IN VIVO AND IN VITRO COMPARATIVE ASPECTS OF SOME PROTEOLYTIC ENZYMES FROM *Mytilus galloprovincialis*

Daniela Mariana MIRCEA[1], Natalia ROSOIU[2], Elena DUMITRESCU[1], Andra OROS[1]

[1]National Institute for Marine Research and Development "Grigore Antipa", Bd. Mamaia 300, Constantza, 900581, ROMANIA
E-mail: mirdana@alpha.rmri.ro
[2] Constantza University "Ovidius", Faculty of Medicine, Str. Ion Voda, No 58, Constantza, 900573, ROMANIA
E-mail: natalia_rosoiu@yahoo.com

ABSTRACT

In natural condition of marine environment, biotic and abiotic parameters interfere during the four seasons. Also, it is known that the metabolism and biochemical behaviour of marine organisms are influenced by seasonal dynamic of abiotic factors and the presence in marine environment of certain xenobiotic substance.

The paper presents a study of enzymatic activity of trypsin, chymotrypsin and pepsin from digestive gland of *Mytilus galloprovincialis* specie in natural conditions of the Black Sea coast in correlation with bioaccumulation level of some heavy metals.

In the same time, these enzymes have been followed up in vitro under the same heavy metals action in different doses. In vivo and in vitro comparative study of mentioned enzymes emphasizes behaviours, which are generated by the presence of some multiple complex factors from natural marine environment and which act together on marine organisms against to the controlled action on them.

KEY WORDS: trypsin, chymotrypsin, pepsin, digestive gland, *Mytilus galloprovincialis*, heavy metals

INTRODUCTION

The most part of heavy metals from seawater is taken by food at digestive level of mussels. Cu, Cd, Pb are the most detected heavy metals in marine environment and organisms, and in certain conditions they may have toxic or lethal effect on alive components of marine ecosystems.

The proteolytic enzymes are linked with quantity and quality of food, and are involved in metabolism of proteins. The heavy metals interaction effect with proteolytic enzymes depends by the metal and enzyme type and also by heavy metal concentration for both “in vivo" and “in vitro” experiments (LE GAL, 1998; MIRCEA *et al*.2005).

MATERIALS AND METHODS

It were used digestive gland from mussels which have been collected from Mamaia Bay, Tomis Port and Agigea, from rocky substratum. The juvenile (20-40 mm) and adult (50-80 mm) mussels were studied in 2003, during the spring and summer period.

Enzymes activity and protein content have been determined in total protein extract of digestive gland (1 g tissue for 10 ml bidistilled water, homogenization, preservation at 4^oC, centrifugation at 4000 x g for 15 min.).

Enzymes activity was analyzed with Anson method cited by ROSOIU in total protein extract (ROSOIU & SERBAN, 1992, 2002; ROSOIU *et al*., 1981).

Pepsin activity was determined by the Anson method, using hemoglobin at pH 2.0 as a substrate; trypsin and chymotrypsin activities were also determined by the Anson method, using adulterated hemoglobin at pH 7.5. The enzymatic activity was expressed as specific activity in nmoles tyrosine/mg protein /minute at 37^oC (ROSOIU & SERBAN, 1992, 2002; ROSOIU *et al*., 1981).

Determination of protein concentration in the total protein extracts was carried out by method of LOWRY *et al.* (IORDACHESCU & DUMITRU,1988).

For experiments “in vitro” it were used pure enzymes (Merck) and hydro soluble salts of Cu^{2+}, Cd^{2+}, Pb^{2+} in different doses.

These determinations were carried out as follows:

- 1.0 ml standard enzyme solution (10 µg/ml) + 1ml of bidistilled water were incubated for one hour in a thermostat at 37^oC, after the activity of the respective enzyme was determined.

- 1.0 ml standard enzyme solution (10 µg/ml) +1 ml metal solution were incubated for one hour in a thermostat at 37^oC, after the activity of the respective enzyme was determined.

The heavy metal concentration of "in vitro" experiment have been chosen taking into account its bioaccumulation level found in digestive gland of mussels (ROSOIU *et al.* 1981).

Heavy metal concentrations (Cu, Cd, Pb -μg/g wet weight) from digestive gland were determined by Atomic Absorption Spectrophotometer in accordance with methods for marine pollution (IAEA,1993).

RESULTS AND DISCUSSIONS

Mytilus galloprovincialis is a very studied marine organisms from different viewpoints and is characterized as following:

- sessile, filter - feeding organism,
- indicator of pollution,
- capacity of bioaccumulation of a large range of xenobiotic substances from marine environment,
- one of the most important benthic invertebrate from Black Sea,
- important source of food for benthos feeding fish,
- its larvae ensure the food for planktivore fish,
- the shells contribute to the formation of the organic fraction of $CaCO_3$ in the unconsolidated sediments on the Black Sea shelf,
- for human food and for obtaining of active biochemical substances (ABAZA,1996-1997).

The paper presents a comparative study of trypsin, chymotrypsin and pepsin activity from digestive gland of juvenile and adult mussels, in natural conditions of marine environment of Black Sea in correlation with bioaccumulation level of Cu, Cd, Pb, "in vivo".

The experiments "in vitro" on pure enzymes had the purpose to show the differences which appear in life and lifeless system.

Achieved results "in vivo"

Cooper level in digestive gland of juvenile mussels ranged from 0.42 – 1.07 μg/g wet weight in spring (Fig.1a – min. in Mamaia Bay, max. in Agigea) and 1.51 – 4.6 μg/g wet weight, in summer (Fig.2a – min. in Agigea, max. in Mamaia Bay).

The distribution of cooper in digestive gland of adult mussels ranged from 0.76 – 0.8 μg/g wet weight in spring (Fig.1a - min. in Mamaia Bay, max. in Tomis Port), and 4.14 – 7.09 μg/g wet weight in summer (Fig. 2a - min. in Mamaia Bay, max. in Tomis Port).

Juvenile and adult mussels registered for Cu, higher values in summer (Fig. 1a, Fig. 2a).

Concentration of cadmium in digestive gland of juvenile mussels ranged from 3.65 –9.79 μg/g wet weight in spring (Fig. 1b – min. in Agigea, max. in Tomis Port) and 0.014 –0.037 μg/g wet weight in summer (Fig.2b – min in Tomis Port, max in Mamaia Bay).

Bioaccumulation level of cadmium in digestive gland of adult mussels ranged from 2.68 – 4.86 μg/g wet weight in spring (Fig.1b – min. in Agigea, max. in Tomis Port) and 0.017 – 0.175 μg/g wet weight in summer (Fig. 2b – min. in Mamaia Bay, max. in Tomis Port).

Juvenile and adult mussels registered for Cd, higher values in spring (Fig. 1b, Fig. 2b).

Lead level in digestive gland of juvenile mussels ranged from 1.56 – 4.01 μg/g wet weight in spring (Fig.1c - min in Tomis Port, max. in Agigea) and 0.21 – 2.34 μg/g wet weight, in summer (Fig.2c - min. in Agigea, max in Mamaia Bay).

Concentration of lead in digestive gland of adult mussels ranged from 0.73 –1.02 μg/g wet weight in spring (Fig. 1c - min. in Agigea, max. in Tomis Port) and 1.89 –2.83 μg/g wet weight in summer (Fig.2c - min. in Mamaia Bay, max. in Tomis Port).

Juvenile mussels accumulated for Pb higher concentrations in spring (Fig. 1c), and the adult ones, in summer (Fig. 2c).

During the spring period, juvenile mussels accumulated Cu, Cd, Pb in higher concentrations than adults (Fig.1 a, Fig. 1 b, Fig.1 c).

In summer, adult mussels accumulated Cu, Cd, Pb in higher concentrations than the juvenile ones (Fig. 2a, Fig. 2b, Fig. 2c).

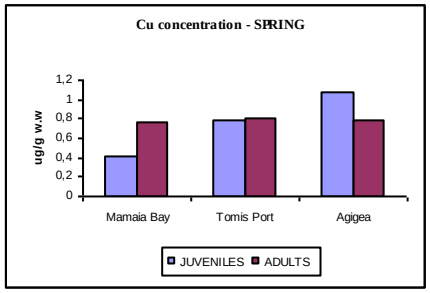

Fig. 1a

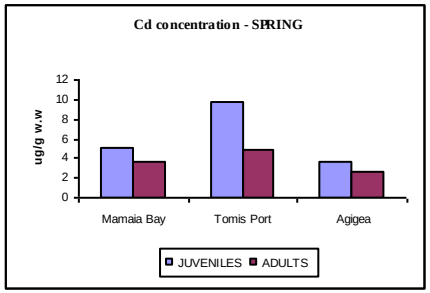

Fig.1b

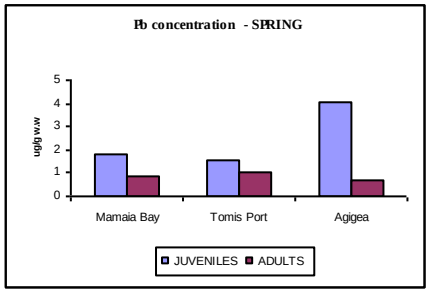

Fig.1c

Fig. 1a,b,c Comparative values of Cu, Cd, Pb concentrations(μg/g wet weight) from digestive gland of juvenile and adult mussels in spring

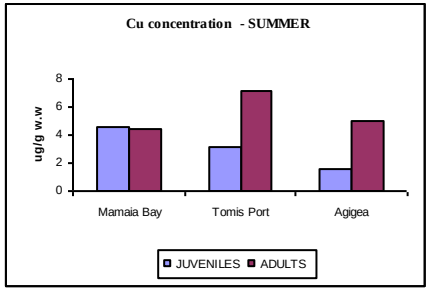

Fig. 2a

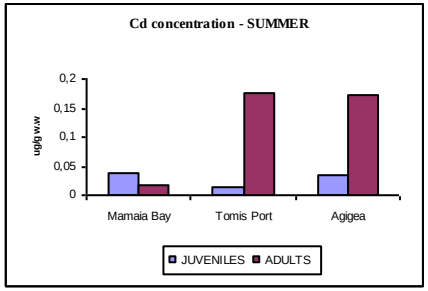

Fig. 2b

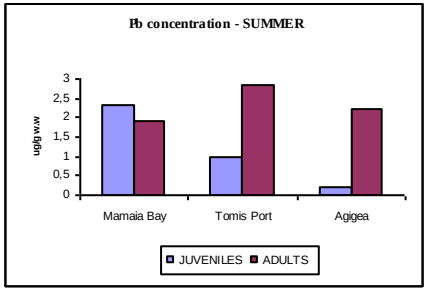

Fig. 2c

Fig. 2a,b,c - Comparative values of Cu, Cd, Pb concentrations(μg/g wet weight) from digestive gland of juvenile and adult mussels in summer

Specific activity of trypsin in digestive gland of juvenile mussels ranged from 0.64 – 107.2 nmoles tyrosine/mg protein /minute at 37^{o}C, in spring (Fig.3a – min. in Tomis Port, max. in Mamaia Bay), and 2.88 – 24.32 nmoles tyrosine/mg protein /minute at 37^{o}C in summer (Fig. 4a - min. in Agigea, max. in Mamaia Bay).

In case of digestive gland of adult mussels, specific activity of trypsin ranged from 8.00 – 50.24 nmoles tyrosine/mg protein /minute at 37^{o}C, in spring (Fig.3a – min. in Tomis Port, max. in Mamaia Bay), and 14.72 – 37.76 nmoles tyrosine/mg protein /minute at 37^{o}C in summer (Fig.4a - min. in Tomis Port, max. in Agigea).

Specific activity of chymotrypsin in digestive gland of juvenile mussels ranged from 6.72 – 78.08 nmoles tyrosine/mg protein /minute at 37^{o}C, in spring (Fig.3b - min. in Tomis Port, max. in Mamaia Bay), and 1.28 – 51.2 nmoles tyrosine/mg protein /minute at 37^{o}C in summer (Fig.4b - min. in Tomis Port, max. in Agigea).

Specific activity of chymotrypsin in digestive gland of adult mussels ranged from 1.92 –33.92 nmoles tyrosine/mg protein /minute at 37^{o}C, in spring (Fig. 3b - min. in Tomis Port, max. in Mamaia Bay), and 3.52 – 5.76 nmoles tyrosine/mg protein /minute at 37^{o}C in summer (Fig.4b – min in Agigea, Mamaia Bay, max in Tomis Port).

Regarding specific activity of pepsin in digestive gland of juvenile mussels, it registered the range 200.36 – 209.95 nmoles tyrosine/mg protein /minute at 37^{o}C, in spring (Fig. 3c - min. in Tomis Port, max. in Mamaia Bay), and 74.43 – 129.81 nmoles tyrosine/mg protein /minute at 37^{o}C in summer (Fig. 4c – min in Agigea, max in Mamaia Bay).

In digestive gland of adult mussels, specific activity of pepsin recorded the range 182.88 – 242.31 nmoles tyrosine/mg protein /minute at 37^{o}C in spring (Fig. 3c - min. in Tomis Port, max. in Mamaia Bay), and 70.59 – 109.81 nmoles tyrosine/mg protein /minute at 37^{o}C in summer (Fig. 4c – min. Mamaia Bay, max in Tomis Port).

During the whole period of observation, juvenile mussels have been the highest values of specific activities for proteolytic enzymes by comparing with adult mussels, in the most of cases (Fig 3a, Fig.3b, Fig 3c and Fig. 4a, Fig. 4b, Fig.4c)

Juvenile and adult mussels registered the highest values of specific activities for all studied enzymes, in spring (Fig. 3a, Fig. 3b, Fig. 3c).

In spring, juvenile and adult mussels had as values for all proteolytic enzymes, min. in Tomis Port and max. in Mamaia Bay.

During the summer period, juveniles registered opposite behaviour regarding studied enzymes, against to the adult ones.

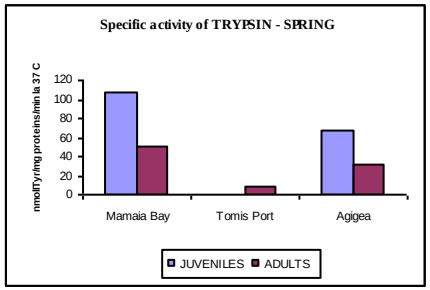

Fig. 3a

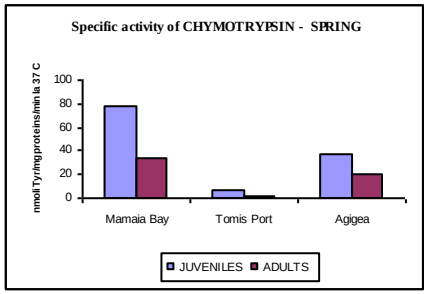

Fig. 3b

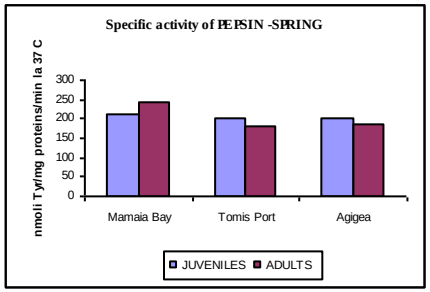

Fig. 3c

Fig. 3a,b,c - Specific activities of trypsin, chymotrypsin and pepsin (nmoles tyrosine/mg protein /minute at 37^{o}C) from mussels digestive gland in spring .

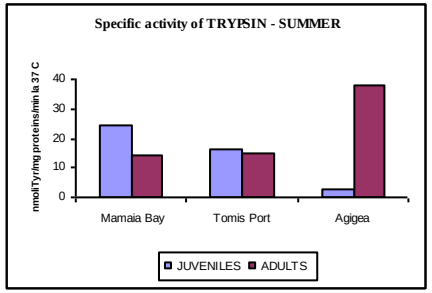

Fig. 4a

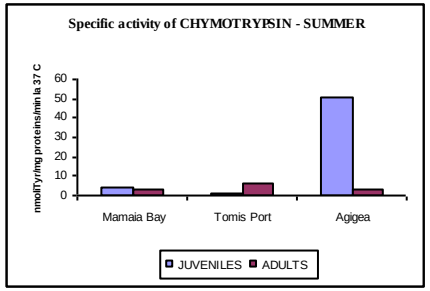

Fig. 4b

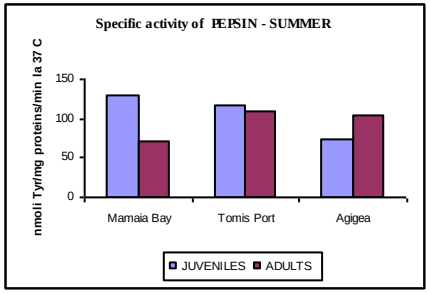

Fig. 4c

Fig. 4a,b,c - Specific activities of trypsin, chymotrypsin and pepsin (nmoles tyrosine/mg protein /minute at 37^{o}C) from mussels digestive gland in summer .

Achieved results "in vitro"

Table 1.

Specific activity of enzymes (nmoles tyrosine/mg protein /minute at 37°C) under the influence of Cu^{2+} in experiment "in vitro"

Cu^{2+} μg	**0**	**0.4**	**7**	**10**	**30**	**60**
Specific activity of Trypsin	44301	46262	48553	58435	66987	52587
Specific activity of Chymotrypsin	11447	13128	13921	18302	17113	14369
Specific activity of Pepsin	17458.5	15947	14385	10088	5445	3901

Table 2.

Specific activity of enzymes (nmoles tyrosine/mg protein /minute at 37°C) under the influence of Cd^{2+} in experiment "in vitro"

Cd^{2+} μg	**0**	**0.01**	**10**	**20**	**40**	**80**
Specific activity of Trypsin	44301	40780	34190	11432	5422	5257
Specific activity of Chymotrypsin	11447	10463	9064	6589	5486	3772
Specific activity of Pepsin	17458.5	12335	9220	8762	5340	3463

Table 3.

Specific activity of enzymes (nmoles tyrosine/mg protein /minute at 37°C) under the influence of Pb^{2+} in experiment "in vitro"

Pb^{2+} μg	**0**	**0.2**	**4**	**10**	**50**	**100**
Specific activity of Trypsin	44301	39433	28602	28239	16781	7771
Specific activity of Chymotrypsin	11447	9591	8686	7408	5535	1142
Specific activity of Pepsin	17458.5	15443	6152	5342	4549	3277

Pure trypsin is activated progresively by Cu ions till 30 μg Cu^{2+} level followed by a decrease at 60 μg Cu^{2+} (Fig. 5a, Table1).

Cd and Pb had an inhibition effect on pure trypsin (Fig. 5b, Fig. 5c, Table 2, Table 3).

Pure chymotrypsin is activated by Cu till 10 μg Cu^{2+} level followed by a decrease at 30 μg Cu^{2+} and 60 μg Cu^{2+} (Fig. 6a, Table1).

Cd and Pb had an inhibition effect on pure chymotrypsin (Fig. 6b, Fig.6c, Table 2, Table 3).

Cu, Cd and Pb manifested an inhibition effect on pure pepsin (Fig. 7a, Fig. 7b, Fig.7c, Table 1, Table 2, Table 3).

CONCLUSIONS

The general tendency of heavy metal accumulation in digestive gland of mussels is to increase from spring to summer (except Cd, which decreases): it is remarked Tomis Port with maximum values registered.

For juvenile mussels, all enzymes have been directly correlated as evolution sense with Cd, Pb, and inverse correlated with Cu.

Proteolytic enzymes from digestive gland of adult mussels had the same tendency regarding Cd, and inverse with Cu and Pb.

Cd and Cu had similar effect for juvenile and adult mussels. Cd was a stimulator, and Cu was an inhibitor for studied proteolytic enzymes.

Pb has a different evolution, juvenile mussels heaving an auto protection behaviour, and a inhibition one for adult mussels.

Agigea may be considered as a reference zone for enzymatic proteolytic behaviour of mussels.

In case of “in vitro” experiment, Cu had an activator effect on trypsin and chymotrypsin till at certain concentration, and after the effect is inverse Cu was an inhibitor for pepsin. “In vivo”, Cu was an inhibitor of enzymes in all situations.

Cd had an inhibitor effect “in vitro”, and “in vivo” was a stimulator for all studied enzymes.

Pb presented an opposite behaviour in case of juvenile (stimulator) and adult (inhibitor) mussels ”in vitro”, Pb manifested an inhibition effect in all cases, for proteolytic enzymes.

The different enzymatic behaviour “in vivo” and “in vitro” is put on the presence in natural marine environment of a various factors (internal or external), which act together on organisms. “In vitro” which is controlled, acts only one external factor.

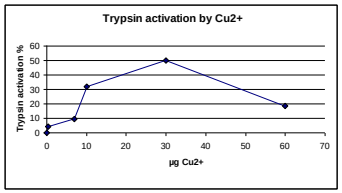

Fig. 5a

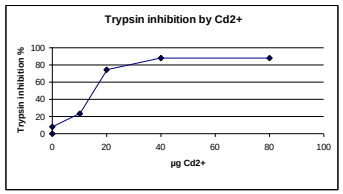

Fig. 5b

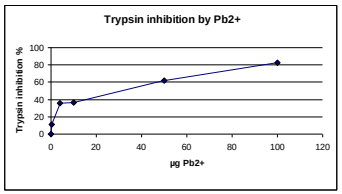

Fig. 5c

Fig. 5a, b, c Evolution of specific activity of trypsin (%) "in vitro" under Cu^{2+}, Cd^{2+}, Pb^{2+} (µg) influence

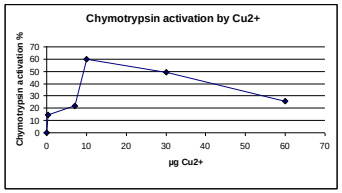

Fig.6a

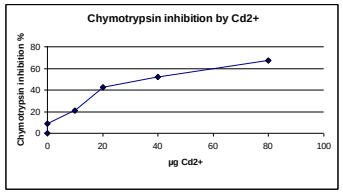

Fig. 6b

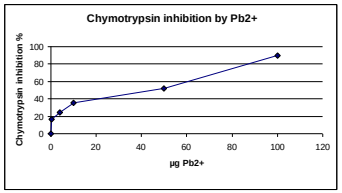

Fig. 6c

Fig. 6 a, b, c Evolution of specific activity of chymotrypsin (%) "in vitro" under Cu^{2+}, Cd^{2+}, Pb^{2+} (µg) influence

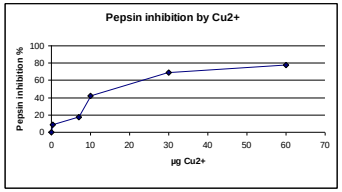

Fig.7a

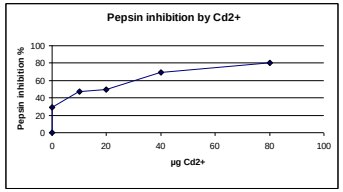

Fig. 7b

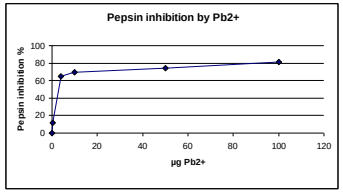

Fig. 7c

Fig. 7 a,b,c Evolution of specific activity of pepsin (%) "in vitro" under Cu^{2+}, Cd^{2+}, Pb^{2+} (µg) influence

REFERENCES

1. ABAZA (V.), 1996-1997 - "Data of mussel stock on the Romanian Black Sea shelf". Recherches Marines, Nr.29-30, pp. 129-139.
2. IAEA,1993 –"Guideline for monitoring chemical contaminants in the sea using marine organisms". Reference methods for Marine Pollution Studies, UNEP/FAO/IOC, 6.
3. IORDACHESCU (D.), DUMITRU (I., F.), 1988 – Biochimie practica – Univ. Bucuresti
4. LE GAL (Y.), 1988 – "Biochimie Marine", Paris, Milano, Barcelona, Mexic
5. MIRCEA (D.M.), ROSOIU (N.),, DUMITRESCU (E.), OROS (A.) 2005 – "Digestive proteolytic potential of *Mytilus galloprovincialis* from the Romanian Black Sea coast", Scientific annals of the Danube Delta Institute, Editura Tehnica, pp. 50-58.
6. ROSOIU (N) , SERBAN (M.), 2002 – "Biochimie – metode si tehnici de laborator", Educational, Editura Paralela 45, Colectia Vademecum, Pitesti,
7. ROSOIU (N.), SERBAN (M.), PANAIT (M.),1981 – Determination of some natural inhibitors of pepsin, trypsin and chymotrypsin in several invertebrate and fish species along the romanian coast of the Black Sea". Recherches Marines, Nr.14, pp.223-237.
8. ROSOIU (N.), SERBAN (M.),1992 – "Substante biologic active din organisme marine". Editura Academiei Romane, Bucuresti.