ISOLATION, CHARACTERIZATION, AND SOME APPLICATIONS OF TRYPSIN FROM GREENLAND COD (GADUS OGAC)

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ISOLATION, CHARACTERIZATION, AND SOME APPLICATIONS OF TRYPSIN FROM GREENLAND COD (*GADUS OGAC*)

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ABSTRACT

Trypsin, E.C. 3.4.21.4, was isolated from the pyloric ceca or the intestines of the Greenland cod (*Gadus ogac*) and purified by the successive steps of ammonium sulfate fractionation, acetone precipitation, and affinity chromatography using soybean trypsin inhibitor coupled to CNBr-activated Sepharose 4B. Some of the physical and catalytic properties of the Greenland cod trypsin were compared with those of commercially available bovine pancreatic trypsin. The Greenland cod trypsin was shown to be homogeneous by analytical polyacrylamide gel electrophoresis and also by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Although certain properties of Greenland cod trypsin were similar to those of bovine trypsin, there were also some significant differences between the two trypsins.

Greenland cod trypsin and bovine trypsin were alike with respect to various criteria. The pH activity profile of Greenland cod trypsin was similar to that of bovine trypsin. Likewise the amino acid composition of Greenland cod trypsin revealed that it was rich in potential acidic amino acid residues as has been reported for trypsin from bovine and other sources. The Greenland cod trypsin was similar to bovine trypsin in being able to hydrolyze ester and amide linkages involving the carboxyl group of arginine. The two trypsins were both inhibited by phenyl methyl sulfonyl fluoride, trasylol and soybean trypsin inhibitor and also by the thiol reagents, 2-mercaptoethanol and dithioerythritol, and were both effective in preventing milk oxidation induced by copper. The molecular weight of Greenland cod trypsin, as determined by sodium dodecyl sulfate polyacrylamide gel

electrophoresis, was similar to values reported for trypsin from bovine and other sources.

The Greenland cod trypsin differed from bovine trypsin in the following respects : the Greenland cod trypsin was most stable at alkaline pH, unlike bovine trypsin which was stable at acid pH; the Greenland cod trypsin was heat labile while bovine trypsin was heat stable. The temperature coefficients and activation energies for the hydrolysis of amide, ester and protein substrates were considerably lower for Greenland cod trypsin than bovine trypsin. The apparent Michaelis-Menten constants (Km') and molecular activities for the hydrolysis of substrates were considerably higher for the cod enzyme than the bovine enzyme. Based on the amino acid compositions, the calculated average hydrophobicity of Greenland cod trypsin was considerably lower than that of bovine trypsin and the cod enzyme contained fewer cysteine residues than bovine trypsin. Greenland cod trypsin activity was depressed to a greater extent by thiol reagents than that of bovine trypsin. Finally, the peptide maps of the two trypsins resulting from the cleavage by papain and cyanogen bromide were different.

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List of Abbreviations

BAEE : benzoyl-l-arginine ethyl ester

BAPA : benzoyl-l-arginine-p-nitroaniline

BT : bovine trypsin (from Sigma Chemical Company)

CD : circular dichromism

DFP : di-isopropyl fluorophosphate

DMS : dimethyl sulfoxide

GCT : Greenland cod trypsin

GPDH : D-glyceraldehyde-3-phosphate dehydrogenase

LDH : muscle type lactate dehydrogenase

MRW : mean residue weight

PMSF : phenyl methyl sulfonyl fluoride

SBTI : soybean trypsin inhibitor

TAME : toluene-sulfonyl-l-arginine methyl ester

TBA : thiobarbituric acid

TCA : trichloroacetic acid

TIU : trypsin inhibitor unit

Chapter 1

INTRODUCTION

1.1 Historical background

The name trypsin was first coined by Kuhne (1877) and was used for many years to describe the entire proteolytic activity of pancreatic juice. It was also the first substance to be classified as an enzyme. However, when other proteolytic enzymes were isolated from the pancreatic juice, the name trypsin was restricted to a single endopeptidase synthesized by the pancreas in the form of an inactive precursor called trypsinogen. Much of the current understanding of trypsinogen and trypsin at the molecular level has been possible as a result of the pioneer work of Northrop et al. (1948).

1.2 Classification and general properties of trypsins

On the basis of the current meaning of the word, the Enzyme Commission of the International Union of Biochemists have assigned trypsin a number E.C.3.4.21.4, to imply that it is a hydrolase acting on peptidyl-peptide bonds within the protein molecule and possessing an unusually reactive serine residue in its active site. Because of the presence of the highly reactive serine residue in the active center of trypsin, the enzyme has been classified with other similar enzymes as serine proteases.

According to Kiel (1971), trypsins have molecular weights ranging from 20,000 daltons to 25,000 daltons, and catalyze preferentially the hydrolysis of ester and peptide bonds involving the carboxyl group of arginine and lysine. The serine hydroxyl group in the active center of trypsin reacts with, and becomes irreversibly inhibited by reagents like diisopropylfluorophosphate (DFP) according to workers like Jansen et al. (1949) or by phenyl methyl sulfonyl fluoride (PMSF) according to Fahrney and Gold (1963). So various workers like Hjelmeland and Raa (1982) and Jany (1976) have used the susceptibility of a particular protease to inhibition by DFP or PMSF as indicative of that particular enzyme belonging to the family of enzymes known as serine proteases. According to Kiel (1971), trypsins show maximum activity towards their substrates within the pH range of 7.0 to 9.0.

1.3 Occurrence of the enzyme in the precursor form

The enzyme is synthesized as the inactive zymogen called trypsinogen. According to Kunitz and Northrop (1936), the enzyme is activated naturally by enterokinase, itself an enzyme secreted in small amounts by the mucous membrane of the stomach. Once some 'active' trypsin is formed from the inactive zymogen precursor by the action of enterokinase, the 'active' trypsin potentiates activation of the rest of the zymogen. According to Davie and Neurath (1955), the activation of the zymogen involves the cleavage of a few amino acid residues from the N-terminal end of the inactive zymogen, a process described as 'limited proteolysis'.

After the new N-terminal has formed from the activation step, the protein is said to undergo conformational changes, according to Neurath *et al.* (1956), leading to a catalytically active configuration. Based on the studies by Matthews *et al.* (1967) and Sigler *et al.* (1968) on the three dimensional structure of α-chymotrypsin, it has been suggested that the conformational changes arise from an ion-pair formation between the positively charged new N-terminal and a negative carboxyl group of aspartate in the interior of the molecule.

1.4 Industrial use of proteolytic enzymes

1.4.1 Criteria for the choice of an enzyme for a particular operation

Proteolytic enzymes, also known as proteases or proteinases, degrade protein molecules by catalyzing hydrolysis of peptide bonds. They belong to the group of enzymes known as hydrolases and are of fundamental importance in several industrial processes. According to Godfrey and Reichelt (1983), over 80 per cent of all industrial enzymes are hydrolases of which approximately 60 percent are proteases. Of the enzymes used as food processing aids, the proteases are used most extensively where they act to improve the quality, stability or solubility of foods, as in baking, brewing, cheesemaking and also meat processing. Some of the features of the proteases, as well as other enzymes which make them useful in industrial applications include the following : (i) they are derived from plant, animal and microbial sources and are invariably non-toxic substances that are able to catalyze specific reactions without eliciting undesirable side reactions ; (ii) they are active at very low concentrations under mild conditions of temperature and pH ; (iii) they can be inactivated after they have been employed to achieve the desired effect in the material being processed.

Even though all living organisms are potential sources of useful enzymes for industrial operations (Godfrey and Reichelt, 1983), the greatest variety of industrial enzymes are presently derived from microbial sources with only a limited number coming from plant and animal sources. Enzymes of plant sources used extensively in industry include papain, ficin, bromelain and amylases of cereal while the animal enzymes of considerable importance are trypsins, lipases and rennets (Godfrey and Reichelt, 1983). In spite of this fact, only very few micro-organisms, 11 fungi, 8 bacteria and 4 yeasts (Godfrey and Reichelt, 1983) are used to produce all the different microbial proteases, because few of these organisms have been stringently evaluated and accepted as safe. It is almost predictable that the use of proteases from plant and animal sources would increase substantially in the forseable future for the following reasons : (i) the few micro-organisms recognised as safe to use as sources of industrial enzymes are almost stretched to the limit, and (ii) even though there may be other potentially safe micro-organisms not in use now, the problems involved in obtaining clearance for their use, as described by Denner (1983), are discouraging to investors which tend to favor the shift to the use of animals and plant materials. Another factor in favor of animals as sources of industrial enzymes is the better economic use of those parts of the animal generally discarded as waste. The utilization of such materials as sources of industrial enzymes would not curtail the availability of food material for human consumption. At the same time, it would serve as a means of minimizing the waste disposal problem of the fishing industry.

According to Yamamoto (1975) and Godfrey and Reichelt (1983), whether or not a particular enzyme would be suitable to use in a particular industrial application would depend on several factors such as the specificity of the enzyme, its tolerance to pH and temperature as well as other factors like the availability of the enzyme, technical service support and cost. Other factors of considerable importance include the presence of inhibitors and / or activators in the processing material. So that if a reaction needs to be carried out at a high temperature, as in the tenderization of meat, a heat stable enzyme like papain would be more suitable to use, a fact that has been utilized by Metz et al. (1975) in the preparation of a barbecue sauce (containing papain). A reaction that proceeds at low pH such as the chillproofing of beer, requires an enzyme that is stable and active under acid conditions. Or if it is desirable to carry out a reaction at lower temperatures, then a protease that is active and stable at lower temperatures should be the enzyme of choice.

1.4.2 **Other applications of proteolytic enzymes in industry**

Proteases are also used to prepare protein hydrolysates from proteinaceous materials like fish and legumes. For instance, Spinelli and Koury (1974) described a process using proteases to modify whole fish or the muscle protein fraction of fish to organoleptically stable fish protein concentrates (FPC). Yokotsuka et al. (1975) developed a process for preparing soy milk from defatted soybeans using an acid protease.

Proteases have also been employed in the leather industry to remove hair or fur from hides and skins and also for bating dehaired hides. For example, Gagne and Neel (1974), developed a process using an alkaline protease immobilized on an insoluble support to dehair or defur animal hides. Monsheimer and Pfleiderer (1976) also described a process for bating dehaired hides with an alkaline protease.

Proteases have also been used to manufacture so-called enzyme detergents. According to Barfoed (1983), the idea of incorporating enzymes in detergents was prompted by the belief that it would facilitate the cleaning of heavily-soiled clothes such as those used by workers in the fishing industry, slaughter houses and hospitals. Because of the nature of detergents, the enzymes that have been used in making enzyme detergents are alkaline proteases. Fukumoto et al. (1974) have developed a process for growing bacteria in the presence of detergent to produce a protease in the detergent. According to Barfoed (1983), a greater proportion of commercially available detergent enzymes are alkaline bacterial proteases of the serine type. Barfoed (1983) also pointed out that the use of such enzymes minimize energy costs by making it possible to clean or wash materials at relatively low temperatures than would otherwise be the case in the absence of the enzymes.

1.4.3 Some other applications of proteases

1.4.3.1 Fermented fish products

One way of preserving or improving the quality of fish is by fermentation with endogenous enzymes or added proteolytic enzymes to convert fish to sauces, pastes, soup stocks, protein concentrates, etc. Another purpose for adding proteases to fish undergoing fermentation is to accelerate the ripening of certain products such as 'matjes'. Proteolytic enzymes that have been used to make fermented fish products have come from either plant, animal or microbial sources. For example, Beddows et al. (1976) described the preparation of fish hydrolysate from mackerel using bromelain as the proteolytic enzyme. Unilever Ltd., of Britain (1975) described a process for the production of salted herring using a mixture of trypsin and chymotrypsin. Murayama et al. (1962) described the use of fungal enzymes for the preparation of fish sauces from several species of fish.

Fermented fish products are very popular in Asian countries and Europe. The processing of fish to fermented fish products is usually done to cut down the waste that arises because they are either not acceptable to consumers or there is a seasonal glut. Equally important is the fact that in the Asian countries, proper fish handling and storage facilities are lacking and fermentation constitutes a major mode of preserving fish to ensure supply in the lean season. Elsewhere, fermented fish products serve mainly as delicacies.

Fermented fish products are grouped into three categories, based on the method used to form the finished product as follows : (i) traditional products in which fermentation is carried out by the enzymes of the flesh and digestive system of the fish being fermented, in the presence of high salt concentration; (ii) traditional products where fermentation is carried out by the combined action of fish enzymes of the flesh and entrails, and microbial enzymes in the presence of salt. In this procedure, the microbial enzymes are added as a starter, i.e. they are usually micro-organisms growing on some form of cereal like cooked rice or maize; and (iii) non-traditional products obtained by accelerating the pace of fermentation either with enzymes or by chemical hydrolysis.

Proteases have also been applied to liquefy fish muscle as a means of extracting protein from fish. One objectionable outcome of this process is the concomitant formation of bitter peptides. In order to overcome the bitterness problem, it has been suggested that the degree of hydrolysis be carefully controlled. Mackie (1974), used various proteases to hydrolyze Atlantic cod (*Gadus morhua*), and found that some of the enzymes, especially trypsin, hydrolyze the muscle protein to a lesser extent than other proteases like pronase and bromelain. One way of circumventing the problem of bitter peptide formation due to excessive proteolysis using enzymes with broad specificities would probably be to use a protease like trypsin, which has a relatively narrow specificity.

1.4.3.2. Prevention of milk oxidation by trypsin

Milk oxidation is an undesirable phenomenon in the dairy industry. According to Anderson (1939), the milk oxidation problem was especially noticeable in the winter months and some of the measures adopted to avoid the development of the problem included the following : (i) elimination of rusty containers in the handling of milk, (ii) the elimination of exposed copper, (iii) discarding the first 10 to 30 gallons of milk flowing through the system in the plant, (iv) sterilizing all equipment with hot water at 82°C instead of using chlorine for that purpose, and (v) pasteurizing all fresh milk promptly.

Anderson (1939) demonstrated that the addition of small amounts of pancreatic enzyme to milk prevented or retarded the development of oxidized flavor and the active component in the pancreatic juice responsible for the prevention of the oxidized flavor was suspected to be trypsin. Since then, workers like Lim and Shipe (1972) and Olson and Brown (1944) have demonstrated that trypsin treatment of raw milk renders the milk resistant to oxidation.

1.5 Some advantages that would be derived from processing foods at low temperatures

Although certain food processing applications require thermostable enzymes, there are several disadvantages associated with processing food at elevated temperatures. Some of the disadvantages in processing food at elevated temperatures include: (i) high energy cost; (ii) destruction of heat labile, essential components in food materials; (iii) proliferation of microbial growth; (iv) destruction of raw materials or products of reaction, and (v) enhancement of undesirable side reactions. Some disadvantages of using thermostable enzymes include the deleterious effects they would have on a finished product if they survive a treatment like pasteurization, commonly used to inactivate enzymes after food processing. On account of the foregoing, thermal instability can be regarded as a desirable property of enzymes in certain food process operations.

1.6 Concept of low temperature adaption

Because groups of living organisms (eg. vertebrate animals) have basically the same functional classes of enzymes to enable them to carry out virtually the same types of chemical reactions, it is to be expected that homologous enzymes from different organisms would have to carry out a given physiological function at strikingly different temperatures. Since several organisms subsist at extremely low temperatures it is of interest to determine how some important biological molecules such as enzymes, have become adapted to such extreme climatic conditions. For instance, the question has been asked if poikilotherms are endowed with enzymes that are more effective catalysts at lower temperatures than their homologs from organisms adapted to the warm environment.

According to workers like Bullock (1955), homologous enzymes from species which are adapted to widely different temperatures hydrolyze their substrates at similar rates, at their respective cellular temperatures. The adaptations to offset the influence of temperature on rates of biological reactions are known as "temperature compensations" and would appear to be important where the reaction rate is first order rather than zero order.

Very little work has been done on extracellular enzymes, though Hofer et al. (1975) investigated the relationship between the substrate binding affinities of very crude preparations of trypsins from various sources and their ambient temperatures. How Hofer et al. (1975) could attribute their findings exclusively to the action of the trypsins (in their very crude extracts) on the synthetic substrate, BAPA, remains a doubtful phenomenon. However, considerable amount of work has been done on intracellular enzymes and according to investigators like Hochacka and Somero (1973), poikilotherms have adjusted their catalytic activity in one, two or all three of the following possible ways : (i) by altering the levels or concentrations of enzymes present in the system; (ii) by changing the type of enzyme present in the system; and (iii) by modifying the catalytic efficiencies of pre-existing enzymes.

The mechanism involving the modification of catalytic efficiencies of enzymes is known as the "modulation strategy", and is thought to involve changes in the kinetic and thermodynamic properties of the enzyme like the substrate turnover numbers, the activation energy (Ea) and hence the activation free energy ΔG, substrate binding affinity (Km') and specificity of the enzyme.

Workers like Somero and Low (1976) and Low et al. (1973) have demonstrated that enzymes from organisms adapted to the cold environment have higher substrate turnover numbers than their homologs from organisms adapted to the warm environment. For example, Low et al. (1973) using intracellular enzymes from rabbit, chicken, tuna, halibut, lobster and cod, demonstrated that the turnover numbers of muscle type lactate dehydrogenase (LDH) from tuna was approximately 4.6 times greater than its counterpart from rabbit at 5°C. Similarly, D-glyceraldehyde-3-phosphate dehydrogenase (GPDH) from cod was about 8 times more active than its homolog from rabbit when comparisons were made at 5°C.

The temperature coefficients (Q_{10}) for the hydrolysis of substrates by cold adapted enzymes have also been demonstrated to be lower than those of their counterparts adapted to the warm environment. For example, while the molecular activity of GPDH from rabbit increased by approximately 30-fold in going from 5°C to 35°C, that of lobster GPDH increased by only 10-fold over the same temperature range (Cowey, 1967). Somero (1975) suggested that the higher molecular activity of the cold adapted enzymes is a consequence of their relatively more flexible structures compared to those of their counterparts from organisms adapted to the warm environment.

Low et al. (1973) and Cowey (1967) have reported that homologous enzymes from organisms adapted to different environments have activation energies that correlate with their respective habitat temperatures. For example, the energies of activation of GDPH from rabbit and lobster were reported by Low et al. (1973) as 19.0 kcal/mole and 14.5 kcal/mole respectively, and the energies of activation of muscle type LDH from rabbit and halibut were reported by the same workers as 13.1 kcal/mole and 9.3 kcal/mole respectively.

Several workers, including Koffler et al. (1957), Ushakov (1964), and Light et al. (1969), have observed an inverse relationship between the catalytic efficiency of homologous enzymes adapted to different temperature regimes and their thermal stability. For example, Kaplan (1965) observed that enzymes from organisms adapted to the cold tend to have lower thermal stabilities than their counterparts adapted to the warm environment.

Some workers, including Cowey (1967) and Hazel and Prosser (1974) suggested that the Km' of enzymes are temperature dependent, with those of cold adapted enzymes generally showing greater positive modulation than their counterparts adapted to the warm environment. The positive correlation between the apparent Km' and temperature in the case of polkilotherms is thought to be of adaptive value, according to workers like Hazel and Prosser (1974) in so far as it assures enhanced substrate binding at lower temperatures which leads to an increase in reaction rates. It is expected that the latter adaptation would be of functional value where substrate concentration in situ is less than "saturated" relative to enzyme concentration.

Carey et al. (1971) and Linthicum and Carey (1972) have observed that the body temperature of polkilotherms are either at or within 1°C of the ambient water temperature. From this, it is implied that the digestive enzymes of fish are adapted to reflect an interdependence of environmental temperature and food utilization.

1.7 Survey of some methods available for the preparation of trypsinogen or trypsin

Methods available for the preparation of the zymogen include the acid extraction of the tissue, followed by ammonium sulfate fractionation and crystallization described by Kunitz and Northrop (1936) and Northrop et al. (1948). Other workers like Tietze (1953) and Balls (1965) prepared homogeneous trypsinogen from the product obtained using the procedure described by Northrop et al. (1948), by recrystallization at pH 7.8 in the presence of trypsin inhibitors. Schroeder and Shaw (1968) also prepared homogenous trypsinogen using chromatography on sulfoethyl- (SE) Sephadex column. Porcine and ovine trypsinogens have also been prepared by workers like Charles et al. (1967) using ammonium sulfate fractionation followed by chromatography on carboxymethyl- (CM) cellulose.

It has been suggested by McDonald and Kunitz (1941) that trypsin prepared by the method of Kunitz and Northrop (1936) has low yield and low specific activity; they attributed this to the partial conversion of some of the zymogen to "inert protein", which cannot be changed to the active trypsin by any known means. According to McDonald and Kunitz (1941), the formation of the "inert protein" from the zymogen is completely prevented if the autoactivation process is made to proceed in the presence of calcium ions. In the presence of calcium ions, trypsinogen is said to be quantitatively converted to the active enzyme, trypsin.

Other investigators have directly isolated the active enzyme without first extracting it as the zymogen. For example, Winter and Neurath (1970) purified a trypsin type enzyme from the starfish *Evasterias trochelli* by preparing an acetone powder of the tissue, or homogenizing the tissue in phosphate buffer, pH 6.5, and fractionating it with ammonium sulfate, followed by chromatography on a dimethylaminoethyl- (DEAE) cellulose column. Camacho et al. (1970) purified two trypsin-type proteases from the pyloric ceca of the starfish *Dermasterias imbricata* by homogenizing the tissue in cold tris buffer and fractionating the supernatant with solid ammonium sulfate. The fraction precipitating between 40% and 60% saturation was collected and redissolved in tris buffer and precipitated with acetone. The acetone fraction was redissolved in the extraction buffer and chromatographed first on a Sephadex G-100 column then on a DEAE cellulose column. Bundy and Gustafson (1973) isolated a trypsin-type protease from *Pisaster giganteus* by preparing an acetone powder of the tissue, then stirring the powder in tris buffer, pH 8.1, and fractionating the supernatant obtained by centrifugation with solid ammonium sulfate. The precipitate formed was dissolved in the extraction buffer, dialyzed against the same buffer and chromatographed on a Sephadex G-100 column. Gates and Travis (1969) isolated trypsin from shrimp by preparing an acetone powder of the digestive glands. The acetone powder was stirred in 0.1M sodium borate buffer, pH 8.0, and centrifuged to obtain a clear supernatant which was fractionated with solid ammonium sulfate and the fraction precipitating between 40% and 60% saturation collected by centrifugation and redissolved in 0.01M tris-HCl buffer, pH 8.0. The solution formed was chromatographed on a Sephadex G-75 column, then on a DEAE-Sephadex A-50 column. Ching-San Chen et al. (1978) purified trypsin type enzymes from the antarctic krill *Euphasia superba* by homogenizing whole krill in 0.1M phosphate buffer, pH 7.0, and fractionating the supernatant formed from the centrifugation of the homogenate with solid ammonium sulfate. The fraction precipitating between 30% and 70% saturation was dissolved in phosphate buffer, dialyzed and precipitated with acetone, then centrifuged to recover the trypsin-containing material which was chromatographed first on a Sephadex G-75 column, then on a DEAE-Sephadex A-50 column. Hjelmeland and Raa (1982) purified two trypsin-type enzymes from capelin *Mallotus villosus* by defatting the digestive tracts with carbon tetrachloride and fractionating the defatted homogenate with solid ammonium sulfate and dissolving the fraction precipitating between 30% and 70% saturation in 0.0125M tris-HCl buffer, pH 8.0. The ammonium sulfate fraction was slowly percolated through an affinity column of benzamidine-CH-Sepharose 4B, then rechromatographed first on a Sephadex G-100 column, then on a DEAE-Sephadex A-25 column. Katoh et al. (1978) described the performance of affinity chromatography columns, and the procedure involved elution of trypsin in a Sepharose 4B-soybean trypsin inhibitor (SBTI) column. Katoh et al. (1978) equilibrated the Sepharose-SBTI affinity column with 0.05M tris-HCl buffer containing 0.5M NaCl and 0.02M $CaCl_2.2H_2O$, pH 7.8, and eluted the trypsin with 5mM HCl.

1.8 Survey of some methods available for determining trypsin activity

As noted elsewhere, trypsins hydrolyze bonds in proteins and peptides involving the carboxyl groups of arginine and lysine, as well as amides and esters of the two amino acids mentioned above. According to Rick (1974), the enzyme hydrolyzes the ester substrates more readily than the amide substrates and peptides least of all. A procedure has been described by

Anson (1939) using hemoglobin as substrate to determine tryptic activity. It involves a preliminary denaturation of the hemoglobin substrate in alkaline urea solution before applying the trypsin. The products of the hydrolysis are solubilized in trichloroacetic acid (TCA) solution and the tyrosine and tryptophan content of the resulting TCA solution is determined using the method of Folin and Ciocalteu (1927). Kunitz (1947) described a procedure involving the use of casein to determine the activity of trypsin; similar to the method of Anson (1939), the products formed from the hydrolysis of casein are solubilized in TCA and the tyrosine and tryptophan content determined by measuring the absorbance of the clear supernatant obtained by centrifugation, at 280 nanometers (nm).

Apart from the protein substrates like hemoglobin and casein, there are available synthetic substrates that are also used to assay for trypsin activity. A commonly used synthetic substrate for the estimation of trypsin activity is benzoyl-DL-arginine-p-nitroanilide (BAPA), a chromogenic substrate whose utilization was first described by Erlanger et al. (1961). In this procedure, trypsin splits the substrate according to the following equation :

$$O_2N\text{-}C_6H_4\text{-}NH\text{-}CO\text{-}CH(NH_2)\text{-}R \quad + \quad H_2O \longrightarrow R\text{-}CH(NH_2)\text{-}COOH \quad + \quad H_2N\text{-}C_6H_4\text{-}NO_2$$

One of the products of the reaction, p-nitroaniline (NA), has a yellow color and absorbs light strongly at 410 nm and this fact is used in measuring either the amount of substrate hydrolyzed or the amount of product formed.

Another synthetic substrate commonly used to determine trypsin activity is Nα-p-toluenesulfonyl-L-arginine methyl ester (TAME). The procedure for its use, described by Hummel (1959), is based on the fact that one of the products of the reaction, toluenesulfonyl-arginine (TA) absorbs light strongly at 247 nm. Trypsin splits the substrate according to the following equation :

$$CH_3-COO-CH(NH_2)-R + H_2O \longrightarrow R-CH(NH_2)COOH + CH_3OH$$

The substrate TAME, has the advantage of being very soluble in water compared to the amide, BAPA.

Another synthetic ester used to assay for trypsin activity is Nα-benzoyl-L-arginine ethyl ester (BAEE). It is split by trypsin according to the following equation :

$$CH_3CH_2-COO-CH(NH_2)-R + H_2O \longrightarrow R-CH(NH_2)-COOH + CH_3CH_2OH$$

The procedure involving the use of BAEE as a trypsin substrate was described by Schwert and Takenaka (1955). One of the products of the reaction, benzoyl-L-arginine (BA) absorbs light strongly at 253 nm and this fact is used to measure the amount of substrate hydrolyzed or the amount of product formed by the action of trypsin. According to Hummel (1959) and Schwert <u>et al</u>. (1948), TAME is much more rapidly hydrolyzed by trypsin than BAEE and it (TAME) is not hydrolyzed by chymotrypsin.

1.9 Purpose of study.

The purpose of the study was to isolate and characterize trypsin from the Greenland cod. Given that this animal has a habitat temperature of 2°C or less throughout the year. It was hypothesized that the trypsin from Greenland cod differs from that of other trypsins thus far characterized by being a more efficient catalyst at low temperature. Furthermore, it was hypothesized that the unique properties of such an enzyme could be exploited in certain food process operations which employ trypsins.

Chapter 2

MATERIALS AND METHODS

2. 1. Biological materials and specimens

The following materials were used for the purposes described below :

2. 1. 1 Greenland cod (*Gadus ogac*)

The Greenland cod fish, whose pyloric ceca and intestines were used as a source of trypsin, was caught from the Northwest river (lake Melville) in Labrador. The first batch of samples were collected in February of 1980. After the fish were caught by hand line, they were immediately dissected and the pyloric ceca and intestines removed and packaged separately then left to freeze at the ambient temperature of about -40°C. The frozen samples were stored at about -20°C and brought to St John's where they were stored at -80°C prior to extraction. Other batches of the Greenland cod were obtained in March of 1981 and September of 1982. These times, the fish were frozen whole and shipped from Labrador to St. John's, where the ceca and the intestines were promptly removed and rapidly frozen in liquid nitrogen before storing at -80°C prior to the extraction of trypsin. The fish caught in February and March were classified as 'winter fish' while those caught in September were classified as 'summer fish'.

2.1.2 Herring (*Clupea harengus harengus*)

The herring used for the making of 'matjes' were obtained fresh from the Lake Group of Companies Ltd., Grand Bank in April of 1981 and iced for approximately 8 h postmortem prior to the preparation of the 'matjes'. The herring were cleaned by removing the scales and fins and rinsing with de-ionized water. Some of the herring were decapitated and eviscerated while others were retained in the round form.

2.1.3 Squid (*Illex illecebrosus*)

The squid used for the fermentation studies were obtained fresh from the Fishery Products Ltd., Holyrood. The squid were cleaned by removing the heads, innards, fins and epithelia and rinsed thoroughly with de-ionized water prior to the immersion in the fermentation medium.

2.1.4 Raw cow's milk

The raw cow's milk used for the oxidized flavor studies was obtained from Kenmount Farms, St. John's.

2.1.5 Atlantic cod (*Gadus morhua*) fish meal

The Atlantic cod fish meal used for the degree of hydrolysis study was obtained from the National Sea Products Ltd., St. John's.

2.2 Chemicals

The following Chemicals used in the study were purchased from Sigma Chemical Company, St. Louis, U.S.A.: Acrylamide, ammonium persulfate, aprotinin (trasylol), Nα-benzoyl-DL-arginine-p-nitroanilide (BAPA), bovine serum albumin, calcium chloride dihydrate, casein purified powder, Coomasie brilliant blue (R-250), copper sulfate pentahydrate, cyanogen bromide activated Sepharose 4B, dithioerythritol, glycine, 2-mercaptoethanol, N,N'-methylene-bis-acrylamide, p-nitroaniline, papain (type III), phenyl methyl sulfonyl fluoride, sodium carbonate, sodium chloride, sodium dodecyl sulfate, sodium hydroxide, soybean trypsin inhibitor (type 1-S), N,N,N',N'-tetramethyl-ethylene diamine (TEMED), Nα-p-toluene-sulfonyl-L-arginine methyl ester, trichloroacetic acid, tris-(hydroxymethyl)-amino methane, trypsin (bovine pancreas, type III).

The chemicals listed below were purchased from Fisher Scientific Company Ltd.: Boric acid, copper acetate, ether (anhydrous), 88% formic acid, 30% hydrogen peroxide.

The following chemicals were purchased from J.T. Baker Chemical Company, Ltd.: Acetic acid (glacial), hydrochloric acid, methanol, potassium tartarate- 4-hydrate, 2-propanol.

Polyoxyethylene lauryl ether (brij 35) was purchased from BDH chemicals and riboflavin (electrophoresis grade) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) low molecular weight standards were purchased from Bio-rad laboratories.

2.3 Methods used in this study

2.3.1 Extraction of trypsinogen or trypsin

2.3.1.1 Treatment of pyloric ceca or intestines prior to extraction

The pyloric ceca or intestines were first converted to powder form by grinding in liquid nitrogen in a Waring blendor.

2.3.1.2 Extraction of soluble material from the pyloric ceca or intestine powder

A modified form of the procedure by Camacho et al. (1970) was used to isolate trypsin. The powder from the ceca or intestine was suspended in 0.05M tris-HCl buffer, pH 7.8, containing 0.5M NaCl and 0.02M $CaCl_2 \cdot 2H_2O$ at a ratio of 1 gram of tissue powder per 5 mL of extraction buffer. The suspension was stirred at 4°C using a magnetic stirrer for 6 h. The 'homogenate' so formed was centrifuged at 3,000 X g in a Sorvall RC-5 refrigerated centrifuge at 4°C for 30 min to rid it of insoluble material to obtain the first supernatant ('Sup' 1). To 'Sup' 1 was added brij 35 to a final concentration of 0.2% and the system was left to stand at 4°C overnight, then centrifuged at 10,000 X g for 30 min in the Sorvall RC-5 superspeed refrigerated centrifuge at 4°C to obtain the second supernatant ('Sup' 2).

2.3.1.3 Fractionation of 'Sup' 2 with solid ammonium sulfate

The 'Sup' 2 was fractionated with solid ammonium sulfate and the fraction precipitating between 40% and 60% saturation (relative to full saturation at 0°C) was collected by centrifugation in the Sorvall RC-5 centrifuge at 4°C and the pellet was redissolved in a minimum amount of cold (4°C) 0.05M tris-HCl buffer, pH 7.8, containing 0.5M NaCl and

0.02M $CaCl_2.2H_2O$. The solution was dialyzed overnight against 3 changes of 6 litres of the extraction buffer.

2.3.1.4 **Acetone precipitation**

The dialyzed solution from the ammonium sulfate step was treated with 3 times its volume of cold acetone (-20°C) and the system was left in a freezer at -20°C for 3 h and the precipitate formed was collected by centrifugation in a Sorvall RC-5 superspeed refrigerated centrifuge at 0°C and dried by first rinsing the precipitate with 20 mL of cold (-20°C) acetone : ether mixture (1:1). The precipitate was collected by centrifugation in a Sorvall RC-5 superspeed refrigerated centrifuge at 0°C. The precipitate was further dried by rinsing with 20 mL of cold ether at -20°C, then centrifuging the material obtained in the Sorvall RC-5 centrifuge at 0°C. The precipitate was then spread thinly in centrifuge bottles and the bottles were placed in front of a fan in the cold room at 4°C. The dried precipitate was redissolved in a minimum amount of extraction buffer and either stored at -20°C until needed to be applied onto the affinity chromatography column, or the preparation was (fully activated, in the case of extracts from the pyloric ceca, and) applied directly onto the affinity column.

2.3.1.5 **Affinity chromatography**

The affinity chromatography column was prepared by coupling SBTI to CNBr activated Sepharose 4B following the procedure developed by Pharmacia Fine Chemicals (1979). The semi purified trypsinogen preparation from the acetone step (in the case of extracts from the ceca) was activated to trypsin by standing at 4°C in the tris-HCl buffer (pH 7.8), for 24 h and was then pumped onto the affinity column at a rate of

15 mL/h. The unbound material was thoroughly washed off the column using the extraction buffer, after which the bound trypsin was eluted with 5mM HCl, using a modified form of the procedure by Katoh *et al.* (1978). Material passing through the column was collected in fractions of 4.8 mL/tube using a LKB Bromma 2112 Redirac fraction collector. The absorbance of the fractions at 280 nm, and the tryptic activity of the fractions absorbing light at 280 nm was assayed using either BAPA or TAME as substrate as described under 2.3.5.1 and 2.3.5.2 respectively. The fractions with tryptic activity toward the synthetic substrates were pooled and either dialyzed against 2mM HCl and lyophilized or were adjusted to pH 7.8 with the extraction buffer and stored frozen at −20°C.

2.3.2 Activation of trypsinogen to trypsin

The fractions from the various purification steps were incubated at 4°C for up to 72 h and their tryptic activities determined at intervals using BAPA as substrate, pH 7.8. The fraction from the affinity column was in 5mM HCl, while the other fractions were all in the extraction buffer (0.05M tris-HCl, containing 0.5M NaCl and 0.02M $CaCl_2.H_2O$, pH 7.8).

2.3.3 Electrophoresis in polyacrylamide gels

2.3.3.1 Establishment of homogeneity of Greenland cod trypsin

Disc electrophoresis was carried out according to the method of Davis (1964). The gel staining solution used contained 0.1% Coomasie blue R-250, 25% (v/v) methanol, 10% (v/v) acetic acid and 0.1% cupric acetate in water. The destaining solution was made up of 25% (v/v) methanol and 10% (v/v) acetic acid in water. The GCT was also electrophoresed using the method of Laemmli (1970).

2.3.3.2 **Molecular weight determination**

The (electrophoretic) method of Laemmli (1970) was used to determine the molecular weight of GCT. Some or all of the following substances were employed as molecular weight standards : phosphorylase. b. bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme. The staining solution contained 0.1% Coomasie blue, 25% (v/v) 2-propanol, 10% (v/v) acetic acid and 0.1% cupric acetate in water. The destaining solution was made up of 25% (v/v) 2-propanol and 10% (v/v) acetic acid in water.

2.3.4 **Protein determination**

Protein was determined using a simplified form of the procedure by Lowry et al. (1951) with crystalline bovine serum albumin as standard. A standard curve was prepared by analyzing samples containing 10 μg to 80 μg of bovine serum albumin.

2.3.5 **Trypsin assay**

The amidase, esterase and peptide hydrolytic activities of both GCT and BT toward synthetic and protein substrates were determined. The synthetic substrates used were BAPA and TAME. The hydrolysis of BAPA was measured by following the change in absorbance at 410 nm in the DU-8 spectrophotometer, based on the method of Erlanger et al. (1961), while the hydrolysis of TAME was measured by following the increase in absorbance at 247 nm in the DU-8 spectrophotometer, based on the method of Hummel (1959). The protein substrate used was casein and a modified form of the procedure by Kunitz (1947) was used.

Procedures in detail :

2.3.5.1 Hydrolysis of BAPA

Unless otherwise specified, the hydrolysis of BAPA was carried out as follows :

Substrate buffer : 0.05M tris-HCl, pH 8.2 containing 0.02M $CaCl_2 \cdot 2H_2O$.

Substrate stock solution : 1mM BAPA was prepared by dissolving 43.5 mg BAPA first in 1 mL of dimethyl sulfoxide (DMS) and 0.1 mL portions made up to 10 mL with the substrate buffer at 25°C.

Enzyme solution : GCT or BT was in 5mM HCl. Wherever possible, the enzyme solutions were adjusted to have approximately the same activity toward BAPA at 25°C. The proportion of reagents in the reaction mixture is specified in appendix A.

2.3.5.2 Hydrolysis of TAME

Unless otherwise specified, tryptic activity toward TAME was determined as follows :

Substrate buffer : 0.046M tris-HCl, pH 8.1, containing 0.01M $CaCl_2$

Substrate stock solution : 0.01M TAME was prepared by dissolving 37.9 mg TAME in 10 mL of de-ionized water.

Enzyme solutions : either the GCT or the BT in 5mM HCl. Whenever possible, the enzyme solutions were adjusted till they had approximately the same activity toward the substrate at 25°C.

The proportion of reagents in the reaction mixture is specified in appendix B.

2.3.5.3 Hydrolysis of casein

Unless otherwise specified, the hydrolysis of casein was carried out as follows:

Substrate buffer : either 0.2M borate-NaOH, pH 9.5, containing 5mM $CaCl_2$, or 0.1M sodium phosphate buffer, pH 7.6.

Substrate stock solution : 1% or 2% casein in substrate buffer. The suspension was heated in a boiling water bath for 15 min to solubilize the casein, then cooled to about 25°C or stored in a refrigerator for a maximum of 7 days. The proportion of reagents in the reaction mixture is as specified in appendix C.

Digestion of casein was stopped by adding 3 mL of 5% TCA and mixing thoroughly. The TCA treated reaction mixture was then held at room temperature (about 25°C) for 1 h and then centrifuged in a bench top Dynac TM centrifuge at 15,600 x g for 20 min. The absorbance of the clear supernatant was read at 280 nm. For the blanks, 3 mL of 5% TCA were added to 1.5 mL of the substrate, followed by 1 mL of the appropriate buffer and 0.5 mL of 5mM HCl and the system thoroughly mixed, then held at room temperature for 1 h, followed by centrifugation in the Dynac TM centrifuge at 15,600 x g for 20 min.

The trypsins were also used separately to hydrolyze urea-treated hemoglobin, squid muscle protein and cod fish meal with a pH stat (Metrohm Herisau Dosimat and Impulsomat E 473).

2.3.5.4 Hydrolysis of Urea-treated hemoglobin

Exactly 0.08 g of native hemoglobin was put in the reaction vessel of the pH stat and to it was added 1 mL of 4M urea solution and the system was equilibrated at 30°C for 30 min. Then, 1 mL of either the GCT or BT solution (whose activities toward BAPA had been adjusted to be approximately the same – Table 3-8) was added to the urea-treated hemoglobin in the reaction vessel of the auto-titrator, and the degree of hydrolysis was followed by titrating with standard 0.059M NaOH to a set pH of 8.0. The reaction was carried out at 30°C and the volume of base consumed was noted. The weight of the reaction mixture in the reaction vessel was also determined.

2.3.5.5 Hydrolysis of fresh squid muscle protein

Extraction of the squid protein was accomplished using a modified form of the procedure by Fujimaki et al. (1970), and it involved shaking 120 g of the minced frozen squid flesh with 3 litres of 0.5M NaOH for 1 h at 30 °C and treating the extraction medium with 1M HCl to lower the pH to 4.5 and leaving the system to stand at room temperature overnight. The precipitate was collected by centrifugation and washed with de-ionized water and lyophilized to obtain the protein powder.

Procedure for the hydrolysis : The procedure used for the hydrolysis of the lyophilized squid protein powder was the same as that described for the hydrolysis of the urea-treated hemoglobin, except that this time, there was no pre-treatment of the sample with urea and also that 0.04 mL of either the GCT or BT solutions was used to digest the protein (Table 3-B).

2.3.5.6 Hydrolysis of cod fish meal

Protein from the cod fish meal was also extracted using a modified form of the procedure by Fujimaki et al. (1970), described under hydrolysis of squid protein and the digestion in the pH stat was also carried out in the same way as was done for the squid protein.

2.3.6 pH studies

The influence of pH on the activity of the trypsins was determined using BAPA or casein as substrate. The influence of pH on the stability of the trypsins was also determined using TAME as substrate.

2.3.6.1 The influence of pH on the activity of trypsins using BAPA as substrate

The pH optimum for the hydrolysis of BAPA was determined by preparing the substrate in various buffer solutions and allowing hydrolysis by the GCT or BT to proceed as described under 2.3.5.1. Compositions of buffer solutions used are specified in appendix D.

2.3.6.2 The influence of pH on the activity of the trypsins using 2% casein as substrate

The pH optima for the hydrolysis of casein by the trypsins were determined by preparing 2% casein in various buffer solutions and allowing hydrolysis by GCT or BT to proceed as described under 2.3.5.3. The compositions of the buffer solutions used are specified in appendix E

2.3.6.3 **The influence of pH on the stability of trypsins**

Either the GCT or the BT was dissolved in de-ionized water, following the procedure of Zwilling et al. (1969), and adjusted till they had approximately the same activity toward TAME and used as the enzyme stock solutions. Then 0.3 mL of the enzyme solutions were made up to 1 mL with the various buffers, as described under 2.3.6.1, at 25°C for 30 min, and the residual tryptic activity assayed as described under 2.3.5.2.

2.3.7 **Temperature studies**

The influence of temperature on the activity and stability of the trypsins was studied using casein and / or BAPA as substrate.

2.3.7.1 **The influence of temperature on the activity of trypsins, using BAPA as substrate**

The procedure used to determine the temperature optimum of either the GCT or the BT is as described under 2.3.5.1, except that the substrates were equilibrated at different assay temperatures before the enzymes were applied.

2.3.7.2 **The influence of temperature on the activity of trypsins using casein as substrate**

The procedure used to determine the temperature optimum of either the GCT or the BT is as described under 2.3.5.3, except that the substrates were equilibrated at different assay temperatures before the enzymes were applied.

2.3.7.3 **The influence of calcium on the activity of the trypsins**

The trypsin solutions were supplemented with solid $CaCl_2.2H_2O$ to final concentrations of 0.02M or 0.2M and applied to BAPA as described under 2.3.5.1 and the release of p-nitroaniline was followed at 410 nm at different temperatures in a Beckman DU-8 computing spectrophotometer.

2.3.7.4 **The influence of temperature on the stability of trypsins**

To determine the thermostability of the trypsins, a modified form of the procedure by Wang and Carpenter (1967), was used and this involved supplementing the trypsin solutions in 5mM HCl with solid $CaCl_2.2H_2O$ to a final concentration of 0.02M and adjusting the enzyme solutions till they had approximately similar activities toward BAPA at 25°C (as specified in the legend to Fig. 3-12). The enzyme solutions were equilibrated at various temperatures for 30 min. then cooled rapidly in an ice bath for 5 min before applying to the substrate at 25°C, as described under 2.3.5.1 to determine the residual tryptic activity.

2.3.8 **Kinetic studies**

The apparent Michaelis-Menten constant (Km') and maximum velocity (V_{max}) of the trypsins were determined using the initial rates of hydrolysis of BAPA or TAME as substrate using Lineweaver-Burk analysis and least squares method of Johansen and Lumry (1961).

2.3.8.1 Using BAPA as substrate

Substrate stock solution : 4.5 mM BAPA stock solution was prepared by dissolving 87 mg BAPA in 2 mL DMS and the solution was made up to 44.5 mL with substrate buffer.

Substrate buffer : 0.05M tris-HCl, pH 8.2, containing 0.02M $CaCl_2.H_2O$.

Enzyme solution : GCT and BT were prepared separately in 5mM HCl and adjusted till they had approximately the same activity toward BAPA at 25°C, as specified under Table 3-14. The different concentrations of the substrates used for the assay are specified in appendix F.

The kinetic parameters - Km' and $V_{max'}$ were estimated at various temperatures to investigate the effect of temperature on those parameters.

2.3.8.2 **Using TAME as substrate**

Substrate stock solution : 10 mM TAME stock solution was prepared by dissolving 37.9 mg of TAME in 10 mL of de-ionized water.

Substrate buffer : 0.046M tris-HCl, pH 8.1 containing 0.0115M $CaCl_2.2H_2O$.

Enzyme solution : GCT and BT were prepared separately in 5mM HCl. The exact quantity of enzyme solution used is specified under Table 3-12. The proportion of reagents in the reaction mixture is specified under appendix G.

The kinetic parameters - Km' and $V_{max'}$ were estimated at various temperatures to investigate the effect of temperature on those parameters. For both BAPA and TAME, the concentrations used were within the range for which steady state kinetics can be applied, based on Whitaker's (1972) recommendations.

2.3.9 Amino acid composition

The amino acid composition of the GCT was determined by hydrolyzing the protein with 6M HCl for 24 h, 48 h and 72 h at 110°C, then separating the amino acids formed on a Beckman 121 MB amino acid analyzer as described in Beckman 121 MB application note 121 MB-TB-017. Tryptophan was determined separately by treating the sample with 3N mercaptoethane sulfonic acid for 24 h according to the method of Penke et al. (1974). Cysteine and methionine were determined after performic acid oxidation using a modified form of the method by Blackburn (1968).

2.3.10 Peptide mapping

The BT was purified to homogeneity by passing through the affinity column and lyophilized to powder form. The purified BT and the GCT were separately hydrolyzed with either CNBr or papain.

2.3.10.1 Using CNBr

A modified version of the procedure by Hofmann (1964) was used to cleave the trypsins and it involved treating 5 mg portions of either the GCT or BT with 2 mL of performic acid (prepared by adding 1 mL of 30% H_2O_2 to 9 mL of 88% formic acid and cooling it to 0°C) and the systems allowed to stand overnight at 4°C. Then the samples were treated with 30 mL of de-ionized water and lyophilized. To the lyophilized samples were added 1 mL of 0.2M HCl and 1 mL of de-ionized water containing 20 mg CNBr. The resulting mixture was kept at 30°C for 30 h, then lyophilized. The freeze dried product was redissolved in 2 mL de-ionized water and lyophilized again to completely remove the reagents. The method of

Laemmli (1970) was used to electrophorese the freeze dried product using 12.5% polyacrylamide as separating gel and 3% polyacrylamide as the stacking gel. The sample buffer comprised 0.0625M tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol and 0.001% bromophenol blue in de-ionized water. The product from the final lyophilization was dissolved in 2 mL of the sample buffer and immersed in boiling water for about 2 min, then cooled rapidly under running tap water and 0.05 mL portions applied to the gels. Electrophoresis was then carried out at a constant current of 3 mA/gel. After the electrophoresis, the gels were fixed with 50% TCA overnight, then stained for 1 h at 37°C with 0.1% Coomasie brilliant blue prepared fresh in 10% (v/v) acetic acid and 20% (v/v) methanol in water. The gels were destained using repeated washings in a solution of 10% (v/v) acetic acid and 20% (v/v) methanol in de-ionized water.

2.3.10.2 Using papain

The method used for the hydrolysis of the trypsins by papain was adapted from the procedure by Cleveland et al. (1977) and it involved dissolving 5 mg of either the affinity purified BT or GCT in 10 mL of sample buffer (0.125M tris-HCl containing 0.5% SDS, 10% glycerol and 0.001% bromophenol blue, pH 6.8). The samples were immersed in boiling water for about 2 min, then cooled to 37°C and 1 mL portions of the cooled enzyme solutions were treated with 0.05 mL of 0.33 μg/mL papain and the digestion was allowed to proceed for 30 min. After the digestion, the system was treated with 2-mercaptoethanol to a final concentration of 2% and the samples were boiled for 2 more min, then cooled rapidly under running tap water.

The samples were electrophoresed by applying 0.05 mL portions to 12.5% gels and electrophoresis was carried out according to the method of Laemmli (1970).

2.3.11 CD spectra of trypsins

Based on the suggestion by workers including Somero (1973) that cold temperature adapted enzymes are more flexible than their warm temperature adapted homologs, the CD spectra of the trypsins were investigated to evaluate the conformation of the two enzymes.

Procedure : A known concentration of GCT or BT in 2mM HCl (0.110 mg/mL BT or 0.121 mg/mL GCT, estimated using the method of Lowry, 1951) was introduced into the sample cell of a Jasco J-20A spectropolarimeter. The settings on the spectropolarimeter were adjusted as follows : a scale of 5 millidegree/cm and a chart speed of 1 cm/min. The wavelength range for which the spectra were obtained was 200 nm to 250 nm, and measurements were taken at various temperatures to determine the influence of temperature on the conformation of the proteins.

2.3.12 The influence of various inhibitors on trypsins

The inhibitors used in this study were PMSF, SBTI and trasylol (also known as aprotinin). The inhibitors were prepared in either 2-propanol or de-ionized water to various concentrations and equilibrated with equal volumes of the trypsin solutions which had been pre-adjusted to have approximately the same activity on BAPA or TAME at 25°C, Table 3-21.

2.3.12.1 Inhibition by PMSF

PMSF inhibition of the trypsins was studied using a modification of the procedure by Fahrney and Gold (1963). The PMSF was dissolved in 10% 2-propanol to a final concentration of 5mM, and equal volumes of the BT and GCT solutions, which had been pre-adjusted to have approximately the same activity toward TAME (Table 3-21), were incubated separately with equal volumes of the PMSF solution at 25°C for 30 min. After the incubation period, portions of the enzyme-inhibitor solution (Table 3-21) were applied to TAME as described under 2.3.5.2.

2.3.12.2 Inhibition by SBTI

SBTI was dissolved in de-ionized water to the following concentrations : 0.025 mg/mL, 0.050 mg/mL, and 0.10 mg/mL. The trypsin solutions were adjusted till they had approximately the same activity toward BAPA and equal volumes of the enzyme solutions were added separately to equal volumes of the SBTI solutions and incubated in an ice bath for 30 min. After the incubation, portions of the enzyme-inhibitor solutions (Table 3-21) were applied to BAPA to determine the residual trypsin activity as described under 2.3.5.1. For the original trypsin activity, 0.5 mL of the trypsin solutions were diluted with 0.5 mL of de-ionized water and incubated in an ice bath for 30 min, and 0.2 mL of the diluted enzyme solution was applied to the substrate as described under 2.3.5.1. For the reference, 0.2 mL of de-ionized water was added to the reaction mixture instead of the enzyme or the enzyme-inhibitor solution.

2.3.12.3 **Inhibition by trasylol**

Trasylol was diluted with de-ionized water to the following concentrations, in TIU/mL : 0.0625, 0.125, 0.250, and 0.500 (where TIU stands for trypsin inhibitor units). The trypsin solutions were pre-adjusted so that equal volumes had approximately similar activities on BAPA at 25°C (Table 3-21), then equal volumes of the enzyme solutions were incubated with equal volumes of the trasylol solution in an ice bath for 30 min and the residual trypsin activity assayed by applying aliquots of the enzyme-inhibitor solution to the substrate as described under 2.3.5.1.

2.3.13 **The influence of thiol reagents on trypsins**

The thiol reagents used in this study were 2-mercaptoethanol (ME) and dithioerythritol (DTE). The thiol reagents were either diluted with or dissolved in de-ionized water to various concentrations and equilibrated with equal volumes of the trypsin solutions which had been pre-adjusted to have approximately the same activity toward BAPA or TAME, as described under 2.3.5.1 and 2.3.5.2.

2.3.13.1 The influence of 2-mercaptoethanol on the activity of trypsins

ME was diluted with de-ionized water to the following concentrations: 1.43M, 0.715M, 0.572M, 0.428M, 0.286M, 0.216M, 0.144M, and 0.070M. Equal volumes of the trypsin solutions were added to equal volumes of the ME and incubated in an ice bath for 30 min, after which 0.20 mL portions of the enzyme-ME solutions were applied to BAPA and the release of p-nitroaniline at 410 nm followed as described under 2.3.5.1. The original activities of the trypsins were determined by adding 0.50 mL of de-ionized water to 0.50 mL the trypsins and incubating the diluted enzymes in an ice bath for 30 min before applying to the substrate.

2.3.13.2 The influence of dithioerythritol (DTE) on the activity of trypsins

The DTE was dissolved in de-ionized water to the following concentrations : 0.50M, 0.25M, 0.10M, 0.05M, and 0.025M. Equal volumes of the freshly prepared DTE solutions were incubated with equal volumes of either the GCT or the BT (which had been pre-adjusted to have approximately similar activity toward TAME at 25°C) in an ice bath for 30 min, after which 0.10 mL portions of the enzyme-DTE solutions were applied to TAME to determine the residual trypsin activity, as described under 2.3.5.2. The original activities of the trypsins were determined by adding 0.50 mL of de-ionized water to 0.50 mL of each of the trypsins and incubating the diluted enzymes in an ice bath for 30 min before applying to the substrate.

2.3.14 Supplementation of fish fermentations with trypsins

The trypsins were used to supplement the fermentation of herring and squid at 10°C.

2.3.14.1 Herring fermentation

The method used was adapted from a patented process for salted herrings (Matjes) - Unilever Ltd. (1975). Freshly caught herring were obtained from the Lake Group of Companies Ltd., Grand Bank in April, 1981 and cleaned as described under 2.1.2. The eviscerated or round herrings were divided into 5 batches and pickled in brine made up of 0.5 kg de-ionized water, 183.33 g NaCl, 38.33 g sugar, 0.55 g sodium nitrate, and 5.0 g benzoic acid. The amount of herring added in each of the 5 batches was approximately 1.5 kg. Batch number 1 (conventional product) had round fish and was not supplemented with trypsin, batch number 2 had eviscerated fish and was also not supplemented with trypsin, batch number 3 had eviscerated fish and was supplemented with semi purified cod trypsin from the ammonium sulfate step, batch number 4 had eviscerated fish and was supplemented with purified GCT and batch number 5 had eviscerated fish and was supplemented with BT. The fermentation was carried out in 2 quart Mason jars with lids.

Enzyme solutions : 34.1 mg of BT at 0.6 BAPA units/mg, 25.5 mg of GCT at 0.8 BAPA units/mg and 57.79 mg of semi-purified GCT at 0.14 BAPA units/mg were added to the respective fermentation vessels. The semi purified cod trypsin was from the ammonium sulfate step (intestine extract) and was in the extraction buffer (0.05M tris-HCl, pH 7.8, containing 0.5M NaCl and 0.02M $CaCl_2$).

2.3.14.2 **Moisture and fat contents of herring**

Moisture or fat content of the fish was determined as follows :

Moisture : Portions were cut from the untreated flesh of 5 herring and mixed thoroughly and the moisture content determined by incubating weighed amounts in an oven at 105°C till a constant weight was attained.

Fat : The fat content was determined using the moisture free herring (from the moisture determinations). One g portions of the dried fish muscle were extracted with 50 mL of ether using a Rafatec fat extractor for 45 min. The ether was evaporated off on a water bath in a fume hood and the fat dried in an oven at 105°C for 2 h.

2.3.15 **Analysis of fermentation brines**

Portions were withdrawn from the fermentation brines from time to time and analyzed for free amino acids, total soluble protein and TCA soluble protein. The pH of the brines were also measured at the same time.

2.3.15.1 Changes in free amino acids

The free amino acids released from the fish into the brines were estimated by centrifuging portions of the brines in a bench top eppendorf centrifuge 5412 at 15,600 x g for 30 min, followed by precipitation of proteins and high molecular weight polypeptides with sulfosalicylic acid and analyzing the clear supernatant obtained after centrifugation for free amino acids using a Beckman 121 MB amino acid analyzer.

2.3.15.2 Total soluble protein

Soluble brine protein was estimated by centrifuging portions of the brines in a bench top eppendorf centrifuge 5412 at 15,600 x g for 30 min, then appropriately diluting the clear supernatant with de-ionized water and reading the absorbancies at 280 nm against de-ionized water as reference. Absorbance at 280 nm was roughly related to protein concentration by assuming that one absorbance unit was equivalent to 1 mg/mL protein.

2.3.15.3 TCA soluble protein

TCA soluble protein in the fermentation brines was estimated by treating portions of the brines with equal volumes of 10% TCA, mixing the system thoroughly and leaving to stand at room temperature (about 25°C) for 30 min, then appropriately diluting the clear supernatant with 5% TCA and reading the absorbance at 280 nm against 5% TCA as reference.

Hitherto, absorbance at 280 nm was roughly related to protein concentration by assuming that one absorbance unit was equivalent to 1 mg/mL protein.

2.3.15.4 pH changes during fermentation

The changes in pH in the fermentation brines were followed using a Fisher accumet pH meter model 140.

2.3.16 Squid fermentation

Fresh squid, procured and cleaned as described under 2.1.3, were brined in the same way as was done for the herring, except that this time there were only 3 batches (the 2 batches that were excluded were the round controls and the eviscerated lots supplemented with the semi purified cod trypsin). Free amino acids, total soluble protein, TCA soluble protein and pH changes in the fermentation brines were determined as described for the herring fermentation. Approximately the same BAPA units of the GCT and the BT (specified under Table 3-23) were used to supplement the fermentation of the squid as was used for the herring. The squid fermentation was also carried out at 10°C.

2.3.16.1 **Residual tryptic activity in squid brines**

Portions of the brines from the various batches were brought up to about pH 8.2 with BAPA substrate buffer and centrifuged in the RC-5 superspeed refrigerated centrifuge at 6000 X g for 30 min to obtain a clear supernatant. Taking the dilution factors into consideration, portions of the clear supernatant containing equal volumes of the brines were added to BAPA and the residual tryptic activity determined, as described under 2.3.5.1 at various intervals in the course of the fermentation. The residual tryptic activity was estimated in all 8 fermentation brines and controls were also run with the BT in the buffer or brine only.

2.3.17 Prevention of copper induced oxidized flavors in raw milk by trypsins

The method used for trypsin inhibition of milk oxidation was adapted from the procedure by King (1962). As mentioned in 2.1.4, the raw milk samples were procured from Kenmount Farms St. John's, and the time elapsed between collection and initiation of experimentation was approximately 1.5 days.

The trypsin solutions in 5mM HCl were adjusted till they had approximately similar activity toward TAME at 25°C.

Various volumes of the enzyme solutions were added to a fixed volume of the raw milk samples as specified in appendix H.

The enzyme and / or water treated samples were stored in the cold room (4°C) for 4 h, then pasteurized by holding in a water bath at 70°C for 45 min. After pasteurization, 5 mL portions of the 10 ppm copper sulfate stock solution were added to the milk samples to raise the copper content by 1 ppm and the samples were cooled and stored at 4°C and examined for malonaldehyde formation as an index of oxidized flavor development, using a modified form of King's (1962) thiobarbituric acid (TBA) method.

2.3.17.1. Measurement of TBA values in trypsin treated and untreated milk samples

Aliquots of 8.8 mL of the copper supplemented milk samples, with or without trypsin, were transferred into Erlenmeyer flasks fitted with glass stoppers and warmed to 35°C, then 0.5 mL aliquots of 1 g/mL TCA solution was added to the samples followed by 1 mL of 95% ethanol. The flasks were stoppered and vigorously shaken for about 10 sec and left to stand at 25°C for 5 min. The samples were then filtered through a Whatman No 42 filter paper and to 4 mL portions of the clear filtrates were added 1 mL of TBA solution prepared by dissolving 1.4 g of TBA in 100 mL of 95% ethanol. The flasks were again stoppered and the contents thoroughly mixed and held in a water bath at 60°C for 1 h, then cooled to room temperature under running tap water and the absorbancies at 532 nm measured in a Beckman DU-8 computing spectrophotometer using de-ionized water as reference. The values obtained were related to the literature values of King (1962) as malonaldehyde was not immediately available to use as a standard.

2.3.17.2 Determination of residual activity of trypsin in milk

The residual activities of the trypsins in the milk samples were estimated before and after pasteurization by adding equal volumes of the trypsin or centrifuged trypsin treated milk samples to TAME and following the hydrolysis at 247 nm in the DU-8 spectrophotometer at 25°C as described under 2.3.5.2.

Chapter 3

RESULTS AND DISCUSSION

3.1 Purification and activation of trypsinogen to trypsin from Greenland cod

Greenland cod trypsin (GCT) obtained from the pyloric ceca or intestines of the Greenland cod was purified as described under 2.3.1. The results of trypsin purification are summarized in Tables 3-1, 3-2, 3-3 and 3-4.

Table 3-1: Purification scheme : GCT from pyloric ceca of fish caught in winter

Step	Total volume (mL)	Total protein (mg)	Total activity (Units)	Specific activity (Units/mg)	Yield (%)	Purification
'Sup' 1	255	1501.4	22.7	0.015	100.0	1.00
'Sup' 2	250	1413.1	22.1	0.016	97.3	1.03
Ammonium sulfate fraction (40%–60%)	50	168.7	9.9	0.059	43.7	3.89
Acetone fraction	20	64.9	20.0	0.308	88.1	20.40
Affinity fraction	38	13.5	10.6	0.780	46.6	51.64

BAPA was used as substrate as described under 2.3.5.1. The weight of pyloric ceca powder used for the extraction was 50 g.

Table 3-2: Purification scheme : GCT from pyloric ceca of fish caught in summer

Step	Total Volume (mL)	Total Protein (mg)	Total Activity (Units)	Specific Activity (Units/mg)	Yield (%)	Purification
'Sup' 1	273	2183.0	40.2	0.018	100.0	1.00
'Sup' 2	265	2154.0	40.7	0.019	101.3	1.03
Ammonium sulfate fraction (40%–60%)	50	220.0	14.8	0.067	36.9	3.66
Acetone fraction	20	97.3	29.7	0.305	74.0	16.60
Affinity fraction	38	20.0	14.9	0.748	37.1	40.66

BAPA was used as substrate as described under 2.3.5.1. The weight of pyloric ceca powder used for the extraction was 50 g.

Table 3-3: Purification scheme : GCT from intestines of fish caught in winter

Step	Total Volume (mL)	Total Protein (mg)	Total Activity (Units)	Specific Activity (Units/mg)	Yield (%)	Purification
'Sup' 1	257	1131.8	70.1	0.062	100.0	1.00
'Sup' 2	250	997.1	68.7	0.069	98.1	1.11
Ammonium sulfate fraction (40%–60%)	50	195.2	26.0	0.133	37.0	2.15
Acetone fraction	20	71.3	22.1	0.310	31.5	5.00
Affinity fraction	38	10.9	8.4	0.770	12.0	12.47

BAPA was used as substrate as described under 2.3.5.1. The weight of the intestine powder used for the extraction was 50 g.

Table 3-4: Purification scheme : GCT from intestines of fish caught in summer

Step	Total Volume (mL)	Total Protein (mg)	Total Activity (Units)	Specific Activity (Units/mg)	Yield (%)	Purification
'Sup' 1	270	1768.5	113.0	0.064	100.0	1.00
'Sup' 2	260	1690.1	111.4	0.066	98.6	1.03
Ammonium sulfate fraction (40%-60%)	50	353.5	48.7	0.138	43.1	2.16
Acetone fraction	20	112.5	37.0	0.329	32.7	5.14
Affinity fraction	38	14.9	12.0	0.800	10.6	12.60

BAPA was used as substrate as described under 2.3.5.1. The weight of the intestine powder used for the extraction was 50 g.

From Tables 3-1 and 3-2, it is apparent that when the pyloric ceca was used as the source of GCT, about 40 to 50 fold purification was achieved, while with the GCT derived from the intestine the purification achieved was only about 12 fold (Tables 3-3 and 3-4).

However, the affinity purified GCT from both the pyloric ceca and the intestines had a similar specific activity. It is also apparent from Tables 3-1, 3-2, 3-3 and 3-4 that more GCT was recovered per gram of tissue from the fish caught in the summer compared with the fish caught in the winter. In addition, more GCT was obtained from the pyloric ceca than the intestines of fish caught in both summer and winter (Table 3-1 vs Table 3-3 and Table 3-2 vs Table 3-4). Further, GCT from the pyloric ceca became fully activated only after the acetone precipitation step as can be seen from the summary of a time course study for the activation of trypsinogen in the various fractions in Fig. 3–1. It is apparent from Tables 3-1, 3-2, 3-3 and 3-4 that the specific activity of GCT from either the pyloric ceca or the intestine ranged from 0.75 to 0.80 BAPA units at 25°C.

It is apparent from Fig. 3-1 that the specific activities of the fractions from 'Sup' 1 up to (and including) the $(NH_4)_2SO_4$ fraction were relatively low and did not appear to increase to any appreciable extent on standing at 4°C for up to 48 h. On the other hand, the specific activity of the acetone fraction showed about 3 fold increase after 18 h incubation at 4°C. A possible explanation for the increase in specific activity is discussed under 3.1.1.3.

The specific activity of the affinity fraction decreased slightly from time zero h to about 24 h at 4°C, then substantially at 72 h at the same temperature, which supports a later finding that GCT is acid labile (as the affinity fraction was in the peak 2 buffer – 5mM HCl, unlike the earlier fractions which were in 0.05M tris-HCl buffer, containing 0.5M NaCl and 0.02M $CaCl_2$).

One explanation for the apparent increase in yield of trypsin from the pyloric ceca after the $(NH_4)_2SO_4$ fractionation step may be that activation of inactive trypsinogen to the active enzyme. This effect was not observed with the trypsin derived from the intestine probably because the inactive zymogen was fully activated to the active enzyme when it was secreted into the intestine.

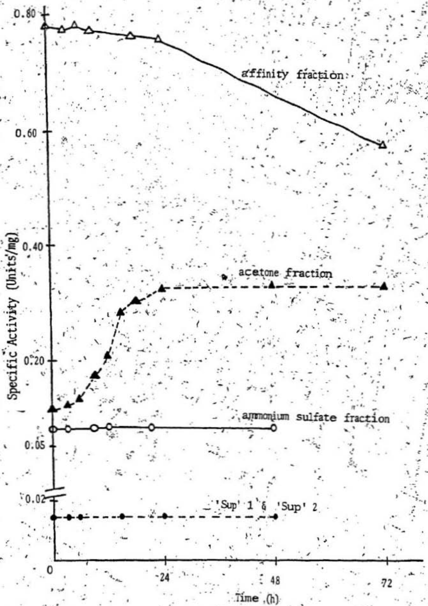


Fig. 3-1: Effect of incubation on activity of various fractions from pyloric ceca

3.1.1 General Discussion: Extraction of trypsin from Greenland cod

Very little is known about the physiology of Greenland cod, but some information is available on a closely related species - the atlantic cod (*Gadus morhua*). While most workers, like Bishop and Odense (1966) and Overnall (1973) agree on the presence of a large bunch of blind sacs (known as the pyloric ceca) in the atlantic cod, opinion seems to be divided as to whether or not the fish has a pancreas. Bishop and Odense (1966) reported that a pancreas is present between the finger-like projections of the pyloric ceca and the intestines of the cod, but Overnall (1973) suggested that the cod has no discrete pancreas. Various workers like Croston (1960) and Ooshiro (1971) have established that the pyloric ceca of various fish (and other animals) is a rich source of digestive enzymes. Croston (1960, 1965), Zendzian and Barnard (1967), Camacho et al. (1970) and Bundy and Gustafson (1973) as well as several other workers have purified trypsin or trypsin-type enzymes from the pyloric ceca of various organisms. The Greenland cod has a distinct pyloric ceca occupying a position between the stomach and the intestine and the tissue from a 1 kilogram fish weighed between 40 g to 70 g, on fresh weight basis.

3.1.1.1 Extraction of trypsin

Initial attempts to use the acid extraction method of Kunitz and Northrop (1936) to extract trypsinogen from the pyloric ceca before activating it to trypsin was unsuccessful and no trypsin activity could be detected when the homogenates or fractions obtained at the various stages of the purification procedure were assayed. This apparent destruction of trypsinogen and hence trypsin by acid extraction supports later findings that

GCT is unstable at acid pH. Consequently, other procedures which employed neutral or alkaline conditions were tried for the purification of GCT. Other workers like Croston (1960), Camacho et al. (1970) and Jany (1976) also observed complete loss of trypsin activity as a result of an acidic extraction method.

The procedure by Camacho et al. (1970), involving the use of ammonium sulfate fractionation, acetone precipitation and chromatography on Sephadex G-100 was adapted for the extraction of GCT. The fraction precipitating between 40% – 60% saturation with ammonium sulfate was collected for further purification in order to minimize co-precipitation of trypsin material with naturally present trypsin inhibitors, whose presence retards or completely prevents autocatalytic activation of inactive trypsinogen to active trypsin. According to Kunitz and Northrop (1936), the naturally present trypsin inhibitors are precipitated from solution at 70% saturation with ammonium sulfate. Additionally, Northrop and Kunitz (1932) observed that the most active precipitate having trypsin activity appeared at about 60% saturation with ammonium sulfate and that further increases in ammonium sulfate saturation did not increase the trypsin activity in the precipitate. The samples were exhaustively dialyzed in order to remove as much of the ammonium sulfate in the sample as possible. The samples were then allowed to autoactivate completely at pH 7.8. before applying to the affinity column to ensure that there was efficient binding between the ligand and GCT so that most of the GCT was recovered in the eluate and not lost together with the unbound materials washed off the column in the form of trypsinogen.

The technique of affinity chromatography was used for further purification of the enzyme. Unlike other chromatographic procedures which separate protein molecules on the basis of size or charge, the affinity technique achieves separation of protein molecules on the basis of their specificity for a particular ligand. With the use of the appropriate ligand, it is possible to exclude other proteins of similar size or charge. Robinson et al. (1971) used chicken ovomucoid-Sepharose 4B affinity column to separate α- and β- trypsins from commercial bovine trypsin and suggested that for trypsin type enzymes that were not inhibited by chicken ovomucoid [eg. starfish trypsin - Winter and Neurath (1970) and human trypsin - Feinstein et al. (1974)], other trypsin inhibitors like pancreatic or soybean trypsin inhibitors could be similarly employed.

Soybean trypsin inhibitor (SBTI) was selected as the ligand for the Sepharose-4B matrix instead of chicken ovomucoid, because certain preparations of the ovomucoid have been implicated by Feeney et al. (1963) to have ovoinhibitor which is capable of inhibiting or binding chymotrypsin. It was decided to allow the zymogen present to be fully activated before loading onto the affinity column since autocatalytic activation has been demonstrated by Northrop and Kunitz (1948) to proceed at slightly alkaline pH. This procedure minimized the loss of GCT in the form of inactive trypsinogen which would not be expected to bind appreciably to the SBTI on the affinity column.

Calcium was also routinely added to the extraction buffer to protect the enzyme from autolysis and conversion to so called "inert proteins" as reported by McDonald and Kunitz (1941), as a precaution just in case.

GCT was similar to other trypsins (like bovine and ovine trypsins) in its requirement for calcium for activity and stability.

3.1.1.2 Evidence of a zymogen

The purification schemes presented in Tables 3-1, 3-2, 3-3 and 3-4 indicate that in samples derived from both pyloric ceca and intestines, there was some trypsin activity at all stages in the purification scheme, based on the capacity of the various fractions to hydrolyze BAPA to some extent. However, the activities obtained with the intestine extracts were considerably higher than those from the pyloric ceca extracts in the early stages of the purification, even though the specific activities of the fractions from the affinity column from either source – pyloric ceca or intestine – were similar. The relatively lower specific activities of the fractions from the pyloric ceca, persisting up to and including the ammonium sulfate extract, could be attributed to one, two or all three of the following : (i) presence of naturally present trypsin inhibitors which prevented either all the extractable trypsin from eliciting the maximum possible activity or inactive zymogen in the extracts from autoactivation by the active trypsin present; (ii) presence of a substantial portion of potential trypsin as inactive trypsinogen; or (iii) a relatively greater amount of non trypsin proteinaceous material in the pyloric ceca extracts.

Kunitz and Northrop (1936) in their preparation of crude trypsinogen from bovine pancreas observed that activation of the zymogen to the active enzyme could not occur due to the presence of naturally present trypsin inhibitors. Even though the presence of naturally present trypsin inhibitors was not specifically looked for, their presence could be assumed based on the fact that they constitute a physiological control mechanism for vertebrates to accomodate the otherwise devastating effects of premature activation of the zymogen.

Workers like Camacho et al. (1970), Bundy and Gustafson (1973) and Jany (1976) observed activation of trypsinogen to trypsin at various stages of their purification of trypsins from the pyloric ceca of various organisms under alkaline conditions. For example Camacho et al. (1970) observed that crude homogenates of pyloric ceca extracts in 0.05M tris-HCl buffer, pH 8.2, did not show any appreciable increase in activity when they were incubated at 20°C for 144 min. whereas crude homogenates supplemented with bovine trypsin more than doubled their activity within 40 min of incubation at 20°C. Bundy and Gustafson (1973) observed that trypsin activity of an acetone extract approximately doubled on standing overnight at 5°C using BAPA as substrate and imputed the activation to elimination or destruction of endogenous trypsin inhibitors. The apparent increase in yield based on total activity recovered in going from the ammonium sulfate fractionation to the acetone step observed in the pyloric ceca extracts, but not the intestine extracts was probably due to activation of zymogens made possible by one, two or all three of the following possibilities : (i) exclusion of some of the naturally present trypsin inhibitors by using 40% - 60% ammonium sulfate saturated fractions; (ii) dialysis of the ammonium sulfate fraction using 6,000 - 8,000 molecular weight cut off dialysis membranes, and (iii) destruction or elimination of any remaining naturally present trypsin inhibitors by the acetone treatment. Activation was not observed with the intestinal extracts because the zymogen is activated to the active enzyme before it is secreted into the intestine to carry out its normal function of hydrolyzing protein molecules.

One way of testing for the presence of the zymogen would be to carry out the purification in the presence of excess trypsin inhibitors (by incorporating the inhibitors in the extraction buffer) and chromatographing the ammonium sulfate fraction on SE-Sephadex at neutral pH, as suggested by Schroeder and Shaw (1968). Alternatively, some bovine trypsin could be added to 'sup'1' to overcome the action of naturally present inhibitors to activate all the zymogen in the pyloric ceca extract before continuing with the subsequent purification steps.

In this work, trypsin inhibitors were not added to the pyloric ceca extracts because the additional problem of separating the inhibitors from the trypsin or trypsinogen was not attractive and would have prolonged the purification process and probably hurt the integrity or recovery of the enzyme through autolysis. Bovine trypsin was not added to activate the zymogen because it was desirous to be able to attribute tryptic activity to proteases from the pyloric ceca without doubt, and also to avoid the possible contamination of GCT with the bovine enzyme. Finally, the delayed activation of the zymogen to the active enzyme, till after the ammonium sulfate fractionation, was probably advantageous in the sense that up to that stage, the enzyme was predominantly in a form that was not self destructing.

3.2 Electrophoresis

The affinity purified GCT was electrophoresed under the following conditions :

3.2.1 SDS – PAGE with Urea

The sample was prepared in sample buffer containing 2-mercaptoethanol, SDS and urea, then boiled in a water bath for approximately 3 min before applying to the gel, using the procedure described by Bethesda Research Laboratories (BRL, 1981).

The results obtained, presented in Fig. 3-2 shows that GCT from either the pyloric ceca or intestines (represented by 'a' and 'c' in Fig. 3-2) appears to be a single polypeptide since it migrated as a single band. Because electrophoresis was allowed to proceed till the dye front moved out of the gels, the R_f values were not estimated since values obtained would not have been very useful in estimating the molecular weights of the protein.

However it is apparent from Fig. 3-2 that GCT migrated to a position approximately halfway between ovalbumin (mol. wt. 45 kdal., and represented by 'y' in Fig. 3-2) and ribonuclease A (mol. wt. 13.9 kdal., and represented by 'z' in figure)

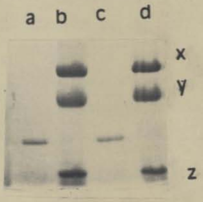


Figure 3-2: SDS - PAGE with Urea (BRL, 1981)

'a' = GCT from the pyloric ceca ; 'b' and 'd' are protein standards ; 'c' = GCT from the intestines ; 'x' = bovine serum albumin ; 'y' = ovalbumin ; 'z' = ribonuclease A.

3.2.2 Determination of molecular weight using SDS – PAGE

The sample was prepared in sample buffer containing 2-mercaptoethanol, and SDS, then boiled in a water bath for approximately 3 min before applying to the gels, using the method of Laemmli (1970).

The results presented in appendix I again shows GCT (on gels 'A' and 'C') as homogeneous and migrating to almost the same distance as the standard labelled 'q' on gel 'B'. The contaminant that appears to be present at the origins of gels A, B, and C were not observed on the original gels and is therefore attributed to optical problems during photography. Other GCT samples run under-similar conditions (and which do not show any contaminants at the origins), are presented in appendix J.

The R_f values were estimated and used to plot a graph of the logarithm of molecular weight of the proteins versus mobility (R_f), appendix K, to estimate the molecular weight of GCT. A summary of the description of the proteins, their R_f values and their corresponding molecular weights is presented in Table 3-5.

The molecular weights of the proteins were also estimated using a Beckman DU-8 computing spectrophotometer in the gel scan mode with molecular weight calculation (appendix L).

It is apparent from Table 3-5 and apendix K that the molecular weight of GCT computed by the DU-8 gel scan program was similar to that estimated graphically, appendix K. It is also apparent from appendix I and Table 3-5 that the molecular weight of GCT was very close to that of the major protein band on the BT gel.

Table 3-5: Summary of R_f values and molecular weights of proteins

Protein(s) on gel	R_f value	Mol. Weight
A ---> GCT from pyloric ceca	0.625	22.60
C ---> GCT from intestine	0.620	22.60
D ---> BT (major band)	0.590	23.82
BT (minor band)	0.625	22.60
B ---> protein standards		
(m) phosphorylase b	0.032	92.50
(n) bovine serum albumin	0.093	66.20
(o) ovalbumin	0.253	45.00
(p) carbonic anhydrase	0.410	31.00
(q) soybean trypsin inhibitor	0.660	21.50
(r) lysozyme	0.810	14.40

3.2.3 Analytical polyacrylamide gel electrophoreses

The samples were prepared in the absence of SDS, 2-mercaptoethanol or urea and a modification of the method of Davis (1964) described by Pharmacia Fine Chemicals (1980) was used.

The results obtained, presented in Fig. 3-3 indicate that GCT purified from either the pyloric ceca or the intestine was also homogeneous on analytical polyacrylamide gels. Fig. 3-3 also shows that the affinity purified BT (which was active on BAPA), was homogeneous on the gels.

The R_f values for the GCT from the ceca and intestines were identical - approximately 0.30 for GCT derived from either the ceca or the intestines. However, the R_f value of the affinity purified BT (0.40) was slightly higher than the value estimated for GCT. The lower R_f value of GCT indicates that the protein migrates slowly on analytical polyacrylamide gels compared to BT, using the method of Pharmacia Fine Chemicals (1980).

3.2.4 General discussion : Electrophoresis of GCT

Greenland cod trypsin from either the pyloric ceca or intestines was homogeneous under the different conditions investigated, which means that the purification procedure was effective in purifying GCT to homogeneity. It also means that GCT is a single polypeptide. The similar R_f values for GCT from both the ceca and the intestines suggests, as expected, that the two are the same molecule.

Figure 3-3 Analytical polyacrylamide gel electrophoresis of trypsins

A and B are GCT from the pyloric ceca and the intestines of Greenland cod respectively ; C is BT from Sigma after affinity chromatography.

The molecular weight of 22.60 kdal estimated for GCT is similar to the values estimated for the major and minor bands derived from BT. The molecular weight estimated for GCT is also similar to values reported for other trypsins. For example, Winter and Neurath (1970) determined the molecular weight of starfish trypsin as 24.43 kdal. Gates and Travis (1969) determined the molecular weight of shrimp trypsin as 25.0 kdal, and according to Kiel (1971), trypsins have molecular weights ranging between 20.0 kdal to 25.0 kdal.

3.3 Trypsin Assay

The time course for the hydrolysis of BAPA, TAME and casein by trypsins were determined at 25°C to determine the specific activity of the trypsins and also the length of time for which reactions were linear. The results obtained are presented in Figs. 3-4, 3-5 and 3-6, and the calculated specific activities are summarized in Table 3-6.

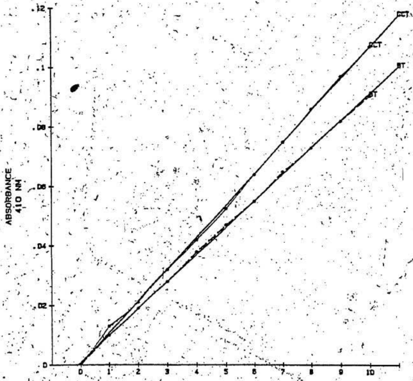


Figure 3-4: Time course : GCT vs BT on BAPA

Legend to Fig. 3-4 : 0.05 mL of 0.104 mg/mL BT or 0.092 mg/mL GCT was added to BAPA (pH 8.2) at 25°C, and the rate of change in absorbance at 410 nm measured at 30 sec. intervals in the DU-8 spectrophotometer, as described under 2.3.5.1. The extra line for each enzyme is the line of best fit.

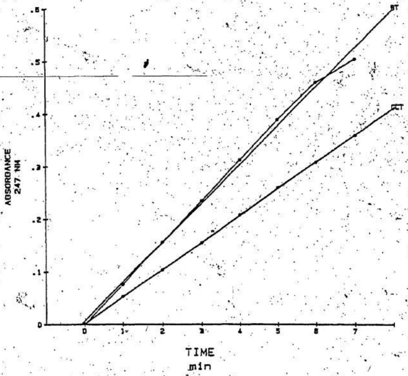


Figure 3-5: Time course : GCT vs BT on TAME

Legend to Fig. 3-5 : 0.02 mL of 0.12 mg/mL BT or 0.05 mL of 0.025 mg/mL GCT was added to TAME (pH 8.1) at 25°C, and the rate of change in absorbance a 247 nm measured at 15 sec. intervals in the DU-8 spectrophotometer, as described under 2.3.5.2. The extra line for each enzyme is the line of best fit.

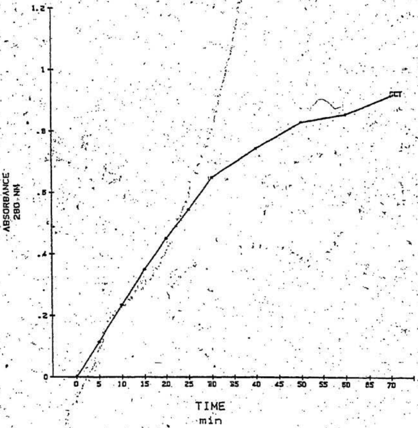


Figure 3-6: **Time course : GCT on 2% casein at 25°C**

Legend to Fig. 3-6 : 0.50 mL of 5.9 μg/mL GCT applied to substrate.

Table 3-6: Summary of specific activities of GCT and BT on various substrates at 25°C

Enzyme	Substrate	Specific Activity[a] (Units/mg enzyme)
GCT	TAME	229.72
	BAPA	0.79
	casein	7.20
BT	TAME	190.82
	BAPA	0.60
	casein	5.82[1]

[1] calculated from pH optimum data at 25°C, pH 7.5. Conc. of BT = 0.50 mL of 7.8 μg/mL stock BT solution.

[a] For TAME ——> as defined by Worthington Enzymes (1978).

Specific activity = Units/mg

$$= \frac{\Delta A_{247\ nm/min} \times 1000 \times 3}{540^{b} \times \text{mg enzyme in assay}}$$

[b] 540 equals the molar extinction coefficient of p-toluene sulfonyl-L-arginine (TA) at 247 nm.

[a] For BAPA (based on Worthington's definition of specific activity of trypsin on TAME).

Specific activity = Units/mg

$$= \frac{\Delta A_{410\ nm/min} \times 1000 \times 3}{8800^{c} \times \text{mg enzyme in assay}}$$

[c] 8800 equals molar extinction coefficient of p-nitroaniline at 410 nm at 25°C, according to Erlanger et al. (1961).

[a] For casein, (using Kunitz's (1947) definition of trypsin activity :

$$TU^{cas}/\mu g \text{ trypsin} = \frac{\Delta A_{280\ nm/min}}{\mu g \text{ trypsin in assay}}$$

therefore, 1 mg trypsin contains :

$$\frac{\Delta A_{280\ nm/min} \times 10^{3}\ TU^{cas}}{\mu g \text{ trypsin}}$$

3.3.1 General Discussion - Trypsin Assay

For both GCT and BT, TAME appeared to be a better substrate than BAPA for the following reasons :

(i) TAME was hydrolyzed at higher rates than BAPA and was the more sensitive assay.

(ii) TAME was readily soluble in water at the temperatures investigated, unlike BAPA which precipitated out of solution at temperatures below 20°C.

However, BAPA was selected for most of the studies because as an amide, it was closer to the natural substrates for trypsin than TAME, an ester. Furthermore, the indicator-like property of BAPA made it possible to confirm, by visual inspection, that reaction had actually taken place, and products had formed from the reactants as a consequence of the trypsin added.

It can also be seen from Table 3–6 that GCT is about 1.3 times more active than BT towards BAPA and about 1.2 times more active than BT towards TAME at 25°C

3.4 Hydrolysis of protein substrates

The trypsins were also applied to digest proteins using a pH stat as described under 2.3.5.4 to 2.3.5.6. The proteins involved were urea-treated hemoglobin and proteins extracted from cod fish meal and squid muscle. The hydrolysis was left to proceed till there was no further base consumption. By measuring the volume of base consumed, the degree of hydrolysis (DH) was estimated using the equation given in a bulletin by Novo Enzymes (1978) as follows :

$$DH = \frac{h \times 100}{h_{tot}}$$

where h is the hydrolysis equivalent of the protein, and h_{tot} is the total hydrolysis equivalent of the protein (given in tables for several proteins). For hemoglobin, h_{tot} was taken as 8.0 and for the cod fish meal and squid muscle protein h_{tot} was taken as 7.3. (Novo Enzymes, 1978)

$$h = B \times 1/\alpha \quad \times N_b/[M \times (\%S/100)]$$

where B = base consumption in mL, α = function of (pH – pK) defined by :

$$\alpha = \frac{10^{(pH - pK)}}{1 + 10^{(pH - pK)}}$$

α^{-1} at 30°C and pH 8.0 is given as 1.40 (Novo Enzymes, 1978). M is the total mass of the hydrolysis mixture (g). S is the substrate concentration in the reaction mixture, and N_b is the normality of the base.

In the case of the hemoglobin, 1 mL of the various solutions was assumed to weigh 1 g. but for the cod fish and squid muscle proteins, the weight of the reaction mixture was determined by weighing the reaction vessel with and without the reaction mixture. The results obtained are summarized in Table 3-7.

Table 3-7: Degree of hydrolysis (DH) of protein substrates by trypsins at 30°C

Substrate	Degree of Hydrolysis (DH)	
	GCT	BT
Urea-treated Hb[a]	1.62	1.45
Cod fish meal[b]	1.67	1.68
Squid muscle protein[b]	3.34	3.32

[a] = only one determination was carried out; the enzyme solutions were adjusted so that equal volumes had approximately the same activity on BAPA at 25°C: 0.20 mL of GCT (equivalent to 15.3 μg protein) hydrolyzed BAPA at a rate $\Delta A_{410\ nm/min}$ of 0.036 at 25°C while 0.20 mL BT (equivalent to 19.9 μg protein) hydrolyzed BAPA at a rate $A_{410\ nm/min}$ of

0.0352 at 25°C. 1 mL of the enzyme solutions were added separately to 0.08 g Hb in 1 mL of 4 M urea.

b => values are averages of two determinations; the enzyme solutions were adjusted to have approximately the same activity on BAPA at 25°C: 0.20 mL GCT (equivalent to 13 μg protein) hydrolyzed BAPA at a rate $\Delta A_{410\ nm/min}$ of 0.0298 at 25°C, while 0.20 mL BT (equivalent to 17μg protein) hydrolyzed BAPA at a rate $\Delta A_{410\ nm/min}$ of 0.0303 at 25°C.

The final composition of the reaction mixture was 0.08 g protein substrate + 1 mL H_2O + 0.4 mL of enzyme solution.

3.4.1 General Discussion : Degree of hydrolysis

It is apparent from Table 3-7 that GCT and BT hydrolyzed the protein substrates to almost the same extent.

3.4.1.1 General comments on the hydrolysis of substrates by trypsins

Although trypsins from different sources appear to have the same common substrates, they do not hydrolyze these substrates to the same extent. For example, Bundy and Gustafson (1973), Camacho et al. (1970) and Kozlovskaya and Elyakova (1974) described trypsins with greater specific activities on their substrates than those obtained with BT. Hjelmeland and Raa (1982) described a trypsin with almost the same activity as BT while Allan et al. (1970) and Zwilling et al. (1969) also described trypsins with substantially lower specific activities than BT.

The capacity of GCT to hydrolyze TAME and BAPA suggests that GCT, like BT, hydrolyses bonds involving the carboxyl groups of arginine. The finding that both GCT and BT hydrolyzed protein substrates – viz., urea-treated hemoglobin, cod fish meal and squid muscle protein to approximately the same extent, suggests that the cleavage specificities of the two enzymes are probably identical.

A way of further testing whether GCT has the same cleavage specificity as BT is to apply GCT and BT (purified to homogeneity) separately to a well defined substrate such as the β-chain of insulin and carrying out gel electrophoresis on the hydrolyzed insulin chain to determine the exact peptides that would be produced as a result of the treatment with the two trypsins.

3.5 The influence of pH on the activity of trypsins at different temperatures using BAPA as substrate

Both GCT and BT appear to have similar pH activity profiles. They are both less active at acid pH and more active at moderately alkaline pH (Fig. 3-7). Temperature did not appear to affect the pH optimum of the hydrolysis of BAPA by GCT, at least for the temperatures investigated (Fig. 3-7). However, the pH optimum of GCT tends to be broader at lower temperatures than at higher temperatures. Furthermore, an increase in temperature appeared to increase the activity of BT more than it did that of GCT. The temperature coefficients (Q_{10}) for the enzyme activities in going from 5°C to 25°C at different pH's are summarized in Table 3-8.

Legend to Fig. 3-7 : (i) 0.20 mL of either 0.094 mg/mL stock GCT or 0.122 mg/mL BT applied to the substrate ; (ii) assays were carried out as described under 2.3.6.1 and 2.3.5.1.

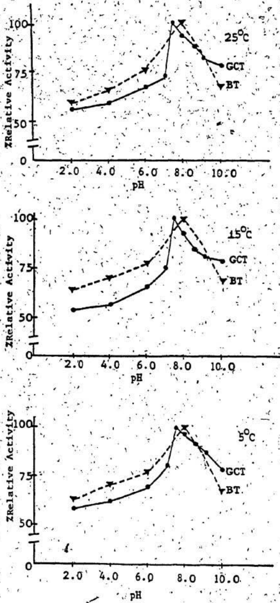


Figure 3-7: The influence of pH on the activity of trypsins at different temperatures on BAPA as substrate.

Table 3-8: Summary of the Q_{10} values of trypsin on BAPA at various pH's

pH	Q_{10} (5°C - 15°C) GCT	BT
2.0	1.57	2.47
4.0	1.57	2.40
6.0	1.63	2.43
8.0	1.61	2.45
10.0	1.48	2.41
	Q_{10} (15°C - 25°C)	
2.0	1.45	2.20
4.0	1.46	2.22
6.0	1.42	2.32
8.0	1.49	2.34
10.0	1.39	2.38

3.5.1 The influence of pH on the activity of trypsins using casein as substrate

The influence of pH on the activity of the trypsins towards casein was determined as described under 2.3.6.2. and 2.3.5.3. The results obtained are presented in Fig. 3-8. The pH activity profiles of GCT and BT are similar in so far as both are more active at moderately alkaline pH and less active at acid pH. However, while BT appeared to hydrolyze casein to the greatest extent at pH 8.0 at all 3 temperatures investigated, GCT appeared to hydrolyze casein greatest between pH 9.0 and 9.5 at the 3 temperatures investigated.

It is also apparent from Fig. 3-8 that while BT was slightly more active than GCT at acid pH, GCT was slightly more active than BT at alkaline pH at the temperatures investigated.

3.6 The effect of pH on the stability of trypsins, on TAME as substrate

The stability of the trypsins was determined as described under 2.3.6.3 and 2.3.5.2 and the results obtained are presented graphically in Fig. 3-9, from which it is apparent that while GCT was more stable at moderately alkaline pH, BT was more stable at acid pH. Overall, therefore, BT was found to be more stable and slightly more active at acid pH while GCT was more stable and slightly more active at alkaline pH, although both enzymes exhibited maximal activity at moderately alkaline pH.

Legend to Fig. 3-8 : 0.50 mL of either 5.9 µg/mL GCT or 7.8 µg/mL BT applied to the substrate and assayed as described under 2.3.5.3 and 2.3.6.2.

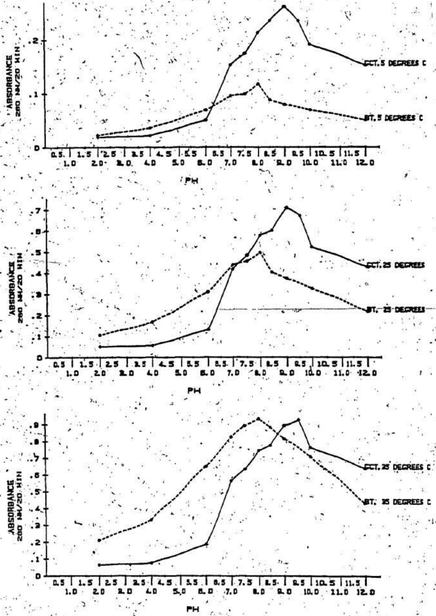


Figure 3–8 : The influence of pH on the activity of trypsins at different temperatures using casein as substrate

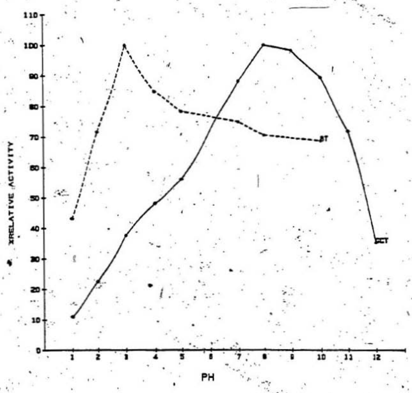


Figure 3-9: The influence of pH on the stability of trypsins

Legend to Fig. 3-9 : approximately 5 mg of lyophilized GCT or BT was dissolved in 10 mL of de-ionized water and 0.30 mL of the enzyme solution was made up to 1 mL with various buffer solutions and incubated at room temperature (about 25°C) for 30 min before applying to the substrate to determine residual trypsin activity. The volume of the resulting buffered enzyme solutions applied to the reaction mixture, with TAME as substrate, was 0.10 mL. Assay was carried out at 25°C as described under 2.3.6.3 and 2.3.5.2.)

3.6.1 General discussion : The influence of pH on activity and stability of trypsins

The pH activity profiles for the hydrolysis of protein, amide and ester substrates by trypsins are generally bell-shaped and reflect maximal enzymatic activity at alkaline pH. Kuhne (1877) observed that trypsin digests its substrates only in alkaline, neutral or very weakly acidic solutions. According to Erlanger et al. (1961), the pH optimum for the hydrolysis of BAPA by BT at 25°C occurs near pH 8.1. Stambaugh and Buckley (1972) reported that the optimum pH for the hydrolysis of BAPA by BT is 8.2. Northrop and Kunitz (1932) observed that BT hydrolyzed casein to the greatest extent in the pH range of 8.0 to pH 9.0. Other workers, including Camacho et al. (1970), Kozlovskaya and Elyakova (1974), Gates and Travis (1969) and Croston (1960) have similarly observed that trypsins hydrolyzed their substrates to the greatest extent at alkaline pH, ranging from pH 7.0 to 9.5.

In spite of the observation that trypsins from various sources (both vertebrate and invertebrate) hydrolyzed their substrates greatest at alkaline pH, trypsins of lower vertebrates and invertebrates appear to be inactivated under acidic conditions unlike trypsins from higher vertebrates. For example, Northrop (1932) and Vithayathil et al. (1961) have reported that bovine and ovine trypsins are stable at acid pH but unstable at alkaline pH while porcine trypsin is stable at both acid and alkaline pH. Camacho et al. (1970), Hjelmaland and Raa (1982), Gates and Travis (1969), Jany (1976) and Ching-San Chen et al. (1978) have described trypsins that are unstable at acid pH but stable at alkaline pH.

A possible explanation for the instability of GCT at acid pH is that the ratio of potential acidic amino acid residues to basic amino acid residues is greater for GCT compared to BT (from the amino acid composition data). Also the fact that GCT has a fewer number of basic amino acid residues suggests that GCT has a relatively fewer number of trypsin-labile bonds. So that at the alkaline pH where trypsins are most active, GCT is less susceptible to autodigestion than BT and its porcine and ovine counterparts and probably makes GCT more stable at alkaline pH than BT.

3.7 The influence of temperature on the activity of trypsins

The trypsins were used to hydrolyze BAPA or TAME at different temperatures as described under 2.3.7.1, 2.3.7.3 and 2.3.8.2. The results obtained (when BAPA was used as substrate) are summarized in Fig. 3-10. Figure 3-10 also illustrates the effect of calcium on the activity of trypsins. The same data were used to obtain Arrhenius plots to estimate the activation energies (E_a) for the hydrolysis of BAPA by trypsins and the results obtained are presented in Table 3-9. Table 3-9 also shows the E_a for the hydrolysis of TAME by the trypsins.

The trypsins were also used to hydrolyze casein as described under 2.3.7.2 at different temperatures and the rate of hydrolysis was measured as the change in absorbance at 280 nm per 20 min ($\Delta A_{280\ nm/20\ min}$).

Legend to Fig. 3–10 : 0.20 mL of either 0.031 mg/mL GCT or 0.038 mg/mL BT applied to the reaction mixture and assays were carried out as described under 2.3.5.1 and 2.3.7.1.

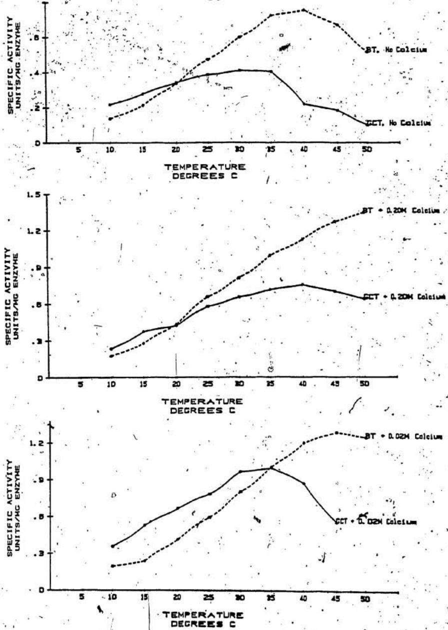


Figure 3-10: The influence of temperature on the activities of trypsins on BAPA as substrate

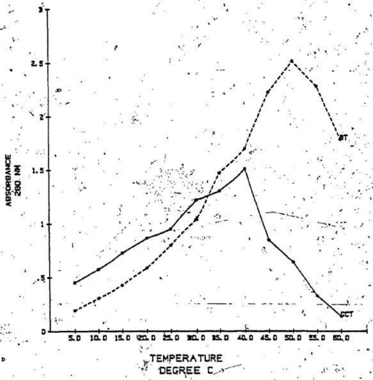


Figure 3-11: Temperature optima of trypsins on 2% casein.

Legend to Fig. 3-11 : 0.50mL of either 0.039 mg/mL GCT or 0.059 mg/mL BT added to the reaction mixture as described under 2.3.5.3 and 2.3.7.2.

The same data were used to obtain Arrhenius plots to estimate the E_a's for the hydrolysis of casein by the trypsins. The data are presented in Fig. 3-11 and Table 3-10.

3.7.1 General discussion : The influence of temperature and calcium on the hydrolysis of BAPA by trypsins

A summary of the temperature optima at which GCT and BT catalyze the hydrolysis of BAPA is presented in Table 3-9. Table 3-9 also shows the E_a values of the trypsin catalyzed reactions estimated using linear regression analysis as well as the r^2 values.

Table 3-9: Summary of the thermal properties of trypsins

Enzyme	Substrate	Conc. of Ca^{++} (M)	Temp. optimum (°C)	E_a (kcal/mole)	r^2
GCT	BAPA	0.00	30.0	6.6	0.985
	BAPA	0.02	35.0	8.2	0.978
	BAPA	0.20	40.0	8.5	0.956
BT	BAPA	0.00	40.0	12.7	0.989
	BAPA	0.02	45.0	13.2	0.981
	BAPA	0.20	50+	13.3	0.988
GCT	TAME	0.01	$35+^x$	8.5	0.967
BT	TAME	0.01	$35+^x$	13.4	0.984

^{x} => temperature range investigated was 5°C to 35°C.

It is apparent from Table 3-9 that both in the presence and absence of calcium, the temperature optimum for the hydrolysis of BAPA by the two trypsins was higher for BT than GCT and also that the temperature optimum for the hydrolysis of the substrate increased with increasing concentration of calcium.

It is also apparent from Table 3-9 that GCT has a lower E_a for the hydrolysis of BAPA or TAME than BT at the various levels of calcium investigated. For instance, in the absence of calcium, the E_a of GCT was approximately equal to 50% of that estimated for BT. Furthermore, increasing concentrations of the calcium appeared to increase the E_a's of both GCT and BT, but the increase in the case of GCT was more pronounced than was observed for BT.

A summary of the temperature optima of the trypsins when casein was employed as substrate is presented in Table 3-10. Table 3-10 also has a summary of the E_a's of the trypsins on casein as substrate.

Table 3-10: Summary of temperature optima of trypsins on casein as substrate

Enzyme	Temp. optimum (°C)	E_a kcal/mole	r^2
GCT	40	7.02	0.984
BT	50	11.14	0.995

It is apparent from Table 3-10 as well as Figs. 3-10 and 3-11 that compared to BT, GCT had a lower temperature optimum and a lower energy of activation when casein was used as substrate. The E_a of GCT was approximately equal to 60% of that found for BT under similar conditions.

3.8 The influence of temperature on the stability of trypsins

The thermal stability of the trypsins was investigated as described under 2.3.7.4 and 2.3.5.1. The activities remaining after incubating the enzymes at different temperatures were used to plot a graph of percent original activity vs temperature and the results are presented in Fig. 3-12, from which it is apparent that GCT was more heat labile than BT. While GCT lost about 50% of its original activity at approximately 50°C, BT retained almost all its activity up to 80°C.

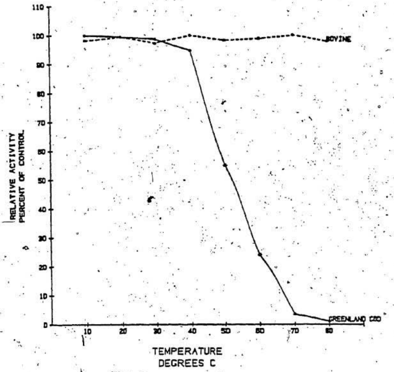


Figure 3-12: Thermostability of trypsins

Legend to Fig. 3-12 : 0.20 mL of either 0.039 mg/mL GCT or 0.052 mg/mL BT in 5mM HCl containing 0.02M Ca^{++}. The enzyme solutions were incubated at various temperatures for 30 min. then cooled rapidly in ice for 5 min before applying to the substrate as described under 2.3.5.1. The original activities of the trypsin solutions were as follows : (i) GCT on BAPA: 0.20 mL hydrolyzed BAPA at a rate $\Delta A_{410\ nm/min}$ of 0.0180 at 25°C. and (ii) BT on BAPA: 0.20 mL hydrolyzed BAPA at a rate $\Delta A_{410\ nm/min}$ of 0.0183 at 25°C.

3.8.1 General discussion : The influence of temperature on the activity and stability of trypsins

3.8.1.1 The influence of temperature on the activity of trypsins

The influence of temperature on the rate of hydrolysis of substrates by trypsins is a combined effect of (i) the influence of temperature on the rate of catalysis, and (ii) the influence of temperature on the rate of denaturation of the enzyme. In the study with BAPA as substrate, the addition of calcium to the enzyme (prior to its application to the substrate) appears to influence both these processes – Fig. 3–10.

As summarized in Table 3-9, the apparent temperature optimum is progressively higher as the concentration of the calcium incubated with the enzyme is increased. This implies that calcium acts to stabilize both BT and GCT from thermal denaturation. In addition, the incubation of the enzyme with calcium appears to stimulate the reaction at temperatures well below that at which denaturation is evident. This suggests that calcium also acts to stimulate the rate of the catalytic reaction. In the presence or absence of calcium, the apparent temperature optimum of GCT was lower than that of BT . This was also the case when casein was used as substrate, Table 3-10.

Sipos and Merkel (1970) observed a concentration dependency of calcium activation of trypsin. Vithayathil et al. (1961) reported that the stability of bovine and ovine trypsins were considerably increased by calcium while the stability of porcine trypsin was only slightly increased. Qua et al. (1981) observed calcium stimulation of protease activity as well as increase in temperature optimum in studies with a halophilic protease produced by a halophilic marine Pseudomonas species. However, other trypsins or trypsin-type enzymes which do not seem to require calcium for stability or activity have been characterized by workers like Camacho et al. (1970) and Gates and Travis (1969).

Abrahamson and Maher (1967) observed a positive correlation between the temperature optimum of pancreatic amylases and body temperature of lizards. Similarly, Light (1964) reported the temperature optima for myosin ATPase activity from several species of lizards from widely different temperature environments were correlated with the body temperature of the lizards. Light et al. (1969) observed that the optimal temperatures for contractility of skeletal muscle from several species of Australian lizards were correlated with the preferred body temperature.

The lower E_a values found for GCT compared to BT is similar to findings made by workers like Cowey (1967) and Low et al. (1973), that enzymes from organisms adapted to cold temperatures generally had lower activation energies than their counterparts from organisms adapted to warm temperatures. For example, Cowey (1967) observed that while GPDH from cod and lobster had E_a's of 14.5 kcal/mole, GPDH of rabbit had an E_a of 19.0 kcal/mole. Based on this finding, Cowey (1967) concluded that cold adapted enzymes show some degree of adaption when compared with their warm adapted counterparts. Low et al. (1973) also found that E_a of muscle LDH from halibut and tuna were 9.30 kcal/mole and 9.35 kcal/mole respectively, while muscle LDH's from chicken and rabbit had E_a's of 11.1 kcal/mole and 13.1 kcal/mole respectively. Other evidences for the direct relationship between habitat temperature and E_a have come from investigation by workers like Somero (1969) using pyruvate kinase from tuna, trout, shrimp, zooarcid, king crab and *Trematomus*, and Kwon and Olcott (1965), using aldolases from rabbit, carp and tuna as well as Hazel (1972) using succinic dehydrogenases from rat, goldfish, frog and *Trematomus*.

However the differences in E_a for the trypsins observed in this study were greater than the differences in E_a's observed by other workers like Cowey (1967) and Low et al. (1973) in their studies with intracellular enzymes. For example, while the E_a of GCT was approximately 40% to 50% lower than that found for BT (depending on the substrate). Low et al. (1973) observed that the E_a's of muscle LDH from halibut and tuna were only about 30% lower than their homolog from rabbit. They also found the E_a's of halibut and tuna were only about 15% lower than their homolog from chicken. Haard et al. (1982), using digestive enzymes (pepsins) from cold adapted fish also reported that the E_a's of the cold adapted extracellular enzymes from fish showed larger differences when their E_a values were compared to that of their warm temperature adapted homolog from porcine (similar to the finding with GCT). They reported that the cold adapted fish pepsins exhibited E_a's ranging from 4.1 kcal/mole to 8.8 kcal/mole in contrast to 11.2 kcal/mole observed for porcine pepsin. Greenland cod pepsin had E_a values of 6.6 kcal/mole at pH 1.9 and 4.2 kcal/mole at pH 3.0 within the temperature range of 10°C to 35°C.

3.8.1.2 The influence of temperature on stability of trypsins

The resistance of BT to thermal denaturation has been demonstrated by several workers. As early as 1932, Northrop and Kunitz demonstrated that when trypsin from bovine pancreas was boiled in dilute HCl at 95°C for 24 min then cooled in ice for only 2 min, there was no loss of activity. Mellenby and Wooley (1913) had previously described this apparent remarkable ability of BT to resist thermal denaturation, and Steigerwaldt (1932) similarly found BT to be extremely resistant to heat denaturation. As a result of subsequent studies, Northrop (1932) established that the BT was denatured on heating but reversed to the native condition very rapidly on cooling and at the same time, completely regained all its enzymic activity.

Vithayathil et al. (1961) observed that the pH range at which reversible heat denaturation occurs with trypsin is between pH 2.0 and pH 2.5. In this study, the observation with BT under much less harsh conditions - Fig. 3-12 - compared to those used by Northrop and Kunitz (1932) is therefore a confirmation of what earlier workers have demonstrated to be a property of BT.

However, this remarkable ability of BT to resist heat denaturation does not appear to be a common property of all trypsins. For example, workers like Bundy and Gustafson (1973), Jany (1976) and Camacho et al. (1970) have all described trypsins that were completely inactivated by heating at lower temperatures - from 45°C to 60°C, for relatively shorter periods of time - from 10 min to 20 min. For these trypsins, restoration of activity was not observed on cooling to 0°C in ice.

The 5mM HCl used as the solvent for trypsins (see legend to Fig. 3-12) was selected in order to curtail autodigestion of the trypsins that has been found to occur at neutral to slightly alkaline pH. Even though GCT was found to be acid labile (from the pH stability study and the preliminary acid extraction procedure) it tolerated mild acidic conditions when calcium ions were present (unlike the conditions for the pH stability and preliminary acid extractions) at least, for a few hours as indicated by the results of the activation study (Fig. 3-1). Presumably, the pH instability was overcome by the calcium ions present and the lower ionic strength of the medium.

Hazel and Prosser (1974) have observed that there is a correlation between heat tolerance by proteins and the temperature of the cells from which they occur. Ushakov (1967) demonstrated from comparative studies of myosin ATPase, aldolase, cholinesterase, adenylate kinase and alkaline phosphatase activities from a variety of species, that proteins from thermophilic species were more heat-stable than proteins from poikilotherms. Abrahamson and Maher (1967) similarly found that the thermostability of pancreatic amylases were correlated with the preferred body temperature. Baslow and Nigrelli (1964) found acetylcholinesterase from warm water teleosts were more heat-stable than acetylcholinesterase from cold water teleosts. Komatsu and Feeney (1970) observed that muscle aldolases from antarctic fishes - *Trematomus* and *Dissothicus* - were more heat-labile than the enzyme from thermophilic bacteria.

Legend to Table 3–11 : (i) 0.05 mL of 13.8 μg/mL GCT solution applied to TAME at 35°C, 25°C and 15°C. (ii) 0.03 mL of 23 μg/mL GCT solution applied to TAME at 5°C. (iii) 0.05 mL of 17.2 μg/mL BT applied to TAME at 35°C and 25°C. (iv) 0.05 mL of 19.5 μg/mL BT applied to TAME at 15°C and 5°C. (v) values are averages of 2 determinations.

3.9 Km' and V_{max} of trypsins using BAPA and TAME as substrates

The V_{max} and Km' of the trypsins were estimated by measuring the initial rates for the hydrolysis of either TAME or BAPA at different substrate concentrations. These determinations were carried out at different temperatures to determine whether the temperature dependency of V_{max} and Km' differs for GCT and BT. The kinetic parameters, V_{max} and Km', were determined by analysis of Lineweaver – Burke plots and by the least squares method of Johansen and Lumry (1961). The results obtained are summarized in Tables 3-11, 3-12, 3-13 and 3-14.

Table 3–11: Trypsin hydrolysis of TAME (pH 8.2) – analysis by Lineweaver-Burke plots

Enzyme	Temp. (°C)	Km' (mM)	V_{max} (Units/μmole)[b]	Q_{10}	[S][a] range (mM)	r^2
GCT	35	0.26	22.89×10^3		0.5 – 1.0	0.990
				1.56		
	25	0.15	14.70×10^3		0.3 – 0.8	0.993
				1.65		
	15	0.12	8.89×10^3		0.3 – 1.0	0.996
				1.71		
	5	0.14	5.21×10^3		0.3 – 1.0	0.983
BT	35	0.04	17.82×10^3		0.3 – 1.0	0.960
				1.95		
	25	0.05	9.13×10^3		0.3 – 1.0	0.950
				2.36		
	15	0.04	3.87×10^3		0.3 – 1.0	0.962
				2.20		
	5	0.05	1.76×10^3		0.3 – 1.0	0.987

[a] [S] = substrate concentration
[b] (units/μmole trypsin)

Table 3-12: Trypsin hydrolysis of TAME - by least squares method of Johansen and Lumry (1961)

Enzyme	Temp. (°C)	Km' (mM)	V_{max} (Units/μmole[b])	Q_{10}	[S][a] range (mM)
GCT	35	0.26	22.93×10^3		0.5 - 1.0
				1.56	
	25	0.15	14.74×10^3		0.3 - 0.8
				1.66	
	15	0.12	8.89×10^3		0.3 - 1.0
				1.70	
	5	0.15	5.25×10^3		0.3 - 1.0
BT	35	0.04	17.77×10^3		0.3 - 1.0
				1.95	
	25	0.05	9.13×10^3		0.3 - 1.0
				2.36	
	15	0.03	3.87×10^3		0.3 - 1.0
				2.22	
	5	0.05	1.74×10^3		0.3 - 1.0

[a] [S] = substrate concentration
[b] (units/μmole trypsin)

Legend to Table 3-12 : data used for Lineweaver - Burke plots used for Table 3-12.

It is apparent from Tables 3-11 and 3-13 that the kinetic parameters estimated for the trypsins by Lineweaver - Burke plots were similar to those obtained by the least square method of Johansen and Lumry (1961), Tables 3-12 and 3-14. It is also apparent from Tables 3-11 to 3-14 that the apparent Km' values were higher for GCT than BT at the various temperatures investigated on both substrates, BAPA and TAME. But while the apparent Km' of the GCT appeared to increase with a temperature increase from 15°C to 35°C (when TAME was used as substrate), and

Table 3-13: Trypsin hydrolysis of BAPA (pH 8.1) – analysis by Lineweaver-Burke plots

Enzyme	Temp. (^{o}C)	Km' (mM)	V_{max} (Units/μmole)	Q_{10}	[S] range (mM)	r^2
GCT	35	1.93	352.0	1.69	0.4 – 1.25	0.998
	25	1.69	208.8		0.4 – 1.25	0.998
BT	35	0.90	54.6	2.09	0.5 – 2.50	0.991
	25	0.97	26.2		0.5 – 2.50	0.997

Table 3-14: Trypsin hydrolysis of BAPA – using least square method of Johansen and Lumry (1961)

Enzyme	Temp. (^{o}C)	Km' (mM)	V_{max} (Units/μmole)	Q_{10}	[S] range (mM)
GCT	35	1.84	339.6	1.64	0.4 – 1.25
	25	1.67	207.0		0.4 – 1.25
BT	35	0.90	54.6	2.04	0.5 – 2.50
	25	1.02	26.8		0.5 – 2.50

Legend to Tables 3-13 and 3-14: (i) 0.20 mL of 17.5 μg/mL GCT solution applied to BAPA at 35°C and 25°C. (ii) 0.20 mL of 35 μg/mL BT solution applied to BAPA at 35°C and 25°C. (iii) values are averages of two determinations.

from 25°C to 35°C (when BAPA was used as substrate), there did not appear to be any definite influence of temperature on the Km' of BT. It is apparent that the Km' values for the BT at different temperatures did not vary appreciably with temperature for both substrates. It is also apparent from Tables 3-11 to 3-14 that both GCT and BT had higher affinities for the ester substrate (TAME) than the amide (BAPA) based on the relative differences in their Km' values.

The Km' values found for BT-BAPA and BT-TAME are similar to values reported in the literature. For example, Erlanger et al. (1961) reported a Km' value of 0.94 mM for BT-BAPA ; Nakata and Ishii (1972) reported a Km' values of 0.76 mM and 0.97 mM for BT-BAPA ; and Koslovskaya and Elyakova (1974) reported Km' values of 0.94 mM for BT-BAPA and 0.05 mM for BT-TAME.

The finding that GCT had higher Km' values for the hydrolysis of BAPA and TAME than BT is similar to findings made by workers including Cowey (1967), Hochacka and Somero (1973), Assaf and Graves (1969) and Ooshiro (1971). These workers observed that the apparent Km' values for the hydrolyses of substrates by cold adapted enzymes were generally higher than those of their homologs from warm adapted species.

Investigations carried out by workers including Ooshiro (1971) indicate that there is a positive correlation between apparent Km' values and temperature, especially for enzymes derived from organisms that are adapted to the cold. The correlation of Km' with temperature (observed with GCT) would be expected to be of adaptive value if at certain periods in the fish's life history substrate concentration becomes limiting, otherwise the significance of enhanced substrate binding at lower temperatures is hard to explain.

Tables 3–11 to 3–14 also show that the turnover numbers for GCT were considerably higher than for BT at the various temperatures investigated for both substrates. It can also be deduced from Tables 3–11 and 3–12 that while GCT was approximately 3 times more active than BT at 5°C, the difference in activity had diminished to about 1.2 times at 35°C. This is consistent with the lower E_a of GCT as discussed under 3.8.1.1. When BAPA was used as substrate for trypsin hydrolysis, it was also observed that the V_{max} and Km' values for the reaction catalyzed by GCT were higher than the values for the reaction catalyzed by BT. Kinetic parameters were not determined for BAPA at lower temperatures since the substrate tended to precipitate out of solution. But while GCT was approximately 8 times more active than BT at 25°C on BAPA, GCT hydrolyzed the same substrate only about 6.2 times more than BT at 35°C. The temperature coefficient was also lower for the hydrolysis of BAPA by GCT than the hydrolysis by BT.

The E_a values (Table 3–9) and the V_{max} values Tables 3–11 and 3–13 were used to estimate the enthalpies (ΔH^*), the free energies (ΔG^*), and the entropies of activation (ΔS^*) of the trypsin catalyzed reactions, using the procedure described by Low et al. (1973). The results obtained are summarized in Table 3–15, from which it is apparent that for the two substrates investigated, the ΔH^*, ΔS^*, and ΔG^* values were lower for the GCT catalyzed reactions than the corresponding values for the BT catalyzed reactions. However, the differences in the ΔG^* values for the reactions catalyzed by the two enzymes were not as pronounced as the differences in the E_a, $\Delta H^{\neq}$ and $\Delta S^{\neq}$ values for the same reactions. It would be predicted from the E_a, and hence the $\Delta H^{\neq}$ that the $\Delta G^{\neq}$ values for the reactions should be considerably lower for the GCT catalyzed reactions than the BT catalyzed reactions (all other things being equal) to enable the former set of reactions to proceed at rates several orders of magnitude higher than the latter set of reactions.

The slight differences in $\Delta G^{\neq}$ values (200 - 600 cal/mole for GCT-TAME vs. BT-TAME reaction and 1,400 - 1,900 cal/mole for GCT-BAPA vs. BT-BAPA reaction) is similar to values described as differences by Low et al. (1973), Asaaf and Graves (1969), and Cowey (1967).

However, the greater negative $\Delta S^{\neq}$ values of the GCT catalyzed reactions compensates for the rather large differences in the $\Delta H^{\neq}$ values to make small the differences in the $\Delta G^{\neq}$ values. The greater negative $\Delta S^{\neq}$ values of the GCT catalyzed reactions also means that the differences in the rates of the reactions catalyzed by the two trypsins should not be as high as would be predicted by the differences in their E_a values. The lower $\Delta H^{\neq}$ values of the GCT catalyzed reactions probably indicates that those reactions are comparatively more temperature-independent than the BT catalyzed reactions. The lower $\Delta G^{\neq}$ values for the GCT catalyzed reactions (noticeably the amidase reaction) indicate that GCT is more efficient than BT in lowering the "energy barrier" to the reactions.

Table 3-15: Summary of thermodynamic activation parameters for trypsin catalyzed hydrolyses of TAME and BAPA

Enzyme	Substrate	Temp (°C)	Ea kcal/mole	ΔH^{*} kcal/mole	$\Delta^{*}S$ e.u.	ΔG kcal/mole
GCT	TAME[1]	35	8.5	7.8	-21.3	14.4
		25	8.5	7.9	-21.2	14.3
		15	8.5	7.9	-21.2	14.0
		5	8.5	7.9	-21.1	13.8
BT	TAME[1]	35	13.4	12.8	-5.6	14.6
		25	13.4	12.9	-5.4	14.5
		15	13.4	12.9	-5.5	14.5
		5	13.4	12.9	-5.3	14.4
GCT	BAPA[2]	35	8.2	7.6	-15.8	12.5
		25	8.2	7.6	-16.8	12.6
BT	BAPA[2]	35	13.2	12.6	-4.2	13.9
		25	13.2	12.6	-6.1	14.4

[1] E_a of trypsin catalyzed hydrolysis of TAME were estimated from the slopes of Arrhenius plots obtained using the V_{max} values in Table 3-10.

[2] E_a values of trypsin catalyzed hydrolysis of BAPA were estimated from the slopes of Arrhenius plots using the initial velocities obtained by investigating the influence of temperature on the activity of the enzymes.

The thermodynamic parameters were estimated according to the following relationships (Low <u>et al</u>., 1973) :

1. $\Delta G^{\ddagger} = \Delta H^{\ddagger} - T\Delta S^{\ddagger}$

2. $\Delta H^{\ddagger} = E_a - RT$

3. $\Delta S^{\ddagger} = 4.576\ (\log K - 10.753 - \log T + E_a / 4.576\ T)$

4. K (in sec^{-1} = $V_{max/mg}$ of enzyme x mol. wt x 1 min/60 se

The Km' and V_{max} values in Tables 3-11 and 3-13 were also used to estimate so-called "physiological efficiency" of the trypsins. The "physiological efficiency" of an enzyme has been defined by Fullbrook (1983) and Mihalyi (1978) as the ratio of its substrate turnover number (V_{max}) to its substrate binding affinity (Km') – i.e. V_{max}/Km'. This means that for a group of enzymes capable of catalyzing the transformation of a particular substrate (or group of substrates) to product(s), the enzyme with the highest V_{max}/Km' ratio would be the most efficient to use to transform the substrate to products. Based on this, the V_{max}/Km ratios for the transformation of TAME and BAPA by the trypsins were compared and the results obtained are summarized in Table 3-16. It is apparent from Table 3-16 that the physiological efficiency generally increased with temperature, except the GCT catalyzed hydrolysis of TAME at 35°C which was lower than the value obtained for the hydrolysis of the same substrate by the same enzyme at 25°C. It is also apparent from Table 3-16 that when TAME was used as substrate, GCT was just slightly more "efficient" than BT at 5°C, while BT appeared to be more "efficient" than GCT at 15°C, 25°C and 35°C. This is in spite of the fact that GCT had higher V_{max} values than BT at all the temperatures investigated. However, when BAPA was used as substrate, GCT was more "efficient" than BT in hydrolyzing the substrate, based on the V_{max}/Km' ratios. It would seem from the observations with TAME and BAPA that the physiological efficiency of the enzyme depends on the type of substrate.

Table 3-16: Summary of the physiological efficiencies of trypsin hydrolysis of TAME and BAPA

Enzyme	Substrate	Assay temp. (°C)	Km' (mM)	V_{max} ($x10^3$)	V_{max}/Km' ($x10^3$)
GCT	TAME	35	0.26	22.89	88.04
		25	0.15	14.70	98.00
		15	0.12	8.89	74.08
		5	0.14	5.21	37.21
BT	TAME	35	0.04	17.82	445.50
		25	0.05	9.13	182.60
		15	0.04	3.87	96.70
		5	0.05	1.76	35.20
GCT	BAPA	35	1.84	0.34	0.18
		25	1.67	0.21	0.12
BT	BAPA	35	0.90	0.05	0.06
		25	1.02	0.03	0.03

Even though GCT had higher Km' and V_{max} values than BT for the hydrolysis of TAME (an ester) and BAPA (an amide), there is an important difference between the two enzymes, in that while the Km' values of GCT appeared to increase with temperature, that of BT did not appear to vary with temperature within the temperature range investigated. In addition, the Km' values for the hydrolysis of BAPA by GCT was about 1.6

to 2 times higher than the values for the hydrolysis of the same substrate by BT, while the Km' values for the hydrolysis of TAME by GCT were 2.8 to 6.5 times higher than the values for the reactions catalyzed by BT. The relatively higher Km' values for the hydrolysis of TAME compared to BAPA by GCT therefore lowers the efficiency of GCT to hydrolyze TAME compared to BT.

Liu and Elliot (1971) compared the hydrolysis of ester and amide substrates by proteolytic enzymes and based on their findings, classified proteolytic enzymes into two categories – (I) those that hydrolyze ester substrates at a rate at least 10^3 times faster than they do towards the corresponding amide substrate, and (II) those that hydrolyze ester and amide substrates at comparable rates. According to Liu and Elliot (1971), most of the serine proteases, like trypsin, belong to the first category of enzymes.

The Vmax/Km' (ester) : Vmax/Km' (amide) ratio for BT, based on the data in Table 3-16, are 7.34×10^3 and 6.95×10^3 at 35°C and 25°C respectively. The Vmax/Km' (ester) : Vmax/Km' (amide) ratio for GCT are 0.48×10^3 and 0.79×10^3 at 35°C and 25°C respectively. These ratios, though different, indicate that both GCT and BT hydrolyze the ester substrate faster than the amide substrate.

The difference in the present study is that TAME is not the corresponding ester substrate for the amide BAPA. Furthermore, Liu and Elliot (1971) did not specify whether their investigation included enzymes from cold adapted species whose Km' values are positively modulated by temperature. If their study covered only enzymes from warm temperature adapted species whose binding affinities are rigidly conserved and do not vary appreciably within a narrow range of temperature (as used in the present study), then their generalization that proteases hydrolyze their ester substrates either 10^3 times faster than their amide substrate or to almost the same extent may not be expected to hold for enzymes that are adapted to the cold (especially where their substrate binding affinities vary with temperature). However, it is clear from Table 3-16 that GCT is similar to BT in so far as both appear to hydrolyze the ester substrate faster than the amide substrate.

The significance of the differences in the Vmax/Km' (ester) : Vmax/Km' (amide) ratios in practical terms can not be rationalized as the substrates used are amides or esters of the α-carboxyl groups of amino acids – substrates that do not occur in proteins, the natural substrates of trypsin.

3.10 CD spectra of trypsins

The CD spectra of the GCT and BT were determined as described under 2.3.11 and the results obtained are summarized in Table 3-17.

The α-helix content was calculated from the following relationships :

$$[\theta] = \frac{\text{chart reading (cm)} \times \text{scale (cm)} \times \text{MRW} \times 10}{\text{cell length (cm)} \times \text{conc. of protein (gm/100 mL)}}$$

$$\%\alpha\text{-helix} = \frac{[\theta]_{obs} - [\theta]_{rc}}{[\theta]_{\alpha\text{-helix}} - [\theta]_{rc}}$$

where, $[\theta]_{obs}$ = molecular ellipticity ; $[\theta]_{rc}$ = ellipticity of the random

Table 3-17: Estimated %α-helix in trypsin at various temperatures

Enzyme	Temp. (°C)	%α-helix
GCT	0.2	7.8
	12.1	7.5
	22.2	7.6
	35.3	7.8
BT	0.2	11.5
	12.1	11.6
	22.2	11.8
	35.4	12.0

coil at that wavelength ; and $[\theta]\ \alpha_{-helix}$ = the ellipticity of the pure 100% helix at the same wavelength.

It was assumed that the two trypsins had negligible or no β-pleated sheets.

3.10.1 General discussion : CD spectra of trypsins

It is apparent from Table 3-17 that the two trypsins are both random coils - i.e. they both have unordered structures. Based on the relatively lower α-helix content of the of the GCT, it can be said that GCT is less ordered than BT. It can also be inferred from Table 3-17 that within the temperature range investigated, neither GCT nor BT underwent any major conformational change that could be detected by the technique. Various workers seem to agree that it is rather difficult to obtain absolute information from CD spectra studies. However, the technique is accepted as a valuable tool when used in comparative studies.

The information in Table 3-17 supports the concept that low temperature adapted enzymes have more flexible structures. This statement is further strengthened by the observation that GCT has fewer disulfide linkages and a relatively lower average hydrophobicity as will be discussed in the next section.

However, caution must be exercised in interpreting the results from the CD spectra study for the following reasons : (i) the study was carried out once and with one sample only, and (ii) the medium in which the trypsins were prepared was acidic. From the pH stability study (under 3.6), BT would be expected to be more stable than GCT under the acidic condition of the experiment. It is not improbable therefore, that the less ordered state of GCT was to some extent due to acid denaturation. However, since the instability of GCT in an acidic environment is dependent on temperature, it would be expected that differences in helical content as a function of temperature would have been observed.

3.11 Amino acid composition of GCT - Residues / Molecule, (based on M. wt. of 23,500 daltons)

The amino acid composition of the GCT was determined after hydrolyses at 24 h, 48 h and 72 h to correct for losses as described under section 2.3.10. The results obtained are summarized in Table 3-18.

Table 3-18: Amino acid composition of Greenland cod trypsin

Amino acid	24 h	48 h	72 h	calculated value	integral value	Residues(#) x mol. wt.
Alanine	16.78	15.95	15.48	16.07	16	1425.44
Arginine	4.43	5.14	5.18	4.92	5	871.00
Aspartic acid	24.01	21.97	22.58	22.85	23	3061.30
Cysteine	8.03	-	-	8.03[a]	8	969.20
Glutamic acid	19.35	18.29	18.37	18.67	19	2795.47
Glycine	29.75	26.53	26.34	27.54	28	2101.96
Histidine	7.32	7.27	7.37	7.32	7	1086.40
Isoleucine	6.15	8.61	8.86	7.87	8	1049.36
Leucine	13.39	14.09	14.24	13.89	14	1836.38
Lysine	5.74	5.95	6.04	5.91	6	877.14
Methionine	3.06	-	-	3.06[b]	3	447.63
Phenylalanine	3.87	3.48	3.41	3.59	4	660.76
Proline	10.77	9.30	9.45	9.84	10	1151.30
Serine	25.90	18.76	17.26	31.70[c]	32	3362.88
Threonine	9.62	9.63	9.45	9.57	10	1191.20
Tryptophan	1.53	-	-	1.53[d]	2	408.48
Tyrosine	7.45	6.59	6.29	6.78	7	1268.33
Valine	10.79	18.29	19.03	16.04	16	1874.40
	Total				218	26,438.63[e]

[a] => determined as cysteic acid after performic acid oxidation.

[b] => determined as methionine sulfone after performic acid oxidation.

[c] ⇒ extrapolated to zero time after hydrolysis.

[d] ⇒ determined after 24 h hydrolysis with 3N mercaptoethane sulfonic acid.

[e] ⇒ Not corrected of water molecules arising from peptide bond formation. So the actual molecular weight is 26,438.63 − [(n−1) x 18] where n = number of residues.

Therefore the actual molecular weight from 218 amino acid residues is equal to 26,439 − [217 x 18] = 22,533 daltons.

Table 3–19 compares certain amino acid residues of GCT with that of BT and trypsins from other sources. It is apparent from Table 3–19 that both the GCT and BT are rich in serine, glycine and the potential acidic amino acid residues.

The minimum molecular weight, based on the number of amino acid residues agrees quite well with values obtained by SDS polyacrylamide gel electrophoresis, viz.: (i) molecular weight of GCT by SDS − PAGE (graphical determination) = 22.5 kdal.; (ii) molecular weight of GCT by SDS − PAGE (DU–8 gel scan program for molecular weight determination = 23.56 kdal.); (iii) molecular weight of GCT by amino acid composition data = 22.53 kdal.

Based on values obtained by the 3 approaches listed above, the average molecular weight of GCT is computed as 22.86 ± 0.60 kdal.

Table 3–19: Comparison of certain amino acid residues from various sources

Amino acid(s)	GCT	BT	Shrimp	Porcine	Human	Crayfish
Total potential acidic amino acid residues	42	36	54	35	42	51
Lysine + Arginine	11	16	8	14	17	7
Total aromatic residues	13	17	19	18	14	21
Serine + Threonine	42	43	34	35	34	32
Isoleucine + Leucine + valine	38	46	42	47	40	49

It is apparent from Table 3–19 that GCT is rich in potential acidic amino acid residues like trypsin from other sources like shrimp, human, crayfish, porcine and bovine. Table 3–19 also reveals that GCT has fewer basic amino acid residues compared to the mammalian trypsins from bovine, porcine and human sources. Furthermore, Table 3–19 shows that GCT has fewer aromatic amino acid residues and fewer hydrophobic amino acid residues (represented by isoleucine, leucine and valine) than trypsins from the other sources referred to in the table.

Table 3-20: Amino acid compositions of trypsins from various sources

Species	Bovine	Porcine	Human	Ovine	Shrimp	GCT
Ala	14	16	13	17	16	16
Arg	2	4	6	4	3	5
Asp + Asn	22	18	21	20	30	23
Half Cys	12	12	8	12	8	8
Glu + Gln	14	17	21	14	24	19
Gly	25	26	20	19	28	28
His	3	4	3	3	5	7
Ile	15	15	12	10	14	8
Leu	14	16	12	14	10	14
Lys	14	10	11	12	5	6
Met	2	2	1	2	2	3
Phe	3	4	4	5	6	4
Pro	9	10	9	9	11	10
Ser	33	24	24	26	24	32
Thr	10	11	10	15	10	10
Trp	4	6	3	–	3	2
Tyr	10	8	7	6	10	7
Val	17	16	16	17	18	16
Total	223	219	201	205	237	218
$H\Phi_{ave}$	1.035	1.081	0.988	0.980	0.906	0.863

[a] lifted from Walsh and Neurath (1964) ; [b] lifted from Travis and Liener (1965) ; [c] lifted from Travis and Roberts (1969) ; [d] lifted from Travis (1968) ; [e] lifted from Gates and Travis (1969).

3.11.1 General discussion – Amino acid composition of trypsins

Greenland cod trypsin is similar to other trypsins in being rich in potential acidic amino acid residues, as well as glycine and serine (Table 3-20). GCT seems to be more like human, shrimp and porcine trypsins based on the similarities in the numbers of certain amino acid residues like alanine, glycine, threonine, cysteine, phenylalanine, tyrosine and valine. For example, like human and shrimp trypsins, GCT has only 8 cysteine residues unlike BT, ovine and porcine trypsins which have 12 cysteine residues. It means that unlike BT which has 6 disulfide linkages, GCT can have a maximum of 4 disulfide linkages, an observation which suggests probable differences in the three dimensional structure of GCT compared to BT. It can also be implied from the observation that GCT has relatively fewer cysteine residues that if all other things were equal, then the greater number of disulfide linkages would make BT more stable or more rigid than GCT. Komatsu and Feeney (1970) observed that aldolases from antarctic fish (which are relatively more heat labile) had lower amounts of cysteine and a higher content of methionine, valine and phenylalanine than aldolase from rabbit (which is relatively more heat stable). This is similar to the results observed for GCT and BT.

The relatively lower average hydrophobicity for GCT than BT based on the amino acid composition data – Table 3-20, suggests that the stabilization of protein molecules by hydrophobic interactions is less in GCT than BT. Bigelow (1967) observed that proteins from thermophilic organisms have distinctly higher hydrophobicities, based on their amino acid composition, than their mesophilic counterparts. Hazel and Prosser (1974) observed that proteins from thermophiles generally contained higher amounts of hydrophobic amino acids than their counterparts from mesophiles.

It must be pointed out however that amino acid composition alone does not reveal which amino acids react with one another in the native enzyme molecule and supplies no definite information about the total number of secondary interactions in the native enzymes.

Knowledge of the primary sequence and three dimensional structure of GCT is needed in order to determine whether the differences in the amino acid composition have any adaptive value. Also, the effects of agents which disrupt hydrophobic interactions in protein molecules on the activity of the enzyme may provide useful conformation regarding the physiological significance of the $H\Phi_{ave}$ for trypsins.

3.12 Peptide Mapping

Peptide maps of the two trypsins were investigated as described under 2.3.10.1 and 2.3.10.2. The results are presented in Figs. 3-13 and 3-14. The results of the papain proteolysis of the trypsins can not be easily interpreted because of the streaking of the bands, Fig. 3-13. Controls were run later to determine whether the trypsins were completely digested or not, and if they were not, which bands on the gels corresponded to undigested trypsins. Very low molecular weight markers were not available for the determination of the relative sizes of the peptides.

It is apparent from Fig. 3–14 also that when CNBr was used to cleave the trypsins, there were up to 4 major bands formed from either GCT or BT. Based on the number of methionine residues in the trypsins, 4 peptides were expected from GCT and 3 from BT as a result of CNBr cleavage. The additional peptide found on the BT gels probably was incompletely digested BT, since its R_f value of 0.31 was quite close to that of the control BT, which was about 0.28.

On the basis of the primary structure of bovine trypsinogen [Kiel (1971)], the 3 peptides from CNBr cleavage of BT should have 86, 74 and 63 residues corresponding to molecular weights of approximately 9.24 kdal, 7.56 kdal and 6.52 kdal respectively. With this in mind, bands k, l and m on C or D (in the figure) may correspond to the 3 peptides that would be predicted from the structure of bovine trypsinogen, refered to earlier on. Since the molecular weight of GCT estimated by polyacrylamide gel electrophoresis was identical to that of BT, some of the GCT bands (at least), may be expected to have lower R_f values than those of BT bands (k, l, and m). The finding that the R_f values of f, g, and i (from GCT) are similar to those of k, l, and m (from BT), probably means that there are segments of GCT that are homologous to segments in BT.

A possible explanation for band 'h' on A or B is that 2 of the methionine residues are probably close to each other in the native GCT molecule, and somehow some of the molecules were not completely cleaved by the CNBr treatment. In other words, band 'h' is probably the 'i' peptide plus an extra piece that was not cleaved – so that this particular band would have 2 methionine residues.

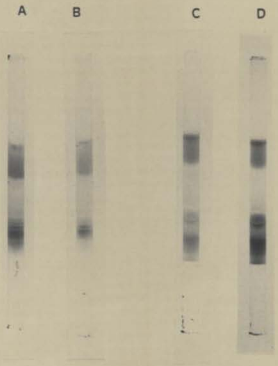


Figure 3-13: Papain proteolysis of trypsins

The results are representative of two runs. Gels A and B have peptides from BT while gels C and D have peptides from GCT.

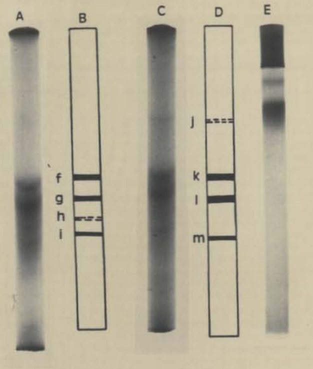


Figure 3-14: CNBr cleavage of trypsins

Bands on gel A are from GCT; bands on gel C are from BT; B and D are a diagrammatic representation of bands on gels A and C (for clarity); and E is BT run as control.

Estimated R_f values of bands on A or B : f = 0.49; g = 0.56; h = 0.62; i = 0.67. Estimated R_f values for bands on C or D : j = 0.31; k = 0.49; l = 0.56; m = o.68.

The results presented in Fig. 3-14 is representative of 2 runs.

The bovine trypsin used for the peptide map study was also purified to homogeneity by passing it through the SBTI–Sepharose 4B affinity column. This was to ensure that all the peptides formed were derived from BT and not some other contaminants present in the commercial preparation.

As indicated in Fig. 3–3, BT purified by affinity chromatography migrated as a single band on polyacrylamide gels using the method of Laemmli (1970).

3.12.1 General Discussion : Peptide Mapping

The rather broad specificity of papain - [capable of cleaving peptide bonds involving the carboxyl groups of arginine, lysine, glutamate, glutamine, histidine, leucine, glycine and tyrosine - according to Kimmel and Smith (1957) and Smith and Kimmel (1960)] - indicates that papain should cleave the trypsins into several very small peptides. However, because the hydrolysis proceeds at different rates, depending on the type of the amino acid involved in the peptide linkage as well as the pH, it is not possible to accurately predict the number of peptides that should arise from using the enzyme (papain) to cleave even a peptide of known amino acid sequence like BT.

However, it is clear from Figs. 3-13 and 3-14 that more peptides were derived from the two trypsins by papain cleavage than by CNBr cleavage. The observation that the peptides from the two trypsins are different suggests that the GCT and BT are not entirely homologous.

3.13 The influence of various inhibitors on trypsins

The influence of the inhibitors - trasylol, SBTI and PMSF on the activities of the trypsins was investigated as described under sections 2.3.12.1, 2.3.12.2 and 2.3.12.3.

A summary of the influence of the inhibitors on the trypsins is presented in Table 3-21.

Table 3-21: Summary of the influence of inhibitors on activities of trypsins

Inhibitor	Concentration	% Residual Activity GCT	BT
Trasylol	–	100.00	100.00
	0.031 (TIU/mL)	81.80	78.00
	0.063 (TIU/mL)	58.86	59.27
	0.125 (TIU/mL)	17.87	18.84
	0.250 (TIU/mL)	2.86	5.23
SBTI	–	100.00	100.00
	6.25 mM	80.10	76.64
	12.50 mM	63.82	61.19
	25.00 mM	22.70	26.48
	50.00 mM	0.00	0.00
5% 2-propanol	–	98.00	100.00
PMSF in 5% 2-propanol	2.5 mM	32.00	27.00

Legend to Table 3-21 :

(i) Values are averages of 2 determinations.

(ii) With trasylol and SBTI, DL-BAPA was used as substrates, as described under section 2.3.5.1. for estimating trypsin activity.

(iii) For PMSF, TAME was used as substrate, as described under section 2.3.5.2. for estimating the trypsin activity.

(iv) The same GCT or BT stock solutions were used for the studies with trasylol and SBTI. The concentration of the stock BT was 97 μg/mL and that of the GCT was 74 μg/mL. For the original activity, equal volumes of the enzyme stock solutions were incubated separately with 5mM HCl followed by incubation in an ice bath for 30 min before addition to the substrate. For BT, 0.20 mL of the diluted enzyme produced an original activity of 0.0171 $\Delta A_{410\ nm/min}$ at 25°C. For GCT, 0.20 mL of the diluted enzyme produced an original activity of 0.0168 $\Delta A_{410\ nm/min}$ at 25°C.

(v) For the PMSF inhibition, the stock enzymes had the following concentrations: GCT = 27.2 μg/mL and BT = 30.80 μg/mL. For the original activity, equal volumes of the two enzymes were incubated separately with 5mM HCl in an ice bath for 30 min before their addition to the substrates. With the GCT, 0.10 mL of the diluted enzyme produced a change in absorbance, $\Delta A_{247\ nm/min}$ of 0.0567 on TAME at 25°C by the procedure described under section 2.3.5.2. For the BT, 0.10 mL of the diluted enzyme produced a change in absorbance, $\Delta A_{247\ nm/min}$ of 0.0533 on TAME at 25°C using the procedure described under 2.3.5.2.

It is apparent from Table 3-21 that the esterase and amidase activities were inhibited to almost the same extent for the two trypsins. The data in Table 3-21 were based on the units of activity in the enzyme stock solutions, which were adjusted to be approximately similar.

3.13.1 General Discussion : The influence of inhibitors on activity of trypsins

Phenyl methyl sulfonyl fluoride (PMSF) has been described by various workers as a serine protease inhibitor. Fahrney and Gold (1963) demonstrated inhibition of certain serine proteases including trypsin by PMSF. Jany (1976) demonstrated that trypsin from a stomachless bonefish was inhibited by PMSF while Hjelmeland and Raa (1972) similarly observed inhibition of capelin trypsins by PMSF. The inhibition of GCT by PMSF suggests therefore that it is a serine protease like BT.

Blow et al. (1974) described SBTI as proteins which bind strongly to trypsin, blocking its active site in the process, based on the crystal structure analyses of a complex of SBTI and porcine trypsin. Stambough and Buckley (1972), Gates and Travis (1969), Camacho et al. (1970), Travis and Roberts (1969), Hjelmeland and Raa (1972) and Bundy and Gustafson (1973) have all described inhibition of trypsins by SBTI.

Trasylol (aprotinin) is also referred to as basic pancreatic trypsin inhibitor, according to Barton and Yin (1973). Workers like Hjelmeland and Raa (1982) have demonstrated inhibition of trypsin by trasylol. Steven and Griffin (1981) demonstrated inhibition of trypsin by trasylol. The inhibition of GCT and BT by SBTI and trasylol suggests that the two trypsins have a similar mechanism of substrate bonding by their active centers.

3.14 The influence of thiol reagents on trypsins

The effect of 2 –mercaptoethanol (ME) on the activities of the trypsins was investigated as described under section 2.3.13.1. The results obtained are presented in Fig. 3–15 from which it is apparent that ME inactivated GCT more than it did BT. GCT was inhibited by 50% at 0.15M ME whereas approximately 1M ME was required to inhibit BT by the same amount. This deduction is based on equivalent units of enzymes. However, it would seem that even when equivalent amounts of the trypsins, in weight, were treated with the same concentration of ME, the GCT activity would still be inhibited to a greater extent than BT. A similar observation was made when dithioerythritol (DTE) was applied to the trypsins, Fig. 3–16. With the DTE, relatively lower concentrations were required to inhibit the trypsins than the ME which suggests that the DTE is a more potent reducing agent than the ME. The GCT was inhibited by 50% by approximately 0.06M DTE whereas approximately 0.14M DTE was required to inhibit BT by the same amount. In the case of the inhibition by DTE also, even though the actual protein content in the GCT was lesser than that of the BT, it still would appear that even when equivalent amounts of the trypsins, in weight, are treated with the same concentration of the DTE, the depression of activity would still be greater with the GCT than the BT.

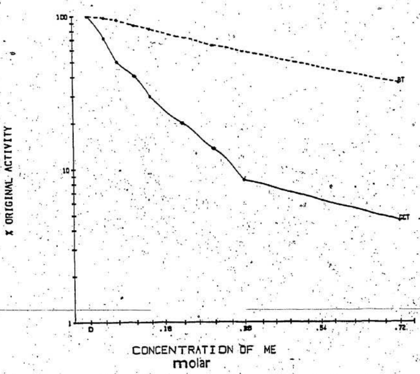


Figure 3–15: The influence of ME on the activity of trypsins

Legend to Fig. 3-15 : (i) stock GCT = 0.058 mg/mL; stock BT = 0.077 mg/mL.; (ii) To determine the original activity, equal volumes of the enzyme stock solution and 5mM HCl were incubated in an ice bath for 30 min, before 0.20 mL of it was applied to BAPA as described under 2.3.5.1. (iii) To estimate the residual trypsin activity, equal volumes of the stock enzyme solution and the ME solution were incubated in an ice bath for 30 min, before 0.20 mL of it was applied to BAPA. The assay was carried out at 25°C. The data used to construct Fig. 3-15 are averages of two determinations. Original activity of GCT on BAPA = 0.0134 $\Delta A_{410\ nm/min}$, and that of BT was 0.0135 $\Delta A_{410\ nm/min}$.

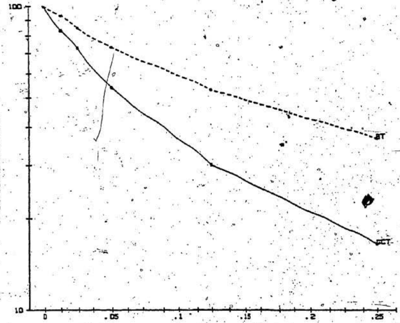


Figure 3–16: **The influence of DTE on the activity of trypsins**

Legend to Fig. 3–16 : (i) stock GCT = 0.036 mg/mL; stock BT = 0.043 mg/mL ; (ii) To estimate the original activity, equal volumes of the stock trypsin solution and 5mM HCl were incubated in an ice bath for 30 min. before 0.10 mL of it was applied to TAME at 25°C. The original activities of the trypsins on TAME were as follows : GCT = 0.075 $\Delta A_{247\ nm/min}$ and BT 0.073 $\Delta A_{247\ nm/min}$. To estimate the residual trypsin activity, equal volumes of the stock trypsin solution and freshly prepared DTE were incubated in an ice bath for 30 min before 0.10 mL portions of it was applied to the substrate at 25°C. The data used to plot Fig. 3–16 are averages of two determinations.

3.14.1 General Discussion : The influence of thiol reagents on activity of trypsins

The observed inhibition of the trypsins by the thiol reagents ME and DTE suggests that preservation of integrity of disulfide linkages in the native enzymes is vital for the catalytic activity of the enzymes.

The greater repression of GCT activity by the thiol reagents suggests that there are either fewer disulfide linkages in GCT whose intergrity need to be preserved for its normal catalytic potential to be realized than BT, or that the essential disulfide linkages maintaining the integrity of the active center of GCT are probably more accessible to reduction by the thiol than BT.

Workers like Steven and Al-Habib (1979), Steven and Podrasky (1978) have similarly observed inhibition of trypsins by thiol reagents. For example, Steven and Al-Habib (1979) demonstrated that dithiothreitol (DTT) was capable of inhibiting trypsin and also that the extent of inhibition increased with increasing concentrations of DTT.

Steven and Podrasky (1978) also observed inhibition of trypsin by thiol reagents like DTT, 2-mercaptoethanol (ME) and cysteine. They observed that for the same amount of trypsin, inhibition by DTT was greatest followed by ME and cysteine in that order. They also observed that inhibition increased with increasing concentration of the thiol reagent and that the amount of DTT required to completely inactivate the enzyme was less than 50% of that required when ME was used, and about 25 times less than the amount required when cysteine was employed for the same purpose. Sondack and Light (1971) also observed inhibition of trypsin by sodium borohydride or dithioerythritol (DTE). They further demonstrated that inhibition by DTE was time and concentration dependent.

3.15 Supplementation of fish fermentation with trypsins

The fermentation of the herrings and squid was carried out as described under 2.3.13.

3.15.1 Herring fermentation

In the case of the herring, the moisture and fat contents of the fresh muscle were determined as described under 2.3.13.2. The results obtained for the moisture and fat determinations were 71.85% (average of 3 determinations) and 10.56% (average of 4 determinations) respectively.

3.15.1.1 pH changes during fermentation

The pH changes during the fermentation of the herring is presented in Table 3-22.

Table 3-22: pH changes in Herring brines

Fish	pH at day 1	2	6	12	18	24	50
Round fish	4.7	4.6	5.0	5.2	5.6	5.8	5.7
Eviscerated control[1]	4.9	4.9	5.1	5.4	5.5	5.8	5.8
Eviscerated fish + crude GCT[2]	4.7	4.9	5.3	5.5	5.7	5.9	6.0
Eviscerated fish + GCT	4.8	4.9	5.1	5.4	5.6	5.7	5.8
Eviscerated fish + BT	4.8	5.0	5.5	5.5	5.7	5.9	5.9

Legend to Table 3–22 : (i) stock BT used to supplement herring fermentation was 3.41 mg/mL and stock GCT used to supplement herring fermentation was 2.49 mg/mL. Stock enzyme solutions adjusted with 5mM HCl till they had similar activity on BAPA at 25°C.

(ii) Activities of diluted enzyme solutions on BAPA : ⇒ 0.05 mL of diluted BT hydrolyzed BAPA at a rate $\Delta A_{410\ nm/min}$ of 0.0117 while 0.05 mL of diluted GCT hydrolyzed BAPA at a rate $\Delta A_{410\ nm/min}$ of 0.0114. The stock enzyme solutions were diluted 25 times before they were applied separately to BAPA.

(iii) Volumes of stock enzyme solutions applied to fish : 10 mL of BT and 10.23 mL of GCT.

(iv) stock crude GCT used to supplement herring fermentation was 3.09 mg/mL ; 0.10 mL of a 10 fold diluted solution hydrolyzed BAPA at a rate $\Delta A_{410\ nm/min}$ of 0.0125 at 25°C. The volume of crude GCT stock solution applied to herring brine was 18.7 mL.

[1] The eviscerated fish used as control were not supplemented with trypsin.

[2] The crude GCT was from the ammonium sulfate fraction.

Legend to Fig 3-17 : (i) amounts of enzymes used to supplement fermentation were as described under Table 3-23 ; (ii) values used to plot figure were averages of 2 determinations ; (iii) assumption made = a change in absorbance of 1 O.D. unit at 280 nm was assumed to be equal to 1 mg/mL protein.

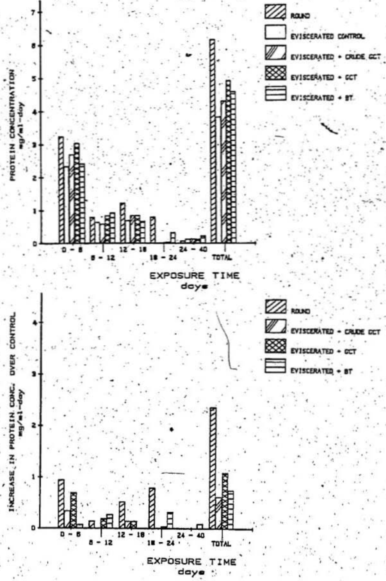


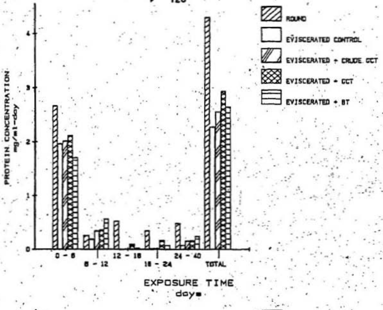
Figure 3-17: Total estimated soluble protein in herring brines

3.15.2 Total estimated soluble protein in herring fermentation brines

The total estimated soluble protein in the herring fermentation brines is summarized in Fig. 3-17.

It is apparent from Fig. 3-17 that the rate of increase of 280 nm absorbing material in the brines was higher in the enzyme supplemented fish systems and the round controls than the gutted control. It is also apparent that trypsin supplementation at the concentration employed in this experiment, did not completely compensate for evisceration over long term fermentation.

During the first 6 days, it especially GCT largely compensates for evisceration more than BT does. It also appears that during the initial stages of the fermentation (about 6 days) GCT was more effective than BT in solubilizing protein from the fish flesh into the brine than BT as expected from the lower Q_{10} values found for GCT. Based on the molecular activities of the two enzymes observed with TAME as substrate, Tables 3-11 and 3-12, GCT would be expected to hydrolyze the proteins of herring 2 to 3 times more efficiently than BT. From Fig. 3-17 however, GCT appears to effect proteolysis approximately 7 times greater than BT, at least for the first 6 days of fermentation. However, the herring is a more complex substrate than TAME (an ester), and the action of the trypsins on the two different substrates might not be expected to be exactly the same. As fermentation progressed, the capacity of GCT to effect proteolysis appeared to diminish when compared to BT and this loss of activity was probably due to the slightly acid pH of the brines and possibly to naturally



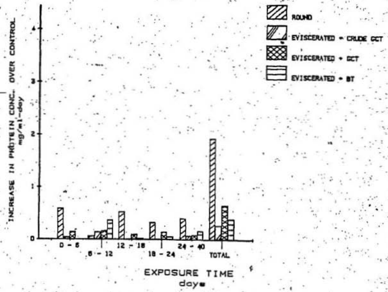


Figure 3-18: TCA soluble protein in herring brines

present and / or end product inhibitors in the brines as discussed under 3.15.5.1.

3.15.3 TCA soluble protein in herring fermentation brines

The 5% TCA soluble protein in the fermentation brines were also determined as described under 2.3.14.3 and a summary of the results is presented in Fig. 3-18.

It is apparent from Figs. 3-17 and 3-18 that the products of trypsin hydrolysis are largely TCA soluble.

A summary of the relative amounts of total estimated soluble as well as 5% TCA soluble protein in the various brines (corrected for control values) after 40 days of fermentation is presented in Fig. 3-19 from which it is apparent that more soluble peptides and amino acids were produced by the round fish than the trypsin supplemented fish systems.

In round herring, the trypsins facilitated the formation of large molecular weight polypeptides in the early days of the fermentation to serve as substrate for other enzymes in the gut and the flesh like chymotrypsins, carboxypeptidases and cathepsins.

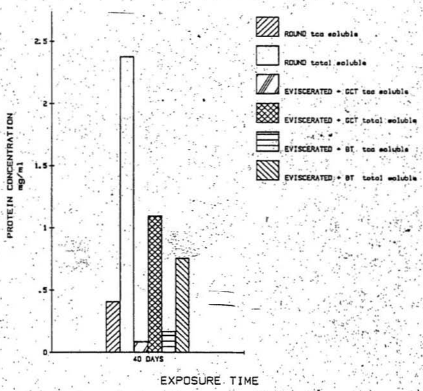
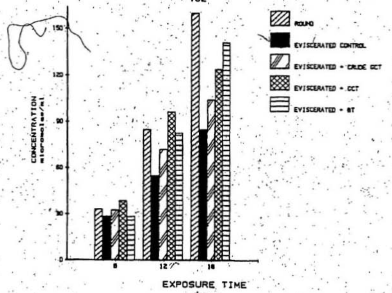


Figure 3-19: TCA vs total estimated soluble protein in herring brines

3.15.4 Free amino acids in fermentation brines

The total free amino acids in the fermentation brines were determined as described under 2.3.14.1 and the results are summarized in Fig. 3-20 from which it is apparent that the round fish fermentation system and the enzyme supplemented systems facilitated the formation of free amino acids in the brines than the ungutted control. Fig. 3-20 also indicates that at day 6, the amount of free amino acids formed in the brines were not very different from that of the gutted control, except the system supplemented with pure GCT, but at day 18 the relative amounts of free amino acids formed in the brines, corrected for the control values, were greatest for the round fish and least for the GCT supplemented system (i.e. not counting the system supplemented with crude GCT) - Fig. 3-20, which supports the finding with the TCA soluble protein in Fig. 3-20.

Even if it is assumed that all the fish had almost the same type and / or level of proteases in the flesh, the round fish had additional proteases in the gut like chymotrypsins, pepsins, carboxypeptidases etc. which probably facilitated the formation of free amino acids to a greater extent than was observed for the other systems. The relatively higher free amino acid content observed with BT at day 18 compared to the GCT was probably due to a diminution in GCT activity due to the unfavorable acid pH of the brine and possibly to the fact that the commercial BT was less pure than the GCT and probably had an active protease contaminant that participated in the hydrolysis of the proteins and peptides to amino acids.



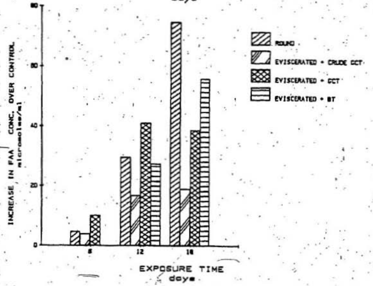


Figure 3-20: Free amino acids in herring fermentation brines

3.15.5 **Major taste active amino acids in brines**

The major taste active amino acids in the brines, as classified by Lee et al. (1982), are summarized in Figs. 3-21 and 3-22 from which it is apparent that more of the taste active amino acids were generated in the round fish and the enzyme supplemented brines than the gutted control, and also that the taste active amino acids increased from day 6 to day 18 in all cases except in the eviscerated control where the level of alanine fell by about 90% in going from day 6 to day 18.

In general, there was more of the taste active amino acids at day 6 with the GCT supplemented system than the BT supplemented system, suggesting again that GCT facilitated the hydrolysis of the fish in the early days of the fermentation to a greater extent than BT. However, at day 18 the situation had virtually reversed with the BT supplemented system having more of the taste active amino acids than the GCT supplemented system. The GCT and BT appeared to cleave more arginine plus lysine bonds than even the round controls (i.e. assuming that the polypeptides from the trypsin cleavages had arginine and lysine as terminal residues which were then cleaved by exopeptidases in the brines), suggesting that the overall greater degree of hydrolysis observed with the round fish was due to the presence other enzymes in the fish gut that were lacking in the GCT and BT systems.

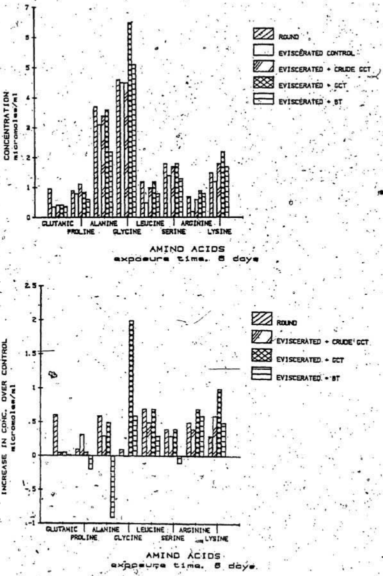
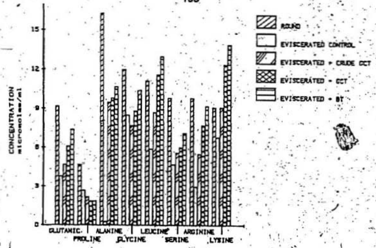


Figure 3-21: Major taste active amino acids in brines after day 6



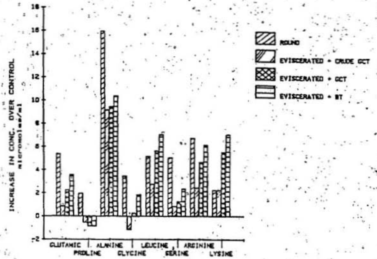


Figure 3-22: Major taste active amino acids in brines after day 18

3.16 Squid fermentation

The trypsins were used to supplement the fermentation of squid as described under 2.3.15 and the changes in pH, soluble protein, free amino acid and trypsin activity in the brines were measured at various time intervals.

3.16.1 pH changes in brines

The pH changes in the fermentation brines were followed as described under 2.3.14.4 and the results obtained are presented in Table 3-23, from which it is apparent that the pH of the brines increased from about pH 4.8 to between 5.7 and 6.0, and that pH change was independent of enzyme supplementation, similar to what was observed with the herring fermentation, Table 3-22.

Table 3-23: pH changes in squid fermentation brines

Sample	pH at Day						
	1	5	8	12	19	26	33
Squid (no enzyme)	4.8	5.3	6.0	6.0	5.9	5.7	5.8
Squid + GCT	5.0	5.4	5.9	5.9	5.7	5.6	5.8
Squid + BT	4.9	5.3	5.9	6.0	5.8	5.6	5.7

Legend to Table 3-23 : (i) concentrations of stock enzyme solutions used in fermentations ⇒ GCT = 1.25 mg/mL and BT = 1.67 mg/mL. (ii) Enzymes adjusted to have approximately the same activity on BAPA at 25°C: 0.10 mL of diluted stock GCT hydrolyzed BAPA at a rate ΔA_{410} nm/min of 0.0285 and 0.10 mL of diluted stock BT hydrolyzed BAPA at a rate $\Delta A_{410\ nm/min}$ of 0.0291. (iii) volumes of stock enzyme solutions applied : GCT = 20 mL and BT = 20.5 mL.

3.16.2 **Total soluble protein in squid brines**

The total estimated soluble protein in the squid brines was determined as described under 2.3.14.2 and the results are summarized in Fig. 3-23 from which it is apparent that the enzyme supplemented systems had more soluble protein in the brines than the unsupplemented system, and also that the amount of protein released increased with time. It also appears that GCT facilitated the release of soluble protein into the brine, at least up to 5 days of the fermentation, after which the release of the soluble protein slowed down relative to the system supplemented with BT, similar to what was observed for the herring fermentation. As can be seen from Table 3-24, the pH of the brines were slightly acidic - which is not very suitable for GCT and the diminution in GCT activity during the latter stages of the fermentation might probably be due to the acidic nature of the brines and possibly also due to the presence of naturally present inhibitors and / or end product inhibitors in the brines. It should be mentioned also that there was a lot of gas production in the fermentation systems from day 26 onwards, indicative of the presence of micro-organisms (halophilic) in the fermentation brines. This development was not noticed in either the herring fermentation or a previous fermentation with squid, carried out in this laboratory by Lee et al. (1982). A possible explanation as to why gas formed in the squid fermentation is discussed under 3.15.5.1.

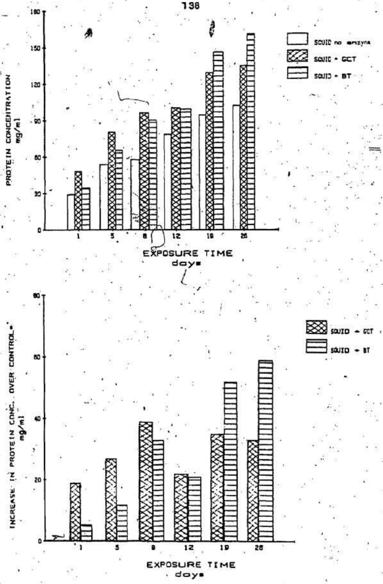


Figure 3-23: Total estimated soluble protein in squid brines

Values used to plot Fig. 3-23 are averages of two determinations.

3.16.3 TCA soluble protein in squid brines

The 5% TCA soluble protein in the squid brines was determined as described under 2.3.14.3 and the results obtained are summarized in Fig. 3-24 from which it is apparent that there was more TCA soluble proteins in the enzyme supplemented brines than the unsupplemented control at all stages of the fermentation investigated. However when the values obtained for the 5% TCA soluble protein (Fig. 3-24) are compared with those for the total soluble protein (Fig. 3-23) it becomes apparent that the products of the trypsin hydrolysis are largely TCA insoluble which indicates that the trypsins probably hydrolyze the protein in the fish to produce predominantly large molecular weight polypeptides rather than small peptides and amino acids.

3.16.4 Free amino acids in squid brines

The results obtained are summarized in Table 3-24, from which it is apparent that the enzyme supplemented systems liberated more free amino acids into the brines than the control.

Table 3-24: Free amino acids in squid brines - (μmoles/mL)

Sample	Exposure time (Days)					
	1	5	12	19	26	33
Control	65.4	84.4	64.5	-	136.4	170.1
Squid + GCT	94.7	109.8	164.9	184.5	181.5	189.8
Squid + BT	81.9	103.2	84.98	181.4	165.7	175.8

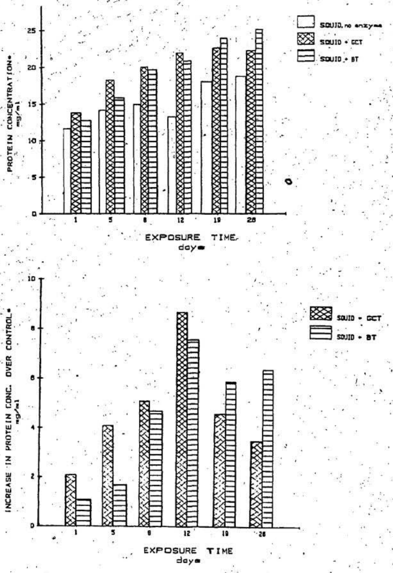


Figure 3-24: TCA soluble protein in squid brines

3.16.5 Major taste active amino acids in squid brines

The major taste active amino acids formed in the squid brines over various time intervals are summarized in Table 3-25, from which it is apparent that the enzyme supplemented samples had more of the taste active amino acids over the period for which measurements were made than the non-enzyme supplemented control.

Table 3–25: Major taste active amino acids in squid brines

Sample	Amino acids in brine (µmoles/mL)													
	Pro		Leu		Ser		Lys		Arg		Ala		Glu	
	at Day													
	5	36	5	36	5	36	5	36	5	36	5	36	5	36
Squid (control)	30	42	3	15	2	1	1	2	2	4	12	25	1	6
Squid + GCT	39	41	4	16	2	4	2	4	3	0.5	16	29	2	11
Squid + BT	37	41	3	14	2	8	2	0.5	2	2	16	25	2	8

However, like the situation observed with the herring, GCT appeared to facilitate the release of the amino acids more rapidly in the early days of the fermentation then slowed down, while BT seemed to steadily increase the accumulation of the amino acids throughout the period for which measurements were made. While GCT seemed to accumulate more of the amino acids in the early days of the fermentation, the system with BT generally either caught up with, or overtook the system with GCT in amino acid accumulation towards the latter stages of the fermentation.

Based on the persistent observation of apparent reduction in GCT activity towards the latter days of fermentation [observed for both herring and squid fermentation as well as in an earlier study by Lee et al. (1982)], the residual activity of the trypsins in the squid brines were estimated as described under 2.3.15.1.

The brines were diluted 10 fold with BAPA substrate buffer to raise the pH to approximately 8.0, then centrifuged at 0°C in a Sorvall RC-5 superspeed refrigerated centrifuge to remove the precipitate. The clear supernatant was used for the estimation of the residual trypsin activity by adding 2 mL portions of the clear supernatant to 1 mL of 1 mM BAPA solution and the rate of release of p-nitroaniline at 410 nm was followed at 25°C in the DU-8 spectrophotometer. For the blanks, 2.0 mL of BAPA substrate buffer was added to 1 mL of 1 mM BAPA solution.

The results obtained are summarized in Table 3-26.

Table 3-26: Trypsin activity in brines at various stages in the fermentation

Sample	Activity (Units/mL)[1] X 10^{-3} at Day								%Original activity (day 19)
	obs	exp	obs	exp	obs	exp	obs	exp	
	1		5		12		19		
Brine + BT (no squid, pH 2.8)	4.3	-	4.1	-	3.8	-	3.7	-	86.1
Brine from squid control, no enzyme	0.2	-	0.6	-	0.3	-	0.5	-	250.0
Brine from GCT supplemented squid	4.0	-	2.9	-	1.7	-	0.9	-	22.5
Brine from BT supplemented squid	3.8	-	3.7	-	2.5	-	2.0	-	52.6
Brine from BT[2] supplemented squid + Brine from GCT supplemented squid	-	-	3.0	3.3	1.9	2.1	1.1	1.5	36.7
Brine from BT[3] (no squid, control) + Brine from GCT supplemented squid	-	-	3.3	3.5	2.1	2.8	1.6	2.3	48.5

Legend to Table 3-26 : (i) values are averages of two determinations;

(ii) [1] $\text{Units/mL} = \dfrac{\Delta A_{410\ nm/min} \times 1000 \times 3}{8800 \times \text{vol. of brine in assay}}$

(iii) [2] equal volumes of the clear supernatants from the BT and GCT supplemented squid brines, and (iv) [3] equal volumes of clear supernatants from BT supplemented brine and GCT supplemented squid brine. (v) 'obs' = observed; and 'exp' = expected.

3. 16.5.1 General Discussion : Supplementation of fish fermentations with trypsins

It appears from Table 3–26 that the decline in trypsin activity was partly due to inhibition by materials in or accumulating in the squid brines, especially because the addition of the clear supernatant from the GCT supplemented squid brine to the supernatant from the BT brine that had no squid was considerably lower than might have been expected if the activity from both sources were additive.

It has been suggested by workers like Uyenko et al. (1952) and Orejana and Liston (1982) that visceral enzymes, particularly trypsin, are principally responsible for the proteolysis that occurs during the fermentation of fish in the presence of high concentration of salt. Unilever (1975) of Great Britain, have described a process for the production of salted herring (matjes) using digestive enzymes - trypsin and chymotrypsin - at 10°C, probably to minimize the undesirable effects that occur when fermentation is carried out at elevated temperatures.

Greenland cod trypsin was therefore applied separately to salted herring and squid to determine if its higher molecular activity at lower temperatures compared to BT could be exploited.

As previously noted, even though GCT seemed to facilitate proteolysis of both the herring and the squid in the early days of the fermentation compared to the eviscerated controls and probably also the BT supplemented systems, the GCT appeared to lose a considerable part of its capacity to effect proteolysis at the latter stages of the fermentation more than the BT supplemented system, and it is suggested that the decrease in GCT activity was probably due to one, two or all three of the following factors : (i) the presence of naturally present trypsin inhibitors in the blood of the fish, as suggested by Orejana and Liston (1982), that somehow inhibited the GCT more than they did BT; (ii) inhibition by end products of the proteolysis, like amino acids and small peptides; and (iii) inactivation due to the acidic pH of the fermentation brines.

It is also suggested that inactivation due to acid pH of brines might be the major cause of the greater depression of GCT activity compared to BT activity since from the pH studies, BT appeared to be relatively more stable and slightly more active at acid pH than GCT. So the exploitation of the higher molecular activity of GCT at lower temperatures was not successfully demonstrated by the fermentation study, and it would probably be more beneficial to employ the GCT in operations that proceed in alkaline pH media, where GCT appears to be more stable and more active than BT. Workers like Orejana and Liston (1982), Raa and Gildberg (1975) and Tarky et al. (1973) have observed that end product inhibition of proteolytic enzymes during fish fermentation does occur.

The experiment on squid fermentation was designed to follow the changes in the activity of the trypsins in the brines, using BAPA as a model substrate, hopefully to find some answers to the problem of decline in rate of proteolysis observed during the herring fermentation. So conditions of the experiment, such as salt, sugar, amount of fish added per unit volume of water etc., were made very similar to those used for the herring fermentation. Which means that the proportion of fish : salt : sugar per unit volume of water in these fermentations was 3 : 0.4 : 0.08, while the proportion of fish : salt : sugar per unit volume of water employed by Lee et al. (1982) in an earlier study with squid was 1.4 : 0.24 : 0.05.

The relatively lower salt content used in this study was probably responsible for halophilic bacteria or yeast growth leading to gas production in the squid brines. The difference in salt content could not be avoided since the procedure used to ferment the herring was adapted from the Unilever (1975) process for making matjes, which was quite different from that used by Lee et al. (1982), and the experiment with the squid was aimed at replicating the herring fermentation study. It appears from this that while the lower level of salt sufficed to support fermentation of the herring without microbial growth, the same salt level was too low to support the fermentation of squid without microbial growth.

3.17 Prevention of the formation copper-induced TBA-reactive substances in raw cow's milk

The determination of the efficacy of trypsins in preventing TBA-reactive substances in raw milk samples was carried out as described under section 2.3.16. To determine the initial activity of the trypsins before applying to the raw milk samples, the stock trypsins were adjusted such that when 5 mL of it were diluted to 50 mL with de-ionized water, 0.100 mL of the diluted enzyme could hydrolyze 1mM TAME at a rate of 0.080 $\Delta A_{247\ nm/min}$ at 25°C.

To determine the activity of trypsins in the milk samples before and after pasteurization. portions of the milk samples were centrifuged in a bench top eppendorf centrifuge at top speed at 4°C for 15 min. then 0.100 mL of the supernatant was applied separately to TAME as described under section 2.3.16.2 at 25°C . A summary of the initial and residual activities of the trypsins applied to the milk is presented in Table 3-27.

Table 3-27: A summary of the initial and residual activities of trypsins applied to raw cow's milk

Enzyme	Initial Activity[a] ΔA_{247} nm/min	Residual Activity $\Delta A_{247nm/min}$	
		Before Pasteurization[b]	After Pasteurization[c]
GCT	0.0793	0.0477	0.000
BT	0.0787	0.0680	0.037

Legend to Table 3-27 : Values are averages of two determinations. and for the residual activities. only the samples with the highest levels of trypsin were tested: stock GCT = 0.0193 mg/mL stock BT = 0.0231 mg/mL.

[a] Initial activity = activity of enzyme before it was added to milk.

[b] Raw milk samples incubated at 4°C for 4h.

[c]Milk pasteurized at 70°C for 45 min.

Table 3-27 indicates that while GCT did not survive the pasteurization treatment, BT did survive the treatment and retained about 47% of its original activity.

The levels of TBA-reactive materials in the milk samples, as measured by the thiobarbituric acid (TBA) method of King (1962), are summarized in Table 3-28.

Table 3-28: Summary of TBA values in milk samples

Enzyme added (% by volume)	Cu (ppm)	$\Delta A_{532\ nm}$ at Day GCT 1	3	12	BT 1	3	12
-	-	0.021	0.052	0.051	0.021	0.052	0.051
-	1	0.033	0.064	0.067	0.033	0.064	0.067
0.032	1	0.014	0.030	0.040	0.020	0.034	0.046
0.064	1	0.013	0.028	0.034	0.014	0.029	0.042
0.128	1	0.009	0.020	0.027	0.010	0.021	0.030
0.256	1	0.010	0.014	0.022	0.011	0.017	0.024
0.512	1	0.012	0.016	0.024	0.012	0.018	0.019
0.640	1	0.009	0.017	0.016	0.009	0.016	0.018

Values are averages of 2 determinations.

Table 3-28 indicates that both GCT and BT suppressed the levels of TBA-reactive substances in the milk samples, at the various enzyme levels and also that the levels of such substances decreased with increasing trypsin concentration.

3.17.1 General Discussion : Prevention of milk oxidation by trypsins

It has been observed by workers like Anderson (1939), Doan and Miller (1940), Olson and Brown (1944) and Foster and Sommer (1951) that bovine pancreas trypsin treatment of milk increases the resistance of milk to oxidized flavor development. However, bovine trypsin has been found to be relatively heat stable and difficult to inactivate after it has been applied to milk samples to prevent or retard oxidized flavor development. For instance, Storrs and Hull (1956) observed that approximately a third of the added trypsin remained active after pasteurization at about 62°C for 30 min. As a result, workers like Weetall (1969) have immobilized trypsin to glass supports and used it to prevent oxidized flavors from developing, then remove the glass bound enzymes from the milk to circumvent the need for inactivation.

Based on the thermal instability of GCT, it was decided to apply it to raw milk to determine whether or not it could prevent the formation TBA-reactive subtances induced by copper and also to determine whether or not it would survive pasteurization treatment. The finding that GCT could not survive the pasteurization treatment after it had been applied to suppress development TBA-reactive substances in the raw milk samples illustrates that advantage could be taken of the lower thermal stability of GCT by the dairy industry to counteract the problem of oxidized flavors instead of BT.

Chapter 4

CONCLUSIONS AND SUGGESTIONS

1. The protease purified from the pyloric ceca or intestine of Greenland cod is trypsin (E.C. 3.4.21.4). This conclusion is based on the findings that : (i) the cod enzyme catalyzes the hydrolysis of trypsin specific amide and ester substrates, (ii) the degrees of hydrolysis of various protein substrates by the cod enzyme were identical to those of bovine trypsin, (iii) it was sensitive to standard trypsin inhibitors including the serine protease inhibitor phenyl methyl sulfonyl fluoride, (iv) the occurrence of the protease in the pyloric ceca in the form of a zymogen, (v) the relative abundance of certain amino acid residues similar to other trypsins and (vi) molecular weight of approximately 23 kdal consistent with trypsin.

2. Greenland cod trypsin is a more efficient catalyst than bovine trypsin under saturating levels of substrate concentration. This conclusion is based on the findings that the cod enzyme has (i) a relatively high specific activity for the amidase, esterase and protein hydrolase reactions, (ii) a relatively high substrate turnover number for the amidase and esterase reactions, (iii) a relatively lower $\Delta G^{\neq}$ for the amidase and esterase reactions, and (iv) a higher "physiological efficiency" for the amidase reaction.

However from the Km' values, BT may be a more afficient catalyst than BT when substrate levels are limiting.

Even though the commercial BT used in some of the studies was not as "pure" as GCT, the fraction of non-trypsin material from the affinity column (peptide mapping and CD spectra studies) was approximately 10% only. Since the removal of the non-trypsin material did not appreciably increase the specific activity of BT (increase due to purification by affinity chromatography was approximately 13%), the statements made under (2) would still be valid if appropriate adjustments are made in the substrate turnover number for the amidase or esterase reactions catalyzed by BT.

3. Catalysis by Greenland cod trypsin is less sensitive to temperature than catalysis by the bovine enzyme. This conclusion is based on the findings that the cod enzyme has (i) a relatively lower Q_{10} for the esterase, amidase and protein hydrolase reactions, and (ii) a relatively lower E_a and ΔH^{*} for the esterase and amidase reactions.

4. The stability of Greenland cod trypsin is different from that of bovine trypsin as demonstrated by differences in their pH and thermal stabilities.

5. The polypeptide chain of Greenland cod trypsin is different from that of bovine trypsin. This conclusion is based on the findings that the cod enzyme had (i) different CNBr peptides, (ii) different amino acid composition, (iii) different hydrophobicity index, (iv) different number of potential disulfide pairs, (v) differences in CD spectra and (vi) different electrophoretic mobility.

The differences in amino acid composition, hydrophobicity index, potential number of disulfide pairs and the CD spectra indirectly suggest that the cod enzyme is less ordered than the bovine enzyme. This less ordered structure of Greenland cod trypsin is probably responsible for some of the observed differences in the thermal stabilities, substrate turnover numbers, E_a's, $\Delta H^{\neq}$'s and $\Delta G^{\neq}$'s of the two proteases.

6. The relatively rapid loss in activity of cod enzyme observed during herring or squid fermentations indicates that it is less suitable than bovine trypsin as a supplement to fish fermentation over long periods of time, but the complete inactivation of cod trypsin by pasteurization after it had successfully been used to modify milk so as to prevent oxidation suggests that it would probably be a more suitable protease to use to prevent milk oxidation.

7. It is suggested that Greenland cod trypsin is potentially useful as an industrial enzyme. Its greater stability and activity at alkaline pH indicate the enzyme may be most suitable for industrial processes like enzymatic hydrolysis of fish protein ; incorporation of the enzyme in detergents for household purposes and the use of the enzyme in the dehairing of hides and bating of leather. Current production practices in these areas utilize bovine trypsin or porcine trypsin or other alkaline proteases of bacterial origin. Other advantages Greenland cod trypsin may have over bovine trypsin as an industrial enzyme in addition to the alkaline stability include (i) its higher molecular activity at lower temperatures, which would make it possible for reactions to be carried out at lower temperatures to cut down energy costs and (ii) its thermal instability which makes it possible for the reaction to be terminated by mild heat treatment.

The advantage Greenland cod trypsin could have over certain alkaline proteases of bacterial origin include the fact that being from animal origin, Greenland cod trypsin would meet with less resistance from the consumer and regulatory agencies than is the case with the bacterial enzyme. The problem of allergic reactions arising from spores produced by some of these micro-organisms also tend to put them at a disadvantage compared to Greenland cod trypsin. However, it is still necessary to carry out further studies on Greenland cod trypsin to determine if its potential can be realized in practice.

The use of Greenland cod trypsin would cut down on the demand for bovine trypsin and also minimize waste in the fish industry and help translate to reality, concepts or headlines such as "wealth from waste" and "garbage is gold".

With the exception of the few investigations carried out by workers like Hofer et al. (1975) – on the relationship between substrate binding affinities of crude trypsin preparations from various sources and their temperature preferenda – ; Owen (1968) on the pepsin digestion of salmonid fish ; and Haard et al. (1982) on cold adapted pepsins from the marine environment, there does not appear to be any definitive work on cold adapted enzymes which are extracellular like trypsin, and no prior studies of this type have been reported on a purified digestive enzyme.

It must be emphasized at this point, however, that not all organisms that inhabit the cold environment show compensatory adjustments in the catalytic efficiencies of their enzymes, even though the evidence available indicate that a great number of animals subsisting under such conditions that have been investigated do show such compensation. For example, the organism may alter the levels of pre-existing enzymes to achieve compensatory adjustments. The potential of altering enzyme levels is expected to play a major role where a particular enzyme or group of enzymes regulate rate limiting steps in a reaction pathway. For example the synthesis of such enzymes may be turned on and their degradation slowed down in the cold; or the synthesis of the enzyme(s) may be turned off and their degradation stepped up at elevated temperatures. It would seem that this strategy would better suit organisms that either inhabit zones whose temperatures oscillate from cold to warm or vice versa, and those other organisms that migrate from one zone to another of different temperature.

Another method of adjusting rates of enzymatic activity involves a different form of a particular enzyme that is a better catalyst at low temperature.

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Appendix

Appendix A

Reagent	Blank (mL)	Test (mL)
H_2O	0.2	–
Substrate	1.0	1.0
Substrate buffer	1.8	1.8
Enzyme solution *	–	0.2

* The enzyme solution was added last to the reaction mixture and the change in absorbance at 15 sec intervals was measured at 410 nm in a Beckman DU-8 computing spectrophotometer.

Appendix B

Reagent	Blank (mL)	Test (mL)
H_2O	0.1	-
Substrate	0.3	0.3
Substrate buffer	2.6	2.6
Enzyme solution *	-	0.1

*The enzyme solution was added last to the reaction mixture and the change in absorbance after 15 sec intervals measured at 247 nm in the Beckman DU-8 computing spectrophotometer.

Appendix C

Reagent	Blank (mL)	Test (mL)
5mM HCl	0.5	-
Substrate buffer	1.0	1.0
Substrate stock	1.5	1.5
Enzyme solution *	-	0.5

* The enzyme solution was added last to the reaction mixture.

Appendix D

pH 2.0 : 0.1M HCl - KCl

pH 4.0 : 0.1M citric acid - NaOH

pH 6.0 : 0.1M citric acid - NaOH

pH 7.0 : 0.1M tris - HCl

pH 7.5 : 0.1M tris - HCl

pH 8.0 : 0.1M tris - HCl

pH 8.5 : 0.1M tris - HCl

pH 9.0 : 0.1M tris - HCl

pH 9.5 : 0.1M tris - HCl

pH 10.0 : 0.1M glycine - NaOH

Appendix E

Composition of buffer solutions* used :

pH 2.0 : 0.2M citrate – HCl

pH 4.0 : 0.2M citrate – NaOH

pH 6.0 : 0.2M citrate – NaOH

pH 7.0 : 0.2M citrate – NaOH

pH 7.5 : 0.2M borate – HCl

pH 8.0 : 0.2M borate – HCl

pH 8.5 : 0.2M borate – HCl

pH 9.0 : 0.2M borate – HCl

pH 9.5 : 0.2M borate – NaOH

pH 10.0 : 0.2M borate – NaOH

pH 11.0 : 0.2M borate – NaOH

pH 12.0 : 0.2M borate – NaOH

* All the buffer solutions contained 5mM $CaCl_2 . 2H_2O$

Appendix F

Substrate stock (mL)	Buffer (mL)	Enzyme (mL)	Substrate conc. (mM)
1.67	1.13	0.20	2.50
1.33	1.47	0.20	2.00
1.00	1.80	0.20	1.50
0.89	1.91	0.20	1.33
0.83	1.97	0.20	1.25
0.67	2.13	0.20	1.00
0.50	2.30	0.20	0.75
0.33	2.47	0.20	0.50
0.27	2.53	0.20	0.40

For the blanks, 0.2 mL of de-ionized water was added to the reaction

Appendix G

Substrate stock (mL)	Buffer (mL)	Enzyme (mL)	Substrate conc. (mM)
0.09	2.81	0.10	0.3
0.12	2.78	0.10	0.4
0.15	2.75	0.10	0.5
0.18	2.72	0.10	0.6
0.21	2.69	0.10	0.7
0.24	2.66	0.10	0.8
0.27	2.63	0.10	0.9
0.30	2.60	0.10	1.0

For the blanks, 0.10 mL of de-ionized water was added to the reaction

Appendix H

Sample	Vol. of Milk (mL)	Vol. of Enzyme (mL)	Vol. of Water (mL)
1	44	-	1.000
2	44	-	1.000
3	44	0.016	0.984
4	44	0.032	0.968
5	44	0.064	0.936
6	44	0.128	0.872
7	44	0.256	0.744
8	44	0.320	0.680

Appendix I

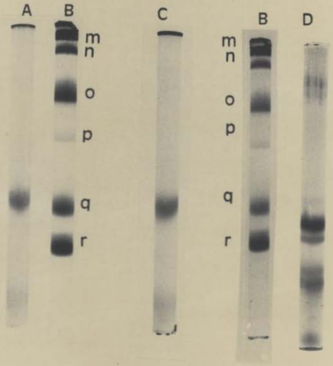

Figure 6-1: Estimation of mol. weight of GCT by electrophoresis (Laemmli, 1970)

'A' = GCT from the pyloric ceca : 'B' = protein standards : 'C' = GCT from the intestines : 'D' = BT from bovine pancreas.

Appendix J

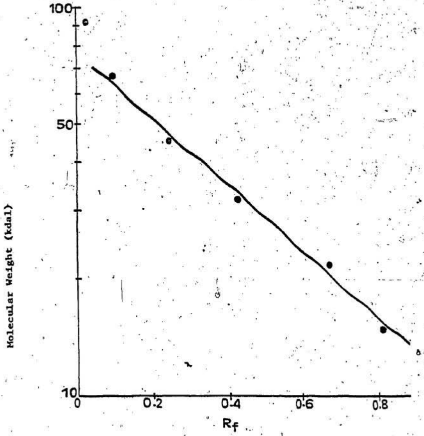

Figure 6-2: Graph of molecular weight vs R_f

The R_f values were estimated as follows :

$$\frac{\text{distance travelled by protein} \times \text{length of gel before staining}}{\text{distance travelled by dye} \times \text{length of gel after staining}}$$

Appendix K

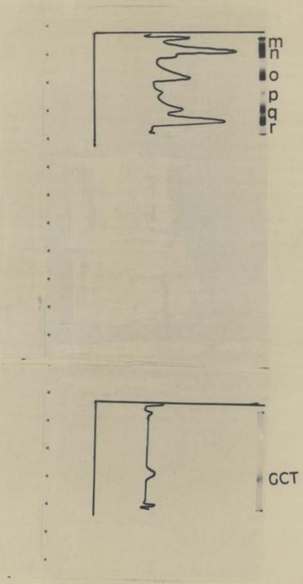

Figure 6-3: Gel scan of proteins with molecular weight calculation

Appendix L

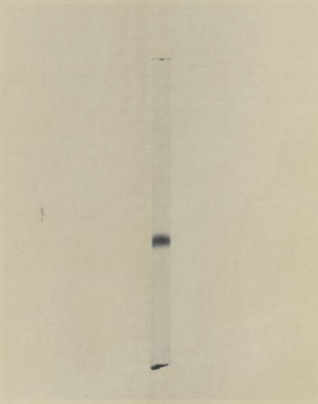

Figure 6-4: SDS polyacrylamide gel of GCT (Laemmli, 1970)