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Purification and Characterisation of Trypsin-Like Enzyme from the Pyloric Caeca of Cod (*Gadus morhua*) II

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ABSTRACT

A trypsin -like enzyme from the pyloric caeca of cod (Gadus morhua) was purified by affinity chromatography on CHOM Sepharose 4B. Some characteristics were established by its catalytic activity on T.A.M.E., typical enzyme substrate, and serine protease inhibitors. The enzyme had an isoelectric point of 5.30 and 5.89 and was very similar in amino acid composition to bovine trypsin, but differed in having a higher relative amount of acidic amino acids and a lower amount of basic amino acids. The enzyme also hydrolysed fish protein substrates.

Key words: Cod, enzyme, trypsin

INTRODUCTION

Trypsin–like alkaline proteinase enzymes have been selected for detailed study and attempts have been made to isolate and characterise them as they constitute the major proteolytic group of enzymes of the pyloric caeca of cod (*Gadus morhua*). Cod has been selected as it could be obtained live by line fishing near Aberdeen. It is also a fish of major industrial importance, and any significant finding obtained would have relevance in the fish processing industry. The pyloric caeca represent the organs which are the major source of alkaline proteinases. They take the form of blind sacs posterior to the stomach. These are intestinal invaginations and the number may vary from one to over one thousand (Bertin, 1958). Histology of cod pyloric caeca has been studied by Bishop & Odense (1966)) who described both exocrine and some endocrine cells in the mesentery, with numerous small pancreatic ducts converging into four or five large ones entering the ileum anterior to the bile duct. The endocrine cells are similar to the islet of Langerhans in mammals and are situated on the highest point of the gall bladder. It is well established that the pyloric caeca are important sources of proteolytic enzymes. Johnston (1941) in assays on crude extracts of pyloric caeca from various species of fish found considerable variation in activity using casein as substrate. Information on alkaline protease is available to a varying extent in the literature. Studies include the following: the

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proteolytic enzymic activity in the pyloric caeca of redfish (Stern & Lockhart, 1953), proteinases in the pyloric caeca of mackerel (Ooshiro, 1971), the cationic chymotrypsin in dogfish (Prahl & Neurath, 1972), and trypsin from the catfish pancreas (Yoshinaka *et al.*, 1983). Noda *et al.* (1982) compared the activities of alkaline and acidic proteinases with bovine trypsin and pepsin and showed that like bovine trypsin the alkaline proteinase from sardines pyloric caeca hydrolysed casein more effectively than other protein substrates. Their properties are similar to those of mammalian trypsin but they differ from it particularly in the acidic-isoelectric point (Murakami & Noda, 1981; Cohen *et al.*, 1981) and by being unstable at low pH values (Camacho *et al.*, 1970; Hjelmeland & Raa, 1982). They are similar to bovine trypsin in being inhibited by soybean inhibitor (Murakami & Noda, 1981) and in being able to hydrolyse trypsin substrates. Usually the enzyme specifications tend to be related to the nature of the amino acid residues, and the enzyme trypsin and trypsin-like enzyme have as specific substrates a positively charged lysine or arginine. Zwilling *et al.* (1969) stated that the trypsin-like proteinases occurring in crayfish (*Astacus leptodactylus*) have cleavage specificity identical to mammalian trypsin. Similar results have been obtained in studies on enzymes isolated from the lower vertebrates by Cohen *et al.* (1981). It is believed that trypsin-like enzymes from pyloric caeca may undergo a variation in concentration over an annual cycle, with the maximum at the end of April and a minimum at the beginning of August (Kashiwada, 1952).

MATERIAL AND METHODS

Thermal stability of proteolytic enzyme: Thermal stability of proteolytic enzyme was tested after 1.0 ml enzymes were pre-incubated for 10 minutes in 3.0mL of Tris-HCl buffer (pH 7.8), containing 0.5M NaCl and 0.02M $CaCl_2$ at the following temperatures of 20, 30, 40, 50, 60, 70 and 80°C, and cooled immediately in an ice bath. Subsequently the enzymes were incubated at 37°C with 1.0mL denatured haemoglobin to determine the residual activity at 280 nm.

Assay of activity of proteolytic enzymes towards synthetic substrates: Activity towards p-tosyl-L-arginine methyl ester (T.A.M.E.) was determined as follows: 2.6 ml of 46mM of Tris-HCl (pH 7.8) containing 11.5mM $CaCl_2$ was added to a cuvette along with 0.3mL of 10mM T.A.M.E. (Walsh, 1970). Then 0.1mL of enzyme solution was added at zero time and mixed immediately. One unit of T.A.M.E. activity was defined as the amount of trypsin or trypsin-like enzyme, which resulted in an increase of one absorbance unit at 247nm per minute. Specific activity was expressed as unit of enzymatic activity per mg protein.

Activity towards N-acetyl-L-tyrosine ethyl ester (A.T.E.E.) was determined using the Bergmeyer *et al.* (1974) procedure. Assays were performed in 50mM phosphate buffer (pH 7.0) with 0.93mM A.T.E.E. as substrate. Enzyme solution (0.20mL) was added at zero time immediately. Absorbance at 237nm was measured at one-minute intervals. One unit of A.T.E.E. was defined as the amount of chymotrypsin or chymotrypsin-like enzymes, which resulted in a decrease of one absorbance unit at 237nm per minute. Specific activity was expressed as unit of enzymatic activity per mg protein.

Effect of inhibitors on enzyme activity: The procedure in this work was similar to that of Gates & Travis (1969) in which one mL of the enzyme solution in the presence of a 100 and 1,000 fold molar excess of phenylmethyl-sulphonyl fluoride; D-L-Arginine; L-1-Tosylamide-2 phenyl-ethylchlorometyl ketone (T.P.C.K.); N-p-Tosyl-L-Lysine chlorometyl ketone (T.L.C.K.); cysteine and 0.25 - 0.025 mg/mL trypsin inhibitor from Soybean (S.B.T.I.) in 0.05M Tris-HCl with 0.02M $CaCl_2$ were mixed and incubated at 30°C for 30 minutes. The activities were assayed with haemoglobin as substrate at 280nm in the usual manner.

Assay of hydrolysis of fish protein substrates by crude extracts from pyloric caeca: The separation of the protein fraction (sarcoplasmic, myofibrillar and connective tissue) was similar to the procedure used by Groninger (1973). Proteinase solution from pyloric caeca of cod were incubated at 40°C for 10 minutes with 1.0mL of the above protein substrates which had been pre-incubated at 100°C for 5 minutes. Buffer was 0.05M Tris-HCl

containing 0.02M $CaCl_2$ (pH 7.8). Each enzyme is expressed as the percentage of each activity on haemoglobin according to the technique of Noda *et al.* (1982).

RESULTS AND DISCUSSION

Thermal stability of proteolytic enzymes: The activity of the trypsin-like enzymes was determined after prior storage for 60 minutes at pH 7.8 over the temperature range of 20 to 70°C. Results in Figure 1 show the effect of storage at those temperatures on their activities towards haemoglobin as substrate under the standardised conditions at 40°C. As is evident from temperatures 20 to 40°C, there was no significant loss of activity, but from 50°C and higher, there was a marked decrease. At around 65°C, the trypsin-like enzyme show half of activity and at 70°C the enzyme almost completely inactivated. These results were similar to those for the anionic trypsin of catfish (Yoshinaka *et al.*, 1984) and of the Arctic fish capelin (Hjelmeland & Raa, 1982) whose enzymes were stable even at 66°C. Yoshinaka *et al.* (1983) while comparing this enzyme with bovine trypsin concluded that the fish trypsin was less stable at temperatures above 50°C. The authors suggested that the difference in thermal stability was reflected in the difference in the pH stability of both enzymes. In works with bovine and pig trypsin Lazdunski & Delaage (1967) stated that the stability was due to interaction of noncovalent bonds such as hydrogen bonds, as well as electrostatic and hydrophobic interactions.

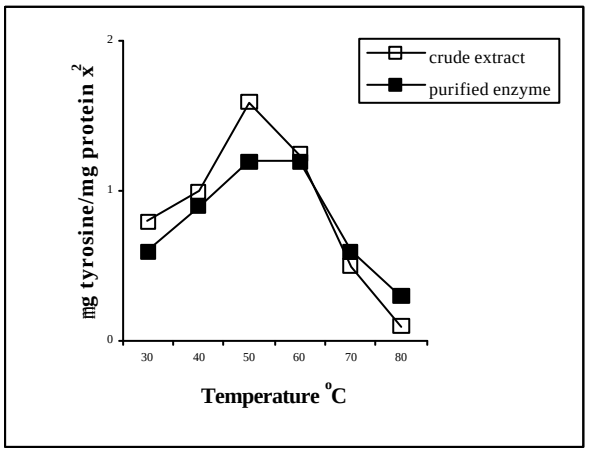

Figure 1 - Effect of temperature on activity of purified and crude enzymes from cod pyloric caeca [Proteolytic activity was measured by Anson (1938) method at 40°C for 15 min. Protein concentration: 1.31mg/mL for crude extract and 0.081mg/mL for purified enzyme]

Whitaker (1972) cited other factors such as substrate, presence or absence of inhibitors and the nature of buffers. In general, enzymes that had a molecular weight lower than 50,000 were less susceptible to modification to their structure by heating. Lazdunski & Delaage (1967) showed that this modification corresponded to a loss of molecular organisation that was manifested by exposure of certain hinge residues of tyrosine and tryptophan, or exposure of peptide bonds of lysine and arginine that can be hydrolysed.

Activity of proteolytic enzymes towards synthetic substrates: The active sites of trypsin and chymotrypsin have much in common with respect to their specificities, with both enzymes catalysing the hydrolysis of a series of substrates such as p-nitrophenylacetate (Hartley & Kilby, 1954) and benzoylalamine (Ravin *et al.*, 1954). There are, however, significant differences as trypsin hydrolyses peptides and ester bonds formed by the carboxyl group of the basic amino acids, lysine and arginine (Neurath & Schewert, 1950) and chymotrypsin primarily catalys the hydrolysis of peptide bonds adjacent to the carboxyl group of the aromatic amino acids tryptophan, tyrosine and phenylalanine (Bergmann & Fruton, 1941; Cunningham, 1965). Knowledge of the conformation of the substrate when located at the active centre of an enzyme is essential for understanding the enzyme specificity, since the conformation adopted by the substrate discloses the stereochemistry of the active centre in view of its complementarity to the substrate. Such compounds, which meet this criteria are of T.A.M.E., which is specific for trypsin activity (Schewert *et al.*, 1948) and A.T.E.E. for chymotrypsin activity (Neurath & Schewert, 1950). They were accordingly used in this work to determine the specificity of the purified enzyme (Figure 2).

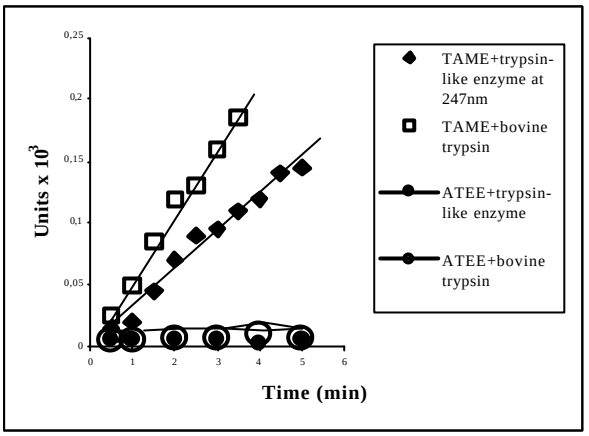

Figure 2 - Tryptic activity from purified enzyme of pyloric caeca of cod and bovine trypsin toward T.A.M.E. and A.T. E. E. [One unit of T.A.M.E. activity was defined as the amount of trypsin-like enzyme which resulted in a increase of one absorbance unit at 247nm. One unit of A.T.E.E. activity was defined as the amount of chymotrypsin-like enzyme resulted in a decrease of one absorbance unit at 237nm.]

The results using the synthetic substrate T.A.M.E. suggested that the fraction isolated after CHOM-CNBr activated Sepharose 4B (Beirão & Mackie, 1994) had relevant tryptic activity. The absence of activity towards the other synthetic substrate A.T.E.E. suggested that it had no chymotryptic activity. Tryptic activity towards specific trypsin substrates was detected in many other fish species such as sardine (Murakami & Noda, 1981) and salmon (Croston, 1965). Similar results were reported for bovine trypsin by Trowbridge *et al.* (1963) and Magalhães-Rocha *et al.* (1980).

Inhibition of proteolytic activity of enzyme: Data on the effect of some inhibitors on the crude and purified enzymes are presented in Table 1. The purified enzyme was inhibited by T.L.C.K., S.B.T.I. and phenyl methyl sulphonyl fluoride, but T.P.C.K. had no inhibitory effect on its proteolytic activity. The active site oriented inhibitor T.L.C.K. for trypsin (Shaw *et al.*, 1965) and T.P.C.K. for chymotrypsin (Schoellmann & Shaw, 1963) were used to identify the enzyme involved in the proteolytic activity. Shaw *et al.* (1965) in their work to obtain evidence on the presence of a histidine residue at the active centre of trypsin stated that T.L.C.K. inactivated trypsin by stoichiometric alkylation at N-3 of a -histidine residue, and that chymotrypsin was not affected. The results of inhibition of proteolytic enzymes by T.L.C.K. provided an additional indication that the binding site of the cod proteolytic enzyme exhibited a higher resemblance to the mammalian trypsin in agreement with other studies on trypsin -like enzymes from fish, viz.: Yoshinaka *et al.* (1984) on catfish; Zwilling *et al.* (1969) on crayfish and Murakami & Noda (1981) on sardines. The results with S.B.T.I. showed that this natural inhibitor strongly inhibited the cod trypsin. The observation was in line with its known effect on other mammalian trypsins (Kunitz, 1946; Camacho *et al.*, 1970; Blow *et al.*, 1974). The stoichiometry of inhibition seemed to be different for T.L.C.K. Soybean trypsin inhibitor has been known as a double-headed inhibitor possessing trypsin binding and chymotrypsin binding (Wu & Laskowski, 1955; Ryan *et al.*, 1965). Baillargeon *et al.* (1980) stated that the reactive site Arg-63-Ile-64 peptide bond of soybean trypsin inhibitor was preferentially and reversibly hydrolysed by trypsin. Table 2 shows the effects of those inhibitors on trypsin-like enzymes from different marine species.

Table 1 - Effect of inhibitors on proteolytic activity of enzymes from pyloric caeca of cod.

Reagents	Concentration (M)	Residual activity (%)*			
		Bovine trypsin	Bovine chymotrypsin	Crude extract	Purified enzyme
T.P.C.K.	10^{-3}	80	5	70	89
	10^{-4}	94	10	77	100
T.L.C.K.	10^{-3}	0	80	79	0
	10^{-4}	27	92	80	2
S.B.T.I.	0.25mg/mL	0	48	40	0

	0.025mg/mL	0	51	60	0
P.M.S.F.	10^{-3}	1	10	47	1.8
	10^{-4}	2.7	19	59	4.0
Control	No Inhibitors	100	100	100	100

Substrates: L-1-Tosylamide-2-phenyl-ethylchloromethyl ketone (T.P.C.K.); N-α-p-Tosyl-L-Lysine chloromethyl ketone (T.L.C.K.); Soybean trypsin inhibitor (S.B.T.I.) and phenyl methyl sulphonyl fluoride (P.M.S.F.).
*Samples were incubated for 15 min at 40°C and residual activity was assayed with haemoglobin as substrate at 280nm.

Concentration of substrate and Michaelis-Menten constant: Figure 3 shows the dependence of the rates of hydrolysis of haemoglobin by cod trypsin-like enzyme and by bovine trypsin on the substrate concentration.
The rates of hydrolysis of urea denatured haemoglobin by both enzymes obeyed Michaelis-Menten kinetics over the concentration of substrate examined. The results are shown in Figure 4.
The Km value of cod trypsin-like enzyme (0.87 x 10^{-5}) on haemoglobin was similar to those of bovine trypsin. In Table 3 values of Michaelis-Menten constants for different substrates are shown.

Table 2 - Comparative studies of inhibitors on the activity of trypsins from different sources.

	Inhibitor (Relative activity %)				
Sample	T.P.C.K.	T.L.C.K.	S.B.T.I.	P.M.S.F.	*
Sardine	100	8	0	-	(1)
Capelin	100	0	0	8	(2)
Crayfish	-	-	-	0	(3)
Catfish	100	0	0	44	(4)
Krill	100	0	0	-	(5)
Shrimp	100	0	0	-	(6)
Bovine	100	0	-	-	(7)
Bovine	-	-	0	-	(8)

*(1) Murakami & Noda (1981); (2) Hjelmeland & Raa (1982); (3) Zwilling *et al*., (1969); (4) Yoshinaka *et al*., (1984); (5) Kimoto *et al*., (1983); (6) Gates & Travis (1969); (7) Mares-Guia & Shaw (1965); (8) Jacobsson (1955).

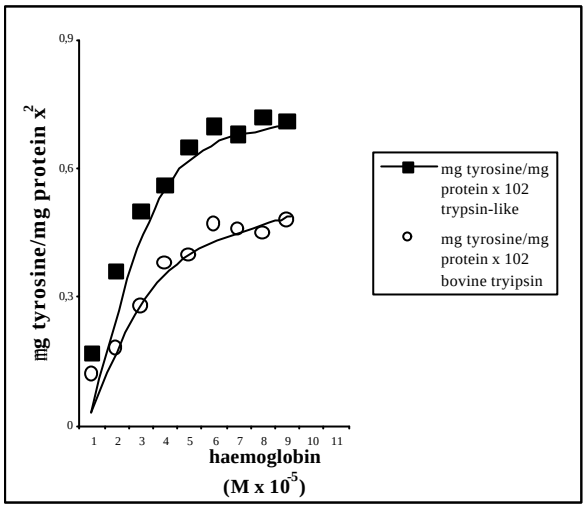

Figure 3 - Effect of substrate concentration on trypsin-like enzyme and bovine trypsin activities when assayed for 10 min at 40°C and pH 7.8 [concentration of enzyme 0.016mg/mL].

Comparative studies between fish trypsin and bovine trypsin have been made by Yoshinaka *et al.* (1984) and Cohen *et al.* (1981) who were stated that the Km for both bovine and fish trypsin are identical or very close.

Activity of crude and purified trypsin-like enzymes towards fish protein substrates: The results for degradation by both the crude and purified enzymes, and bovine trypsin are given in Figures 5, 6 and 7. These results showed that the

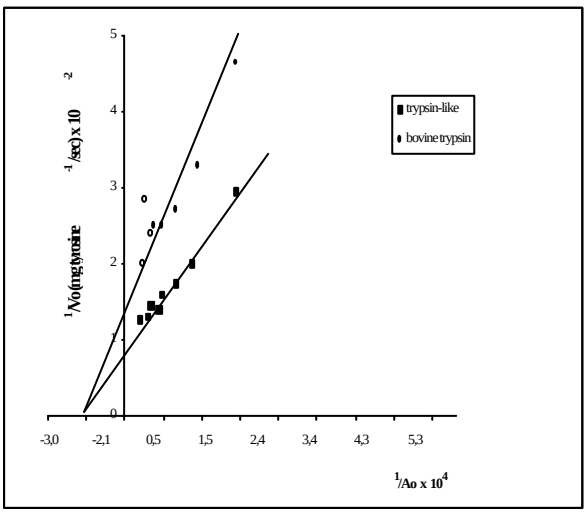

Figure 4 - Lineweaver-Burk plot for the hydrolysis oh haemoglobin by cod trypsin-like enzyme and bovine trypsin.

influence of pH was greater towards tryptic activity and that the myofibrillar proteins were degraded at the same rate as haemoglobin by both the purified fish trypsin-like enzyme and bovine trypsin, suggesting that they were good substrates for the tryptic activity.

Sarcoplasmic protein when compared with myofibrillar protein seemed to be very resistant to proteolytic degradation.

Table 3 - Comparative values of Km for some trypsins.

Trypsin source	Substrate	Km values	
Bovine	casein	$4.4x10^{-5}$	(1)
Krill	haemoglobin	$3.12x10^{-5}$	(2)
Bovine	B.A.E.E.	$1.8x10^{-5}$	(3)
Mackerel	casein	$9.1x10^{-4}$	(4)
Bovine	T.A.M.E.	0.029mM	(5)
Catfish	T.A.M.E.	0.029mM	(5)
αBovine	T.A.M.E.	$0.099x10^{-4}$	(6)
βBovine	T.A.M.E.	$0.150x10^{-4}$	(6)

(1) Vithayathill *et al*. (1961); (2) Fik (1984); (3) Inagami & Sturtevant (1960); (4) Pan & Kuo (1983); (5) Yoshinaka *et al*. (1984); (6) Magalhães-Rocha *et al*. (1980).

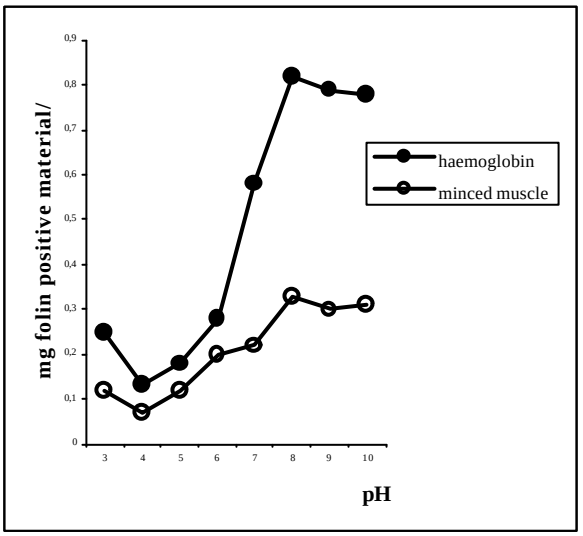

Figure 5 - Hydrolysis of haemoglobin and minced muscle by crude extract from cod.

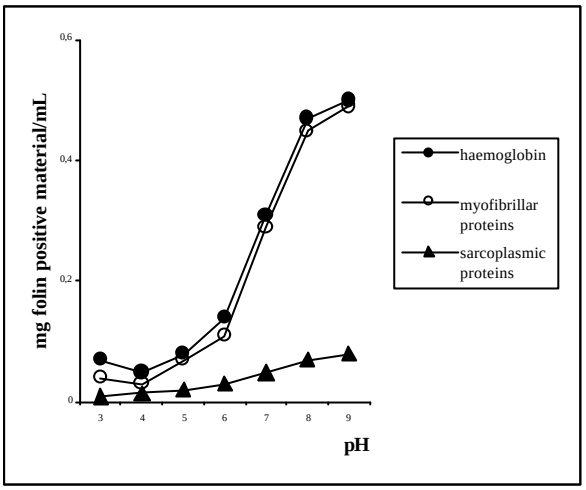

Figure 6 - Hydrolysis of haemoglobin, myofibrillar proteins and sarcoplasmic proteins by purified trypsin-like enzyme of cod (concentration of enzyme 0.065mg/mL).

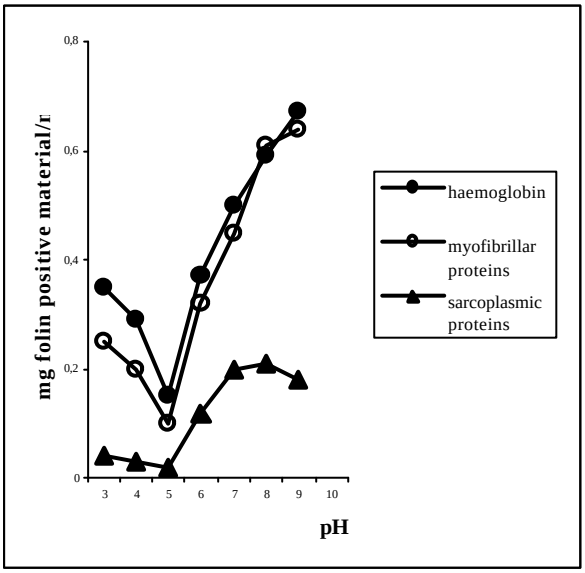

Figure 7 - Hydrolysis of haemoglobin, myofibrillar proteins and sarcoplasmic proteins by bovine trypsin (concentration of enzyme 0.065mg/mL).

The difference between myofibrillar and sarcoplasmic protein might lie in the presence of an inhibitor of serine proteinase, as suggested by Hara *et al.* (1985) in studies on white croaker muscle and by Waxman & Krebs (1979) on bovine cardiac muscle. A similar conclusion was reached by Hjelmeland & Raa (1982) using capelin muscle as trypsin-type substrate. The authors found that myofibrillar proteins were degraded to a greater extent than the sarcoplasmic proteins, which had a high resistance to degradation.

When sarcoplasmic and myofibrillar proteins were mixed together and treated with trypsin, the rates of degradation decreased. This protective action of the sarcoplasmic fraction may explain the relatively slow degradation of minced muscle by tryptic enzymes at alkaline pH. The results of this work are of particular interest, when it is considered that kamaboko production relies on the use of water-washed proteins with the myofibrillar protein as the principal protein involved in the gel setting process. The hydrolysis involves the action of selected proteolytic enzymes to split specific peptide bonds in proteins, resulting in a decrease in the size of protein, with consequent changes in solubility and functional properties.

This work involved the studies using specific synthetic substrates in order to identify the presence of different enzymes in the proteolytic activity of the purified fraction. Positive activity towards a trypsin-specific substrate (T.A.M.E.) indicated in this respect that the enzyme was trypsin-like. However, T.A.M.E. was hydrolysed much less by cod trypsin-like enzyme than by bovine trypsin. Active site-oriented inhibitors for trypsin or chymotrypsin were used to elucidate the tryptic characteristics of the purified enzyme.

The purified enzyme, such as mammalian trypsin was inhibited strongly by T.L.C.K. but not by T.P.C.K. Soybean trypsin inhibitor also inhibited the enzyme, confirming the serine protease characteristic. The activity of the trypsin-like enzyme towards fish muscle substrate was low, with the muscle tissue showing high resistance. Myofibrillar proteins seemed to be a better substrate, as they were degraded at the same rate as haemoglobin. In summary, there is considerable reason to believe that the heat stable alkaline enzyme is responsible for the decrease noted in gel strength of fish gel kamaboko.

RESUMO

A tripsina do ceco pilórico do bacalhau (*Gadus morhua*) foi purificada por cromatografia de CHOM Sepharose 4B. Algumas características foram determinadas como atividade catalítica para T.A.M.E., substratos enzimáticos e inibidores de proteases. A enzima mostrou ponto isoelétrico de 5,30 e 5,89 e composição de aminoácidos similar à tripsina bovina, mas diferiu por ter um alto

percentual de aminoácidos ácidos e baixo valor em aminoácidos básicos. A tripsina do bacalhau também hidrolisou substratos de proteínas de pescados.

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