methylated hemoglobin was at pH 2.0. The optimum pH for the "gastricsin fraction" with methylated hemoglobin was quite sharp and was at 0.5 pH units lower than that found with porcine pepsin A. The pH optimum for the "gastricsin fraction" with APDT was quite broad and extended from pH 1.7 to pH 2.7.

A Greenland cod protease mixture was prepared by repeated chromatography of a stomach homogenate on Sephadex G100 as described in section 2.2.1.2. The pH optima of this mixture are shown in Figure 3-15. Very broad optima were obtained extending from pH 1.5-3.5 using either casein or hemoglobin as substrates. The optima obtained with methylated substrates again differed between substrates. The optimum activity obtained with methylated casein was at pH 3 while the optimum with methylated hemoglobin was at pH 4. The pH optima for the peptide substrates Z-trp-ala, Z-tyr-ala and Z-tyr-leu were at pH 2-2.5.

Activated Greenland cod gastric proteases were purified from a crude stomach homogenate by Sephadex G100, chromatofocusing, and Sephadex G75 chromatography as described earlier and were used in the following experiments. The pH optima of the Greenland cod protease 1 are shown in Figure 3-16. The pH optimum was at pH 3.5 with hemoglobin as the substrate and was at pH 3.0 with

Figure 3-14: pH Profiles for Porcine "Gastricsin Fraction"

The activity of porcine "gastricsin fraction" was measured with different substrates and at different pH at 27° C as described below. **A** (▲) hemoglobin substrate, (●) casein substrate. Triplicate tubes containing 1.5 ml of 2% protein substrate and .075 ml of 0.2M citrate HCl buffer (adjusted to pH values below 2 with HCl and above 2 with NaOH) were incubated with 15-65 μg of enzyme for 20 min. The reaction was stopped by adding 1.5 ml of 12% TCA. Specific activity is expressed as ΔA_{280}/h/mg protein. **B** (■) methylated hemoglobin as substrate, (◆) methylated casein as substrate. Triplicate assay mixtures containing 0.5 ml of 0.3% methylated protein, 0.25 ml of 0.2 M citrate HCl buffer and 10 μg enzyme were incubated at 27 ° C for 10 min. The reaction was stopped by adding 0.5 ml of 0.1M sodium borate and the free amino groups formed by the proteolysis were detected with TNBS as described in section 2.2.4.2. Specific activity is expressed as μmoles of peptide bonds hydrolysed/h/mg protein. **C** (△) Triplicate assays were performed with APDT as the substrate. 2mM APDT (0.25 ml) plus 0.2M citrate HCl buffer (.05 ml) plus 30 μg of enzyme were incubated in a total volume of 1 ml for 2 h. The reaction was stopped by adding 0.5 ml of ninhydrin reagent and the color was developed as described in section 2.2.4.4. Specific activity is expressed as μmoles of peptide bonds hydrolysed/h/mg protein.

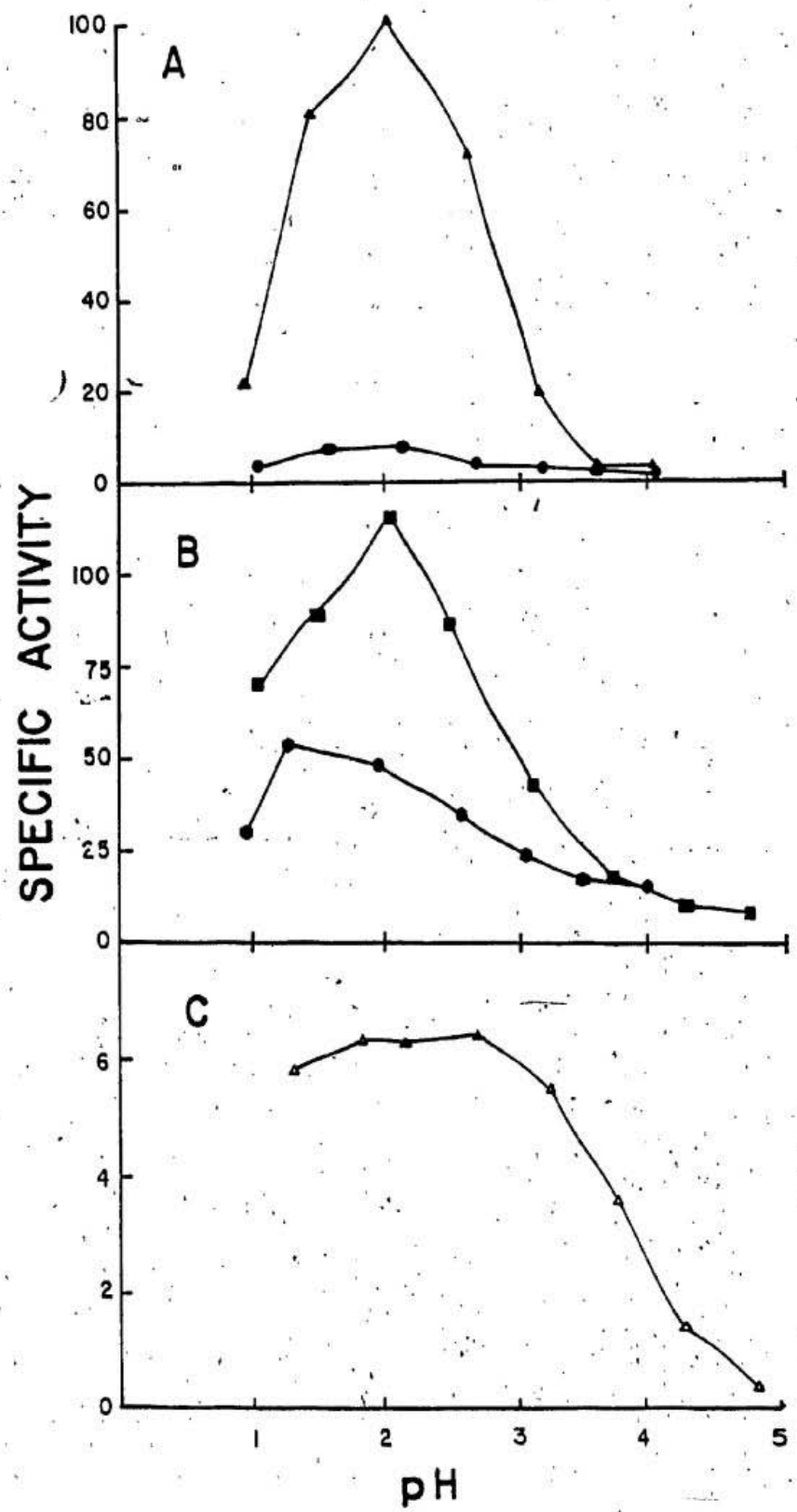


Figure 3-14: pH Profiles for Porcine "Gastricsin" Fraction.

Figure 3-15: pH Profiles for Greenland Cod Protease Mixture

The activity of a mixture of Greenland cod proteases was measured with different substrates and at different pH at 27° C as described below. **A** (▲) hemoglobin substrate, (●) casein substrate. Triplicate tubes containing 1.5 ml of 2% protein substrate and .075 ml of 0.2M citrate HCl buffer (adjusted to pH values below 2 with HCl and above 2 with NaOH) were incubated with 30-60 μg of enzyme for 20 min. The reaction was stopped by adding 1.5 ml of 12% TCA. Specific activity is expressed as ΔA_{280}/h/mg protein. **B** (■) methylated hemoglobin as substrate, (◆) methylated casein as substrate. Triplicate assay mixtures containing 0.5 ml of 0.3% methylated protein, 0.25 ml of 0.2 M citrate HCl buffer and 35 μg enzyme were incubated at 27 ° C for 10 min. The reaction was stopped by adding 0.5 ml of 0.1M sodium borate and the free amino groups formed by the proteolysis were detected with TNBS as described in section 2.2.4.2. Specific activity is expressed as μmoles of peptide bonds hydrolysed/h/mg protein. **C** (○) Z-tyr-ala as substrate, (△) Z-tyr-leu as substrate, (□) Z-trp-ala as substrate. Triplicate assays were performed with the different peptide substrates. 2mM peptide (0.5 ml) plus 0.2M citrate HCl buffer (.05 ml) plus 90 μg of enzyme were incubated in a total volume of 1 ml for 285 min. The reaction was stopped by adding 0.5 ml of ninhydrin reagent and the color was developed as described in section 2.2.4.4. Specific activity is expressed as μmoles of peptide bonds hydrolysed/h/mg protein.

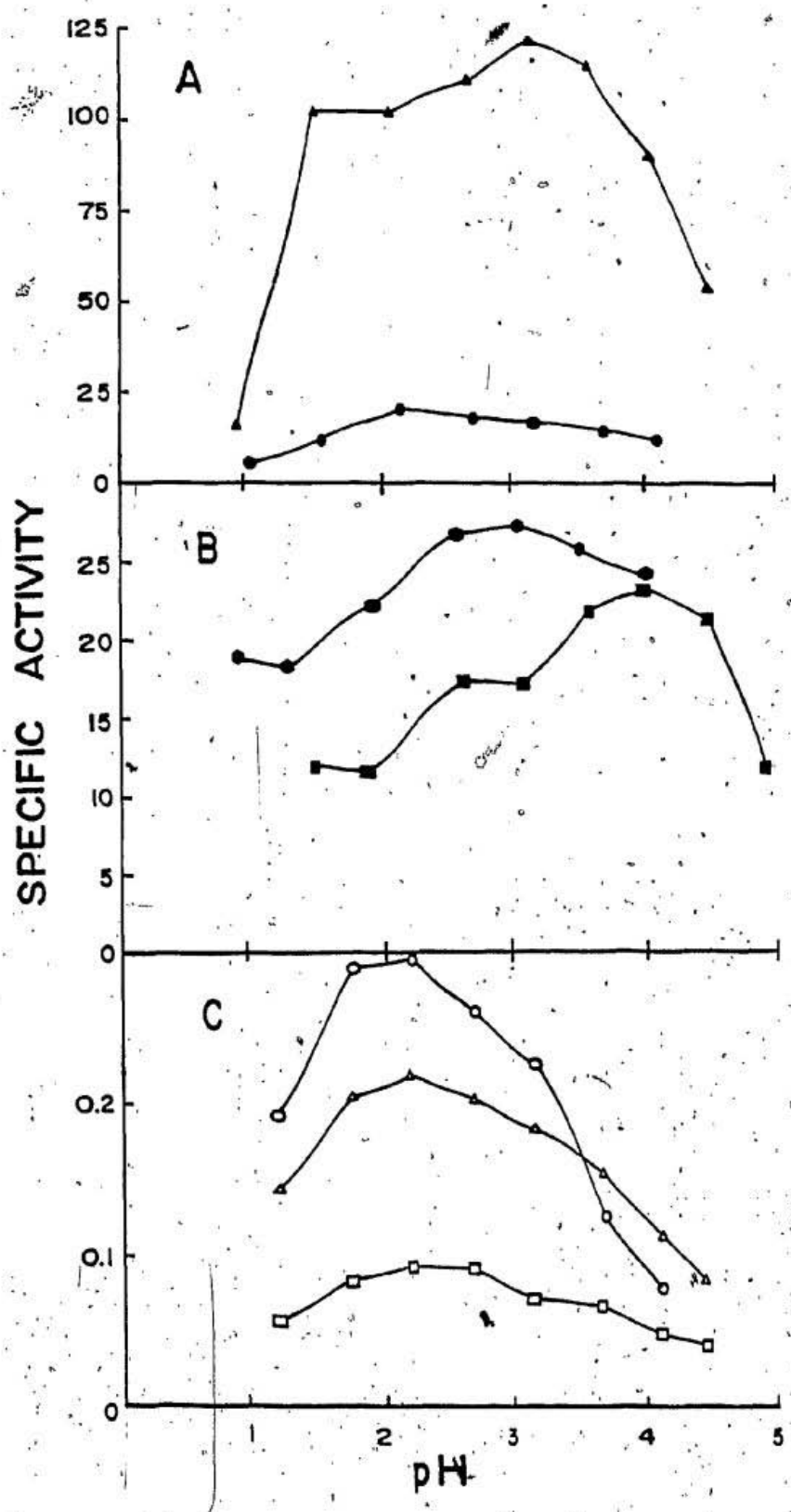


Figure 3-15: pH Profiles for Greenland Cod Protease Mixture

casein as the substrate. The pH profiles with the methylated substrates were characterized by a plateau area from 1.5-3 followed by an increase in activity to a maximum at pH 4-4.5. Of all the peptide substrates, protease 1 was active only with APDT and had optimum activity at pH 2.3-3.5.

Greenland cod protease 2 had a pH optimum at pH 2.5-3 with hemoglobin and pH 2.5 with casein as the substrate (Figure 3-17). The pH optima shifted with the methylated substrates to pH 4 for methylated hemoglobin and pH 3.5 for methylated casein with a tendency for a minor optimum at pH 1. Protease 2 had a single sharp optimum at pH 2 with the peptide derivative Z-tyr-ala.

Greenland cod protease 3 had optimum activity with hemoglobin at pH 2.5-3 and with casein as substrate at pH 3 (Figure 3-18). The pH optimum with methylated casein was at pH 3.5 while with methylated hemoglobin the maximum activity was at pH 4 with a minor second optimum at pH 1.5. The peptide derivative Z-tyr-ala gave a sharp optimum at pH 2.5 with protease 3. The data for the pH optima of the gastric proteases are summarized in Table 3-2.

3.3.2. Specific Activity of the Proteases on Various Substrates

The specific activities of all the protease preparations with the various substrates are shown in Table 3-3. Cod protease 1 had similar activity on 2% hemoglobin as did the porcine enzyme preparations while protease 2 and 3 had respectively 40% and 25% of the activity of protease 1. "Gastricsin fraction" was about 50% more active than pepsin A with casein as the substrate. Cod proteases 1 and 2 were 70% as active and protease 3 was 45% as active as porcine pepsin

Figure 3-16: pH Profiles for Greenland cod Protease 1

The activity of Greenland cod protease 1 was measured with different substrates and at different pH at 27° C as described below. **A** (▲) hemoglobin substrate, (●) casein substrate. Triplicate tubes containing 1.5 ml of 2% protein substrate and .075 ml of 0.2M citrate HCl buffer (adjusted to pH values below 2 with HCl and above 2 with NaOH) were incubated with 15 μg of enzyme for 30 min. The reaction was stopped by adding 1.5 ml of 12% TCA. Specific activity is expressed as ΔA_{280}/h/mg protein. **B** (■) methylated hemoglobin as substrate, (◆) methylated casein as substrate. Triplicate assay mixtures containing 0.5 ml of 0.3% methylated protein, 0.25 ml of 0.2 M citrate HCl buffer and 15 μg enzyme were incubated at 27 ° C for 75 min. The reaction was stopped by adding 0.5 ml of 0.1M sodium borate and the free amino groups formed by the proteolysis were detected with TNBS as described in section 2.2.4.2. Specific activity is expressed as μmoles of peptide bonds hydrolysed/h/mg protein. **C** (△) Triplicate assays were performed with APDT as substrate. 2mM APDT (0.25 ml) plus 0.2M citrate HCl buffer (.05 ml) plus 100 μg of enzyme were incubated in a total volume of 1 ml for 210 min. The reaction was stopped by adding 0.5 ml of ninhydrin reagent and the color was developed as described in section 2.2.4.4. Specific activity is expressed as μmoles of peptide bonds hydrolysed/h/mg protein.

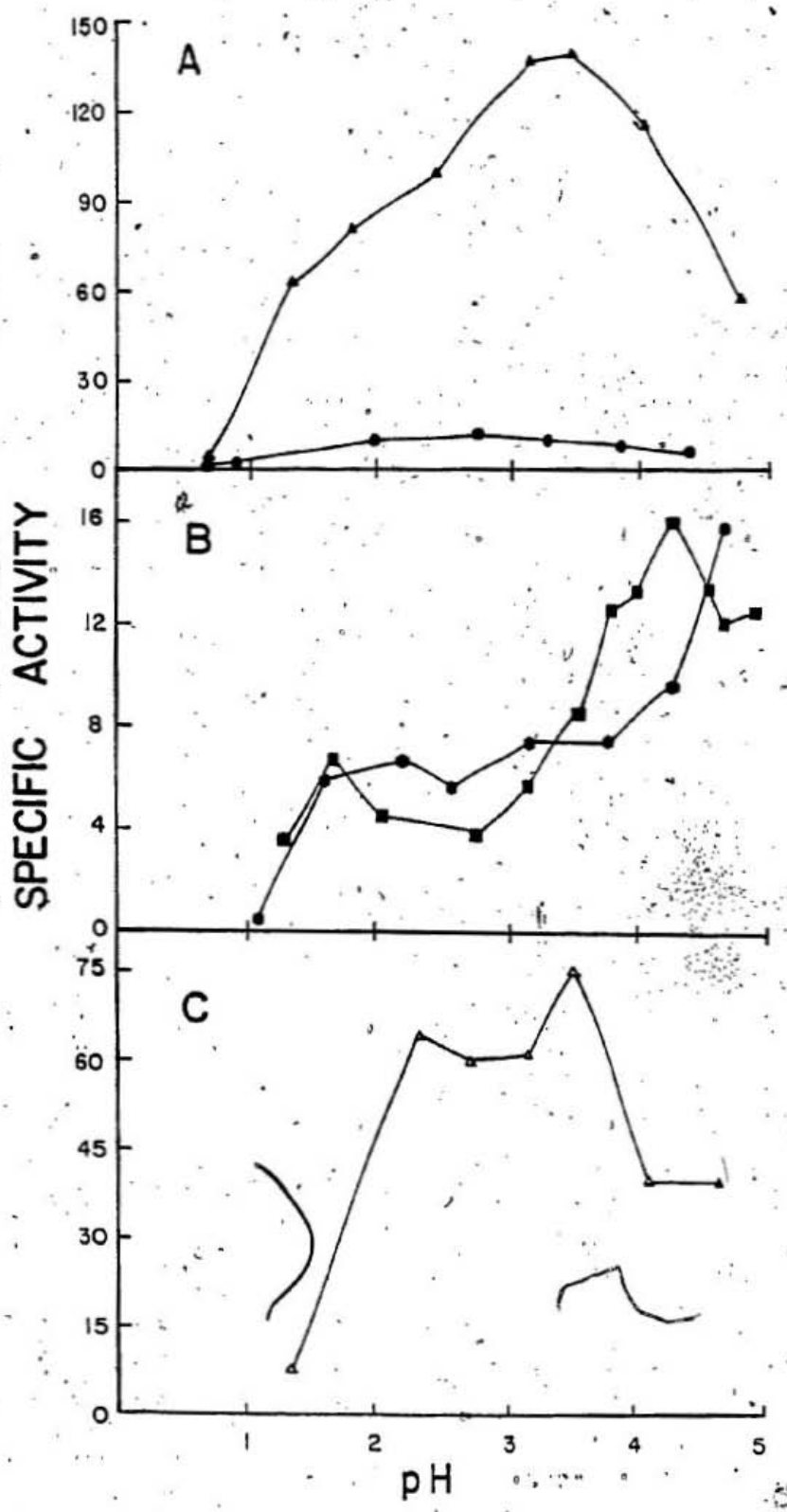


Figure 3-16: pH Profiles for Greenland cod Protease 1

Figure 3-17: pH Profiles for Greenland cod Protease 2

The activity of Greenland cod protease 2 was measured with different substrates and at different pH at 27° C as described below. **A** (▲) hemoglobin substrate, (●) casein substrate. Triplicate tubes containing 1.5 ml of 2% protein substrate and .075 ml of 0.2M citrate HCl buffer (adjusted to pH values below 2 with HCl and above 2 with NaOH) were incubated with 60 μg of enzyme for 30 min. The reaction was stopped by adding 1.5 ml of 12% TCA. Specific activity is expressed as ΔA_{280}/h/mg protein. **B** (■) methylated hemoglobin as substrate, (◆) methylated casein as substrate. Triplicate assay mixtures containing 0.5 ml of 0.3% methylated protein, 0.25 ml of 0.2 M citrate HCl buffer and 60 μg enzyme were incubated at 27 ° C for 45 min. The reaction was stopped by adding 0.5 ml of 0.1M sodium borate and the free amino groups formed by the proteolysis were detected with TNBS as described in section 2.2.4.2. Specific activity is expressed as μmoles of peptide bonds hydrolysed/h/mg protein. **C** (○) Triplicate assays were performed with Z-tyr-ala as substrate. 2mM peptide (0.5 ml) plus 0.2M citrate HCl buffer (.05 ml) plus 60 μg of enzyme were incubated in a total volume of 1 ml for 120 min. The reaction was stopped by adding 0.5 ml of ninhydrin reagent and the color was developed as described in section 2.2.4.4. Specific activity is expressed as μmoles of peptide bonds hydrolysed/h/mg protein.

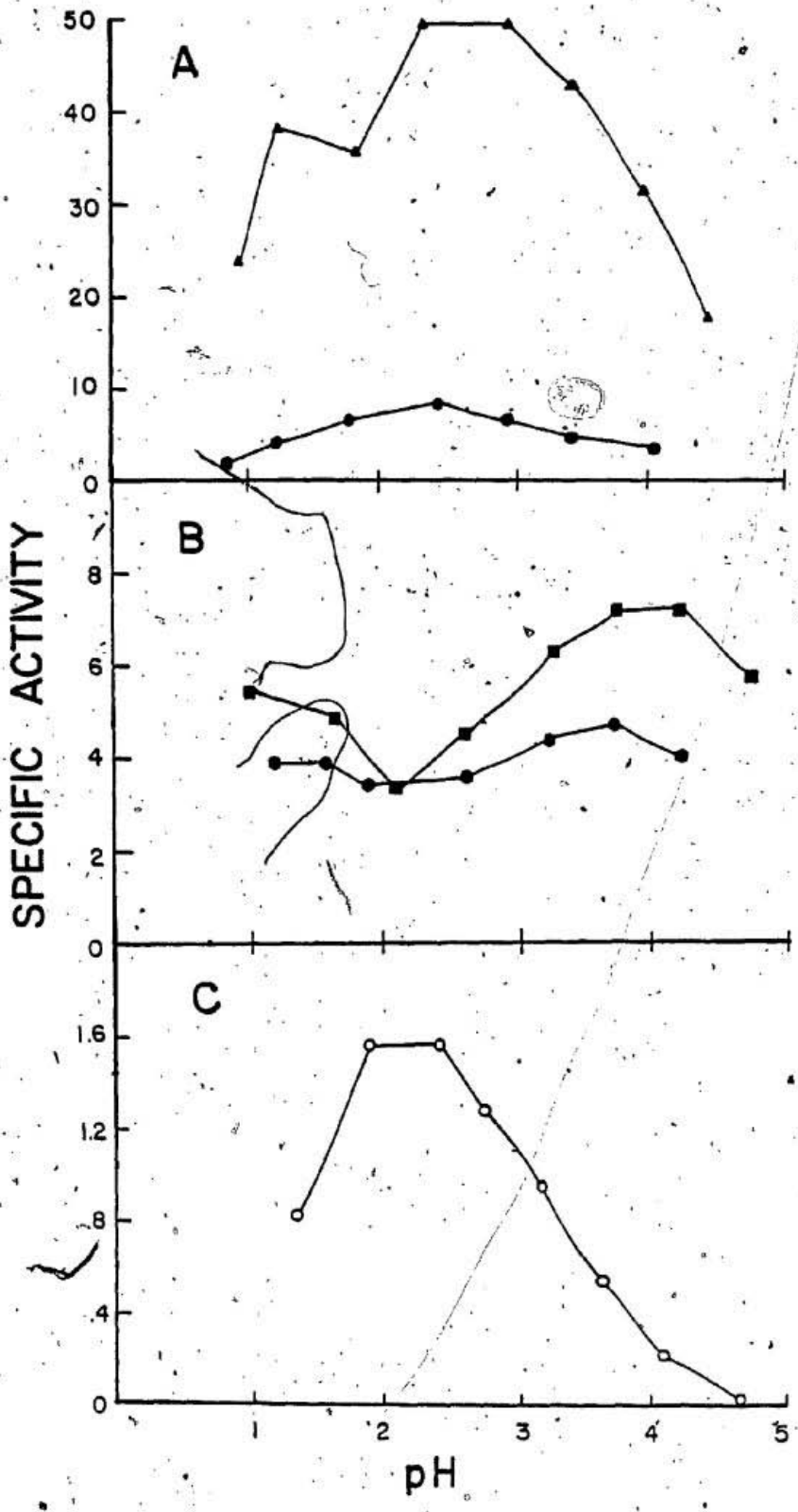


Figure 3-18: pH Profiles for Greenland cod Protease 2

Figure 3-18: pH Profiles for Greenland cod Protease 3

The activity of Greenland cod protease 3 was measured with different substrates and at different pH at 27° C as described below. **A** (▲) hemoglobin substrate, (●) casein substrate. Triplicate tubes containing 1.5 ml of 2% protein substrate and .075 ml of 0.2M citrate HCl buffer (adjusted to pH values below 2 with HCl and above 2 with NaOH) were incubated with 70 μg of enzyme for 30 min. The reaction was stopped by adding 1.5 ml of 12% TCA. Specific activity is expressed as ΔA_{280}/h/mg protein. **B** (■) methylated hemoglobin as substrate, (◆) methylated casein as substrate. Triplicate assay mixtures containing 0.5 ml of 0.3% methylated protein, 0.25 ml of 0.2 M citrate HCl buffer and 45 μg enzyme were incubated at 27 ° C for 20 min. The reaction was stopped by adding 0.5 ml of 0.1M sodium borate and the free amino groups formed by the proteolysis were detected with TNBS as described in section 2.2.4.2. Specific activity is expressed as μmoles of peptide bonds hydrolysed/h/mg protein. **C** (○) Triplicate assays were performed with Z-tyr-ala as substrate. 2mM peptide (0.5 ml) plus 0.2M citrate HCl buffer (.05 ml) plus 225 μg of enzyme were incubated in a total volume of 1 ml for 120 min. The reaction was stopped by adding 0.5 ml of ninhydrin reagent and the color was developed as described in section 2.2.4.4. Specific activity is expressed as μmoles of peptide bonds hydrolysed/h/mg protein.

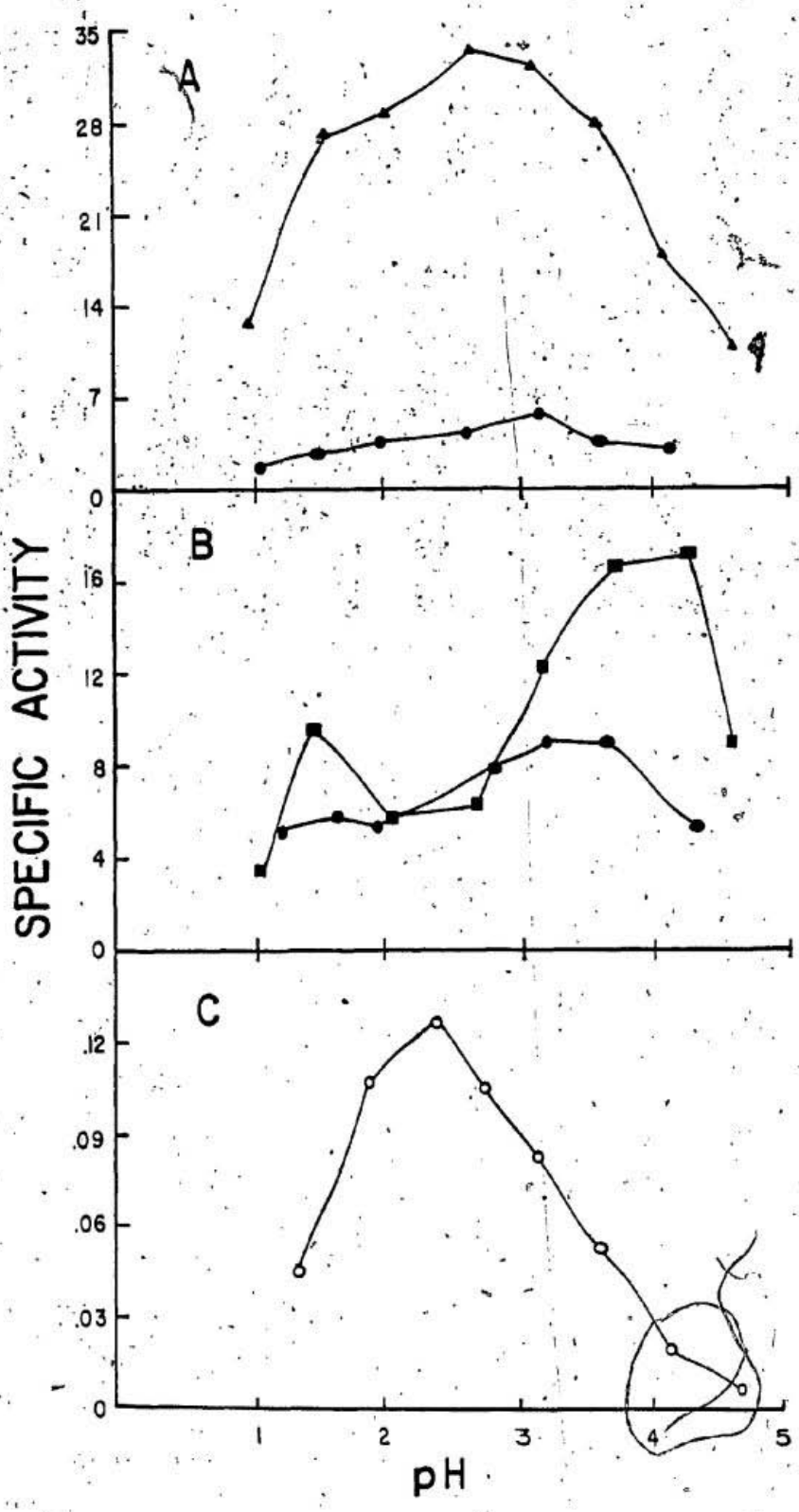


Figure 3-17: pH Profiles for Greenland cod Protease 3

Table 3-2: pH Optima of Gastric Proteases with Various Substrates

Substrate	Pepsin A	C P 1	C P 2	C P 3
Hemoglobin	2	3.5	2.5-3	2.5-3
Casein	2	3.0	2.5	3
Methylated Hemoglobin	2.5	4-4.5	4	4
Methylated Casein	1.5	4.5	3.5	3.5
APDT	1.5-2	2.5-3.5		
Z-tyr-ala			2	2.5

The data for the "gastricsin-fraction" was not substantially different from that of pepsin A and was therefore not included here. C P 1 = cod protease 1, C P 2 = cod protease 2, C P 3 = cod protease 3.

A. Thus it can be seen that protease-1 is more like the porcine gastric proteases than the other Greenland cod proteases in the activity with protein substrates. The activities of porcine pepsin A and "gastricsin fraction" are quite similar on the methylated substrates. All of the cod proteases also have quite similar activities on the methylated substrates. Hdwever, the cod proteases had only 5% of the activity on methylated hemoglobin and 11% of the activity on methylated casein compared to the porcine enzymes. The activity of the cod protease mixture with the protein substrates is greater than the sum of the activities of the individual cod enzymes. This synergism is probably due to the differences in specificity of the different cod proteases (as demonstrated with the peptide substrates) which results in more complete digestion of protein substrates by the gastric protease mixture.

The activities of the various proteases with the different peptide substrates were quite different. Porcine pepsin A was the most active with APDT and porcine "gastricsin fraction" had 65% of the activity of pepsin with this substrate. Cod protease 1 was the most active of the cod proteases with APDT but was only 1% as active as porcine pepsin A with this substrate. Protease 2 was 40% as active and protease 3 was only 3% as active as cod protease 1 with APDT. Protease 2 was the most active of all the proteases with Z-trp-ala, Z-tyr-ala, Z-tyr-leu, Z-tyr-ser and Z-tyr-thr. Cod protease 3 was 3-8% as active as protease 2 while protease 1 had no detectable activity with any of this group of substrates. Porcine "gastricsin fraction" was active only with Z-tyr-ser, having 23% of the activity of cod protease 2 with this substrate. Porcine pepsin A was active with Z-tyr-ser and Z-tyr-thr having respectively 18% and 39% of the activity of cod protease 2. Thus, of the cod proteases, protease 1 was the most similar to the porcine enzymes in its activity on various substrates. It is noteworthy that Z-tyr-thr was a substrate for porcine pepsin A but not for porcine "gastricsin fraction". An examination of the clotting activity of the various proteases is quite instructive. Gastricsin fraction was the more active of the porcine enzyme preparations and pepsin A had only 45% of the activity of "gastricsin fraction" with this assay. However, the cod proteases were on the average 8 times more active in the milk clotting assay than porcine "gastricsin fraction", with protease 1 being the most active and protease 2 being the least active.

The relative activities of the various gastric proteases with the different substrates are expressed as a ratio of the proteolytic activity with hemoglobin as

Table 3-3: Specific Activities of Gastric Proteases with Various Substrates

Substrate	Porcine Pepsin	Gastricsin Fraction [a]	Cod Protease Mixture	Cod Protease 1 [c]	Cod Protease 2 [c]	Cod Protease 3 [c]
2% hemoglobin	90.74	86.26	128.71	102.24	42.59	25.84
2% casein	10.91	15.05	19.84	7.29	7.21	4.50
2% gelatin	<0.4	<0.8	<1,0	N.D.	N.D.	N.D.
0.4% methylated hemoglobin	169.44	134.51	23.03	6.76	7.12	9.21
0.4% methylated casein	65.58	62.35	26.69	8.50	7.52	4.93
0.4% methylated gelatin	<3.0	<3.0	<3.0	N.D.	N.D.	N.D.
0.5 mM APDT	8.166 [b]	5.296	.078 [b]	.068	.027	.002
1 mM Z-trp-ala	<.005	<.0003	.190	<.0008	.135	.008
1 mM Z-tyr-ala	<.005	<.0003	.698	<.0008	.774	.064
1 mM Z-tyr-leu	<.003	<.009	.066	<.0008	.065	.003
1 mM Z-tyr-ser	.025	.032	N.D.	<.0008	.137	.004
1 mM Z-tyr-thr	.027	<.004	N.D.	<.0008	.07	.002
milk clotting	246.4	549.8	N.D.	6227.4	2905.1	3664.2

[a] 0.5% hemoglobin and casein were used; [b] 0.4 mM APDT was used; [c] 1.4% casein, 0.26% methylated hemoglobin, 0.18% methylated casein were used. N.D. = not determined; temperature = 26 ° C. Units of activity were; for hemoglobin and casein, ΔA_{280}/h/mg enzyme; for all others except milk clotting, μmoles of bonds hydrolysed/h/mg enzyme; for milk clotting, clotting units/mg enzyme. A milk clotting unit = amount of enzyme that will clot 10 ml of milk in 100 sec at 27 ° C.

the substrate in Table 3-4. Cod protease 1 had a relative casein activity that was only 50% of that of all other protease preparations. The relative activities of the cod proteases were 100 times less with methylated hemoglobin and APDT, and 5-10 times less with methylated casein than the corresponding relative activities of the porcine protease preparations. The relative activity of Cod protease 2 with peptide substrates (other than APDT) was at least 10 times greater than any other of the proteases. Cod protease 3 was active with a variety of peptide substrates but had no greater relative activity with peptide substrates than the porcine protease preparations. A comparison of the relative milk clotting activity (CU/PU) of the different proteases also reveals that the cod proteases would be more suitable than the porcine enzymes in clotting milk. Protease 3 had the highest CU/PU ratio which was 52 times that of porcine pepsin.

3.3.3. Measurement of the Degree of Hydrolysis of Hemoglobin

The degree of hydrolysis (DH) of 2% hemoglobin by each of the proteases is summarized in Table 3-5. The reactions were carried out at pH 3 using a pH stat as outlined in section 2.2.4.5. Porcine pepsin hydrolysed the hemoglobin to a similar extent as did porcine "gastricsin fraction". Protease 3 was the most effective enzyme at hydrolysing the hemoblobin and produced 1.9 times the degree of hydrolysis as did porcine pepsin. The DH values for cod protease 1 and 2, although apparently larger, were not significantly different ($P<.05$) from porcine pepsin. Generally speaking, the cod proteases more completely hydrolyse the hemoglogin substrate than the porcine enzymes. This is probably indicative of the much wider substrate specificity of the cod proteases. This is in agreement with the activity of the cod proteases with the large number of peptide substrates (Table 3-3).

Table 3-4: Relative Specific Activities of the Gastric Proteases Compared to Proteolytic Activity with Hemoglobin

Substrate	Porcine Pepsin	Gastricsin Fraction	Cod Protease Mixture	Cod Protease 1	Cod Protease 2	Cod Protease 3
casein	0.12	0.17	0.15	0.07	0.17	0.17
methylated hemoglobin	1.86	1.56	0.18	0.07	0.17	0.37
methylated casein	0.72	0.72	0.21	0.08	0.18	0.19
APDT	0.09	0.06	0.0006	0.0007	0.0006	0.0001
Z-trp-ala			0.0015		0.0032	0.0003
Z-tyr-ala			0.0054		0.0182	0.0025
Z-tyr-leu			0.0005		0.0015	0.0001
Z-tyr-ser	0.0003	0.0004			0.0032	0.0002
Z-tyr-thr	0.0003				0.0016	0.0001
milk clotting	2.72	6.39		60.91	68.21	141.80

3.4. Determination of Kinetic Parameters

The effect of changing the substrate concentration on the initial velocities of the various enzyme preparations were measured using a variety of substrates. The estimates for Km and Vmax were calculated by fitting the data to the Michaelis-Menton model and are reported in Tables 3-6 to 3-11. In some experiments with hemoglobin or casein as the substrate and measuring the activity by estimating the TCA soluble reaction products (section 2.2.4.1) the measured rate of activity

Table 3-5: PH Stat Measurements of the Degree of Hydrolysis of Hemoglobin

Enzyme	Degree of Hydrolysis (%)
Porcine "Gastricsin Fraction"	3.70 ± 0.80 (4)
Porcine Pepsin A	3.73 ± 1.02 (6)
Cod Protease 1	4.34 ± 0.66 (3)
Cod Protease 2	5.34 ± 0.04 (2)
Cod Protease 3	7.03 ± 0.52* (3)

Values are expressed as the mean ± standard deviation with the number of determinations in parenthesis. * significantly different from porcine pepsin ($P<.001$). All enzyme preparations were purified as described in the text. Hydrolysis was measured at 25 °C pH 3. for 24-48 h until the reaction was complete.

actually decreased at the highest substrate concentrations. This effect was more pronounced at colder temperatures and fitted a Substrate Inhibition model very well. This was not felt to be actual substrate inhibition but more a function of the TCA solubles assay technique used for the reasons outlined below.

The digestion of proteins by gastric proteases can be thought to occur in two stages. In stage 1, the large protein molecules are cleaved to form smaller fragments, but these fragments are still too large to be soluble in TCA. In stage 2, these fragments are split to form smaller peptides that are soluble in TCA. At very high substrate concentrations, the production of small TCA soluble peptides will be competitively inhibited by the presence of an excess of large protein molecules. This inhibition may be more intense at colder temperatures due to slower rates of diffusion of the protein molecules and slower overall reaction rates.

The concentration of TCA used in the assay is important, since the more concentrated the TCA solution used, the smaller the size of the peptides that will be soluble in it. In this regard, the concentration of TCA was doubled from that normally used by most workers when it was found that some of the cod gastric proteases were not totally inhibited at a final TCA concentration of 3%. In addition, the initial temperature of the TCA/protein mixture may affect the solubility of the peptides. These phenomena may explain the lack of other reports of this apparent substrate inhibition in the vast literature where Anson's (1938) method was used. It was concluded therefore that the data at high substrate concentrations that showed this unusual effect should not be used in the calculation of Km' and Vmax.

Statistical comparisons of selected Km' or Vmax were made using the t test and calculating the t values using the following equation;

$$t = (X_1 - X_2)/\sqrt{2s^2}$$

where

$$s^2 = (n_1-2)s_1^2 + (n_2-2)s_2^2/(n_1 + n_2 - 4)$$

X_1, X_2 are the mean of Km' or Vmax s_1, s_2 are the corresponding standard errors n_1, n_2 are the number of values plotted in the corresponding Lineweaver Burke plots. The number of degrees of freedom are n_1+n_2-4

3.4.1. Estimates of Vmax and Km'

The Vmax and Km' obtained at different temperatures for porcine pepsin are reported in Table 3-6. The Vmax with hemoglobin and methylated hemoglobin as substrates were greater than the Vmax with casein and methylated casein, respectively, by about the same extent. The Km' for hemoglobin were similar to the Km' obtained with casein, but the Km' for methylated hemoglobin were twice those obtained with methylated casein. The physiological efficiency (Vmax/Km') were remarkably constant at the different temperatures with hemoglobin and casein as the substrates. However, with the other substrates physiological efficiency tended to decrease with decreasing temperature.

The estimates for Vmax and Km' for porcine "gastricsin fraction" obtained at different temperatures are given in Table 3-7. The Vmax with hemoglobin and casein as the substrates were about the same as those obtained for porcine pepsin A. As found for porcine pepsin A, the Vmax for "gastricsin fraction" with hemoglobin and methylated hemoglobin were higher than those obtained with casein and methylated casein. The Vmax with APDT were similar for porcine pepsin A and "gastricsin fraction". However, estimates for Km' for porcine "gastricsin fraction" were significantly lower (P ranged from $<.05$ to $<.001$) than those for porcine pepsin A. The estimates for both Vmax and Km' with APDT as the substrate were similar for both porcine pepsin A and "gastricsin fraction". The physiological efficiency of this enzyme preparation tended to increase with decreasing temperature with hemoglobin as the substrate. The physiological efficiency decreased with decreasing temperature with casein and methylated hemoglobin as the substrates and was independent of temperature with methylated casein and APDT as the substrates.

The kinetic parameters at different temperatures for a Greenland cod protease mixture that has been purified by repeated chromatography on Sephadex G100 are given in Table 3-8. The Vmax with hemoglobin were higher than those obtained with casein as the substrate while the Vmax with methylated hemoglobin were significantly lower ($P<.025$) than those with methylated casein at 25 °C. Vmax obtained with most substrates for the cod protease mixture were comparable to those obtained for porcine pepsin A and "gastricsin fraction" the most notable exception was the very low Vmax with methylated hemoglobin. The Vmax for casein with the cod protease mixture was higher ($P<.05$) than the

Table 3-6: Estimates of Kinetic Parameters for Porcine Pepsin A

Substrate	Temp	Vmax	Q_{10}	Km'	N	Vmax/Km'
hemoglobin	27.2	119.3±13.0		.561±0.87	6	212.7
			1.11			
hemoglobin	15.2	89.4±3.6		.431±0.24	5	207.4
			2.53			
hemoglobin	5.0	34.6±4.3		.139±.036	5	248.9
casein	27.2	28.8±4.4		.955±.173	5	30.2
			2.22			
casein	15.1	10.7±1.1		.332±.051	5	32.3
			2.46			
casein	5.4	4.49±0.52		.113±.030	5	39.7
methylated hemoglobin	27.0	238.6±21.2		.143±.017	6	1668.5
			2.80			
methylated hemoglobin	15.1	71.5±46.7		.126±.106	5	567.4
			2.90			
methylated hemoglobin	5.0	24.9±6.4		.028±.014	6	889.3
methylated casein	27.2	83.3±15.4		.065±.018	6	1281.5
			1.79			
methylated casein	15.1	38.4±3.6		.040±.006	6	960.0
APDT	27.0	11.24±.450		.146±.009	5	77.0
			1.51			
APDT	16.1	6.81±.839		.113±.023	6	60.3
			1.88			
APDT	5.2	3.33±.631		.085±.026	5	39.2

Units for Vmax are; for hemoglobin and casein, ΔA_{280}/h/mg enzyme; for all other substrates, μmoles of bonds hydrolysed/h/mg enzyme. Units for Km' are; for protein substrates, % ; for peptide substrates mM. The data for Km' and Vmax are expressed as the mean ± standard error calculated from one set of triplicate measurements of initial velocity versus substrate concentration. N = the number of measurements on each V vs [S] curve. Temp = temperature ° C. All rate measurements using [S] values greater than 0.5% were omitted from the calculations of Vmax and Km' with casein and hemoglobin as substrates due to possible artifacts as discussed in the text.

Table 3-7: Estimates of Kinetic Parameters for Porcine "Gastricsin Fraction"

Substrate	Temp	Vmax	Q_{10}	Km'	N	Vmax/Km'
hemoglobin	27.2	162.9±12.3		.436±.046	5	373.6
			2.02			
hemoglobin	15.2	67.2±6.3		.148±.028	5	454.1
			2.61			
hemoglobin	5.0	26.3±2.9		.026±.016	5	1011.5
casein	27.2	20.5±2.0		.103±.034	5	199.0
			1.55			
casein	15.1	10.9±1.7		.088±.028	3	123.9
			3.10			
casein	5.4	3.63±.12		.081±.006	3	44.8
methylated hemoglobin	27.0	169.1±10.3		.107±.009	6	1580.4
			1.84			
methylated hemoglobin	15.1	77.1±14.2		.062±.016	5	1243.5
			3.22			
methylated hemoglobin	5.0	23.7±2.04		.023±.004	5	1030.4
methylated casein	27.2	83.7±6.92		.074±.009	6	1131.1
			2.37			
methylated casein	15.1	29.2±3.82		.030±.007	6	973.3
APDT	27.7	9.27±1.712		.242±.061	6	38.3
			1.80			
APDT	14.7	3.96±.434		.096±.018	6	41.3
			1.63			
APDT	5.2	2.56±.224		.073±.011	5	35.1

Units for Vmax are; for hemoglobin and casein, ΔA_{280}/h/mg enzyme; for all other substrates, μmoles of bonds hydrolysed/h/mg enzyme. Units for Km' are; for protein substrates, %; for peptide substrates mM. The data for Km' and Vmax are expressed as the mean ± standard error calculated from one set of triplicate measurements of initial velocity versus substrate concentration. N = the number of measurements on each V vs [S] curve. Temp = temperature ° C. All rate measurements using [S] values greater than 0.5% were omitted from the calculations of Vmax and Km' with casein and hemoglobin as substrates due to possible artifacts as discussed in the text.

Vmax with casein for porcine pepsin. However, the Vmax for the cod protease mixture with the peptide substrate Z-tyr-ala was lower ($P<.001$) than the Vmax for the porcine enzymes with APDT. Similar Km' for the cod protease mixture were obtained with casein and hemoglobin. The Km' with Z-tyr-ala for the cod protease mixture was 7-10 times higher than the Km' with APDT for the porcine enzymes. The physiological efficiency decreased with decreasing temperature with hemoglobin, methylated hemoglobin, and Z-tyr-ala while the physiological efficiency with casein as the substrate was not affected by temperature.

The estimates for the kinetic parameters for cod protease 1 at different temperatures are listed in Table 3-9. The Vmax with hemoglobin was very low compared to the porcine gastric protease preparations and the cod protease mixture but were twice those obtained with casein as the substrate for this enzyme. In constrast, the Vmax obtained with methylated hemoglobin were lower than those obtained with methylated casein as the substrate. The Vmax for cod protease 1 with APDT was only 10% of the Vmax for porcine pepsin with APDT. The Km' for cod protease 1 with hemoglobin as the substrate were greater than those obtained with casein as the substrate. The Km' for protease 1 with hemoglobin were similar to those obtained for the porcine enzymes with hemoglobin. The Km' for protease 1 with methylated hemoglobin were similar to those with methylated casein. The Km' with APDT was greater for cod protease 1 than for porcine pepsin A. The physiological efficiency tended to decrease with decreasing temperature for all substrates.

The kinetic parameters of cod protease 2 at different temperatures are given

Table 3-8: Estimates of Kinetic Parameters for Cod Protease Mixture

Substrate	Temp	Vmax	Q_{10}	Km'	N	Vmax/Km'
hemoglobin	27.2	194.1±78.2		1.37±.676	6	141.7
			2.39			
hemoglobin	15.2	67.7±6.07		.674±.083	6	100.4
			2.57			
hemoglobin	5.0	25.8±.780		.324±.016	6	79.6
casein	27.2	47.9±4.93		1.13±.135	5	42.4
			1.70			
casein	15.1	23.3±4.91		.594±.162	5	39.2
			5.93			
casein	5.4	4.05±.459		.101±.027	5	40.1
methylated hemoglobin	27.0	5.02±3.18		.044±.043	5	114.1
			1.73			
methylated hemoglobin	15.1	2.44±1.34		.022±.023	5	110.9
			1.37			
methylated hemoglobin	5.0	1.76±.306		.024±.008	5	73.3
methylated casein	27.0	67.8±18.9		.395±.124	6	171.6
Z-tyr-ala	25.5	4.864±.181		1.689±.090	5	2.880
			4.35			
Z-tyr-ala	14.6	1.027±.204		1.247±.384	5	0.824
			1.72			
Z-tyr-ala	5.6	.633±.149		1.230±.431	5	0.515
Z-trp-ala	27.0	.161±.030		.619±.205	4	0.26

Units for Vmax are; for hemoglobin and casein, ΔA_{280}/h/mg enzyme; for all other substrates, μmoles of bonds hydrolysed/h/mg enzyme. Units for Km' are; for protein substrates, % ; for peptide substrates mM. The data for Km' and Vmax are expressed as the mean ± standard error calculated from one set of triplicate measurements of initial velocity versus substrate concentration. N = the number of measurements on each V vs [S] curve. Temp = temperature ° C. All rate measurements using [S] values greater than 0.5% were omitted from the calculations of Vmax and Km' with casein and hemoglobin as substrates due to possible artifacts as discussed in the text.

Table 3-9: Estimates of Kinetic Parameters for Cod Protease 1

Substrate	Temp	Vmax	Q_{10}	Km'	N	Vmax/Km'
hemoglobin	26.2	33.1±14.7		.857±.469	5	38.6
			1.85			
hemoglobin	15.2	16.3±5.94		.630±.301	5	25.9
			2.70			
hemoglobin	5.6	6.30±3.60		.529±.412	5	11.9
casein	26.2	15.7±.405		.317±.011	5	49.5
			2.41			
casein	15.1	5.88±.297		.150±.013	5	39.2
methylated hemoglobin	25.4	17.7±8.73		.468±.257	6	37.8
			1.76			
methylated hemoglobin	15.2	9.84±8.77		.398±.402	6	24.7
			2.04			
methylated hemoglobin	5.7	5.07±4.99		.372±.418	6	13.6
methylated casein	25.4	23.6±3.30		.305±.051	6	77.4
			0.66			
methylated casein	5.7	18.1±17.3		.487±.502	6	37.2
APDT	27.0	.114±.016		.310±.060	5	.368

Units for Vmax are; for hemoglobin and casein, ΔA_{280}/h/mg enzyme; for all other substrates, μmoles of bonds hydrolysed/h/mg enzyme. Units for Km' are; for protein substrates, % ; for peptide substrates mM. The data for Km' and Vmax are expressed as the mean ± standard error calculated from one set of triplicate measurements of initial velocity versus substrate concentration. N = the number of measurements on each V vs [S] curve. Temp = temperature ° C. All rate measurements using [S] values greater than 0.5% were omitted from the calculations of Vmax and Km' with casein and hemoglobin as substrates due to possible artifacts as discussed in the text.

in Table 3-10. The Vmax obtained with hemoglobin were 8-10 times greater than those obtained with casein and 3-5 times greater than those obtained for protease 1 with hemoglobin. The Vmax for protease 2 with Z-trp-ala were 5 times those for protease 1 with APDT. The Km' for cod protease 2 with hemoglobin were 10-20 times greater than those with casein and 5-10 times greater than the Km' for cod protease 1 with hemoglobin. The Km' of all the peptide substrates listed in Table 3-10 were similar, with the possible exception of Z-tyr-ser. The physiological efficiencies with hemoglobin and casein as the substrate decreased with decreasing temperature.

The kinetic parameters for cod protease 3 determined at different temperatures are found in Table 3-11. As found for the other proteases, the Vmax with hemoglobin as substrate were greater than the Vmax with casein as the substrate. The Vmax for Z-tyr-ala hydrolysis for cod protease 3 were similar to Vmax for protease 2 with Z-tyr-ser and much less than Vmax for the porcine enzymes with APDT. The Km' with hemoglobin were much higher than those obtained with casein as the substrate ($P<.025$). This was also true for cod protease 1 and 2 but not for porcine pepsin A. The Km' for Z-tyr-ala with cod protease 3 were higher than for any of the other peptide substrates with any of the enzymes. The physiological efficiency of cod protease 3 did not decrease with decreasing temperature for the protein substrates. However, with Z-tyr-ala as the substrate physiological efficiency decreased with decreasing temperature.

To summarize of all of this kinetic data, the Vmax and Km' for each of the gastric proteases are compared in Table 3-12. This table is meant for general discussion only so the values are expressed as low, moderate and high. The values for the "gastricsin preparation" were similar to those of porcine pepsin A except for a somewhat lower Km' for casein and were not included in this table. Porcine pepsin has generally high Vmax and low Km' with all substrates, except for a high Km' with casein. The cod protease mixture had moderate to high Vmax and high Km' for all substrates except methylated hemoglobin. Cod protease 1 had low to moderate Vmax and Km' with all substrates. Cod protease 2 had both high Vmax and Km' with hemoglobin as the substrate and moderate to low Vmax and Km' with the other substrates. Cod protease 3 had low to moderate Vmax and Km' with hemoglobin and casein, high Vmax and Km' with methylated hemoglobin and low Vmax and high Km' with the peptide substrates.

Table 3-10: Estimates of Kinetic Parameters for Cod Protease 2

Substrate	Temp	Vmax	Q_{10}	Km'	N	Vmax/Km'
hemoglobin	27.4	184.7±97.3		7.71±4.31	6	24.0
			3.37			
hemoglobin	15.2	44.9±9.72		2.80±.692	6	16.0
			2.31			
hemoglobin	5.7	20.5±7.03		2.48±.982	6	8.27
casein	27.4	16.7±1.99		.412±.064	5	40.5
			2.22			
casein	15.2	6.17±.670		.257±.040	5	24.0
			2.71			
casein	5.7	2.40±.614		.144±.064	5	16.7
methylated hemoglobin	15.2	6.16±6.05		.263±.318	6	23.4
			1.55			
methylated hemoglobin	5.7	4.18±4.61		.170±.234	6	24.6
methylated casein	25.9	66.6±76.1		1.43±1.68	6	46.6
			2.80			
methylated casein	15.2	22.2±77.5		.751±2.77	6	29.6
Z-trp-ala	26.0	.542±.071		.324±.055	6	1.67
Z-tyr-ser	26.0	.348±.393		1.20±1.50	6	.290
Z-tyr-thr	26.0	.072±.036		.331±.213	6	0.218
Z-tyr-leu	26.0	.099±.043		.339±.186	6	0.292

Units for Vmax are; for hemoglobin and casein, ΔA_{280}/h/mg enzyme; for all other substrates, μmoles of bonds hydrolysed/h/mg enzyme. Units for Km' are; for protein substrates, % ; for peptide substrates mM. The data for Km' and Vmax are expressed as the mean ± standard error calculated from one set of triplicate measurements of initial velocity versus substrate concentration. N = the number of measurements on each V vs [S] curve. Temp = temperature ° C. All rate measurements using [S] values greater than 0.5% were omitted from the calculations of Vmax and Km' with casein and hemoglobin as substrates due to possible artifacts as discussed in the text.

Table 3-11: Estimates of Kinetic Parameters for Cod Protease 3

Substrate	Temp	Vmax	Q_{10}	Km'	N	Vmax/Km'
hemoglobin	26.9	49.6±11.6		1.85±.517	6	26.8
			0.99			
hemoglobin	15.2	42.9±28.8		1.99±1.48	5	21.6
			3.16			
hemoglobin	5.7	14.3±5.04		.732±.329	5	19.5
casein	26.9	7.47±.545		.144±.018	5	51.9
			1.79			
casein	15.2	3.57±.110		.063±.005	5	14.5
			2.35			
casein	5.7	1.96±.220		.018±.012	5	108.9
methylated hemoglobin	26.9	64.7±30.1		1.92±.980	9	33.7
			1.11			
methylated hemoglobin	15.3	50.1±17.4		1.81±.695	9	27.7
methylated casein	26.9	30.7±21.8		.981±.725	6	31.3
			1.25			
methylated casein	15.3	21.2±34.2		.785±1.34	6	27.0
Z-tyr-ala	26.9	.307±.434		2.23±3.35	6	0.138
			2.87			
Z-tyr-ala	15.4	.093±.048		.818±.478	6	0.114
			1.44			
Z-tyr-ala	5.6	.066±.090		.864±1.35	6	0.076
Z-trp-ala	26.9	.018±.004		.189±.059	6	0.095

Units for Vmax are; for hemoglobin and casein, ΔA_{280}/h/mg enzyme; for all other substrates, μmoles of bonds hydrolysed/h/mg enzyme. Units for Km' are; for protein substrates, % ; for peptide substrates mM. The data for Km' and Vmax are expressed as the mean ± standard error calculated from one set of triplicate measurements of initial velocity versus substrate concentration. N = the number of measurements on each V vs [S] curve. Temp = temperature ° C. All rate measurements using [S] values greater than 0.5% were omitted from the calculations of Vmax and Km' with casein and hemoglobin as substrates due to possible artifacts as discussed in the text.

As can be seen from the above data, the Km' and Vmax for the different gastric proteases are quite different with the different substrates. The cod protease mixture generally has a high Vmax and high Km' on the different substrates while the porcine proteases have high Vmax but low Km'. Thus, the physiological efficiency for porcine pepsin are generally much higher than those for the cod proteases. In terms of the efficiency of digestion of protein by the different animals, the rather low physiological efficiency of the cod proteases may be offset by the wider substrate specificity of these enzymes compared to the porcine gastric proteases.

Table 3-12: Summary of Kinetic Parameters of the Gastric Proteases with Different Substrates

Enzyme	Hemoglobin		Casein		Methylated Hemoglobin		Peptides	
	Vmax	Km'	Vmax	Km'	Vmax	Km'	Vmax	Km'
Porcine Pepsin	H	L	M	H	H	L	H	L
Protease Mix	H	H	M	H	L	L	M	H
Protease 1	L	L	M	M	L	L	L	M
Protease 2	H	H	M	M	L	L	L	M
Protease 3	L	M	L	L	H	H	L	H

Values for methylated casein were not included since the high standard errors of the estimates for Vmax and Km' made comparisons difficult. L= low, M= moderate, H= high and refer to comparisons among the different substrates for the same enzyme.

3.4.2. Activation Energies of the Gastric Proteases

The activation energies (Ea) for the hydrolysis of various protein and peptide substrates were calculated from Arrhenius plots using Vmax obtained at various temperatures. The data are summarized in Table 3-13. The activation energies obtained for the different gastric proteases were all quite high. The Ea for the "gastricsin fraction" were the highest on the average while the Ea for cod protease 1 and 3 were generally the lowest. However, since only a few values of Vmax at different temperatures were determined, the interpretation of this data cannot be taken too far. It seems intuitively reasonable, however, that the gastric proteases of the Greenland cod may have lower activation energies due to the cold temperature habitat of this species. In this regard, somewhat lower Ea were obtained with certain preparations of Greenland cod gastric proteases. There may also be quantitative differences in the amount of gastric proteases present in the Greenland cod compared to an animal which lives in a warmer environment. These data are included in Appendix 6.

3.5. Structural Features of the Proteases

3.5.1. Thermal Stability

A comparison was made of the thermal stability properties of the various proteases using the procedures outlined in section 2.2.7.1. Figure 3-19 shows the effects of heat treatment on the activity of a mixture of cod proteases at various pH values and porcine pepsin A and "gastricsin fraction at pH 1.9. As can be seen, there was little difference in the thermal stability of porcine pepsin A and "gastricsin fraction", with 1/2 of the activity remaining after treatment at 57 °C.

Table 3-13: Activation Energy for the Hydrolysis of Protein and Peptide Substrates

Enzyme	Substrate	Activation Energy	r^2	N
Porcine Pepsin	hemoglobin	49.78	.9971	9
	casein	59.02	.9999	3
	methylated hemoglobin	71.02	.9998	3
	APDT	44.56	.9894	7
Gastricsin Fraction	hemoglobin	121.85	.9617	3
	casein	54.55	.9618	3
	methylated hemoglobin	61.49	.9803	3
	APDT	45.73	.964	7
Protease Mixture	hemoglobin	54.55	.9939	3
	casein	77.54	.9247	3
	methylated hemoglobin	33.06	.9648	3
	Z-tyr-ala	64.00	.9498	4
Protease 1	hemoglobin	55.51	.9845	3
	methylated hemoglobin	43.72	.9987	3
Protease 2	hemoglobin	70.68	.9871	3
	casein	61.91	.9988	3
Protease 3	casein	43.72	.9996	3
	Z-tyr-ala	50.36	.9212	3
Mucosal Homogenate	hemoglobin	43.68	.9832	5

The Vmax from Tables 3-6 to 3-11 and other data where applicable were used in Arrhenius plots to determine the Activation energies (Ea) for the various gastric proteases. Activation energies are in kjoul/mol. The temperature range was 0-40 °C. The r^2 values are the correlation coefficients for the linear regression analysis of the Arrhenius plots. The Ea for porcine pepsin with hemoglobin was calculated using the velocities obtained with 2% hemoglobin (3% final TCA concentration) at pH 1.9.

In comparison, the cod protease mixture at pH 1.9 had lost 1/2 of its activity at only 41 °C. It is interesting to note that the protease mixture had greater thermal stability at pH 3 losing 1/2 of the activity at 52 °C at this pH. As has been reported for many acid proteases, the thermal stability of the activated protease mixture was lower at pH 6.5 while a nonactivated mixture of the zymogens from the Greenland cod had higher thermal stability than active protease mixture at pH 1.9. The thermal stabilities of porcine pepsin A and "gastricsin fraction" were greater than those of the cod proteases by a similar amount.

3.5.2. pH Stability

The pH stabilities of the various proteases were measured by storing the enzymes solutions from pH 2 to 8 at 5 °C for 96 hours (Figure 20) as outlined in section 2.2.7.2. The data for porcine pepsin and "gastricsin fraction" are shown in Figure 3-20A. The pH stability of pepsin A was marginally less than that of porcine "gastricsin fraction". The pH stability of the cod proteases are shown in Figure 3-20B. Protease 1 was the least stable of the cod proteases in this experiment. Protease 2 was the most stable of the cod proteases while the stability profile of protease 3 closely resembled that of porcine pepsin A. However, all the cod proteases gradually lose activity as the pH rises while the pH profiles of the porcine enzymes have very flat plateau regions right up until inactivation increased rapidly with increasing pH.

Figure 3-19: Thermal Stability of the Gastric Proteases

Enzyme preparation (30-50 μg) in pH 1.9 or 3 HCl or 50 mM Tris phosphate pH 6.5 were incubated at different temperatures for 1 h and then cooled in ice. Triplicate aliquots (50 μl) were then assayed for proteolytic activity with hemoglobin as the substrate as described in section 2.2.4.1. (▲) cod protease mixture at pH 6.5 which had been first activated at pH 2, 30 min at 5° C, (●) nonactivated cod protease mixture pH 6.5, (■) cod protease mixture pH 1.9, (△) cod protease mixture pH 3, (○) porcine pepsin A pH 1.9, (□) porcine "gastricsin fraction" pH 1.9.

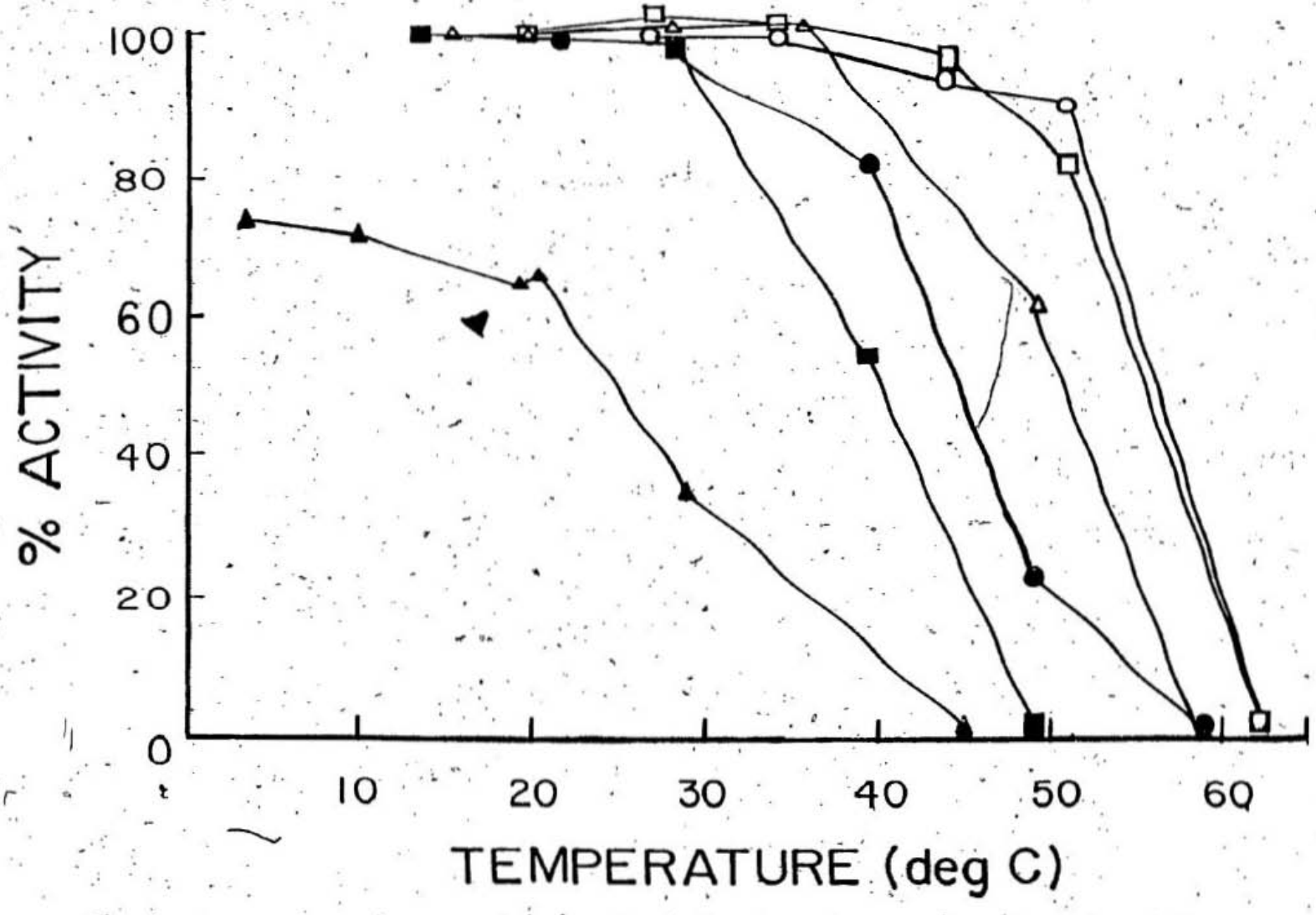


Figure 3-19: Thermal Stability of the Gastric Proteases

Figure 3-20: pH Stability of the Gastric Proteases

Enzyme preparation (60-70 μg) were mixed with 25 μl of 0.2M citrate buffer (pH 2-6.5 with NaoH) or 0.2M Tris HCl buffer (pH 6.5-8) and sufficient water was added to bring the volume to 225 μl. After 96 h at 5 $^{\circ}$ C, triplicate aliquots (40 μl) were then assayed for proteolytic activity with hemoglobin as the substrate as described in section 2.2.4.1. The activity after treatment was expressed as a percentage of the activity of untreated enzyme which was frozen at pH 2.5. **A** (▲) porcine pepsin A, (●) porcine "gastricsin fraction", **B** (△) cod protease 1, (○) cod protease 2, (□) cod protease 3.

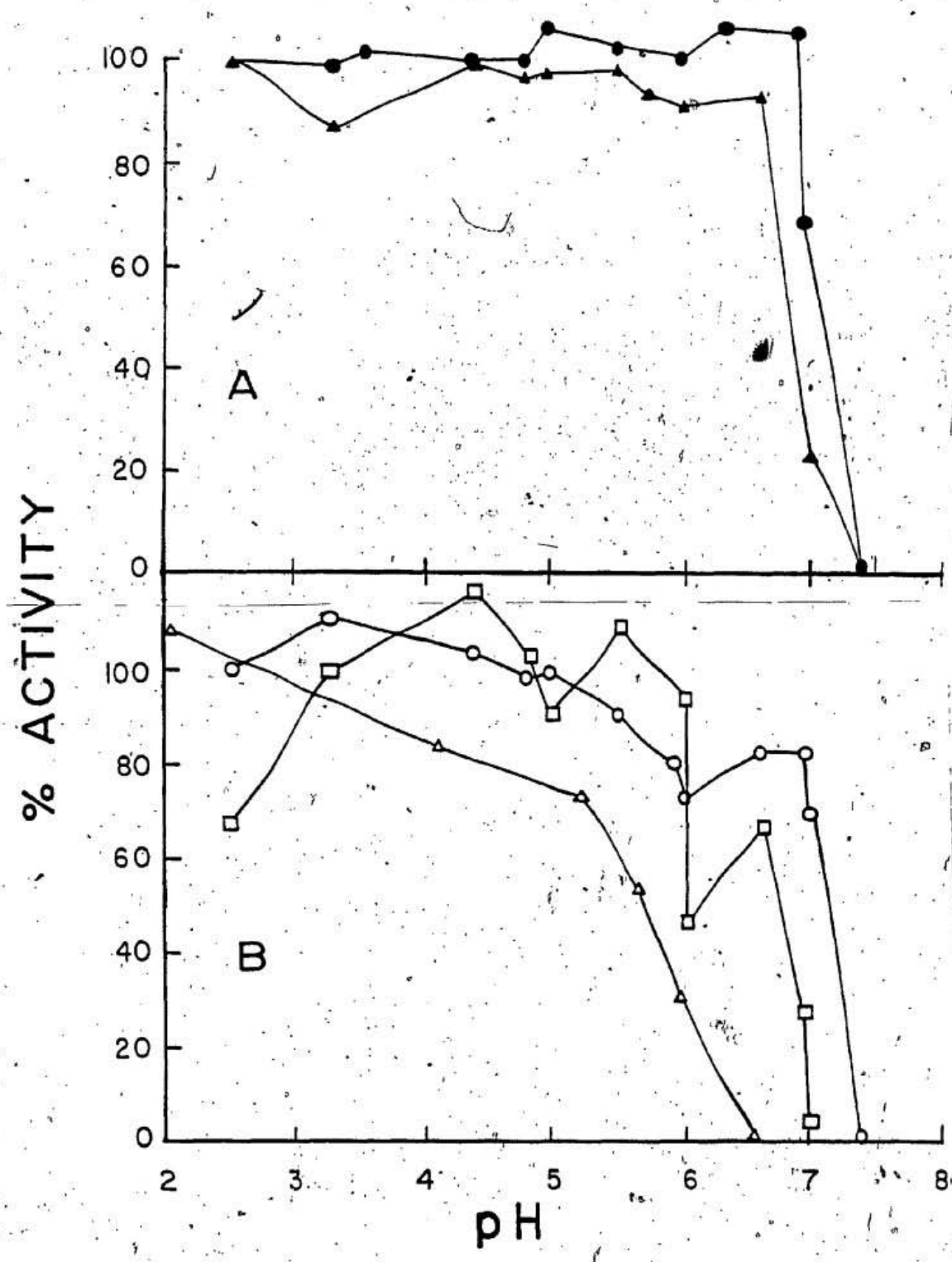


Figure 3-20: pH Stability of the Gastric Proteases

3.5.3. Effect of NaCl

The effect of sodium chloride on the activities of all proteases was investigated since it was reported by Sanchez-Chiang and Ponce (1981, 1982) that NaCl activates the proteases isolated from the hake *Merlucius gayi*. The results are shown in Figure 3-21. Both porcine pepsin A and porcine "gastricsin fraction" showed slight inhibition at higher levels of sodium chloride while cod protease 3 was unaffected by the presence of NaCl. However, a 2-fold enhancement in the rates of proteolysis by cod proteases 1 and 2 were found with 25 mM NaCl in the reaction medium. Thus, Greenland cod proteases 1 and 2 are stimulated by salt as are many fish gastric proteases, such as those from *Merlucius gayi*, while cod protease 3 is more like the porcine enzymes since it is unaffected by salt.

3.5.4. Immunological Comparisons

The cross-reactivity of the various proteases with four different antibody preparations was investigated in order to estimate the structural similarities of the various enzymes. Two types of studies were carried out as described in the section 2.2.6 (1) immunochemical titration and (2) ouchterlony double diffusion tests.

The immunochemical titrations of the proteases with a purified IgG fraction containing antibody against "gastricsin fraction" are shown in Figure 3-22. The data are plotted as the percentage of the activity remaining after incubation with IgG versus the ratio of IgG to enzyme (mg/mg). Since the gastric proteases studied here have approximately the same molecular weight, these ratios can be directly compared among the various enzymes. It can be seen that a similar percentage of the total precipitation of pepsin A and "gastricsin fraction" occurs

Figure 3-21: Effect of NaCl on the Activity of the Gastric Proteases

Triplicate aliquots (10-50 μg) of enzyme preparation were assayed for proteolytic activity with 2% hemoglobin solution pH 2 (dialysed extensively against pH 2 HCl) in the presence of 0-65 mM NaCl by measuring the TCA soluble reaction products as described in section 2.2.4.1. Activity is expressed as ΔA_{280}/h/mg enzyme. (○) porcine pepsin A, (□) porcine "gastricsin fraction", (▲) cod protease 1, (●) cod protease 2, (■) cod protease 3.

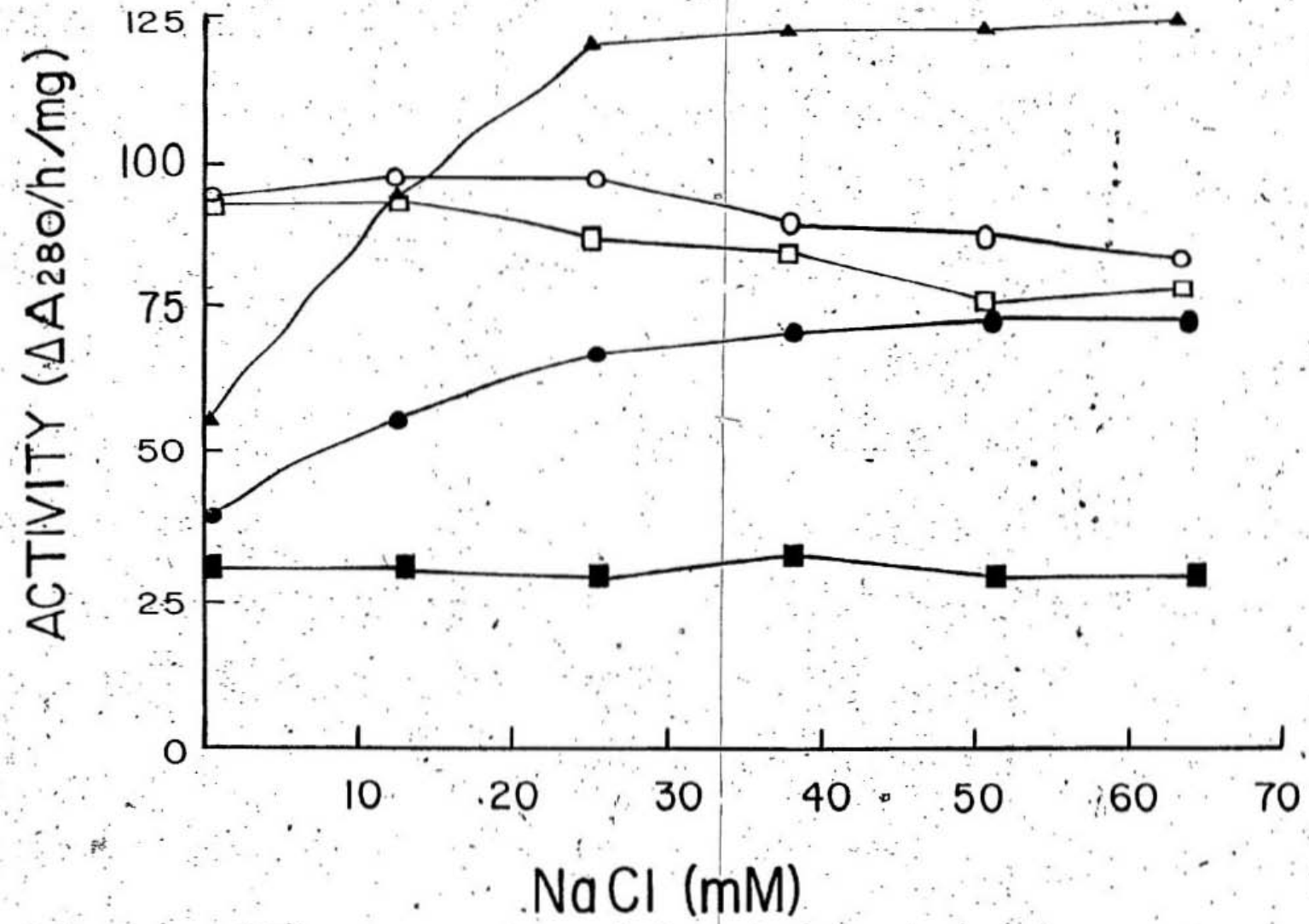


Figure 3-21: Effect of NaCl on the Activity of the Gastric Proteases

at all IgG/enzyme ratios while the cod proteases are not affected. However, pepsin A is more completely removed from solution than "gastricsin fraction" with the anti-gastricsin fraction IgG. The immunochemical titrations of the proteases with anti-pepsin A IgG are quite similar (Figure 3-23). The cod gastric proteases were not affected by this IgG preparation, and the percentage of the total amount of precipitation of porcine "gastricsin fraction" and porcine pepsin A was the same at all IgG/enzyme ratios. The reaction with the anti-pepsin IgG occurred at lower IgG/enzyme ratios and left a lower residual activity compared to the immunochemical titrations with the anti-gastricsin fraction IgG. This indicates that the titers of the two IgG preparations are different. Direct comparisons can therefore only be made among the different proteases within a particular figure and not between figures.

Antibodies were also raised against purified proteases T16 and T25 isolated from psychrotrophic pseudomonads (Jackman *et al.*, 1983) and the IgG fractions were isolated as described in Appendix 4. The effects of titrating the various proteases with anti-T16 IgG preparation are illustrated in Figure 3-24. "Gastricsin fraction" was precipitated at a ratio of IgG/enzyme of about 4 but this precipitation was decreased at higher ratios. Cod protease 1 and porcine pepsin gave very similar patterns of reaction except that the cod protease 1 was more completely precipitated at higher IgG/enzyme values. Cod protease 2 and 3 were unaffected by the anti-T16 preparation. In contrast to all the other IgG preparations, the anti-T25 IgG preparation reacted with all the proteases at different IgG/enzyme ratios (Figure 3-25). Cod protease 1 was precipitated at the lowest concentration of antibody and was completely precipitated at an

Figure 3-22: Immunochemical titration of the Gastric Proteases with anti-Gastricsin Fraction IgG.

Antibodies were raised in rabbits against purified porcine "gastricsin fraction" and the IgG fraction of the serum was isolated. Purified enzyme preparations (30-90 μg) having activity with hemoglobin as substrate as indicated in Table 3-3 were incubated with various amounts of IgG fraction in 15 mM sodium phosphate buffer pH 5.5 in a total volume of 0.4 ml at 5 °C for overnight. After centrifugation the supernatant was assayed in triplicate for proteolytic activity with 2% hemoglobin as substrate. The data are plotted as the percentage of the proteolytic activity remaining after incubation with IgG versus the ratio of IgG/enzyme used (mg/mg). (Δ) porcine pepsin A, (▲) porcine "gastricsin fraction", (O) cod protease 1, (■) cod protease 2, (●) cod protease 3.

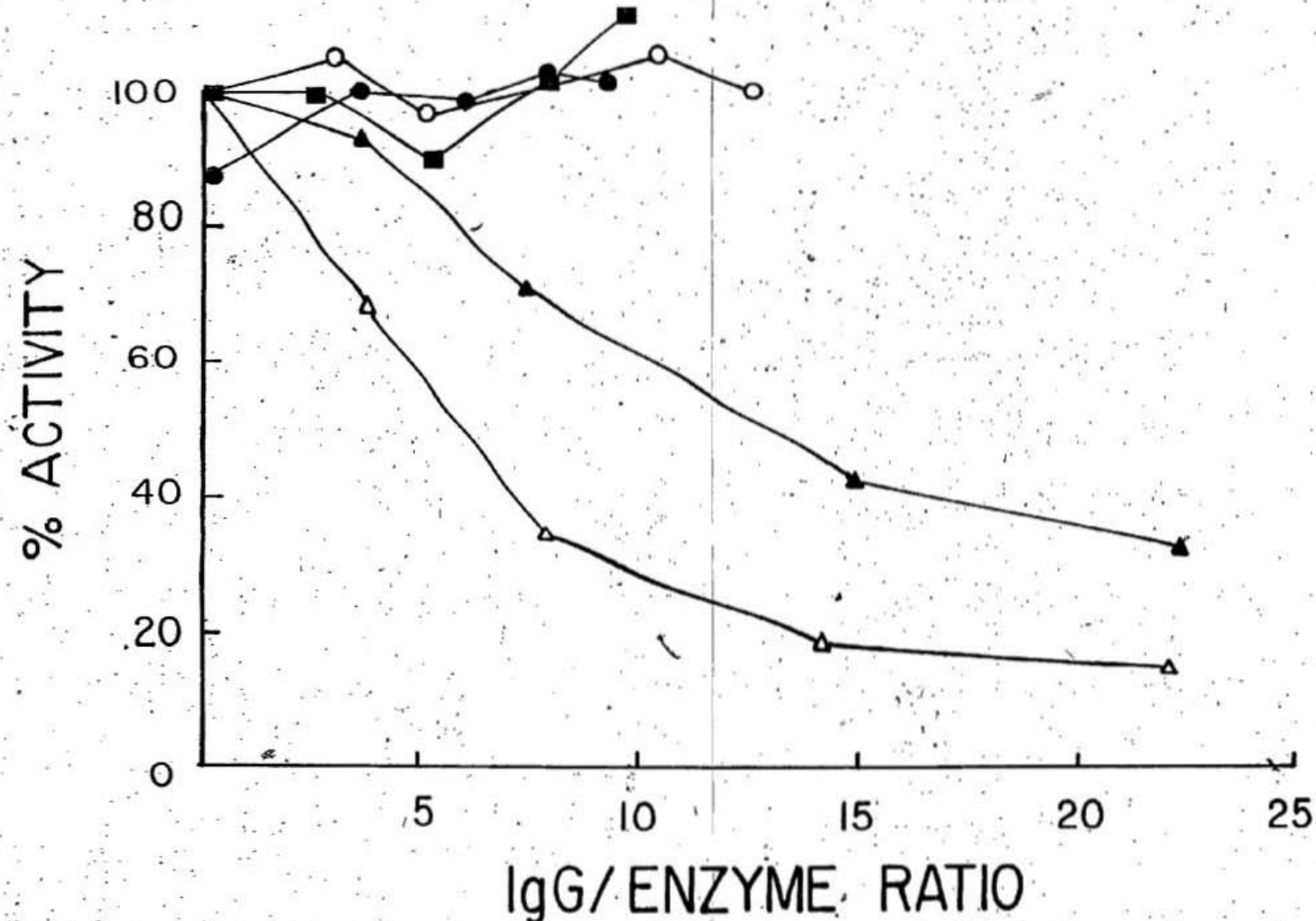


Figure 3-22: Immunochemical titration of the Gastric Proteases with anti-Gastricsin Fraction IgG

Figure 3-23: Immunochemical titration of the Gastric Proteases with anti-Pepsin A IgG

Antibodies were raised in rabbits against purified porcine pepsin A and the IgG fraction of the serum was isolated. Purified enzyme preparations (30-90 µg) were incubated with various amounts of IgG fraction in 15 mM sodium phosphate buffer pH 5.5 in a total volume of 0.4 ml at 5 °C for overnight. After centrifugation the supernatant was assayed in triplicate for proteolytic activity with 2% hemoglobin as substrate. The data are plotted as the percentage of the proteolytic activity remaining after incubation with IgG versus the ratio of IgG/enzyme used (mg/mg). (Δ) porcine pepsin A, (▲) porcine "gastricsin fraction", (O) cod protease 1, (■) cod protease 2, (●) cod protease 3.

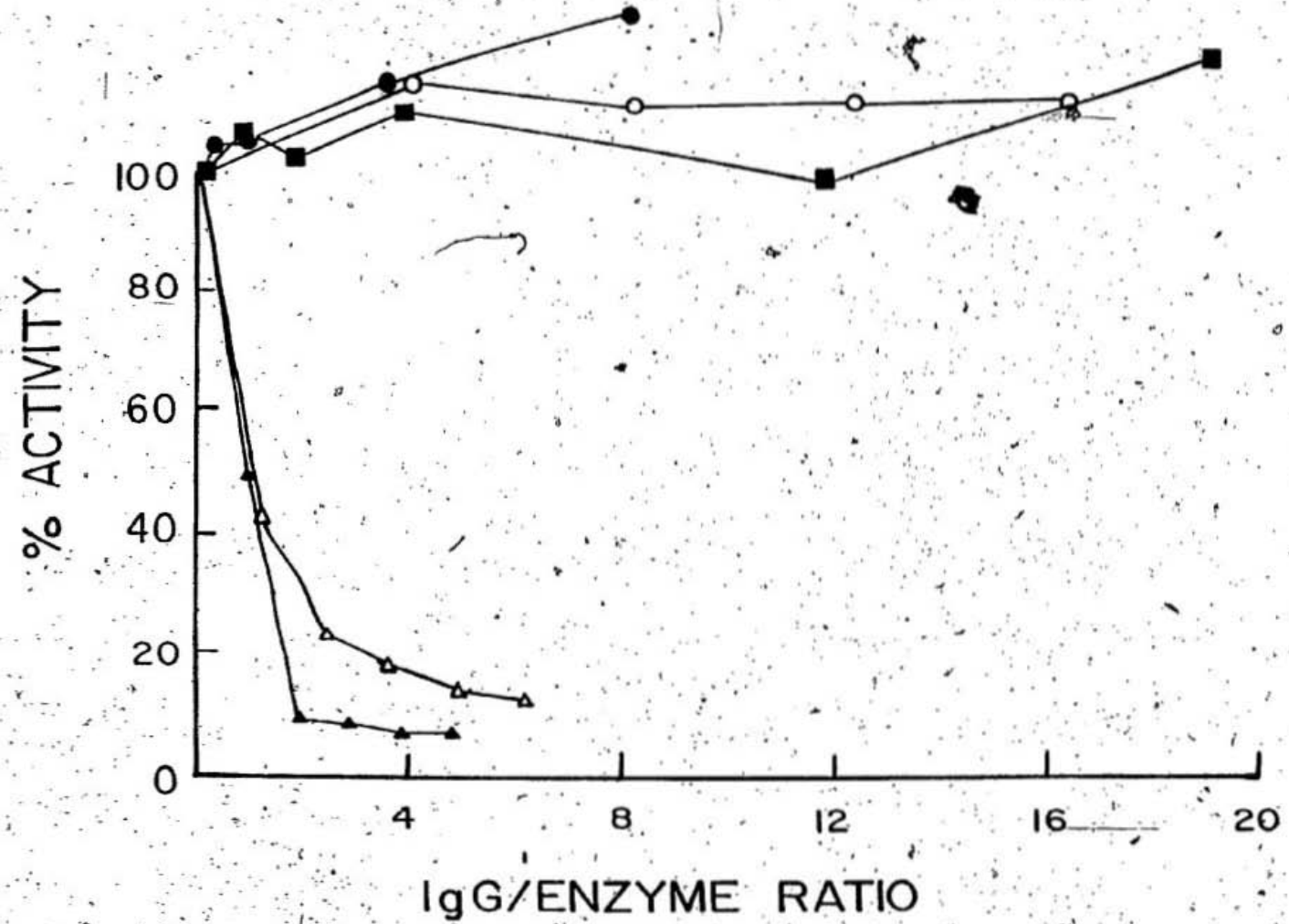


Figure 3-23: Immunochemical titration of the Gastric Proteases with anti-Pepsin A IgG

IgG/enzyme ratio of 5. Porcine "gastricsin fraction" and cod protease 3 gave very similar reaction profiles and were completely precipitated at an IgG/enzyme ratio of\20. Cod protease 2 and porcine pepsin A reacted in a very similar manner in this immunochemical titration study.

Attempts were also made to use Ouchterlony double diffusion gels with multiple outer wells to detect structural similarities and differences among the various proteases. The results of such a study using anti-porcine pepsin A preparation are shown in Figure 3-26. The absence of any spur formation at the junction of the precipitin lines for porcine pepsin A and "gastricsin fraction" indicates that this IgG preparation detects no structural differences between pepsin A and "gastricsin fraction". As was found in the immunochemical titration studies, there was no reaction between the IgG preparation and any of the cod proteases. Ouchterlony double diffusion experiments were performed using anti-T25 IgG preparation and the various gastric proteases. However, precipitin lines formed only with T25 protease and not with any other of the gastric proteases in these experiments. Additional experiments where 4% polyethylene glycol was included in the agar to improve the sensitivity also failed to produce precipitin lines with any of the other gastric proteases.

3.5.5. Molecular Weight Estimates

The molecular weights of the cod proteases and their zymogens were estimated by SDS polyacrylamide gel electrophoresis (SDS-PAGE) as described in section 2.2.3.1. A calibration mixture of proteins of known molecular weight was run in parallel tubes and a calibration curve of the logarithm of the molecular

Figure 3-24: Immunochemical titration of the Gastric Proteases with anti-T16 IgG

Antibodies were raised in rabbits against purified pseudomonad T16 protease and the IgG fraction of the serum was isolated. Purified enzyme preparations (30-90 μg) were incubated with various amounts of IgG fraction in 15 mM sodium phosphate buffer pH 5.5 in a total volume of 0.4 ml at 5 °C for overnight. After centrifugation the supernatant was assayed in triplicate for proteolytic activity with 2% hemoglobin as substrate. The data are plotted as the percentage of the proteolytic activity remaining after incubation with IgG versus the ratio of IgG/enzyme used (mg/mg). (△) porcine pepsin A, (▲) porcine "gastricsin fraction", (○) cod protease 1, (■) cod protease 2, (●) cod protease 3.

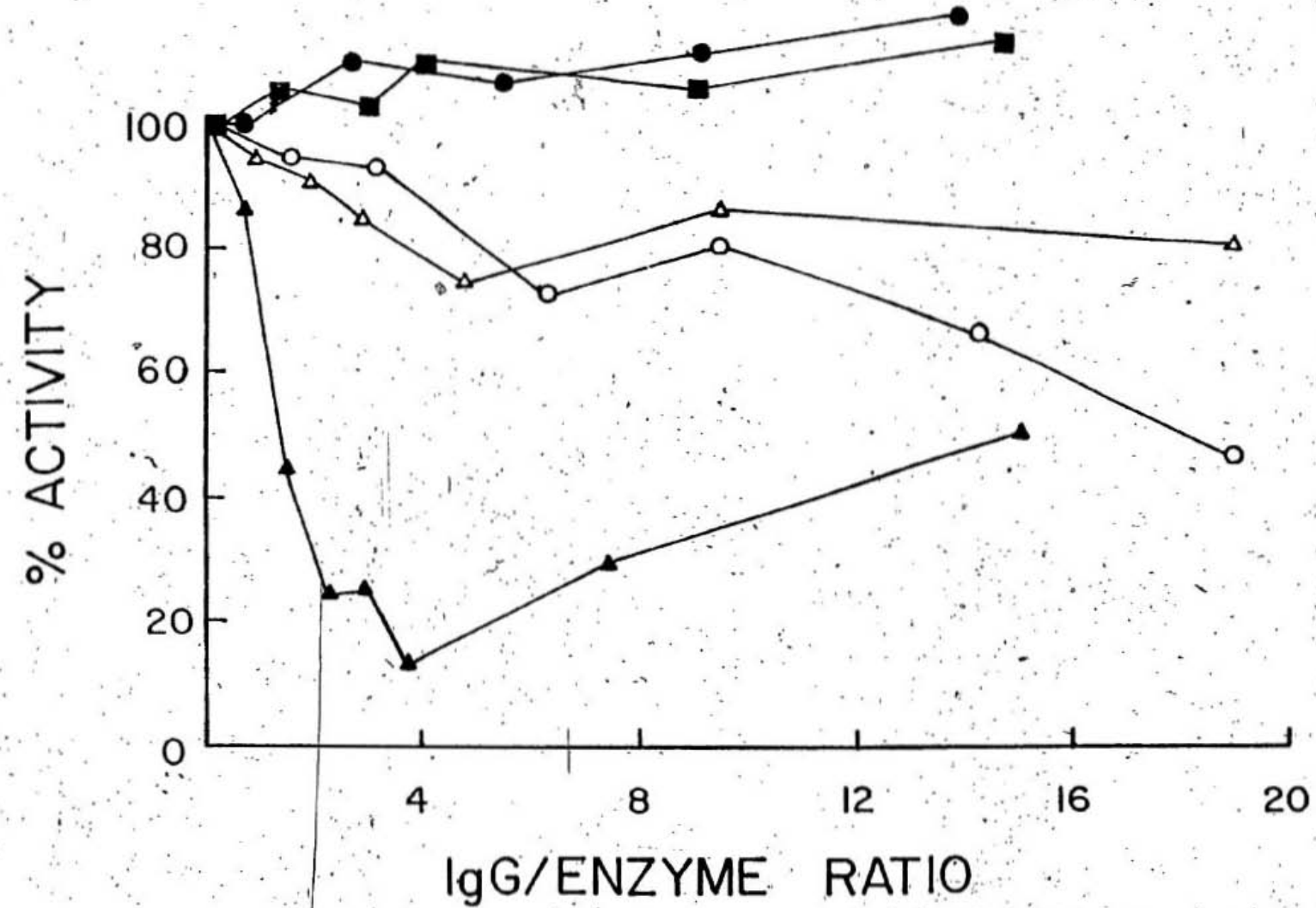


Figure 3-24: Immunochemical titration of the Gastric Proteases with anti-T16 IgG

Figure 3-25: Immunochemical titration of the Gastric Proteases with anti-T25 IgG

Antibodies were raised in rabbits against purified pseudomonad T25 protease and the IgG fraction of the serum was isolated. Purified gastric protease preparations (30-90 μg) were incubated with various amounts of IgG fraction in 15 mM sodium phosphate buffer pH 5.5 in a total volume of 0.4 ml at 5 °C for overnight. After centrifugation the supernatant was assayed in triplicate for proteolytic activity with 2% hemoglobin as substrate. The data are plotted as the percentage of the proteolytic activity remaining after incubation with IgG versus the ratio of IgG/enzyme used (mg/mg). (△) porcine pepsin A, (▲) porcine "gastricsin fraction", (○) cod protease 1, (■) cod protease 2, (●) cod protease 3.

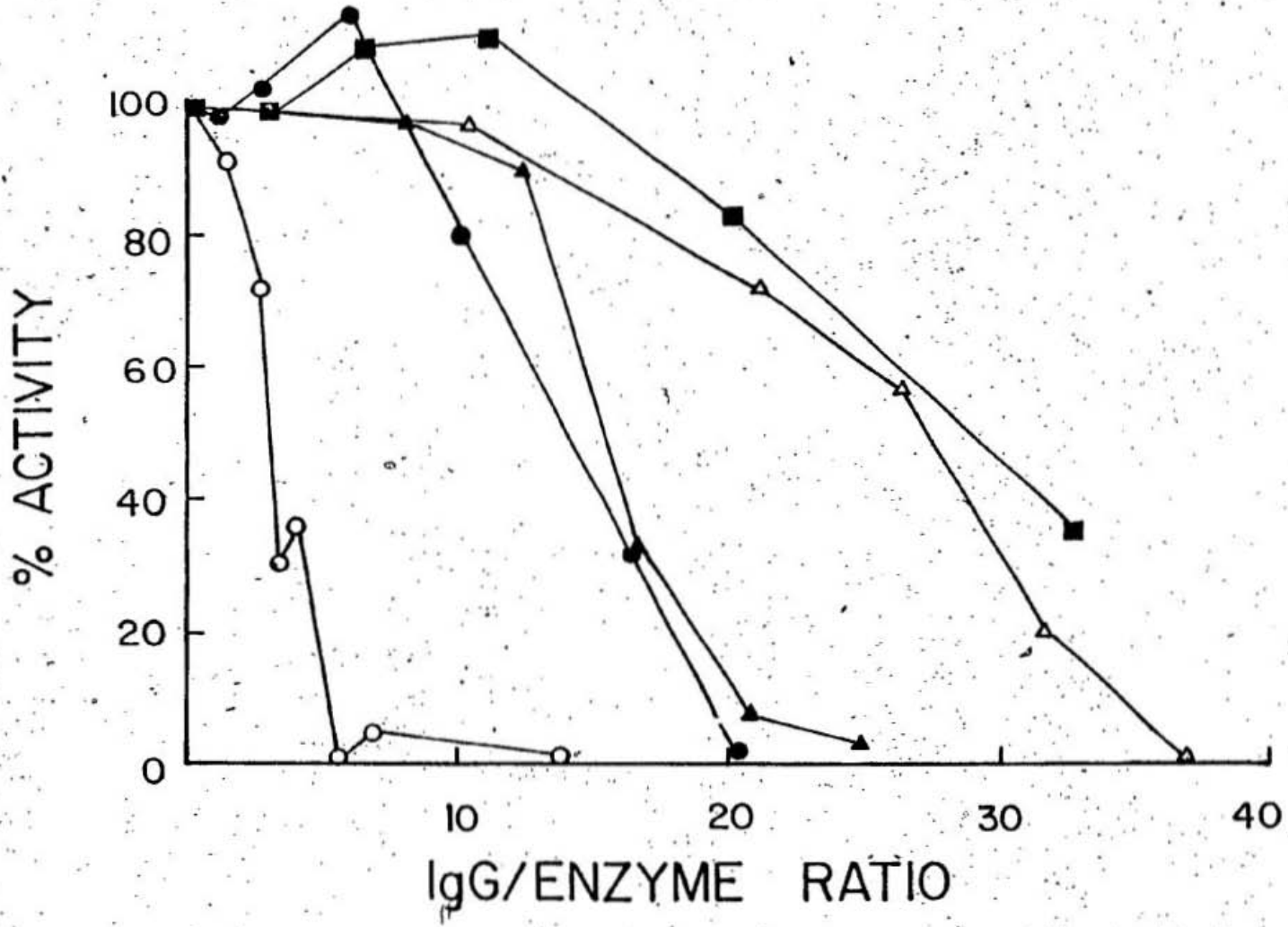
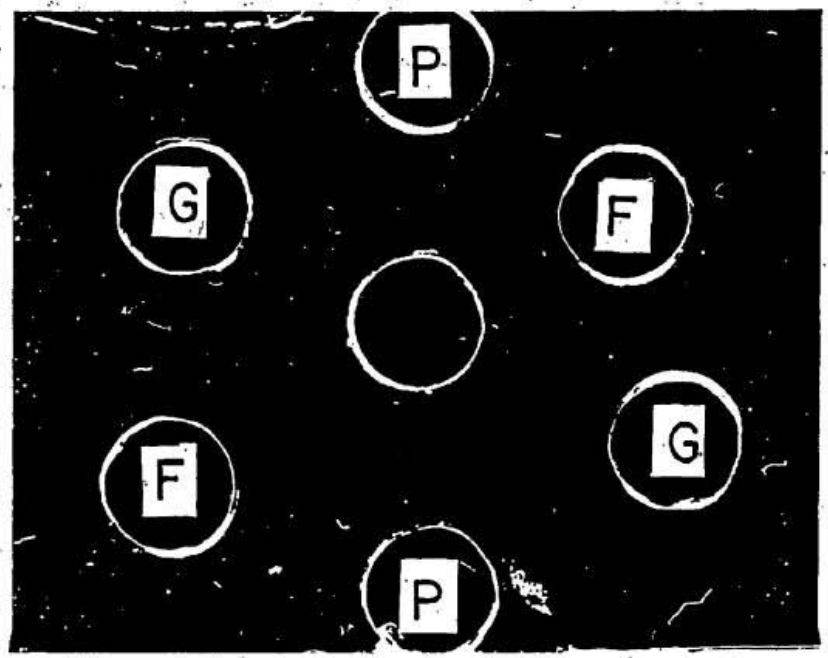


Figure 3-25: Immunochemical titration of the Gastric Proteases with anti-T25 IgG

Figure 3-26: Ouchterlony Double Diffusion of anti-Pepsin A IgG and the Gastric Proteases

Wells (0.4 cm) were punched approximately 0.5 cm apart in agar gels on microscope slides prepared as described in Appendix 5. The outer wells were filled with different enzyme solutions; **P** porcine pepsin A, **G** porcine "gastricsin fraction", **F** Greenland cod protease mixture prepared by chromatography of a crude stomach homogenate on Sephadex G100. The inside well contained anti-pepsin A IgG preparation. The intensity of the precipitin bands was enhanced by once refilling the wells before they were completely emptied.


P
G
F
F
G
P

weight versus Rf was constructed. This was used to estimate the molecular weight of the cod proteases once their Rf values were measured. The results of a number of these runs are shown in Table 3-14. The different cod proteases had very similar molecular weights in the 36-38 kdal range with zymogen forms that were 5.9-7.4 kdal larger as determined by this method. The porcine gastric proteases had molecular weights similar to the Greenland cod gastric proteases.

The molecular weights of the cod proteases were also determined by gel filtration. A 1.5 x 80 cm column of Sephadex G75 was calibrated with proteins of known molecular weight. A plot of the logarithm of the molecular weight versus the retention coefficient Kav for these standards is shown in Figure 3-27. This calibration curve was used to estimate the molecular weights of the cod proteases and a zymogen mixture. The results of this experiment are included in Table 3-14. The molecular weight estimates obtained by this method were lower than those from SDS-PAGE.

3.5.6. Amino Acid Composition

The amino acid composition of the Greenland cod proteases and porcine "gastricsin fraction" were determined using the amino acid analyser. The data for the analysis after 24 h, 48 h and 72 h were worked up as described in section 2.2.8 and relating the data to whole numbers of alanine residues. The results are given in Table 3-15. Cod protease 1 was higher in threonine, alanine, lysine, histidine and arginine and lower in serine, glutamate, isoleucine, phenylalanine, cystine and methionine than the other proteases. The low content of cystine was quite striking and has not been reported for the gastric proteases of any other species to

Table 3-14: Estimates of the Molecular Weights of the Gastric Proteases

From SDS-PAGE

Cod Protease 1	zymogen	42,434 ± 2,516
	activated	36,358 ± 3,529
Cod Protease 2	zymogen	41,751 ± 1,051
	activated	35,898 ± 426
Cod Protease 3	zymogen	45,320 ± 1,714
	activated	37,970 ± 3,120
Porcine pepsin A	activated	37,600
Gastricsin fraction	activated	36,960

From Gel Filtration

Cod Protease 1	25,609
Cod Protease 2	23,442
Cod Protease 3	23,907
Zymogen Mix	34,860

From Amino Acid Analysis

Cod Protease 1	37,099
Cod Protease 2	37,458
Cod Protease 3	37,458
Gastricsin Fraction	35,933

Values for the molecular weight of the Greenland cod gastric proteases and their zymogens by SDS-PAGE are expressed as the mean ± standard error of three determinations. Molecular weight estimates from gel filtration are from a single experiment.

Figure 3-27: Calibration Curve of Sephadex G75 Column

The following proteins were chromatographed on a 1.6 x 90 cm column of Sephadex G75 equilibrated and eluted with pH 2.5 HCl and the elution volume (Ve) of each was noted;(◐) RNAase, (■) chymotrypsinogen A, (●) ovalbumin and (▲) aldolase. The void volume (Vo) of the column was determined using blue dextran. The retention coefficient Kav was calculated for each protein usind the following formula;

$$Kav = (Ve - Vo)/(Vt - Vo)$$

where Vt is the total volume of the column. The regression coefficient for the plot of the logarithm of the molecular weight versus Kav was .9958

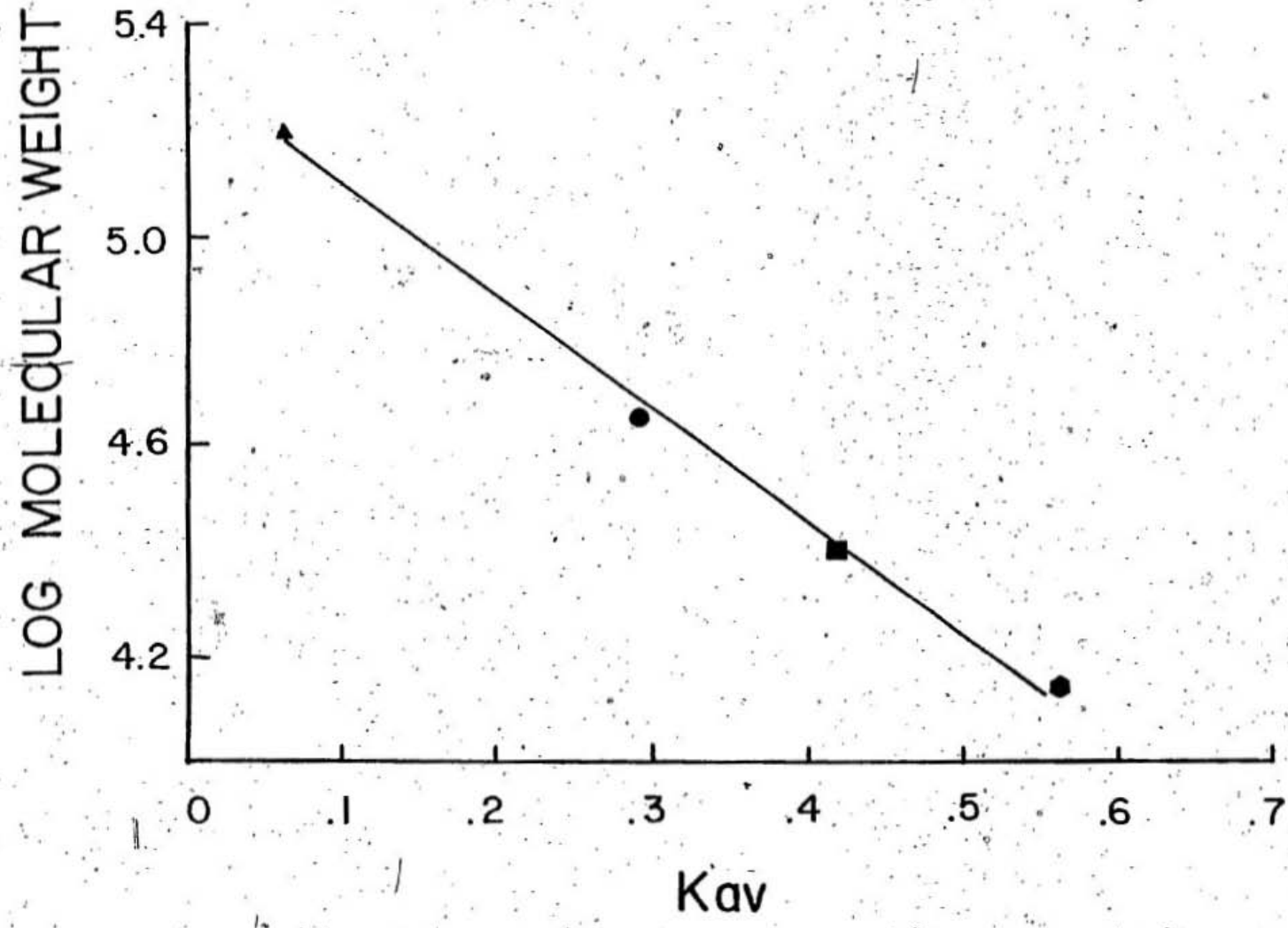


Figure 3-27: Calibration Curve of Sephadex G75 Column

date. Cod protease 2 was higher in valine and lower in proline and histidine than the other cod proteases. Protease 3 was higher in aspartate and glutamate and lower in glycine, lysine and arginine than the other cod proteases. Porcine "gastricsin fraction" was lower in alanine, valine, lysine, histidine and arginine than the other cod proteases. Porciné "gastricsin fraction" differs from porcine pepsin A mainly in the content of serine, proline, tryptophan, cystine and methionine. Obvious differences exist between the amino acid composition data of the "gastricsin fraction" isolated in this study and the literature data for porcine gastricsin and pepsin C. The isoleucine/leucine and aspartate/glutamate ratios were distinctively lower with gastricsin than pepsin A or chymosin.

Hydrophobicity indices for the gastric proteases were calculated according to Bigelow (1967) and are included in Table 3-15. The indices for porcine pepsin A and gastricsin were similar, with the "gastricsin fraction" being more like pepsin A. The highest hydrophobicity index was obtained for bovine chymosin. Cod protease 1 had the lowest index while the indices for cod protease 2 and 3 were intermediate between that of pepsin A and chymosin.

The amino acid composition data can be compared using the Metzger Difference Index (Metzger *et al.*, 1968, Woodward, 1978). An estimate of the differences in the amino acid composition of two proteins is obtained by summing the absolute values of the differences between the mole fractions for each of the amino acids in the proteins. For two polypeptides of completely different amino acid compositions, *eg.* poly-lysine and poly-glutamic acid, this value would be 2 while for two identical proteins the value would be 0. A Metzger Difference Index

Table 3-15: Amino Acid Composition of Gastric Proteases

A A	C P 1	C P 2	C P 3	"G F"	Pep C	Gast	Pep A	Chym
asx	33	37	43	46	28	26	41	30
thr	29	22	25	27	25	23	26	18
ser	36	48	45	40	35	32	45	27
glx	23	30	36	28	41	39	26	29
pro	21	16	19	22	18	15	15	12
gly	41	42	37	43	32	31	36	25
ala	45	22	22	18	21	19	16	13
val	30	36	31	21	20	19	21	21
ile	17	24	24	23	14	13	23	15
leu	19	17	16	28	34	30	27	19
tyr	13	11	12	17	18	16	16	15
phe	14	17	18	14	21	19	14	14
lys	11	9	6	2	4	4	1	8
his	9	3	6	2	1	1	1	4
arg	12	7	4	3	4	4	2	5
trp	1	2	3	3	6	3	5	4
cys	2	6	5	4	6	(6)	6	6
met	4	9	8	2	4	(4)	4	7
total	**360**	**358**	**360**	**342**		**304**	**325**	**272**
H I	773.4	843.2	839.2	813.2		804.5	817.2	888.3
ile/leu	.89	1.41	1.50	.83	.41	.43	.85	.79
asx/glx	1.43	1.23	1.30	1.64	.68	.67	1.58	1.03

Amino acid compositions of the purified Greenland cod proteases and "gastricsin fraction" were determined by triplicate acid hydrolysis at each of 24 h, 48 h and 72 h hydrolysis times at 110 °C. Cysteine and methionine were determined on 24 h acid hydrolysates after oxidation of the samples with performic acid in duplicate. Tryptophan was determined after 24 h hydrolysis in 3N mercaptoethane sulfonic acid in duplicate. The data are reported as moles of amino acid per mole of protein. Values in parenthesis were assumed. A A = amino acid, H I = hydrophobicity index, C P 1 = cod protease 1, C P 2 = cod protease 2, C P 3 = cod protease 3, "G F" = "gastricsin fraction" isolated in this study, Pep C = pepsin C (Ryle and Hamilton, 1966), Gast = porcine gastricsin (Chiang *et al.*, 1967), Pep A = porcine pepsin A (Fox *et al.*, 1977), and Chym = bovine chymosin (Foltmann, 1970)

ranging from 0 to 100 is obtained by multiplication of these values by 50. The Difference Indices for the comparisons of the different proteases are given in Table 3-16. Cod protease 2 and 3 were very similar and were more like chymosin than pepsin or gastricsin. Chymosin, pepsin and gastricsin were more closely related to each other than to cod protease 1. The porcine "gastricsin fraction" was more like pepsin A than gastricsin as judged by the Metzger Difference Index.

Table 3-16: Metzger Difference Indices for the Comparison of the Amino Acid Composition of Gastric Proteases

	Pep A	"G F"	Gast	C P 1	C P 2	C P 3
Bovine Chymosin	12.09	13.32	10.75	15.75	11.05	9.33
Porcine pepsin A		5.26	12.34	18.69	11.66	11.11
Porcine Gastricsin		12.43		16.85	12.88	10.90
Cod Protease 1					14.13	14.63
Cod Protease 2						5.71

C P 1 = cod protease 1, C P 2 = cod protease 2, C P 3 = cod protease 3, "G F" = "gastricsin fraction" isolated in this study, Gast = porcine gastricsin (Chiang *et al.*, 1967), Pep A = porcine pepsin A (Fox *et al.*, 1977), and Chym = bovine chymosin (Foltmann, 1970)

Chapter 4

DISCUSSION

4.1. General Considerations

4.1.1. Purification of Greenland Cod Proteases

The purification procedures for the Greenland cod gastric proteases resulted in enzyme preparations that were 6-200 times higher in specific activity than the starting homogenate (Table 3-1). The techniques of gel filtration, ammonium sulfate fractionation and ion exchange chromatography have been used by most workers in the purification of pepsinogens (eg. Ryle, 1970 and Kassel and Meitner, 1970). In this work, chromatofocusing gave superior resolution of the different proteases compared to flat bed isoelectric focusing. Another advantage of chromatofocusing is that the proteins are eluted from the column at their respective isoelectric points. This provides a label for each of the fractions which aids in their identification. However, the various proteases in samples taken at different times of the year had somewhat variable isoelectric points. These differences have been summarized in Appendix 6. It was unclear whether these differences were a seasonal phenomenon or due to differences in the handling of the samples. Varying degrees of phosphorylation or carbohydrate content may be responsible for these differences as was found with bovine gastricsin (Martin *et al.*, 1982). This area was not investigated further since the main thrust of this work

was the comparison of the cod gastric proteases to the porcine gastric proteases. It is apparent, however, that the isoelectric points of the Greenland cod gastric proteases and their zymogens are much higher than the isoelectric points of porcine pepsin and pepsinogen.

4.1.2. Differences in Properties of Porcine Gastricsin and the "Gastricsin Fraction" Isolated in this Study

A number of differences have been found between the properties of porcine "gastricsin fraction" isolated in this work and the properties of porcine gastricsin reported in the literature (Chiang *et al.*, 1967). This was somewhat suprising since the same purification methods were used in the present study as by these workers and similar elution profiles were obtained from the ion exchange column. In addition, the yield of "gastricsin fraction" obtained in this study (3-7%) from 1:10,000 crude pepsin was similar to the reported yield of gastricsin (5%) by Chiang *et al.*, 1967. The molecular weight of gastricsin was lower than the molecular weight of pepsin in all studies. The differences in the gastricsin preparations are discussed below.

Striking differences were found in the specificity of the "gastricsin fraction" isolated in this study and that reported in the literature. In the early work on the classification of gastricsin (Richmond *et al.*, 1958), the pH optimum for gastricsin with hemoglobin as the substrate was pH 3 while the pH optimum for pepsin was 2. However, more recent studies (Ward *et al.*, 1978) have shown that the pH optimum with hemoglobin as substrate can be affected by the salt concentration. This has also been observed for human pepsin (Samloff and Dadufalza, 1977). The

pH optimum for both "gastricsin fraction" and pepsin A with hemoglobin as the substrate was consistently found at pH 2. The pH optimum may also be affected by the treatment of the hemoglobin solution. In fact, a higher pH optimum was obtained with "gastricsin fraction" compared to pepsin A with methylated hemoglobin as the substrate. The pH optimum for pepsin A and "gastricsin fraction" was the same with methylated casein as the substrate.

Literature reports on the substrate specificity of gastricsin differ from the data obtained in this study. Tang *et al.* (1959) reported that APDT, which was a good substrate for pepsin A, was a very poor substrate for gastricsin. However, the "gastricsin fraction" isolated in the present work was able to hydrolyse APDT, but at 1/2 the rate of pepsin A. The substrates Z-tyr-ala, Z-trp-ala, Z-tyr-leu, Z-tyr-thr and Z-tyr-ser were all reported to be good substrates for porcine gastricsin and poor substrates for pepsin A (Tang, 1970). However, the "gastricsin fraction" isolated in this study was active only with Z-tyr-ser and pepsin A was active with Z-tyr-ser and Z-tyr-thr. Greenland cod proteases 2 and 3 were able to hydrolyse all of these peptide substrates. This result is particularly interesting in light of a later report (Sanchez-Chiang and Ponce, 1981) that gastric proteases from the fish *Merluccius gayi* (which these authors have termed "gastricsins") were unable to hydrolyse Z-tyr-ala or Z-trp-ala. It has also been reported in the literature that porcine gastricsin was slightly less active than pepsin in the milk clotting assay. The "gastricsin fraction" isolated in this study clotted milk at twice the rate of pepsin A. The amino acid composition of the "gastricsin fraction" also differed from the literature values for the amino acid composition of gastricsin and porcine pepsin C (Tang, 1970; Ryle, 1970). In

particular, the "gastricsin fraction" used in this study had higher aspartate, glycine, isoleucine and lower glutamate, alanine, leucine, phenylalanine, lysine, cystine and methionine than the literature values. The Metzger Difference Index between porcine pepsin A and the "gastricsin fraction" was much lower than the Difference Index between porcine pepsin A and the gastricsin reported in the literature. Also, the immunological properties of porcine pepsin A and the "gastricsin fraction" were not distinctive.

It is therefore apparent that there are many differences in the properties of the "gastricsin fraction" isolated in this study and those reported in the literature. It is clear, however, that the "gastricsin fraction" isolated in this study is different from pepsin A. "Gastricsin fraction" had a slightly lower pH optimum than pepsin A with methylated hemoglobin as substrate. Pepsin A was more active than "gastricsin fraction" with APDT and Z-tyr-thr and was only half as active as the "gastricsin fraction" in the milk clotting assay. The physiological efficiency (Vmax/Km') for "gastricsin fraction" with both hemoglobin and casein were higher than those for pepsin A. "Gastricsin fraction" had a higher average activation energy than pepsin A and pepsin A was marginally less pH stable than "gastricsin fraction". "Gastricsin fraction" was preferentially precipitated by anti-T16 antibody and had a slightly lower molecular weight than pepsin A. The amino acid composition of the "gastricsin fraction" isolated in this study and pepsin A were also different. A reaction of identity was found between porcine pepsin and gastricsin on the Ouchterlony double diffusion plates. This means that the antibodies in this preparation of anti-pepsin IgG have been raised against common structural features in both proteins. If a spur had formed at the junction

of the precipitin lines of two adjacent proteins, it could be assumed that the proteins have structural differences. The lack of spur formation, however, may only mean that the appropriate antibodies against the unique structural features of the proteins were not present.

There may be many reasons for the differences in the properties of the gastricsin isolated in this study and the literature data. The most obvious reason might be differences in the 1:10,000 pepsin used in this study and that used by Chiang *et al.* (1967). These commercial preparations may have been treated differently or changes in the preparations (such as autolysis or oxidation) may have occurred in storage. This study leaves some doubt on the presence of gastricsin in 1:10,000 pepsin. The very similar properties of the "gastricsin fraction" and pepsin A leads one to postulate that the "gastricsin fraction" isolated in this study is actually a modified form of pepsin A. The availability of a commercial supply of purified gastricsin would be helpful in resolving these questions. In any case, it is apparent that the "gastricsin fraction" is not the same enzyme as the porcine gastricsin reported in the literature and the literature data will be referred to in any comparisons involving porcine gastricsin.

4.1.3. Activation of Zymogens

The rapid activation at low temperatures of the zymogens of the Greenland Cod gastric proteases was not unexpected since the habitat temperature of this fish is consistently low (Bobbitt and Akenhead, 1966). However, although porcine pepsinogen was slow to activate at 0 °C, it was activated rapidly at 35 °C. This is clearly not a disadvantage for the pig since it maintains its body temperature at

37 °C. The differences in the activation rates of the different cod proteases at low temperatures are interesting. Since protease 1 was activated at a significantly slower rate at 0 °C than the other proteases, this might be a disadvantage for the cod. However, it is highly probable that the rate of activation of protease 1 is much faster *in vivo* due to the presence of active proteases in the stomach.

4.1.4. Specificity of the Proteases

A large number of substrates were used to examine the substrate specificity of the various proteases. The traditional method for the measurement of rates of proteolysis is the increase in A_{280} of TCA soluble reaction products. However, this method does not directly measure the rate of hydrolysis of peptide bonds and the rates obtained by this method are not directly comparable to those obtained by other methods. The use of methylated protein substrates allows the number of peptide bonds hydrolysed to be directly measured since the method detects the free amino groups formed by proteolysis. It should be remembered that both methylated and non-methylated proteins are comprised of a number of different peptide bonds. Thus the actual substrate of the enzyme may change as the reaction proceeds. The enzyme will hydrolyse those bonds that are the preferred substrate at first and then switch to another type of bond as the preferred substrate is depleted. Also, since the chain length of the peptides produced by hydrolysis gets shorter as the reaction proceeds, secondary binding of the substrate to the enzyme may become a factor as the reaction proceeds. As mentioned in the Introduction, these secondary binding sites can play an important role in the rate of hydrolysis of the substrate.

The substrate specificity of a protease can be determined by analysis of the hydrolysis products of a small protein substrate of known sequence (such as the β chain of insulin). This method is tedious and the interpretation may be complicated by the variable effects of the secondary binding of the substrate as the reaction proceeds. Another method used to determine the substrate specificity of proteases is to use a number of small peptides. However, the reaction rates with these small peptides are generally slow due to the complete lack of secondary binding of the substrates. For this reason the hydrolytic activities with peptide and protein substrates have been referred to as "peptidase" and "protease" activities, respectively, by some workers.

Measurements of the degree of hydrolysis (DH) of hemoglobin by the various proteases also gives an indication of the substrate specificity of the enzymes. A protease that has a low DH will also have a narrow substrate specificity. Thus, when the particular peptide bonds in hemoglobin that are substrates for the enzyme are completely hydrolysed, the reaction ceases. A protease that could hydrolyse a larger number of different types of peptide bonds would result in a higher DH. Obviously, direct comparisons of DH among different proteases can be made only with the same protein substrate since different proteins are composed of different peptide bonds. It is therefore apparent that the cod proteases generally have a broader substrate specificity than the porcine proteases since they have higher DH (Table 3-5). However, while cod protease 3 had the highest DH, the activity of this enzyme on a number of peptide substrates was less than the activity of cod protease 2. Thus small differences in the rate of peptide hydrolysis are not as important as the overall specificity for a large number of

peptides in determining the DH of an enzyme. In addition, the rate of peptide bond hydrolysis can be much different in a protein compared to a peptide substrate due to secondary binding effects.

The degree of hydrolysis of hemoglobin by the gastric proteases were greater (2X for porcine pepsin, 4.5X for cod protease 3) than those obtained with either bovine trypsin or trypsin isolated from Greenland cod (Simpson, 1983). This result reflects the very narrow substrate specificity of trypsin compared to pepsin which has a very broad substrate specificity. The value of h_{tot} for hemoglobin was taken at 8.0 meq/g since this value represented the average of a number of proteins. However, if one corrects for the presence of heme, the value of h_{tot} would be decreased by only 3.8% to 7.695 meq/g and the DH would be increased accordingly. This small difference was not considered significant enough to correct the assumed value for h_{tot}.

The ratio of the milk clotting activity to the proteolytic activity (CU/PU) of the proteases is the major indicator of the chymosin-like properties of an enzyme. A high CU/PU ratio means that the enzyme is suitable for the milk clotting process of cheese making since the curd yield is higher and fewer off-flavors will be produced in the curd due to nonspecific proteolysis during and after the clotting process.

4.1.5. Kinetic Parameters

Initial velocity measurements at different substrate concentrations were fitted to a Michaelis Menton model and Km' and Vmax were calculated. The Vmax for all enzymes were higher with the protein substrates than the peptide substrates. The Vmax with hemoglobin was higher than the Vmax with casein using the TCA solubles method but Vmax for methylated casein were similar to those for methylated hemoglobin. This is probably due to a lack of aromatic amino acids in casein which would result in low rate measurements with casein using the TCA method. However, several changes have been observed in the amino acid composition of the proteins after the methylation procedure and these changes may have affected the enzyme rates. The methylation of hemoglobin results in a 5% decrease in the content of leucine and phenylalanine, a 10% decrease in the content of serine and valine, a 15% decrease in the content of threonine and, most importantly, a 65% decrease in the content of tyrosine. This data was obtained by 24 h acid hydrolysis of the hemoglobin before and after methylation (data not shown).

A digestive enzyme has very a different function from that of an intracellular enzyme. A digestive enzyme should function in such a way as to digest food in the fastest and most efficient way possible. Any discussion of kinetic parameters of gastric proteases with protein substrates is complicated by the multiplicity of different peptide bonds present in the protein. A high Vmax or turnover number is clearly desirable for rapid digestion but the effect of Km' is not clear. Km' measured with a protein substrate represents the cumulative

effects of a series of individual Km' for the different peptide bonds present in the protein. However, if Km' is regarded operationally as the concentration of substrate that produces half maximal velocity, a digestive enzyme with a low Km' would be functioning at a faster rate with a smaller amount of food present. The simultaneous variations in both Vmax and Km' make the interpretation of the physiological significance of these parameters difficult. It has been noted in some systems, however, that the physiological efficiency (Vmax/Km') tends to be a constant. In this regard, the physiological efficiencies of the enzyme β-lactamase (EC 3.5.2.6) from different species of bacteria were very similar while the Km' were not (Pollack, 1965). However, this constancy of the values for the physiological efficiency has not been found with all the gastric proteases and different substrates in this study.

The physiological function and the desirable kinetic properties of an intracellular enzyme may be quite different from a digestive enzyme. A very high Km would be disadvantageous for a rate-limiting enzyme in a metabolic pathway. It is important for these enzymes to have Km in the range of the physiological levels of their substrates so that small changes in concentration of the substrate result in large changes in catalytic rate of the enzyme. In this way the rate-limiting enzymes regulate an entire metabolic pathway. A high Km for these enzymes would make them insensitive to small changes in substrate concentration. The function of a digestive enzyme is quite different and requires only that digestion proceed at as fast a rate as possible.

The activation energies for proteolysis were calculated using the Arrhenius equation;

$$\ln(k_2/k_1) = Ea\,(T_2-T_1)/R(T_2-T_1)$$

where k_1 and k_2 represent velocity constants at absolute temperatures T_1 and T_2. The results in Table 3-13 indicate that there was no significant difference between the activation energies of porcine pepsin and the cod proteases. Over the course of this work some differences have been noted in the various samples of Greenland cod stomachs (Appendix 6). With some samples, differences were also found in the activation energies of the proteases and on occasion low activation energies were obtained. In this regard, it is noteworthy that Greenland cod trypsin has a much lower activation for catalysis than does bovine trypsin (Simpson and Haard, 1984, 1984a). This was not pursued further since it was not the main thrust of this work.

The effect of temperature on the rate of a reaction can also be estimated by measuring the Q_{10}. The Q_{10} is the factor by which a reaction rate is increased for a 10 °C rise in temperature and a low Q_{10} is indicative of a low Ea. Q_{10} values for the temperature ranges 5-15 °C and 15-25 °C are included in Tables 3-6 to 3-11. No distinct differences were seen between the Q_{10} values of the different gastric proteases.

4.1.6. Structural Features of the Proteases

The cod proteases were more susceptible to inactivation by heat than the porcine enzymes. This has been found in many animals which live at colder habitat temperatures. Thus proteolytic enzymes from the pyloric caeca of cod, herring and mackerel are more heat labile than bovine trypsin (Rossebo and

Underdal, 1972) and the thyroid protease of burbot from cold waters had lower thermal stability than that from burbot in a warmer habitat (Wiggs, A., 1974). In addition, the gastric proteases from tuna, cod *Gadus callaris* and other fish species mentioned in the introduction have lower thermal stability. Trypsin has been isolated from the Greenland cod (Simpson and Haard, 1984a) and has lower thermal stability than bovine trypsin. The thermal stability of the Greenland cod proteases was greater at pH 3 and much less at pH 6.5 compared to pH 1.9 (Figure 3-19). This result is in agreement with the pH stability data (Figure 3-20) which indicated that the enzymes are unstable at pH values less than 2 and greater than 7. The instability of the Greenland cod gastric proteases to alkali contrasts with the increased stability to alkali of the gastric proteases of many of the lower vertebrate species (Ward *et al.*, 1978).

The effect of the concentration of salt on the activity of the proteases was very marked. Cod protease 1 and 2 showed a 2-fold stimulation in activity in the presence of 25 mM NaCl, cod protease 3 was not affected and the porcine enzymes were slightly inhibited. The activating affect of salt on gastric proteases has also been reported for the gastric proteases of a number of fish species including "gastricsins I and II" from the hake *Merluccius gayi* (Sanchez-Chiang and Ponce, 1981a). The "gastricsin I" of this species was preferentially activated by monovalent chlorides (Li, Na, K, and NH_4) while the "gastricsin II" was preferentially activated by divalent salts (Ca, Ba, Mg and Be) while sodium and potassium acetate inhibited the activity of both enzymes. In a later paper (Sanchez-Chiang and Ponce, 1982) these authors report that "pepsin-like" enzymes from this species are also stimulated by salt. The activating effect of salts

was to increase Vmax and lower Km' for the hydrolysis of hemoglobin. These authors have interpreted the results to be due to the hydration of the salt, thus binding the water molecules and changing the hydrophobicity of the system to favor hydrophobic interactions between the enzyme and the substrate. This interpretation is probably an oversimplification and further work on the nature of the enzyme-substrate interactions with these enzymes is necessary. It does appear, however, that the hydrophobic interactions between enzyme and substrate are of a different nature with enzymes such as cod proteases 1 and 2 that are stimulated by salt than with the gastric proteases that are unaffected by salt. It may be significant that the activity of the Greenland cod gastric proteases that have the highest isoelectric points are stimulated by salt while the cod gastric protease of low pI was unaffected by salt.

The immunological work indicates that there are similarities in the structures of the different proteases. Porcine pepsin and "gastricsin fraction" both reacted with either anti-pepsin or anti-gastricsin IgG preparation. Using anti-T16 IgG, "gastricsin fraction" was shown to be unique, cod protease 1 was similar to porcine pepsin and the other proteases did not react. All the proteases were inhibited to the same extent when anti-T25 IgG was used. The ratios of IgG/enzyme that gave complete inhibition were similar for cod protease 3 and porcine "gastricsin fraction" and for cod protease 2 and porcine pepsin.

The molecular weights of the proteases were all in the range of 35-37 kdal as determined by SDS-PAGE (Table 3-14). However, the molecular weight estimates obtained by gel filtration were significantly lower than the values from SDS-

PAGE. Long rod-shaped molecules can show this effect due to the difficulty of the molecules in penetrating the gel bed. However, pepsin A has a prolate elipsoid shape (roughly egg-shaped) which would not result in unusually slow elution from the gel. The effect may be due to interactions between the proteases and the Sephadex gel structure which slowed the elution of the enzyme and gave the appearance of a smaller molecular weight. This difference between molecular weights determined by gel filtration and SDS page was also found with gastric proteases from *Merluccius gayi* (Sanchez-Chiang and Ponce, 1981) and seal (Shamsuzzaman and Haard, 1984).

The amino acid compositions of the various proteases can be compared by calculating the Metzger Difference Index (Table 3-16). Cod protease 3 was the most similar to the porcine enzymes in amino acid composition as indicated by the lowest Difference Index followed by cod protease 2 and 1. The lowest Difference Index for each of the cod proteases compared to the mammalian gastric proteases was with bovine chymosin. This result is quite interesting when one considers the high milk clotting activity of the cod proteases. The Difference Indices between chymosin, gastricsin and pepsin were in the same range as the Difference Index for the cod proteases compared to pepsin, gastricsin and chymosin. Cod protease 2 and 3 were very similar but distinct from cod protease 1.

4.2. Comparison of the Cod Proteases and Porcine Proteases

4.2.1. General Discussion

The cod proteases are similar to the porcine proteases in a number of ways. They are all gastric proteases with pH optima in the acidic region. They all have inactive zymogen precursors that are activated by exposure to acidic conditions. They all have rather broad specificity and are active with protein and peptide substrates. Immunological studies indicated that the enzymes share several structural features. The molecular weights of the proteases are quite similar and the amino acid compositions are not grossly different.

Overall, the cod proteases have few properties which are pepsin-like, and more properties that are chymosin-like and gastricsin-like. The low activity of the cod proteases with APDT is a gastricsin-like property while the high milk clotting and CU/PU ratios are more like chymosin. The rather high isoelectric points of both the zymogens and the activated proteases from the Greenland cod are more like gastricsin and chymosin than pepsin. Also, the cod proteases generally have much higher pH optima than porcine pepsin. The cod proteases were less active with the methylated protein and peptide substrates than porcine pepsin. The cod proteases generally have a wider substrate specificity than porcine pepsin as indicated by both the data in Table 3-3 and the higher degree of hydrolysis of hemoglobin (Table 3-5). Both the pH stability and the thermal stability of the cod proteases were lower than those of porcine pepsin. The stimulatory effect of NaCl on two of the cod proteases makes them different from both porcine pepsin and gastricsin but similar to other fish proteases which are thought to be gastricsin-

like (Sanchez-Chiang and Ponce, 1981a). The cod proteases were also shown to be different from porcine proteases with 3 out 4 IgG preparations.

4.2.2. Cod Protease 1 and the Porcine Proteases

Cod protease 1 had several characteristics that are similar to the porcine enzymes. Its zymogen was the slowest of all the cod proteases to activate and the enzyme was the most active of the cod proteases with APDT (a good pepsin substrate). It also behaved in a similar manner to porcine pepsin with the anti-T16 IgG preparation. Cod protease 1 also had several unique properties that made it different from the porcine enzymes. Its amino acid composition was the most different from the porcine enzymes and the zymogen form of protease 1 had the highest isoelectric point of the cod enzymes. Also, the pH optima of the porcine enzymes and cod protease 1 were very different. Cod protease 1 was more like fish "gastricsins" in its stimulation by NaCl. The Km' with APDT for cod protease 1 was greater than that for porcine pepsin.

4.2.3. Cod Protease 2 and the Porcine Proteases

Cod protease 2 also had some characteristics similar to the porcine enzymes. Cod protease 2 and porcine pepsin were completely inhibited by anti-T25 IgG preparation at similar IgG/enzyme ratios. However, it had a broad specificity with the various peptide substrates, all of which were reported to be good substrates for human and porcine gastricsin (Chiang *et al.*, 1967). Cod protease 2 also had several properties that were different from the porcine enzymes. It reacted differently from the porcine enzymes with anti-T16 IgG preparation. Its zymogen was activated at a very fast rate while porcine pepsinogen was activated

at a slow rate. In addition, it was stimulated by NaCl to the same extent as the fish "gastricsins" (Sanchez-Chiang and Ponce, 1981a).

4.2.4. Cod Protease 3 and the Porcine Enzymes

Cod protease 3 has many properties that make it similar to the porcine enzymes. It had the lowest isoelectric point of all the cod proteases. Cod protease 3 and the porcine enzymes were not affected by NaCl. In addition, cod protease 3 had a broad specificity for the peptide substrates that have also been reported to be good substrates for porcine and human gastricsin. Cod protease 3 had a similar pH stability profile to porcine pepsin. However, cod protease 3 reacted differently from porcine pepsin with anti-T16 IgG preparation. It also had the highest milk clotting activity of all the cod proteases and can therefore be thought of as the most chymosin-like of the cod gastric proteases.

4.2.5. Comparison of Gastric Proteases in Greenland Cod and Other Fish Species

A number of common features exist among the gastric proteases of the various fish species discussed in the literature (section 1.2.7) and these have been summarized in Table 2-2. Fish gastric proteases generally have higher pH optima or retain their activity at a higher pH than does porcine pepsin. Fish proteases have lower thermal stability and temperature optima, are more active with hemoglobin as a substrate and less active with peptide substrates and in the milk clotting assay than porcine pepsin. The activity of many fish gastric proteases is also stimulated by the presence of salt. The amino acid compositions of fish gastric proteases reveal a larger number of basic residues, resulting in higher

isoelectric points for these proteins. Gastric proteases from some fish species also have a higher cystine content than porcine pepsin. These differences in amino acid content may be directly responsible for the increased stability to alkali found in many fish species.

The Greenland cod gastric proteases share many of these general properties of other fish gastric proteases. However, Greenland cod gastric proteases did not have an increased stability to alkali shown by gastric proteases from other fish. The high activity of the cod proteases in the milk clotting assay was very unique. In this regard, the Greenland cod gastric proteases are more chymosin-like than the gastric proteases from other fish species. Also in contrast to most other fish gastric proteases, the Greenland cod gastric proteases were not more active than porcine pepsin with hemoglobin as the substrate.

Although some workers have classified some gastric proteases from fish as gastricsins (Sanchez-Chiang and Ponce, 1981), most authors have been reluctant to do so. As can be appreciated from the previous discussion , the definition of what exactly constitutes a gastricsin is not clear. Undoubtedly, future detailed research on the characterization of the gastric proteases from other fish and other lower vertebrate species will lead to the classification of these enzymes. It seems clear, however, that any classification of enzymes must rely heavily on the catalytic function of the enzymes and their substrate specificity and less on kinetic properties that can be readily affected by the assay conditions used. The gastric proteases from the Greenland cod have many properties that resemble each of the gastric proteases, pepsin, gastricsin and chymosin and these are summarized in Table 4-1.

Table 4-1: Comparison of the Properties of the Greenland Cod Gastric Proteases with the Gastric Proteases of Other Species

	Pep A	Gast	Chym	Fish	Unique
Kinetic Characteristics					
Activity on protein substrates	1		?		2,3
Activity with peptide substrates	1	2,3		?	
Milk clotting activity			1,2,3		
pH optima protein substrates		2,3	1		
pH optima peptide substrates	2	1,3		?	
Activation rate of zymogen				?	1,2,3
Vmax and Km				?	1,2,3
Activation energy and Q_{10}	1,2,3	?	?	?	
Structural Characteristics					
Thermal stability				1,2,3	
pH Stability	1,2,3				
Effect of NaCl	3			1,2	
Molecular weight	1,2,3		1,2,3		
Amino acid composition					1,2,3
Isoelectric point			2,3	?	1
Immunological relatedness				?	1,2,3

The numbers 1, 2, and 3 indicate that cod protease 1, 2 or 3 have properties similar to pepsin A (Pep A), gastricsin (Gast), chymosin (Chym), gastric proteases from other fish (Fish) or whether the characteristic is unique to the Greenland cod. "?" indicates that no clear distinction could be made.

4.2.6. Conclusions

This thesis was based on the hypothesis that the gastric proteases from the Greenland cod had the basic properties common to all gastric acid proteases but also had properties unique to gastric proteases from other fish species. The results and the discussion presented here have supported this hypothesis. In particular, it was found that the Greenland cod gastric proteases have broad substrate specificity and are active over a wider range of pH than porcine pepsin. This broad substrate specificity results in more complete hydrolysis of protein substrates. The zymogens of the cod gastric proteases are activated at much lower temperatures than porcine pepsinogen. This represents a cold temperature adaptive feature of these enzymes. In addition, the Greenland cod gastric proteases are less thermally stable than porcine pepsin, a property that has been found for enzymes from other poikilothermic animals. However, in contrast to the gastric proteases of most other fish species, the gastric proteases from the Greenland cod are not more stable to alkali than mammalian pepsin, but show a similar pH stability as porcine pepsin. A unique adaptive feature of the Greenland cod gastric proteases 1 and 2 is the 2-fold stimulation of the hydrolysis of protein by 25 mM NaCl. This property requires further study to determine the effect of NaCl on pH optima as well as Vmax and Km with different substrates. These properties, together with the high milk clotting activity and CU/PU ratios of the Greenland cod gastric proteases may make these enzymes useful in certain industrial processes.

It is apparent that the different Greenland cod gastric proteases have some

properties in common with pepsin (APDT hydrolysis), some properties in common with gastricsin (pH optima with hemoglobin, activity on peptide substrates) and some properties in common with chymosin (high milk clotting activity). From the data presented here, it cannot be conclusivel stated that a particular cod protease is more like pepsin A, gastricsin or chymosin. It may be that the Greenland cod gastric proteases represent less differentiated forms of proteases which are more closely related to a common ancestral protease from which mammalian pepsin A, gastricsin and chymosin have evolved. However, speculation as to the evolutionary relationships of these proteases must await further conclusive structural studies such as peptide mapping, conclusive immunological studies and, ultimately, the determination of the complete amino acid sequences.

4.2.7. Future Research

At this point, it is appropriate to pose a number of questions that have arisen out of this work in order to provide direction for future research. The further characterization of the Greenland cod gastric proteases could proceed in many directions. The distribution of pepsinogen isoenzymes could be investigated. What is the seasonal distribution of pepsinogen isoenzymes? What is the nature of the differences in isoenzymes, ie. Are the differences due to small changes in amino acid composition, degrees of phosphorylation or carbohydrate content? Do the kinetic parameters of the cod proteases differ throughout the year? Does a pepsin B (peptidase) type of protease active only on Z-glu-tyr or APDT exist in the Greenland cod? What is the distribution of the various isoenzymes in the different regions of the stomach? It may also be useful to fractionate the Greenland cod gastric proteases on a DEAE column to allow a more direct comparison with the literature.

The kinetic parameters of the Greenland cod gastric proteases could be further investigated. In particular, the stimulation of the activity of proteases 1 and 2 in the presence of salt is very interesting. What is the nature of this stimulation? What is the effect of salt on Vmax, Km, pH optimum and thermal and pH stability? Is the stimulation of activity found with peptide as well as protein substrates, ie. does salt affect the secondary binding characteristics of large substrates? How does the nature of the substrate affect the stimulation by salt? In this regard, the approach taken by Fruton (1976) using a variety of peptide substrates could be used. What are the structural features of cod protease 3 that cause the lack of stimulation by salt? A study of the possible synergism of a mixture of the Greenland cod proteases would be of interest. What role does each of the proteases play in the various stages of hydrolysis of the protein, *ie.* do the various proteases preferentially hydrolyse peptides of different chain length? The inhibition of the Greenland cod proteases by EPNP, DAN and pepstatin and the possible effect of salt on this inhibition would be of interest.

A number of investigations on the structure of the Greenland cod proteases could be done. What is the extent of α helix and other tertiary structures in the enzymes? How are these features affected by temperature, pH and salt concentration? What are the primary sequences of the Greenland cod gastric proteases? In this regard, a comparative study of the Metzger Difference Index versus the percentage of sequence difference of the acid proteases would be of interest.

Chapter 5

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Chapter 6

APPENDIX

6.1. Appendix 1 : Preparation of SDS and Ornstein-Davis Gels

6.1.1. SDS Gels

Solutions required are:

1. Acrylamide 30g , bisacrylamide 0.8g make up to 100 ml with water
2. Tris base 18.17g , 6N HCl 20-25ml ,10% SDS 4ml make up to 100 ml pH 8.8
3. Tris base 6.06g , 10% SDS make up to 100 ml , pH 6.8 with HCl
4. Tris base 12g , glycine 57.6g make up to 4l with water (running buffer)
5. Glycerol 5ml , 2-mercaptoethanol 2.5 ml ,10% SDS 15 ml , solution (3) 6.25 ml dilute to 50 ml with water. (sample buffer)
6. Ammonium persulfate 200 mg , dissolve in 2 ml water (make fresh)

Procedure: Clean gel tubes in DECON or equivalent, rinse well and dry in the oven. Cover one end of the tube with Parafilm and place the tubes in a level gel rack. Mix the lower gel solution, degas, fill the tubes to the 10 cm mark using a pasteur pipette and layer .1% SDS on the top. When the gel has hardened,

To prepare 12 gels of 7.5% acrylamide:

Lower Gel	Upper Gel
water 19.7 ml	water 3.25 ml
soln B 10.0 ml	soln C 1.25 ml
soln A 10.0 ml	soln B 0.5 ml
soln F 0.25 ml	soln F 15 ul
TEMED 15 ul	TEMED 5 ul

(TEMED = tetramethylethylenediamine)

remove the excess liquid with a pasteur pipette. Mix the upper gel components, degas, pipette on 250 μl and layer on .1% SDS. When the gel has hardened, remove the upper liquid and place the tubes in the electrophoresis unit.

Samples are prepared by heating 1-5 μg of protein in 100-500 μl of solution E for 2-5 min at 90 °C. Once the samples have cooled, add 5 μl of .1% bromophenol blue tracking dye and apply to the gel. Carefully layer on running buffer and fill the apparatus with this buffer. Run the gels at 1 ma/tube until the tracking dye enters the lower gel when the current is increased to 2 ma/tube.

6.1.2. Ornstein-Davis Gels

Solutions required are:

1. 1N HCl 48 ml, Tris base 36.6 g, TEMED 0.23 ml make up to 100 ml, pH 8.9
2. 1N HCl 48 ml, Tris base 5.98 g, TEMED 0.46 ml make up to 100 ml, pH 6.7
3. Acrylamide 28 g, bis acrylamide 0.735 g, make up to 100 ml.
4. Acrylamide 10 g, bis acrylamide 2.5 g, make up to 100 ml.
5. Riboflavin 4 mg, water to 100 ml
6. Sucrose 40 g, make up to 100 ml.
7. Tris base 2.4 g, glycine 11.52 g, make up to 4l, pH 8.3 (running buffer)
8. Ammonium persulfate 0.14 g, make up to 100 ml.

To make enough gel solution for 12 gels:

Lower Gel		Upper Gel	
soln A	5 ml	soln B	.25 ml
soln C	10 ml	soln D	0.5 ml
water	5 ml	soln E	.25 ml
soln H	20 ml	soln F	1.0 ml

Procedure: Clean the gel tubes in DECON or equivalent, rinse them well and dry in the oven. Cover one end of the tubes with a double layer of Parafilm and place in a level gel rack. Fill tubes with lower gel solution to the 10 cm mark with a

pasteur pipette. Carefully layer 50 µl water over the gel and leave 30-60 min to polymerize. Remove water with pasteur pipette, add 200 µl of upper gel solution, layer water on the top and polymerize the gels in front of a flourescent lamp. When gelled remove water with pasteur pipette and place in electrophoresis apparatus.

To prepare samples, add 20 µl solution A, 50 µl solution F and up to 100 µl sample or 20 µl solution A, 150 µl sample and 2 drops of glycerol plus 5 µl of 0.1% bromophenol blue tracking dye. Pipette on 200 µl sample per tube and carefully overlay the running buffer. Run at 1 ma per tube until sample enters the running gel and then run at 2 ma per tube until electrophoresis is complete.

6.2. Appendix 2 : Methods for Estimating Protein Concentration

6.2.1. Biuret Method

Prepare the following solutions;

1. **Biuret reagent** 1.5 g cuprous sulfate is dissolved in 400 ml water. Then dissolve in 6 g of sodium potassium tartrate. Add 300 ml of 10% NaOH and make up to 1l with water.

2. **5% DOC** dissolve 5g sodium deoxycholate (taurocholate) in 100 ml water

3. **Standards** for most proteins use fat free bovine serum albumin as a standard, for hemoglobin solutions use purified hemoglobin as a standard. make up a 10 ml solution of the standard.

Prepare a standard curve by mixing 0, .2, .4, .6, .8, ml of standard protein solution with .2 ml 5% DOC and sufficient water to 1.0 ml. For test solutions mix up to 0.8 ml of sample with .2 ml of 5% DOC. Perform all assays in duplicate. Add 4 ml of biuret solution and let stand at room temperature for 30 min. Measure the absorbance at 550 nm and construct a standard curve of A_{550} versus mg protein.

6.2.2. Coomassie Blue G250 Method

The following solutions are required;

1. **Dye Solution** Dissolve 0.6 g of Coomassie Brilliant Blue G250 (Pierce Chemical Co.) in 1l of 3% perchloric acid and filter through Whatman #1 filter paper. Measure the absorbance of the solution at 465 nm and dilute with 3% perchloric to give a value of 1.3-1.5.

2. **Standards** 1 mg/ml fat free bovine serum albumin; dilute by 1:20 to give a 50 ug/ml solution.

Prepare a standard curve using 0, .2, .4, .6, .8, 1.0 ml of standard protein and sufficient water to 1 ml. For test solutions use up to 1 ml sample. To each tube, add 1 ml of the dye solution and mix well. Measure the absorbance at 465 nm and 600 nm with water as a reference. Calculate the ratio of A_{600}/A_{465} for the samples and the blank. Subtract the blank ratio and construct a standard curve of the corrected ratios versus the μg of protein.

6.2.3. MicroKjeldahl Method

The following solutions are required;

1. **Digestion mixture** The following chemicals are combined in the order given: potassium sulfate 40 g, selenium oxychloride 2 ml, water to 250 ml and sulfuric acid 250 ml.

2. **Nessler's Reagent** Sigma ammonia color reagent (65 g/l double iodide of mercury and potassium)

3. **Standard Protein Solution** fat free bovine serum albumin 0.626 mg/ml (equivalent to 100 μg N/ml)

Construct a standard curve using 0, .2, .4, .6, .8, 1.0 ml of protein standard with sufficient water to 1 ml. Use up to 1 ml sample. Add .2 ml of digestion mixture to all tubes and heat to 310 ° C for at least 2 hr (usually overnight). When the tubes have cooled, dilute the acid digest to 10 ml with water and transfer 1 ml aliquots to another set of tubes. Add 0.33 ml of water to each tube, mix well and add 0.67 ml Nessler's reagent. Measure the absorbance at 500 nm and plot a standard curve of A_{500} versus μg of protein.

6.3. Appendix 3 : Preparation of Methylated Protein Substrates

Dissolve 1.5 g of protein in 150 ml of 0.1M borate buffer pH 9.0. For colored proteins such as hemoglobin, the solution is bleached by heating to 50 °C and adding 4.5 ml of 30% hydrogen peroxide. Cool the solution to 0 °C, stir rapidly and add 600 mg of sodium borohydride with a few drops of 2-octanol to prevent foaming. Add 37% formaldehyde (6 ml) in 100 µl increments over 30 min. Acidify the mixture to pH 6 with acetic acid and dialyse against water. The solution can then be freeze dried or the protein concentration can be determined by the biuret method.

6.4. Appendix 4 : Purification of the IgG Fraction from Rabbit Serum

Solutions required;

1. sodium sulfate 25% solution in water
2. sodium chloride .85% solution
3. sodium phosphate buffer 17.5 mM pH 6.3

Procedure:

1. **Precipitate with 18% Sodium Sulfate** To 20 ml of whole serum add 51.4 ml 25% sodium sulfate with constant stirring at room temperature. Centrifuge the mixture at 10,000 x g for 10 min and dissolve the pellet in a measured amount of saline. Determine the volume of the pellet by subtracting the volume of saline added from the final volume obtained.

2. **Precipitate with 16% Sodium Sulfate** Add 25% sodium sulfate dropwise under constant stirring. The volume of sulfate used is determined by;

$$\text{vol sulfate} = 1.78 \times \text{final vol} - 2 \times \text{pellet vol (from step 1)}$$

Centrifuge the mixture at 10,000 x g for 10 min at 25 ° C. Dissolve the pellet in the minimun amount of saline and dialyse overnight against phosphate buffer pH 6.3 in the cold.

3. **DEAE Cellulose Chromatography** Determine the concentration of protein in the crude preparation by the Coomassie Blue G250 method. Prepare a column of DEAE cellulose (10 mg protein/ml column material) which has been

adjusted to pH 6.3 with 0.2M sodium dihydrogen phosphate and washed with 17.5 mM phosphate pH 6.3. Apply the dialysed sample to the column and elute with 17.5 mM phosphate pH 6.3 at room temperature. Monitor the absorbance at 280 and combine the peak fractions. Determine the concentration of protein in the IgG fraction.

6.5. Appendix 5 : Preparation of Ouchterlony Double Diffusion Plates

Solutions required:

1. **Adhesive agar** Noble agar (Difco) 0.1 g, glycerine 0.05 ml, dissolve in 100 ml water by boiling

2. **Running agar** Noble agar 1.5 g, water 75 ml 0.02% sodium azide and 25 ml of barbital buffer (32.1% tris, 13.7% barbital, 54.2% sodium barbital pH 7.2; Gelman Instruments Co.) dissolve by boiling.

Procedure: Wash slides well. Pour adhesive agar over the slides and dry at 37 ° C. Insert the slides in the frame and pour the running agar to fill the wells (approximately 3 ml). Punch out wells in the desired pattern after the agar has hardened.

6.6. Appendix 6 ; Differences Found in Proteases from Different Samples

6.6.1. Isoelectric points of Greenland Cod Proteases

Sample Date	pI (nonactivated)	pI (activated)
Feb., 1980	7.5, 6.1-6.2, 5.15, 4.4-4.5	6.0, 5.2, 4.5, salt peak
Sept., 1981	7.5, 5.75, 5.3, 4.8	
Mar., 1982	7.65, 5.9-6.0, 4.9-5.1	
Nov., 1982	7.6, 5.45, 4.65	4.85, 4.40, salt peak
Apr., 1983	8.0, 6.25, 5.45, 5.20	5.85, 4.95, 4.5, salt peak
Jun., 1983	8.0, 5.9-6.0, 5.15, 4.6	5.8, 5.05, 4.5; salt peak

"salt peak" represents activity remaining on the chromatofocusing column after the gradient was complete which was eluted with 1M NaCl.

6.6.2. Activation Energies of Greenland Cod Proteases

Peak Number from chromatofocusing	Isoelectric Point	Activation Energy (kjoul/mol)
1	7.5	49.95
2	6.2	28.88
2	5.75	54.76
3	4.8	38.00
3	5.3	25.16