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Partial characterization of hepatopancreatic and extracellular digestive proteinases of wild and cultivated *Octopus maya*

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Abstract:

Abstract Proteinases from hepatopancreas (HP) and gastric juice (GJ) from wild and cultured red octopus (*Octopus maya*) were characterized. Hepatopancreas assays revealed optimal activity at pH 4, 9–10 and 10 for wild and pH 3, 8, and 9, for cultured octopuses, for total proteinases, trypsin and chymotrypsin, respectively. In the gastric juice, maximum activity was recorded at pH 6, 8, and 7 for total proteinases, trypsin, and chymotrypsin, respectively for both wild and cultured octopus. A reduction on enzyme activity of 70 and 20% was observed in HP and GJ extracts, respectively when protease inhibitor Pepstatin A was used. That result suggests that the main proteases in the HP were aspartic acid proteinases type (possibly Cathepsin D) and some of them were present in the GJ. Dissociating discontinuous polyacrylamide gel electrophoresis showed activity bands between 20 and 28, 30 and 34, 35 and 45, 60 and 70 kDa, and a last one between 75 and 100 kDa. We concluded that extracellular digestion of *O. maya* takes place in an acid environment, around pH 6. In contrast, intracellular digestion in the HP is developed at pHs between 3 and 4, where cathepsin D could be the most important enzyme for *O. maya*.

Keywords: Cephalopods ; Digestive proteinases ; Hepatopancreas ; Gastric juice ; *Octopus maya*

1. Introduction

The red octopus (*Octopus maya*) is an endemic species that inhabits the coasts of the Peninsula of Yucatan and represents an important fishing resource (Voss and Solís-Ramírez, 1966, Solís-Ramírez and Chávez, 1986; Solís-Ramírez, 1988; Cabrera and Defeo, 2001; Pérez Lozada et al*.,* 2002). *O. maya* has a great potential for aquaculture because this species reaches the juvenile stage 30 days after hatching, whereas other species go through a 60-day planktonic life before their settlement as juveniles (Moguel et al., 2010). At present, there is no adequate artificial feed that can be used to foster octopus culture at an industrial level. Different types of artificial feeds were fed to *O. maya* juveniles with limited success. Rosas et al., (2007) reports that the main limitation of the use of pellets to feed *O. maya* juveniles was related with the low digestibility of the diets (lower than 20%). Aguila et al., (2007) used artificial diets supplemented with different concentrations of fish hydrolysate (CPSP). Results of this study showed a positive growth rate when octopuses were fed with a diet supplemented with 15% of CPSP, suggesting that with that diet the food digestibility can be enhanced. Other studies done on *O. maya* and *O. vulgaris* reported the effect of the type of binder used to prepare the artificial diets. In *O. maya* juveniles, the digestibility of crab paste was improved using gelatin besides sodium alginate, indicating that octopuses can digest feed better when a proteinaceous binder is used (Rosas et al., 2008).

It is known that octopuses are carnivorous, for which proteolytic enzymes are determinant in the digestion and availability of nutrients (Boucher-Rodoni and Mangold, 1985; Aguila et al*.,* 2007; Domingues et al*.,* 2007). Digestibility depends on digestive enzymes that act in the gastric juice to activate chymotrypsin. Digestion in cephalopods is a two-parts process: extracellular and intracellular (Boucher-Rodoni et al., 1987;

Koueta and Boucaud-Camou, 1999; Koueta et al., 2000). The extracellular enzymes secreted by the hepatopancreas produce the first hydrolysis on the ingested food (Perrin, 2004). The gastric juice, containing the necessary enzymes for the extracellular digestion, is mixed with the ingested food in the crop and carried to the hepatopancreas to be absorbed. The intracellular digestion of the chymo takes place in the hepatopancreas where the molecules are hydrolyzed to be transported or stored (Boucaud-Camou et al., 1976). At present, the low digestibility of the formulated diets could be explained by the incapacity of the extracellular digestive enzymes to hydrolyze adequately the nutrient content in the diet. The high quantity of feces observed in octopuses fed elaborated diets with high protein and energy content supports that hypothesis (Rosas et al., 2009, 2010).

Enzymes in the hepatopancreas are of proteolytic nature. (Boucaud-Camou and Boucher-Rodoni, 1983). Although trypsin and chymotrypsin (alkaline proteinases) have been the most studied enzymes in cephalopods (Mangold, 1989; Ezquerra et al., 2002) there are other enzymes, such as cysteine proteinases, that have a high proteolytic activity (Cárdenas-Lopez and Haard, 2005). In the jumbo squid *Dosidiscus gigas*, cysteine proteinases peaked proteolysis at pH values of 3 to 4 and 5 to 6, suggesting cysteine cathepsins (B, H, and L) as the main enzymes in the hepatopancreas of jumbo squid (Cardenas-Lopez and Haard, 2005). Recently a cathepsin L was identified in the jumbo squid that had an optimal pH of 4.5 and optimal temperature of 55 °C (Cardenas-Lopez and Haard, 2009). Unfortunately there are no studies on the enzyme activity of the gastric juice and, in consequence, we do not know the characteristics of the digestive enzymes that modulate external digestion and chymo formation in cephalopods.

The pH is one of the main factors affecting enzymatic activity, modulating the secondary conformation of the molecule or modifying the electric charge of the enzyme and/or of the substrates (Murray et al., 2001; Le Bihan et al., 2006;). In an attempt to characterize the digestive enzymes, in the present study the effect of the pH on the activity of the digestive proteinases present in the gastric juice and hepatopancreas tissue of *O. maya* was evaluated, in both wild and cultivated octopuses. At the same time, the molecular weight of the enzymes of the gastric juice was determined electrophoretically.

2. Materials and Methods

2.1. Origin of animals

The study was performed in the Unidad Multidisciplinaria de Docencia e Investigación, Facultad de Ciencias (UMDI, Sisal, Yucatan, Mexico) of the Universidad Nacional Autónoma de México. Wild *O. maya* (N = 15; 536.01 ± 125.89 g) were caught using artisan lines, with only fresh crabs as bait and without hook, in front of Sisal harbor (Yucatán, Mexico). Octopuses were transported from the port to the laboratory situated 300 m inland, in a 400-L circular tank with sea water. Laboratory animals were individually placed in 80-L tanks for acclimation during 7 days at 28 ± 1°C, 34 salinity, oxygen dissolved higher than 5.5 mg L^{-1} and pH > 8. A PVC tube of 4” diameter was offered as a shelter. During the acclimation time, octopuses were fed dead *C. sapidus* crabs.

Cultured *O. maya* (N = 15; 11.90 ± 2.08 g) were obtained from spawning of wild mature females under controlled conditions. Hatchlings (100 ± 18 mg living weight; 80

120 octopuses m^{-2}) were obtained from *O. maya* system production. In that system
121 octopuses were cultivated in 8 m^2 (1600-L) tanks during two months until animals
122 reached 2g of living weight and maintained at 28 ± 1°C, 34 PPT salinity, oxygen
123 dissolved higher than 5.5 mg L^{-1} and pH > 8. A photoperiod of 10 h light and 14 h
124 darkness was provided. During culture period, animals were fed crab pieces *ad libitum*
125 and provided with a clean *Megalongena corona bispinosa* conch (spiral-shell) to serve
126 as a refuge. Production tanks were connected to a flow through sea water system and
127 coupled to skimmer and anthracite earth filter. Survival of 40% was obtained in the
128 production tanks and it was a consequence of octopus cannibalism. When octopuses
129 reached 2 g of living weight, they were re-distributed in three 7-m^2 sea water outside
130 tanks (0.7 octopuses m^{-2}) where animals were fed crab pieces. Natural sea water was
131 supplied to tanks and connected to a recycling system. Only a 10% of total seawater
132 was renewed daily. In that system sea water was maintained at 28 ± 2°C, 34 PPT
133 salinity, oxygen dissolved higher than 5.5 mg L^{-1} and pH > 8. Experimental tanks were
134 placed below a plastic mesh as a shade (90%). Pieces on "T" form (38 mm diameter)
135 were provided as refuges. To reduce the interaction between octopuses, a mesh was
136 used as a cap in two sides of the "T" pieces. Animals were kept in these tanks during
137 one month until sampling. Due to cannibalism reduction, *O. maya* juveniles survival was
138 75%. Growth rate was not recorded during the experiment, to avoid excessive
139 manipulation. Animals were weighed only at the sampling time.
140
141 2.2. Enzyme preparations
142 Hepatopancreas (HP) and gastric juice (GJ) samples were obtained directly from
143 the experimental animals. Before sampling, octopuses (24-h fasting animals) were

anesthetized in a cold sea water bath (15 ^{o}C) for 2 to 5 min. Samples were stored at -80 ^{o}C until analysis. The enzymatic HP extract was obtained from 60 mg of tissue homogenized in 1 mL^{-1} distilled water. Homogenates were centrifuged (16 170 *g* at 4 ^{o}C) for 30 min. To obtain GJ samples, the octopuses were stimulated to segregate it by placing a crab piece in a mesh bag for 15 min in the octopus tank. After the cold seawater anesthesia, the GJ was obtained directly from the crop (anterior stomach) using a 3-cm sterile syringe for the wild and of 1-cm for cultured octopuses. Gastric juice was centrifuged in the same conditions as the HP extracts.

2.3. Protein quantification and enzyme activity assays

Total soluble protein was evaluated with the Coomassie blue dye method according to Bradford (1976) using serum bovine albumin as standard. The activity of total proteinases, trypsin and chymotripsin, was evaluated using Stauffer´s universal buffer (1989) prepared in a wide pH range from 2 to 11. Total proteinases were measured using the methods of Anson (1938), whereas Charney and Tomarelli (1947) and Delmar et al., (1979) methods were used for trypsin and chymotripsin, respectively.

Total proteinases activity was assayed using hemoglobin (1%) as substrate. Briefly, 20 µL of the enzyme extract (dilution 1:10) was mixed with 0.5 mL of buffer, 0.5 mL of freshly prepared substrate in buffer at the corresponding pH and incubated for 10 min at 37 ^{o}C. The reaction was stopped by adding 0.5 mL of 20% (w/v) trichloroacetic acid (TCA) and cooling for 15 min at 4 ^{o}C. The precipitated undigested substrate was separated by centrifugation for 15 min at 13 370 *g*. The absorbance of the supernatants was measured spectrophotometrically at 280 nm against the substrate without enzyme extract (blank). All determinations were done in triplicates and included blanks, which

consisted of buffer, substrate and TCA without enzyme extracts. Blanks were incubated as above mentioned and read at each pH. In order to establish the possible presence of acid enzymes (cathepsin D), the enzymatic extracts were incubated with pepstatin A (1 mM dissolved in dimethylsulfoxide (DMSO) as inhibitor of aspartic proteinases at pH 3 (hepatopancreas) and pH 6 (GJ).

Trypsin activity was assayed using Nα-Benzoyl-DL-arginine 4-nitroanilide-HCl (BAPNA), 1 mM) as substrate. For this, 167 µL of the enzyme extract (dilution 1:100) was mixed with 833 µL of fresh substrate dissolved in buffer (at each experimental pH) and incubated for 60 min at 25 ^{o}C. The absorbance was measured spectrophotometrically at 410 nm against the substrate without enzyme extract. Chymotrypsin activity was assayed using N-Succinyl-Ala-Ala-Pro-Phe p-nitroanilide (SAAPNA1.142 mM) as substrate. The method is the same as that used for trypsin, except that the reaction was incubated for 30 min at 37 ^{o}C. Before dissolving into the buffer, BAPNA and SAAPNA were dissolved in DMSO. One unit of enzymatic activity was defined as the change in absorbance per minute per milligram protein of the enzyme used in this assay (ΔAbs min^{-1} mg protein^{-1}).
All determinations were done in triplicates and included blanks, which consisted of buffer and substrate without enzyme extracts and incubated at specific times and temperatures at each pH.

2.4. SDS-PAGE and substrate SDS-PAGE

Proteins and enzyme of GJ from wild octopuses were separated by discontinuous dissociating 12% PAGE-gelatin according to Laemmli (1970). The stacking gel was

obtained by adding 0.65 mL of 30% polyacrylamide (PAA) to 0.5 M Tris-HCl (pH 6.8) and the resolving gel by adding 2 mL PAA, 0.5 mL of 1% gelatin to 1.5 M Tris-HCl (pH 8.8). To run the gel, a Tris-glicine-SDS 1X was prepared. The samples were prepared with mercaptoethanol at a concentration of 5, 10, and 15 µg protein. A low molecular weight standard (BIO-RAD, 161-0305) was placed on the gel (BIO-RAD, 165-1932, size of the gel 8 x 16 cm) and run with the sample at 4 ^{o}C and15 mA. After electrophoresis, the gel was washed with distilled water and incubated over night in Stauffer's universal buffer, pH 3, with 2% Triton 100 at 37 ^{o}C. Afterwards, the gel was washed again with distilled water and stained with 0.25% Coomassie Brillant Blue R250 in an aqueous solution of 45% methanol and 10% glacial acetic acid for at least 3 h and then distained with the same solution without dye.

2.5. Statistical analysis

All the enzymatic assays were performed in triplicate. Data were expressed as mean ± standard error of the mean (SEM). Differences among means were analyzed by ANOVA followed by Tukey´s multi-comparison test when the Cochran test showed homogeneity of variances. When variances were different, a Kruskal-Wallis test was applied. Differences are reported as statistically significant when $P<0.05$. Statistical differences of data sets in tables and graphs are indicated by different letters. Statistical analysis was carried out using the Statgraphics 4.1 software, Statistical Graphics Corp.

RESULTS

A higher protein concentration was registered in gastric juice (GJ) of wild octopuses than that observed in the cultivated ones ($P < 0.05$; Table 1).

Total proteinases

In wild octopuses, a higher proteinases activity of the HP was registered at pH 2 and 11 ($P<0.05$; Table 2). However, those activities were not considered into the analysis taking into account that enzymes of DG and GJ could be destroyed at such extreme pHs. In consequence, the maximum enzyme activity of HP was considered that obtained at pH 4 (Fig. 1a; $p < 0.05$). In cultured animals, a higher proteinases activity of HP was registered at pH 3, followed by the activity observed at pH 2 and 4 (Fig. 1b; $P<0.05$). For GJ, the maximum activity was registered at pH 6 both wild and cultured octopuses (Tables 2 and 3; Figs 1c and d; $P < 0.05$).

A high HP trypsin activity was observed in wild octopuses at pHs 9 -10 and in cultured octopuses at pH 8 (Figs. 2a and b; $P < 0.05$). In GJ the maximum activity of trypsin was located at pH 8, both wild and cultured animals (Figs. 2c and d; $P < 0.05$).

The maximum chymotrypsin activity of the HP of wild octopuses was registered at pH 10 while in cultured animals it was registered at pH 9 (Figs 3 a and b; $P < 0.05$). In contrast, a maximal activity of chymotrypsin was obtained in the GJ at pH 7, both wild and cultured animals (Tables 2 and 3; Figs. 3c and d; $P < 0.05$).

Inhibition

A 72% reduction in proteinases activity was observed in the HP when pepstatin A was used. Using the same inhibitor, a reduction of 18% was observed in the GJ's proteinases activity (Table 4).

241 Zymogram at pH 3

242 Gastric juice zymogram of wild octopuses showed bands of enzyme activity and
243 proteins between the 20 and 28 kDa, 30 and 34 kDa, 35 and 45 kDa, 60 and 70 kDa
244 and a last one between 75 and 100 kDa (Fig. 4).

245

246 Discussion

247 The digestive proteinases present in marine species have been characterized according
248 to their biochemical properties (Ribero *et al.,* 1999; Fu *et al*., 2005; García-Esquivel and
249 Felbeck, 2006;). In fact, there are studies that demonstrate that the digestive enzymes in
250 the HP of cephalopods are essentially acid (jumbo squid and sepia) (Cárdenas-Lopez
251 and Haard, 2005; Perrin et al., 2004; Cárdenas-Lopez and Haard, 2009).

252 Cephalopods digestion includes pancreatic secretions of extracellular enzymes
253 and intestinal absorption of small molecules (in cecum and hepatopancreas). Although
254 at present, intracellular digestion has been demonstrated in several cephalopod species
255 (Boucaud-Camou and Boucher-Rodoni, 1983), we don't know reports on the activity of
256 total proteinases, trypsin, and chymotrypsin in the GJ of cephalopods, although Bider
257 (1950) made a review in which some aspects of the digestive enzymes present along
258 digestive tract.

259 In the present study and for the first time, we show the enzyme activity in the HP
260 and GJ of octopus and its relation with the pH. Also, we observed that the digestive
261 enzymes activity measured in the HP is different from that observed in the GJ,
262 suggesting that intracellular and extracellular enzymes are different. Proteinases from
263 the HP of *O. maya* show an optimal activity at pH 3 and 4, indicating the presence of
264 lysosomal proteinases associated with the intracellular digestion of the chymo. The 72%

inhibition observed when an inhibitor of aspartic proteinases was used suggests the possible presence of cathepsin D in the HP of *O. maya*. In jumbo squid, two peaks of enzyme activity were observed; one between 3 and 4 and the other between pH 5 and 6 suggesting the presence of cathepsins B, H, and L (Cárdenas-López and Haard, 2005). These authors concluded that the relatively high proteolytic activity observed in jumbo squid's HP at pH 5 was due to cathepsin L, which was recently confirmed (Cárdenas-López and Haard, 2009). In *S. officinalis*, HP's acidic phosphatases had higher activity than alkaline enzymes, suggesting that non proteolytic enzymes work in an acid environment in the digestive gland of that species (Perrin et al. 2004). In *S. officinalis,* it was shown that the activity of acid phosphatases was 1000 times higher than that of trypsin and chymotrypsin, being chymotrypsin the most active alkaline enzyme of that species (Perrin et al. 2004). Thus, the presence of both aspartic and cysteine proteinases (cathepsins) or acid phosphatases put in strong evidence that in cephalopods (at least in jumbo squid, sepia, and *O. maya*) the enzyme activity of HP is carried out at a low pH environment, where the intracellular digestion process of chymo could be more efficient (Boucaud-Camou and Boucher-Rodoni, 1983).

The acid conditions found in the GJ in this study are new data for cephalopods. An 18% inhibition of the enzyme activity in the GJ in the presence of a cathepsin D inhibitor indicates that aspartic proteinases are less represented in the extracellular digestion than in the intracellular digestion. In the GJ, a maximal proteinase activity was observed at pH 6 suggesting that, besides cathepsin D, trypsin, and chymotrypsin, there are other enzymes in the GJ that command the extracellular digestion in *O. maya*. Although, at the present, we do not know the enzymes' composition of the GJ, it is interesting to note that cathepsins B, H, and L have an optimal pH at 6, 6.8, and

5.5,respectivelly; we obtained a pH close to 6 for a maximal enzyme activity in the GJ of *O. maya*, that suggest that cathepsins could be important enzymes in GJ. Other cathepsins have also been identified in vertebrates; cathepsin X was recently demonstrated as an extracellular enzyme with an optimal pH of 5.1 (Nägler *et al*., 2006). Recently, evidence has been accumulating that cysteine proteinases are secreted from a large number of cell types exhibiting an extracellular function in vertebrates and invertebrates, supporting the hypothesis that the extracellular enzymes of the GJ of *O. maya* could be cathepsins (Wilson et al., 1998; Nägler et al., 2006).

In the mix of the enzymes of the GJ of *O. maya* proteins showed molecular weights between 30 and 70 kDa. In gastric juice a mix of enzymes and proteins are observed. According with other studies we can assume that some of bands observed were enzymes because that reacted against the substrate putted into the electrophoresis gel. Perera *et al*. (2008) performed a partial characterization of proteinases in the spiny lobster and found bands of activity between 14 and 45 kDa, identifying trypsin isoforms. A study in the sea cucumber (Fu et al., 2005), confirmed the existence of at least three proteinases, whose molecular weights were 20.6, 39.1, and 114.1 kDa, respectively. Furthermore, the 20.6 kDa protease was confirmed to be a metalloprotease and the 39.1 kDa protease, a serine protease (Fu et al., 2005). Chymotrypsin was isolated from the viscera of the Monterey sardine with an approximate molecular weight of 26 kDa (Castillo et al., 2006), and, in the blue abalone, enzymes with molecular weights of 28.1, 29.5, 30 y 32 kDa were also obtained (Hernández et al., 1998). All these results demonstrate that digestive enzymes in aquatic organisms have low molecular weights. Also cathepsins have low molecular weights. Cathepsin B is around 37 kDa whereas cathepsin H and L have a molecular

weight around 23 kDa (Aranishi et al., 1992; Wilson et al., 1998; Cardenas-Lopez and Haard, 2009).

From the results obtained, we concluded that extracellular digestion of *O. maya* takes place in an acid environment, around pH 6, where enzyme activity of proteinases is maximal. In contrast, intracellular digestion in the HP is developed at pHs between 3-4, where cathepsin D could be the most important enzyme for *O. maya*. Taking into account the GJ's characteristics, cathepsins could be hypothesized as the most important extracellular enzymes in this cephalopod species. According with the inhibition experiment results it is possible to propose that other cathepsin besides cathepsin D could be present on GJ of *O. maya.*

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Figure legends

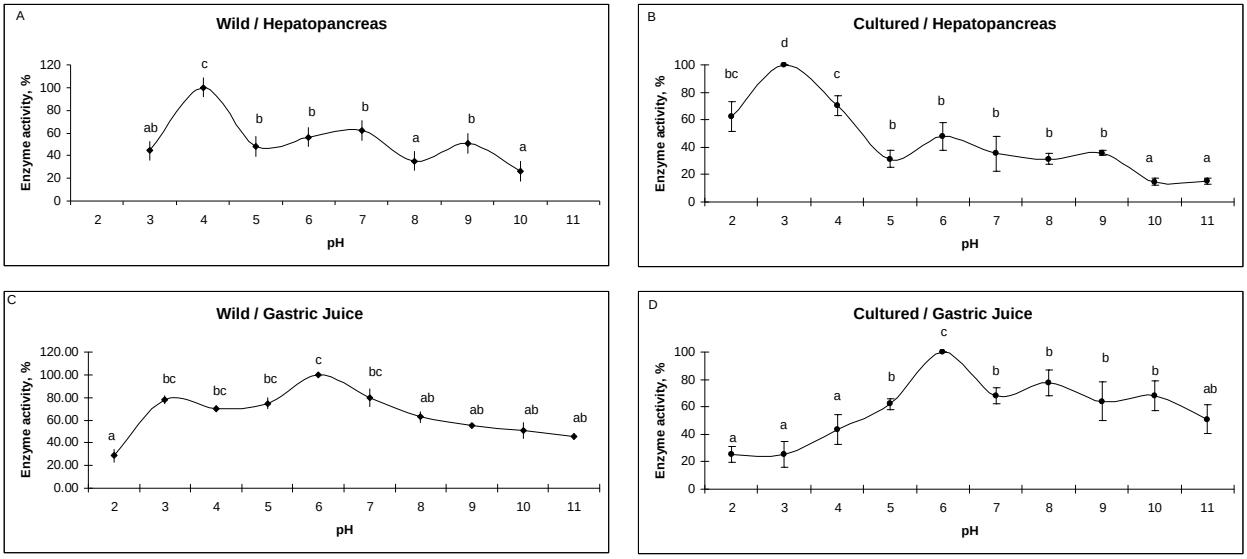
Figure1. Effect of pH on total proteinases activity of wild (a) and cultured (b) hepatopancreas (HP) and wild (c) and cultured (d) gastric juice of *O. maya*. Values as mean ± S.E.. Different letters means statistical differences at $P < 0.05$ level.

Figure 2. Effect of pH on trypsin activity of wild (a) and cultured (b) hepatopancreas (HP) and wild (c) and cultured (d) gastric juice of *O. maya*. Values as mean ± S.E.. Different letters means statistical differences at $P < 0.05$ level.

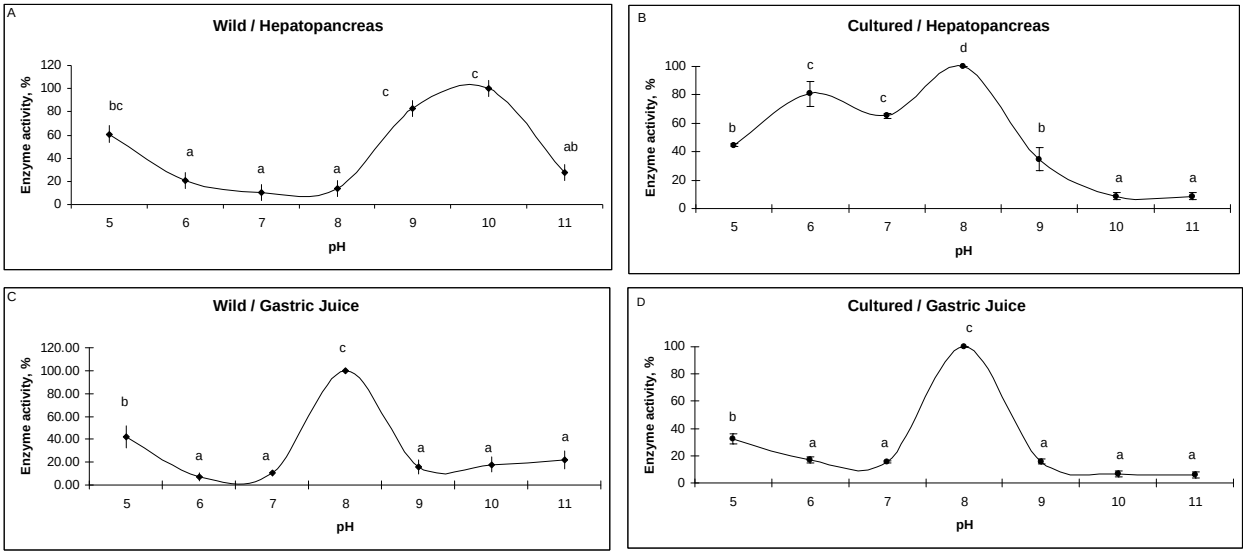
Fig. 3. Effect of pH on chymotrypsin activity of wild (a) and cultured (b) hepatopancreas (HP) and wild (c) and cultured (d) gastric juice of *O. maya*. Values as mean ± S.E.. Different letters means statistical differences at $P < 0.05$ level.

Figure 4. Zymogram of proteinases present in the gastric juice of wild *O. maya*. A: 5 µg, B: 10 µg and C: 15 µg of protein.

Figure 1.


A
Wild / Hepatopancreas
Enzyme activity, %
pH
B
Cultured / Hepatopancreas
Enzyme activity, %
pH
C
Wild / Gastric Juice
Enzyme activity, %
pH
D
Cultured / Gastric Juice
Enzyme activity, %
pH

489 Figure 2.



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493 Fig. 3.

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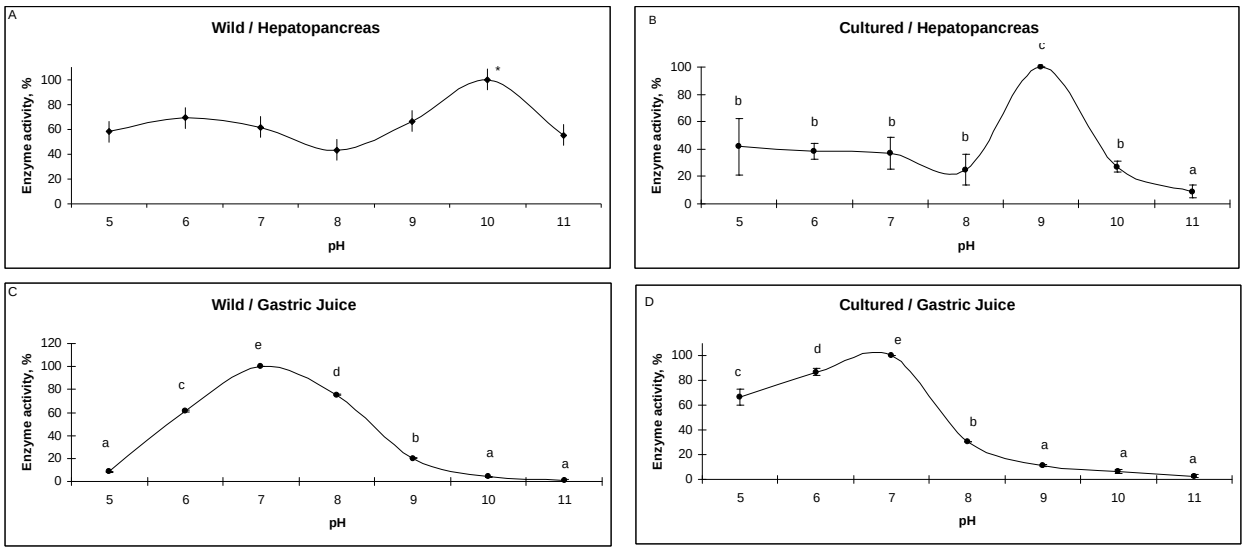

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498 Fig. 4

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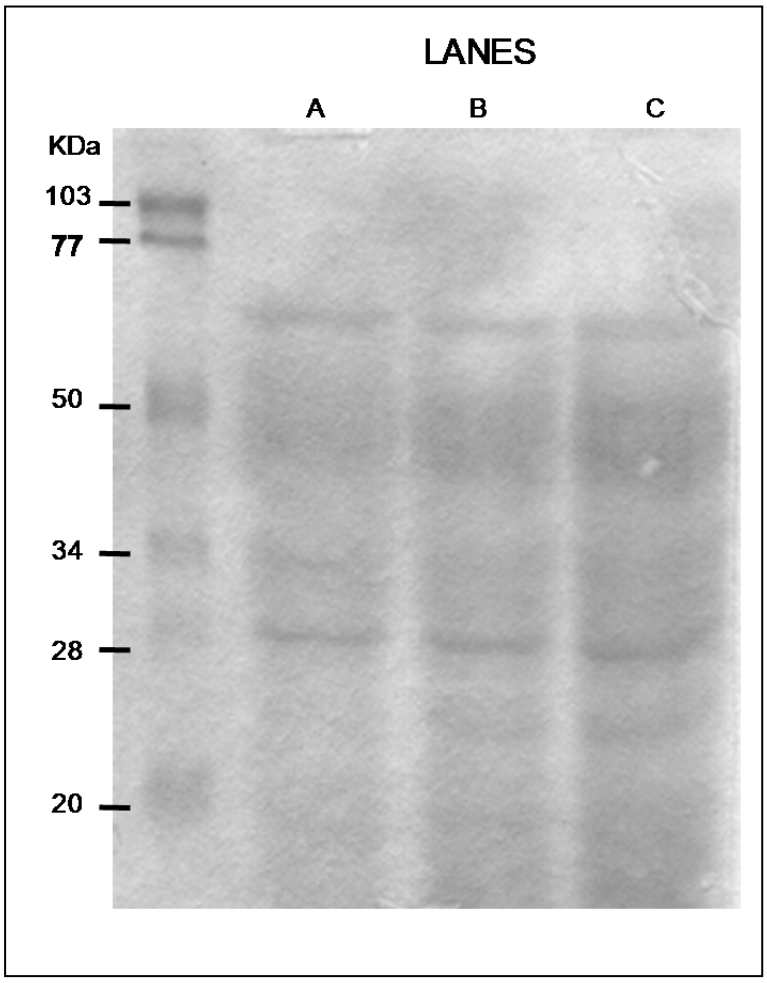

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522 Table 1. Values of soluble protein of the evaluated samples. The results are the mean ±
523 SEM of five determinations.

Sample	Soluble protein (mg/mL)
TS	33.33 ± 2.4
TC	16.65 ± 0.5
JS	67.70 ± 6.3*
JC	23.16 ± 2.0

524

525 TS: Tissue of hepatopancreas of wild octopuses; TC: Tissue of hepatopancreas of culture octopuses; JS:
526 Gastric juice of wild octopuses; JC: Gastric juice of culture octopuses. (*) denote significant differences
527 from all other samples at $P<0.05$.

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Table 2. Values of enzymatic activity (UI/mg protein) total proteinases, trypsin, and chymotrypsin present in hepatopancreas and gastric juice of wild *O. maya*.

	pH									
Sample	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
Hepatopancreas										
Proteases	37190 ± 865^{b}	7789 ± 292^{a}	17521 ± 4754^{a}	8442 ± 1181^{a}	9870 ± 4831^{a}	10893 ± 3592^{a}	6167 ± 2207^{a}	8901 ± 2894^{a}	4625 ± 2047^{a}	20892 ± 5750^{ab}
Trypsin	-	-	-	17.50 ± 2.43^{bc}	5.91 ± 1.67^{a}	3.05 ± 1.87^{a}	4.01 ± 1.33^{a}	23.90 ± 3.19^{c}	28.77 ± 3.16^{c}	7.90 ± 2.00^{ab}
Chymotrypsin	-	-	-	126 ± 29.4	150 ± 2.9	133 ± 30.2	94 ± 33.7	144 ± 36.9	217 ± 46.7	110 ± 55.5
Gastric juice										
Proteases	810 ± 221.0^{a}	2119 ± 196.1^{bc}	1920 ± 253.2^{bc}	2021 ± 162.6^{bc}	2724 ± 278.4^{c}	2134 ± 95.1^{bc}	1694 ± 157.6^{ab}	1504 ± 158.8^{ab}	1383 ± 233.2^{ab}	1254 ± 187.1^{ab}
Trypsin	-	-	-	0.34 ± 0.04^{a}	0.05 ± 0.01^{a}	0.08 ± 0.02^{a}	0.91 ± 0.27^{b}	0.11 ± 0.01^{a}	0.13 ± 0.01^{a}	0.17 ± 0.04^{a}
Chymotrypsin	-	-	-	0.84 ± 0.06^{ab}	6.18 ± 0.28^{c}	10.07 ± 0.52^{e}	7.60 ± 0.40^{d}	2.04 ± 0.11^{b}	0.41 ± 0.03^{a}	0.08 ± 0.06^{a}

Values ± SEM

Table 3. Values of enzymatic activity (UI/mg protein) total proteinases, trypsin, and chymotripsin present in hepatopancreas and gastric juice of culture *O. maya*.

	pH									
Sample	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
Hepatopancreas										
Proteases	10566 ± 918.4^{cd}	17460 ± 1502.3^{e}	12070 ± 161.3^{d}	5296 ± 619.0^{ab}	8032 ± 1044.3^{bcd}	5794 ± 1668.1^{ab}	5374 ± 239.4^{ab}	6307 ± 856.5^{abc}	2503 ± 225.9^{a}	2737 ± 618.1^{b}
Trypsin	-	-	-	5.91 ± 1.87^{ab}	10.11 ± 2.35^{ab}	8.61 ± 2.68^{ab}	13.41 ± 4.52^{b}	4.38 ± 1.50^{ab}	0.98 ± 0.07^{a}	0.96 ± 0.06^{a}
Chymotrypsin	-	-	-	14.23 ± 6.6^{ab}	11.35 ± 1.1^{ab}	10.10 ± 1.0^{a}	6.00 ± 2.5^{a}	31.90 ± 8.6^{b}	8.30 ± 1.9^{a}	2.17 ± 0.7^{a}
Gastric juice										
Proteases	741 ± 166.5^{a}	690 ± 132.9^{a}	1252 ± 283.4^{a}	1953 ± 440.3^{ab}	3117 ± 604.8^{b}	2097 ± 415.9^{ab}	2325 ± 336.2^{ab}	1865 ± 330.0^{ab}	2052 ± 431.4^{ab}	1501 ± 297.9^{ab}
Trypsin	-	-	-	0.24 ± 0.015^{b}	0.12 ± 0.010^{a}	0.12 ± 0.001^{a}	0.75 ± 0.036^{c}	0.12 ± 0.008^{a}	0.05 ± 0.019^{a}	0.04 ± 0.019^{a}
Chymotrypsin	-	-	-	0.81 ± 0.060^{c}	1.07 ± 0.056^{d}	1.23 ± 0.029^{d}	0.38 ± 0.009^{b}	0.14 ± 0.007^{a}	0.08 ± 0.020^{a}	0.03 ± 0.014^{a}

Values ± SEM

Table 4. Inhibitor pepstatin A effect on total proteinases activity in wild octopuses.

Activity	UI/mg protein	
	Gastric juice	Hepatopancreas
Without inhibitor	3486.15	17460.00
With inhibitor	2860.03	4904.51
% Inhibition	17.96	71.91