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Sea Urchins Proteins, Enzymes, their Complexes and Functioning

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Abstract

Currently, there is little information about the structure and functioning of proteins and enzymes of sea urchins. However, eggs and spermatozoa of the sea urchin Strongylocentrotus intermedius are the exceptionally convenient model for studying the patterns of development from embryo to the body as well as some other biological processes of living organisms at different stages of their development. All available published data on different enzymes and proteins of the sea urchins are discussed. The dynamics of relative change in the activity of several repair enzymes in the developing embryos during 26 stages of urchin was analyzed. It has recently been shown that living organisms contain not only very stable complexes of various proteins already described in the literature but also previously undiscovered very durable protein complexes. It has been shown that sea urchins also contain previously undescribed highly stable complexes of protein and enzymes. A comparison between very stable protein unusual complexes from sea urchin eggs, female placenta, and human milk was carried out.

Keywords: Eggs of sea urchin; Developing embryos; Repair enzyme activities; The stable soluble protein complexes

Abbreviations: AP: Apurinic/Apyrimidinic; BER: Base Excision Repair (Reversal Base Restoration); CK: Creatine Kinase; EDTA: Ethylenediaminetetraacetic Acid; kDa: Kilo Daltons; MM: Molecular Mass; PAGE: Polyacrylamide Gel Electrophoresis; SPCs: Stable Protein Complexes from the Placenta, Milk, and Urchin Eggs; NMP: Nucleoside Monophosphate; Rhz: Sphingolipid Rhizochalin; SDS: Sodium Dodecyl Sulfate; StAP: Alkaline Phosphatase of S. intermedius

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Introduction

The study of the molecular mechanisms of the most important biochemical processes in humans is sometimes not possible without the use of animal models, including marine creatures. Analysis of the data obtained from sea-animal and human models can complement and enhance studies on general molecular mechanisms of many components including proteins and their complexes functioning.

This review describes all available published data on different enzymes and proteins of the sea urchins and the results of a comparison study on various proteins, peptides, and their complexes from sea urchins and humans.

Back in the last century, important vantages of sea urchins as an object of biological research were disclosed, namely, the potential of getting large batches of gametes and synchronously upcoming embryos, the simplicity in their incubation using controlled conditions, as well as the ease of surveillance and processing of the obtained data. Sea urchins present one of the most prevalent and important squads of invertebrates in the orarian zones of the World Ocean that have a momentous role in maritime ecosystems [1-3]. Their eggs and spermatozoa are well known as an exceptionally convenient model for studying the patterns of development from embryo to the body, which is very difficult to achieve in humans. In this review, we analyze data on the investigation of some enzymes, proteins, and previously undescribed soluble multiprotein complexes of the sea urchin *Strongylocentrotus intermedius* eggs and spermatozoa. We also discuss the published data related to proteins and enzymes of sea urchins.

Literature Review

Analysis of dynamics of enzymes during embryonic development

Genomes of all living organisms exist in a dynamic balance between various continuous DNA damages and removal of the damage due to an operation of known DNA repair enzymes [4]. The diminution in DNA repair ability is eventually manifested in different forms of mutagenesis, carcinogenesis, or cell death, which are associated with a number of human and other organisms' pathologies. DNA repair is critical for quickly proliferating cells. DNA damages inhibit replication progress and can be transformed into mutations during replication of decisively differentiated cells that occasionally need to maintain their genome integrity throughout a body's life. In most organisms, several pathways have been identified, including forwarding reversal base restoration (BER), nucleotide resection, mismatch repair, non-homologous final coupling, and recombination restoration [4]. BER, the most common type of removal of small non-bulky DNA lesions, which have great importance to multicellular organisms, refereed from the embryonic lethality of knockouts destroying the entire path [5]. During the BER, several DNA glycosylases excise damaged bases from DNA, and then apurinic/apyrimidinic (AP) endonuclease splits DNA at the AP site forming free 3'-OH groups, which are then used by DNA polymerase for including normal deoxynucleotides. Finally, DNA ligase recovers the initially damaged strands [4].

In actively proliferating cells, resembling the cells of developing embryos, DNA repair is key to the preclusion of the cumulation of mutations and timing of cell division. Since sea urchins are synchronously developing embryos, they can be used in large quantities in different experiments. Thus, they represent a very commodious model for studying the dynamics of changes in the functioning of the repair enzymes during their development. Revealing of embryonic substances, including repair enzymes, proteins, as well as their complexes, is momentous for understanding embryo development and their function in all organisms, including humans.

Samples of early embryos of the sea urchin *Strongylocentrotus intermedius* (12 of 26 stages of development) were collected and lysed according to [6-8]. All 26 stages of the sea urchin development are given in **Table 1** [9], and some of them are shown in **Figure 1** [9].

The relative activity of several repair enzymes was estimated by oligonucleotide-based analysis, which is easy to interpret and oft

Table 1 Developmental stages of sea urchin [9].

Embryo collection time, hr: min post-fertilization	Stage number*	Stages
30 seconds 0:20	1*	1a. Fertilized egg: formed fertilization shell 1b. Fertilized egg: formed gealine layer
0:30 0:50	2	2a. The stage of a narrow "strip" (prophase of the first division-cleavage) 2b. The stage of a wide and dumbbell-shaped strip (metaphase - telophase of the first division
1:07	3	Cleavage (2 blastomeres)
1:45	4	Cleavage (4 blastomeres)
2:23	5	Cleavage (8 blastomeres)
3:00	6	Cleavage (16 blastomeres)
3:38	7	Cleavage (32 blastomeres)
4:20	8	Early blastula 1. Blastomeres retain their rounded shape
5:13	9	Early blastula 2. Blastomeres partially lose their rounded shape
7:15	10	Middle blastula 1. Blastomeres have lost their round shape
9:30	11	Middle blastula 2. Hatching - the embryos retain their spherical shape
11:00	12	Late blastula 1. Single cells of primary mesenchyme appear
12:00	13	Late blastula 2 (mesenchymal blastula)
15:00	14	Early gastrula 1. The beginning of invagination of the vegetative wall of the embryo
17:00	15	Early gastrula 2. The appearance of the primordial bowel rudiment (archenteron)
18:00	16	Middle gastrula 1. Archenteron reaches approximately the center of the blastocele.
19:20	17	Average gastrula 2. Archenteron reaches its final size.
20:30	18	Late gastrula 1. The emergence of the secondary mesenchyme and the beginning of the formation of ectodermal belts of cilia
23:25	19	Late gastrula 2. The onset of skeletal formation

Embryo collection time, hr: min post-fertilization	Stage number*	Stages
25:00	20	Prism 1. Larvae take on a characteristic angular shape
27:30	21	Prism 2. The digestive tract consists of poorly delimited anlages of the esophagus, stomach, and intestines
29:50	22	Early pluteus 1. The mouth opening appeared. Chromatophores have appeared
34:30	23	Early pluteus 2. The sections of the digestive tract are clearly delineated.
39:35	24	Early pluteus 3. The first pair of arms increased noticeably, a bookmark of the second pair appeared - oral
54:00	25	Middle pluteus 1. Both pairs of arms are extended
84:00	26	Middle pluteus 2. Transition to active feeding

*The stages of development of the hedgehogs that were used in work [10] are marked in bold.

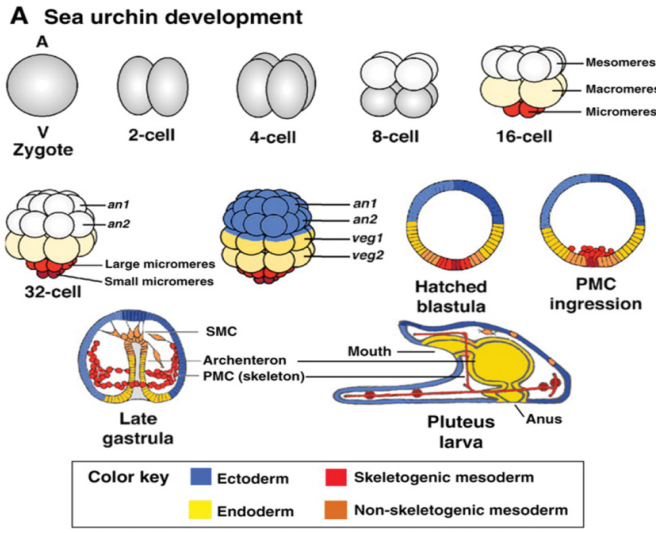

Figure 1 Several of the 26 stages of sea urchin development.

used in DNA repair investigations in cell extracts of different organisms [10]. Relative activity of uracil–DNA glycosylase, 8-oxoguanine–DNA glycosylase, apurinic/apyrimidinic endonuclease, and DNA polymerase during sea urchin embryo development was analyzed. These sea urchin enzymes have not yet been described in the literature.

The uracil–DNA glycosylase harshly upsurge after the prism stage **(Figure 2A),** when the first signs of differentiation of the digestive tract appear, while the specific activity of 8-oxoguanine–DNA glycosylase **(Figure 2B)** constantly runs low over the course of the development [10]. The AP endonuclease activity was gradually increasing but fallen at the last stage mid-pluteus 2 (during the transition of larvae to active feeding) **(Figure 2C).** At the first stages of divisions (prior to completion of synchronous cleavage of the cells), the DNA polymerase activity was high, and then it decreases repeatedly, demonstrating a transient peak at stages of late blastula after hatching **(Figure 2D).** The development of sea urchin embryos seems to be depending on various factors of DNA-damage in early stages within the protective envelope and later as a free-floating larva, with hatching requiring adaptation to the shift in genotoxic stress conditions [10]. No correlation was revealed between the enzymatic activity dynamics and the known gene expression data from developing related species, *Strongylocentrotus purpuratus* [10]. The results suppose that the base excision repair enzymes may be regulated in the embryos of the urchins at the level of covalent modification or protein stability [10]. It is possible that, in some sense, such changes in the emergence or disappearance of repair enzymes can occur after the fertilization of human cells. These data appear to be useful for further investigation of DNA repair processes not only in the case of sea urchins and other sea organisms but also in various animals that may be very similar.

There is also some other evidence of DNA repair in sea urchins. Treatment of sea urchin embryos with genotoxic agents (methyl methanesulfonate or bleomycin) led to DNA damages and to the cell cycle delay [11]. At low toxic agent doses, embryos were capable to repair the DNA damage, while at high concentrations, they show morphological and biochemical changes as well as apoptosis. Embryos extracts obtained support *in vitro* repair of oligonucleotides containing damages. The data demonstrate that urchin embryos comprise enzymes and proteins important for DNA repair [11].

The other approach used for the search of repair enzymes of sea urchins is an investigation of effects of UV-irradiation, hydrogen peroxide, etc., on larvae of the urchin *Strongylocentrotus purpuratus*. Ultraviolet radiation effects on the urchin embryos and led to the significant formation of DNA pyrimidine dimers [12]. Embryos of sea urchin after ultraviolet irradiation showed significantly higher concentrations of proteins with antioxidant activities and p53 and p21 transcription activators. The observed cellular processes lead to apoptosis, and a significant increment in DNA strands breaks in the nuclei of developing embryos treated by ultraviolet light. Cell death and reduced survival of sea urchin embryos are summoned by both indirect and direct effects of ultraviolet radiation.

It was shown that ultraviolet radiation acts for proteins via a few cellular pathways, which indicates that the mechanisms engaged in UV-induced developmental delay in the urchin embryos are integrated into multiple pathways for cellular stress, protein turnover as well as translation, signal transduction, cytoskeletal dynamics, and general metabolism [13]. The multiple responses

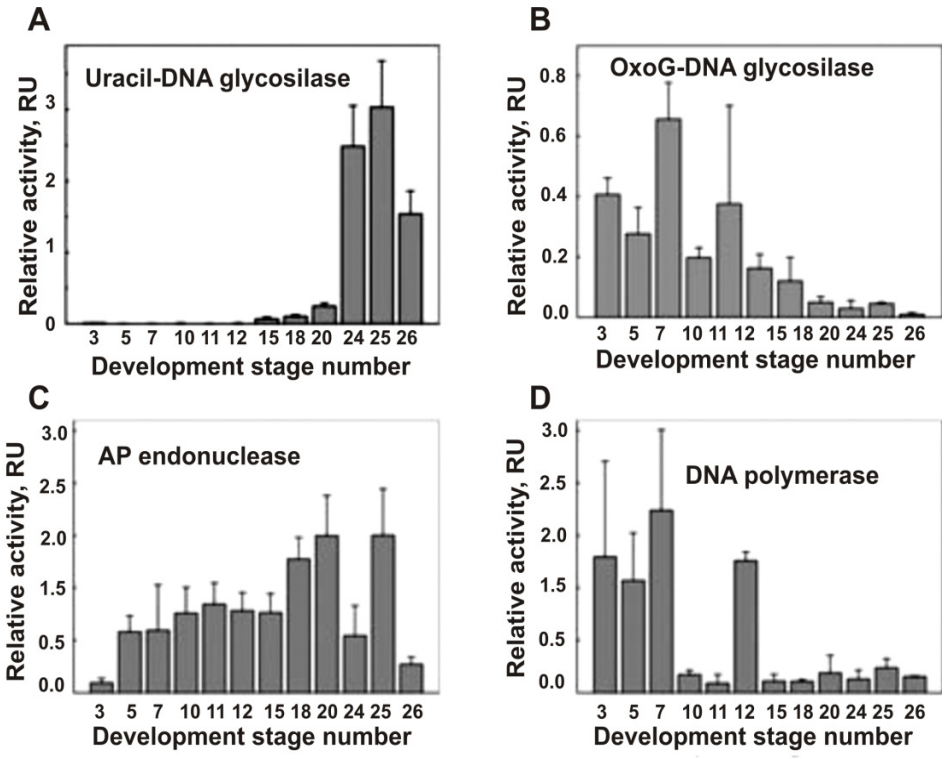

Figure 2 Dynamics of changes in the relative activities of repair enzymes and DNA polymerase during the 26 stages of the development of the sea urchin embryos [10].

of the urchin larvae to DNA damage may represent the specific ways for protecting the population of these animals from acute genotoxic stress.

The capability of the genome protection from harmful DNA damage is very important to maintaining the stability of the genome and defense against diseases. Many echinoderms, including sea urchins, are marked by the absence of tumor diseases. In [14], DNA damage was induced in coelocytes and larvae of adult sea urchins under the influence of various genotoxicants, and the ability to DNA repair was estimated during a 24-hour recovery period. Larvae were more susceptible than coelocytes, with a higher degree of initial DNA damage and increased mortality levels for 24 hours after their treatment with different genotoxicants. Surviving larvae were capable to repair damage within 24 hours effectively. Temporal expression profiles of 17 genes of DNA repair proteins showed a large induction gene in whole cells compared to larvae, with especially high expression of several of them. Thus, DNA repair ability is very important for understanding the effects of various genotoxicants on organisms and ways of DNA recovery [14].

According to published data, there is evidence of changes in the relative amount and activities of some other enzymes during embryonic development. The turnover and localization of the DNA-dependent (cytosine-5)-methyltransferase were analyzed during the development of the sea urchin embryo *Paracentrotus lividus* [15]. It was shown that the enzyme is, to varying degrees, needed at various stages of embryonic development. The switching point occurs at the blastula stage, where the transferase presents in all embryonic cells and removes a peptide of about 45 kDa from the amino-terminal region of the 190 kDa protein. The 145 kDa transferase form demonstrates its modified enzymatic properties, and the enzyme is rapidly destroyed during several hours before gastrulation. At later stages, the transferase is synthesized again, but only in definite types of cells, including neurons. These data demonstrate that enzyme concentration is differently critical for different differentiated cells under a developing the urchin embryo [15].

It was shown that the sea urchin *Strongylocentrotus intermedius* contains O-hydrosilhydrolases: very active 1,3-β-D-glycanase and α-D-mannosidase and two enzymes with lower activities - β-D-glucosidase and β-D-galactosidase [16]. The dynamics of change in the activities of the enzymes at different stages of embryo development were investigated. At all stages of embryo development, a high level of α-D-mannosidase and 1,3-β-D-glycanase was found. Activity β-D-glucosidase was low. 1,3-β-D-glycanase was found in unfertilized eggs, and its activity decreased only after the formation of 2, 4, and 8 blastomeres and at later stages [16].

Proteolytic hydrolysis of sperm histones of male sea urchin occurs due to the activation of maternal cysteine protease during fertilization [17]. This protease is necessary for the remodeling of male chromatin and for the development of the cell cycle in lately formed embryos. This protease is presented in the nucleus of unfertilized eggs and is promptly absorbed in the male pro-

nucleus after insemination. This enzyme persists bound to chromatin at the S phase of the first cell cycle; then, it moves to the mitotic spindle in the M phase and, after cytokinesis, moves to the nuclei of the daughter cells. A high identity of the protease sequence with cathepsin proteolytic enzymes of different organisms was found. Phylogenetic analysis clearly shows that this histone sperm enzyme is a subtype of cathepsin L [17].

Thus, there are still relatively little data in the literature on various repair and other enzymes that are involved in the development of sea urchins. At the same time, this particular model is extremely promising for studying the patterns of development from embryo to the body. However, in the literature, there is data on some other proteins and enzymes of sea urchins, which can be used for understanding possible differences in the structure and biological functions of their in marine organisms and in humans, as well as for medicine and other purposes.

Sea Urchin Enzymes

The literature describes the features of the functioning of DNases in humans and animals (e.g., [18-22]). These DNases are Mg^{2+}-dependent. However, DNases of sea urchins differ markedly from those in mammals, which is of particular interest. Ca^{2+}, Mg^{2+}- and Ca^{2+}, Mn^{2+}-dependent DNases were revealed in sea urchins. The complexation between Ca^{2+}, Mg^{2+}-dependent DNase from sea urchin embryos with double-stranded DNAs was shown using immunological electron microscopy [18]. After the hydrolysis reaction, in the DNA-DNase complex, the enzyme will stay bound with the terminal fragment of DNA. It was shown that acid DNases from marine organisms interact specifically with the local conformation of B-DNA [19].

Ca^{2+}, Mn^{2+} and Ca^{2+}/Mg^{2+} dependent acid DNases were obtained from spermatozoa of the sea urchin *Strongylocentrotus intermedius* [6]. Ca, Mg-DNase is shown to be a nuclear protein with molecular mass (MMs) ~63 kDa with pH-optimum at pH 7.5. The enzyme activity declines in the following order: Ca^{2+}+Mg^{2+} > Mn^{2+} ≈ Ca^{2+}+Mn^{2+} > Mg^{2+}+EGTA > Ca^{2+}. It has maximal activity in seawater. The Ca,Mn-DNase (25 kDa optimal pH = 8.5) is answerable for the internucleosomal splitting of spermatozoa chromosomal DNA. The enzyme activity decreases in the following order: Ca^{2+}+Mn^{2+} > Ca^{2+}+Mg^{2+} > Mn^{2+} > Mg^{2+}+ EGTA. The enzyme is inactive in seawater. Spermatozoa acid A-DNase with MM of 37 kDa (optimum pH=5.5) is not a nuclear protein. A-DNase cannot be activated by bivalent metal ions. Mechanisms of the hydrolysis of double-stranded DNA were established for these three DNases.

Participation of Ca, Mg-DNase in spermatozoa apoptosis has been shown using its strong inhibitor - Zn^{2+} ions [20]. It was demonstrated that sphingolipid rhizochalin (Rhz) induced apoptotic changes of nuclear chromatin, internucleosomal DNA cleavage, and activation of several caspases in spermatozoa. In the presence of Zn^{2+} ions, there was blocking of Rhz-induced DNA fragmentation and internucleosomal cleavage of HeLa S and Vero E6 cell nuclei chromatin by highly purified Ca, Mg-DNase.

A new alkaline phosphatase (salt resistant, pH optimum = 8.0-8.5, from eggs of the sea urchin *Strongylocentrotus intermedius* (StAP) was shown to have a unique property to split substrates in seawater with high activity [21]. Copper, zinc, cadmium, and lead in different concentrations (15-150 µg/l) added to seawater, or standard buffer completely suppress the phosphatase activity. StAP is very sensitive to the presence in seawater of not only metals but also different detergents, pesticides, and various oil products. Seawater samples taken from aquatic areas of the Troitsy Bay of the Peter the Great Bay of Japan Sea demonstrate inhibition of the phosphatase activity; the same was demonstrated for some samples of freshwaters. In contrast to phosphatases of mammals, sea urchin enzyme suppression assay is highly sensitive and technically easy-to-use, allowing testing a great number of samples contaminations [21].

Later, it was shown that these alkaline phosphatase and Ca,Mg-DNase inhibition tests are very productive for analysis of the total pollution of natural marine ecosystems [22]. The sea water samples containing various pollutions were getting together in different places. The sensitivity of the alkaline phosphatase analysis of water pollutes was comparable with the known standard sea urchin sperm cell toxicity test. It was shown that a complex of these two methods could be useful to evaluate marine water areas and to estimate the biological conditions of invertebrates adapting to various anthropogenic and environmental effects.

Several other sea urchins enzymes are described in the literature. Gonad's RNase of immature stage sea urchins showed a constant level of activity [23]. RNase activity of mature males and females of the sea urchin species *Anthocidaris crassispina* and *Hemicentrotus pulcherrimus* (optimal pH = 5.0) demonstrated that its average specific activity at the immature stage of the female *H. pulcherrimus* rapidly increased from 7.4 to 62.8 units/mg, while in males *H. pulcherrimus*, it decreased from 7.4 to 1.9 units/mg. The same phenomenon was observed in sea urchin *A. crassispina*. It was determined that this sea urchin enzyme is an RNase T2 type [23].

Two different DNA ligases are described at the early sea urchin embryogenesis [24]. Light form (50kDa) is revealed in unfertilized eggs (oocyte form), and a heavier ligase (110kDa) is found at the two-cell stage (embryonic form). α-Amylase is an enzyme catalyzing the hydrolysis of starch and other polysaccharides containing α-1,4-glycosidic bonds. Amylase is present in some different organisms, where it carries out the processes of hydrolysis of oligo- and polysaccharides. α-Amylase was isolated from the digestive tract of sea urchins, *Strongylocentrotus nudas* [25]. Chloride ions activate the enzyme and shift the optimal pH from 6.0 (-NaCl) to 7.3 (50mM NaCl). The enzymatic properties of sea urchin alpha-amylase were almost the same as in mammalian α-amylases.

Cellulases are several enzymes produced chiefly by fungi, bacteria, and protozoans, that catalyze the decomposition of cellulose and of some related polysaccharides. Cellulases of the glycoside-hydrolase-9 family are known to be widespread in metazoan [26]. Cellulase (54 kDa) was isolated from the Japanese purple sea urchin *Strongylocentrotus nudus*, and its catalytic properties and the primary structure of the protein were determined. The enzyme was highly active in the hydrolysis of carboxymethyl cellulose [26].

Sea urchin eggs activation at fertilization in deuterostomes needs an increase in intracellular Ca^{2+}, which is appeared from the egg's endoplasmic reticulum [27]. In sea urchins, Src kinase (SpSFK1) is necessary for a PLCgamma-associated signaling event that triggers Ca^{2+} release. The function of the Src kinase in the initiation is the release of Ca^{2+} during fertilization. The sequence analysis of the *Strongylocentrotus purpuratus* genome results in the identification of additional SFK kinases of Ca^{2+} transmission and activation of sea urchin eggs. The cloning and analysis of four additional SFKs and testing of their functions at the initial release of Ca^{2+} during fertilization were performed. While two new SFKs (SpFrk and SpSFK3) are required for the release of Ca^{2+}, SpSFK5 seems unnecessary for the early events of transition from an egg to an embryo. SpSFK7 may be involved in preventing the premature release of Ca^{2+}. The complexation analysis shows that only SpSFK1 is able to bind directly with PLCgamma. One SpSFK and PLCgamma are located according to immunolocalization studies in the cortex of the egg and at the site of interaction between the sperm and the eggs. Together, these data show that more than one SFK enzymes are involved in the release of Ca^{2+} during fertilization [27].

The *Strongylocentrotus purpuratus* sea urchin sperm flagella creatine kinase (CK) is both the main component of the sperm tail membrane and cytosolic enzyme [28]. Three pools of a kinase called CK I, CK II, and CK III were discovered. The relative activity of these three types of kinases can be expressed approximately as 1:10:1. However, they functionally differ in their ability to bind to lipids. The data are consistent with the fact that the CK II association with a membrane is a two-step process, including the pH-dependent intramolecular process. The CK membrane association, together with the microtubule association, may represent the mechanism necessary for the specific accumulation of the kinase in the flagellum [28].

In the sperm of the sea urchin, *Strongylocentrotus purpuratus*, a functional shuttle of phosphocreatine, which is necessary for the existence of mitochondrial and cytosolic isoforms in different places, required for sperm motility [29]. Thus, the myristoylated and non-myristoylated forms of creatine kinase exist side by side in the flagellum of the sea urchin, and myristoylation is essential for its efficient association with liposomes.

Multiprotein complex analysis

In living organisms, many protein complexes with very different functions have been discovered [30]. Macromolecular complexes are necessary for the retention of biological processes, but their abundance among animals and other organisms is not yet fully understood. In addition to the already discovered very stable complexes of the ribosome and other type, biological fluids of various organisms can contain other very stable complexes with many different functions. Moreover, such complexes, which not yet known in the literature, can be similar in structure and function in mammals, marine and other organisms, or differ greatly. Comparison of stable protein complexes from different organisms can help to identify general and specific patterns of their existence and biological function in organisms of different levels of development and organization. Moreover, it is very possible that some of the complexes can be detected at the beginning only using model animals like sea urchins and only then revealed in humans.

By the combination of biochemical fractionations with quantitative mass spectrometry, the analysis of soluble multiprotein complexes in various multicellular animals was carried out [31]. Using a combination of several approaches, a draft conservation map was created that includes over one million predicted interactions leading to the formation of various complexes with a high degree of confidence. Clustering has revealed a wide spectrum of conservation, ranging from ancient eukaryotic communities to rarer innovations of multicellular animals. The completeness, centrality, and modularity of these reconstructed interactions, according to the author's point of view, demonstrate their fundamental mechanistic importance and adaptive value for animal and other organisms cell systems. However, the structure and biological functions of real-life complexes can be established only after their isolation and detailed analysis.

In comparison with individual proteins and enzymes, their complexes usually possess polyfunctional properties and different biological functions. The formation of stable complexes might be an important system mechanism for the expansion of diversity and the biological functions of different proteins and other substances in different organisms, including humans. For example, the majority of cellular processes are dependent on enzymes that usually interact, forming larger temporary or stable protein complexes-associates to increase the efficiency, specificity, and speed of metabolic pathways [30].

At present, some new stable complexes of sea urchins and humans have been obtained and characterized. We have analyzed the likelihood of the presence of very stable protein complexes in the placenta [32,33], milk [34,35] of women, and in the eggs of sea urchins [36]. Previously undescribed highly stable protein complexes were first found in the female placenta [32,33].

Human proteins associated with placental membranes were recently analyzed by SDS-PAGE and MALDI mass spectrometry [37]. Overall, 733 unique proteins, including 34 protein complexes, were identified. In our works, we searched for new stable complexes in sea urchin eggs (*Strongylocentrotus intermedius*) not described in the literature and compare them with human complexes.

The human placenta is a specific organ protecting, feeding, and regulating the growth of the embryo. It appears that the revealing and characterization of different components of the placenta, including various proteins and possible multiprotein complexes, is very important for understanding the placenta functions. A very stable multiprotein complex (SPC, ~1000 kDa) from the soluble fraction of homogenates of human placentas [32,33] was isolated. The SPCs were well separated by gel filtration on Sepharose-4B from other proteins of the placental extracts **(Figure 3A).** The presence of stable multiprotein complexes was analyzed in human milk [34,35] and eggs of sea urchins [36], which were also separated by gel filtration **(Figures 3B and 3C).** In all cases, separation of multiprotein complexes with molecular weights of ~1000±100 kDa occurred.

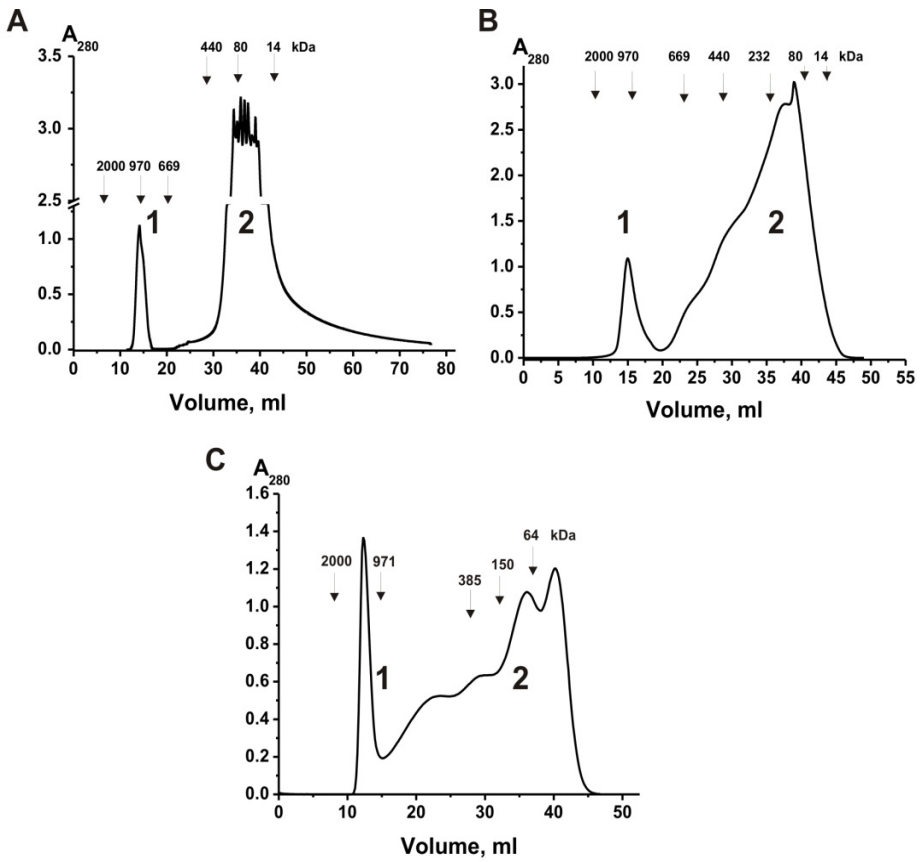

Figure 3 Separation of very stable protein complexes (SPCs, peaks 1) from other proteins (peaks 2) by gel filtration on a Sepharose 4B column plasmas of human placenta (A), milk (B), and the extract of urchins eggs (C). In all cases, the molecular weights of very stable protein complexes were close to 1000 ± 100 kDa [32-37].

The analysis of complexes stability by light scattering (LS) demonstrated that the SPCs are very stable in the presence of guanidinium chloride, Triton X100, $MgCl_2$, NaCl, acetonitrile, and other compounds in high concentrations **(Figure 4).** The complexes dissociate efficiently only in the presence of 8 M urea supplemented with 0.5 M NaCl and 50 mM ethylenediaminetetraacetic acid (EDTA) **(Figures 4A and 4B)** [33-35] or additionally with Tween or Triton **(Figure 4C)** [36]. Such very stable complexes are unlikely can be formed due to a random association of different proteins.

According to SDS-PAGE analysis and data of MALDI mass spectrometry, the complex from the placenta contains many glycosylated peptides and small proteins with 4-10 kDa MMs as well as twelve proteins with higher MMs: alkaline phosphatase, hemoglobin, chorionic somatomammotropin hormone, heat shock protein beta-1, cytoplasmic actin, human serum albumin, peroxiredoxin-1, 78 kDa glucose-regulated protein, disulfide isomerase A3, serotransferrin, annexin A5, and immunoglobulin G (IgGs). Each of these twelve proteins and enzymes has different and important own biological functions in living organisms. It was shown that the placenta SPC possesses two DNA-recognizing sites demonstrating different affinities for a 12-mer oligonucleotide [32]. The placenta SPCs demonstrated nine different enzymatic activities: catalase, peroxidase (H_2O_2-dependent), oxidoreductase (H_2O_2-independent), DNase, RNase, ATPase, phosphatase, protease, and amylase [32,33]. The splitting of $r(pC)_{23}$, $r(pU)_{23}$, and $r(pA)_{23}$ by SPCs results the formation of 1-22-mer oligonucleotides, while splitting of mirR137 results in its site-specific hydrolysis at 3A-4U > 9U-10A > 8U-9U ≥ 15U-16A specific cleavage sites. Some of these enzymatic activities of SPCs are absent in the individual proteins included in the complex [32,33]. However, new active centers of the complex can be formed at the junctions of various proteins of the placenta SPC. The combination of proteins with different functions in one complex can significantly expand its biological functions due to the emergence of its multifunctionality.

For newborns, mother's milk is more than various nutrients source; it provides a wide array of many different components stimulating the growth of newborns and protecting children from bacterial and viral infections. Therefore, the identification of female milk components, including proteins and their complexes, is very important for understanding milk functions. A very stable multiprotein complex (SPC; ~1000 kDa) from 15 samples of mother's milk was isolated as described above. The relative content of the SPCs varied from 6 to 25% of the total different milk proteins. Lactoferrin (LF) and α-lactalbumin were major proteins of all fifteen SPCs, while human milk albumin and β-casein were

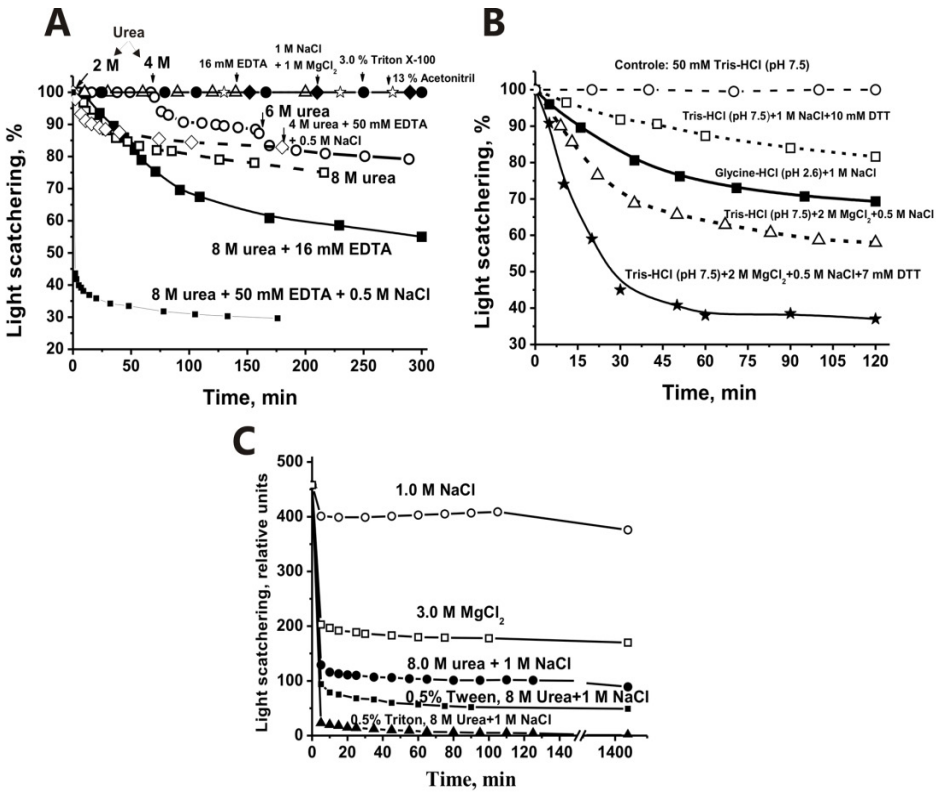

Figure 4 Typical examples of overtime changes in the relative light scattering (LS) intensity of three SPCs (0.5 mg/ml) from human milk (A), placenta (B), and sea urchin eggs (C) under different conditions. All components of the reaction mixtures are shown in Panels 1-3 [32,34,36].

presented as moderate or minor proteins; the relative content of IgGs and secretory immunoglobulin A (sIgAs) varied greatly in different SPC preparations. All SPCs efficiently split DNA and maltoheptaose [34,35].

LF is known as the protein of the acute phase response and unspecific defense against various types of viral and microbial infections [34,35]. LF is found in human epithelial secretions, barrier body fluids, and in the secondary granules of leukocytes. Therefore, lactoferrin and its complexes with other proteins may be formed not only in milk; they can be important for LF different forms functioning not only in milk but also in other various biological fluids and cells [34,35].

The association of enzymes and proteins can include different metal ions, which may be essential for proteins complexations and for their biological functions. However, there is no published data concerning metal ions of any protein complexes. Content of different microelements in the SPCs and milk was evaluated using two-jet plasma atomic emission spectrometry [35]. The relative content of various elements in mother's milk decreased, on average, in the following order: Ca > P > Mg > Al ≥ Zn ≥ Fe > Cu > B (0.76-3500 μg/g of dry milk powder). The content of some elements in milk was very low (Sr > Mn > Cr > Ba > Pb > Ag > Ni > Cd; <0.03-0.5 μg/g). The relative amount of eight elements in SPCs was by a factor of 1.2-9.6 higher than in females milk and increased in the order: Ca ≈ Mg < P < Al < Fe < Pb < Ba < Cr < Cd < Zn. The content of eight metals in SPCs was 12.3-110-fold higher: Cu (12.3) > B (19.7) > Ag (28.7) > Ni (38) ≥Sr (110) [35]. Thus, it was shown that during the complexes formation, the increase in the relative content of different metal ions in some complexes is possible. Such an increase in the content of metal ions in the complexes can be important for the processes of their association, proteins, and enzymes conformations, their enzymatic activities, and biological functions.

We have proposed that such very stable protein complexes can be in different liquids and cells, including sea urchin eggs [36]. Identification and characterization of embryonic peptides and proteins and their different complexes seem to be very important for an understanding of embryo proteins functions. Three preparations consisting of a mixture of eggs of 10 different female sea urchins were used [36]. Soluble fractions of the eggs homogenates were obtained and subjected to gel filtration on the Sepharose 4B, as described above **(Figure 3C).** A nearly symmetrical protein peak with high molecular mass (~1100 ± 100 kDa) was separated from other different peaks of proteins. According to the data of the LS method, the sea urchin SPCs are stable in 20 mM Tris-HCl, pH 7.5, containing 1 M NaCl, and to a lesser extent in the presence of 3 M $MgCl_2$ usually destroying electrostatic contacts between different proteins. Similar to very stable protein complexes from human milk and placentas [32-34], the sea urchin eggs complex was destroyed only by 8 M urea supplemented with 1.0-3.0 M NaCl,

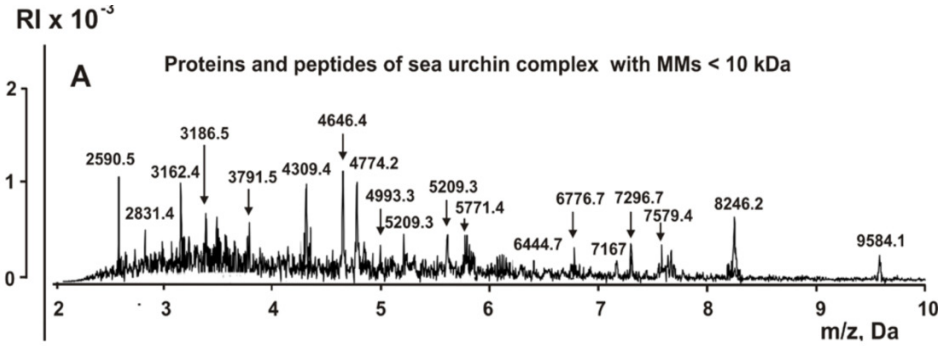

Figure 5 MALDI mass spectra of small proteins and peptides corresponding to SPC of urchin eggs.

but better dissociated after the addition of Triton or Tween [37] **(Figure 4C).** According to SDS-PAGE data, this complex contains many major, moderate, and minor proteins with MMs from 10 to 95 kDa. Thus, all three complexes from different sources were of approximately the same stability and comparable size.

MALDI-TOF mass spectrometry assay is a semi-quantitative approach giving information mainly on only the MMs of analyzed compounds. This method in the analysis of MMs of proteins mixtures has some restrictions. If in the used conditions any of the mixture's protein is present in the increased concentration and well crystallizes on MALDI-target, the signal of this protein can be very high, when the signals of other proteins may be low or even completely suppressed. Exactly this situation was observed during the MALDI MS analysis of urchin SPCs.

Proteins and peptides of lower MMs (<10 kDa) are usually soluble in acetic acid, and during gel staining with Coomassie, they are washed out of the gel. Therefore, three different sea urchin SPC preparations were first boiled in buffer containing 1% SDS and 50 mM DTT, and then the samples were subjected to sequential filtration using membranes skipping proteins with different MMs. Finally, the fractions containing proteins ≤10 kDa were obtained and analyzed using the MALDI mass spectrometry approach. The fraction ≤10 kDa contains more than 20 sufficiently distinct peaks of peptides and short proteins **(Figure 5).** Currently, evidence has been obtained that SPCs of milk and the placenta, similar to sea urchin complex, also contain many small proteins and peptides (< 10 kDa).

The SPCs were first boiled in buffer containing 1% SDS and 50 mM DTT, and then the samples were subjected to sequential filtration using membranes skipping proteins with MMs < 10 kDa. Finally, these fraction components were analyzed using MALDI mass spectrometry [36]. Furthermore, peaks correspond to the fraction of 10-30 kDa. Fraction ≤100 kDa contains approximately 20 proteins with MMs 29.4-96.1 kDa [36].

Thus, SPCs consist of many proteins and peptides. The small gel fragments after SDS-PAGE corresponding to major and medium proteins were isolated. We tried to identify these proteins by MALDI mass spectrometric analysis (MS and MS/MS) of their tryptic hydrolyzates. All proteins of SPCs from milk and the placenta were successfully identified (see above).

Good MS and MS/MS spectra were obtained for several urchin SPC proteins. It was impossible, however, to identify the majority of the proteins due to the lack of data on such proteins in the sea urchins and other invertebrate's databases. Assuming possible homology of proteins of the sea urchin eggs and other organisms, we used other databases. One protein has homology with keratin (type II cytoskeletal 1; *Homo sapiens*). The second one was homologous to alkaline phosphatase (*Pseudomonas fluorescens*), while the third protein demonstrated homology with human lactotransferrin. A homolog of glucose-6-phosphatase of *Strongylocentrotus purpuratus* corresponds to the fourth protein, while the homolog of the *Alveinella pompejana* protein (cDNA clone CAGA18424 5', mRNA sequence) to the fifth protein. Other medium and major proteins were impossible to identify even by their potential homology with proteins of other various organisms.

The presence in the placenta [33] and sea urchin [36] native SPCs of alkaline phosphatase was additionally confirmed by the detection of these complexes' phosphatase activity. Thus, it was shown, for the first time that sea urchin's eggs similar to human milk and placenta contain a very stable protein complex of high MM ≈ ~1100 kDa consisting of many peptides and proteins [34,35]. Thus, only five proteins of sea urchin's eggs SPCs were identified. In addition, to large proteins SPCs contain many different small proteins and peptides with MMs from 2 to 9.5 kDa. It should be especially noted that very stable complexes have been found in both humans and sea urchins. However, these complexes contain different peptides and proteins. This suggests that they can have very different functions in these organisms. Analysis of their possible biological functions looks very important [36,37].

Conclusion

This review combines for the first time the available results of the study of various enzymes, proteins, peptides, and their stable complexes from sea urchin, the analysis of which was only possible due to cooperation between two laboratories of two different institutes.

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