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Methodological approach to the isolation of functionally active proteins from the tissues of marine hydrobionts: an example of *Adamussium colbecki*

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Abstract Enzymes from cold-adapted organisms have significant application potential. Because of their unique properties they have been found to be useful in various industries. Despite indisputable practical interest, cold active enzymes also represent a valuable model for fundamental research into protein folding and catalysis. Many investigators have focused their attention on marine hydrobionts, which are growing in importance as a promising source of enzymes. The nature of the source not only determines the availability and the cost of biomolecules of interest but also determines the choice of method for their extraction.

A simple and convenient methodological approach of two-stage extraction of proteins has been tested on the Antarctic marine hydrobiont—*Adamussium colbecki*. This method extracts enough effective protein directly from primary raw materials, as well as when using leftover crude precipitates. The electrophoretic pattern of proteins showed the presence of molecules in a wide range of molecular weights in the samples of *A. colbecki* after the first and the second stage of extraction. The general proteolytic activity in the first and the second extracts were examined using a zymogram technique. Our experiments revealed that the second extract of *A. colbecki* contained thermo stable protease exhibiting a molecular weight of 95 kDa in a gelatin zymogram. Further biochemical assays, using different substrates, were conducted to partially identify the types of hydrolases present in the first and the second extracts. Our results revealed the presence of enzymes with collagenolytic and some amylolytic activities preserved in the second extracts. But no esterase or amidase trypsin-like activities were found in the second extract, in contrast to the first extract where this type of activity was significant.

Keywords **Antarctic marine hydrobiont, protein extraction, enzyme activity determination**

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1 Introduction

Proteases constitute an important class of hydrolytic enzymes that are found in all life forms. They are involved in the production of hormones and pharmacologically-active peptides as well as in various cellular functions. These functions include protein digestion, protein turnover, cell division, blood clotting cascade and signal transduction. Nowadays proteases are the most important group of industrial enzymes. They have wide range of applications in diverse fields such as detergent manufacture, leather processing, silk degumming, food and dairy, baking, pharmaceutical industries, waste management and others[1-3]. Enzymes can be obtained from various sources and the nature of the source determines the availability, the cost and the ease of processing. Marine organisms have attracted the attention of scientists as potential objects for biochemical studies as well as for industrial purposes. The importance of marine hydrobionts (sponges *D. arenaria*, *S. microspinosa*, *S. mirabilis*, *P. angulospiculatus*, molluscs *C. farreri*, C. purpurascens, crab *T. tridentatus*, sea urchin

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A. membranaceus, sea cucumbers *A. japonicus*, *L. grisea*, Antarctic krill *E. superba*, anemones *C. gigantean, A. sulcata*, jellyfish *R. esculentum* et al.) as sources of structurally diverse bioactive compounds is growing rapidly[4-7]. Because they live in challenging environmental conditions, hydrobionts from the Antarctic region are characterized by considerable structural and functional diversity of biologically active compounds, and the presence of enzymes with unique structures and activities. So enzymes produced by cold-adapted hydrobionts display a greater proteolytic activity towards native protein substrates, lower activation energy for catalysis, and often have high catalytic activity at low temperatures[8-9]. Furthermore, the relatively low thermal stability at elevated temperatures often observed in enzymes in cold adaptive organisms, may also be beneficial in some applications as these enzymes can be efficiently and sometime selectively inactivated by moderate heat input. The ability to heat-inactivate cold-active enzymes has particular relevance to the food industry where it is important to prevent any modification of the original heat-sensitive substrates and product[2,10]. In molecular biology, heat-labile enzymes are advantageous to obtain irreversible enzyme inactivation by mild heat treatment without interference with subsequent reaction[9,11]. Due to their unique properties, cold-adaptive enzymes represent a valuable model for fundamental research into molecular mechanisms of enzyme catalysis.

Most enzymes from aquatic hydrobionts can be also found in terrestrial organisms. However, some differences in molecular weight, amino acid composition, pH optimum, temperature optimum, stability, inhibition characteristics and kinetic properties have been observed[12-15]. Considering these differences the optimization of methodological approaches for extraction and purification of metabolites from aquatic hydrobionts is an important task of modern biochemistry.

The main goal of the present work, therefore, was to optimize conditions for maximum extraction of salt- and water-soluble proteins from tissue of marine hydrobionts while preserving their functional activities. Special attention was paid to testing different hydrolytic activities in the resultant extracts of hydrobiont tissue. In our work we used specimens of a marine hydrobiont from the Antarctic region — *Adamussium colbecki* (the Antarctic scallop). The endemic scallop *A. colbecki* is one of the most common bivalves in Antarctica which is widely spread not only along the coast, but also can be found at depths from 3 m to nearly 1500 m down into the ocean[16]. This hydrobiont appears to be a very important species of the Antarctic benthic marine ecosystems, mainly in relation to its functional role in the transfer of energy from the water column to the benthos[17] and because of its ability to accumulate of xenobiotics and metals[18].

2 Material and methods

The specimens of *A. colbecki* (n = 35) were collected near the island Galindez (65°15′ S, 64°15′ W) of the Argentine Islands archipelago. The materials were collected by the XVII and XVIII Ukrainian Antarctic expeditions on March 2012 — April 2013, March 2013 — April 2014 respectively. After collection the scallops were immediately frozen in liquid nitrogen to prevent enzyme deterioration and stored at –80°C. The samples were brought to the laboratory frozen. The mass of hydrobiont was measured and the samples were homogenized with sequential addition of liquid nitrogen and an extraction buffer of 0.1 M Na-phosphate containing 0.15 M NaCl, 0.15 mM EDTA, pH 7.4. In order to prevent enzymatic degradation of proteins, the serine protease inhibitor PMSF was added to the samples. All procedures were performed at 4°C. We used the detergent Triton X-100 to provide more complete extraction, in particular the release of membrane-bound molecules. Samples were homogenized for 5 min and then centrifuged at 10000 g for 60 min at 4°C. Supernatants from this process (the first supernatants) were selected, packaged in bottles and lyophilized. At the second phase of extraction, 1 M acetic acid in the ratio of 1:5 was added to the precipitates. The suspensions were kept at room temperature for 1 h with continuous stirring. Then the suspensions were placed in a water bath at 90°C for 45 min. It should be noted that heating of the samples may be an additional step of purification of the extracts from ballast heat sensitive proteins and polypeptides. After cooling the samples were centrifuged at 10000 g for 45 min. The precipitates were discarded and the supernatants were re-centrifuged at 10000 g for 60 min at 4°C. These second supernatants were also lyophilized. The lyophilizates can be stored for a long time at a temperature of –20°C without losing the functional properties of the proteins and peptides. For the determination of enzymatic activity the lyophilizates were dissolved in 0.05 M Tris-HCl buffer (pH 7.4) and used as an enzyme source after gel filtration chromatography with using as a chromatographic matrix Sephadex G-25 (BioLogic LP, Bio Rad, USA). The method of Bradford was used for quantitative determination of proteins[19]. Bovine serum albumin was used as a standard in these determinations. Sodium dodecyl sulfate-polyacrylamaide gel electrophoresis (SDS-PAGE) of both extracts was carried out using 5% (w/v) stacking gel and 10% (w/v) separating gel[20]. SDS-PAGE was performed using Mini-Protean Tetra System (Bio Rad, USA). Zymography was done according to the method of Ostapchenko et al.[21]. Separating (12% w/v) gel was polymerized in the presence of gelatin (1 $mg \cdot ml^{-1}$). Areas of gelatin digestion were visualized as non-staining areas in the gel. The resultant electrophorograms were analyzed with the help of the TotalLab 2.04 program. The electrophorograms presented in Figure 1 are typical for the series of repeated experiments (at least three in each series). Total proteolytic activity was determined using 1% casein as a substrate and monitored at a wavelength of 280 nm as described in Munilla-Moran and Stark[22]. Collagenolytic activity was assessed with help of native collagen type I following the method described by Moore and Stein[23].

The released amino acids from collagen were detected. The liberated amino acids were measured in relation to L-leucine in the presence of ninhydrine. Trypsin-like amidase activity was measured using *N*-α-benzoyl-L-Arg-*p*-nitroanilide (L-BApNA, 0.3 mM) as substrate[24]. The basis of this assay is the colorimetric estimation of the amount of *p*-nitroaniline released as a result of enzymatic hydrolysis of L-BApNA. For the determination of esterase activity *N*-α-p-tosyl-L-Arg methyl ester (L-TAME; 1 mM) was used[24]. Amylolytic activity was estimated using 1% starch as substrate. In each experiment (n = 4) all samples were tested in triplicate. Results are expressed as means ± SE.

3 Results and discussion

At the first stage of our work we optimized conditions for effective extraction of native proteins from the tissue of *Adamussium colbecki*. Currently, a large number of methods are available to extract proteins from biological materials but most of them are based on using polar organic solvents[25-28]. Taking into account that organic solvents can cause irreversible protein denaturation and loss of functional properties we did not apply any solvents at the first stage of extraction. On the basis of the literature[29] and the results of our previous work[30] about the presence in the tissues of hydrobionts of salt- and water-soluble protein fractions, we used 0.1 M Na-phosphate buffer, pH 7.4, as the extraction medium. However, some percentages of proteins (5%—15%) may be very tightly associated with the post-extraction cellular debris, and may thus not be extractable at all[31]. So, the leftover precipitate can be a rich source of biologically active substances, and in particular peptides. Marine-derived polypeptides have shown numerous bioactivities such as antihypertensive, antioxidative, anticoagulant and antimicrobial[32]. Hence, we used the crude precipitates that remained after the first extraction stage to obtain protein and peptide fractions. We applied the method based on the use of acetic acid aqueous solution as an extractant[33]. This method works quite well for the isolation of proteins from plants[34]. Under these conditions the extraction of mainly acid-soluble peptides occurs. It enabled fractions enriched by peptides to be obtained. On the other hand, as most polysaccharides, steroids, flavonoids, vitamins and glycosides are practically insoluble in acetic acid aqueous solution, it also serves as an additional factor that helps to obtain relatively pure protein and peptide fractions.

SDS-PAGE analysis was performed to estimate the efficiency of the extraction procedure and to obtain information about the protein and peptide composition in tissue of *A. colbecki*. A typical electrophorogram is shown in Figure 1. The results of electrophoretic protein separation of both investigated extracts revealed the presence of proteins with molecular weights ranging from 20 to 115 kDa. To establish the exact molecular weights of identified protein fractions the electrophorograms were analyzed using the TotalLab 2.04 program. The analysis identified 10 protein bands (with molecular weights of 22, 49, 67, 78, 90, 97, 99, 101, 105, 121 kDa) and 9 protein bands (with molecular weights of 20, 27, 37, 43, 58, 64, 71, 76, 83 kDa) in the first and the second extract respectively. Despite a similar number of fractions, the molecular-weight composition of proteins in the analyzed extracts was different. From our observations it can be concluded that the second extract was characterized by a lower number of high-molecular weight protein bands compared with the first extract which had a considerable number of high-molecular weight fraction proteins. A considerable portion of middle-molecular fractions in the second extract can be explained by the hydrolysis of proteins during the second stage of extraction.

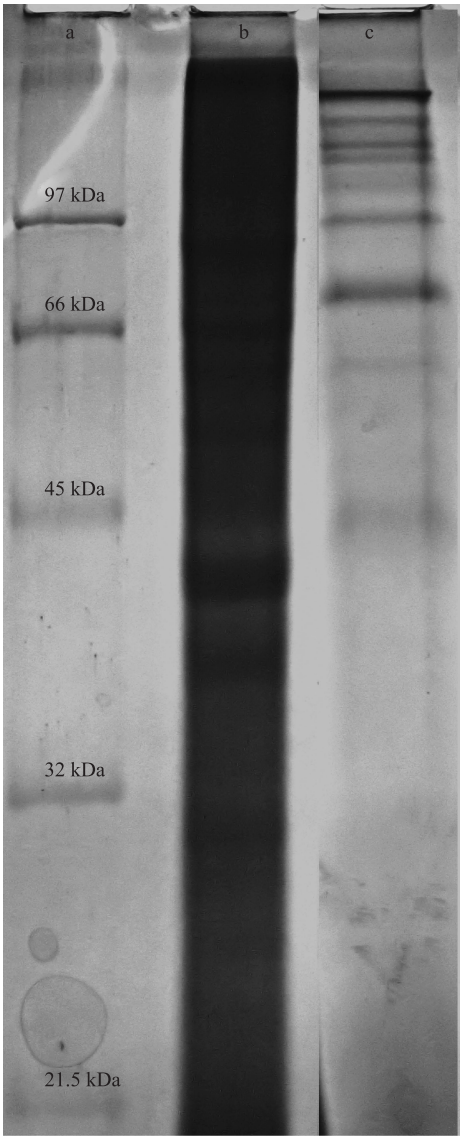

Figure 1 Typical electrophorogram of protein separation: **a**, molecular weight markers; **b**, the second extract of *A. colbecki*; **c**, the first extract of *A. colbecki*

The variety of proteins with different molecular weights suggests the presence of functionally active molecules in the

analyzed extracts. As marine organisms are characterized by high enzyme content, in particular hydrolases, we tested our samples for the presence of enzymatic activity. For the identification of proteolytic active enzymes and the determination of their apparent molecular weights we applied the zymography technique which is described[21] as a simple, sensitive, quantifiable and functional assay to analyze active enzymes in biological samples. It should be noted that preparation of separating gel in the presence of gelatin allows identification of enzymes belonging to the proteases family, in particular serine proteases as well as metalloproteases. The appearance in gelatin zymogram clear areas (Figure 2) indicates the presence of active proteolytic enzymes in the both extracts of *A. colbecki*.

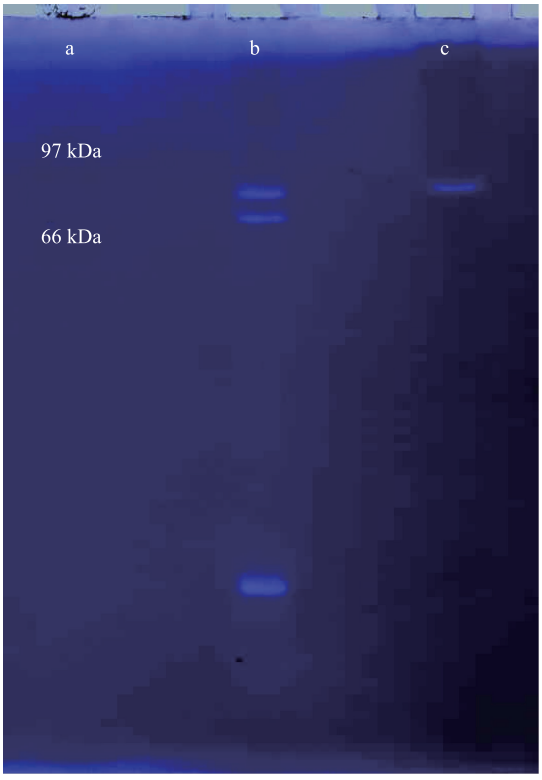

Figure 2 Typical zymogram of protein separation: **a**, molecular weight markers; **b**, the first extract of *A. colbecki*; **c**, the second extract of *A. colbecki*

Three active enzymes were revealed in the first extract (Figure 2, line b). It should be emphasized that despite the use of highly aggressive treatment (heating at 90°C in acetic acid) for obtaining the second extract, one active area was still detected in gelatin zymogram (Figure 2, line c). The estimated molecular weights of the enzymes are approximately 94, 86 and 31 kDa for the first extract and 95 kDa for the second extract. Based on the coincidence of molecular weights and visual similarity of active area in region of 95 kDa in both extracts we suggest that these are due to the same enzyme. Our data about molecular weight and thermo stability of the revealed protease are consistent with other observations[35].

In accordance with the modern concept of adaptive strategies for low temperature functioning[36], improved catalytic efficiency in cold active enzymes is accompanied by loss of structural stability that results in limited thermal stability and rapid denaturation by mild heat treatment. From this position, our observation about the presence of a thermo stable enzyme in the second extract of *A. colbecki* is quite interesting. Further research to identify and characterize this enzyme is recommended.

In order to obtain information about the composition of enzymes present in tissues of *A. colbecki*, and considering the presence of enzymatic activity in the second extract, we tested our samples for the presence of the main types of digestive activities. At first, we determined caseinolytic activity which mediated by different types of proteases and therefore can be regarded as a total protease activity. Our results (Table 1) revealed significant caseinolytic activity in the first extract of *A. colbecki*. Slight total proteolytic activity was also identified in the second extract.

Because the total activity is mediated by a number of digestive enzymes working together, the proteases from *A. colbecki* extracts were further characterized using different substrates. Two synthetic substrates, the amide L-BApNA and the ester L-TAME, were used to identify the presence of trypsin-like proteases. Highest enzymatic activity was observed when L-TAME was used as substrate, which may indicate the presence of trypsin-like enzymes with the predominant substrate specificity due to esters rather than amides. No esterase or amidase trypsin-like activity was detected in the second extract. Taking this into account, coupled with the presence of expressed enzymatic

Table 1 Enzymatic activity in the extracts of A. *colbecki*

	Total protease activity (Units/mg protein)	Amylolytic activity (Units/mg protein)	Collagenolytic activity (Units/mg protein)	Trypsin-like (esterase) activity (Units/mg protein)	Trypsin-like (amidase) activity (μmol p–nitroaniline/ min x mg) protein
First extract	22.3 ± 2.34	1.23 ± 0.018	38.5 ± 3.42	10.6 ± 0.89	4.2 ± 0. 23
Second extract	1.87 ± 0.02	0.4 ± 0.011	17.3± 1.14	-	-

Notes: Values are means ± SE; n=4.

activity towards collagen, we speculate that the active area (95 kDa) in the zymogram of the second extract is mediated by collagenases belonging to a group of metalloproteases but not serine collagenolytic proteases. As known from the literature, the molecular weight range of collagenolytic-serine proteases is approximately 24—36 kDa whereas metallocollagenases have molecular weights from 30 to 150 kDa[14]. It should be noted that among three types of digestive activities, the collagenolytic activity was highest in both extracts. Unexpectedly, we identified some amylolytic activity in the second extract of *A. colbecki*, the value of which was 0.4±0.011 Units/mg protein. Further studies of purification and characterization of individual enzymes from tissue of marine hydrobionts, using different substrates and specific inhibitors, are in progress.

4 Conclusion

Our results demonstrate an efficient protein extraction procedure. Our method of two-stage extraction is characterized by using inexpensive reagents, a small number of isolation stages and a lack of critical parameters. Application of this method for extraction of proteins from tissue of hydrobionts is suitable for obtaining molecules with a wide range of molecular weights and with preserving their functional activity at both stages of extraction which was confirmed in our studies an example of other marine hydrobionts[37-38].

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References

1 Newman D J, Cragg G M. Marine natural products and related compounds in clinical and advanced preclinical trials. J Nat Prod, 2004, 67(8): 1216–1238
2 Margesin R, Feller G. Biotechnological applications of psychrophiles. Environ Technol, 2010, 31(8-9): 835–844
3 Joshi S, Satyanarayana T. Biotechnology of cold-active proteases. Biology, 2013, 2(2): 755–783
4 Newman D J, Cragg G M, Snader K M. Natural products as sources of new drugs over the period 1981-2002. J Nat Prod, 2003, 66(7): 1022–1037
5 Byun H G, Lee J K, Park H G, et al. Antioxidant peptides isolated from the marine rotifer, Brachionus rotundiformis. Process Biochem, 2009, 44(8): 842–846
6 Pedpradab P, Molex W, Nukoolkarn V, et al. Biological activities of extracts from Andaman Sea sponges, Thailand. Eur Asian J BioSci, 2010, 4: 63–69
7 Ngo D H, Wijesekara I, Vo T S, et al. Marine food-derived functional ingredients as potential antioxidants in the food industry: an overview. Food Res Int, 2011, 44(2): 523–529
8 Siddiqui K S, Cavicchiol R. Cold-adapted enzymes. Annu Rev Biochem, 2006, 75: 403-433
9 Feller G. Psychrophilic enzymes: from folding to function and biotechnology. Scientifica, 2013, 2013: 512840
10 Gerday C, Aittaleb M, Bentahir M, et al. Cold-adapted enzymes: from fundamentals to biotechnology. Trends Biotechnol, 2000, 18(3): 103–107
11 Kobori H, Sullivan C W, Shizuya H. Heat-labile alkaline phosphatase from Antarctic bacteria: rapid 5′ end-labeling of nucleic acids. Proc Natl Acad Sci U S A, 1984, 81(21): 6691–6695
12 Saborowski R, Sahling G, Navarette del Toro M A, et al. Stability and effects of organic solvents on endopeptidases from the gastric fluid of the marine crab Cancer pagurus. J Mol Catal B: Enzym, 2004, 30(3-4): 109–118
13 Lane A L, Moore B S. A sea of biosynthesis: marine natural products meet the molecular age. Nat Prod Rep, 2011, 28(2): 411–428
14 Salamonea M, Cuttittab A, Seiditac G, et al. Characterization of collagenolytic/proteolytic marine enzymes. Chem Engin Trans, 2012, 27: 1–6
15 Genta-Jouve G, Thomas O P. Biosynthesis in marine sponges: the radiolabelling strikes back. Phytochem Rev, 2013, 12(3): 425–434
16 Schiaparelli S, Linse K. A reassessment of the distribution of the common Antarctic scallop Adamussium colbecki (Smith, 1902). Deep-Sea Res II, 2006, 53(8-10): 912–920
17 Cerrano C, Calcinai B, Bertolino M, et al. Epibionts of the scallop Adamussium colbecki (Smith, 1902) in the Ross Sea, Antarctica. Chem Ecol, 2006, 22(S1): S235–S244
18 Bonacci S, Corsi I, Focardi S. Cholinesterase activities in the adductor muscle of the Antarctic scallop Adamussium colbecki. Antarct Sci, 2006, 18(1): 15–22
19 Bradford M M. A rapid and sensitive method for quantities of utilizing the principle of protein binding. Analyt Biochem, 1976, 86: 193–200
20 Laemmli U K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 1970, 227(5259): 680–685
21 Ostapchenko L, Savchuk O, Burlova-Vasilieva N. Enzyme electrophoresis method in analysis of active components of haemostasis system. Adv Biosci Biotechnol, 2011, 2(1): 20–26
22 Munilla-Moran R, Stark J R. Protein digestion in early turbot larvae, Scophthalmus maximus (L.). Aquaculture, 1989, 8(3-4): 315–327
23 Moore S, Stein W H. A modified ninhydrin reagent for the photometric determination of amino acids and related compounds. J Biol Chem, 1954, 211(2): 907–913
24 Xavier L P, Almeida Oliveira M G, Guedes R N C, et al. Trypsin-like activity of membrane-bound midgut proteases from Anticarsia gemmatalis (Lepidoptera: Noctuidae). Eur J Entomol, 2005, 102(2): 147–153
25 Mitra S K, Walters B T, Clouse S D, et al. An efficient organic solvent based extraction method for the proteomic analysis of Arabidopsis plasma membranes. J Proteome Res, 2009, 8(6): 2752–2767
26 Barbin D F, Natsch A, Müller K. Improvement of functional properties of rapeseed protein concentrates produced via alcoholic processes by thermal and mechanical treatments. J Food Process Pres, 2011, 35(3): 369–375
27 Kim E Y, Kim D G, Kim Y R, et al. An improved method of protein isolation and proteome analysis with Saccharina japonica (Laminariales) incubated under different pH conditions. J Appl Phycol, 2011, 23(1): 123–130
28 Zhen Y, Shi J S. Evaluation of sample extraction methods for proteomic analysis of coniferous seeds. Acta Physiol Plant, 2011, 33(5): 1623–1630
29 McCloud T G. High throughput extraction of plant, marine and fungal specimens for preservation of biologically active molecules. Molecules, 2010, 15(7): 4526–4563
30 Savchuk A N, Gladun D B, Grebinik D N, et al. Marine hydrobionts

as an alternative source of protein molecules of directed action. J Biopharmaceut, 2014, 6: 12–17 (in Russian)

31 Nguyen R T, Harvey H R. Preservation of protein in marine systems: Hydrophobic and other noncovalent associations as major stabilizing forces. Geochim Cosmochim Acta, 2001, 65(9): 1467–1480

32 Kim S K, Wijesekara I. Development and biological activities of marine-derived bioactive peptides: a review. J Funct Foods, 2010, 2(1): 1–9

33 Skripnikov A Y, Anikanov N A, Kazakov V S. The search for and identification of peptides from the moss Physcomitrella patens. Russ J Bioorg Chem, 2011, 37(1): 95–104

34 Shvab O V, Trishkin S V, Shepel' E N, et al. Analysis of peptides from animal and plant tissues. Bioorg Khim, 1999, 25(1): 20–24 (in Russian)

35 Fukuda M, Hasegawa Y. Protease activity of a 90-kDa protein isolated from scallop shells. Turk J Fish Aquat Sci, 2014, 14(1): 247–254

36 D'Amico S, Claverie P, Collins T, et al. Molecular basis of cold adaptation. Philosoph Trans Roy Soc London B Biol Sci, 2002, 357(1423): 917–925

37 Gladun D V, Raksha N G, Savchuk O M, et al. Hydrolytic enzymes of marine organisms as an instrument for investigation of protein–protein interaction. FEBS J, 2015, 282(1):

38 Raksha N G, Gladun D V, Savchuk O M, et al. Collagenolytic activity in tissue extract of parborlasia corrugatus from antarctic region. Biomed Res Ther, 2015, 2: 21