Folia biologica (Kraków), vol. **52** (2004), No 3-4

# Lysozyme-like Activity in Eggs and in Some Tissues of Land Snails *Helix aspersa maxima* and *Achatina achatina*

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*Accepted September 6, 2004*

FIOŁKA M. J., WITKOWSKI A. 2004. Lysozyme-like activity in eggs and in some tissues of land snails *Helix aspersa maxima* and *Achatina achatina*. Folia biol. (Kraków) **52**: 233-237.

Antibacterial lysozyme-like activity against *Micrococcus luteus* in eggs and some tissues of snails *Helix aspers maxima* and *Achatina a*chatina was detected in a turbidimetric standard assay. The bacteriolytic activity in *Helix aspersa maxima* was higher than in *Achatina achatina*. After the application of the bioautography technique, several lytic zones of *Micrococcus luteus* were observed in both studied species. Electrophoresis in denaturing conditions followed by immunodetection of lysozyme using EWL antibodies indicated the presence of several lysozyme forms in the tested snails.

Key words: Antibacterial immunity, lysozyme-like activity, eggs of snails.

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Molluscs comprise more than 150 000 species and they are one of the largest phylums in the animal kingdom. Most data on defense reactions of these animals have been obtained from studies of only a limited number of representatives of *Pulmonata* and *Bivalvia* that are of economical significance (mussels *Mylitus*, oysters *Crassostrea*, and vineyard snail *Helix pomatia*) or of medical importance (molluscs that are intermediate hosts of human and domestic animal parasites: *Biomphalaria*, *Bulinus*, *Lymnaea*) (YAKOVLEVA *et al.* 2001). However, information pertaining to the defense reactions of other representatives often is fragmentary or completely absent.

The immune defense system of molluscs consists of cellular and humoral mechanisms. The main mechanisms of neutralization and elimination of potential pathogens in molluscs are based on phagocytosis and encapsulation. Granular hemocytes are the most numerous cell type of molluscan blood active in cellular defenses (RATCLIFFE & ROWLEY 1981). Molluscan humoral immunity is brought forth by lysozyme activity, lectin and the phenyloxidase system (GLIŃSKI & JAROSZ 1997). Other less known agents of humoral immunity include mercenens, paolins (LIE *et al*. 1965), the acute phase reactants (MANDAL *et al.* 1991), α-2 macroglobulin (BENDER *et al*. 1992; THOGERSEN *et al*. 1992; FRYER & BAYNE 1996) and multifunctional binding proteins with antiprotease activity (BORTH 1992).

Lysozyme, a bacteriolytic factor found in all representatives of *Mollusca*, acts by splitting the bond between N-acetylglucosamine and N-acetylmuramic acid present in the cell wall of Gram-positive bacteria and in some fungi it binds chitin – a major constituent of the cellular wall. Lysozyme is synthesized in and secrected from hemocytes. This protein has a relatively low molecular weight (about 15 kDa), and it is stable in low pH and high temperature. Physical differences may be species-specific in molluscs. For example, FENG (1974) found oyster hemolymph lysozyme asociated with acidic proteins but not with basic ones. Lysosomes of hemocytes in clams and oysters (EBLE & TRIPP 1968) contain lysozyme, among lysosomal enzymes.

Unlike insects, there is no evidence that points to a drastic increase in the activity of hemolymph lysozyme after bacterial infection in molluscs (GLIŃSKI & JAROSZ 1997). However, hemolymph of untreated *Crassostrea virginica* showed not only antibacterial activity against *M. luteus*, *B. subtilis*, and *B. megaterium*, but also against *E. coli* and some other Gram-negative bacteria (RODRICK & CHENG, 1974; GOTZ & TRENECZEK 1991). The kuruma shrimp (*Marsupenaeus japonicus*) lyso-

zyme from hemocytes displayed lytic activity against several *Vibrio species* and fish pathogens, including *Vibrio penaeicida* (HIKIMA *et al*. 2003). Lysozyme from starfish *Asterias rub*ens is a new form, called type i (invertebrate) lysozyme, which differed from both types c (chicken) and g (goose) known in other animals, as well as from plant and bacterial phage lysozymes (BACHALI *et al.* 2004).

The eggs of molluscs contain some biologically active substances for their protection. The eggs of sea hare of the species *Aplysia* (subclass *Opithobranchia* of the *Mollusca*), contain a lectin that can agglutinate marine bacteria (KAMIYA & SHIMIZU 1981). An antitumor factor, aplysianin E, was also found in these eggs (KISUGI *et al.* 1987). An antineoplastic and antibacterial glycoprotein purified from the eggs of *Aplysia kurodai* completely suppressed growth of *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* and *Candida albicans* (IIJIMA *et al.* 1994).

The main physical barriers covering the soft body of snails are the shell and mucus. Mucus protects the body against desiccation and sometimes contains lectins, protease inhibitors and antibacterial factors that destroy bacteria (GLIŃSKI & JAROSZ 1997).

In this paper, the results of studies on antibacterial lysozyme-like activity in eggs and in some tissues of two species of land snails are presented.

## Material and Methods

The studies were performed on freshly laid eggs of two species of land snails: *Helix aspersa maxima* and *Achatina achatina*. The eggs came from breeding stock maintained at the Department of Biological Basis of Animal Production of the University of Agriculture in Lublin. The snails were raised indoors in plastic pens (*Helix*) or in an aquarium with soil (*Achatina*), in natural daylight, controlled room temperature and humidity and fed *ad libitum* with a snail commercial fodder, fresh lettuce and apples.

The masses of eggs were collected using a Hamilton syringe and put in precoolled Eppendorf tubes in an ice bath. After measuring protein concentration, the protein solution was added to sample buffer for electrophoretic characterization or to sodium phosphate buffer for bacteriolytic assay.

Mucus was collected from the soft body of *Achatina achatina* to Eppendorf tubes, whereas hepatopancrea of *Helix aspersa maxima* was analyzed after isolation and homogenization in phosphate buffer pH 6.4.

Bacteriolytic activity against Gram-positive bacteria was determined by turbidimetric standard assay (SHUGAR 1952) with freeze-dried cell walls of *Micrococcus luteus* (Sigma), using a suspension of cell walls (1mg/ml) in 33 mM phosphate buffer pH 6.4. The rate of cell wall hydrolysis was measured after 15 sec incubation at 25$^{o}$C in a 450 nm in Bio-Rad spectrophotometer. The enzyme activity was calculated in units/mg. The concentration of protein was estimated using the Bradford reagent (Bio-Rad) (BRADFORD 1976). Bovine serum albumin was used as the standard.

Analytical disc electrophoresis of native proteins was carried out according to REISFELD *et al.* (1962) with a 15 % separation gel at pH 4.3. The acid gel was 1.5 mm thick. Electrophoresis was performed for 1 h at 150 volts. The lysozymes were visualized using an activity assay after first washing the gel in 33 mM phosphate buffer, pH 6.4 for 30 min. This was followed by overlaying the gel with 10 ml phosphate buffer containing $2.5 \times 10^5$ cells of *Micrococcus luteus*. The lysozyme bands were detected after 48 h by clearning of bacteria in the overlaid agarose gel. Chicken egg white lysozyme (EWL) (Sigma) was used as a standard.

After acid electrophoresis, the gel was scanned with a Sharp XJ 330 scanner and analyzed using an Image Master TM 1D Amersham Pharmacia Biotech. Programme.

SDS-polyacrylamide gel electrophoresis was performed by the method of LAEMMLI (1970) in 12% acrylamide gels. Samples were heated at 100$^{o}$C for 10 min in sample buffer. Low molecular standards (Bio-Rad) were rabbit muscle phosphorylase b (94 kDa), bovine serum albumin (67 kDa), hen egg ovalbumin (43 kDa), bovine carbonic anhydrase (30 kDa), soybean tripsin inhibitor (20.1 kDa) and hen egg white lysozyme (14.4 kDa).

After SDS-PAGE protein samples were electroblotted onto Immobilon membrane (Millipore) for 90 min at 150 V. For identification of the lysozyme, polyclonal antibodies to EWL were used for immunoblotting. As second antibodies, alkaline phosphatase-conjugated goat anti-rabbit IgGs were used. Immunoreactive bands were visualized by incubation with p-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate.

## Results and Discussion

Lysozyme is a part of the defense mechanism against bacteria in most animals (JOLLES & JOLLES 1984) including molluscs (for a review see GLIŃSKI & JAROSZ 1997; PŁYTYCZ *et al.* 1999). In the present study, lysozyme-like activity was

Table 1

Lysozyme-like acivity in eggs and in some tissues of snails *Helix aspersa maxima* and *Achatina achatina*

| Tissues of snails | Lysozyme-like activity (U/mg) | Concentration of protein (mg/ml) |
|---|---|---|
| Eggs of *Helix aspersa maxima* | 1060.40 | 33.76 |
| Eggs of *Achatina achatina* | 505.30 | 48.49 |
| Hepatopancrea of *Helix aspersa maxima* | 690.10 | 64.05 |
| Mucus of soft body *Achatina achatina* | 518.80 | 0.35-6.94 |

detected in the egg masses of two land snails, *Helix aspersa maxima* and *Achatina achatina*. Using turbidimetric standard assay, it was found that the lysozyme-like activity in eggs of *Helix aspersa* (1060.40 U/mg) was twice higher than in *Achatina* eggs (505.30 U/mg) (Table 1). Lysozyme-like activity in the mucus of the soft body of *Achatina* (518.80 U/mg) was comparable to activity in egg masses of this species, but lysozyme-like activity in hepatopancrea in *Helix aspersa* was lower than in *Helix* eggs (690.10 U/mg). Afterwards, the activity in the obtained samples was tested by the bioautography technique after electrophoretic resolution of native proteins in the acidic polyacrylamide gels (Material and Methods). Several lytic zones of *Micrococcus luteus* were observed in the case of both

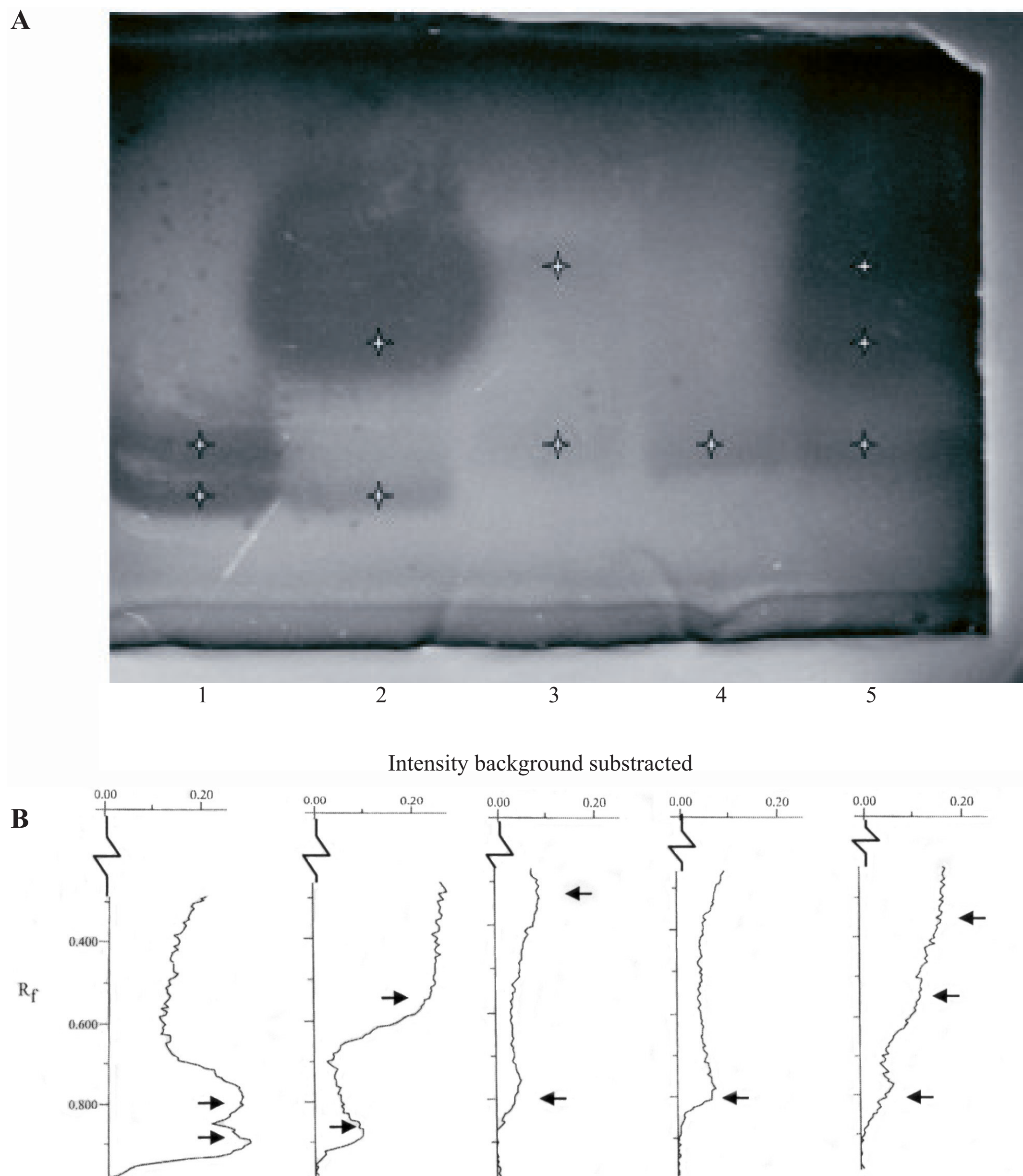


Fig. 1. Detection of lysozyme-like activity by bioautography. The bands on the electrophoregram (A) or the peaks on the densitogram (B) are marked with stars or arrows, respectively; EWL (lane 1), *Achatina achatina* eggs (lane 2), *Helix aspersa maxima* eggs (lane 3), *Helix aspersa maxima* hepatopancrea gland (lane 4), *Achatina achatina* soft body mucus (lane 5).

studied species (Fig. 1). Especially, wide lytic zones more acidic in charge in comparison to EWL were visible. The obtained results indicate the existence of lysozyme in several forms, differing in electric charge. However, taking into consideration that electrophoresis was performed in non-denaturating conditions, the possibility that the enzyme is associated with different egg proteins can not be excluded.

The lysozymes isolated from invertebrates generally have a molecular weight of about 15 kDa and belong to the C-type (chicken-type) lysozyme, with some exceptions. Lysozyme from the eggs of the insect *Ceratitis capitata,* with a molecular weight about 23 kDa, shows high sequence homology at the N-terminal end with bacterial phage lysozyme, but not with chicken lysozyme (JOLLES & JOLLES 1984). Lysozyme isolated from the snail *Helix pomatia* also possesses a high molecular weight of about 24 kDa. Lysozyme purified from the mussel *Mytilus edulis* has a mass of about 18 kDa and a pI of 9.2, which is considerably lower than the isoelectric point of hen lysozyme (JOLLES & JOLLES 1984). Therefore, electrophoresis in denaturing conditions (SDS/PAGE) was performed, followed by immunodetection of lysozyme using EWL antibodies. The obtained results are presented in Figure 2. In the case of *Helix aspersa maxima* eggs, three protein bands of Mr 13-17 kDa were recognized by the antibodies. Also, antibodies against EWL recognized three proteins of Mr 34-42 kDa in the homogenate of *Helix aspersa maxima* hepatopancrea. Additionally, three positive immunoreactive signals were observed in the case of *Achatina achatina* eggs. However, in this case the molecular mass of the proteins was calculated at about 40-66 kDa. In the mucus of the *A. chatina* soft body, two immunoreactive proteins were detected using antibodies, one with a molecular mass corresponding to EWL (14.4 kDa) and a second with molecular mass of about 43 kDa. Alltogether, the data indicate the presence of several lysozyme forms in the studied tissues of snails.

It is worth mentioning that several forms of lysozyme exist in insects. In *Drosophila melanogaster* for example, seven genes coding digestive lysozyme were described (DAFFRE *et al.* 1994), but in the cricket *Gryllus bimaculatus* three forms of haemolymph lysozyme were purified (SCHNEIDER 1984).

Activity against different bacteria strains, but not against *Micrococcus luteus*, in egg masses of molluscs was found by BENKENDORFF *et al.* (2001). Antibacterial activity tested against 3 human pathogenic bacteria: *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*, was detected in egg masses from 32 species of molluscs, from 2 classes and 18 families. Antibacterial activity in molluscan egg masses was found to extend across the marine, estuarine, freshwater, and terrestrial environments. However, the antibacterial and antifungal role of lysozyme in molluscs is still not well characterized.

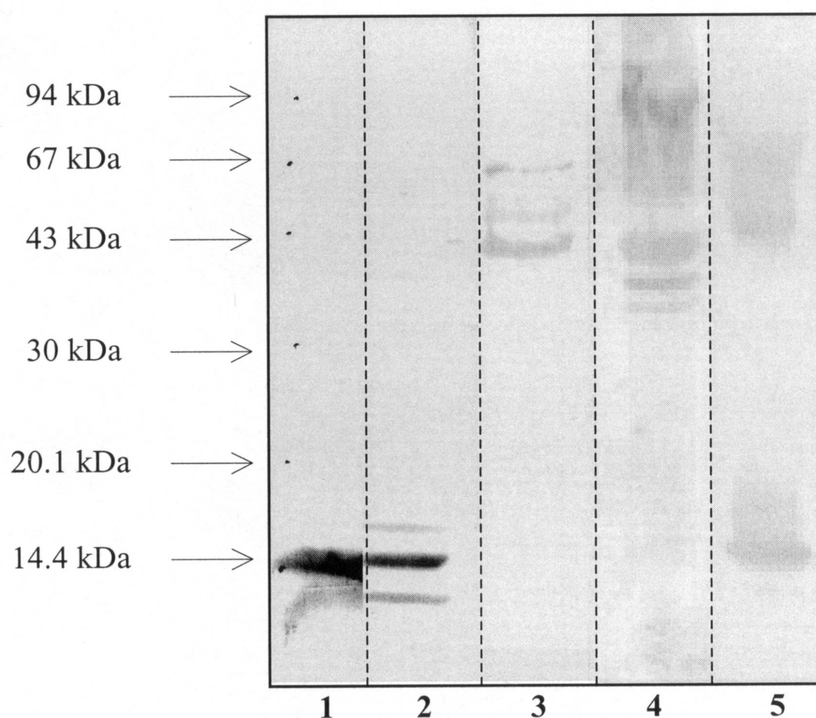


Fig. 2. Immunoblotting with monoclonal antibody against chicken egg white lysozyme (EWL); low molecular standards (lane1), *Helix aspersa maxima* eggs (lane 2), *Achatina achatina* eggs (lane 3), *Helix aspersa maxima* hepatopancrea gland (lane 4), *Achatina achatina* soft body mucus (lane 5).

## Acknowledgements

The authors would like to thank Mr Konrad CYZMAN from the Department of Biological Bases of Animal Production of the University of Agriculture in Lublin for the eggs of snails.

## References

BACHALI S., BAILLY X., JOLLES J., JOLLES P., DEUTSH J. S. 2004. The lysozyme of the starfish *Asterias rubens*. A paradygmatic type i lysozyme. Eur. J. Biochem. **271**: 237-242.

BENKENDORFF K., DAVIS A. R., BREMNER J. B. 2001. Chemical defense in the egg masses of benthic invertebrates: an assessment of antibacterial activity in 39 mollusks and 4 polychaetes. J. Invertebr. Pathol. **78**: 109-118.

BENDER R. C., FRYER S. E., BAYNE C. J. 1992. Proteinase inhibitory activity in the plasma of a mollusk: evidence for the presence of -macroglobulin in *Biophalaria glabrata*. Comp. Biochem. Physiol. **102B**: 821-824.

BORTH W. 1992. $\alpha_2$-Macroglobulin, multifunctional binding protein with targeting charactetristics. FASEB J. **6**: 3345-3353.

BRADFORD M. M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**: 248-254.

DAFFRE S., KYLSTEN P., SMAKOVLIS C., HULTMARK D. 1994. The lysozyme locus in *Drosophila melanogaster*: an expanded gene family adapted for expression in the digestive tract. Mol. Gen. Genet. **242**: 152-162.

EBLE A. F., TRIPP M. R. 1968. Enzyme biochemistry of phagosome in oyster leukocytes. Bull. NY Acad. Sci. **B13**: 93.

FENG S. Y. 1974. Lysozyme-like activities in the hemolymph of *Crassostrea virginica*. Contemp. Top. Immunobiol. **4**: 225-231.

FRYER S. E., BAYNE C. J. 1996. Host-parasite interactions in molluscs. (In: Invertebrate Immunology. B. Rinkevich, W.E.G. Muller eds Springer, Berlin, Heidelberg, New York): 131-153.

GLIŃSKI Z., JAROSZ J. 1997. Molluscan immune defenses. Arch. Immunol. Therap. Exp. **45**: 149-155.

GOTZ P., TRENECZEK T. 1991. Antibacterial proteins in insects other than *Lepidoptera* and *Diptera* and in some other invertebrates. (In: Immunology of Insects and Other Arthropods. Gupta A. P. Boca Raton ed. Ann. Arbor, London): 323-370.

HIKIMA S., HIKIMA J., ROJTINNAKORN J., HIRONO I., AOKI T. 2003. Characterization and function of kuruma shrimp lysozyme possessing lytic activity against *Vibrio* species. Gene **316**: 187-195.

IIJIMA R., KISUGI J., YAMAZAKI M. 1995. Antifungal activity of aplysianin E, a cytotoxic protein of sea hare (*Aplysia kurodai*) eggs. Dev. Comp. Immunol. **19**: 13-19.

JOLLES P., JOLLES J. 1984. What's new in lysozyme research? Always a model system, today as yesterday. Molec. Cell. Biochem. **63**: 165-189.

KAMIYA H., SHIMIZU Y. 1981. A natural agglutinin inhibitable by D-galacturonic acid in the sea hare *Aplysia* eggs: characterization and purification. Bull. Jpn. Soc. Sci. Fish. **47**: 255-259.

KISUGI J., KAMIYA H., YAMAZAKI M. 1987. Purification and characterization of aplysianin E, an antitumor factor from sea hare eggs. Cancer Res. **47**: 5649- 5653.

LAEMMLI U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227**: 680-685.

LIE C. P., PRESCOTT B., EDDY B., CALDES G., GREEN W. R., MARTINO E. C., YOUNG A. M. 1965. Antiviral activity of paolins from clams. Ann. NY Acad. Sci. **130**: 374-382.

MANDAL C., BISWAS M., NADPUKAR A., SAILEN M. 1991. Isolation of a phosphoryl choline-binding protein from the hemolymph of the snail *Achatina fulica*. Dev. Comp. Immunol. **15**: 227-239.

PŁYTYCZ B., GLIŃSKI Z. F., JAROSZ J., KSIĄŻKIEWICZ-KAPRALSKA M., MARKOWSKA M., SKWARŁO-SOŃTA K. 1999. Comparative Immunology. Jagiellonian University, Kraków ed. (In Polish).

RATCLIFFE N. A., ROWLEY A. F. (eds) 1981. Invertebrate Blood Cells. Academic Press, London.

REISFELD R. A., LEWIS U. T., WILLIAMS E. 1962. Disc electrophoresis of basic proteins and peptides on polyacrylamide gels. Nature **195**: 282- 283.

RODRICK G. E., CHENG Th. C. 1974. Kinetic properties of lysozyme from the hemolymph of *Crassostrea virginica*. J. Invertebr. Pathol. **24**: 41-48.

SCHNEIDER P. M. 1984. Purification and properties of three lysozymes from hemolymph of the cricket, *Gryllus bimaculatus* (De Geer ). 1985. Insect Biochem. **15**: 463-470.

SHUGAR D. 1952. The measurement of lysozyme activity and the ultra-violet inactivation of lysozyme. Biochim. Biophys. Acta **8**: 302-309.

THOGERSEN I. B., SALVESEN G., BRUCATO F. H., OIZZO S. V., ENGHILD J. J. 1992. Purification and characterization of an $\alpha$-macroglobulin proteinase inhibitor from the mollusc *Octopus vulgaris*. Biochem J. **285**: 521-524.

YAKOVLEVA N. V., SAMOILOVICH M. P., GORBUSHIN A. M. 2001. The diversity of strategies of defense from pathogens in molluscs. J. Evol. Biochem. Physiol. **37**: 358-367.