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# Characterization of Collagenolytic/Proteolytic Marine Enzymes

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Blue Biotechnology an exponential growth research/applicative area found its success especially in biomedical applications.There are many example of biotechnology applications of enzyme extracted from marin organism. Molecules having biological activity, antibiotics, anti-tumor molecules, inhibitors of inflammation and in other processes were found and isolated in different marine species. Our interest is focalized on proteolityc enzymes, prevalently in the direction of their stability and opportunity to be used at low temperature.
Numerous tons of fish are harvested each year, including viscera, is considered as waste during aquatic product processing. These, by products of the seafood industry, are protein-rich but are discarded without any attempt at recovery (Gildberg et al., 2002). High activity of fish enzymes at low temperatures is desirable for industrial applications, especially in certain food processing operations that require low temperatures. Furthermore, the relatively low thermal stability often observed in fish enzymes, may also be beneficial in such applications as the enzymes remained can be inactivated more readily, with less heat treatment.
We are interested to investigate the marine enzyme extracted by tissues. Moreover with the new molecular biology technology we will clone single gene and to provide marine organism enzymes by in vitro synthesis systems both in eukaryotic and prokaryotic system.
We have partially characterized/purified marine enzymes extracted form hepatopancreas of *Eriphia verrucosa and Palimurus elephas*. Our preliminary results had shown the presence of several collagenolytic, proteolytic, caseinolytic activities in extracts obtained from the marine organisms; having potentiality applications in industrial/biomedical applications.

## 1. Introduction

Marine organisms are rich potential sources of several enzymes that may have some unique properties of interest for both basic research and industrial applications. The kinetic chromogenic Limulus amebocyte lysate (LAL) assay is the most widely used assay for endotoxin measurement for environmental samples. This assay uses an endotoxintriggered enzyme cascade from the Atlantic horseshoe crab (*Limulus polyphemus*) to cleave a colorimetric substrate (Hochstei, 1987). Recently recombinant A recombinant factor C (rFC) assay that uses rFC reagent produced from the cDNA of the Mangrove horseshoe crab (*Cacinoscorpius rotundicauda*) was recently developed (Thorne et al.,

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2010). Main digestive proteinases detected in the hepatopancreas of fish are trypsin and chymotrypsin. Trypsin has been extracted, purified and characterized in various fish species like true sardine and yellowfin tuna (Klomklao et al., 2006; Kishimura et al., 2006). Moreover same enzyme has been cloned and synthesized in vitro model. In the past, decapods enzymes obtained from hepatopancreas were used in would healing therapy; and have been reported their ability to remodelling the fibrin clot during wound healing process, such as to promote tissue repair in burnings, ulcerations and other. Generation of bioactive peptides is another relevant application of marine enzyme. For example bioactive peptides with antioxidant properties derived from different proteins by enzymatic hydrolysis have become a topic of large interest for pharmaceutical, health food and processing/preservation industries. Antioxidant activity has been reported for protein hydrolysates obtained from different sources such as whole capelin, squid skin Pacific whiting (Klompong et al., 2009; Kim et al., 2001).
One significant application of the enzyme is tissue dissociation. Collagen degradation is due to a limited number of proteases that can generate collagen degradation. The most prominent enzymes used in this application were collagenases obtained from the *Clostridium histolyticum* human pathogenic microorganism. Crude collagenase is well suited for tissue separation since it contains the enzyme required to attack native collagen and reticular fibers in addition to the enzymes, which hydrolyze the other proteins, polysaccharides and lipids in the extracellular matrix of connective and epithelial tissues. The utilization of purified enzyme blends consisting of collagenase class I (CI) and II (CII) from *Clostridium Histolyticum* are an essential step within the current standard procedure isolation of cells from tissue usable in cells therapy. Purified collagenase alone is usually inefficient in dissociating tissues due to insufficient hydrolysis of all collagenous polypeptides and its limited activity against the high concentrations of non-collagen proteins and other macromolecules found in the extracellular matrix.. In addition to collagenase, neutral protease plays an prominent role in islet isolation. Their accelerates tissue dissociation but their could result in a decrease in islet yield through islet fragmentation and disintegration (Bucher et al., 2004). A correct balanced of enzyme blend is an essential step in cell isolation. Clostridium Histolyticum enzymes have a particularly strong enzymatic activity, but always present some toxic effects reducing the efficiency in cell purification (Salamone et al., 2010). We have partially characterized/purified marine enzymes extracted form hepatopancreas of different species: *Palimurus elephas, Eriphia verrucosa*. We have analyzed collagenase and proteases extracted from the hepatopancreas samples, and compared their activity to collagenases from Clostridium hystoliticum.

## 2. Experimental

### 2.1 Marine organisms, organs selection and extraction

*E. verrucosa and P. elephs* were obtained from the fish market of Mazara. The samples were packed in polyethylene bags, placed in ice and transported to the research laboratory within 30 min. The internal organs were separated and only the hepatopancreas was collected and then stored in sealed plastic bags to 20 °C until used for enzyme extraction. Collected hepatopancreas samples from E. *verrucosa and P. elephs*, were washed with PBS and homogenized, after that samples were centrifuged to 10,000 g and the supernatants aliquoted and used as the “crude protease extracts”. Obtained samples were analyzed by SDS-PAGE and gelatin zymography.

### 2.2 Polyacrylamide gel electrophoresis and zymography

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out for the determination of purity and molecular weight of the purified enzyme, as described by Laemmli (1970), using 5 % (w/v) stacking and 15 % (w/v) separating gels. Samples were prepared by mixing the purified enzyme at 1:5 (v/v) ratio with distilled water containing 10 mM Tris-HCl (pH 8.0), 2.5 % SDS, 10 % glycerol, 5 % β-mercaptoethanol and 0.002% bromophenol blue. After electrophoresis, the gels were stained with 0.25 % Coomassie Brilliant Blue R-250 in 45 % ethanol, 10 % acetic acid and destained with 5 % ethanol and 7.5 % acetic acid. The molecular weight of the enzyme was estimated using a molecular weight markers. Zymography was performed on native-PAGE.

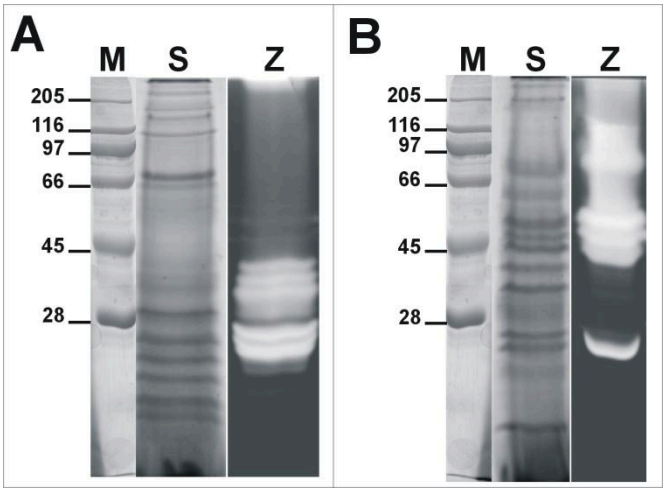
### 2.3 Collagenolitic activity

To investigate the collagenolytic activity we measured them using a modification of the collagen digestion method of Mandl, et al. (1953) in which the enzyme is incubated for 5 h with native bovine achilles tendon collagen at 37 °C. The extent of collagen digestion is determined using the Moore and Stein colorimetric ninhydrin process (Moore and Stein, 1948) The amino acids released are expressed as micromoles leucine per milligram dry weight of collagenase. One unit equals one micromole of L-leucine equivalents released from collagen in 5 h at 37 °C, pH 7.5, under the specified conditions.
Four tube containing 25 mg of bovine collagen (Sigma) were incubated in 5.0 ml of 0.05 M TES buffer and incubate at 37 °C for 15 min each of four test tubes includes at least two tubes to serve as blanks which will contain no enzyme. Start the reaction by adding 0.1 mL of enzyme dilution to appropriate tubes. After 5 h collagenase reaction was stopped by transferring 0.2 mL of solution (leaving behind the collagen) to test tubes containing 1.0 mL of ninhydrin-citric acid mixture. Include an enzyme blank (collagen incubated with 0.1 ml TES buffer in place of enzyme). Heat for 20 min in a boiling water bath. After cooling, dilute with 5 mL of 50 % n-propanol. Let stand for 15 min and read absorbance at 600 nm. From an L-leucine standard curve determine micromoles amino acid equivalent to leucine liberated. Caseinase activity of the same enzyme blend is determined using the above assay and substituting 25 mg vitamin free casein for collagen. The reaction is stopped after 5 h by the addition of 0.5 mL of 50 % trichloroacetic acid. After centrifugation, 0.2 mL of the supernatant is transferred to 1.0 mL of ninhydrin and treated as above. Caseinase activity is calculated as collagenase activity.Collagenolityc activity was also evaluated by the Enzymatic Assay of Collagenase using Carbobenzoxy-Gly-Pro-Gly-Gly-Pro-Ala-OH as substrate (Grassmann and Nordwig, 1960). Crude enzyme extract from Eriphia verrucosa epatopancreas was diluted in Citrate Buffer; 0.1 mol/L, pH 6.3 contains Calcium acetate 0.01 mol/L for quantitative collagenase-determination.

## 3. Results

### 3.1 Identification of gelatinolitic enzyme using gelatine zimography

We have identified different enzyme present on the total protein extracted from hepatopancres of *Eriphia verrucosa and Palinurus elephas*. As shown in Figure 1, lines As and Bs describe the proteins patterns of the two different analyzed species; while, lines Az and Bz shown proteolytic enzymes having gelatinolytic activity. As possible to discern from the pictures, different patterns in proteins and in enzymatic activities are present in the two different analyzed species extracts; moreover, the enzymatic activity detected were obtained performing the experiments to 4 °C.




In A samples obtained from E. verrucosa; in B samples obtained from P. elephas.
In the lines S the SDS-PAGE analysis, in which samples were stained with Blue Comasie G-250.
In the lines Z the gelatin zymographies, in which samples were stained with Blue Comassie R-250.
In the lines M are the markers, on the left molecular weight is specified in KDa.

*Figure 1:SDS-PAGE and gelatin zymography analyses of E. verrucosa and P. elephas extracts*

### 3.2 Comparative analyses of proteolytic activity on different substrates

We have compared the collagenolytic activity of marine enzymes both on linear peptides than on non-soluble collagen from bovine tendon. In the first case (Figure 2) we have analyzed the catalytic marine enzyme activity in comparison with the recombinant class I e class II Clostridium hystolyticum collagenases (ABIEL srl). In these experiments we have used the linear Carbobenzoxy-Gly-Pro-Gly-Gly-Pro-Ala-OH peptide as substrate, a specific substrate for collagenases. As shown in Figure 2, the collagenolytic activities available in the marine enzymes are more active in digestion compared to recombinant collagenases, used as control.

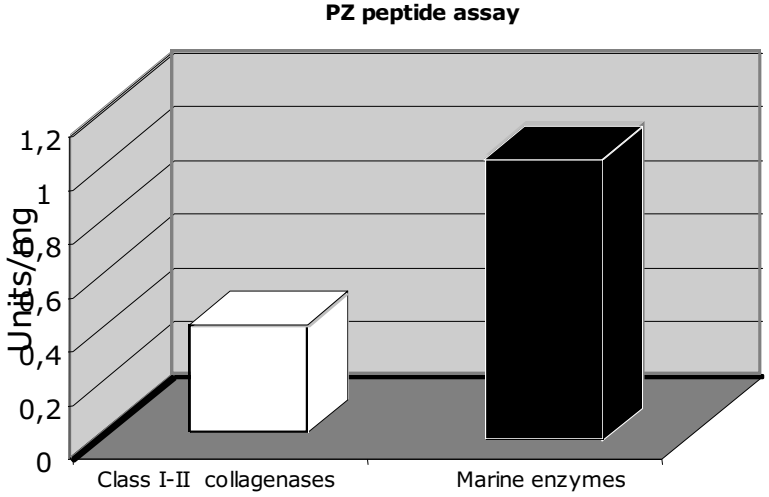



*Figure 2 – Enzymatic activity of recombinant collagenases and Marine enzymes (P. elephas) extracts on PZ peptide. Recombinant class I-II collagenases and P. elephas extract were tested on PZ peptide digestion using the same concentration. The Units are valued as the amount of One unit equals one micromole di Gly-Pro-Ala from Z-Gly-Pro-Gly-Gly-Pro-Ala in 1 minute at pH 6.3 at 37 °C.*

Since, the linear substrate used is also recognized by gelatinases, enzymes acting on denatured collagens, we applied a more specific method to value collagenase activity in this case we use the non-soluble collagen from bovine tendon; to this assay we have associate, also, a test to in quantify the analyzed enzymes ability to digest another substrate, the casein. As shown in Figure 3, the enzymatic activity on "native" collagen fibers, from bovine tendon, present in the marine analyzed extracts is one third respect to recombinant class I and II from *C. hystoliticum*. In a similar approach, we have measured the proteases activity of the same blend enzyme used on bovine tendon collagens on the casein substrate. As shown, in Figure 3, caseinolytic activities were detected in the marine enzymes; while any casein degradation activity was detected in recombinant collagenases. These experiments were performed both to 37 °C than 4 °C, showing the same catalytic activity on casein digestion (data not shown).

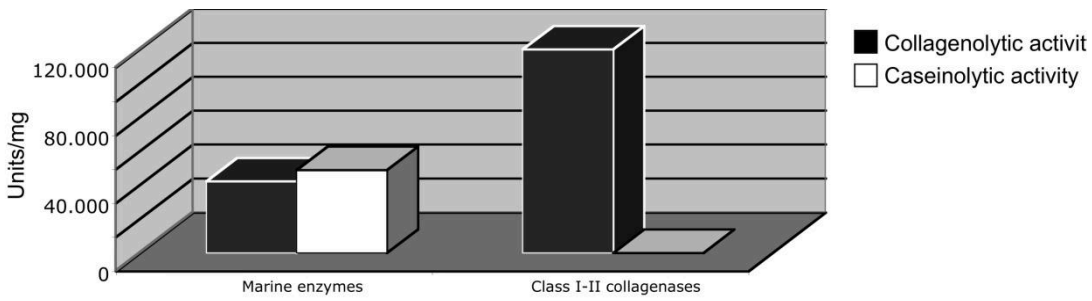


*Figure 3 – Enzymatic activity of recombinant collagenases and Marine enzymes (P. elephas) on bovine tendon collagen "native collagen" and casein substrates. Recombinant class I-II collagenases and P. elephas extract were tested using the same concentration. One unit equals one micromole of L-leucine equivalents released from collagen or casein in 5 hours at 37°C, pH 7.5, under the specified conditions*

## 4. Conclusions

In the present study, enzyme with gelatinolytic activity from hepatopancreas of *E. verrucosa and P. elephas* was partially purified. At least six different enzymatic activity were described in a range between 45 and 20 kDa in *E. verrucosa*; while 97 and 28 kDa in *P. elephas*. The enzymatic activity of identified proteolytic enzymes is not affected by low temperature, they work in a temperature range between 37 to 4 °C. We have quantify the collagenases activity using to separate quantification assays: the Pz liberalized peptide, an evaluation performed on linear peptide that show preferentially the gelatinolytic activities; and the non-soluble collagen from bovine tendon, this put in star light the ability of enzymes to perform their effect on threedimensional form of the substrate. In these experiments, the molecules that we have as refer enceabout enzymatic activity were the *Clostridium hystoliticum* collagenases. In the marine extracted enzymes also casein activities were detected; also, in this case, a large range of temperature action was found. Further researches, however, are needed to determine the properties of individual components from *E. verrucosa* and *P. elephas* usable as possible biotechnological and biomedical tool.

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