ISOLATION AND CHARACTERIZATION OF MILK COAGULATING GASTRIC ENZYMES FROM BIGEYE TUNA (*THUNNUS OBESUS*)

JOAQUIM F. DA PONTE TAVARES & JOSÉ A. BETTENCOURT BAPTISTA

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Six proteolytic active fractions were separated from bigeye tuna stomachs by preparative IEF rotofor, and purified by gel permeation and ion-exchange chromatography. The presence of NaCl in the extraction process markedly enhanced the yield of tuna gastric enzyme. Molecular weight of the most active pure fraction by SDS-PAGE corresponded to a 31.000 daltons. The amino-acid composition of the same fraction differed slightly from literature values, and its thermostability determined by DSC, indicated peak denaturation temperature of 61.85° C. The tuna protease activity at 14-26° C was less temperature dependent than commercial rennet and completely lost the activity above 40° C of temperature.

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Seis fracções proteolíticas activas de estômagos de atum foram separadas com o recurso ao "Rotofor IEF" e purificadas pelas técnicas cromatográficas de permeação de gel e de permuta iónica. A utilização de NaCl aumentou o rendimento do processo de extracção. O peso molecular da fracção pura mais activa, determinado pela técnica "SDS-PAGE", foi de 31.000 daltons. A composição amino-ácida obtida da mesma fracção diferiu da referida na literatura e a sua termoestabilidade, determinada por "DSC", evidenciou um pico de degradação à temperatura de 61.85 °C. A actividade da protease extraída de estômagos de atum, às temperaturas de 14-26 °C, mostrou-se menos dependente da temperatura do que a do "rennet" comercial e perdeu toda a sua actividade acima de 40 °C.

Joaquim F. Ponte Tavares, Universidade dos Açores, Rua da Mãe de Deus, 58, PT-9500 Ponta Delgada, Açores, Portugal. - José A. Bettencourt Baptista, Department of Molecular and Medical Genetics, University of Toronto, Toronto, Ontario M5S 1A8, Canada. Present address: Universidade dos Açores, Rua da Mãe de Deus, 58, PT-9500 Ponta Delgada, Açores, Portugal.

INTRODUCTION

The increased production of fish offal in the fish industries presents a growing pollution and disposal problem, and consequently, the major concerns of the world leading fish processing and exporting countries, is the management and development of new products using underutilized fish. There has also been an increased interest in the search for rennet substitutes, as genetically engineered recombinant enzymes, because of a decline in the number of calves slaughtered and an increase in demand of proteases for cheese production (DE-KONING et al. 1978). Rennet substitutes of microbial origin have been accepted by the industry, although they have not been totally satisfactory (ERNSTROM 1974), and plant proteases generally appear to give rise to a softer

curd than calf rennet (OOSTHUIZEN & SCOTT-BLAIR 1963). Another alternative could be the extraction of milk clotting enzymes from fish stomach mucosa. In addition, the potential exists for establishing a new local food-related industry, which could be considered either, in less developed countries, as an alternative to produce rennet substitutes for domestic use, or eventually, to export for cheese-producing nations. At the same time the utilization of a waste material to produce a value-added product could likely increased overall fish processing. Availability of an inexpensive rennet would encourage cheese manufacturers to convert milk into a more stable and preserved food product (EDELSTEN & JENSEN 1970; GREEN & STACKOPOOLE 1975). The purpose of the present study was to isolate and characterize the major active protease from bigeye tuna.

MATERIALS

Spray dried non-fat dry milk was purchased from new Dundee Creamery, New Dundee, Ontario, Canada. Commercial rennet was provided by Dairy and Food Laboratories (Waukesha, Wiscosin). Protease substrate gel tablets, chemicals for isoelectric focusing (Bio-Lyte 3/10 ampholytes) and molecular weight calibration standards for SDS gel electrophoresis, were provided by Bio-Rad, Richmond CA. DEAE-Sephadex A-50 and Sephadex G-100 were obtained from Pharmacia. MW 12,000-14,000 cut-off tubing of 45 mm diameter (spectra/por standard cellulose tubing) was purchased from Spectrum Medical Industries, California. Unless otherwise specified, all reagent grade chemicals were obtained from E. Merck, Darmstadt, Germany, and Sigma Chemicals Company, St. Louis, MO, USA.

Bigeye tuna (*Thunnus obesus*, 30-50 kg/fish), caught from fishery zones in the North Atlantic Ocean in proximity of the Azores Islands, was obtained from the University of the Azores. The stomachs were removed from the fish after it was caught, frozen rapidly, and stored at - 20° C.

METHODS

EXTRACTION OF TUNA FISH PEPSINOGENS

The stomachs, were partially thawed, split, cleaned, and briefly rinsed 3 times in tap water. The inner mucosa linings were peeled away from outer muscular layer and chopped in small pieces, using a sharp knife. The material was homogenised, using a combination meat grinder/Waring commercial blender, with equal weight of distilled water (DW), containing 25% w/w of total weight (tissue plus DW) of NaCl, and maintained at 4° C. On the following day, the homogenate was stirred for 1 min. at 0°C, using a Polytron tissue homogenizer, and the solid material was removed by centrifugation, utilizing a Beckman L8-70 ultracentrifuge unit at 35,000 x g, for 60 minutes at 4° C. The pellet was twice more extracted as described above and the clear supernatants, called "crude pepsinogen", were combined, concentrated approximately 10 fold, using a Büchi rota-evaporator at room temperature or partially freeze dried in a Labconco lyophilizer, and stored at -20° C for further purification.

ISOELECTRIC FOCUSING AND ELECTROPHORESIS

The crude pepsinogen was exhaustively, about six times, dialysed (spectro/por standard cellulose membrane tubing of 45 mm i.d., MW cut-off 12,000-14,000) against distilled water, for 48 hrs. at 4° C, to remove the substantial amount of NaCl. This procedure insures that the nominal pH range of the ampholyte will extend over the full length of the focusing chamber, and then the maximum voltage can be applied. A narrow range ampholyte (bio-Lyte 3/10, Bio-Rad, Richmond, CA) was mixed with desalted tuna crude pepsinogen to a final concentration of 2% (w/v). The sample volume (50 ml) was fractionated using a Bio-Rad preparative IEF rotofor apparatus, originally designed by EGAN et al. (1984) and adapted by Bio-Rad Laboratories, equipped with Bio-Rad computer in order to control the electrophoresis

power of 12 watts. The temperature of the system was maintained at 4° C with a circulating water bath. The different enzymes from crude pepsinogen were separated according to the isoelectric point (Ip), in a reproducible pH gradient. However, ampholytes can interfere with some assays, amino acids analyses, or subsequent purifications steps. In order to remove the ampholytes, each one of the most active fractions was again dialysed into a high salt solution, such as 1 M NaCl to strip electrostatically bound ampholytes from enzymes by ion exchange, and then against distilled water prior to lyophilization.

The molecular weight components of the active fractions, obtained from rotofor, were estimated by polyacrylamide gel electrophoresis, in presence of 2.5% of sodium dodecyl sulphate and 5% 2-mercaptoethanol, by comparison with a calibration mixture, according to the PhastSystem Development Technique (Pharmacia). Electrophoresis was done with Phast gel gradient 8-25 using phast gels buffer strips. Molecular weight standards for SDS-PAGE gel were: phosphorilase b (94,000), ovalbumin (67,000), carbonic anhydride (31,000), soybean trypsin inhibitor (20,100), and α-lactalbumin (14,400). After electrophoresis, gels were fixed with 12.5% trichloroacetic acid for 15 min. and stained with Coomassie brilliant blue G250 for 1 h (DIEZEL et al. 1972). The molecular weights were also confirmed by a Beckman HPLC System, model 324 on a (60 cm x 7.5 mm i.d.) Spherosel TSK 3000 SW column, equilibrated and eluted with 0.15 M KH_2PO_4 + 0.15 M Na_2SO_4, at pH 5.0, versus molecular weight standards.

ASSAY OF PROTEOLYTIC ACTIVITY

To determine the total activity of the tuna protease extracts, the zymogen was activated prior to use, by adding 0.1 N HCl to pH 3.0-4.0, holding this pH at room temperature for 60 minutes, and readjusting the pH to 5.0 with 0.1 N NaOH (BREWER et al. 1984).

The activity of the bigeye tuna protease was assayed by the measurement of milk coagulation time versus a known liquid standard rennet of 230 IMCU/ml (20% chymosin, 80% bovine pepsin in a solution of 16% NaCl and 0.5% sodium benzoate), using a Nameter Viscometer (Nameter Co, Edison N.J.), equipped with IBM compatible computer via an analog-to-digital converter, which measures the increasing of milk viscosity due to clot formation. Fresh skim milk powder (97% T.S.) at pH 6.3, was reconstituted to 12% (w/v) in 10 mM $CaCl_2$ (BERRIDGE 1952, 1955), and stored overnight at 4° C. This substrate was warmed and maintained, at coagulation temperature of 30° C, for one hour prior to tuna enzyme extract or standard rennet addition (suitably diluted in order to produce visible clotting in approximately 6 minutes). The milk clotting time was also measured, using the apparatus described by SOMMER & MATSEN. (1935), and modified by RAND & ERNSTROM (1964). The protease activity, determined in MCU/ml, was carried out at 30° C in 25 ml portions of substrate, in wide mouthed bottles. One ml of diluted enzyme was added, and the clotting time, in minutes, was measured by the number of revolutions (20 rpm). The appearance of the first visible flakes of curd on the moving glass surface was taken as the end point of the determination. All the assays were performed in triplicate including the blanks.

Another method used to evaluate the proteolytic activity of tuna gastric enzymes was the Bio-Rad's protease substrate gel tablets, that provided a rapid and a convenient process for preparing agar diffusion plates. The tablets produce a 1% agar gel containing a bovine casein preparation in a Tris buffered physiological saline solution at pH 7.2. Protease diffusion into the substrate gel was accompanied by digestion of the casein, and the size of the transparent ring around the samples wells in a turbid gel measure the enzyme proteolytic activity.

GEL PERMEATION CHROMATOGRAPHY

The most active fractions, determined by Bio-Rad's protease substrate gel tablets, from crude pepsinogen rotofor separation, were combined, and further purified by gel permeation chromatography, on a Sephadex G-100 (30 cm x 15 mm i.d.) column, from Pharmacia, eluted with

50 mM Tris-Chloride buffer, at pH 7.0. The sample, dissolved in 3 ml of the eluent buffer, was loaded on the column, after its equilibration, and the effluent was collected every 5 minutes. Elution was carried out, using a Isco peristaltic pump at a flow rate of 0.5 ml/min and the detection was accomplished with an optical unit model UA-5 (280 nm) recording absorbance monitor (Isco, Lincoln, NE). Alternate fractions were assayed for proteolytic activity and the pooled active fractions were combined, immediately dialysed against distilled water and lyophilized.

Ion-exchange Chromatography

Ion-exchange technique take advantage of the anionic properties of aspartic proteases. The combined fractions from gel permeation chromatography were repurified on (30 cm x 18 mm i.d.) DEAE-Sephadex A-50 column, from Pharmacia, pre-equilibrated with 20 mM Tris-Chloride buffer at pH 7.0. After sample loading, the column was eluted at a rate of 0.5 ml/min with the starting buffer, followed by a step gradient with 0.25 M NaCl in 20 mM Tris-Chloride buffer, pH 7.0, as an eluent. A second step gradient, was finally applied using 0.50 M NaCl in 20 mM Tris-Chloride buffer, until the end of the separation, maintaining the same pH. Fractions were collected every 15 minutes and the estimation of the enzyme was determined according to Anson (1938) by the absorbance at 235 and 280 nms, using a Shimadzu UV-VIS spectrophotometer, model 260. The pooled material was assayed for proteolytic activity, and the active fractions were combined, dialysed, and lyophilized. The final purification was performed by high performance liquid chromatography, with a HEMA BIO-DEAE column (10 cm x 8 mm i.d.) using a linear gradient of 0-100% B in 30 minutes between A = 20 mM Tris/Cl (pH 7.0) and B = A + 0.5 N NaCl (pH 7.0), at a flow rate of 0.5 ml/min. The purity of the collected material was confirmed by diode array UV detector, comparing the UV-profile, at the upslope, top and downslope of the peak.

Determination of Amino-acid Composition

The amino acid composition of the purified tuna protease fraction after hydrolysis, were determined using the Beckman DABS Amino Acid Analysis Kit, according to Nolan & Dovichi (1987). This methodology is applied for both primary and secondary amino acids, using 4-dimethylaminoazobenzene-4-sulfonyl (dabsyl) chloride (2 mg/ml, in acetone) in the presence of 0.5 ml of 0.5 M sodium bicarbonate (final pH adjusted to 8.5-9.0). The kit included an amino acid calibration standards and the analysis were carried out in a Beckman HPLC system, model 324. An Ultrasphere C_{18} column (250 x 4.6mm i.d.) was used with a gradient elution, according to Beckman DABS-Amino Acid Kit, between (A) 10 mM citric acid and 4% dimethylformamide (DMF), pH 6.50 +/- 0.05 and (B) 300 ml of "A" plus 700 ml Acetonitrile-with 4% DMF, with detection at 436 nm.

RESULTS AND DISCUSSION

Extraction of Tuna Protease

The yield of extraction, increased with increasing amount of sodium chloride from 15% to 25% (w/w). According to Tavares et al. (in press), salt concentration of 15% (w/w) slightly accelerated activation, whereas of 25% (w/w) markedly enhanced the yield of tuna protease, compared with 0.20 M sodium phosphate at pH 7.2 (Brewer et al. 1984). An extraction of 25% (w/w) NaCl at pH 5.0, from a tuna stomach of 343 g produced a total recovered yield of 482 MCU.

Purification of Bigeye Tuna Proteases

Separations, based on the isoelectric properties, have been successful in obtaining active aspartic proteases from several sources (Egan et al. 1984). The method can be used, as long as the enzyme is both stable and soluble at its isoelectric point. The bioactivity, after the

separation, was maintained, possibly because the proteases were in solution at low temperature (4° C), probably in their native conformation, and organic solvents were not used. The fraction from the first IEF rotofor separation showing the highest activities, in Bio-Rad protease substrate tablets, within pH range of 3.94 to 6.50, were combined to be reapplied to the rotofor cell for further separation.

The most active fractions (# 18 and # 19) from the second crude pepsinogen IEF rotofor separation, were combined and applied to a gel permeation column. The eluted fractions, numbered 50 to 90, containing the protease free of small contaminant molecules, were pooled dialysed and lyophilized, (Fig. 1). The resulting enzymatic material were repurified by ion-exchange chromatography using the starting buffer until fraction # 23, followed by a step gradient until fraction # 34 and a final step gradient until the end of the separation, fraction # 52. The most active fraction emerged as a medium peak with its centre at fraction # 41. A small enzyme peak (or shoulder), containing potential proteolytic activity, preceeded the main peak, and emerged from the fractions #s 35-39 (Fig. 2). The combined material, was submitted to HPLC final purification, and the most active fraction that emerged from the column between 18-24 minutes, as a single symmetrical peak, was used for amino-acid composition and for thermostability studies by differential scanning calorimeter (Fig. 3).

Molecular Weight Estimates

The molecular weights of the bigeye tuna protease fractions, from rotofor separations (#s 13, 14, 15, 16, 18 and 19), were estimated by SDS-PAGE. The results are shown in Fig. 4, and exhibited a strong band, which corresponds to a molecular weight close to 31,000 daltons. Porcine pepsin and cod gastric protease have molecular weights slightly higher (Squires et al. 1985).

The molecular weights determined by HPLC - size-exclusion chromatography were higher than those determined by SDS-PAGE. Long rod-shaped molecules can show this effect to difficulty of the molecules in penetrating the pores of the gel structure packing. A similar difference between molecular weights determined by SDS-PAGE and gel filtration was also found with gastric protease from seal (Shamsuzzaman & Haard 1984).

Comparison of Milk Clotting Enzymes

The effects of proteolytic activity of tuna gastric and commercial rennet enzymes on milk clotting were measured at different pHs by relative clotting time. The milk coagulation time for both had a pH dependence profile similar for pH values ranging 5.5-6.3, but the first one was less sensitive to losses of activity above pH 6.4 (Fig. 5).

Amino Acid Composition

The amino acid composition of the most active pure fraction, isolated from tuna gastric proteases, summarized in Table 1, was compared with data published by Tanji et al. (1988). Bigeye tuna

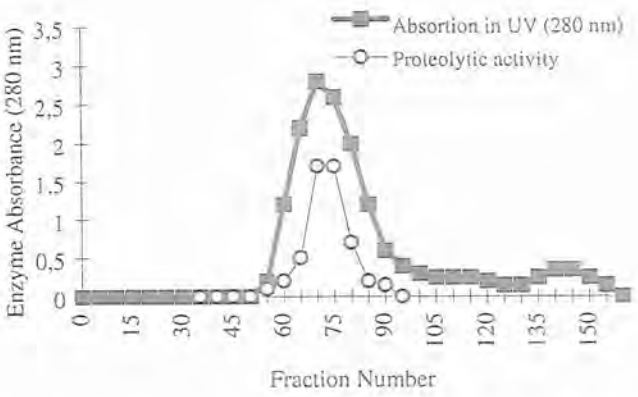

Fig. 1- Gel permeation of tuna proteases on a Sephadex G-100 (30 cm x 15 mm i.d.) column. Assay conditions: Eluent 0.05 M Tris/Chloride, pH 7.0, temperature 4° C., flow rate 0.5 ml/min., detection by UV (280 nm).

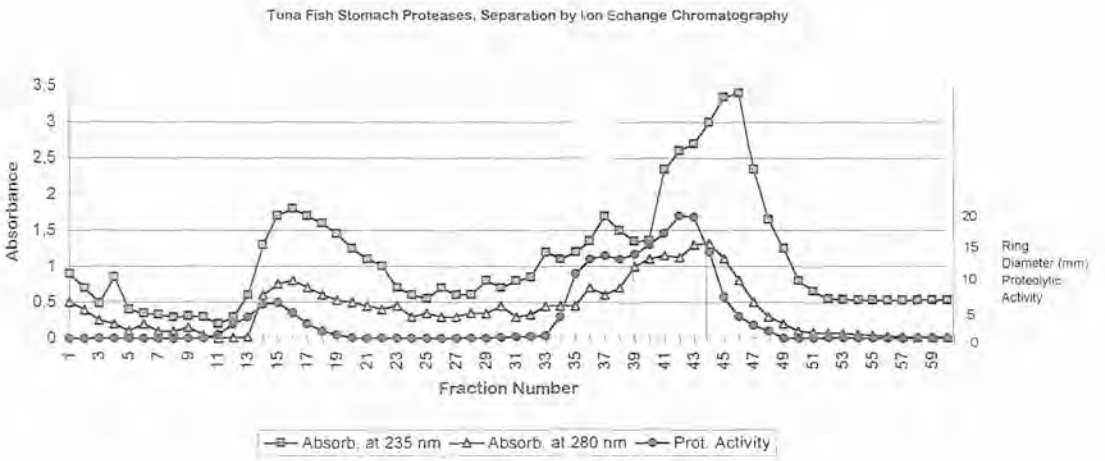

Fig. 2- Ion-exchange chromatography of tuna proteases on a DEAE cellulose (30 cm x 18 mm i.d.) column. Assay conditions: 20 mM Tris/Chloride, pH 7.0 until fraction # 23. Step gradient with 0.25 M NaCl in 20 mM Tris/Chloride, pH 7.0 until fraction # 34 and a final step gradient with 0.5 M NaCl in 20 mM Tris/Chloride, pH 7.0 until the end (fraction # 52). Flow rate 0.5 ml/min., and detection by UV (280 nm).

differred slightly somewhat in containing less serine, cysteine, isoleucine, and more basic amino acids, lysine and arginine, possibly because of different environments were the fish was caught. The amino acid composition of the various proteases were also compared by SQUIRES et al. (1985), that found similar differences between gastric proteases from the same species.

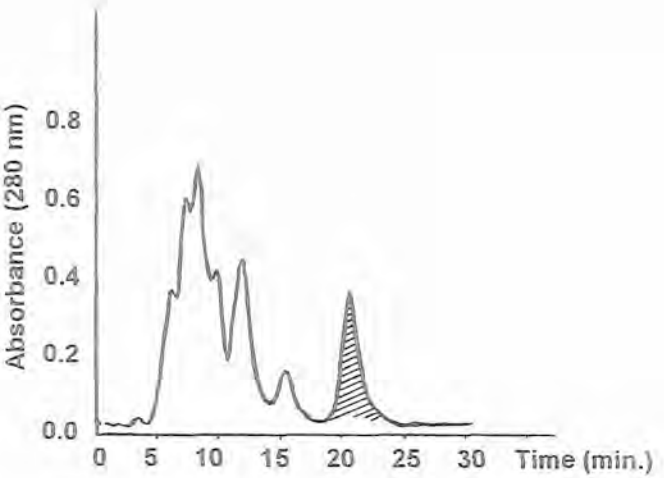

Fig. 3 HPLC-IEC of tuna protease fraction on HEMA BIO-DEAE (10 cm x 8 mm i.d.) column. Assay conditions: Mobile phase: A = 20 mM Tris/Cl (pH 7.0) and B = A + 0.5 N NaCl (pH 7.0). Linear gradient elution of 0-100% B in 30 minutes. Flow rate 0.5 ml/min., and detection by UV (280 nm).

THERMOSTABILLITY

Recent studies, on the circular dichroism (CD) spectra of enzymes, have born out the conclusions that the enzymatic properties, such as thermostability and specificity, are determined to a large extent by the primary structure of the particular enzyme.

The influence of assay temperature on clotting time is summarized in Fig. 6. Data shows that in low temperature renneting milk (14-26° C) for cheese making, less tuna protease would be required to facilitate renneting than calf rennet. One approach to study kinetic stability of the aspartic proteases, is a technique called differential scanning calorimeter (DSC). It is used to measure the temperature and heat flow associated with transitions in materials, as a

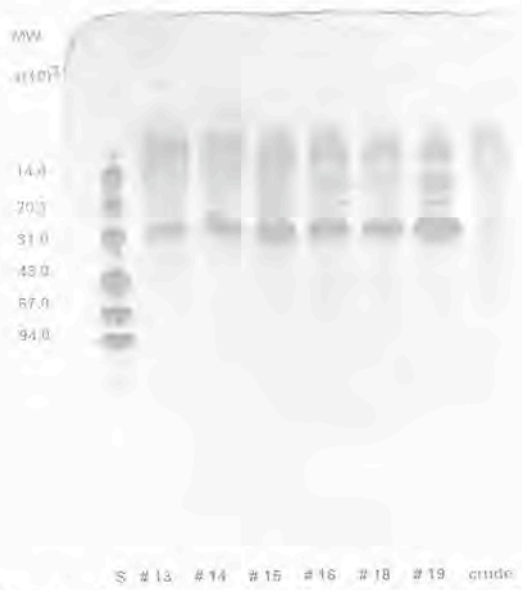

Fig. 4- SDS-PAGE of six most active fractions of desalted aspartic proteases from tuna gastric enzymes, separated using IEF with a range 3-10 ampholyte. The gel was loaded with 0.5 µl of the sample quantity (3 mg/ml, dry weight). Lane "S" contained low molecular weight standards: alpha-lactalbumin (14,400), trypsin inhibitor (20,100), carbonic anhydride (31,000), ovalbumin (67,000) and phosphorilase-b (94,000). Stained with coomassie blue.

Table 1

Amino Acid composition of the tuna gastric protease fraction compared to data published in literature (TANJI et al. 1988).

Tuna protease	Mole % (Experiment)	Mole % (Literature values)
Asp	10.7	8.55-12.62
Thr	6.7	5.10-8.06
Ser	7.2	9.10-13.85
Glu	11.2	6.39-12.83
Pro	3.6	3.60-5.28
Gly	10.7	8.90-11.73
Ala	9.9	4.78-12.50
Cys	0.0	0.56-1.97
Val	6.2	6.25-10.06
Met	1.9	1.11-2.57
Ile	3.9	4.28-7.08
Leu	7.3	4.44-9.78
Tyr	2.6	3.07-6.30
Phe	3.5	3.89-6.25
His	2.4	0.31-2.50
Lys	6.7	0.31-3.06
Arg	5.7	0.62-3.90

function of time and temperature. Such measurements provide quantitative and qualitative information about physical and chemical changes, that involve endothermic or exothermic processes, or changes in heat capacity. Using this methodology the purified tuna protease was dialysed, liophylized and diluted to a concentration of 10 mg/ml. Sample volume of 10 µl was hermetically sealed in aluminun pan, and 10 µl of distilled water was sealed in the reference pan. The activation energy of denaturation was generated using the Arrhenius expression. A sigmoidal baseline option was used, since a substantial change in heat capacity occurred during the process. DSC thermogram of the most active tuna gastric enzyme fraction, indicates peak denaturation temperature of 61.85° C, and the activation energy of denaturation was 0.4128 J/g, (Fig. 7).

CONCLUSIONS

1. Tuna gastric enzyme induced clotting of milk, via initial limited proteolysis of one of the casein

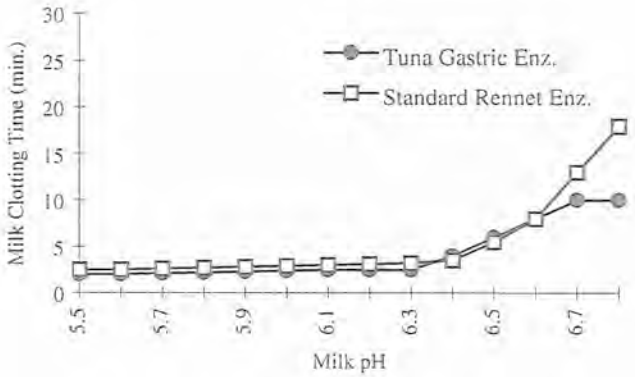

Fig. 5- Comparison of milk clotting activity of tuna protease relative to commercial rennet at different pH values. Assays conditions were 12% milk solids, 10 mM $CaCl_2$, and incubation temperature of 30°C. Data are means of triplicate determinations.

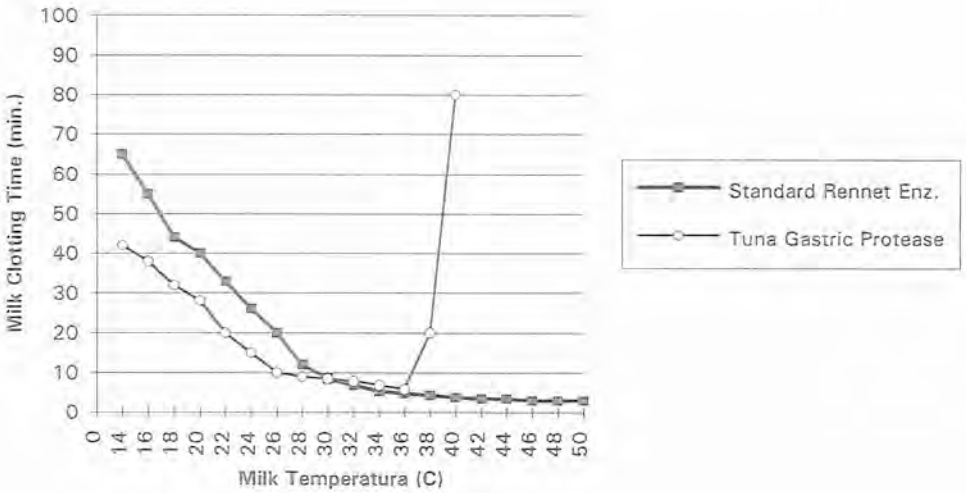

Fig. 6- The influence of assay temperature on the clotting time. Assay conditions were 12% milk solids, 10 mM $CaCl_2$, pH 6.3, and incubation temperature in the range 14-50° C. Data are means of triplicate determinations.

fractions (k-casein), and subsequent aggregation of casein micelles.

2. It is our belief that there exists an enormous potential for the recovery and use of gastric proteases from bigeye tuna fish. In spite of the potential diversity of microbial enzymes, very few species are used to produce industrial enzyme, because the cost of getting a microorganism stringently evaluated and accepted as safe is substantial.

3. The finding that bigeye tuna protease can more efficiently clot milk at low temperature, indicates its usefulness in cold renneting milk.

4. Bigeye tuna protease and commercial rennet where similarly effective at mild temperatures, but the former enzyme can be inactivated above 40° C. This phenomenon represents an advantage in some experiments (e.g., prevention of oxidised flavour in milk), since residual enzyme can be eliminate by pasteurization, and would not be

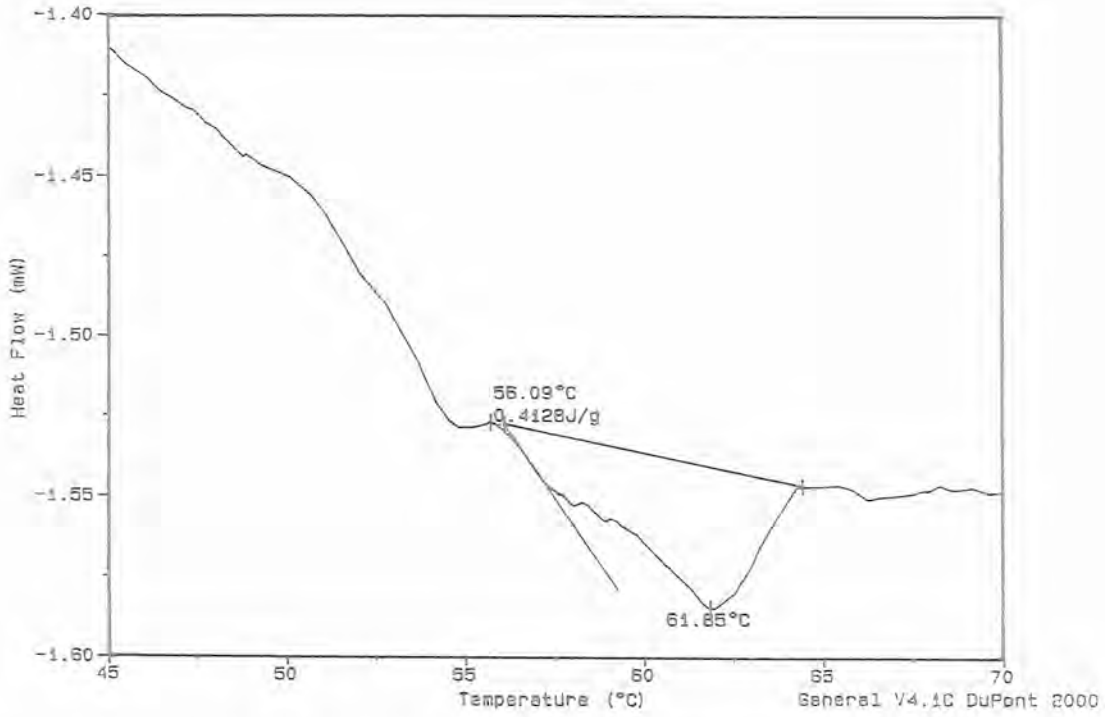

Fig. 7. Differential scanning calorimeter measurement of the most active tuna gastric protease, at a heating rate of 5° C/min., from 45 to 70°C. Trace was average of three scans.

present to cause subsequent hydrolysis of milk proteins. Thermostability studies, lend support to the theory of the evolutionary adaptation to various thermal environments. Generally with the enzymes, there is a direct relationship between the habitat temperature of the organism, from which the enzyme is derived, and its thermal stability.

5. The milk pH influence on the milk coagulation by tuna gastric enzyme had a pH dependence profile similar to that of calf rennet for pH values, ranging 5.5-6.3, but the first one was less sensitive to losses of activity above pH 6.4.

6. The colby Cheese manufactured with bigeye tuna fish protease was tested by a sensory panel constituted by 100 consumers. The data evaluated by analysis of variance was already published by TAVARES et al. (1993).

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