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A Single Step Purification of Gastricsin-Like Proteinase from Atlantic Cod (*Gadus morhua*)

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Abstract: A gastricsin-like proteinase was purified from gastric mucosa of Atlantic cod by a single step purification scheme on ion-exchange of Amberlite CG-50. The purification was very efficient as the recovery was 205% and the purification factor was 1796-fold. The enzyme preparation was homogeneous as observed by SDS-PAGE and isoelectric focusing. The enzyme has an estimated molecular weight of 34 kDa and the pI of 4.4.

Key words: Enzyme, purification, cod, protease

Introduction

During the last two decades, aquatic organisms have been recognized as a new source of protease as well as other biochemical (Sikorski *et al*., 1994). This development is partly motivated by the decrease in fish production all over the world since 1986 due to over-fishing. Maximum utilization of the catch may provide new-products and sources of income for the fisheries industries.

Cold-adapted digestive enzymes from fish is worth considering as a new source of industrial enzyme based on the following reason:

1) Fish-derived enzymes have some unique physical, chemistry and catalytic properties such as lower heat stability, high molecular activity at low temperature and an increased ability to catalyze the hydrolysis of native protein substrates when compared with protease from mammals, thermophilic organisms and plant source (Simpson and Haard, 1984).
2) Fish viscera is abundant and wasted. They are usually used as fish meal or disposed off. Utilization of fish offal is important economically and environmentally. Better utilization of fish offal may minimize the disposal problem and over-fishing as well as recovering valuable biochemical, such as proteases. The dumping of fish offal also might create environmental problems because they contains high fat and proteins which are easily degraded, producing organic compounds (Suparno and Ponermo, 1992). Safety or legislative issues regarding the use of fish-derived enzymes are also minimum as fish are considered a natural source (Reece, 1988). In addition, there is no religious constraint (e.g. halal or kosher) on fish derived enzymes
3) Fish viscera is rich with proteases. For example, Atlantic cod stomach may contain about 2 g pepsin per kg (Gildberg *et al*., 1990) while the cod intestine contains about 1 g trypsin-like enzymes (trypsin, chymotrypsin and elastase) per kg (Asgeirsson *et al*., 1989).

Many work on fish digestive enzymes have been reported on their purification and characterization (Norris and Ellam, 1940; Xu *et al*., 1996; Bjarnason and Asgeirsson, 1993; Kristjansson *et al*., 1995), application (Haard, 1992; Stefansson and Steingrimsdottir, 1990; Raa, 1990; Brewer *et al*.,1984), three dimensional structure (Smalas *et al*., 1994) and cDNA (Gudmundsdottir *et al*., 1993, 1994).

Fish pepsin is one of the successful enzyme that find its place in industry. In recent years, fish pepsins have been used for hydrolysing the supportive tissue that envelops salmon roe and roe sacks in Scandinavia, Canada, Japan (Raa, 1990; Gildberg and Almas, 1986; Gildberg, 1988), production of caviar from orange roughy and salmon in New Zealand (Xu *et al*., 1996) and recovering carotenoid pigments from crustacean waste for use in the feed of farmed fish and seafood (Simpson and Haard, 1985; Cano-Lopez *et al*., 1987). This study suggests an efficient purification scheme for Atlantic cod pepsin.

Gastric proteases have been isolated from various cold water fish (Gildberg, 1988; Gildberg and Raa, 1983) including salmon (Norris and Ellam, 1940; Sanchez-Chiang *et al*., 1987), dogfish (Merrett *et al*., 1969), trout (Owen and Wiggs, 1971), hake (Sanchez-Chiang and Ponce, 1981), sardine (Noda and Murakami, 1981), American smelt (Haard *et al*., 1981), Polar cod (Arunchalam and Haard, 1985), Greenland cod (Squires *et al*., 1986a; 1986b), Atlantic cod (Almas, 1990; Brewer *et al*., 1984; Martinez and Olsen, 1989; Haard, 1986; Gildberg and Almas, 1986; Reece, 1988; Gildberg *et al*., 1990; Gildberg, 1992), finfish orange roughy (Xu *et al*., 1996) and capelin (Gildberg and Raa, 1983).

Many studies have been carried out in isolating and studying pepsin from Atlantic cod including those of Almas (1990), Reece (1988), Brewer *et al*. (1984) and Haard (1986). Atlantic cod pepsin was reported to have a molecular weight of 41 000 daltons. Atlantic cod pepsin as well as other cold-adapted fish pepsin such as those from American smelt (Haard *et al*., 1981), Polar cod (Arunchalam and Haard, 1985), Greenland cod (Squires *et al*., 1986a; 1986b) and capelin (Gildberg and Raa, 1983). The aim of this work is to isolate fish pepsin using a purification scheme that have not been reported previously.

Materials and Methods

Whole fresh Atlantic cod (*Gadus morhua*), caught from North Sea were supplied by a wholesaler in Bridlington. Amberlite CG-50 type II (200-400 mesh) was purchased from Supelco UK, Sigma-Aldrich Co. Ltd., Dorset, UK. Electrophoresis reagents and protein standards for isoelectric focusing (IEF Mix, pH 3.6-9.6), SDS-PAGE (Dalton VII-L), gel filtration (MW-GF-70 Kit) were obtained from Sigma Chemical Co., Dorset, UK. Other reagents were (analytical grade) obtained from Merck Ltd., Lutterworth, UK.

The whole viscera were removed and separated into stomach and intestine. They were kept frozen at -30 °C until used.

Partially thawed cod stomachs were split, emptied and briefly rinsed with distilled water. The tissues were then cut into small pieces and then homogenized with 50 mM Tris-HCl pH 7.3 (0.5 g tissue/ml) using waring blender (Waring Product Division Dynamic Corporation of America, New Hartford, Connecticut) for 3 min. The homogenate was centrifuged for 30 min at 15 000 *g* at 4 °C

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(Centrifuge Model J2-HS, Beckmann, USA) to remove the insoluble material. The supernatant was then freeze dried. This lyophilized sample was designated as crude pepsinogen.
The resin was obtained in powder form. The resin (48.1 g) was soaked in large amount of 0.2 M citrate buffer, pH 2.1 with several changes of buffer. Then the resin was poured slowly in the clean column (2.6 x 32 cm^2). The column was equilibrated with 0.2 M sodium citrate buffer, pH 2.1 at a flow rate of 0.5 ml/min.

Ionic exchange: A sample containing 1 g crude cod pepsinogen was dissolved in 10 ml of 0.2 M sodium citrate buffer, pH 2.1 and introduced to the column. The flow rate was 0.5 ml/min and fractions of 12 ml were collected. The elution buffer was then changed to 0.2 M sodium citrate buffer, pH 3.8. The elution with pH 3.8 was continued until the effluent pH has reached 3.8 and its absorbance at 280 nm is less than 0.1. Elution was resumed with 0.2 M sodium citrate buffer, pH 4.2 until the effluent pH has reached 4.2. Pepsin peak, if any should be eluted with this buffer. Finally 0.2 M sodium citrate buffer, pH 4.6 was used to elute any gastricsin peak. The proteolytic peak was pooled, dialyzed in cold against several changes of distilled water and freeze-dried for further analysis.

Enzyme assays: Hemoglobin was used as a substrate to measure pepsin activity essentially as described by Anson and Mirsky (1932). In the assay used, 0.1 ml of the enzyme solution was added to 0.9 ml hemoglobin solution (0.2% hemoglobin in 0.01 N HCl). After 30 min incubation at 37 °C, the reaction was stopped by adding 0.4 ml trichloroacetic acid (20 % w/v). The mixture was then centrifuged at 12 000 rpm for 5 min and the absorbance of the supernatant was measured using a spectrophotometer (Cecil, Model CE 292, Cecil Instrument, Cambridge, England) at 280 nm. One unit of enzyme activity was defined as the amount of enzyme required to increase the absorbance of TCA soluble material by 1.000 absorbance unit at 280 nm in 30 min at 37 °C. Protein determination was carried out using modified Lowry method as described by Peterson (1977).

Determination of molecular weight: SDS-PAGE was carried out using 7.5-16.5 % gradient gel on a discontinuous buffer system according to the procedure by Laemmli (1970). Hoefer SE 600 Series gel electrophoresis units (Hoefer Scientific Instruments, San Francisco, California) was used. The protein bands were stained with Coomassie Blue. For molecular weight determination by gel filtration, Sephadex G-75 was used.

Electrofocusing: Electrofocusing (pH range of 3-10) was performed in 0.5 mm thin-layer polyacrylamide gels (PAGE) using LKB 2217 Ultrophor Electrofocusing Unit (LKB Produkter AB, Bromma, Sweden). Protein bands were stained with Coomassie blue after fixing. Proteolytic activity after focusing were detected by hemoglobin. The focused gel was placed in 2 % hemoglobin dissolved 0.1 M glycine buffer, pH 2.6 for 20 min at 4 °C followed by incubation for 37 °C for 1 hr. The gels were then stained with Coomassie blue after fixing.

Results

Purification: An efficient single step purification on Amberlite CG-50 was employed to purify an acidic protease from Atlantic cod.

Determination of molecular weight: SDS-PAGE shows two bands, the enzyme band and a very low molecular weight bands which was probably autolysis product. SDS-PAGE analysis gave a molecular weight of 34430 ± 1400 Da. On the other hand, gel filtration gave very low molecular weight of 2 955 Da for activity fraction.

Electrofocusing: Isoelectric focusing has two bands, but only one activity band with hemoglobin and caseogram. The band with no proteolytic activity may be autolysis product and it may be correspond to the low molecular weight protein band in SDS-PAGE. The enzyme has a pI of 4.6.

Discussion

The predominant gastric proteases in vertebrates are pepsin A and pepsin C or gastricsin. Both of them are secreted in the form of zymogens in gastric mucosa. Gastric mucosa is rich in pepsinogen, while gastric juice is rich in pepsin. Since the identity of cod gastric proteases is unknown, the word pepsin and pepsinogen used in this study refer to word pepsin and pepsinogen in general and not to word pepsin A and pepsinogen A. Hemoglobin assay detects pepsin activity of both pepsinogen and pepsin.
Extraction of cod pepsinogen from mucosa was carried out at pH 7.3 to avoid any activation and autolysis. Centrifugation was carried out to remove insoluble particle prior to freeze drying. Although precipitation with ammonium sulphate is common practice in the purification of pepsin, it was omitted in order to avoid the need for dialysis which may lead to activity loss.
Pepsin A and gastricsin from human, porcine and bovine gastric juice have been separated and quantified using a weak cationic exchange column of Amberlite CG-50 (Richmond *et al.*, 1958; Tang, 1970). The elution of the enzymes is pH dependent. Pepsin A was eluted earlier at lower pH (pH 4.0) while gastricsin appeared later at higher pH (pH 4.4). Cod pepsin behaved more like gastricsin from human, porcine and bovine sources (Fig. 1). Like these enzymes, elution of cod pepsin occurred at pH 4.4. The enzyme preparation, unlike crude cod pepsinogen, was able to clot milk. This indicates that cod pepsinogen was activated during the isolation. The recovery of greater than 100 % (Table 1) could be due to the salt activation by citrate buffer or removal of inhibitors. This purification method proved to be more successful than previously reported methods for purifying fish pepsins (Table 2). It is very promising to purify fish pepsin in a big scale using this purification scheme.
The molecular weight estimations of cod pepsin were obtained from three different methods (Table 3). SDS-PAGE analysis of Amberlite CG-50 isolated enzyme shows one band (Fig. 2). The molecular weight of pepsin by separation on Sephadex G-75 and FPLC was very low compared to the value given by SDS-PAGE. These results suggested that gel filtration cannot be used to determine the molecular weight of cod pepsin. Molecular weight determination of fish pepsins by gel filtration has been reported to give low values (Sanchez-Chiang and Ponce, 1981; Gildberg and Almas, 1986). One possible explanation for this incorrect molecular weight of fish pepsins is that fish pepsins are glycoproteins (Gildberg *et al.*, 1990; Xu *et al.*, 1996). Interactions between carbohydrate residues of fish pepsins and the polysaccharides of the gel matrix would lead to lower than expected elution volume. However, the molecular weight estimate of cod pepsin by gel filtration was very low compared to that of capelin pepsin I and II (23 and 27 kDa on Sephadex G-75) and salmon pepsin I and II (27 and 32 kDa on Sephadex G-100) (Gildberg and Raa, 1983; Sanchez Chiang *et al.*, 1987). These results shows that cod pepsin was retarded more on the gel filtration column.
The correct molecular weight estimation by gel filtration on FPLC could be made by adding 2 % triton X-100 (reduced) in the enzyme sample (Table 3). It is likely that low pressure gel filtration can be improved the same way. The concentration of triton X-100 used was above its critical micelles concentration, thus micelles of about 90 kDa were formed and eluted in void volume. The molecular weight of cod pepsin on SDS-PAGE was consistent with those reported by other workers i.e., 34-36 kDa (Gildberg *et al.*, 1990; Martinez and Olsen, 1989).
The focused gels were stained for both protein and protease band (Fig. 3). Protein bands were stained with Coomasie blue

Table 1: Purification of pepsin from the gastric mucosa of Atlantic cod

Purification step	Total activity (units)	Total protein (mg)	Specific activity (units/mg protein)	Recovery (%)	Purification (-fold)
Crude pepsinogen	745.0	50.000	14.9	100	1
Amberlite CG-50	1205.6	0.057	26 769.2	205.6	1796

Table 2: Comparison of the efficiency of the purification scheme of fish gastric proteinases

Reference	Source	Recovery	Precipitation	Purification scheme
Gildberg and Raa (1983)	Arctic fish capelin	14.4	$(NH_4)_2SO_4$	DEAE-cellulose (Whatman DE 52), CM-cellulose (Whatman DE 52), gel filtration
Gildberg *et al.* (1990)	Atlantic cod	34	$(NH_4)_2SO_4$	S-sepharose
Arunchalam and Haard (1985)	Polar cod	>90%	Nil	CBZ-D-phenylalanine-TETA sepharose 4B
Noda and Murakami (1981)	Sardine	5.3	$(NH_4)_2SO_4$	CM-cellulose (1 or 2x), gel filtration (2x)
Tanji *et al.* (1988)	Bluefin tuna	69	$(NH_4)_2SO_4$	DEAE cellulose, gel filtration, Q-Sepharose Fast Flow, Mono Q HR 5/5
Sanchez-Chiang and Ponce (1981)	Hake	13.6	$(NH_4)_2SO_4$	DEAE-cellulose, DEAE- Sephadex A-50
Twining *et al.* (1983)	Rainbow trout	3.5	$(NH_4)_2SO_4$	Polylysine-Sepharose, DEAE-Sephacel, Sephadex G-200, DEAE- Sepharose

DEAE – Diethylaminoethyl CM – Carboxy methyl CBZ - Carbamazepine TETA – Triethylene tetramine

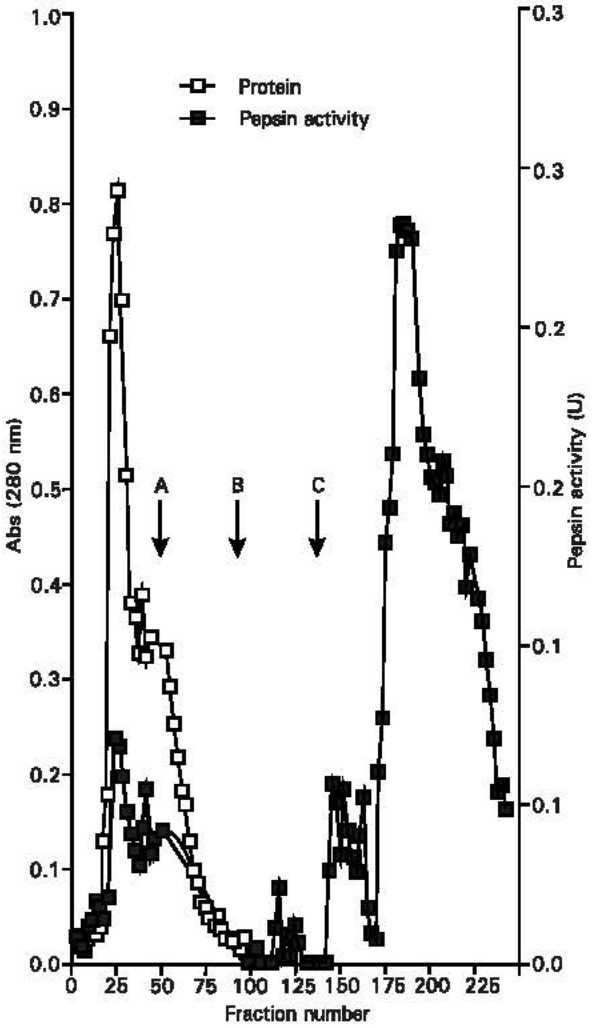

Fig. 1: Ionic exchange chromatography of crude cod pepsinogen on amberlite CG-50. Changes in buffers is marked with arrows, where A, B and C refer to 0.2 M citrate buffers of pH 3.8, 4.2 and 4.6. The flow rate was 0.5 ml/min and 12 ml fraction were collected. Protein content was not determined after fraction 88.

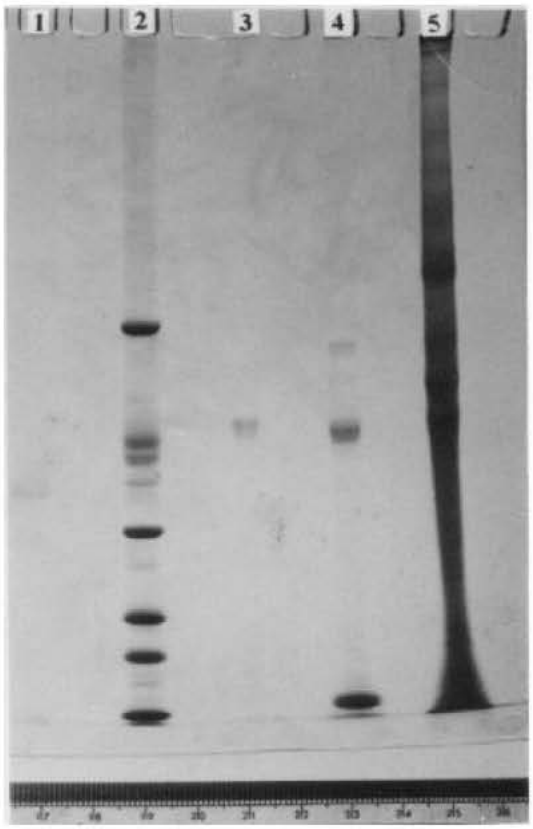

Fig. 2: SDS-PAGE analysis of amberlite CG-50 isolated cod pepsin (lane 1). Other lanes represent crude pepsinogen (lane 5), molecular weight marker (lane 2) and cod pepsinogen (lane 3).

Table 3: Comparison of molecular weights of cod pepsin obtained by three different methods

Methods	Molecular weight (Da)
SDS-PAGE	32 870±160
Low pressure gel filtration	2 510±200
FPLC	4 040
FPLC (with 2% Triton X-100 (reduced))	33 320

FPLC = fast protein liquid chromatography
SDS-PAGE = sodium dodecyl sulphate polyacrylamide gel

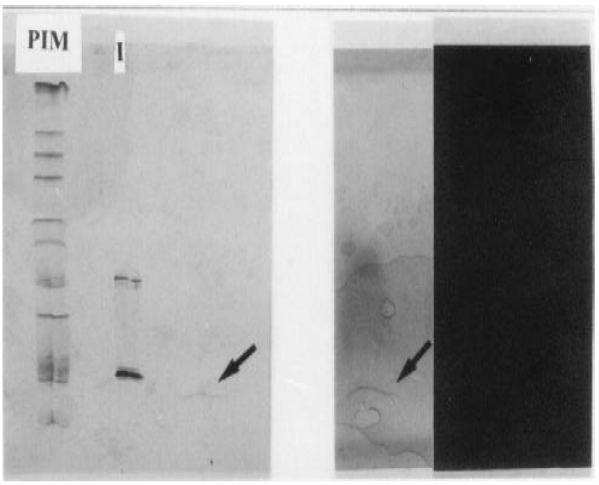

Fig. 3: Stained isoelectric focusing gels showing protein bands (blue bands with clear background) and protease bands (clear bands with blue background) for cod pepsin. Pim is pi markers and I is trypsin inhibitor.

staining, while protease bands were detected by incubation of the focused gels with hemoglobin solution. Cod pepsin has one protein band and one activity band. The protein band with protease activity corresponded to the pI of 4.4. This pI value was close to that reported by Reece (1988) for an Atlantic cod pepsin isozyme which is between 3.8-4.7. Fish pepsins have higher pI than mammalian pepsins (Gildberg *et al.*, 1990; Reece, 1988). This difference is partly due to higher content of basic amino acids in fish pepsins and also because phosphate may be present in the molecular structure of mammalian pepsins (Herriot, 1962).

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