RECOVERY OF DIGESTIVE ENZYMES FROM ATLANTIC COD (Gadus morhua) AND THEIR UTILIZATION IN FOOD PROCESSING

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Recovery of Digestive Enzymes From Atlantic Cod (*Gadus morhua*) and Their Utilization in Food Processing

BY

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ABSTRACT

An extraction process for the recovery of proteolytic enzymes from the digestive tract of cod (*Gadus morha*) is described. Processing variables were optimized by employing a rotatable experimental design with computer graphics-assisted response surface methodology (RSM). A simple extraction procedure was developed which effectively isolated both acid and alkaline proteases from cod viscera with recovery yields of 52% and 30%, respectively. Further purification and characterization revealed that the crude acid proteases consisted of three types of gastric enzymes designated as acid proteases A, B, and C. Acid protease B was classified as fish pepsin II. Acid proteases A and C possessed properties similar to chymosin and gastricsin, respectively. The partially purified alkaline proteases possessed properties of trypsin-like enzymes and acted on N-benzoyl-L-tyrosine ethyl ester (BTEE), a synthetic substrate for chymotrypsin.

Utilization of the crude isolated proteases was tested in milk-clotting for cheese making, as well as preparation of protein hydrolysates from under-utilized fish species. Cod pepsin was capable of clotting milk efficiently at low temperatures, which shows its potential use in cold renneting of milk. Capelin protein hydrolysate with a recovery yield of 55.8% was obtained when crude cod gastric proteases were used over a 4 h hydrolysis period at ambient temperatures.

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List of Abbreviations and Symbols

AE	Acidic proteolytic enzymes
APDT	Acetyl-L-phenylalanyl-L-diiodotyrosine
BAEE	Benzoyl-L-arginine ethyl ester
BAPNA	α-benzoyl-DL-arginine-p-nitroanilide
BE	Alkaline proteolytic enzymes
BTEE	N-benzoyl-L-tyrosine ethyl ester
BU	Unit of BTEE activity
CPH	Capelin protein hydrolysate
CPU	Casein proteolysis unit
D	Dalton (Unit of molecular weight)
DFP	Diisopropyl fluorophosphate
DMSO	Dimethyl sulphoxide
FPH	Fish protein hydrolysate
HPU	Haemoglobin proteolysis unit
MCU	Milk clotting unit
ME	2-Mercaptoethanol
M_r	Relative molecular mass
PAA	Polyacrylic acid
pI	Isoelectric point
PMSF	Phenylmethylsulphonyl fluoride

RA	Relative activity
RSM	Response surface methodology
SAS	Statistical analysis system
SBTI	Soy bean trypsin inhibitor
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SGP	Seal gastric proteases
TCA	Trichloroacetic acid
Tris	Tris[hydroxymethyl] amino methane
β_k	Regression coefficients
$[E]$	Enzyme concentration (w/v)
$H_{\phi ave}$	The average hydrophobicity of a protein
K_m	Michaelis-Menten constant
K_{cat}	Protein turnover constant
R^2	Correlation coefficient
$[S]$	Substrate concentration
V_{max}	Maximum velocity of enzyme reaction

Chapter 1

Introduction

The management of the fishery resources and new product development using underutilized species are major concerns for the world leading fish-producing and fish-exporting countries. One of the potential problems associated with increased fishing is increased production of processing discards and fish offal. Approximately 100 million metric tons of fish, invertebrates, and molluscs are landed annually. The utilization of the catch is far from optimal, as only about 70% of the total landings are used for human consumption (Sikorski and Pan, 1993). Of this, the average process yield is 15–30%. Thus, there is a large amount of processing by-products available.

In Canada, approximately 50% of the fish processing discards are used in production of fish meal, while the remainder represents a disposal and pollution problem (Simpson and Haard, 1987). Although there are many approaches to further utilize fish processing wastes, interest has been expressed in isolating added-value compo-

nents from such raw material. Digestive proteases are a possible group of compounds which could be effectively isolated and characterized.

Enzymes play an important role in industrial manufacturing and processing, particularly in the food industry (Knorr and Sinskey, 1985). Enzyme production is currently experiencing unprecedented growth and expansion (Wasserman, 1990). The value of the world enzyme market has increased from $ 220 M in 1960, $ 400 M in 1970, $ 550 M in 1980, $ 1,000 M in 1985 to an approximate value of $ 2,000 M in 1990 (Chaplin and Bucke, 1990). This rapid increase is due to the availability of a large number of enzymes at relatively low cost, and their potential use in a variety of applications. Of the enzymes in use, proteases constitute an important group with global sales representing nearly 60% of the total enzyme market value (Godfrey and Reichelt, 1983). Utilization of proteases in the food industry includes cheese manufacturing(Visser, 1981; Law and Goodenough, 1991), meat tenderization (Fawcett and McDowell, 1987; Etherington, 1991), beer chill-proofing(Lea, 1991), flavour development in processing(In, 1990), modification of viscoelasticity of bread dough(Hamer, 1991), as well as production of the functional proteins (Feeney, 1977; Whitaker, 1980; Han, 1989; Shahidi *et al.*, 1993). Proteolytic enzymes have clinical importance in the production of protein hydrolysates for therapeutic diets (Free, 1980; Adler-Nissen, 1986), and are also valuable as laundry detergent additives or in enzyme-based cleaners for ultrafiltration systems (Smith and Bradley, 1987).

Proteases from various sources differ greatly in their catalytic and physical prop-

erties; suitability of a particular enzyme for a specific industrial application depends on several factors including specificity, reaction conditions, response to inhibitors and activators, cost and the availability of the enzyme and technical service support.

Earlier investigations on fish proteases were focused on the influence of enzymes on the properties and quality of fish during processing and storage. Only a few studies have been carried out to explore the possibility of enzyme utilization in food production, such as processing of fish products. In recent years some interesting new applications of enzymes in fish processing have emerged. This is, in part, due to an increased knowledge of enzymes and their uses, but also because many enzymes, including fish enzymes, have become commercially available.

The application of marine enzymes in a variety of applications is of interest. Proteolytic enzymes from cold-adapted fish viscera are more active at relatively low temperatures and are less stable thermally than their counterparts from warm-blooded land-based animals and plant sources (Simpson and Haard, 1987). These low-temperature-active and thermally unstable enzymes are expected to have sufficiently unique properties to justify their application in certain food processing operations.

Cod viscera (stomach, pyloric caeca and intestines) constitute approximately 7% of the fish weight. Therefore, a large quantity of viscera is available for recovery of proteolytic enzymes. Recently, crude cod pepsin preparations have been produced commercially by a Norwegian Company (Marine Biochemicals, Tromso) (Stefansson and Steingrimsdottir, 1990). In Iceland, extensive research and development projects

in the area of enzyme production and utilization have been conducted. The main emphasis is on the production of enzymes from thermophilic and psychrophilic microorganisms and from the viscera of cod, *Gadus morhua*, (Stefansson, 1988). This latter research group is interested in other proteolytic enzymes such as trypsin and chymotrypsin (Stefansson and Steingrimsdottir, 1990).

The currently established extraction procedures can only isolate one of the diverse variety of digestive enzymes in cod viscera. There are many unresolved concerns associated with enzyme characterization and their catalytic mechanisms. Furthermore, establishing an isolation technology that can co-extract most of the enzymes presented and suitable for scaling-up is wanted. The present study has focused on presenting a simple and effective procedure for the isolation of both acid and alkaline proteases from cod viscera. The extracts could then be used directly for food processing or may be further purified for specific applications. Use of the isolated proteolytic enzymes in food processing was also considered.

The objectives to the present study were: (1) to establish an isolation process that can co-extract acid and alkaline proteases from cod viscera, adaptable to large scale production; (2) to optimize processing conditions for the co-extraction procedure established; (3) to purify (or partially purify) and characterize the enzymes isolated in order to evaluate the co-extraction procedure; and (4) to apply isolated proteases for milk-clotting in cheese making as well as production of fish protein hydrolysates.

Chapter 2

Literature Review

There is a renewed interest in the study of proteolytic enzymes, mainly due to the recognition that proteolysis plays an essential role in many cellular processes such as digestion, translocation, protein turnover, secretion of proteins, and the activation of many toxins of importance to food scientist, such as the neurotoxins synthesized by *Clostridium botulinum* (Loffler, 1986). Enzyme technology has evolved to become an integral part of the food industry, and enzyme production is currently experiencing unprecedented growth and expansion. Recent technological advances in enzymology suggest that there may be no limit to the kinds of reactions enzymes can catalyze (Wasserman, 1990).

Among commercially available enzymes, proteases constitute approximately half of the world enzyme production. The term proteolytic enzyme or protease is synonymous with peptidase and these terms refer to all enzymes which catalyze the

hydrolysis of peptide bonds in polypeptides and proteins. All proteases are 'intracellular' at some stage in their existence (Bond and Butler, 1987). Some proteases are synthesized for export to extracellular spaces, and exert their biological action as discrete entities outside cells.

From an analysis of their in-vitro properties, proteases have been classified in a number of ways. On the basis of the pH range over which they are active, proteases are classified as acidic, neutral and alkaline. Based on their ability to hydrolyze specific proteins, they are classified as keratinase, collagenase, elastase, etc.. According to their similarities to well-characterized enzymes, proteases are classified as trypsin, chymotrypsin, cathepsin, chymosin, etc.. However, the most satisfactory classification is based on their mechanism of action (Hartley, 1960). This classification which is used by the Enzyme Commission consists of four distinct classes of serine, cysteine, aspartyl and metalloproteases.

Proteases may also be subdivided into exopeptidases, whose action is directed by the amino- or carboxy-terminus of the peptide (EC 3.4. 11-19), and endopeptidases, which can cleave peptide bonds internally and usually can not accommodate the charged amino- or carboxy-terminal amino acids near the active site (EC 3.4. 21-24, 99). It has been suggested that the term endopeptidase be used synonymously with protease, and this recommendation is adopted throughout in this thesis.

2.1 Proteolytic Enzymes From Digestive Tracts of Marine Fish

Studies on protease activity of enzymes present in fish viscera began in the nineteenth century. Stirling (1884) showed that extracts from cod and herring stomach were able to digest fibrin in the presence of dilute hydrochloric acid. Vonk (1929) found that the pepsin content of pike stomach was higher than that in the stomach of mammals investigated. He suggested that fish produced more pepsin to compensate for reduced activity due to low secretion of gastric acid (Vonk, 1929). Since salmon pepsin was first crystallized in 1939 (Norris and Elam, 1940), gastric enzymes from several fish species have been purified and studied (Norris and Methies, 1953; Kubota and Ohnuma, 1970; Noda and Murakami 1981). Proteolytic enzymes which have been widely investigated include serine (e.g. trypsin-like) proteases, and aspartic proteases (e.g. pepsin, cathepsin D etc.), although other types of proteases such as pancreatic metalloproteinase (Yoshinaka *et al.*, 1984; 1985a and b) have occasionally been examined.

There have been several excellent reviews on the proteolytic enzymes mentioned above (Barrett, 1979; Foltmann, 1981; Bond and Butler, 1987; Gildberg, 1988; Haard, 1992). Since the present study includes aspartyl (pepsin, or pepsin-like enzyme) and serine proteases (trypsin, or trypsin-like enzymes), the following review focuses on these two classes of proteases.

2.1.1 Aspartyl proteases

Aspartyl proteases (EC 3.4. 23) constitute one of the four main classes of enzymes present in eukaryotes that act on interior peptide bonds of proteins and oligopeptides under acid conditions. Because of their optimal action under acidic conditions, aspartyl proteases are referred to as acid proteases. Since it was found that the two functional groups of catalytic site of these enzymes are always aspartic acid carboxyl groups, the new name aspartyl protease has been adopted. The term 'aspartyl protease' is more appropriate than 'acid protease' because some enzymes such as rennin, now known to be an aspartyl protease, do not necessarily belong to acid protease group and their optimum pH is around 6-8 (Fruton, 1987).

The aspartyl proteases of gastric juice are all secreted as inactive precursor-zymogens. These zymogens are irreversibly converted into active enzymes by releasing peptide chains (activation segments) from NH_2-terminal segments (Foltman and Pedersen, 1977). No zymogens for microbial aspartyl proteases have been found.

Aspartyl proteases include pepsin, renin, cathepsins D and E, as well as some microbial proteases. Members of the pepsin group include pepsins A, B, C, and D (Foltmann, 1981). Chromatographic separation of the components has shown that the predominant pepsin A (usually denoted pepsin) of adult mammals is accompanied by pepsin C (the currently-preferred name is gastricsin), as well as by the minor components denoted pepsin B and pepsin D (Ryle, 1970). These various aspartyl proteases have in common the property of cleaving proteins and suitable oligopeptides

at pH 1.5 – 5.5. A widely used diagnostic test is their inhibition by the naturally occurring peptide pepstatin and by active-site-directed diazo compounds. Available structural data indicate that all aspartyl proteases belong to one family (Huang *et al.*, 1980). The enzymes generally have molecular weights in the range of 30,000 to 40,000 D, and possess bilobal structures comprised of mainly β-sheet with a cleft that contains the active site (Blundell *et al.*, 1980).

It is difficult to obtain a highly purified gastric proteases due to the occurrence of autodigestion. The most homogeneous pig pepsin is obtained by a short activation of purified pepsinogen, followed by column chromatography at 4^0 C. Under these conditions, minimal autodigestion of the active pepsin occurs (Foltmann, 1981). At present, the preferred method for the preparation of apparently homogeneous pepsin is rapid activation of crystallized pepsinogen, and passage of the mixture through sulphoethyl Sephadex C-25 to remove peptides and then through Sephadex G-25 to remove salts (Fruton, 1987). However, acceptable results have also been obtained from starting materials containing active enzymes (Foltmann, 1981).

Scientists from different research areas have different interests in these enzymes. Biochemists are mainly interested in the pepsin and pepsin-like enzymes from the stomach of vertebrates, including mammals. However, both pepsin-like enzymes and cathepsin D have been subject to considerable investigation by marine biologists and other researchers; cathepsins are considered to be important in keeping quality of fish during processing and storage. Recently, food scientists have engaged in the recovery

of marine enzymes for their subsequent utilization in the food industry (Haard, 1992).

Gastric digestion is considered to be the only physiological role of fish pepsins (Gildberg, 1988). Therefore, fish species which lack a stomach or have a stomach without secretion of gastric acids do not have pepsin (Kapoor *et al.*, 1975). In such fish, both trypsin and cathepsins take part in digestion at close to neutral pHs (Jany, 1976). It is now widely accepted that cathepsins play an important role in digestion in many invertebrates (Vonk and Western, 1984). Since cathepsin D is one of the major tissue proteases (Barrett, 1977) and possibly the most abundant acid proteases in vertebrates (Ikeda *et al.*, 1986), the action of this proteolytic enzyme in fish has been studied for several decades.

Most fish belonging to the *Osteichthyes* and *Selachii* classes possess stomachs which secrete HCl and pepsinogen (Mernett *et al.*, 1969; Twining, *et al.*, 1983; Arunchalam and Haard, 1985), and it has been characterized from cod (Brewer *et al.*, 1984), salmon (Norris and Elam, 1940), dogfish (Mernett *et al.*, 1969), hake (Sanchez-Chiang and Ponce, 1981), and trout (Owen and Wiggs, 1971). Aspartic proteases from the gastric mucosa of harp seal (*pagophilus groenlandicus*) have also been purified and characterized. Four zymogens of acidic proteases A, B, C, and D were isolated (Shamsuzzaman and Haard, 1983). Three zymogens of gastric proteases have been isolated from the stomach mucosa of Greenland cod (*Gadus ogac*) by exclusion chromatography and chromatofocusing (Squires *et al.*, 1986a).

Research has proven that many fish species secrete at least two pepsins with

different pH optima (Noda and Murakami, 1981; Gildberg and Raa, 1983; Martinez and Olsen, 1986). Apart from the confusion in the mammalian gastric proteins, these pepsins are usually referred to as fish pepsin I and pepsin II (Gildberg, 1988). Fish pepsins which have been investigated and classified into these two groups are summarized in Table 2.1.

2.1.2 Serine proteases

The serine proteases comprise a large group of enzymes which is distinguished by the reactivity of a serine residue in the active site (Hartley, 1960). Most members of the group are endopeptidases. The serine proteases also exhibit strong esterolytic activity toward esters analogous to the specific peptide substrate - a fact with little physiological importance, but one used by biochemists in kinetic studies (Mathews and van Holde, 1990). A common test for these enzymes is the inhibition of their hydrolase activity by the reaction of serine residue with diisopropyl fluorophosphate (DFP) (Walsh and Wilcox, 1970).

Comparisons of relative rates of catalysis using a variety of synthetic substrates have lead to the definition of the specificities of representative members of the class. These range from the narrow specificity of the trypsins, which is directed toward the bond on the carboxyl side of arginine and lysine, to the rather broad specificity of the subtilisins which will attack bonds between a wide variety of amino acids. Serine proteases that have been characterized in prokaryotes and eukaryotes belong to

Table 2.1: **Differentiation of fish pepsins**

ITEMS	Pepsin I	Pepsin II	References
Molecular weight (D)[a]	$> 35 \times 10^3$	$\leq 35 \times 10^3$	Gildberg, (1988)
Relative amount:[b]			
in Sardine	major	minor	Noda and Murakami (1981)
in Capelin	major	minor	Gildberg and Raa (1983)
Isoelectric point (pI)[c]	6.5–7.0	close to 4.0	Gildberg (1988)
pH optimum:[d]	(3–4)	(2–3)	Gildberg (1988)
Cod	3.6 - 3.7	2.5 - 2.8	Bjelland *et al.* (1988)
Sardine	4.0	2.0	Noda and Murakami 1981
Capelin	3.7	2.5	Gildberg and Raa, 1983
pH stability in strong acid and neutral conditions	less stable	stable	Gildberg (1988)
Temperature optimum:			
Capelin	low	high	Gildberg and Raa (1983)
Cod	low	high	Martinez and Olsen (1986)
Specific activity (U/mg):[e]			
Atlantic cod	3,000	2,000	Gildberg and Almas (1986)
Capelin	1,100	2,000	Gildberg and Raa (1983)
Influence of NaCl[f]:			
American salmon	activated	unaffected	Sanchez-Chiang *et al.*(1987)

[a] According to SDS-polyacrylamide gel electrophoresis. The symbol D refers dalton.
[b] The seasonal variations in relative amounts may be substantial (Squires *et al.*, 1986a);
[c] Mammalian pepsins have much lower pI than fish pepsins, and values as low as 1.0 have been reported (Tiselius *et al.*, 1938);
[d] pH optimum was measured using haemoglobin as a substrate;
[e] The activities were determined by 1 h incubation at pH 3.0 and 25^0 C using haemoglobin as a substrate.
[f] Influence of NaCl on fish gastric proteases were also reported by Squires *et al* (1986b) and Sanchez-Chiang and Ponce (1982) using different classification systems.

the chymotrypsin and the subtilisin families (Barrett, 1986). Only the chymotrypsin family has been found in eukaryotes. The chymotrypsin family includes many extracellular proteases such as trypsin, elastase, thrombin, plasma kallikrein, plasma coagulation proteases, and cellular protease (Bond and Butler, 1987).

Serine proteases are widely distributed in nature. A survey of proteolytic enzymes in various species of fish digestive tracts has revealed that serine proteases are distributed in fish intestine with a high activity at alkaline rather than neutral pH. In teleost fishes, which do not have a distinct pancreas, serine proteases have been found in the pyloric caeca, but only in the active form (Walsh and Wilcox, 1970). Proteolytic enzymes distributed in the intestinal organs of marine vertebrates were generally similar to those of mammals, such as trypsin- or chymotrypsin-like enzymes in the intestine and pancreas, and pepsin-like enzymes in the stomach.

Trypsin- and chymotrypsin-like enzymes have a molecular weight of approximately 25,000 D (Prahl and Neurath, 1966; Reek and Neurath, 1972; Cohen and Gertler, 1981), a high isoelectric point (pI), and their action is inhibited by phenylmethylsulphonyl fluoride (PMSF) (Fahrney and Gold, 1963; Jany, 1976), soybean trypsin inhibitor (SBTI), and amino acid derivatives of chloromethyl ketone (Gates and Travis, 1969; Camacho *et al.*, 1970; Bond and Butler, 1987). It is believed that the structure of the active site of fish pancreas chymotrypsin is similar to that of other vertebrate forms of the enzyme (Barnard and Hope, 1969). Chymotrypsin from the pancreas of carp has been found to be similar to mammalian enzymes in both physical and ki-

netic properties, including inhibition patterns (Cohen and Gertler, 1981; Cohen *et al.*, 1981). However, chymotrypsins isolated from the pyloric caeca of herring and capelin (Kalac, 1978) and from mackerel (Ooshriro, 1968) were found to have greater relative activities on peptide and casein substrates than bovine α-chymotrypsin. The activity of dogfish chymotrypsin was two to three times higher than bovine chymotrypsin in the hydrolysis of collagen and other proteins (Ramakrishna and Hultin, 1987a and b).

Trypsin has been found in pancreatic tissues of all the species, from invertebrates to mammals (Walsh, 1970; Kapoor *et al.*, 1975). Trypsin-like proteolytic enzymes have been purified and characterized in several fish species including African lungfish (Reeck and Neurath, 1972; de Haen *et al.*, 1977), mackerel (Pyeun and Kim, 1986), herring (Kalac, 1978), carp (Cohen and Gertler, 1981; Cohen *et al.*, 1981), sardine (Murakami and Noda, 1981), capelin (Hjelmeland and Raa, 1982), catfish (Yoshinaka *et al.*, 1984, 1985a), chum salmon (Uchida *et al.*, 1984a and b), eel (Yoshinaka *et al.*, 1985c), Greenland cod (Simpson and Haard, 1984a and b), Atlantic cod (Overnell, 1973; Simpson *et al.*, 1990), and anchovy (Martinez *et al.*, 1988). A commercially available procedure for the recovery of crude trypsin or trypsin-like enzymes from cod *Gadus morhua* has been reported to be in progress (Stefansson and Steingrimsdottir, 1990).

2.2 Cold-adapted Fish Proteases

Poikilothermic organisms from temperate regions usually undergo anesthesia when encountering environmental temperatures near 0^0 C. However, certain poikilotherms remain active at such low temperatures (Simpson and Haard, 1987). The process by which a species adapts to a specific thermal environment over many generations has been described as 'evolutionary temperature compensation' (Hazel and Prosser, 1974). As a consequence of cold adaptation, digestive proteolytic enzymes from cold-adapted fish are more active at low temperatures than their counterparts derived from vertebrate and thermophilic organisms (Hultin, 1978; Haard *et al.*, 1982; Arunchalam and Haard, 1985; Simpson and Haard, 1987).

Trypsin from cod (*Gadus morhua*) was more heat labile with a relatively higher activity at low reaction temperatures than bovine trypsin, which was considered to be better suited to produce fish protein hydrolysates at low temperatures and for preventing copper-induced oxidation of milk (Simpson and Haard, 1984a). In comparison with mammalian pepsins, pepsins from cold and temperate water fish have a higher activity at low temperatures (Kitamikado and Tachino, 1960; Gildberg and Raa, 1983), and express a much lower temperature coefficient (Haard *et al.*, 1981; Brewer *et al.*, 1984). This particular temperature property of proteolytic enzymes from cold-adapted fish has been exploited in certain food processing operations (Haard, 1992).

2.2.1 Enzymic adaptability on genetic basis

Attempts have been made to elucidate the biochemical properties of cold-adapted fish proteases as well as the nature of enzymic adaptation to the cold environment. Poikilotherms adapted to cold temperatures exhibit enzymic adaptability by increasing the levels of enzymes present in the system, or by evolutionary adaptability of preexisting enzymes (Somero, 1969; Somero, 1971). The conservation of a basic physiological function, together with the achievement of cold adaptability may be the result of appropriate alterations in the primary structure of the enzyme because the primary structure determines the distribution of possible tertiary structures in certain environments. A model of mutation-adsorption has been introduced to elucidate the principle that gradual transformation of shape and function results from changes in primary structure (Conrad, 1979). The main conclusion in this model is that a subsystem is embodied in the molecule which serves as a buffer, absorbing mutation or other forms of genetic variations and expressing these as variations in features of the shape critical for function. The condition for an effective evolutionary response to selection is gradualism of function change in response to primary structure changes (Conrad, 1979).

Protein engineering has revealed that the function of enzymes, including thermostability, temperature optimum and kinetic properties could be controlled in a predictable fashion by applying site-directed mutagenesis (Ulmer, 1983). Site-directed mutagenesis permits a single or a few selected amino acid residues in a specific enzyme

to be precisely altered (Nosoh and Sekiguchi, 1991).

2.2.2 Relationships between molecular structure, stability and catalytic efficiency of enzymes

Under conditions of low temperatures, the catalytic efficiency of most cold-adapted poikilothermic enzymes is higher than that of their homologs derived from warm-temperature-adapted organisms (Low and Somero, 1976; Simpson and Haard, 1984b). The greater catalytic efficiency of enzymes from cold-adapted poikilotherms at low reaction temperatures has been attributed to their relatively more flexible structures which permit them to undergo conformational changes that favour a higher reaction rate (Somero, 1975).

The greater stability of proteins from thermophilic organisms has been attributed to the presence of greater hydrogen bonding (Koffler, 1957), more extensive hydrophobic interaction (Singleton and Amelunxen, 1973), and more pronounced disulphide linkages (Komatsu and Feeney, 1970). The folding of a polypeptide chain is driven by the strong tendency of sequestering hydrophobic side chains from solvent, which are followed or accompanied by various non-covalent interactions such as H-bonding, electrostatic and hydrophobic interactions, and van der Waals contacts, between side chains and between main chain and side chains of polypeptides and proteins (Nosoh and Seikiguchi, 1991). The three-dimensional structure is finally formed with a delicate balance between the stabilizing factors driven by these non-covalent interactions

and the destabilizing effect of thermal energy. Heat, or denaturants such as urea and guanidine hydrochloride cause cooperative unfolding of the protein (Tanford, 1968). No generalized mechanism for protein stability has yet been presented (Nosoh and Sekiguchi, 1991).

A detailed investigation on comparative properties of Greenland cod and bovine trypsins indicated that Greenland cod trypsin had a less-ordered structure than bovine trypsin (Simpson and Haard, 1984b). Furthermore, it was noted that 1) at 0.2 to 35.3^0 C, Greenland cod trypsin contained 7.3-7.8% $\alpha-$helix and 92.2-92.7% random coil, whereas bovine trypsin contained 11.5-12.0% $\alpha-$helix and 88.0-89.5% random coil; 2) Greenland cod trypsin had only 8 cysteine residues as opposed to 12 in bovine trypsin; and 3) the average hydrophobicity ($H\phi_{ave}$) of Greenland cod trypsin (0.86 kCal/residue) was less than that of bovine trypsin (1.04 kCal/residue).

Enzyme-catalyzed reactions occur only after the contact of enzyme on an appropriate position of the substrate molecule. It has been reported that substrate binding affinity of enzymes, both intracellular and extracellular proteases, is temperature-dependent (Hazel and Prosser, 1974). Hultin (1978) observed five different types of temperature dependent affinity relationships for enzymes from different sources. Digestive enzymes with temperature-dependent K_m values have been discussed and reviewed by Simpson and Haard (1987).

The different binding affinity of substrate for enzymes has been explained in terms of enzyme-substrate interactions which are stabilized by a set of weak bonds which

exhibit opposing stability characteristics as a function of temperature (Simpson and Haard, 1987). It is considered that the formation of hydrogen bonds and electrostatic interactions proceed exothermically and will therefore be more important at low temperatures. In contrast, hydrophobic bonds form endothermically and prevail at elevated temperatures. Amino acid composition and average hydrophobicity data have demonstrated that proteins from thermophilic organisms have distinctly higher hydrophobicities than their mesophilic counterparts (Bigelow, 1967; Hazel and Prosser, 1974).

2.2.3 Multiple forms of enzymes and thermal compensation

It has been a common view that thermal compensation mechanisms in poikilotherms allow their metabolic processes to continue at rates that are relatively independent of temperature. Poikilotherms adapted to cold environments exhibit enzymic adaptability by: (1) increasing the concentrations of enzymes present in the system, (2) changing the type of enzyme present in the system, and (3) evolutionary adaptability of preexisting enzymes (Somero, 1969; Hochacka and Somero, 1971; Owen and Wiggs, 1971). Poikilotherms exhibit these different thermal compensation mechanisms in response to environmental changes.

The most immediate response of a poikilotherm to a cold environment is to generate more enzymes for keeping its metabolic rates relatively constant. Quantitative differences have been observed when measuring the same fish species at different tem-

peratures (Smit, 1967; Owen and Wiggs, 1971). The increased pepsinogen quantities observed as a result of cold acclimation in these studies provide at least partial compensation for lowered temperatures. Since pepsinogen secreting cells discharge all accumulated zymogen granules upon stimulation (Hirschowitz, 1957), it is considered that the increased amount of pepsinogen would enhance digestion rates in cold acclimated fish species.

Existence of multiple forms of fish pepsinogens is also related to temperature compensation. Arunchalam and Haard (1985) observed that Arctic cod pepsin had two isozyme forms having marked differences in K_m. One isozyme form of Arctic cod pepsin had a K_m similar to that of porcine pepsin and the other one had a very high K_m with a correspondingly high V_{max}. The digestive enzymes and their corresponding genes have been modified during acclimation and mutation, from which their thermal and kinetic properties have changed and quantities have been altered to adapt to the new environment. The occurrence of multiple forms of gastric proteases which has been reported for other marine species include: sardine (Noda and Murrakami, 1981), harp seal (Shamsuzzaman and Haard, 1984), Atlantic cod (Brewer *et al.*, 1984), Greenland cod (Sqiures *et al.*, 1986a), and dogfish (Guerard and Gal, 1987).

2.3 Utilization of Proteases From Marine Fish

The use of enzymes is well established and widespread within the food industry. The properties of enzymes make them ideal tools for the manipulation of biological materials. However, of the thousands of enzymes described so far by biochemists, only a mere handful of enzymes are actually used commercially in the food industry. This is due to a wide range of reasons including unsuitable reaction conditions, instability of the enzyme during processing, or the prohibitive cost involved in obtaining large amounts of sufficiently pure enzymes.

Traditionally, enzymes have been used for various food processing applications such as meat tenderization (Fawcett and McDowell, 1987; Etherington, 1991), baking (Hamer, 1991), and cheese production (Visser, 1981; Law and Goodenough, 1991). However, enzymes can theoretically be used in almost all food processing operations in which a biochemical or chemical reaction takes place. Among the enzymes employed in the food industry, proteases are most extensively used for improving the quality, stability, and functionalities of food products. The topic on proteolytic enzymes from marine organisms and their application in food processing has been reviewed by Mohr (1980), Simpson and Haard (1987), Stefansson and Steingrimsdottir (1990), and Haard (1992). Utilization of proteases discussed here are limited to the use of isolated proteases as rennet substitutes for milk-clotting and for preparation of fish protein hydrolysates.

2.3.1 Utilization of fish proteases as rennet substitutes

The use of biocatalysts in the food industry, especially in preparation of dairy products, is one of the oldest examples of biotechnology. A typical case is the use of proteolytic enzymes in the production of cheese. One of the key steps in cheese manufacturing is the enzymatic coagulation of milk. Addition of appropriate enzymes to milk leads to partial proteolysis of κ-casein which destabilizes the casein micelle and brings about coagulation of the milk proteins to form the curd. The ideal enzyme for this conversion is rennet (mainly chymosin) which is obtained commercially from the fourth stomach (abomasum) of unweaned calves. Chymosin is a highly specific endoproteinase, which splits the κ-casein into a glycomacropeptide and para-κ-casein by selectively catalyzing the hydrolysis of the bond between phenylalanine 105 and methionine 106 (Berridge, 1951; Fox, 1969; Dalgleish, 1982). Calf rennet, consisting mainly of chymosin with a small but variable proportion of pepsin, is a relatively expensive enzyme for the commercial production of cheese.

Attempts have been made to find various substitutes for calf rennet due to a decline in the number of slaughtered calves and an increase in the demand for cheese (de Koning, 1978). Rennin substitutes obtained from microbial origin have now been accepted by the industry. However, a limitation of microbial rennet is the relatively broad specificity of the enzymes present. This is one of the major problems associated with the use of proteases for coagulating milk. Since microbial proteases are not as specific as rennet and bring about more hydrolysis, it may produce bitter

peptides or lead to destabilization of the curd gel. Another problem associated with the development of microbial rennets is temperature stability. Chymosin is a relatively unstable enzyme that loses most of its activity during completion of its function in milk-clotting. The enzyme from microbial sources such as *Mucor miehei*, however, retains its activity after clotting and is still active at the maturation stages of cheese-making which may produce bitter off-flavours. Attempts have been made to clone chymosin into *Escherichia coli* and *Saccharomyces cerevisiae* and the enzyme has been secreted in an active form only from the latter (Chaplin and Bucke, 1990).

Chicken pepsin has also been employed as a rennet substitute (Emmons et al., 1976; Stanley and Emmons, 1977; Gordin and Rosenthal, 1978). However, cheddar cheese prepared with this enzyme may acquire intense off-flavours. Use of gastric proteases from marine species as a rennet substitute has also been examined. Fish stomach contains substantial amounts of proteases active at less acid conditions than mammalian pepsins. More recent research has revealed that many fish species secrete at least two pepsins with different pH optima (Noda and Murakami, 1981; Gildberg and Raa, 1983; Martinez and Olsen, 1986). Some gastric proteases from marine species have characteristics similar to that of chymosin in clotting milk at higher pHs (Shamsuzzaman and Haard, 1983; Brewer *et al.*, 1984). It has been reported that dogfish pepsin II can clot milk effectively even at pH 6.8 (Guerard and Garl, 1987).

A crude preparation of gastric proteases from harp seal (*Phoca groenlandica*) was found to coagulate milk over a wider pH range than porcine pepsin (Shamsuzzaman

and Haard, 1983). Simple gastric mucosal extracts from young harp seal contained four zymogens of acidic proteases A, B, C, and D. Protease A was a chymosin-like rather than a pepsin-like enzyme (Shamsuzzaman and Haard, 1984). Cod pepsin, which exhibits gastricsin-like properties, is well suitable for the cold renneting of milk because of its relatively low temperature coefficient (Brewer *et al.*, 1984). However, the use of cod pepsin immobilized on Sepharose (Haard 1986) and seal gastric protease immobilized on chitin (Han and Shahidi, 1993) to perform cold renneting is not feasible since the high molecular activity of enzymes at low temperatures appear to be lost as a consequence of immobilization. It is considered that immobilizations in any way will greatly inhibit the structural flexibility of gastric proteases thus reduce their activities. To date, there has been no successful attempt to use immobilized proteases for milk-clotting in cheese making. The search for a low cost rennet substitute is continuing.

Very recently, using protein engineering technology, a new chymosin substitute named 'chymogen' has become commercially available in Denmark (Brusgaard, 1992; Halfhide, 1992). Compared to chymosin from other sources, the engineered enzyme produced from *Aspergillus niger* has advantages including vegetarian improved, secure supply with a pure chymosin, and comparable performance in cheese making to that of calf rennet (Halfhide, 1992).

2.3.2 Recovery of Fish Proteins by Enzymic Hydrolysis

There has been much interest in developing new products from protein resources available from the sea. The effective utilization of underutilized species of fish and fish processing waste will have an immediate impact on the world's animal feed and human food supplies. Therefore, more emphasis should be placed on the 'total utilization' of present resources.

Enzymatic hydrolysis of food proteins is a widely used method for improving their solubility and solubility-dependent functional properties (Adler-Nissen, 1976, 1986; Han, 1989; Shahidi *et al.*, 1993). However, use of enzymes in fish processing is minimal compared to other fields of food processing. Preparation of fish protein hydrolysates using proteolytic enzymes is the first case to be considered.

Fish protein concentrates may be produced by various processes to afford products with different properties and commercial values for a variety of applications. Most of the existing processing methods can be classified into chemical (acidic or alkaline extraction), physico-chemical (aqueous extraction or organic solvent extraction), and biological (enzymatic and microbial hydrolyis). However, severe conditions of alkaline treatment of fish proteins may in extreme cases cause changes that may be undesirable from a nutritional point of view (Franzen and Kinsella, 1976; Feeney, 1977; Sikorski and Naczk, 1981; Robbins and Ballew, 1982). The loss of functional properties when employing organic solvents and high lipid residues in the aqueous extraction procedures are considered practical difficulties in organic solvent extraction.

Application of enzyme technology in fish processing has attracted considerable interest for converting wastes and underutilized species of fish into protein concentrates. The treatment of fish or fish processing wastes with proteolytic enzymes represents an interesting alternative to mechanical methods for separating flesh from bones using deboning machines (Mohr, 1977) and chemical methods for the preparation of fish protein concentrates.

The production of fish protein hydrolysates using proteolytic enzymes has been investigated since the 1960's. In 1961, the term fish protein concentrates (FPC) was adopted by the Food and Drug Administration (FDA) to replace the earlier name of "fish flour" (Shenouda and Pigott, 1975). Since then, considerable research on preparation of fish protein hydrolysates using different proteolytic enzymes has been reported. One disadvantage of protein hydrolysates relates to their bitterness caused by the presence of bitter peptides. In the late 1970's, Alcalase was found to be useful for producing bland protein hydrolysates from deboned cod offal and fresh herring (Lalasidis *et al.*, 1978; Lalasidis and Sjoberg, 1978), sardine (Sugiyama *et al.*, 1991) as well as other fish species(Hevia *et al.*, 1976; Hale and Bauersfield, 1978; Thankamma *et al.*, 1979).

There are two types of processes for the production of fish protein hydrolysates namely autolytic process and accelerated hydrolysis (Mohr, 1977). The autolytic process depends on the action of the digestive enzymes of the fish itself which lasts from a few days to several months, depending on the process. There are no enzyme

costs involved for autolytic process and it is simple to operate. However, prolonged digestion normally results in problems related to the functional properties of protein hydrolysates and the time of the processing cycle.

Accelerated hydrolysis using commercial proteases offers far better possibilities than autolysis does, since it allows control of product properties. The relative activities of more than twenty commercially available proteolytic enzymes have been determined using washed and freeze-dried fish proteins (Hale, 1969). Enzymatic hydrolysis of fish proteins using enzymes of animal, plant, or microbial origins has been thoroughly studied (Hale, 1969; Cheftel *et al.* 1971; Wessels and Atkinson, 1973; Hale, 1974; Mackie, 1974; Hevia *et al.* 1976; Hale and Bauersfield, 1978; Sugiyama *et al.*, 1991) and excellent reviews have been written on this topic (Finch, 1971; Mohr, 1977; Sikorski and Naczk, 1981). However, accelerated hydrolysis is generally a more complex process, and the cost of enzymes may influence the economical aspects and commercial viability of the process.

Although the accelerated hydrolysis of fish proteins using commercial proteases has been studied, the use of proteolytic enzymes from the digestive tracts of marine species has not received adequate attention. Based on the consideration that proteolytic enzymes present in the digestive tracts of marine species are able to digest their feed (mainly small fish), the use of proteolytic enzymes isolated from cod and seal digestive tracts for preparation of fish protein hydrolysates from capelin was investigated in this study.

Chapter 3

Materials and Methods

3.1 Materials

3.1.1 Sample collection

Cod viscera

Atlantic cod (*Gadus morhua*) were obtained fresh from Conception Bay, Newfoundland. The viscera were removed, split, cleaned, and the liver and fat were discarded. The cleaned viscera were vacuum packaged and stored at - 60^0 C until use.

Male and spent capelin

Male and spent capelin (*Mallotus vilosus*) were collected at Bay Bulls, Newfoundland in June. The whole fish was washed, vacuum packaged, and stored at -60^0 C until use.

3.1.2 Chemicals

Casein was obtained from BDH Chemicals (Toronto, ON). Polyacrylic acid was obtained from Aldrich Chemical Company (Milwaukee, WI). DEAE Sephadex A-50 was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Calf chymosin (E.C.3.4.23.4), bovine serum albumin (BSA), α-benzoyl-DL-arginine p-nitroanilide (BAPNA), haemoglobin (washed and dialyzed), tris(hydroxymethyl)- aminomethane, benzoyl-L-arginine ethyl ester (BAEE), N-benzoyl-L-tyrosin ethyl ester (BTEE), pepstatin, porcine pepsin, and other chemicals were purchased from Sigma Chemical Company (St. Louis, MO).

3.1.3 Equipment

Major equipment used in this study were: Sorvall superspeed RC2-B automatic refrigerated centrifuge (Ivan Sorvall Inc., Norwalk, U S A); Fisher accument pH meter, Model 805 MP with gel-filled polymer-body combination electrode (Fisher Scientific Company, Ottawa, ON); HP 8452A diode-array UV/Visible spectrophotometer with HP 89531A UV/Vis operating software and GP-100 Graphics printer (Hewlett-Packard Company, San Francisco, CA); Gel electrophoresis apparatus GE-2/4; and Electrophoresis constant power supply ECPS 2000/300 (Pharmacia Fine Chemicals, Uppsala, Sweden).

3.2 Experimental Procedures

3.2.1 Co-extraction of acid and alkaline proteases from cod viscera

Figure 3.1 summarizes the procedure used for the co-extraction of proteolytic enzymes from digestive tracts of Atlantic cod (*Gadus morhua*). Frozen viscera were thawed at 4^0 C overnight and minced with ice-cold distilled water at a 1:4 ratio (w/v) for 3 min. A 1.0 N solution of HCl was added to the mince to adjust the pH to 6.0, and the mixture was then blended for 50 min at room temperature. After blending, the slurry was centrifuged at 13,200 x g for 20 min at 4^0 C, and the aqueous phase was separated to isolate acid and alkaline proteases.

A 1.0% solution of polyacrylic acid was added to the aqueous extract to give a 0.05 to 0.15% solution, similar to the proccedure of Reece (1988). The mixture was allowed to stand for 30 min, centrifuged at 12,100 x g for 15 min at 4^0 C and the supernatant was decanted. Crude acidic proteases could be obtained by a simple ultrafiltration of the supernatant to a specific concentration. In this study, the supernatant was made 63% saturated with solid ammonium sulphate at 4^0 C and centrifuged at 13,200 x g for 30 min. The precipitate was dissolved in a minimum amount of 0.1 M acetate buffer at pH 6.0, and dialyzed against the same buffer overnight to obtain crude acid proteases.

The polyacrylic acid precipitate was redissolved in 50 mM Tris buffer at pH 7.8.

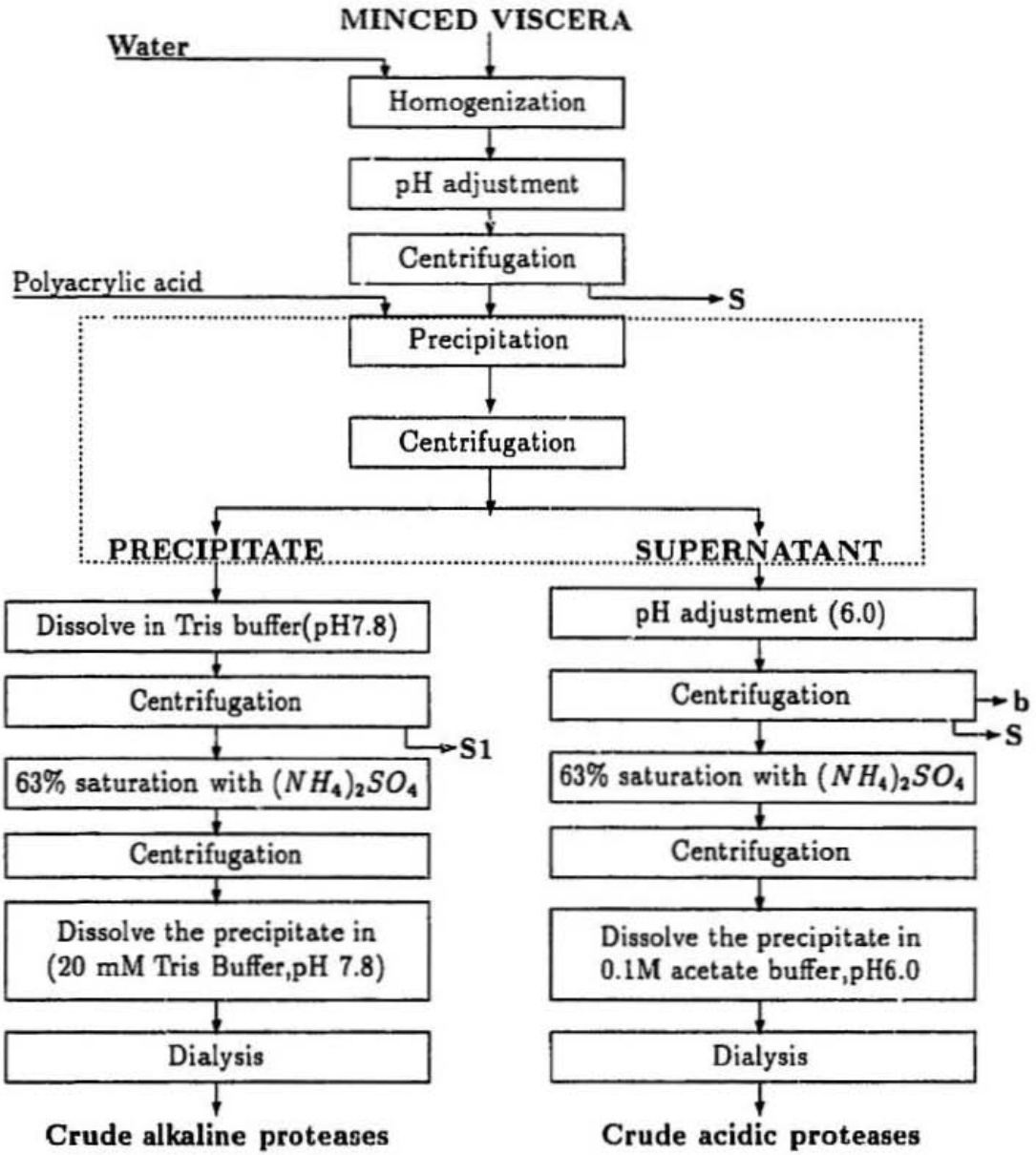


Figure 3.1: **Flow sheet for co-extraction of acid and alkaline proteases from Atlantic cod viscera**[a].

[a] Abreviations are: **S**, sludge; **S1**, sludge that can be further treated for recovering precipitate (polyacrylic acid); **b**, The supernatant can also be concentrated by ultrafiltration or other means to obtain crude acid proteases. The dashbox includes key steps for whole procedure of co-extraction procedure.

The precipitate formed was removed by centrifugation and the supernatant was made 63% saturated with solid ammonium sulphate at 4^0 C. The precipitate formed was dissolved in a minimum of 20 mM Tris buffer, pH 8.2 containing 5 mM calcium chloride, and dialized overnight against the same solution to obtain crude alkaline protease.

3.2.2 Purification of proteolytic enzymes

Gel permeation chromatography

Crude enzyme preparations, prepared as described in Section 3.2.1 (30 mL of crude acid or alkaline proteases), were subjected to gel permeation chromatography in a 3.2×100 cm Sephadex G-75 column. For acid proteases, the column was equilibrated and eluted with 0.1 M acetate buffer, pH 6.0. For alkaline proteases, the column was equilibrated and eluted with 50 mM Tris buffer, pH 7.8. The proteolytic activity of the fractions was measured as described later (Section 3.3.3). Fractions containing proteolytic activity were collected for further use. A portion of the partially purified enzymes was concentrated by freeze drying or ultrafiltration.

Ion-exchange chromatography

DEAE-Sephadex A-50 was packed into a 2.5 × 40 cm column for the purification of acid proteases. The column was equilibrated with 0.1 M acetate buffer, pH 6.0. Enzyme collected from Sephadex G-75 gel chromatography was applied to the column

which was then eluted with the same buffer until the A_{280} of the fractions and their proteolytic activity approached zero. The column was then eluted sequentially with 0.2, 0.4, and 0.8 M sodium chloride in 0.1 M acetate buffer at pH 6.0, 5.5, and 5.0, respectively. Aliquots from alternate fractions were assayed for proteolytic activity. Fractions containing proteolytic activity were collected for characterization.

3.2.3 Production of fish protein hydrolysates

Production of capelin protein hydrolysates (CPH) was carried out as summarized in Figure 3.2. The ground frozen fish samples were thawed, mixed with an equal weight of water and homogenized in a Waring blender for approximately 2 min. The pH value of the suspension was adjusted to the optimum value of the protease under investigation (pH 3.0 for crude cod acid proteases and seal gastric proteases; pH 8.5 for cod alkaline proteases and Alcalase; pH 7.0 for Neutrase.). Hydrolysis was carried out for 60 to 240 min at room temperature with pH-stat control. The enzymes were inactivated by heating to 85^0 C for 5 min. The sludge was removed by suction filtration. The pH of the resultant filtrate was adjusted to 5.5 with 4 N NaOH or HCl. The hydrolysate was dehydrated by freeze-drying. The ratio of total Kjeldahl nitrogen in the final product to that originally present in the ground fish was calculated as the yield of protein.

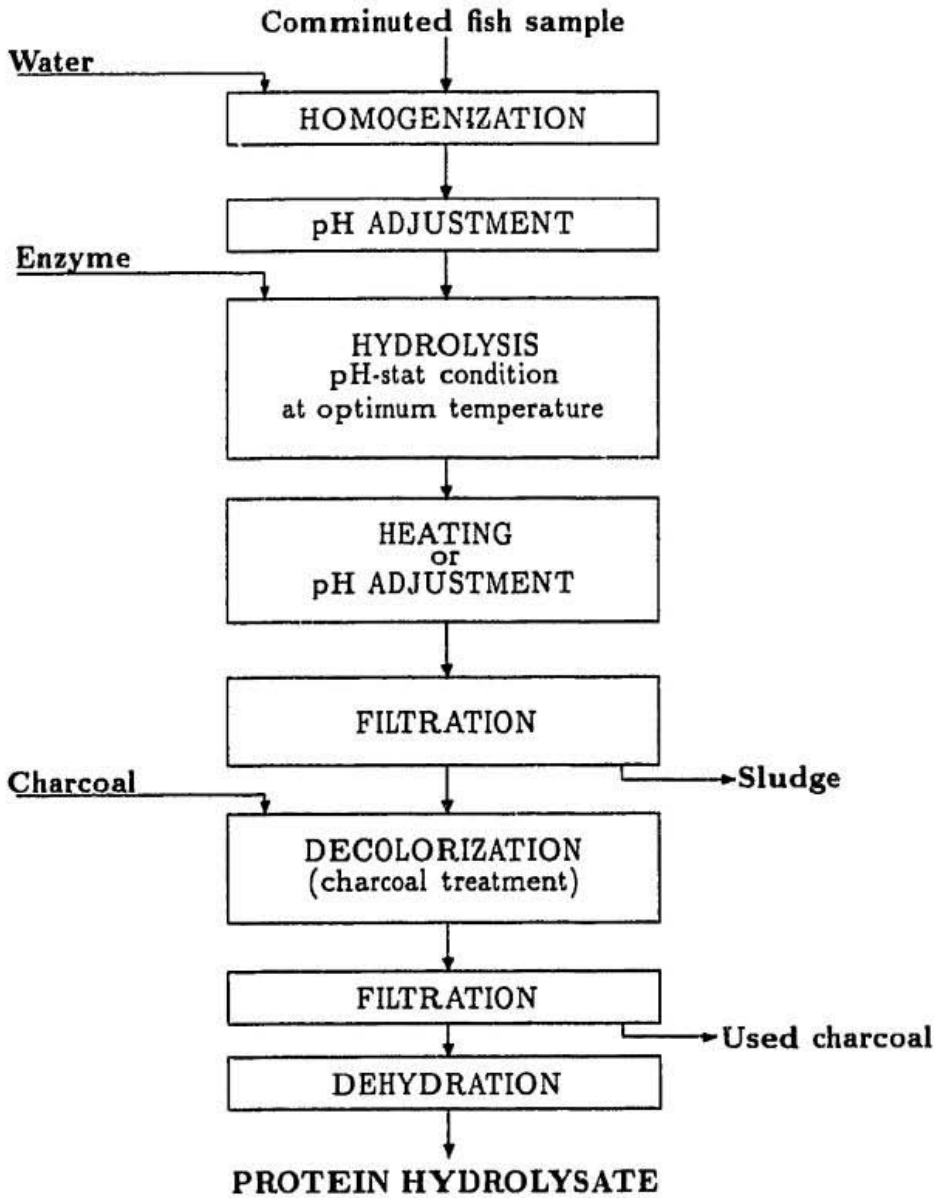


Figure 3.2: **Flow sheet for preparation of protein hydrolysate.**

3.3 Analytical Methods

3.3.1 Protein determination

Protein content during extraction and purification of enzymes was determined according to the method of Lowry *et al* (1951) using bovine serum albumin as a standard (Appendix A). The reaction mixture was composed of 1.0 mL protein solution and 4.0 mL working solution. The working solution was prepared by adding 1 mL 1% sodium tartrate and 0.5% $CuSO_4$ to 49 mL 2% Na_2CO_3 in 0.1 M NaOH. The absorbance at 660 nm was read after the addition of Folin reagent for 30 min at ambient temperature. Protein content during column chromatography was measured and expressed as absorbance at 280 nm using a 0.1 M acetate buffer, pH 3.0, as a blank (Appendix B). Total crude protein (N $\times$6.25) content was determined by the Kjeldahl method (AOAC, 1990).

3.3.2 Determination of lipid content

The total lipid in fish samples and protein hydrolysates was extracted according to the method of Bligh and Dyer (1959) and Woyewoda *et al.* (1986). Ten grams of solid sample (50 mL for liquid samples) was homogenized in chloroform/methanol/water (1:1:1, v/v/v). The mixture was held overnight for separation. The chloroform layer was separated and the solvent was removed by evaporation (evaporated at room temperature for two hours, and then heated at 95^0 C for 30 min).

3.3.3 Overall proteolytic activity

Proteolytic activity using bovine haemoglobin substrate

Proteolytic activity of acid proteases was determined using bovine haemoglobin substrate as described by Anson (1938) and Ryle (1970) with minor modifications as described below. The reaction mixture was composed of 0.2 mL enzyme solution, 1.0 mL 0.2 M acetate buffer, pH 3.0, and 0.6 mL 1.5% (w/v) bovine haemoglobin solution. The reaction was terminated by addition of 2.5 mL solution of 5.0% (w/v) trichloroacetic acid (TCA) after 10 min of reaction at 25^0 C. Blanks were obtained by adding TCA to the enzyme prior to the addition of substrate. After 30 min standing at room temperature, the solution was filtered through a Whatman No. 3 filter paper and the absorbance of the TCA-soluble material was read at 280 nm. A linear relationship existed at $A < 0.45$. One haemoglobin unit (HU) was defined as the amount of protease which increased the absorbance at 280 nm of TCA-soluble material by 0.001 unit per min under the above experimental conditions.

Proteolytic activity using a casein substrate

Proteolytic activity using a casein substrate was determined under the conditions described above for haemoglobin substrate except for employing different buffers (pH 3.0 for acid proteases, and pH 8.2 for alkaline proteases). One casein unit (CU) was defined as the amount of protease which increased the absorbance at 280 nm of TCA-soluble material by 0.001 unit per min under the above experimental conditions.

3.3.4 Assay of pepsin activity using a synthetic substrate

A synthetic substrate, acetyl-L-phenylalanyl-L-diiodotyrosine (APDT) was used for measurement of pepsin activity according to the method described by Ryle (1970). A 0.5 M HCl solution was added to the enzyme preparation to bring the mixture to pH 2.0. The acidified enzyme solution was incubated at room temperature for 10 min before performing the measurement of enzyme activity. The reaction mixture contained 0.5 mL of enzyme solution, 0.25 mL of HCl and 0.25 mL of APDT solution. After 20 min reaction at 25^0 C, 1.0 mL of ninhydrin reagent, prepared according to the method described by Ryle (1970), was added to the test mixture. All test tubes were placed in a boiling water bath for exactly 15 min and were then cooled under a stream of cold water. The reaction mixtures were diluted with 5 mL 60% ethanol and tubes were then shaken thoroughly. The absorbance of solutions at 570 nm was read against a water blank. One APDT unit was defined as the quantity of the enzyme required to increase the absorbance at 570 nm by 0.001 units per min under the experimental conditions.

3.3.5 Trypsin esterase activity

Trypsin esterase activity of alkaline proteases was measured using benzoyl-L-arginine ethyl ester (BAEE) as a substrate according to the method described by Rick (1965). To a 3.0 mL cuvette, 2.8 mL of 1 mM BAEE in 50 mM Tris buffer, pH 8.0, containing 20 mM $CaCl_2$ was added. A 0.2 mL aliquot of properly diluted enzyme solution was

then added at zero time and mixed immediately. The absorbance at 254 nm was measured continuously for a period of 10 min. One unit of BAEE activity was defined as the amount of trypsin or trypsin-like enzymes which increased the absorbance at 254 nm by 0.001 per min. Specific activity was expressed as units of enzymatic activity per mg enzyme.

3.3.6 Chymotrypsin esterase activity

Chymotrypsin esterase activity was measured by the method of Walsh (1970) with minor modifications. N-Benzoyl-L-tyrosine ethyl ester (BTEE) was used as substrate. To a 3.0 mL cuvette, 1.5 mL of 0.1 M Tris buffer, pH 8.2, containing 50 mM $CaCl_2$, and 1.4 mL of 1 mM BTEE in 50% methanol was added. 0.1 mL of enzyme solution was then added at zero time and mixed immediately. The absorbance at 256 nm was measured at 1 min intervals. One BTEE unit (BU) was defined as the amount of chymotrypsin-like enzyme which increased the absorbance at 256 nm by 0.001 unit per min.

3.3.7 Trypsin amidase activity

The amidase activity of trypsin was measured by the method of Arnon (1970) with minor modification, using α-benzoyl-DL-arginine p-nitroanilide (BAPNA) as a substrate. One gram of BAPNA was dissolved in 50 mL dimethyl sulphoxide (DMSO) and diluted to 500 mL with water. The reaction mixture contained 0.2 mL of par-

tially purified cod alkaline protease solution, 2.0 mL of 0.1 M Tris-HCl buffer, pH 8.2 containing 5 mM $CaCl_2$, and 1.0 mL BAPNA solution. The mixture was incubated for 40 min at 25° C. The reaction was stopped by the addition of 1.0 mL 30% (v/v) acetic acid solution and the amount of p-nitroanilide released was measured at 410 nm against a blank (the blank was prepared with the addition of 1.0 M 30% acetic acid solution before that of BAPNA solution). One BAPNA unit was defined as the amount of enzyme which increased the absorbance at 410 nm by 0.001 per min under the above assay conditions.

3.3.8 Milk-clotting activity

Milk-clotting activity was assayed by the procedure of Manji *et al* (1988) with minor modifications as described below. Enzyme solution (0.2 mL) at concentrations of 0.1 to 1.0 mg/mL was added to 30 mL of reconstituted milk consisting of 12% Carnation instant skim milk powder in 0.01 M $CaCl_2$ solution at an appropriate pH (from 5.50 to 6.70, adjusted by HCl or NaOH). The mixture was then gently and constantly swirled until the first appearance of a white precipitate at the bottom of the beaker. The time for flecks to appear was taken as the milk-clotting time (MCT). One milk-clotting unit (MCU) was defined as the amount of enzyme that clotted 10 mL of the reconstituted milk in 100 s at 25° C (Squires *et al.*, 1986a; Manji *et al.*, 1988).

3.3.9 Milk-clotting unit to proteolytic unit ratio

The ratio of milk-clotting unit to proteolytic unit (MCU/PU) was used to identify suitable proteases as milk-clotting agents in cheese-making (Puhan and Irvine, 1973; de Koning *et al.*, 1978). Proteases which were successful in cheese-making had a relatively high milk-clotting to proteolytic activity ratio (Visser, 1981).

3.3.10 pH optima and stability

The effect of pH on the hydrolysis rate of haemoglobin and casein was determined using various buffers during the hydrolysis period. The procedures for measuring the rate of proteolytic hydrolysis were the same as those described above (Section 3.3.3). To determine pH stability, the proteases were incubated in various buffers at different pH conditions (pH 1.4 to 8.5 for acid protease and 3.0 to 11.0 for alkaline protease) for 2 h at 25^0 C, before the residual activities were determined using the methods described in Section 3.3.3. The universal buffers employed were prepared according to the method described by Teorell and Stenhagen (1938).

3.3.11 Temperature optimum and thermal stability

The effect of temperature on the rate of hydrolysis of casein and haemoglobin was determined at various temperatures varying from 5 to 80^0 C. To determine thermostability, proteases were incubated at various temperatures for different periods (from 2 to 60 min) before residual activities were determined using standard methods

described in Section 3.3.3.

3.3.12 Inhibition of enzyme activity

The enzyme to be tested was pre-incubated with each inhibitor in a total volume of 0.40 mL buffer (0.1 M acetate pH 3.0 for acid proteases, 0.05 M Tris buffer for alkaline proteases) for 20 to 60 min at 20^0 C prior to the addition of substrate solution for assay of the remaining activity. Soya bean trypsin inhibitor (SBTI) was dissolved in enough deionized water at concentrations of 0.025, 0.050, and 0.100 mg/ml. The alkaline protease solutions were added separately to equal volumes of the SBTI solutions and incubated in an ice bath for 30 min. After incubation, residual trypsin activity was determined using BAPNA as substrate under conditions described above.

Pepstatin was dissolved in DMSO and diluted to 1.0×10^{-5} M as stock pepstatin solution. Acid proteases were incubated separately with different concentrations of pepstatin solution for 30 min at room temperature. After incubation, the residual proteolytic activity was determined using a 1.5% solution of haemoglobin as substrate under standard assay conditions as described in Section 3.3.3.

3.3.13 Estimation of relative molecular mass, M_r

The relative molecular mass (M_r) of the Atlantic cod gastric proteases was estimated by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS), and by gel permeation chromatography using a 1.5 × 100 cm column packed

with Sephadex G-75.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed at pH 8.3 using a 10% separating gel and a 3.75% stacking gel in a discontinuous buffer system, according to the method of Laemmli (1970). The gel was stained by 0.1% (w/v) Coomassie Brilliant Blue R250. Protein standards used for determining the relationship between mobility on gels and M_r, are presented in Figure 4.10.

The Sephadex G-75 gel filtration chromatography was performed according to the method of Whitaker (1963) and Andrews (1965). The column was equilibrated and eluted with 0.1 M acetate buffer of pH 5.5. The proteins were column chromatographed and the elution volume of each protein determined. The column was calibrated by using bovine albumin, egg albumin, chicken ovalbumin, soybean trypsin inhibitor, equine myoglobin, and α-lactalbumin. The M_r of a protein was determined from a plot of logarithm of M_r versus the distribution coefficient (K_d). The value of K_d for a given solute is defined by the relationship (Price and Stevens, 1989):

$$K_d = \frac{V_e - V_0}{V_i - V_0}$$

where V_e is the elution volume of the molecule estimated, V_i is the elution volume of a small molecule (vitamin B_{12}) which is totally included by the column, V_0 is the elution volume of a molecule (dextran blue with a M_r of 2×10^6) which is completely excluded by the column.

3.4 Experimental Design and Optimization of Co-extraction Conditions

Optimum solutions can be obtained by various approaches. The classical approach to optimization is to test one variable at a time or alternatively modify the variables by so-called "back-and forth" method. These approaches require a large number of experiments and often do not consider the interactions among the variables.

More sophisticated techniques applied in conjunction with response surface methodology (RSM), are canonical, ridge regression, steepest ascent or descent methods (Myers, 1971). The principles and functions of RSM were first developed by Box and Wilson (1951). RSM has been modified and expanded into a powerful tool for emperical model development and optimization (Khuri and Cornell, 1987). RSM can be defined as a statistical method that uses quantitative data from appropriate experimental designs to determine and simultaneously solve multivariate equations (Giovanni, 1983). These equations can be graphically represented as response surfaces which can be used to describe how the test variables affect the response, to determine the interrelationships among the test variables, and to describe the combined effect of all test variables on the response.

Computer graphics-assisted optimization approach is considered to be more advanced in the investigation and optimization of complex systems. In the graphical approach, the predictive models are used to creat contour surfaces or contour lines

within the experimental region by the computer. The contour surfaces and contour plots are created on three- or two-coordinate diagrams, respectively. These surfaces and plots present information for two factors and one of more responses, and are resonably accurate (depending upon the representational accuracy of the model) within the experimental region.

The regions of optimum response(s) are judged by visual inspection of the contour surfaces. This method reduces the possibilities of "unrealistic"solutions, since only the regions within the experimental space are examined, and allows simultaneous optimization of several competing responses by simple surperimposition.

3.4.1 Experimental design

Rotatable experimental design was performed to optimize co-extraction conditions which allow both acid and alkaline proteases to be recovered at optimum levels. Samples for the co-extraction were treated under different conditions and the results were evaluated according to response-surface-methodology (RSM) (Floros and Chinnan, 1988). A rotatable experimental design of three factors (pH value; concentration of polyacrylic acid; and temperature) and three levels with three replicates at the centre point was used (Box and Behnken, 1960). The basic theoretical aspects, the fundamental assumptions and the experimental implications of RSM have been discussed elsewhere (Myers, 1971; Thompson, 1982).

The results collected from three-factor-three-level rotatable experiment were sub-

jected to regression analysis (least squares) to generate regression coefficients for model fitting and further optimization. A model (or mathematical function) was established for describing the relationships between process variables and output responses in the procedure of co-extraction of acid and alkaline proteases from cod viscera. The co-extraction process was assumed to be a system of three input factors (pH values in precipitating solution, pH; polyacrylic acid concentration in the solution, C; and operation temperature of solution, T) and two output responses (acid proteases in supernatant, AE; alkaline proteases in precipitate, BE). It was further assumed that two mathematical functions, $(f_k, k = 1, 2)$, exist between each of the response variable (Y_k) and the three independent process variables.

$$Y_k = f_k(pH, C, T) \tag{3.1}$$

Due to the unknown form of the function f_k, second order polynomial equations were used to approximate the functions of Equation 3.1.

$$Y_k = \beta_{k0} + \sum_{i=1}^{3} \beta_{ki} X_i + \sum_{i=1}^{3} \beta_{kii} X_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{kij} X_i X_j \tag{3.2}$$

where $\beta_{k0}, \beta_{ki}, \beta_{kii}, \beta_{kij}$ are constant coefficients and X_i, X_j are the coded independent processing variables, linearly related to pH, C, and T.

A total of 15 runs were performed in random order (overall randomization) to estimate the 10 constants. The constants (regression coefficients) were calculated using RSREG (Response Surface REGression) procedure of the Statistical Analysis

Table 3.1: **Process variables and their levels in rotatable experiment design[a].**

Input process variables	Symbol		Levels		
	Coded	Uncoded	-1	0	1
pH value	X_1	pH	3.40	4.30	5.20
PAA[b] concentration (%)	X_2	C	0.05	0.10	0.15
Temperature (^oC)	X_3	T	5	15	25

[a]Variables refer to processing conditions during co-extraction as described inside the dashbox in flow sheet (Fig.3.1).
[b] Polyacrylic acid.

Chapter 4

Results and Discussion

4.1 Optimization of Processing Conditions for Co-extraction of Proteolytic Enzymes From Viscera of Cod (*Gadus morhua*)

Numerous reports on the extraction, purification and characterization of fish proteases have appeared over the last two decades. Most of these have focused on methods and procedures to obtain purified enzymes and their subsequent characterization and classification, regardless of the number of steps or complications involved. However, in this study emphasis was primarily on designing a simple and effective isolation procedure for maximum recovery of proteases from fish viscera. It was also of interest to adapt the process for large-scale commercial application.

As it can be seen from the flowsheet (Fig.3.1), the key for isolating alkaline proteases from the bulk solution was the conditions for forming polyacrylic acid (PAA)-enzyme complex from which alkaline proteases were precipitated from the bulk solution. The precipitation of PAA-enzyme complex may be affected by many processing conditions including pH, concentration of PAA, temperature, ionic strength, etc. In order to approach optimum conditions for forming PAA-enzyme complex, a response surface methodology (RSM) described in Section 3.4 was employed and the three-variable-three-level rotatable experimental design (Table 3.1) was performed to collect data for the optimization.

4.1.1 Model Fitting

Results of the three-variable-three-level rotatable experimental design are summarized in Table 4.1. The recovery of enzymes varied considerably under different experimental conditions. The experimental data were then subjected to least-squares regression analysis to generate regression coefficients and to determine the significant variables/interactions. The RSREG procedure of the Statistical Analysis System (SAS Institute Inc., 1990) was used to fit the experimental results to second order polynomial of Eq. 3.2, to perform the regression analysis, and to plot three-dimensional response surfaces and contour maps.

Table 4.1: **Experimental data for the three-factor-three-level response surface analysis for model co-extraction system.**

Treatment	FACTORS[a]			RESPONSES[b]	
	X_1 pH	X_2 C (%)	X_3 T (^{0} C)	Acid Protease in solution	Alkaline Protase in precipitate
1	1	1	0	0.5819	0.0984
2	1	-1	0	0.9421	0.1124
3	-1	1	0	0.4411	0.1027
4	-1	-1	0	0.6521	0.2473
5	1	0	1	0.7563	0.1275
6	1	0	-1	0.7624	0.1342
7	-1	0	1	0.4782	0.1781
8	-1	0	-1	0.4939	0.1965
9	0	1	1	0.4273	0.0733
10	0	1	-1	0.4414	0.0886
11	0	-1	1	0.6835	0.1831
12	0	-1	-1	0.6954	0.1618
13	0	0	0	0.6097	0.1687
14	0	0	0	0.5928	0.1722
15	0	0	0	0.5940	0.1795

[a] See Table 3.1 for detailed information on factors and their levels used in this experiment.

[b] Responses were expressed with proteolytic activities (absorbance of TCA-soluble protein hydrolysates at 280 nm) measured under standard conditions as described in Materials and Methods (Section 3.3.3). A final concentration of 0.5% haemoglobin or casein was used as substrate for acid and alkaline proteases, respectively. A 0.1000 absorbance unit for acid proteases corresponded to an extraction of 205 HU/g cod viscera, and for alkaline proteases corresponded to that of 83 CU/g viscera. Results are mean values of duplicate determinations.

The regression coefficients (β_k) obtained by fitting the experimental data (Table 4.1) to the second-order response surface models (Eq. 3.2) are presented in Table 4.2. Two models describing the relationship between enzyme (acid and alkaline proteases) recovery and processing conditions (pH, PAA concentration, and temperature) were obtained. The models developed were tested by analysis of system variance (response variables for model system). Results indicate that the developed models are highly significant with satisfactory correlation coefficients (R^2) (Table 4.3). By performing the F-test for the test of lack-of-fit, the models developed had no significant lack of fit at both 5% and 1% levels (Table 4.3), suggesting that the models adequately represented true relationships between process variables and response variance (Draper and Smith, 1981; Floros and Chinnan, 1988; SAS, 1990).

4.1.2 Overall effect of process variables

The overall effect of process variables (pH, PAA concentration, and temperature) on the output responses were further analyzed and the results are shown in Table 4.3. Values of pH and PAA concentration were shown to be important factors which significantly (at 1% probability level) affected the recovery of acid and alkaline proteases. Temperature (T) was not an important factor for the co-extraction process as it did not significantly affect any of the two responses (Table 4.3).

Table 4.2: **Regression coefficients for second order polynomials in model co-extraction system.**

Regression coefficient[a] β_k	Acid protease (AE) $k = 1$	Alkaline protease (BE) $k = 2$
β_{k0}	1.1928**	0.5463**
β_{k1}	-0.4026*	-0.1079
β_{k2}	1.0906	-1.0774
β_{k3}	0.0086	0.0044
β_{k11}	0.0718**	-0.0005
β_{k22}	-0.8289*	0.7261 **
β_{k33}	-1.0667	-13.123**
β_{k12}	0.0003	0.0003
β_{k13}	-0.0011	-0.0183
β_{k23}	-0.0003*	-0.0001*

[a] Coefficients of Eq. 3.2. Numbers 1, 2, and 3 refer to pH, concentration of PAA, and temperature, respectively. See Appendix III for detailed information of calculated results.

** Significant at 1% probability level.

* Significant at 5% probability level.

Table 4.3: **Analysis of variance for response variables and overall effect of process variables.**

SOURCE	Degrees of freedom	Sum of squares: Acid protease (AE)	Sum of squares: Alkaline protease (BE)
Model	9	0.2896**	0.0317**
Linear	3	0.2659**	0.0226**
Quadratic	3	0.0181**	0.0045**
Crossproduct	3	0.0056	0.0046**
Residual	5	0.002321	0.000343
Lack of fit	3	0.002144	0.000282
Pure error	2	0.000178	0.000061
Process variables			
pH values	4	0.1375**	0.0123**
PAA concentration(%)	4	0.1518**	0.0232**
Temperature (^{0} C)	4	0.0046	0.0011
Correlation coefficient (R^2)		0.9920	0.9893

** Significant at 1% probability level.

* Significant at 5% probability level.

Based on the predictive models of recovery of acid and alkaline proteases (coefficients presented in Table 4.2), computer-generated three-dimensional response surfaces were obtained, as shown in Fig. 4.1. All surfaces were generated for a constant temperature (T = 25^0 C), since temperature had no significant effect on the process. A temperature of 25^0 C was chosen since it approximated the ambient temperature.

The effect of pH on co-extraction process was somewhat complicated. The isoelectric points, pI, of cod (*Gadus morhua*) gastric proteases are approximately 3.8 to 4.7 (Reece,1988). For Greenland cod gastric proteases, the pI values of three zymogens were reported to be > 7.5, 5.8–5.9, 4.9–5.4, respectively (Squires *et al.*, 1986a). It can be seen that at the same concentration of PAA, a higher precipitation pH value would keep more acid proteases in the bulk solution (Fig. 4.1.A). A decrease in precipitation pH resulted in a decrease in the recovery of acid proteases since under acidic conditions autodigestion of the enzymes is promoted. Since fish gastric proteases are most stable in pH range of 4.5 to 6.0 (Table 2.1), neutral conditions for extraction and purification of gastric proteases is recommended. A more acidic precipitation condition may promote the formation of protein-PAA complex and thus reducing the recovery of acid protease due to precipitation or co-precipitation.

In contrast, alkaline proteases would precipitate when co-extraction pH is more acidic. Theoretically, too low pH values during co-extraction may inactivate alkaline proteases since they are commonly unstable under acid conditions. However, results of this experiment indicated that alkaline proteases were not easily inactivated dur-

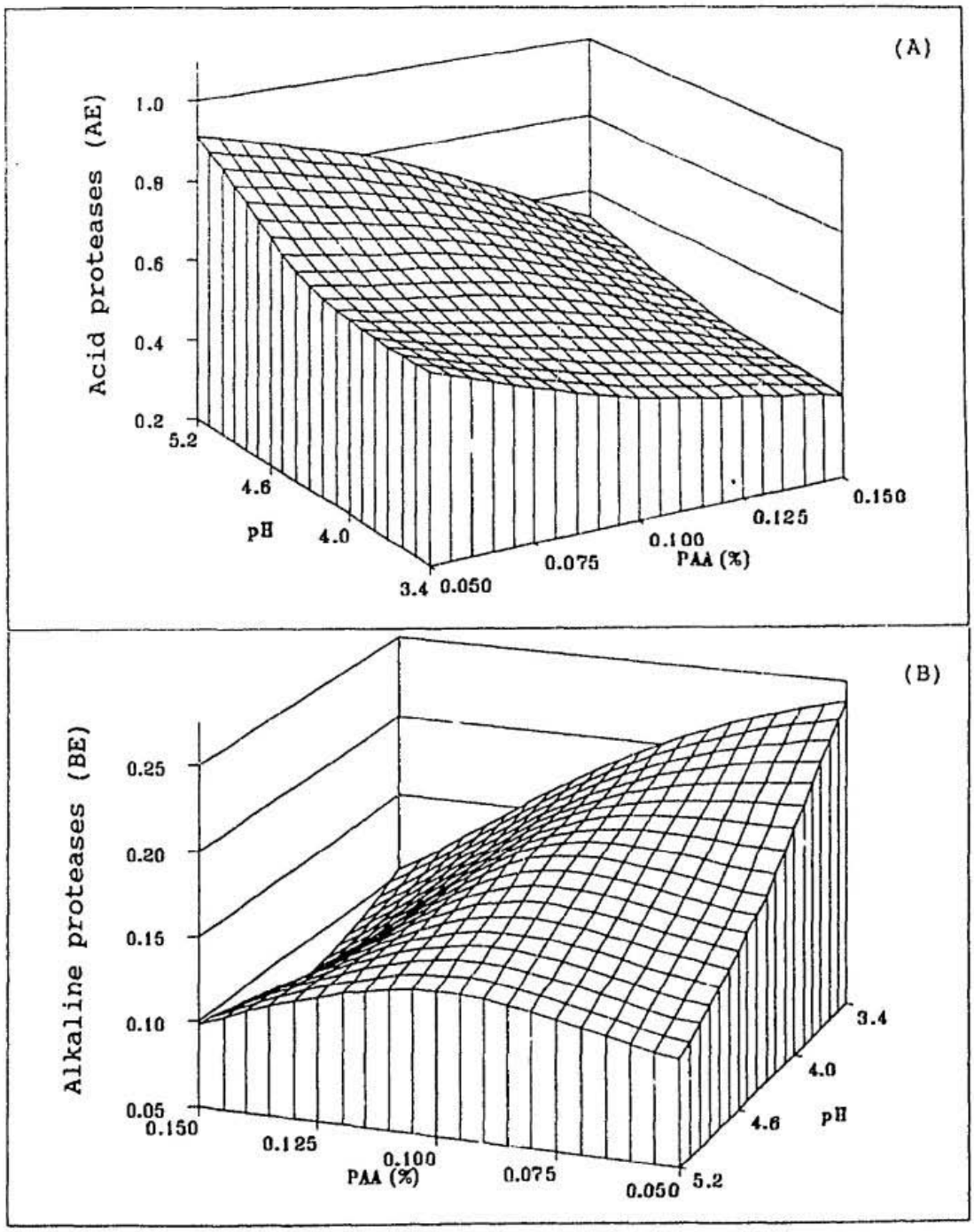

(A)
Acid proteases (AE)
1.0
0.8
0.6
0.4
0.2
5.2
4.6
pH
4.0
3.4
0.050
0.075
0.100
PAA (%)
0.125
0.150
(B)
Alkaline proteases (BE)
0.25
0.20
0.15
0.10
0.05
0.150
0.125
0.100
PAA (%)
0.075
0.050
5.2
4.6
pH
4.0
3.4

Figure 4.1: **Response surface of enzyme recovery at different pH and polyacrylic acid (PAA) concentration.**
(A), Recovery of acid proteases (AE) in supernatant; (B), Recovery of alkaline proteases (BE) in precipitate. Recovery of proteases was represented as proteolytic activities, absorbance of TCA-soluble material at 280 nm, using haemoglobin as a substrate for acid protease and casein for alkaline protease measured under standard conditions as described in Materials and Methods (Section 3.3.3). Data from Table 4.2.

ing the co-extraction procedure as a result of formation of enzyme-PAA complex. A decrease in PAA concentration resulted in an increase in acid protease recovery (Fig. 4.1). This is not unexpected since all enzymes were retained in bulk solution as no precipitation occurred. However, an increase in PAA concentration did not result in an increase in the recovery of alkaline proteases. A higher recovery of alkaline proteases was found when less than 0.1% PAA concentration at pH values lower than 4.0 were employed.

4.1.3 Location of optimum processing conditions

Using the procedure described by Myers (1971), the stationary points may locate outside the range of three-dimensional space. Results from computer fitted models show that the stationary points are saddle points (with no unique minimum or maximum, see Appendix III) for both of the described models calculated from the experimental data. For this reason, graphical multi-response optimization techniques (Floros and Chinnan, 1988) and computer generated contour plots (Fig. 4.2) were used for locating optimum processing conditions for the co-extraction of acid and alkaline proteases.

Since the optimal responses for both AE and BE would not locate exactly in the same area as described by RSREG analysis, constraints were set to optimize the recovery of both acid and alkaline proteases from the co-extraction process. Proteolytic activity of acid proteases in supernatant was set at $> 350 HU/mL$ (corresponding

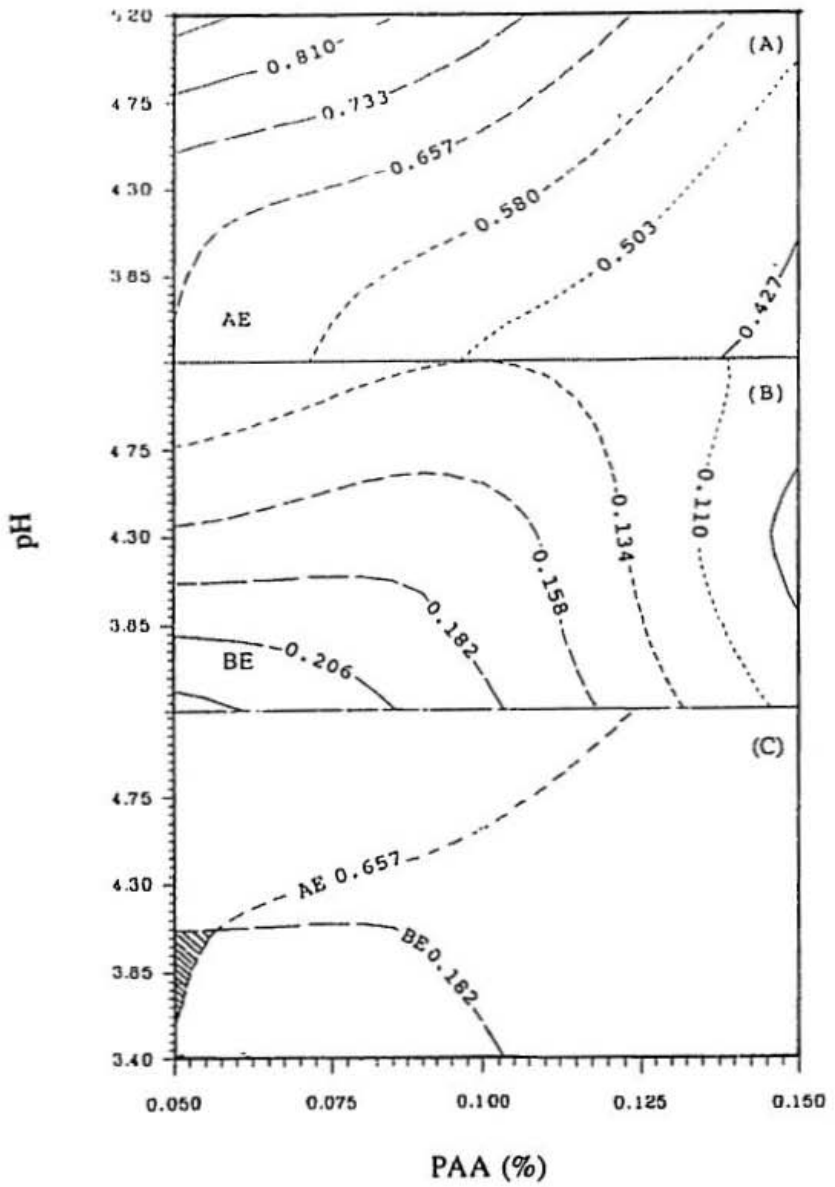

(A)
0.810
0.733
0.657
0.580
0.503
0.427
AE
(B)
0.134
0.110
0.158
0.182
0.206
BE
(C)
AE 0.657
BE 0.182
4.75
4.30
3.85
3.40
0.050
0.075
0.100
0.125
0.150
pH
PAA (%)

Figure 4.2: **Contour plot of influence of pH and polyacrylic acid (PAA) concentration on the co-extraction of acid and alkaline proteases.**
(A). Contour plot for recovery of acid proteases; (B). Contour plot for recovery of alkaline proteases; (C). Superimposed contour plot of constrained region. (AE, Activity of acid proteases; BE, Activity of alkaline proteases.)

to an absorbance value of 0.700 at 280 nm), and of alkaline protease was set at $> 90 CU/mL$ (corresponding to an absorbance value of 0.180 at 280 nm).

Selected optimum conditions for the process may be based on the region which could satisfy the stated constraints (Floros and Chinnan, 1988). Superimposing the individual contour plots for the response variables, acid protease recovery (AE) and alkaline protease recovery (BE), results in the identification of a region which may satisfy all constraints as shown in Fig. 4.2.C. The shaded area in the lower left of the superimposed plot covers all possible variances in which all constraints are satisfied. Adequacy of the model equations for predicting optimum response values was tested using a set of predicted optimum processing conditions (pH 3.85, PAA concentration 0.055%, temperature 25^0 C). This set of conditions was determined to be optimum by the RSM optimization procedure. Since the auto-activation of pepsinogen occurs at pH 4.0-4.6 (Foltmann, 1981), no residual pepsinogen is expected to remain in the enzyme preparation from the co-extraction system. The predicted and experimental values for acid and alkaline proteases at these conditions are given in Table 4.4. The experimental and predicted values were reasonably close. Thus, the model developed is a good predictor of the two responses at optimum constraints.

Table 4.4: **Predicted levels of process variables and results of confirmed experiments.**

ITEMS	Variables	Optimum range
Predicted process conditions[a]	PAA concentration (%, w/v)	0.050 - 0.060
	pH	3.60 - 4.10
	Temperature (^{o} C)	15
Predicted output response[b]	Acid protease in supernatant	> 0.657
	Alkaline proteases in precipitate	> 0.182
Experimental values of process conditions	PAA concentration (%, w/v)	0.055
	pH	3.85
	Temperature (^{o} C)	25
Results of experimentation[b]	Acid proteases in supernatant	0.838 ±0.086
	Alkaline protease in precipitate	0.214 ±0.031

[a] PAA, Polyacrylic acid.

[b] Optimal ranges are represented as proteolytic activities, absorbance of TCA-soluble protein hydrolysates at 280 nm, using haemoglobin as a substrate for acid proteases and casein for alkaline proteases measured under standard conditions as described in Materials and Methods. A 0.100 absorbance unit for acid proteases corresponded to an extraction of 205 HU/g cod viscera and for alkaline proteases corresponded to that of 83 CU/g viscera.

4.2 Co-extraction of Crude Acid and Alkaline Proteases and Their Properties

4.2.1 Co-extraction of crude acid and alkaline proteases

The result of co-extraction and purification of proteolytic enzymes from cod viscera, using optimized process conditions described above, are summarized in Table 4.5. Both acid and alkaline proteases were recovered effectively by the co-extraction procedure established. One kilogram of cod viscera yielded approximately 10 g of crude acid proteases with 52% recovery of proteolytic activity and 2.2 g of crude alkaline proteases with 30% recovery of proteolytic activity. The total proteolytic units include both zymogens, unactivated proteases, and activated proteases due to extraction conditions. Results from this study indicated that the extraction procedure developed may have potential adaptability for industrial applications.

Further purification allowed better recovery of acid proteolytic activity as shown in Table 4.5. One kilogram of cod viscera yielded approximately 1.7 g of gastric proteases with 160% recovery in proteolytic activity compared to the crude homogenate. The total proteolytic activity was increased up to about four times after DEAE Sephadex ion-exchange chromatography. This revealed that some dramatic changes may have occurred during the ion-exchange chromatography (see Section 4.3).

Table 4.5: **Co-extration and purification scheme for acid and alkaline proteases from Atlantic cod viscera[a].**

STEPS[b]	Units (U/mL)[d]	Protein[c] (mg/mL)	Specific Activity (U/mg)[d]	Total Volume (mL)	Purify (Fold)	Total protein (mg)	Total unit[d] (×1000)	Recovery (%)
Crude P								
A	386	5.86	65.9	930	1.0	5449.8	360.00	100
B	114.5	5.86	19.5	930	1.0	5449.8	106.49	100
Acid P								
A1	419	5.30	79.1	820	1.20	4346.0	343.58	95.7
A2	1171.6	12.9	90.8	160	1.38	2064.0	187.46	52.2
A3	329.5	3.12	105.6	460	1.60	1435.2	151.57	42.2
A4-A	177.8	0.198	898	658	13.6	130.3	116.99	32.6
A4-B	410.8	0.184	2233	976	33.9	179.6	400.94	111.9
A4-C	194.6	0.090	2162	344	32.8	31.0	66.94	18.6
Alkal. P								
B1	107.3	2.16	49.5	330	2.54	712.8	35.41	33.3
B2	528.9	7.52	70.3	60	3.61	451.2	31.73	29.9
B3	103.0	1.31	78.6	300	4.00	393.0	30.90	29.0

[a] Based on 200 g cod viscera sample under optimized co-extraction conditions.
[b] Abreviations in this column refer to: P, protease; A, acid protease; B, alkaline protease; A1, supernatant after polyacrylic acid (PAA) precipitation; A2, sodium sulphate fraction; A3, collection of Sephadex G-75 gel filtration; A4, collections of DEAE-Sephadex chromatography, A4-A, B, and C are three different acid proteases obtained from the purification step; B1, PAA removed alkaline protease; B2, 63% sodium sulphate fraction; B3, collections of Sephadex G-75 gel filtration. Refer to Fig. 3.1 for detailed information.
[c] Protein content was measured at 280 nm using bovine serum albumin as a standard.
[d] Presented unit of proteolytic activity using haemoglobin and casein as substrate for acid and alkaline proteases, respectively. All assays for proteolytic activity were performed under standard conditions as described in Materials and Methods (Section 3.3.3).
[e] refers to purification fold.
[f] refers to recovery of proteolytic activity.

4.2.2 Properties of crude cod acid and alkaline proteases

pH Profile of crude enzyme preparations

The pH profiles of crude acid and alkaline proteases are shown in Figure 4.3. The optimum pH for the hydrolysis of haemoglobin by crude acid proteases was in the range of 3.0 to 3.5, and for hydrolysis of casein by crude alkaline proteases was close to 9.0 (Fig. 4.3.A). It has been reported that many fish species secrete at least two pepsins with different pH optima (Noda and Murakami, 1981; Gildberg and Raa, 1983; Shamsuzzaman and Haard, 1984; Martinez and Olsen, 1986; Squires *et al.*, 1986a), and that different pH optima cover one broad pH optimum at about 3.0 for a haemoglobin substrate (Donta and van Vunakis, 1970; Sanchez-Chiang and Ponce, 1981).

The crude acid proteases isolated were stable in acid, but unstable at alkaline pH (Fig 4.3.B) as observed for most fish pepsins. The stability of the acid proteases was maximum at pH range of 4.0 to 6.0. A considerable loss of proteolytic activity occurred when the enzyme preparations were incubated at their optimum reaction pH. The activity of the enzyme was completely lost at pH > 8.0.

In contrast to the acid proteases, alkaline proteases were unstable at acidic pH and stable at alkaline pH (Fig. 4.3.B). The maximum stability of crude alkaline proteases was close to pH 9.0. Unlike trypsins from higher vertebrates such as bovine or porcine, trypsins from invertebrates and lower vertebrates are generally unstable at acid pH but stable at alkaline pH (Camacho *et al.*, 1970; Bundy and Gustafson, 1973;

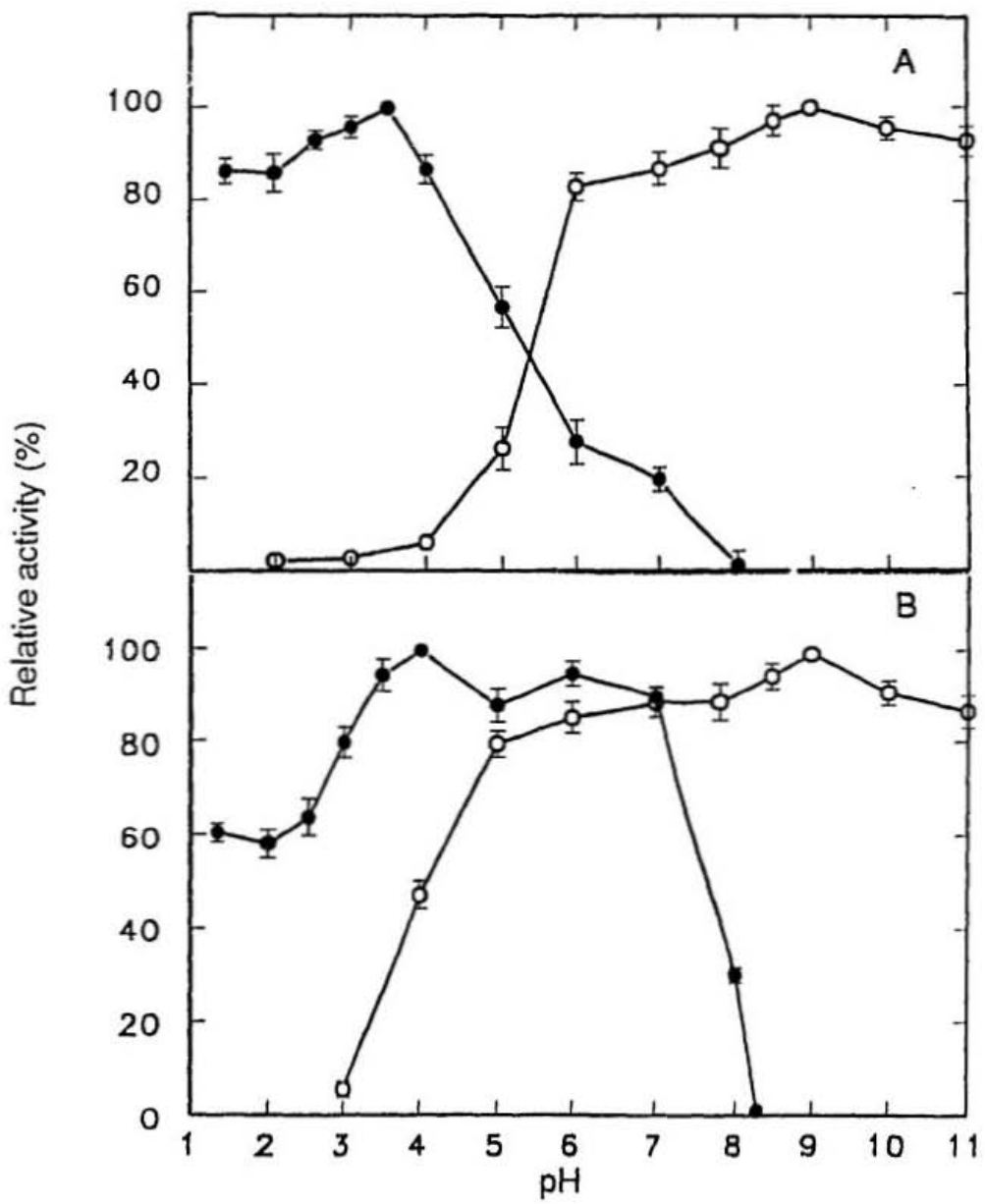

A
B
Relative activity (%)
100
80
60
40
20
0
1
2
3
4
5
6
7
8
9
10
11
pH

Figure 4.3. pH profiles of crude cod acid (●) and alkaline (○) proteases. A, Optimal reaction pH for crude enzyme preparations. B, Influence of pH on enzyme stability. The proteolytic activities were measured using haemoglobin as a substrate for acid proteases and casein for alkaline proteases under standard assay conditions as described in Section 3.3.3. The enzyme concentration (determined by Lowry assay) was 2.65 mg/mL of acid proteases in 0.1 M acetate buffer, pH 3.0; 2.16 mg/mL of alkaline proteases in 50 mM Tris buffer, pH 7.8. The highest activity measured under the experimental conditions was expressed as 100% relative activity (RA). 100% RA corresponded to 87 HU/mg (acid proteases) and 56 CU/mg (alkaline proteases) in Figure A, 78 HU/mg (acid proteases) and 52 CU/mg (alkaline proteases) in Figure B.

Hjelmeland and Raa, 1982; Simpson and Haard, 1984a; Simpson *et al.*, 1990). The reason for lower stability of fish trypsins at acid pH as compared with trypsins from vertebrate is thought to be due to a higher ratio of potential acidic to basic residues for fish trypsins as compared with vertebrate trypsins (Simpson *et al.*, 1990).

Further characterization of co-extracted proteases is described in Section 4.3. However, pH profiles of the crude enzyme preparations confirmed that both acid and alkaline proteases were isolated from the co-extraction procedure. The alkaline proteases were recovered upon PAA precipitation from which the acid proteases remained in the bulk solution and was partially purified (1.20 fold). According to UV spectrum (Fig. 4.4), nucleotides and nucleic acids were not precipitated from the bulk solution as noted from the ratio of absorbances at 280/260 nm (0.839 for acid proteases and 0.989 for alkaline proteases) which increased significantly for alkaline proteases after PAA precipitation. Results given in Table 4.5 indicate that the alkaline proteases were purified 2.54 fold after this operation.

Temperature optima and thermal stability

Both acid and alkaline proteases obtained from the co-extraction process possessed around 90% relative activities at ambient temperatures (Fig. 4.5). The temperature optimum for the hydrolysis of haemoglobin by crude acid proteases was close to 42^{0} C, approximately 20^{0} C below the temperature optimum of mammalian pepsins.

Different temperature optima have been reported by authors using different buffer systems: Haard (1986) found that the maximum initial rate of haemoglobin diges-

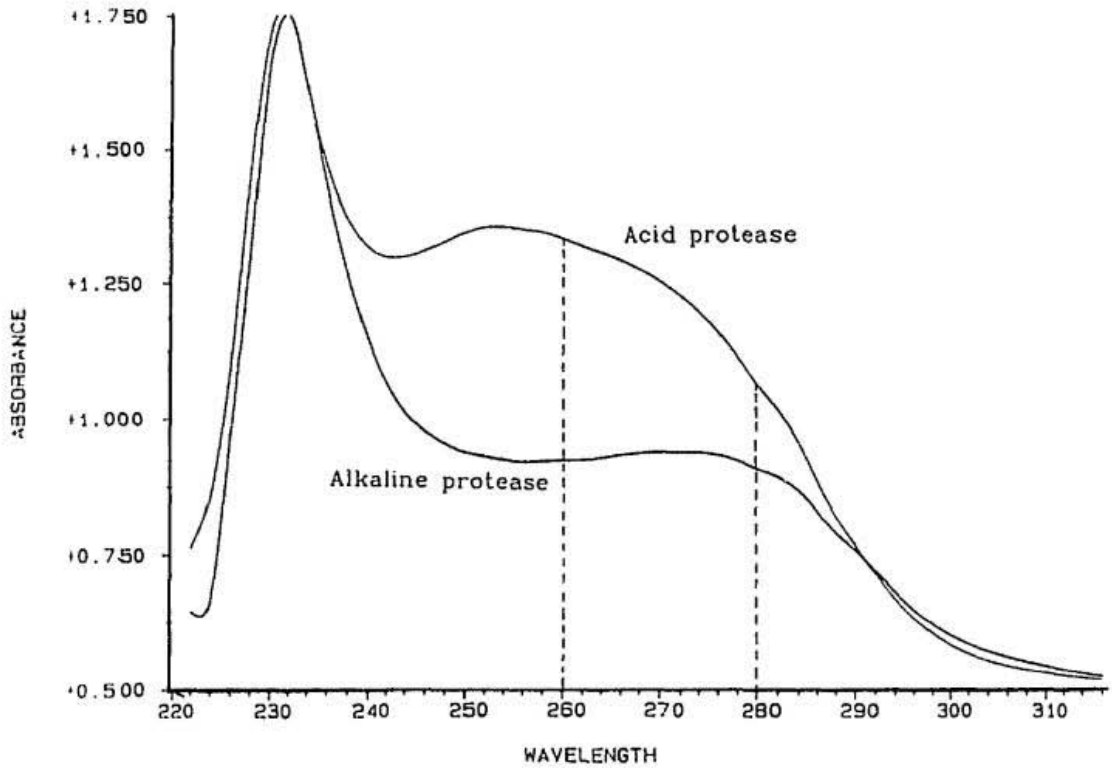


Figure 4.4: **UV spectrum of acid and alkaline proteases after co-extraction.**

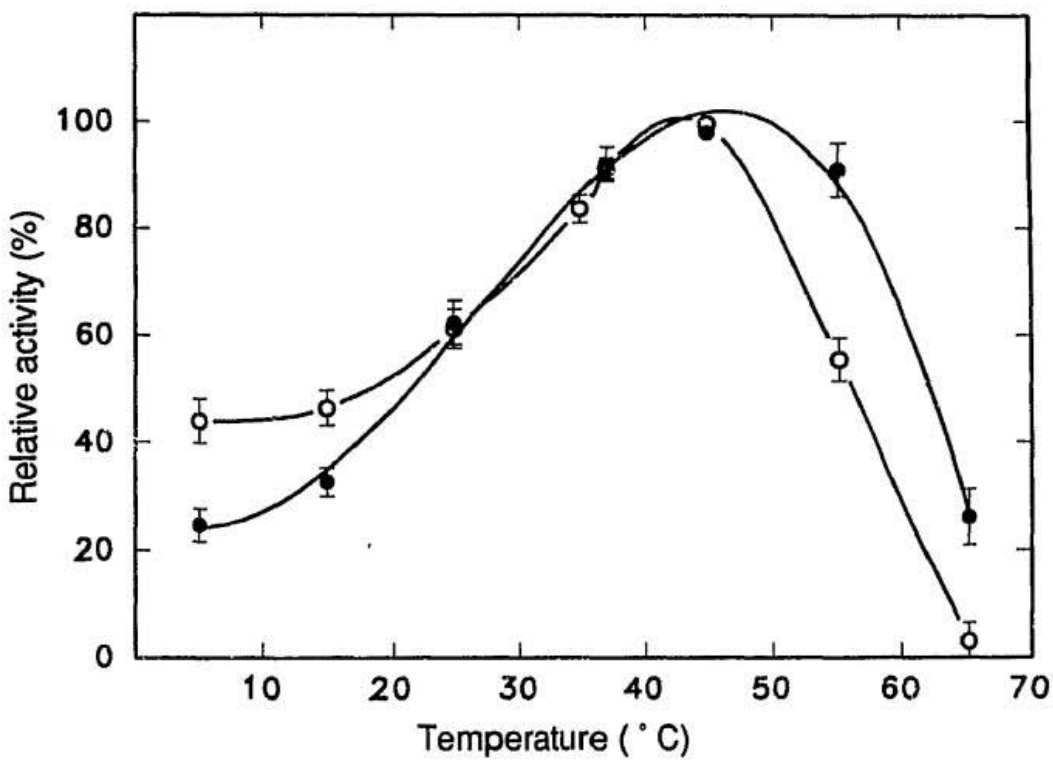

Relative activity (%)
100
80
60
40
20
0
10
20
30
40
50
60
70
Temperature (° C)

Figure 4.5. **Optimal reaction temperatures of crude cod acid (•) and alkaline (o) proteases.**

The enzyme preparations (2.65 mg/mL of acid proteases in 0.1 M acetate buffer, pH 3.0; or 2.16 mg/mL of alkaline proteases in 50 mM Tris buffer) were incubated with substrates at 5 – 60^0 C. All other conditions for the measurement of residual activity were the same as those described in Section 3.3.3. Haemoglobin was used as a substrate for acid proteases and casein for alkaline protease. 100% relative activity corresponded to 132 HU/mg for acid proteases and 84 CU/mg for alkaline proteases. Results are mean values of triplicate determinations.

tion by Atlantic cod pepsin at pH 1.9 was 35^0 C. Martinez and Olsen (1986) found temperature optima of 35 and 40^0 C for Atlantic cod pepsin I and II at an incubation pH of 3.0 for 60 min. Under similar conditions, a temperature optimum of 42^0 C was determined in a crude preparation of cod pepsin (Bjelland *et al.*, 1988). Experiments with American smelt pepsins found that the temperature optimum was increased from about 32^0 C at pH 1.9 to about 37^0 C at pH 3.0 (Haard *et al.*, 1981). It is considered that the temperature optimum will be highest if the incubation pH is in the middle of the pH stability range.

The temperature optimum for the hydrolysis of casein was close to 43^0 C by crude alkaline proteases (Fig 4.5). Bjelland *et al.* (1988) obtained similar results for trypsins from Atlantic cod.

The thermal stability of proteolytic enzymes is closely related to their temperature optimum, above which rapid inactivation occurred. The crude acid proteases lost approximately 10% of their original activity when incubated at 37^0 C for 60 min (Fig. 4.6). It has been reported that during a long period of incubation, heat denaturation of Atlantic cod pepsins began at 30^0 C (Haard *et al.*, 1982).

The crude alkaline proteases were unstable above their optimum reaction temperature. The enzyme lost approximately 20% of their original activity when incubated at 40^0 C for 60 min (Fig 4.6). According to the results obtained by Simpson and Haard (1984b), Greenland cod trypsin began to lose its activity at 40^0 C due to heat inhibition. Atlantic cod trypsin had a similar thermal stability and was unstable

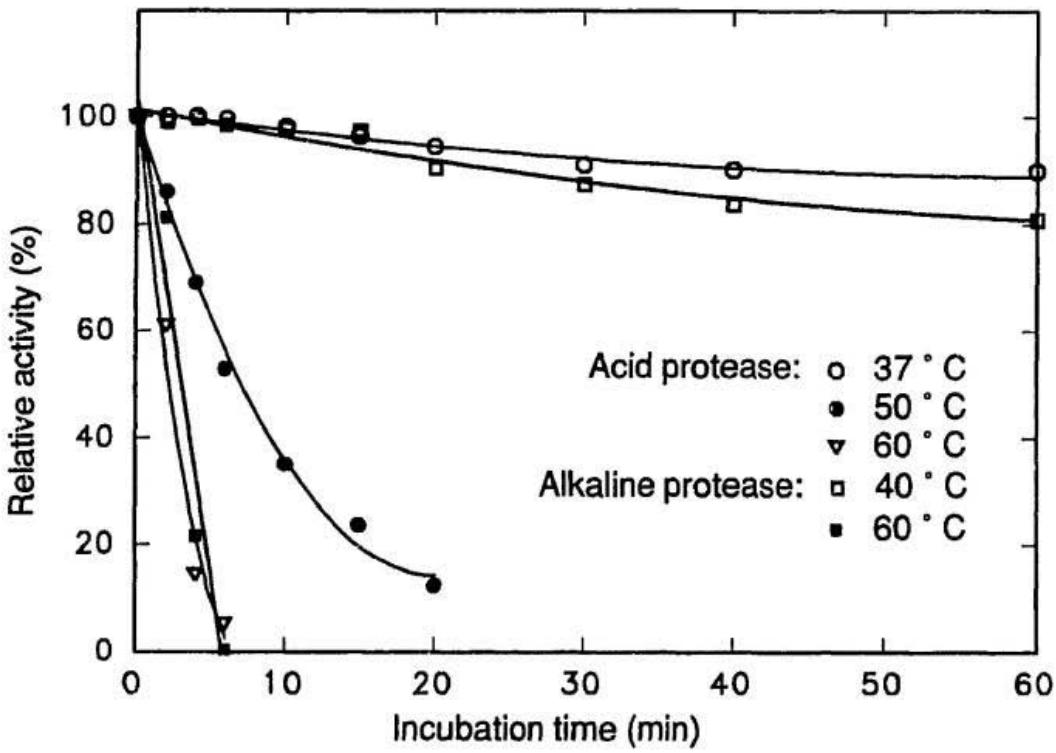

Relative activity (%)
100
80
60
40
20
0
0
10
20
30
40
50
60
Incubation time (min)
Acid protease:
37 ° C
50 ° C
60 ° C
Alkaline protease:
40 ° C
60 ° C

Figure 4.6: **Influence of temperature on the stability of crude enzymes.** Both crude acid proteases (2.65 mg/mL in 0.1 M acetate buffer, pH 3.0) and alkaline proteases (2.16 mg/mL in 50 mM Tris buffer, pH 7.8) were incubated at different temperatures for different periods before their residual activity was measured (Enzyme concentrations were determined by Lowry assay). The residual proteolytic activity was measured under standard assay conditions as described in Section 3.3.3, using haemoglobin as a substrate for acid proteases and casein for alkaline proteases. 100% relative activity corresponded to 42 HU/mg for acid proteases and 49 CU/mg for alkaline proteases.

above 40^0 C (Simpson *et al.*, 1990). There is little difference in thermal stability of trypsins from Greenland and Atlantic cod.

High catalytic activity at low temperatures reflects considerable molecular flexibility and hence, low temperature optimum as well as low thermostability. Results obtained by Simpson *et al.* (1990) showed that Atlantic cod trypsin has only 10 one-half cystine residues and differed in this respect from both Greenland cod trypsin (8 one-half cystine residues) and bovine trypsin (12 one-half cystine residues). This indicates that Atlantic cod trypsin can potentially have a maximum of 5 disulphide bonds. Thus, the contribution of disulphide linkage to the mechanical properties of Atlantic cod trypsin may be less than that of bovine trypsin but higher than that of Greenland cod trypsin.

Ease of heat denaturation and high enzymic activity at low temperatures are unique properties of cold adapted fish proteases. Atlantic cod proteases, both gastric- and trypsin-like enzymes, showed these properties, indicating their possible advantage in certain food processing. Utilization of these crude enzyme preparations in milk-clotting for cheese making and production of capelin protein hydrolysates are described in Section 4.5.

4.3 Partial Purification and Characterization of Acid Proteases From Atlantic Cod

The purification and characterization of fish gastric proteases have been reported by many authors over the last two decades. Squires *et al.* (1986b) have classified the three isolated gastric proteases from Greenland cod. The Greenland cod gastric proteases represent primitive less-differentiated forms of gastric proteases than the pepsin, gastricsin, and chymosin from mammals. It is, therefore, difficult to strictly classify the cod proteases as pepsins, gastricsins, or chymosins since they exhibit a blend of characteristics from these three groups (Squires *et al.*, 1986b). For characterizing the enzymes obtained from the co-extraction, further purification of enzymes was carried out. However, unlike other enzymes, gastric proteases are very difficult to purify since they undergo the autodigestion as mentioned in Section 2.1.1. Therefore, no attempt was made in this study to obtain highly purified proteases.

Several guidelines were set to compare the characteristics of different enzymes obtained. Two designation systems (cod proteases 1, 2, and 3, designated by Squires *et al.* (1986a); fish pepsins I and II as described in Section 2.1.1) are used as a comparison in the following discussion. No attempt has been made in this study to classify the gastric proteases isolated into the major groups of gastric proteases characterized for mammals.

4.3.1 Multiple forms of gastric proteases

The crude cod acid proteases co-extracted were further purified using G-75 gel filtration and DEAE-Sephadex ion-exchange chromatography as described in Section 3.2.2. Fractions (48 to 80) from G-75 gel filtration which possessed proteolytic activity showed only one major peak at 280 nm (Fig. 4.7), indicating that acid proteases from cod viscera had similar molecular weights. Two large protein peaks which did not show proteolytic activity were removed after gel filtration.

The fractions collected from G-75 gel filtration were applied to DEAE-Sephadex ion-exchange column (2.5 × 40 cm), and three peaks having acid protease activity were eluted from this column (Fig. 4.8). The three peaks with proteolytic activity were designated as acid proteases A, B, and C, and their characteristics are summarized in Table 4.6. Similar results have been reported by Squires *et al.* (1986a) who had isolated three gastric proteases from Greenland cod using different extraction and purification procedures (Extracted at pH 7.4 Tris buffer; purified by repeated Sephadex G-100 exclusion chromatography and polybuffer exchange chromatofocusing. In order of decreasing isoelectric points, the three enzymes were designated as Greenland cod proteases 1, 2, and 3, respectively). Among the three acid proteases isolated, acid protease B was dominant. It has been reported that pepsin II is dominant in Atlantic cod (Martinez and Olsen, 1986). Based on their relative amounts, the acid protease B belongs to fish pepsin II and acid proteases A and C belong to fish pepsin I groups.

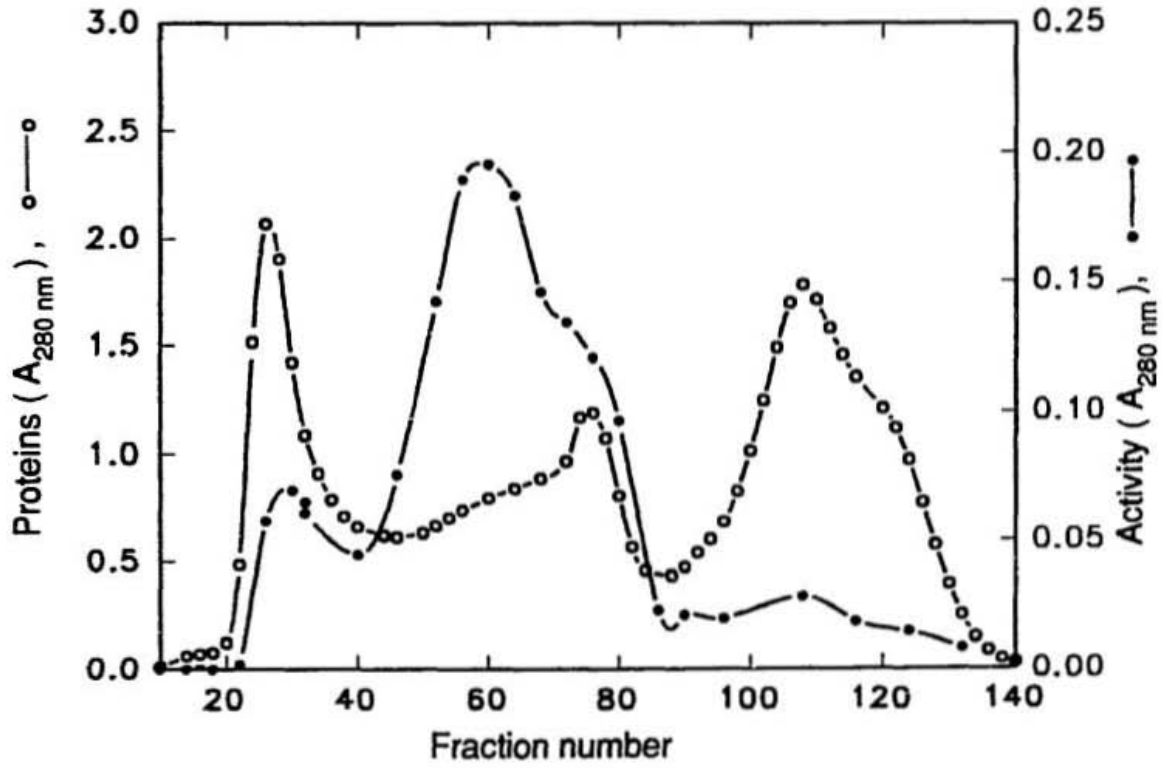

Proteins ($A_{280\ nm}$), o—o
Activity ($A_{280\ nm}$), •—•
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0.25
0.20
0.15
0.10
0.05
0.00
20
40
60
80
100
120
140
Fraction number

Figure 4.7: **Sephadex G-75 gel filtration chromatography of crude acid proteases from Atlantic cod viscera.**
(The column was equilibrated and eluted with 0.1 M acetate buffer, pH 6.0. The proteolytic activity was measured using haemoglobin as a substrate under standard assay conditions as described in Materials and Methods (Section 3.3.3))

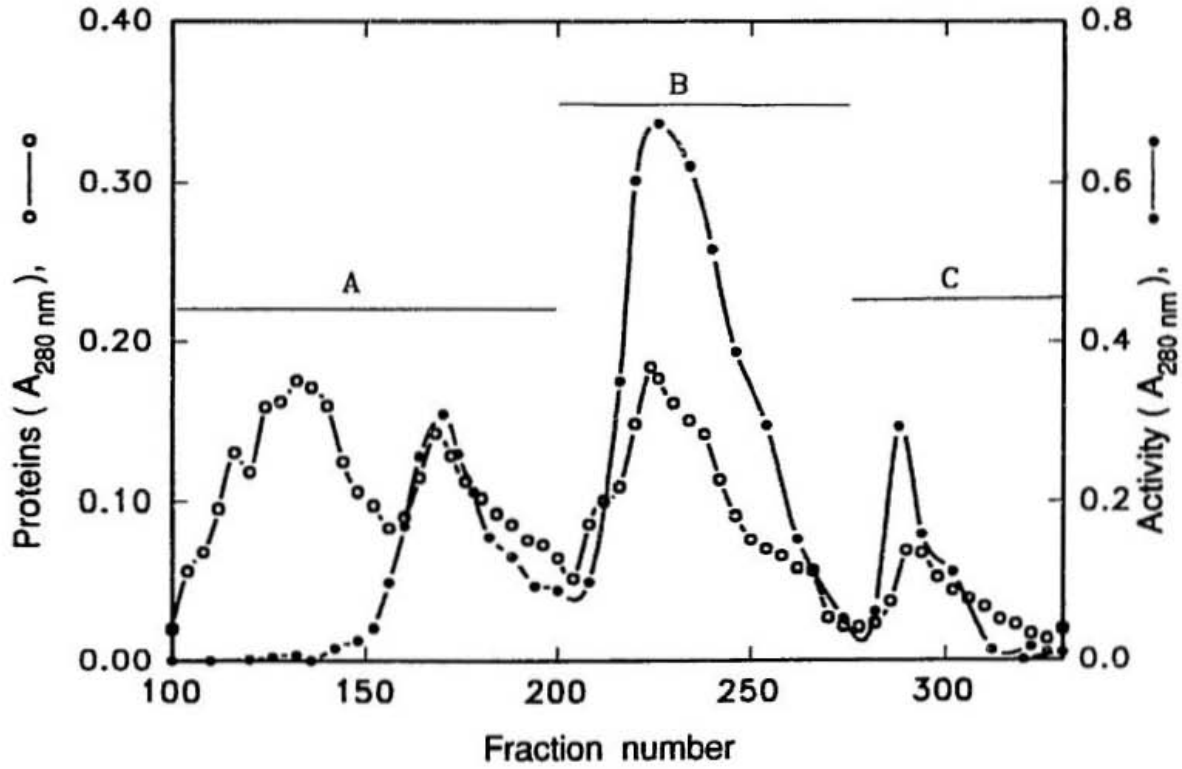

0.40
0.30
0.20
0.10
0.00
0.8
0.6
0.4
0.2
0.0
100
150
200
250
300
A
B
C
Proteins ($A_{280\ nm}$), o—o
Activity ($A_{280\ nm}$), •—•
Fraction number

Figure 4.8: **DEAE Sephadex A-50 ion-exchange chromatography of Atlantic cod gastric proteases.**
(The column, 2.5 × 40 cm in size which was put in cold room (4^0 C), was eluted with 0.1 M acetate buffer, pH 6.0, until the absorbance at 280 nm approached zero. The proteases were eluted separately with the following buffers: A, 0.1 M acetate buffer containing 0.2 M NaCl, pH 6.0; B, 0.1 M acetate buffer containing 0.4 M NaCl, pH 5.5; C, 0.1 M acetate buffer containing 0.8 M NaCl, pH 5.0. The flow rate was 0.4 mL/min. Fraction size was 4.0 mL. Proteolytic activity of the eluted fractions were measured using haemoglobin as a substrate under standard assay conditions as described in Materials and Methods (Section 3.3.3).)

Table 4.6: **Summary of characteristics of the three gastric proteases.**

Properties	Acid Protease A	Acid Protease B	Acid Protease C
Molecular weight:			
By SDS-PAGE[a]	37,700	32,900	32,300
By Gel chromatography[b]	36,500	31,400	28,500
Substrate specificity:			
Specific activity for haemoglobin[c]	898	2233	2162
Specific activity for casein [c]	230	657	430
Specific activity for APDT[d]	0.0235	0.0291	0.0007
Milk clotting activity (MCU)[e]	2.50	2.21	1.19
Optimal pH for haemoglobin[f]	3.2	2.5	3.7
pH stability at 25^0 C:[g]			
Fairly stable	around 7	3-6	5-6.5
Labile	1–4	1-3	3-5
Rapid inactivation	> 8.5	> 7.0	< 3.0, > 7.7
Pepstatin inhibition (%):[h]			
0.025 (μg/mL)	50.4	78.4	55.3
0.05 (μg/mL)	7.9	50.8	10.9
0.5 (μg/mL)	0.0	1.2	0.0

[a] Data from Figure 4.10.
[b] Data from Figure. 4.9.
[c] Data from Table 4.5.
[d] Measured under conditions described in Section 3.3.4.
[e] Measured and calculated according to the method described in Section 3.3.8.
[f] Data from Figure. 4.11.
[g] The residual activity was determined using 1.5% haemoglobin as a substrate under conditions described in Section 3.3.3. Refer to Figure 4.11 for detailed data.
as a substrate under standard assay conditions as described in Materials and Methods (Section 3.3.3);
[h] Represented as percentage of remaining activity. See Figure 4.13 for detailed information.

The occurrence of multiple forms of gastric proteases has been reported for other fish species. Two acid proteases have been purified from the digestive tracts of sardine (Noda and Murrakami, 1981) and dogfish (Guerard and Gal, 1987). Four zymogens of acid proteases have been isolated from the gastric mucosa of harp seal (Shamsuzzaman and Haard, 1984). Brewer *et al.*(1984) reported that two pepsins were isolated from Atlantic cod. Using electrofocusing chromatography, four different gastric proteases were isolated from Atlantic cod (Reece, 1988). Squires *et al.* (1986a) reported that three zymogens of gastric proteases of the Greenland cod were isolated by exclusion chromatography and chromatofocusing. As described in Section 2.2.3, the occurrence of multiple forms of gastric proteases may account for the enzyme adaptability of poikilotherms resulting from the divergent evolution (all were presumably derived from a common ancestral protease). The posttranslational modifications (such as glycosylation) may also account for the occurrence of multiple forms of gastric proteases.

Ion-exchange chromatography greatly increased the proteolytic activity of purified proteases. The recovery of proteolytic activity of acid proteases A and B were increased dramatically after this chromatographic separation (Table 4.5). This increase in proteolytic activity of purified acid proteases is perhaps due to the presence of salt (NaCl) in the elution buffer (from 0.1 to 0.6 M) used in the purification procedure.

4.3.2 Effect of NaCl on activities of gastric proteases

Several authors have reported the stimulation of fish pepsins with salts in hydrolysing proteins. Haard (1986) reported that hydrolysis of casein by Atlantic cod pepsin at pH 5.5 was stimulated approximately 50% by addition of 50 to 250 mM $CaCl_2$ to the reaction mixture, and stimulation of cod pepsin catalyzed protein (including haemoglobin) hydrolysis was most pronounced in the presence of 25 to 100 mM NaCl. The activating effect of salts was found to increase V_{max} and lower K_m for the hydrolysis of haemoglobin. Squires *et al.* (1986b) reported that in the presence of 0.2 M NaCl, the K_m for the hydrolysis of haemoglobin by Greenland cod protease 2 was decreased by 50%, while the V_{max} was increased by 50%. They found that Greenland cod proteases 1 and 2 were stimulated by salt, similar to some other fish gastric proteases, while cod pretease 3 was more like the porcine pepsin which is unaffected by salt.

The activating effect of salts on gastric proteases from other fish species has also been reported. Sanchez-Chiang and Ponce (1981) suggested that salts stimulate protein hydrolysis by gastricsins, but not by pepsins. Based on this suggestion, Haard (1986) considered that gastric proteases isolated from the stomach linings of Atlantic cod might be classified better as gastricsins than pepsins. However, in a later paper, Sanchez-Chiang and Ponce (1982) reported that pepsin-like enzymes from this fish species were also stimulated by salt. Results from this study demonstrated that proteolytic activity of all three gastric proteases were stimulated by different salt

concentrations after ion-exchange chromatography (Table 4.5). It should be noted that presence of some proteolytic inhibitors in the crude enzyme preparations may be partially involved. Purification with ion-exchange chromatography removes such inhibitors, thus enhancing the proteolytic activity of purified enzymes. Naturally-occurring trypsin inhibitors in crude trypsin preparations from cod pyloric caeca have been reported by Simpson and Haard (1984a).

4.3.3 Estimation of relative molecular mass

The relative molecular mass (M_r) of cod acid proteases were estimated by Sephadex G-75 gel filtration on a 1.5 × 100 cm column, and by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in a discontinuous buffer system. Estimated by gel filtration, the M_r of acid proteases A, B, and C isolated from DEAE-Sephadex A-50 ion-exchange chromatography were 36500, 31400, and 28500, respectively (Fig 4.9). The corresponding M_r values determined by SDS-PAGE were 37700, 32900, and 32300, respectively (Fig. 4.10).

Under experimental conditions described in Section 3.3.13, SDS-PAGE patterns for acid proteases A, B, and C are presented in Fig 4.10. Therefore, the isolation procedure established in this study and subsequent separation by DEAE-Sephadex A-50 ion-exchange chromatography were satisfactory. Thus, it is possible to obtain purified gastric proteases with acceptable purity from starting materials containing the active enzymes (Foltmann, 1981).

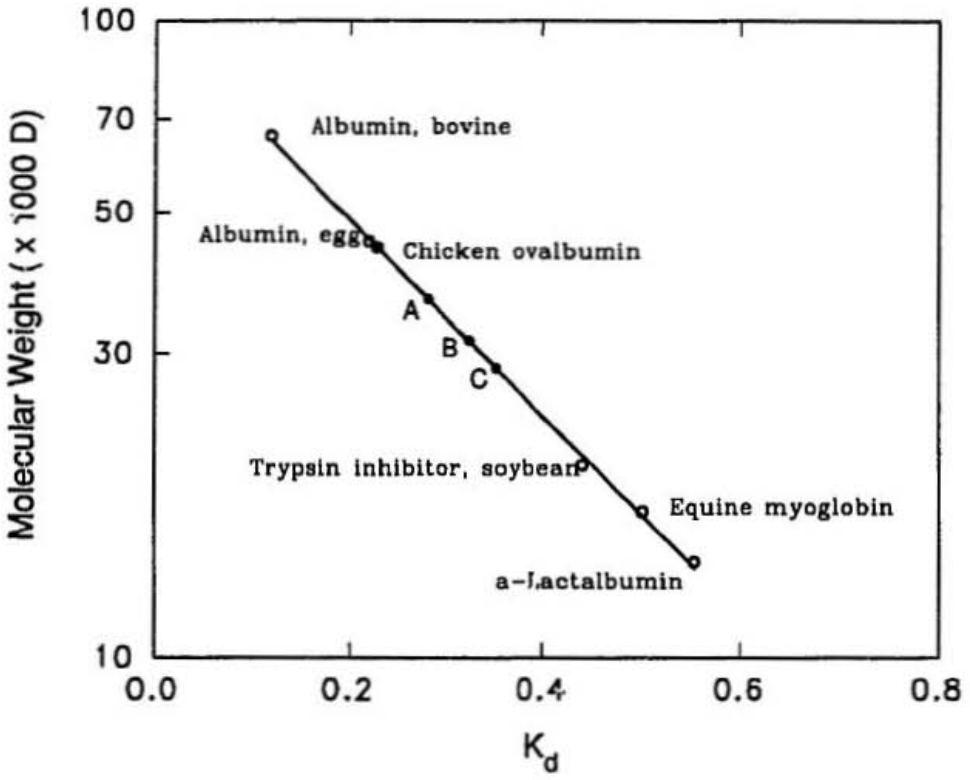

100
70
50
30
10
Molecular Weight (x 1000 D)
Albumin, bovine
Albumin, egg
Chicken ovalbumin
A
B
C
Trypsin inhibitor, soybean
Equine myoglobin
a-Lactalbumin
0.0
0.2
0.4
0.6
0.8
K_d

Figure 4.9: **Estimation of relative molecular mass (M_r) of enzymes by gel permeation chromatography**
($logM_r = -1.5396K_d + 4.9949$ ($R^2 = 0.9994$). The K_d values for A, B, and C are 0.2807, 0.3233, and 0.3508, corresponding to M_r values of 36500, 31400, and 28500, respectively. A, B, and C refer to Atlantic cod acid proteases A, B, and C, respectively. K_d is the distribution coefficient for a solute as defined in Section 3.3.13. Original data are presented in Appendix D.)

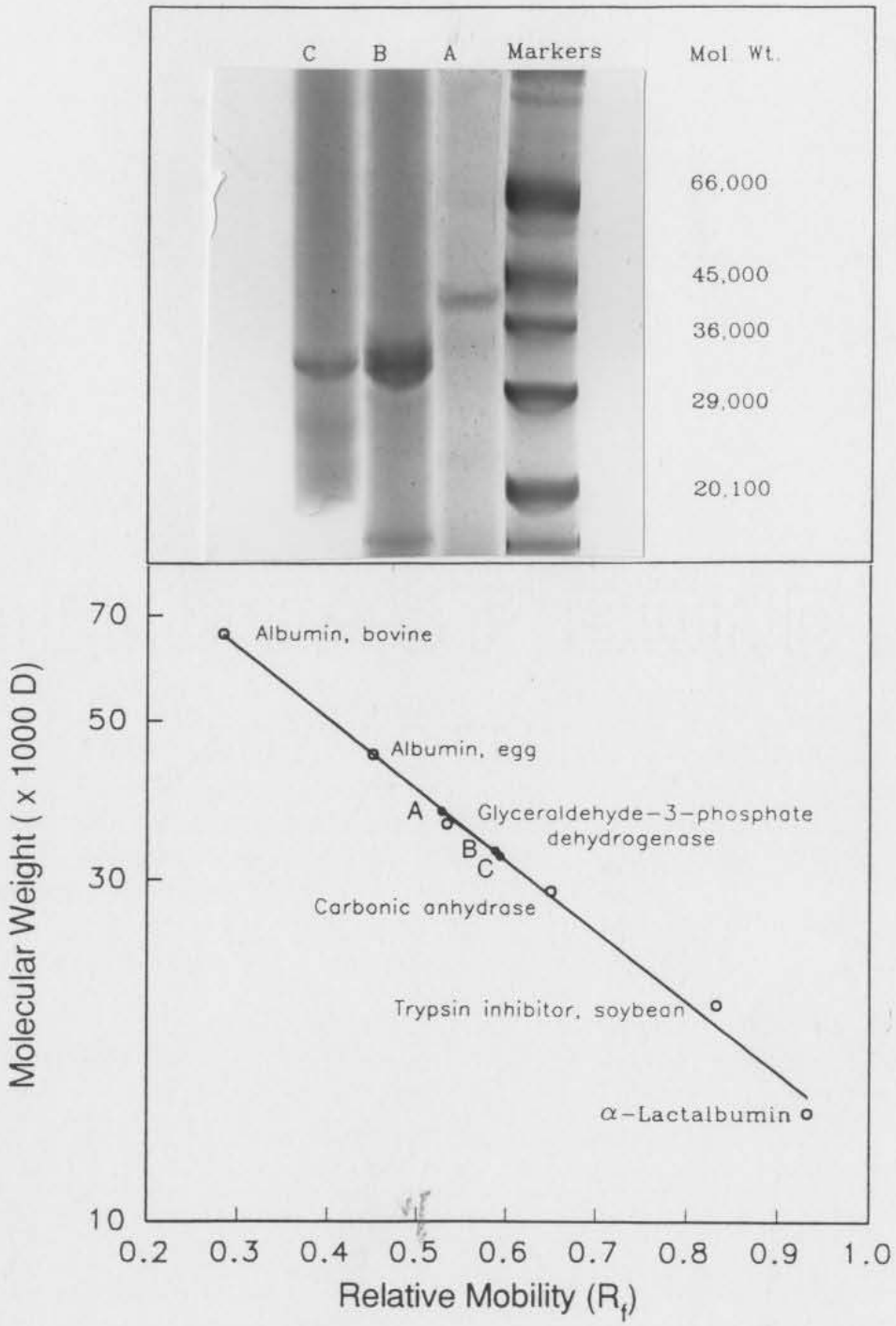

C
B
A
Markers
Mol Wt.
66,000
45,000
36,000
29,000
20,100
70
50
30
10
Albumin, bovine
Albumin, egg
A
Glyceraldehyde-3-phosphate dehydrogenase
B
C
Carbonic anhydrase
Trypsin inhibitor, soybean
α-Lactalbumin
Molecular Weight (x 1000 D)
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
Relative Mobility (R_f)

Figure 4.10: **Estimation of relative molecular mass (M_r) by SDS-PAGE** ($log M_r = -0.989 R_f + 5.098$ ($R^2 = 0.997$). R_f values for A, B, and C are 0.527, 0.587, and 0.595, corresponding to M_r values of 37700, 32900, and 32300, respectively. A, B, and C refer to Atlantic cod acid proteases A, B, and C, respectively. SDS-PAGE refer to sodium dodecyl sulphate polyacrylamide gel electrophoresis. Original data are presented in Appendix E.)

The M_r of gastric proteases and of microbial acid proteases range from approximately 31000 to 40000 (Fruton, 1987). Using SDS-PAGE, all fish pepsins investigated had a M_r value close to 35000 and pepsin I being slightly large than pepsin II (Gildberg, 1988). Based on this consideration, acid protease A is similar to fish pepsin I and acid proteases B and C are similar to fish pepsin II. Estimated by SDS-PAGE and amino acid analysis, Greenland cod gastric proteases have very similar M_r values varying between 36000 and 38000, which are similar to that of porcine pepsin. However, the M_r of Greenland cod gastric proteases estimated by gel filtration were lower (from 23442 to 25609) than those from SDS-PAGE and amino acid analysis (Squires *et al.*, 1986a). The M_r values estimated by SDS-PAGE and gel chromatography for Atlantic gastric proteases in this study did not show such a great difference as those obtained from Greenland cod.

4.3.4 pH profiles of partially purified cod gastric proteases

The optimum reaction pH was 3.2 for acid protease A, 2.5 for acid protease B and 3.7 for acid protease C, using haemoglobin as a substrate (Fig 4.11.I). It has been reported that many fish species secrete at least two pepsins with different pH optima(Noda and Murakami, 1981; Gildberg and Raa, 1983; Martinez and Olsen, 1986). These pepsins are usually referred to as pepsin I and pepsin II. Pepsin I has a pH optimum for haemoglobin digestion in the range of 3 to 4, whereas pepsin II is most active in the range of 2 to 3 (Gildberg, 1988). Cod pepsin II was found to have a pH

optimum slightly higher (around 2.6) than that of pig pepsin, whereas cod pepsin I had a pH optimum in the same range (around 3.7) as squid cathepsin D (Bjelland *et al.*, 1988). However, some authors have considered that an optimum reaction pH of 3.6 for protein hydrolysis is rather a typical property of gastricsin and chymosin than that of pepsin which normally has an optimum reaction pH of 2.0 with protein substrates (Shamsuzzaman and Haard, 1984).

Classification of the pepsins and pepsin-like enzymes based on their optimum pH activity was proposed by Foltmann and Pedersen (1977). The three gastric proteases showed great differences in their stabilities at various pHs. Previous investigations have shown that fish pepsin I is less stable than pepsin II in both strong acid and neutral conditions (Gildberg, 1988). The present study (Fig. 4.11.II) indicates that protease C is less stable than protease B under strong acid and is less stable than protease A in neutral conditions. According to their pH stability, protease C belongs to fish pepsin I and the other two proteases belong to fish pepsin II. Acid protease A shows increased stability to alkali, which is similar to chymosin from this point of view (Foltmann and Pedersen, 1977).

4.3.5 Catalytic properties and specific activity

The catalytic properties of enzymes is the basis for their classification. The specific activities of the three different gastric proteases isolated from cod are summarized in Table 4.6. All of the three gastric proteases had different specific activities against

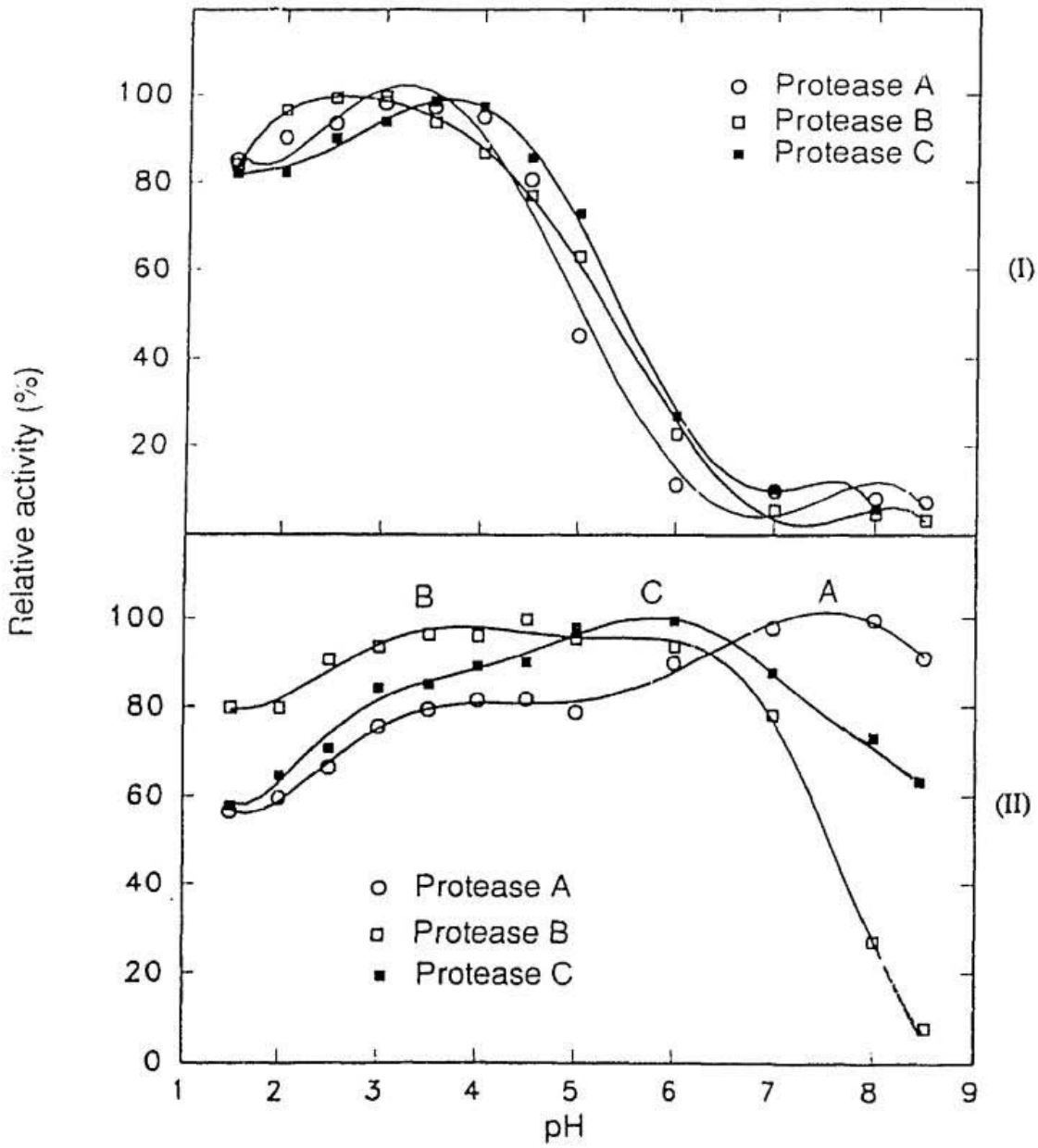

Relative activity (%)
100
80
60
40
20
o Protease A
□ Protease B
■ Protease C
(I)
B
C
A
100
80
60
40
20
0
o Protease A
□ Protease B
■ Protease C
(II)
1
2
3
4
5
6
7
8
9
pH

Figure 4.11: **pH profiles of three gastric proteases.**
I, Optimal reaction pH of cod gastric proteases. II, The influence of pH on enzyme stability. Enzymes with concentrations presented in Table 4.5 were used. All proteolytic activities were measured using haemoglobin as a substrate. The highest activity measured under the experimental conditions was expressed as 100% relative activity (RA). 100% RA correspnoded to 940 HU/mg, 2310 HU/mg, and 2350 HU/mg in Figure (I); 1220 HU/mg, 2240 HU/mg, and 2150 HU/mg in Figure (II) for acid proteases A, B, and C, respectively. Enzyme concentrations were determined by Lowry assay (1951). Results are mean values of duplicate determinations.

haemoglobin and casein substrates. They digested haemoglobin about 3-6 times faster than casein. The specific activities of the three Atlantic cod gastric proteases were similar to gastric protease 2 and 3 from Greenland cod in which the ratio of HU/CU was less than 6.0 (Greenland cod pepsin 1 was found to have a ratio of HU/CU of greater than 12 (Squires *et al.*, 1986b). Many authors have reported that fish pepsins digested haemoglobin 3-10 times faster than casein and fish myofibrillar proteins (Gildberg and Raa, 1983; Bjelland *et al.*, 1988).

Specific activities of 3000 and 2000 HU/mg have been measured for pepsin I and II from Atlantic cod respectively, using haemoglobin as substrate (Gildberg and Almas, 1986). The three purified gastric proteases (A, B and C) obtained in this study had a specific activity of 898, 2233 and 2162 U/mg, respectively, using haemoglobin as a substrate (Table. 4.6). Using specific activity as a guideline, the three gastric proteases isolated by ion-exchange chromatography possessed activities close to that of purified homogeneous, especially for acid proteases B and C. However, it should be noted that the specific activity of the individual enzymes appears to vary with the season of catch (Squires *et al.*, 1986a).

Acid protease C has a very low activity torward APDT, which is similar to gastricsin (Ryle 1970). The ratio of MCU/PU for gastric proteases of Greenland cod was 30-50 times higher than that of porcine pepsin and thus was considered to be like chymosin (Squires *et al.*,1986a). However, this study did not find such a high milk-clotting activity for Atlantic cod gastric proteases (Table 4.6).

Gastric proteases of Greenland cod have a broader substrate specificity than porcine pepsin since they hydrolyze haemoglobin to a greater extent (Squires *et al.*, 1986a). However, the activities of purified Atlantic cod gastric proteases were significantly inhibited by their reaction substrate, haemoglobin, at high concentrations (Fig. 4.12). The high concentration of globin substrate inhibition is one of the properties of pepsin-like enzymes (Hartsuck and Tang, 1978).

The globin and the activation peptide of pepsinogen compete for the same active site, thus inhibiting the intermolecular pepsinogen (intermediate δ) activation (Hartsuck and Tang, 1978). The proteolytic activities in this study were measured after a 30 min incubation of the enzyme preparations in pH 3.0 acetate buffer at 25^0 C. Hence, there is no reason for the existence of an unactivated pepsinogen in the reaction mixture since the autocatalytic activation may begin at pH 4.4–4.6 (Herriott, 1939). It is considered that both substrate and product of hydrolysis influence on the activity of proteolytic enzymes, especially for purified protease with high concentration of substrate or hydrolytic product.

4.3.6 Inhibition of proteolytic activities by pepstatin

Pepstatin, an isovaleryl derivative of a pentapeptide (Iva-Val-Val-Sta-Ala-Sta-OH) produced by strians of *Streptomyces*, is a well known aspartyl protease inhibitor. Statine [Sta, (3S, 4S)-4-amino-3-hydroxy-6-methylheptanoic acid] is an unusual amino acid contributing to the inhibition of proteolytic activities of gastric proteases (Rich

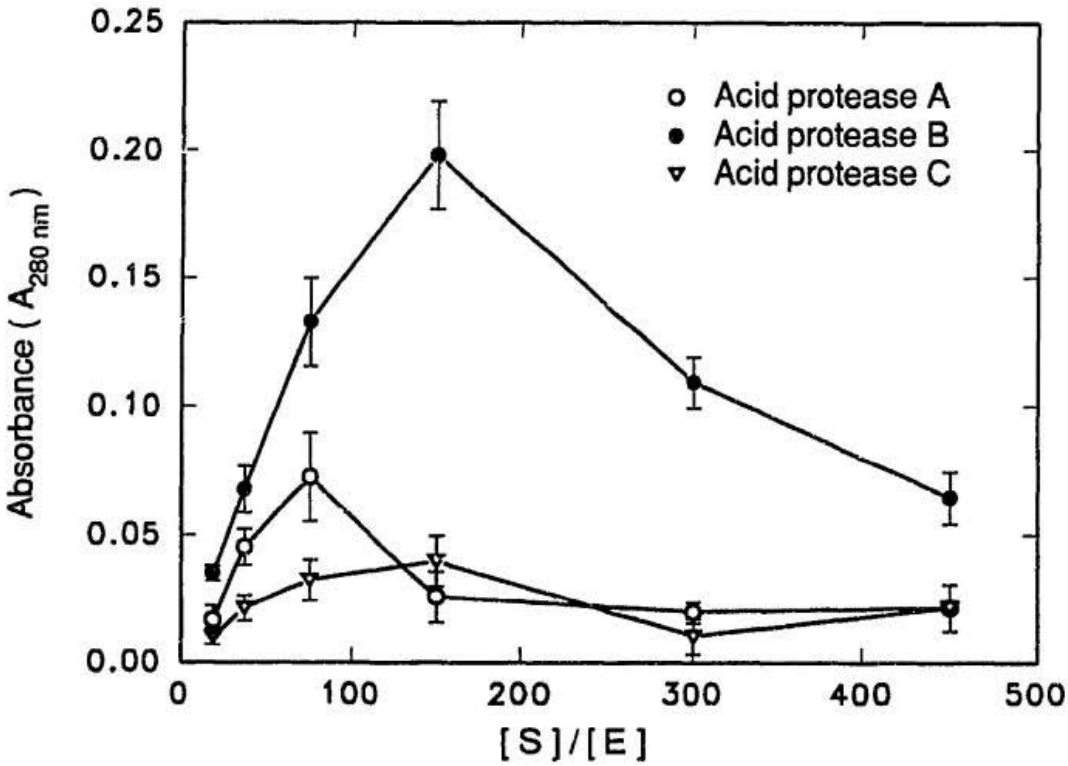

0.25
0.20
0.15
0.10
0.05
0.00
Absorbance ($A_{280\ nm}$)
0
100
200
300
400
500
[S]/[E]
Acid protease A
Acid protease B
Acid protease C

Figure 4.12: **Substrate inhibition of three cod gastric proteases.** (Proteolytic activities of individual gastric protease at different haemoglobin concentrations described in the figure were measured in 0.1 M acetate buffer, pH 3.0, at 25^0 C. Units for [S] and [E] are percent concentrations (w/v). Results are mean value of triplicate determinations.)

et al., 1985). An outstanding structural feature of the pepstatin molecule is the preponderance of hydrophobic groups which can tightly bind at the active site of aspartyl proteases, in which the extended active site is strongly hydrophobic in nature (Fruton, 1987).

All of the three gastric proteases for proteolysis were progressively inhibited by increasing concentrations of pepstatin (Table 4.6). The proteolytic activities of the three enzymes were progressively reduced with the increase of inhibitor concentration. A total inhibitory effect on proteolysis was obtained at 0.5 μg/mL of pepstatin concentration (Figure 4.13). Results of pepstatin inhibition of the three acid proteases indicated that all of the three acid proteases belonged to the group of aspartyl proteases.

It has been reported that at a concentration of inhibitor sufficient to abolish completely the activity of pig pepsin or human pepsin, human gastricsin was inhibited to 50%, while calf chymosin was only slightly inhibited (Marciniszyn *et al.*, 1977). These differences indicate that although various aspartyl proteases may be alike in their requirement for two aspartyl carboxyl groups in the bond-breaking phase of enzymic catalysis, these enzymes appear to differ in their binding of peptide ligands (including peptide substrates) at their active sites (Fruton, 1987). The physio-chemical properties of pepstatin are currently unclear, and the exact mechanisms of pepstatin inhibition on the aspartyl proteases are still under investigation (Fischer, 1987).

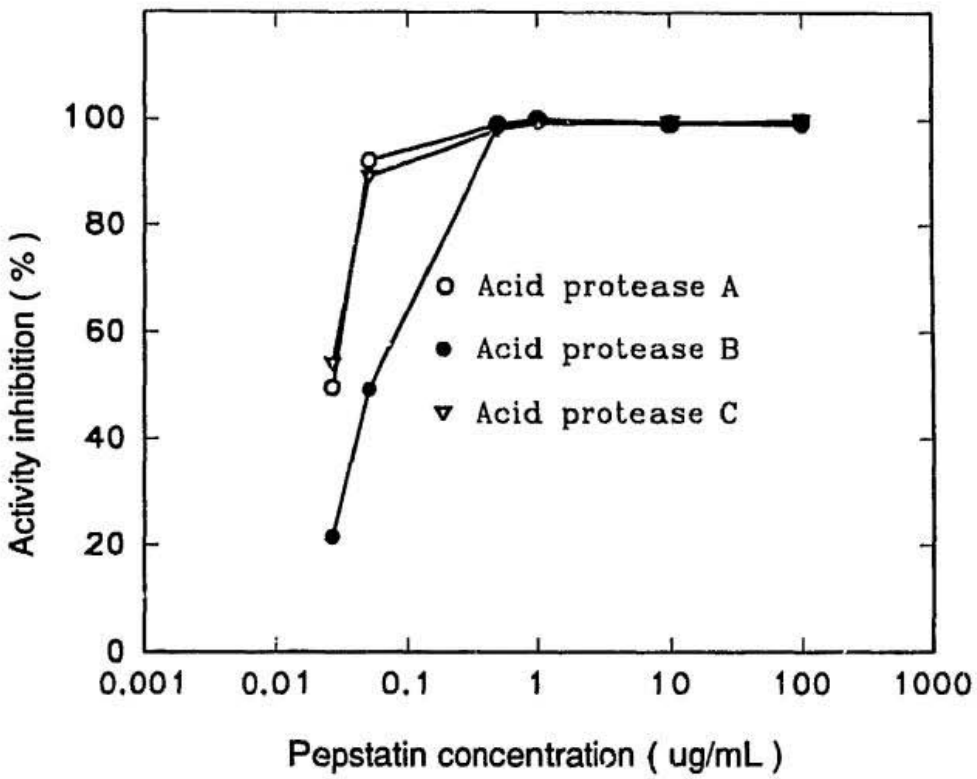

100
80
60
40
20
0
Activity inhibition (%)
0.001
0.01
0.1
1
10
100
1000
Pepstatin concentration (ug/mL)
Acid protease A
Acid protease B
Acid protease C

Figure 4.13: **Inhibition of pepstatin on proteolytic activities**
All enzyme preparations were adjusted to the same concentration as determined by Lowry assay (0.09 mg/mL in 0.1 M acetate buffer, pH 3.0). The adjusted enzyme preparations were incubated separately with different concentrations of pepstatin solution as presented in the figure for 0 min at room temperature. The residual activity was determined using a 1.5% solution of haemoglobin as substrate under standard assay conditions as described in Section 3.3.3. Enzyme activity at zero pepstatin concentration was 510 HU/mg, 1240 HU/mg, and 2120 HU/mg for acid proteases A, B, and C, respectively. Results are mean values of duplicate determinations.)

4.3.7 Conclusions on characterization of acid proteases

All of the three cod acid proteases (acid proteases A, B, and C), obtained by co-extraction process and subsequent purifications in this study, are aspartyl proteases as they were all inhibited progressively by increased concentrations of pepstatin, a well known aspartyl protease inhibitor. This conclusion is further confirmed by molecular weight (around 35,000 D), pH profile, and specific activity on different substrate studies (see Table 4.6). In comparison with other fish gastric proteases (Table 2.1), acid protease B belongs to fish pepsin II. Acid protease A and C share characteristics of fish pepsins I and II as summaried in Table 4.7.

Acid protease A shows increased activity to alkali, has relatively higher activity on milk-clotting and is slow moving in electrophoresis, which is similar to the characteristics of chymosin (Foltmann and Pedersen, 1977). Acid protease C has very low activity on APDT, high pH optima on haemoglobin digestion, which is similar to gastricsin. The main part of the enzymes, acid protease B, possesses all properties of fish pepsin II. The detailed mechanism for the occurrence of multiple forms of gastric proteases and their mixed characteristics is unclear.

Table 4.7: **Conclusions in characterization of cod gastric proteases[a].**

Properties	Acid Protease A	Acid Protease B	Acid Protease C
General properties:			
Molecular weight (D)[b]	I	II	II
Optimal pH for haemoglobin[c]	I (1)	II (2)	I (3)
Relative amount[d]	I	II	I
Substrate specificity:			
HU/CU[e]	(3)	(2)	(1)
Specific activity on APDT	High	High	Low
Milk clotting activity (MCU)	(1)	(3)	(2)
pH stability at 25^0 C:[f]	I/II	II	I

[a] Results are based on a comparasion of characteristics of Atlantic cod gastric proteases with those of (1) Greenland cod gastric proteases 1, 2, and 3 (Squires *et al.*, 1986a; 1986b), and (2) fish pepsins I and II (see Table 2.1 for detailed information).
[b] Pepsin I is slightly large than pepsin II (Gildberg, 1988).
[c] Pepsin I has pH optimum for this haemoglobin digestion in the pH range of 3–4, whereas pepsin II is most active in the pH range of 2–3 (Bjelland *et al., 1988*).
[d] Pepsin II is dominant in cod (Martinez and Olsen, 1986).
[e] HU/CU, ratio of haemoglobin activity to casein activity.
[f] Pepsin I is less stable than pepsin II in both acid and neutral conditions (Gildberg, 1988).

4.4 Partial Purification and Characterization of Alkaline Proteases From Atlantic Cod

4.4.1 Partial purification and general characteristics

The crude alkaline proteases co-extracted from cod viscera were partially purified by Sephadex G-75 gel permeation chromatography and their properties were studied (Table 4.8). The pH profiles of alkaline proteases are discussed in Section 4.2.2. Alkaline proteases showed an optimum pH at around 8.5-9.0 on casein hydrolysis. The alkaline proteases were unstable in acid, especially when pH was < 4.0. The maximum stability of alkaline proteases was close to pH 9.0. Similar results in pH profiles for trypsins have been reported by Camacho *et al.* (1970), Simpson and Haard, (1984a), and Simpson *et al.* (1990).

The temperature optimum for the hydrolysis of casein by cod alkaline proteases was close to 43^0 C, similar to that obtained for Atlantic cod trypsin by Bjelland *et al.* (1988). The alkaline proteases were unstable at temperatures above their optimum reaction temperature. Even at the optimum reaction temperature, the enzymes began to lose their activity after a 60 min incubation period. similar patterns in optimum reaction temperature and thermal stability for trypsins from both Atlantic (Simpson *et al.*, 1990) and Greenland cod (Simpson and Haard, 1984b) have been reported.

Table 4.8: **Characteristics of the isolated alkaline proteases[a].**

Properties	Cod alkaline protease
pH optimum (on casein hydrolysis)[b]	pH 8.5-9.0
pH stability[b]	pH 8.0-9.5
Temperature optimum[c]	43^0 C
Specific activity against casein[d]	78.6 (CU/mg)
Specific activity against BAPNA[e]	34.8 (U/mg)
Specific activity against BAEE[f]	66.3 (U/mg)
Specific activity against BTEE[f]	72.9 (U/mg)
SBTI inhibition (0.025 M, 30 min)[g]	98% inhibition
Inhibition by 0.2 M ME[h]	50% inhibition

[a] Refer to Materials and Methods (Section 3.3.3; 3.3.5; 3.3.6; 3.3.7) for detailed information on assays of specific activities and their unit definitions.
[b] Data from Figure 4.3.
[c] Data from Figure 4.5.
[d] Data from Table 4.5.
[e] Measured and calculated according to the method edscribed in Section 3.3.9. BAPNA refers to α-benzoyl-DL-arginine p-nitroanilide.
[f] BAEE and BTEE refer to benzoyl-L-arginine ethyl ester, N-benzoyl-L-tyrosine ethyl ester, respectively. Data from Figure 4.14.
[g] Data from Figure 4.15. SBTI refers to soybean trypsin inhibitor. The remained activity was measured using BAPNA as substrate according to the method described in Section 3.3.12.
[h] ME refers to 20mercaptoethanol. Data from Figure 4.16.

4.4.2 Catalytic properties

The enzyme preparation obtained showed amidase (against α-benzoyl-DL-arginine p-nitroanilide, BAPNA), esterase (against benzoyl-L-arginine ethyl ester, BAEE, Figure 4.14), and proteolytic (against casein) activities, characteristics of trypsin-like enzymes (Rich, 1965; Cohen and Gertler, 1981; Uchida *et al.*, 1984b; Simpson *et al.*, (1990). The partially purified alkaline proteases were also active when added to N-benzoyl-L-tyrosine ethyl ester (BTEE) (Figure 4.14), a synthetic substrate for chymotrypsin (Barnard and Hope, 1969; Walsh, 1970; Kalac, 1978). Trypsins having activity on BTEE have also been reported by other authors. Chen *et al.* (1988) reported that a crystalline hog trypsin obtained from Sigma Co. had a similar activity pattern as chymotrypsins on BTEE. However, since the crude alkaline proteases are actually a mixture of different enzymes (most likely chyomtrypsin family of serine class), it is possible that the alkaline proteases so obtained may contain trypsin and chymotrypsin-like enzymes.

4.4.3 Influence of inhibitors on proteolytic activities

The activities of alkaline proteases towards casein and BAPNA were inhibited by 0.025 M soybean trypsin inhibitor (SBTI) (Fig. 4.15). The classification of a protease is usually determined according to its inhibitors (Barrett, 1977). SBTI inhibition has been considered as a key guideline for characterization of trypsin or trypsin-like enzymes. Inhibition of trypsins by SBTI has been described by Gates and Travis

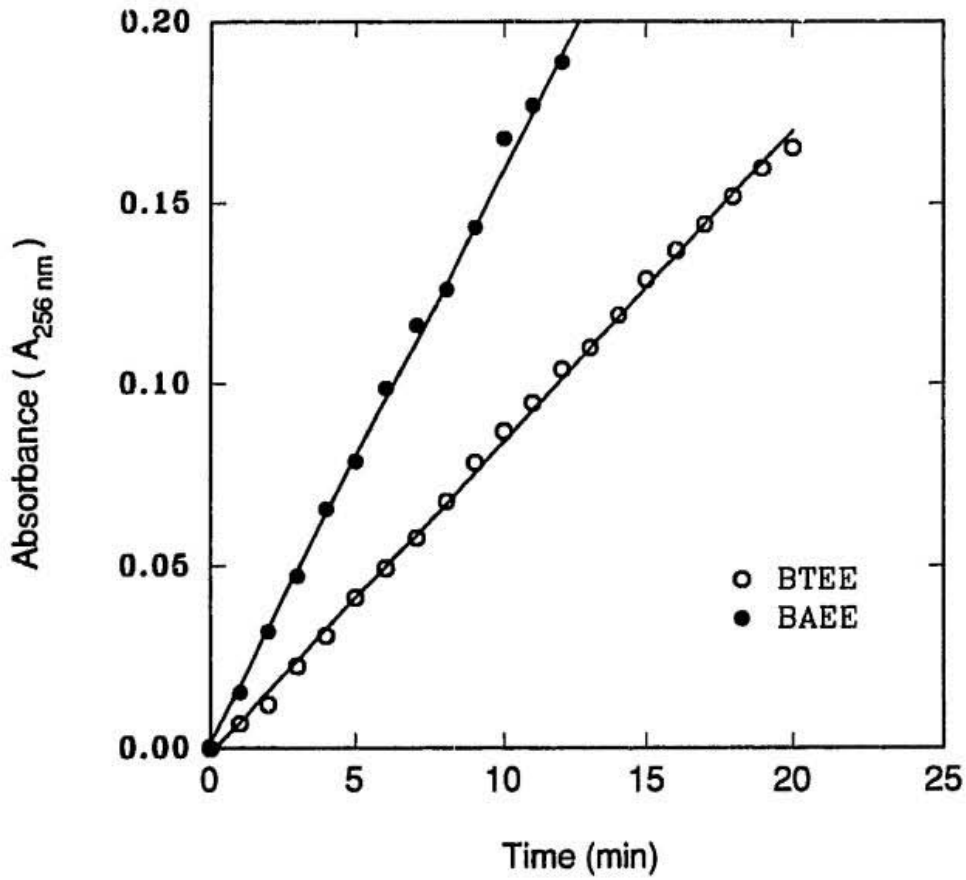

0.20
0.15
0.10
0.05
0.00
Absorbance ($A_{256\ nm}$)
0
5
10
15
20
25
Time (min)
BTEE
BAEE

Figure 4.14: **Hydrolysis of cod alkaline protease on synthetic substrate, benzoyl-L-arginine ethyl ester, BAEE, and benzoyl-L-tyrosine ethyl ester, BTEE.**
The experiment was carried out at 25^0 C using the method described in Materials and Methods (Section 3.3.5). Enzyme concentration was determined by Lowry assay (1.31 mg/mL in 50 mM Tris buffer, pH 7.8).

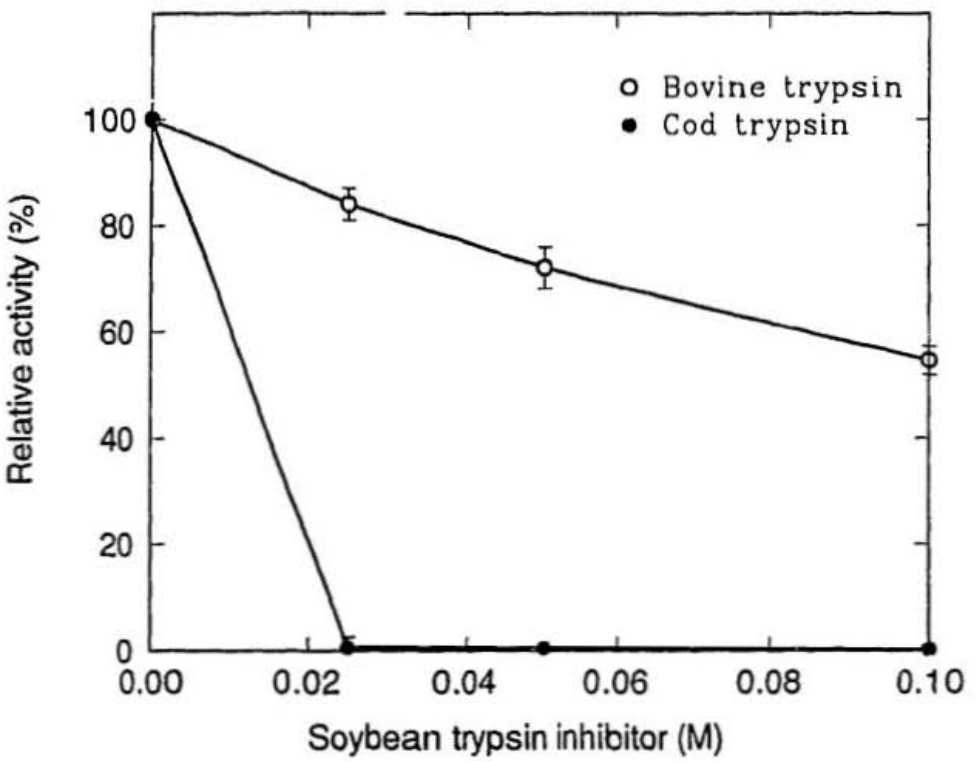

Bovine trypsin
Cod trypsin
Relative activity (%)
100
80
60
40
20
0
0.00
0.02
0.04
0.06
0.08
0.10
Soybean trypsin inhibitor (M)

Figure 4.15: **Inhibition of alkaline proteases by soybean trypsin inhibitor.** The orignal enzyme concentrations were determined by Lowry assay and were adjusted to 1.0 mg/mL in 50 mM Tris buffer, pH 7.8. Residual activity was measured using BAPNA as a substrate according to the method described in Section 3.3.7. 100% relative activity corresponded to 35 U/mg for cod trypsin, and 128 U/mg for bovine trypsin.

(1969), Travis and Roberts (1969), Camacho *et al.* (1970), Stambaugh and Buckley (1972), and Hjelmeland and Raa (1982). It has been reported that SBTI are proteins which can bind strongly to the active site of trypsin and thus block its activity. Inhibition of alkaline proteases by SBTI indicates the trypsin-like nature. However, since SBTI would also affect other serine proteases, inhibition by SBTI alone is not sufficient enough for designating the nature of trypsin. Other guidelines should also be considered for the characterization of enzymes.

The amidase activity of Atlantic cod trypsin was inhibited by up to 50% when a 0.2 M solution of 2-mercaptoethanol (ME) was used, whereas 1.0 M ME was required to obtain the same level of inhibition for bovine trypsin (Fig. 4.16). Cod trypsin was more susceptible to ME than bovine trypsin. Inhibition of bovine trypsin by thiols has been reported by Sondack and Light (1971), Steven and Podrasky (1978), and Steven and Griffin (1981). The observed inhibition of trypsins by high concentrations of thiols suggests that preservation of integrity of disulphide linkages in the native enzymes is vital for their stability and catalytic activity. Inhibitions of the amidase and esterase activities of both bovine trypsin and Greenland cod trypsin by thiol reagents have been reported; cod enzyme was more susceptible to these inhibitors. Presence of fewer potential disulphide pairs in cod enzyme as compared with those in that from bovine was considered responsible for this effect (Simpson and Haard, 1984a). The greater sensitivity of cold-adapted fish trypsin to thiols indicates the presence of fewer potential disulphide pairs existing in the fish enzyme, which makes

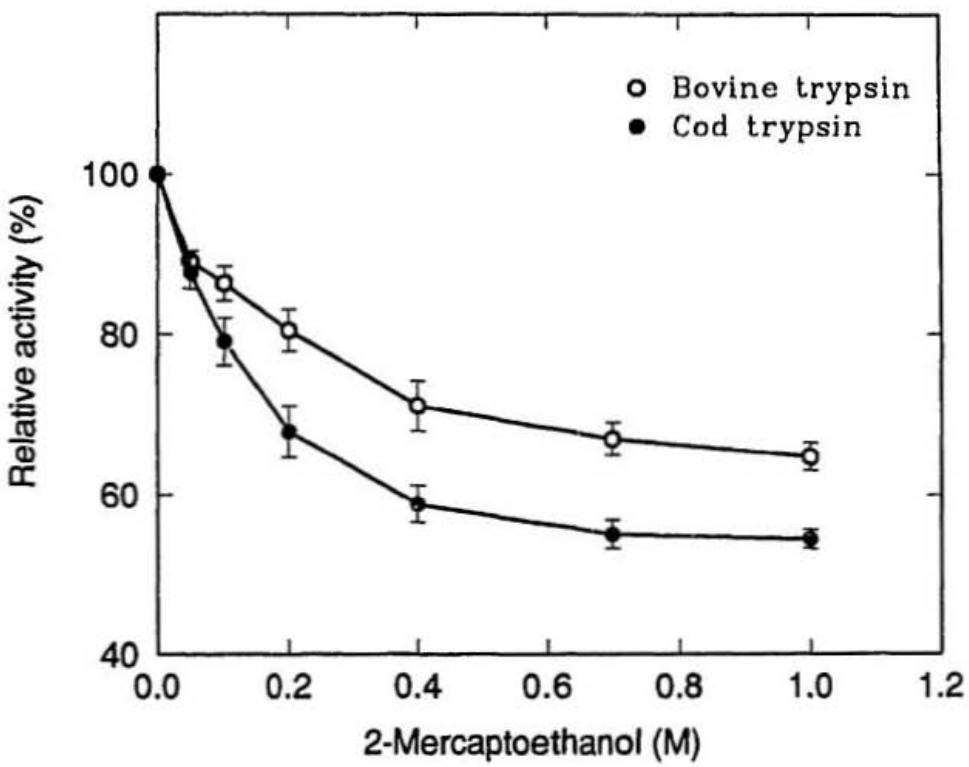

Bovine trypsin
Cod trypsin
Relative activity (%)
100
80
60
40
0.0
0.2
0.4
0.6
0.8
1.0
1.2
2-Mercaptoethanol (M)

Figure 4.16: **Influence of 2-mercaptoethanol (ME) on the activity of cod trypsin and bovine trypsin.**
The enzyme concentrations were determined by Lowry assay and were adjusted to 1.0 mg/mL in 50 mM Tris buffer, pH 7.8. The values of 100% activity were the same as described in Figure 4.15. The residual trypsin activities were determined using BAPNA as a substrate at 25^0 C by the method described in Section 3.3.7. Results are mean values of triplicate determinations.)

its molecular structure more flexible and thus more heat labile.

4.4.4 Conclusions on alkaline proteases co-extracted

Based on the results presented above, it is concluded that the partially purified alkaline proteases co-extracted from the digestive tract of Atlantic cod (*Gadus morhua*) are similar to Greenland cod trypsin (Simpson and Haard, 1984a) and Atlantic cod trypsin (Simpson *et al.*, 1990). The partially purified enzyme preparation contained activities of both trypsin- and chymotrypsin-like enzymes. The enzyme so obtained was extremely heat labile and unstable under acid conditions. It possessed high catalytic efficiency at ambient temperatures similar to that reported for trypsins from other cold adapted poikilothermic organisms. Since properties of cod trypsin have been well investigated (Simpson and Haard, 1984a, 1984b; Simpson *et al.*, 1990), no attempt was made to duplicate these studies.

Results of this study show that both aspartyl proteases (mainly gastric proteases) and serine proteases (mainly trypsin and chymotrypsin) can be co-extracted. With minor modification, it is expected that the co-extraction procedure so established could also be used for isolating both acid and alkaline proteases from digestive tracts of other marine species.

4.5 Use of Fish Proteases In Food Processing

4.5.1 Proteolytic enzymes as rennet substitutes

Milk-clotting activity of different proteases was investigated using reconstituted milk. A differential response to the effect of pH was noticed during the clotting process. Influence of pH on the clotting time was investigated using crude Atlantic cod pepsin and crude gastric protease from harp seal (Han and Shahidi, 1993).

The milk clotting activity of crude acid proteases of cod was relatively low compared to that of crude seal gastric proteases (SGP) and calf chymosin. In addition, cod acid proteases did not clot milk efficiently at pH values above 6.4 (Fig. 4.17). The same observation was made by Brewer *et al.*(1984). However, cod acid proteases clotted milk more efficiently than calf chymosin at 15^0 C. According to Brewer *et al.*(1984) cheeses prepared with cod pepsin were judged to be acceptable by a sensory panel and the intensity of cheddar flavour for enzymes used was in the order of: calf rennet > cod pepsin > porcine pepsin. On the other hand, Squires *et al.* (1986a) reported that cod gastric proteases had much wider substrate specificity than porcine pepsin. A broad specificity for an enzyme makes it unsuitable as a rennet substitute for cheese making because this will result in lower yield of curd and excessive proteolysis which may produce off-flavour during aging of the cheese (Brewer *et al.*, 1984). It is, therefore, difficult to reach a conclusion on the suitability of using crude cod gastric proteases as rennet substitutes for milk-clotting in cheese making. How-

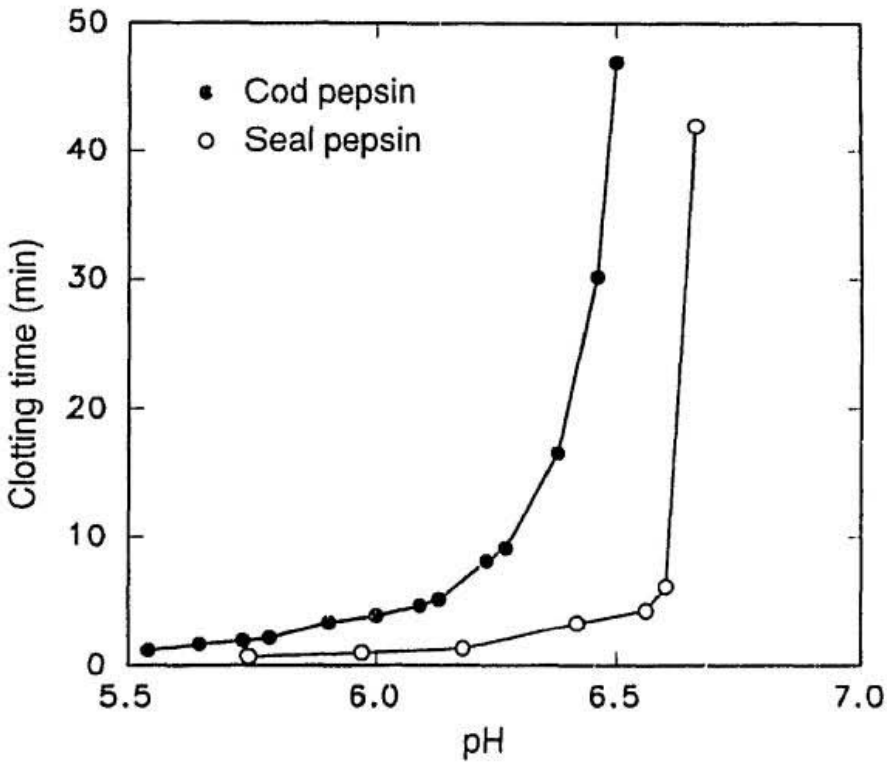

Cod pepsin
Seal pepsin
Clotting time (min)
50
40
30
20
10
0
5.5
6.0
6.5
7.0
pH

Figure 4.17: **Influence of pH values on milk-clotting time at 25^0 C.** Cod acid protease was a partially purified cod gastric protease after G-75 gel filtration. The enzyme concentrations were determined by Lowry assay and were adjusted to 3.0 mg/mL in acetate buffer, pH 6.0. The ratio of enzyme activity (HU) to milk (g) was 4.3 HU/g for cod pepsin and 4.0 HU/g for seal pepsin. The pH values of reconstituted milk were adjusted with 2.0 M HCl or NaOH. Duplicate determinations were made at each pH value.

ever, the fact that cod pepsin can clot milk efficiently at low temperatures shows its potential application in cold renneting of milk.

The milk-clotting time was reduced by increasing the concentration of the added enzyme. No significant increase was observed when the ratio of enzyme activity (HU) to milk (g) was larger than 10 HU/g (Fig. 4.18). Since part of the enzyme used will remain in the coagulated milk and contributes to the development of flavour in the final product, use of an adequate concentration of enzyme is necessary to control flavour characteristics of the products.

Using purified enzymes at relatively low dosage may have advantages in reducing undesirable flavour as well as other disadvantages resulting from broad specificity when using crude enzyme preparations. The purified cod gastric proteases showed different milk-clotting activities as described in Table 4.6. Acid protease A had the highest milk-clotting activity among the three gastric proteases isolated. Acid protease C showed relatively low activity in milk-clotting. Since a broad hydrolysis in milk-clotting may result in undesirable results in the final product, use of purified rennet substitutes is strongly recommended. Based on the consideration of milk-clotting activity and stability in neutral pH, cod acid protease A is considered to be the first candidate as a rennet substitute for milk-clotting in cheese making. Acid protease B had the highest hydrolytic activity on casein and had similar milk clotting activity as acid protease A, which is also a good candidate as a rennet substitute. No evaluation on qualities of final products was performed in this study.

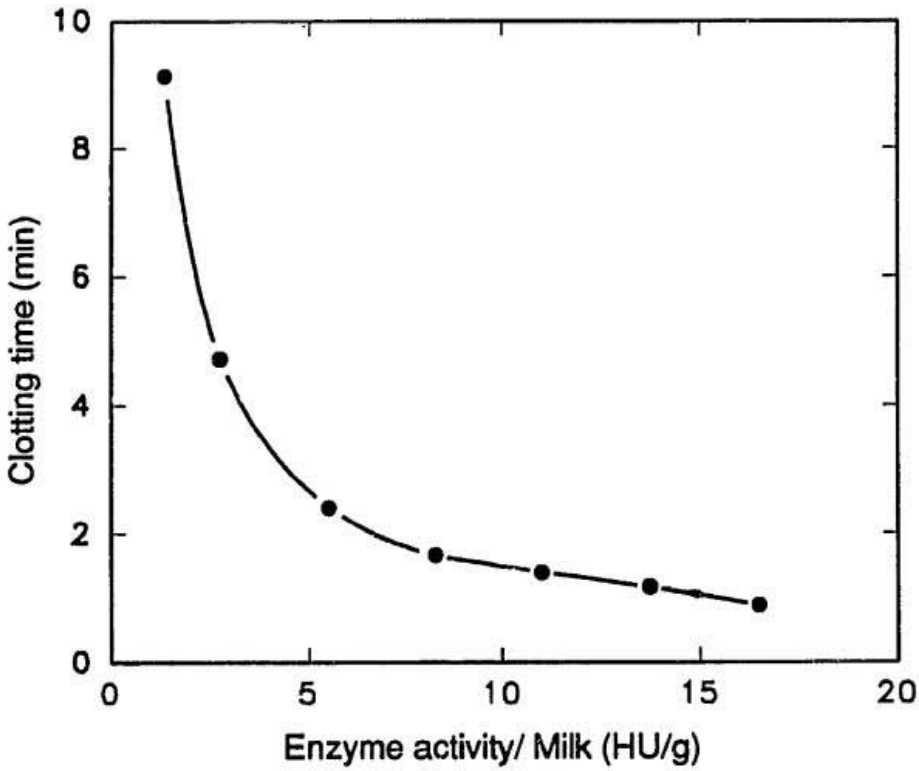

10
8
6
4
2
0
Clotting time (min)
0
5
10
15
20
Enzyme activity/ Milk (HU/g)

Figure 4.18: **Influence of enzyme concentration on the milk-clotting time.** The enzyme (3.12 mg/mL in 0.1 M acetate buffer, pH 6.0) was obtained from partially purified cod gastric protease after G-75 gel filtration. The experiment was carried out at room temperature. Results are mean values of duplicate determinations.)

4.5.2 Preparation of fish protein hydrolysates

Fish protein hydrolysate produced by protease treatment is sometimes called "biological fish protein concentrate" (Haard, 1992). The production of fish protein hydrolysates by protease treatment is a means to transform low-value pelagic fish and fish offal into protein concentrates with better functionality than fish meal. The process for the preparation of fish protein hydrolysates commonly involves limited hydrolysis of ground fish with proteases. A major problem encountered with enzyme treated fish protein hydrolysates is the formation of bitter tasting hydrophobic peptides (Mohr, 1980). Hydrolysis with proteases that have a narrow specificity for peptide bonds may prevent the formation of bitter peptides (Adler-Nissen, 1986; Simpson and Haard, 1987). Compared with currently applied microbial proteases, fish proteases have relatively narrow specificity. Therefore, fish proteases may serve as better candidates for production of fish protein hydrolysates.

Protein recovery with different proteolytic enzymes

Crude enzymes preparations (both acid and alkaline proteases) isolated from cod viscera were used for the preparation of fish protein hydrolysate (FPH) from capelin. Results so obtained were compared with FPHs prepared using seal gastric proteases as well as commercial enzymes. The yields of capelin protein hydrolysate using different proteolytic enzymes is summarized in Table 4.9.

Table 4.9: **Protein recovery by various proteolytic enzymes and compositions of capelin protein hydrolysates[a].**

PROTEINASES	Protein recovery[c] (%)	Main composition of protein hydrolysates[b]			
		Protein[c] (%)	Lipid (%)	Moisture (%)	Ash (%)
Cod AE	55.8±1.4	69.4±1.1	0.08±0.03	5.0±0.3	16.9±1.9
Cod BE	26.7±2.2	87.7±1.3	0.78±0.01	5.3±0.3	6.2±0.9
Crude SGP	49.2±3.1	70.6±2.3	0.45±0.01	5.7±0.2	19.3±0.8
Neutrase	51.6±1.9	65.9±0.7	0.21±0.01	5.3±0.1	22.5±2.6
Alcalase	57.9±1.0	67.2±1.5	0.18±0.00	6.3±0.1	19.8±1.8
Autolysis	22.9±1.6	64.3±3.3	1.51±0.02	5.8±0.2	17.7±1.8

[a] For this set of experiments, the ratio of enzyme to substrate was 30AU/kg proteins in initial materials. The hydrolysis was carried out at 25^0 C for 4 h. Data are mean values of triplicate determinations.

[b] Calculated on dried protein hydrolysate samples.

[c] Based on the Kjeldahl measurement ($N \times 6.25$).

Results in Table 4.9 indicate that the type of enzyme employed exerts an important effect on the yield and properties of the final product. Furthermore, conditions of hydrolysis such as pH, temperature, duration of hydrolysis as well as concentration of enzyme, are quite important in the extraction of proteins.

The type of enzyme used has a significant influence on the yield of protein hydrolysate as well as the functional properties of the product so obtained. It is a common observation that endopeptidases having a low specificity, such as bacterial proteases, are generally more effective in solubilizing fish proteins than highly specific endopeptidase such as trypsin. Among the three crude fish proteases (cod acid proteases, cod alkaline proteases, and seal gastric proteases) used in this study, cod acid protease gave the highest protein recovery (Table 4.9). More proteins were hydrolyzed and solubilized by cod acid protease than those by crude seal gastric proteases.

The lowest protein recovery yield was observed when using crude cod trypsin. It has been reported that the sarcoplasmic fraction of fish muscle is relatively resistant to degradation by digestive enzymes and trypsin. Gildberg and Raa (1979a and b) observed that enzymatic degradation of fish muscle to water-soluble components may occur at a faster rate in the neutral/slightly acid pH range, than at the alkaline pH optimum of proteases. They also reported that sarcoplasmic proteins of fish muscle, like minced fish, were somewhat resistant to degradation by a crude mixture of digestive enzymes and by trypsin, and inhibited the enzymatic degradation of myofibrils as well as other proteins such as haemoglobin (Raa and Gildberg, 1982).

Mechanism of enzyme hydrolysis

The digestion system is actually a very complicated mixture in which the number of substrates included and the products to be produced are unclear. Enzymatic hydrolysis of the ground fish mixture results in the detachment of tissues from the matrix and dissolution of a portion of the released tissue into soluble peptides.

Figure 4.19 shows the SDS-PAGE profiles of capelin protein hydrolysates (CPH) in aqueous phase obtained by using different enzyme systems. No autodigestion of the largest or smallest proteins occurred during the first 4 h of incubation at room temperature. Cod pepsins digested the largest proteins first. It is known that an increase in length of the substrate chains increases K_{cat} of pepsin without decreasing the K_m value (Antonov, 1977; Fruton, 1977). The microbial protease, Alcalase, had much broader hydrolysis specificity than those of mammalian pepsins. No bands emerged from the stained gel for CPH produced by Alcalase assisted hydrolysis, indicating that all proteins have been digested into small peptides (molecular weight less than 14,000 D). Thus, Alcalase had the best capacity for protein solubilization and resulted in highest protein recovery among the enzymes employed in this study. Studies on SDS-PAGE profiles of the aqueous phase from hydrolysis of cod frames with papain at 80° C showed that after 30 min of hydrolysis the majority of peptides in solution had a molecular weight of less than 14,000 D (Levin *et al.*, 1989).

The kinetics of the enzymic reaction can be easily studied in a pH-stat controlled experiment. The amount of alkali or acid added to keep the pH constant is near-

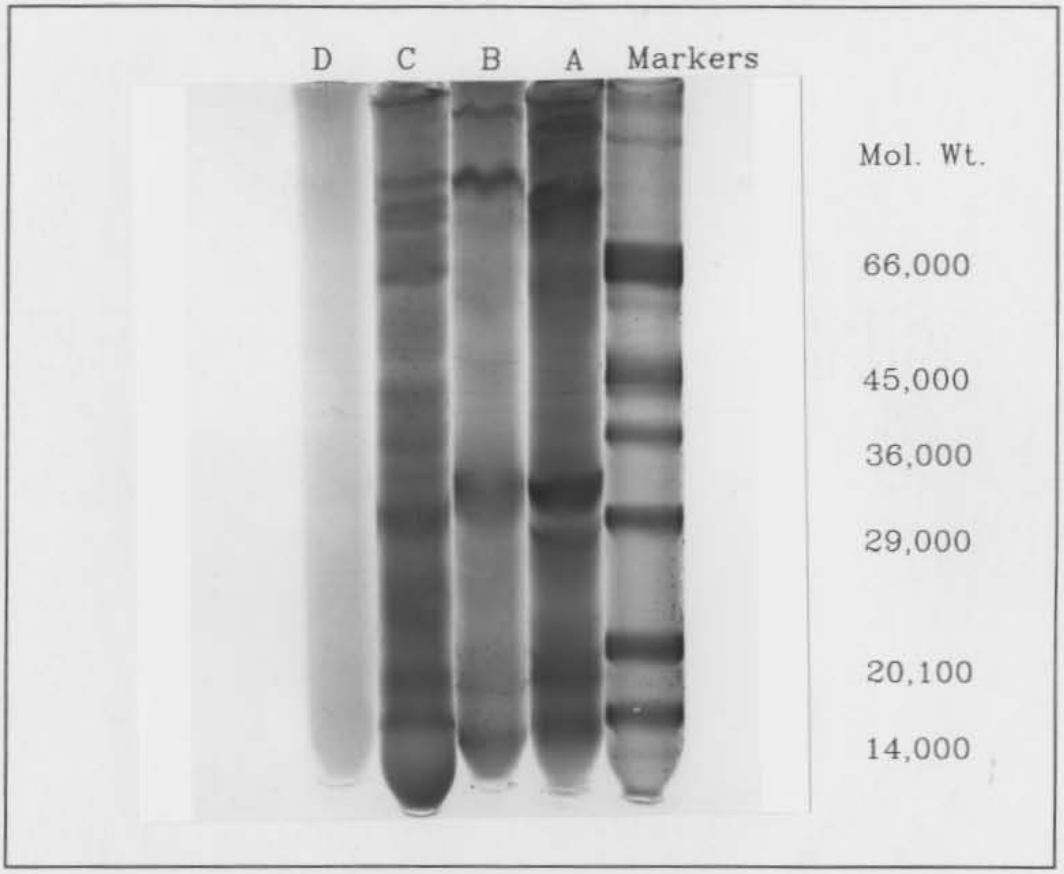

D
C
B
A
Markers
Mol. Wt.
66,000
45,000
36,000
29,000
20,100
14,000

Figure 4.19: **SDS-PAGE of capelin proteins and their hydrolysates** (A, Capelin proteins at zero time of hydrolysis; B, Capelin protein hydrolysates (CPH) from autodigestion; C, CPH prepared by cod pepsin hydrolysis; D, CPH prepared by Alcalase hydrolysis. All of the samples were prepared under conditions described in Table (4.9). SDS gels were prepared according to the procedure described in Section 3.3.12, similar to that for cod pepsins.)

proportional to the number of peptide bonds broken. The pH-stat records of two digestions with different proteolytic enzymes (crude cod acid protease and crude seal gastric protease) showed clearly that after the addition of the protease, the hydrolysis of the fish particles was characterized by an initial rapid phase during which a large number of peptide bonds were ruptured per unit time (Fig. 4.20). The rate of enzymatic hydrolysis subsequently decreased, and the hydrolysis entered a stationary phase during which no apparent hydrolysis took place.

The bulk of soluble protein hydrolysate was released during the initial step of hydrolysis. It is of interest to note that no increase (sometimes even decrease) in the release of soluble hydrolysates was observed when additional enzyme was added to the reaction mixture during the stationary phase of hydrolysis (results not shown). Inhibition of hydrolysis by products might be responsible for this observation (Mohr, 1977). Although plastein reaction may also occur, presence of a high concentration of soluble peptides in the reaction mixture significantly reduces the rate of hydrolysis and the yield of soluble protein recovery.

At a molecular level, conformation of protein molecules dictate the extent and pattern of their breakdown by proteases (Rutman and Heimlich, 1974; Adler-Nissen, 1976). However, studies have shown that the highly organized arrangement of proteins in the intact tissue does not reduce accessibility of protein towards proteolytic attack (Mohr,1977). Under the hydrolysis conditions employed in this study, it is considered that fish proteins were not denatured or underwent any conformational

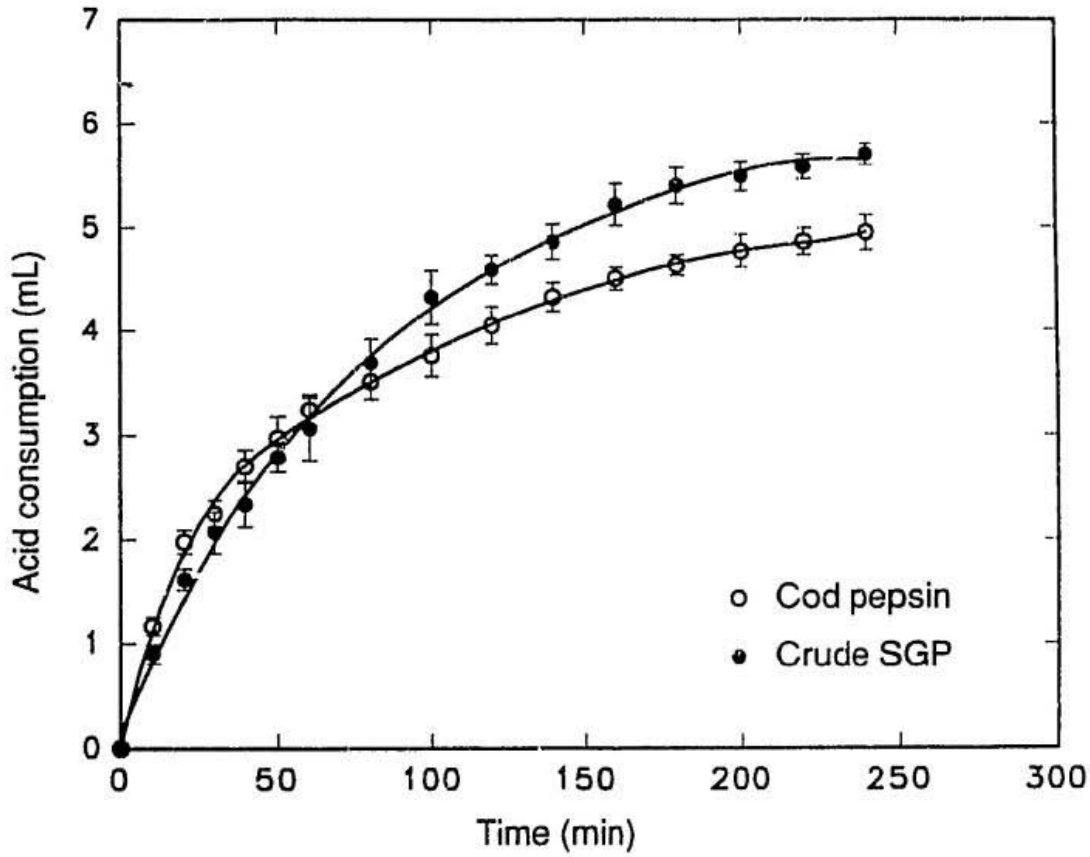

Acid consumption (mL)
Time (min)
Cod pepsin
Crude SGP
0
1
2
3
4
5
6
7
0
50
100
150
200
250
300

Figure 4.20: **Hydrolysis of capelin proteins with different enzymes.** The initial reaction mixture contained 50 g ground fish sample, 50 mL water, and 1.0 mL enzyme solution (5.0 mg/mL in 0.1 M acetate buffer, pH 6.0). The hydrolysis was controlled by pH-stat method with the addition of 2.0 M HCl to keep the pH at 3.0. Both experiments were performed at 25^0 C. Results are mean values of triplicate determinations.)

changes during the hydrolysis (all experiments were performed at pHs ranging from 3.0 to 8.2 at ambient temperatures). The hydrolysis proceeded as proteases were mixed with substrates both in molecular and particle states (Mohr, 1977).

Properties of protein hydrolysates

Protein hydrolysates prepared with varying degrees of hydrolysis possess different functional properties. Some functional properties of capelin protein hydrolysate so obtained were investigated and results are presented elsewhere (Shahidi *et al.*, 1992; 1993).

The proximate composition of protein hydrolysates depended greatly on the type of proteolytic enzyme used. Treatment with proteases reduced the residual lipid in the final hydrolysates (Table 4.9.) This is considered an important factor for storage stability of products. Although some investigators in the 1970's found that over 90% proteins could be recovered by aqueous extraction (Chu and Pigott, 1973; Shenouda and Pigott, 1975; 1976), the retention of lipids in the final product prevented extensive use of the recovered proteins. Since the polar and neutral fish lipids interact with some proteins such as actin to form lipid-actin complex, a major obstacle in the aqueous extraction procedure is to break the lipid-protein complex. The high lipid content in the final products leads to their instability and deterioration of their functional properties and loss of nutritional value.

Protease treatment provides a basis for the removal of structural lipids from a protein system. During hydrolysis, rapid changes occur in the structure of fish muscle

tissues. Electron microscopy of thin sections of cod muscle reveals that the myofibrillar proteins are extensively degraded during hydrolysis, whereas the elaborate membrane system of the muscle cell seems to be comparatively resistant to breakdown(Mohr, 1977). As hydrolysis proceeds, these membranes tend to round up and form vesicles. The structural lipids incorporated into membrane systems are retained and become enriched in the insoluble vesicles as hydrolysis proceeds. The lipid content of protein hydrolysate may be reduced by removing these structural lipids in membrane particle residues.

The protein hydrolysates have an isoelectric point of around pH 6.6, however, only about 26 percent of total crude proteins in the hydrolysate were precipitated at their isoelectric point. These proteins remained insoluble when the pH was further increased. The insoluble fraction of protein hydrolysate in neutral or alkaline pHs is designated as alkaline-insoluble proteins, the amount of which depended on the season of catch (result not presented).

Autolysis and its influence on the production of fish protein hydrolysates

There are many factors to be taken into consideration when investigating production of protein hydrolysates. The type of enzyme, pH, ratio of enzyme to substrate, and reaction temperature are important functions to be considered. The presence of proteolytic enzymes in the viscera of fish had a considerable influence on the production of fish protein hydrolysates (Gildberg, 1982). Results from this study showed that the autolytic enzymes of fish perform a very important function during the produc-

tion of fish protein hydrolysates (Table 4.9). Using autolytic enzymes alone without adding proteases produced protein hydrolysates with around 23% protein recovery at pH 3.0, at ambient temperature for 4 h. Prolonged autodigestion period will make more proteins be solubilized.

The protein hydrolysis of whole fish by native or autolytic enzymes enhanced the overall extraction of the fish protein at both acid and alkaline pHs, especially when the process was carried out at ambient temperatures (result not shown). The autolytic proteolytic enzymes degrade or modify fish proteins in both acid and alkaline extraction media. Alkaline extraction condition would favour further protein hydrolysis by autolytic enzymes (such as trypsin and chymotrypsin) of the viscera which exhibit greater activity at alkaline pH values. Acid extraction condition would favour enzyme activity of the catheptic and pepsin-like enzymes found in varying amounts in muscles and viscera of different species of fish. Enhanced protein solubilization may be attained by pre-digesting the fish mince prior to enzyme addition, which results in higher yields of protein extraction than procedures when no pre-digestion period is used (results not shown). Hence, it is important to control the duration between homogenization and addition of enzymes in order to ensure uniform quality of protein hydrolysates.

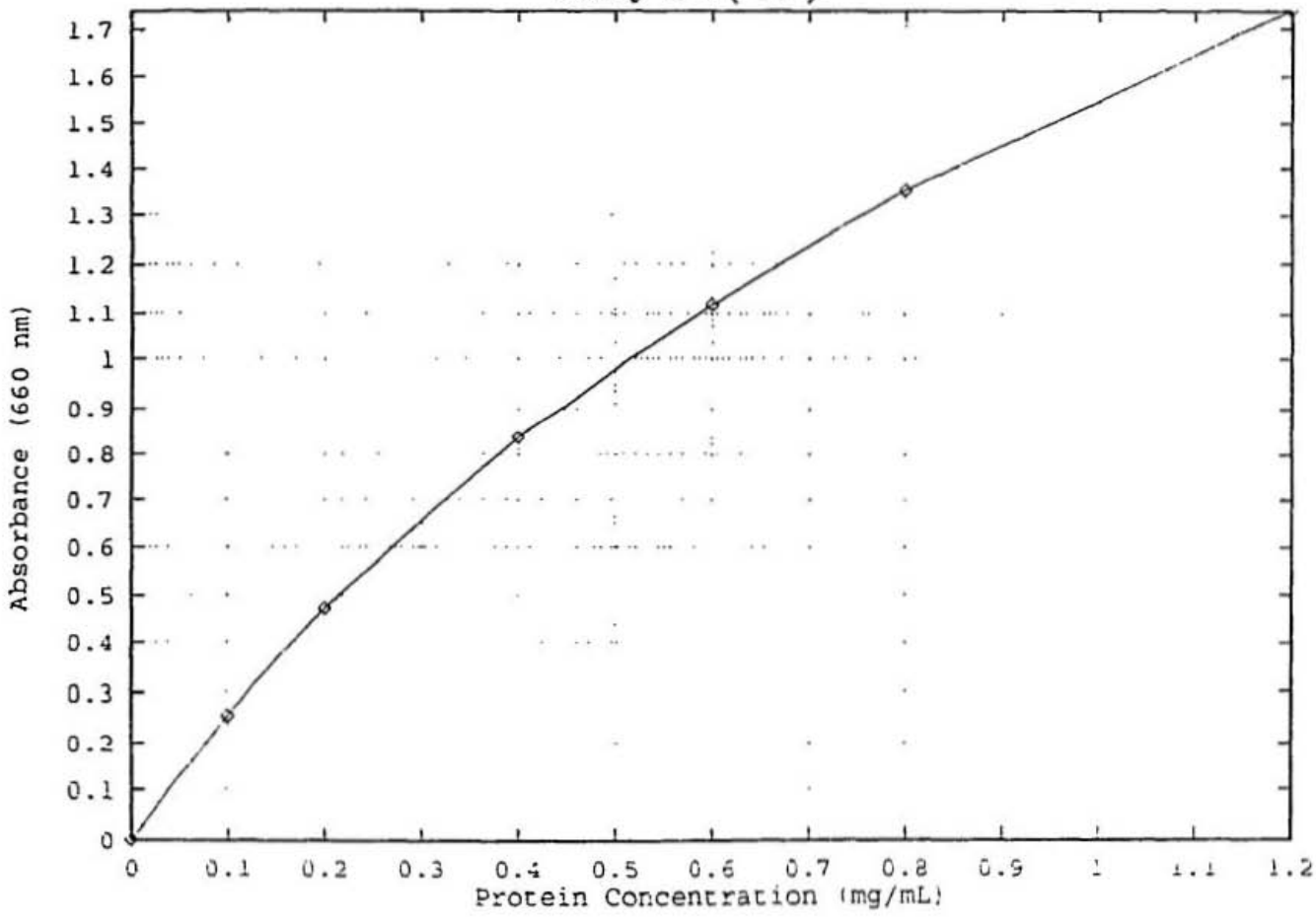
4.6 Conclusions

The co-extraction procedure established in this study allows effective isolation of both acid and alkaline proteases from cod viscera. Optimization of the co-extraction conditions allows both acid and alkaline proteases to be isolated with maximum recovery (recovery yields of 52% for acid proteases and 32% for alkaline proteases). The acid proteases contained three different gastric proteases designated as acid proteases A, B and C. In comparison with other designated groups for fish gastric proteases, acid protease B is characterized as fish pepsin II. Acid proteases A and C have properties similar to chymosin and gastricsin, respectively. Most of the properties of Atlantic gastric proteases (acid proteases A, B, and C) were similar to those from Greenland cod (designated as proteases 1, 2, and 3). The ion-exchange chromatography designed for the isolation of different gastric proteases was effective with high resolution in separation. The partially purified alkaline proteases co-extracted contained mainly trypsin with chymotrypsin activity.

Results of this study indicate that cod viscera is an excellent source of proteolytic enzymes. Crude or purified enzymes so obtained may be employed as a substitute for cold renneting in cheese making operations and for preparation of fish protein hydrolysates from processing discards and underutilized species.

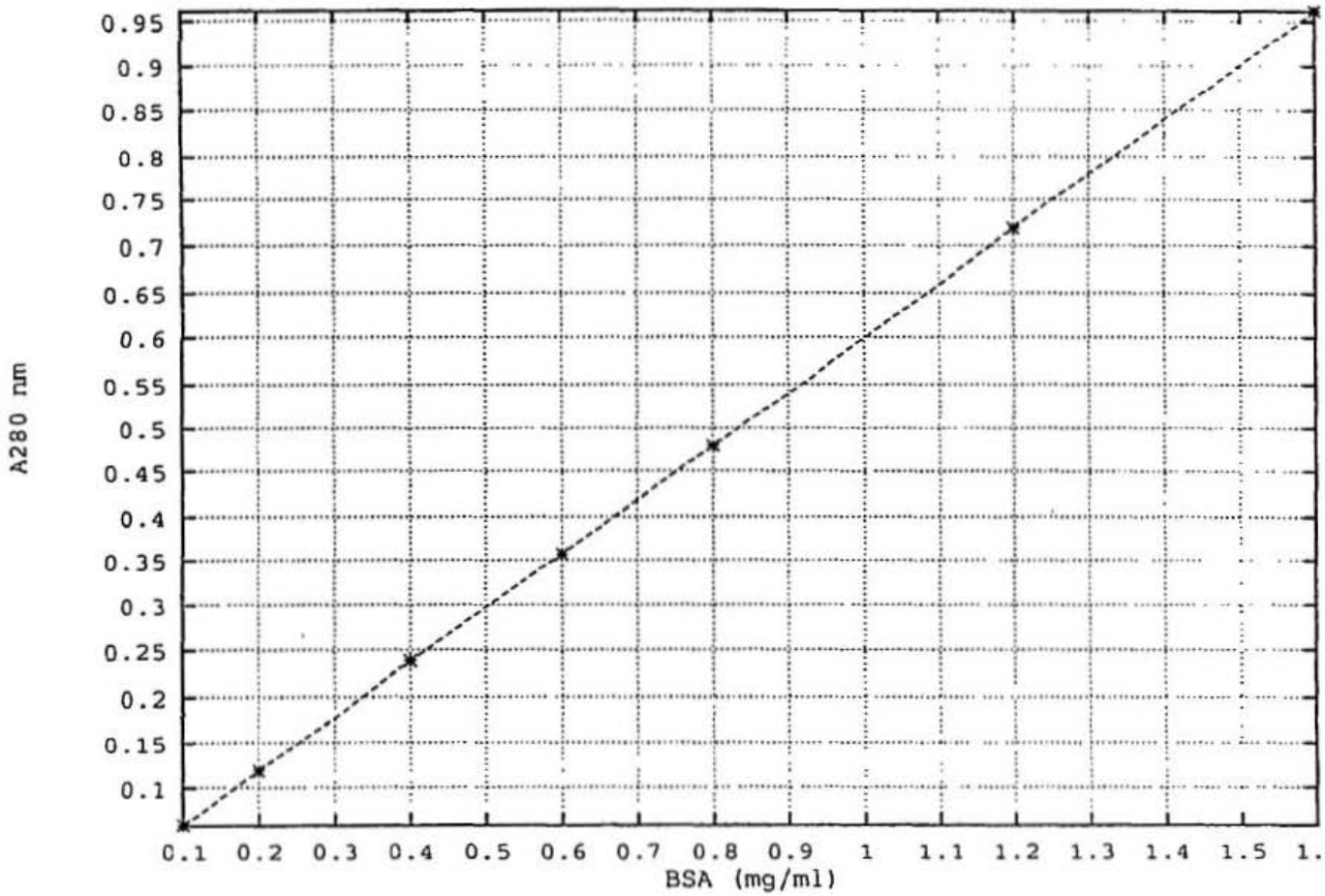
Appendix A.

Concentration of bovine serum albumin (BSA) measured by method of Lowry *et al* (1951).



Appendix B.

Absorbance of bovine serum albumin (BSA) at 280 nm



Appendix C.

1. Calculated results from rotatable experimental design by SAS program.

1. For acid proteases in supernatant.

Response Surface for Variable Y: AE

Response Mean	0.610140
Root MSE	0.021547
R-Square	0.9920
Coef. of Variation	3.5315

Regression	Degrees of Freedom	Type I Sum of Squares	R-Square	F-Ratio	Prob > F
Linear	3	0.265878	0.9108	190.9	0.0000
Quadratic	3	0.018123	0.0621	13.012	0.0085
Crossproduct	3	0.005589	0.0191	4.013	0.0844
Total Regress	9	0.289590	0.9926	69.305	0.0001

Residual	Degrees of Freedom	Sum of Squares	Mean Square	F-Ratio	Prob > F
Lack of Fit	3	0.002144	0.000715	8.035	0.1127
Pure Error	2	0.000178	0.000088923		
Total Error	5	0.002321	0.000464		

Parameter	Degrees of Freedom	Parameter Estimate	Standard Error	T for H0: Parameter=0	Prob > |T|	Estimate from Coded Data
INTERCEPT	1	1.192801	0.293343	4.066	0.0097	0.598833
X1	1	-0.402579	0.123051	-3.272	0.0222	0.122175
X2	1	1.090556	1.411465	0.773	0.4747	-0.135175
X3	1	0.008646	0.006560	1.318	0.2447	-0.005975
X1*X1	1	0.071770	0.013844	5.184	0.0035	0.058133
X2*X1	1	-0.828889	0.239412	-3.462	0.0180	-0.037300
X2*X2	1	-1.066667	4.485381	-0.238	0.8215	-0.002667
X3*X1	1	0.000267	0.001197	0.223	0.8325	0.002400
X3*X2	1	-0.001100	0.021547	-0.0511	0.9613	-0.000550
X3*X3	1	-0.000343	0.000112	-3.056	0.0282	-0.034267

Factor	Degrees of Freedom	Sum of Squares	Mean Square	F-Ratio	Prob > F	
X1	4	0.137480	0.034370	74.029	0.0001	pH
X2	4	0.151771	0.037943	81.724	0.0001	PAA (%)
X3	4	0.004645	0.001161	2.501	0.1709	T (C)

Canonical Analysis of Response Surface
(based on coded data)

Factor	Critical Value Coded	Uncoded	
X1	-2.828362	1.754474	pH
X2	-5.549846	-0.177492	PAA (%)
X3	-0.141692	13.583076	T (C)

Predicted value at stationary point 0.801579

2. For alkaline proteases in precipitate.

Response Surface for Variable Y: BE

Response Mean	0.148293
Root MSE	0.008277
R-Square	0.9893
Coef. of Variation	5.5816

Regression	Degrees of Freedom	Type I Sum of Squares	R-Square	F-Ratio	Prob > F
Linear	3	0.022591	0.7045	109.9	0.0001
Quadratic	3	0.004492	0.1401	21.853	0.0027
Crossproduct	3	0.004640	0.1447	22.574	0.0025
Total Regress	9	0.031722	0.9893	51.447	0.0002

Residual	Degrees of Freedom	Sum of Squares	Mean Square	F-Ratio	Prob > F
Lack of Fit	3	0.000282	0.000093944	3.094	0.2538
Pure Error	2	0.000060727	0.000030363		
Total Error	5	0.000343	0.000068512		

Parameter	Degrees of Freedom	Parameter Estimate	Standard Error	T for H0: Parameter=0	Prob > |T|
INTERCEPT	1	0.546301	0.112686	4.848	0.0047
X1	1	-0.107913	0.047270	-2.283	0.0713
X2	1	-1.077361	0.542206	-1.987	0.1036
X3	1	0.004381	0.002520	1.739	0.1426
X1*X1	1	-0.000535	0.005318	-0.101	0.9238
X2*X1	1	0.726111	0.091969	7.895	0.0005
X2*X2	1	-13.123333	1.723034	-7.616	0.0006
X3*X1	1	0.000325	0.000460	0.707	0.5113
X3*X3	1	-0.000140	0.000043076	-3.240	0.0229

Factor	Degrees of Freedom	Sum of Squares	Mean Square	F-Ratio	Prob > F	
X1	4	0.012256	0.003064	44.723	0.0004	pH
X2	4	0.023175	0.005794	84.565	0.0001	PAA (%)
X3	4	0.001134	0.000284	4.138	0.0757	T (C)

Canonical Analysis of Response Surface
(based on coded data)

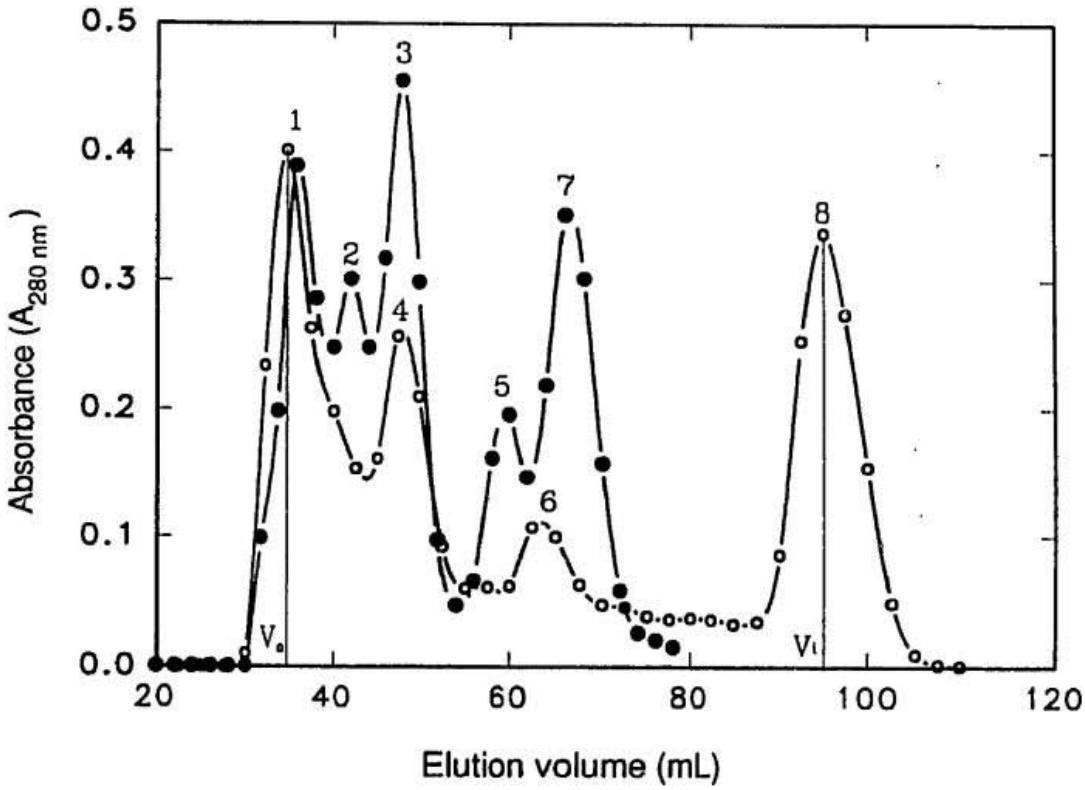
Factor	Critical Value Coded	Uncoded	
X1	3.419235	7.377312	pH
X2	1.062249	0.153112	PAA (%)
X3	-0.075432	14.245679	T (C)

Predicted value at stationary point 0.096975

Appendix D

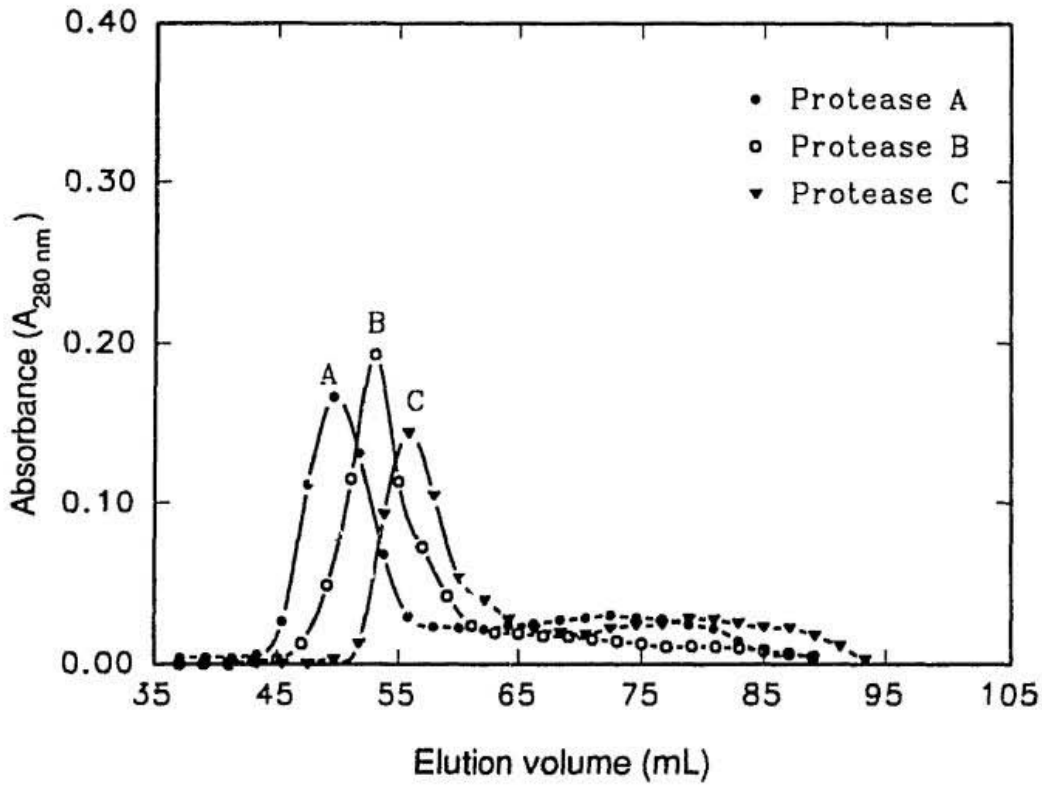
Estimation of relative molecular mass (M_r) of proteins by gel permeation chromatography.

1. Calibration of Sephadex G-75 gel permeation column



(The proteins and markers used in order of number presented were: 1), Dextran blue, 2 $\times 10^6$; 2), Bovine albumin, 66,000; 3), Egg albumin, 45,000; 4), Chicken ovalbumin, 44,000; 5), Soybean trypsin inhibitor, 20,100; 6), Equine myoglobin, 17,000; 7), α-lactalbumin, 14,200; 8), Vitamin B_{12}. The column, 1.5 $\times$ 100 cm in size, was eluted with 0.1 M acetate buffer, pH 5.5 at ambient temperature. The flow rate was 4.0 mL/h.)

2. Gel permeation chromatography of cod gastric proteases on Sephadex G-75 column



(The elution conditions were the same as those for the calibration of the column. The proteolytic activity was checked using haemoglobin as a substrate under standard assay conditions as described in Section 3.3.3 (the reaction time was 60 min). The K_d values for acid proteases A, B, and C are 0.2807, 0.3233. and 0.3508, respectively.)

Appendix E

Estimation of relative molecular mass (M_r) of proteins by SDS-PAGE[a]

Proteins (or enzymes)	No	Migration distance		Relative mobility		M_r
		Proteins	Dye	R_f	R_f, (ave.)	
Bovine albumin	1	1.70	6.00	0.283		
	2	1.70	6.10	0.279	0.282	66,000
Egg albumin	1	2.70	6.00	0.450		
	2	2.75	6.10	0.451	0.451	45,000
G-3-PD[b]	1	3.20	6.00	0.533		
	2	3.25	6.10	0.533	0.533	36,000
Carbonic	1	3.90	6.10	0.650		
anhydrase	2	3.95	6.10	0.648	0.649	29,000
Soybean trypsin	1	5.00	6.00	0.833		
inhibitor	2	5.10	6.10	0.836	0.835	20,100
α-lactalbumin	1	5.60	6.00	0.933		
	2	5.70	6.10	0.934	0.934	14,200
Acid protease	1	3.10	5.90	0.526		37,740[c]
A	2	3.10	5.95	0.528	0.527	(37,700)[d]
Acid protease	1	3.50	6.00	0.583		32,923[c]
B	2	3.60	6.10	0.590	0.587	(32,900) [d]
Acid protease	1	3.50	5.90	0.593		32,322[c]
C	2	3.55	5.95	0.597	0.595	(32,300) [d]

[a] SDS-PAGE refer to sodium dodecyl sulphate polyacrylamide electrophoresis. The calibration equation was calculated by Sigma Plot regression:
$log M_r = -0.989 R_f + 5.098$ ($R^2 = 0.997$).

[b] Glyceraldehyde-3-phosphate dehydrogenase.

[c] Calulated results from calibration equation.

d Results presented in the text of this thesis.

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