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EXTRACTION AND ACTIVITY OF REGULATORY PEPTIDES FROM SEA URCHIN EGGS

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Experiments were performed to extract low molecular weight peptides from the eggs of the sea urchin *Strongylocentrotus pallidus* using sterilizing microfiltration and solid-phase extraction, along with analytical gel chromatography and spectroscopy. The peptides extracted had molecular weights of 360 – 1200 Dal and were divided into two fractions: acidic peptides contained a predominance of glutamic and aspartic acid residues, along with aromatic amino acid residues; alkaline peptides contained mainly lysine and arginine residues and lacked aromatic amino acids. Assessment of the biological activity of these extracts demonstrated high specificity in stimulating the development of explants in organotypic cultures of spleen, testicle, myocardium, and cerebral cortex tissues.

Key words: Regulatory peptides, sea urchin eggs, microfiltration, solid-phase extraction, organotypic tissue culture.

Endogenous regulatory peptides (ERP), in particular geroprotective peptides, are contemporary therapeutic agents which produce no significant side effects. Most applied studies in this area have addressed the extraction and purification of ERP from mammalian organs and tissues and the use of these extracts in medical practice [1]. The restricted availability of the necessary raw materials has stimulated studies seeking chemical or genetic engineering synthesis of peptide agents [2]. An alternative pathway to developing the corresponding pharmacognosy and medical biotechnology consists of widening the range of raw sources by using organs and tissues from invertebrates. Immunostimulatory β-thymosines from sea urchins are now known [3], as are nucleoprotein complexes from baker's yeast and activated lymphocytes [4]. Evolutionarily, sea urchins are closer to the higher eukaryotes than *Drosophila* and *C. elegans,* as they have calcareous shells with mobile plates and needles [5]. Sea urchin eggs contain the Ca-binding protein calmodulin, which plays a leading role in forming the shell at the early stages of development of this organism [6].

Calmodulin is a single-chain protein present in all organisms in the animal and plant worlds and consists of 148 amino acid residues, of which 25% are Glu and Asp residues and 10% are Lys and Arg. These residues, which have charged side groups, are located close to each other, thus forming a chain of charged blocks and clusters [7]. The regulatory functions of calmodulin are known to be expanded when it binds with ubiquinone [8]. Subsequent degradation of calmodulin on proteosomes appears to lead to the formation of a pool of endogenous regulatory di-, tri-, and tetrapeptides which originated in the calmodulin chain in the form of charged blocks and clusters. These observations provide grounds for seeking low molecular weight biologically active peptides with regulatory and geroprotective activity in sea urchin eggs and extracting them preparatively.

EXPERIMENTAL SECTION

Raw material for extracting regulatory peptides consisted of sea urchin eggs (SUE) from *Strongylocentrotus pallidus*, which lives on the continental shelf of the northern Russian seas. Peptides were extracted and purified using the following stages:

1) Extraction of the peptide components of SUE using buffer solution pH 3.8 with an eggs:extractant ratio of 1:10.

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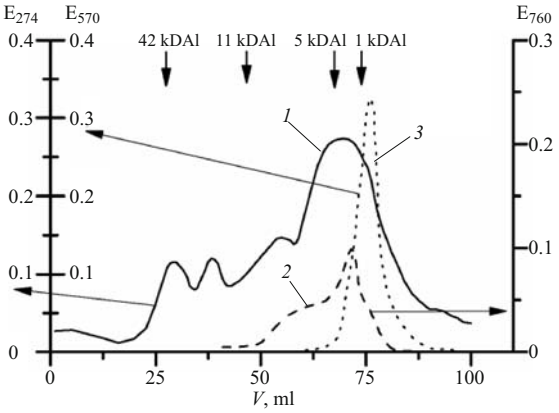

Fig. 1. Gel chromatography of an extract of sea urchin eggs after sterilizing microfiltration on Sephadex G-50 superfine. The ordinate shows the optical density of the eluate at 274 nm (*1*), the eluate coloration intensity with Folin's reagent at 760 nm (*2*), used to assess protein concentration by the Lowry method, and the eluate coloration intensity with ninhydrin at 570 nm (*3*), used to assess the concentration of α-amino nitrogen.

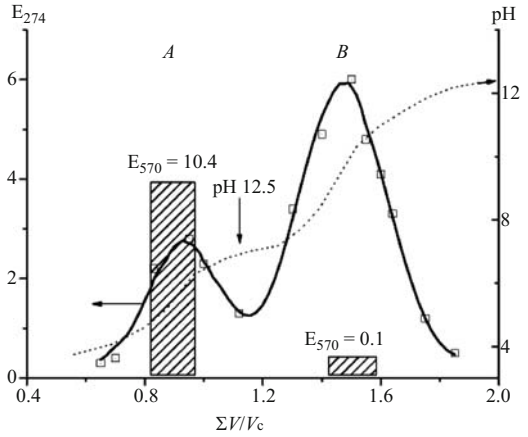

Fig. 2. Plot showing the two-step (isocratic) desorption of peptide components from cation exchange resin Dowex 50 WX 8 using neutral (*A*) and alkaline (*B*) desorption buffers (the arrow shows acidification of the alkaline buffer). The levels of substances containing α-amino nitrogen in peaks *A* and *B* are indicated by the shaded columns.

2) Microfiltration of the extract using a tangential regime in a Pellicon 2 sterilizing module with a nominal pore size of 0.22 μm and a filtration rate of 1.8 – 2.5 liter/h.

3) Solid-phase extraction (sorption) of peptides from the filtrate on cation exchange resin Dowex D50 WX 8 in a countercurrent regime with a suspended solid phase layer.

4) After sorption and washing of the cation exchange resin, the sorbent was used as the stationary phase in a low-pressure chromatography column. Peptides were desorbed and fractionated using a displacement ion exchange chromatography regime [9]. Displacement was performed in two stages – with neutral and alkaline buffer solutions for group fractionation of peptides based on the acidity of their ionogenic side groups [10].

Chromatography was performed at rates not exceeding $4\ ml \cdot h^{-1} \cdot cm^{-2}$. pH was measured at the column output, along with optical density at 260 and 274 nm, and the concentration of α-amino acids was monitored using the Moore and Stein method. Protein concentrations were measured by the Lowry method [11] in fractions with an optical density at 280 nM greater than 0.10.

The molecular weights of extracted peptides were assessed using a standard gel chromatography method on Sephadex G-50 "Superfine" on a column of size 1.6 × 45 cm. The molecular weights of peptide preparations were measured using calibration curves plotted for calibration standards, i.e., cytochrome c (Sigma, 11,600 Dal), Thymalin (5000 Dal) and vasopressin (1000 Dal) (OOO Samson-Med), along with tryptophan and phenylalanine (Reanal). Peptide fractions were dried in a vacuum evaporator at temperatures not exceeding 50°C.

The biological activity of preparations were assessed in organotypic tissue cultures of spleen, testicle, ovary, myocardium, and cerebral cortex explants from 3-month-old Wistar rats as described previously [12]. Petri dishes containing experimental explants were loaded with 3 ml of nutrient medium supplemented with agents over the concentration range 0.01 – 0.5 ng/ml. Petri dishes containing control explants were loaded with 3 ml of nutrient medium without additions. Explants were cultured at 37°C and growth was assessed after 3 days by phase contrast microscopy with a micro-video attachment. The PhotoM 1.2 program was used to determine the growth area index (AI) of each specimen as the ratio of the area of the whole explant (including the cell outgrowth zone) to the area of the central zone. The AI of control explants was taken as 100% and the activities of study compounds were also expressed as percentages.

TABLE 1. Characteristics of the Complex Peptide Preparation.

Preparation	pH *	Concentration of	
		peptides, Wadell method, mg/mg dry substance	α-amino nitrogen, mg/mg dry substance, in terms of leucine
Complex (A + B)	6.05	0.31	0.39

* Solution, 1 mg/ml

RESULTS AND DISCUSSION

Figure 1 shows a gel chromatogram of the acid extract of sea urchin eggs after sterilizing microfiltration, which provides evidence that this extract contained protein and peptide components with molecular weights ranging from 300 to 40,000 Dal. Use of Dowex 50 WX 8 as sulfocation exchange resin allowed the molecular weight range of the sorbed substances to be decreased to 300 – 2000 Dal because of the high cross-linking density of this sorbent and the steric inaccessibility of its sorption centers for large molecules [9]. The process of desorption of low molecular weight components in the displacement ion exchange chromatography regime presented in Fig. 2 shows that the use of two buffer solutions

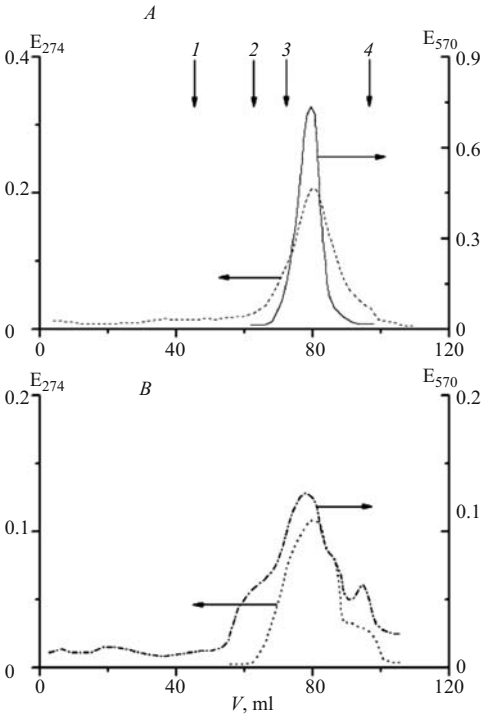

Fig. 3. Gel chromatograms of preparations A and B after two-step desorption of peptides (see Fig. 2) on Sephadex G-50 eluted with 0.1 N sodium chloride. Eluting solution flow rate was 3.0 ml · h^{-1} · cm^{-2} of column cross-section. Arrows above plots show the retention volumes of the reference components: *1*) cytochrome c (11.6 kDal), *2*) thymalin (5 kDal); *3*) vasopressin (1 kDal); *4*) tryptophan (204 Dal).

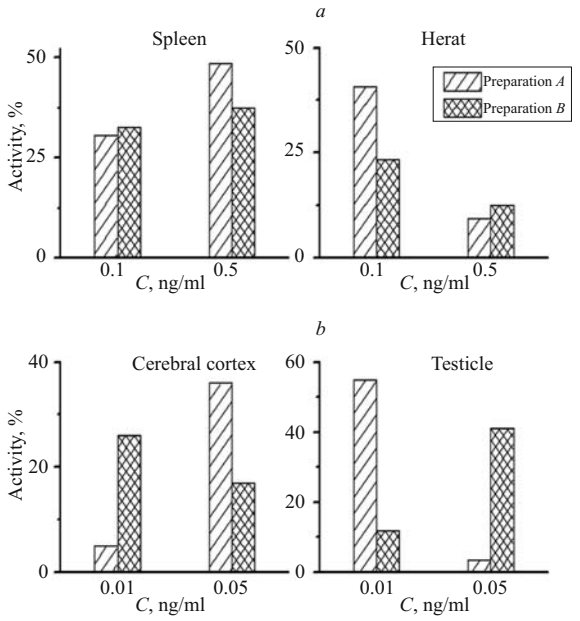

Fig. 4. Stimulation of cell proliferation in different tissues by regulatory acidic (*a*) and alkaline (*b*) peptides from sea urchin eggs ($p < 0.05$).

– neutral and alkaline – for peptide displacement yielded a group separation of peptides in terms of their electrochemical characteristics. The first peak (*A*) desorbed from the column at pH 4.0 – 4.4, i.e., in conditions in which dissociation of the protein carboxyl groups of glutamic and aspartic acids started to occur. This peak contained desorbed peptides with structures dominated by glutamic and aspartic acid residues. The second peak, at pH 9.5 – 10.5, contained alkaline peptides desorbed in that pH range due to the onset of deprotonation of the amino groups of lysine and arginine residues [6, 10]. Table 1 shows mean values for the complex sea urchin egg peptide preparation.

Figure 3 shows gel chromatograms of peptides in the first and second peaks desorbed from the cation exchange resin. These provide evidence that these peaks lack components with molecular weights exceeding 1200 Dal and that the first peak, but not the second, contains aromatic amino acids (optical density at 274 nm).

Peptide preparations obtained after drying the first and second peaks were used to evaluate their effects on the development of explants in organotypic cultures of various tissues. Figure 4 presents data on the stimulating actions of thee preparations on spleen, testicle, ovary, myocardium, and cerebral cortex explants. At ulatralow concentrations, alkaline peptides had the most marked effects on cerebral cortex explants, while acid peptides had the greatest effects on testicle explants. Stimulation of cell proliferation in the lymphoid tissue of the spleen showed little dependence on peptide acidity or concentration. These preparations had regulatory activity in relation to reproductive system tissues, stimulating proliferation in testicle tissues and inhibiting proliferation in ovary tissues (not shown). In the latter case, reductions in the numbers of cells in explant growth zones appeared to result from an increase in apoptosis in ovary tissues [12].

REFERENCES

1. V. Kh. Khavinson and V. N. Anisimov, *Peptide Bioregulators and Aging* [in Russian], Nauka, St. Petersburg (2003).
2. V. T. Ivanov and Yu. A. Berlin, *Bioorgan. Khim.*, **26**(10), 752 – 755 (2000).
3. D. Safer and P. K. Chowrashi, *Cell Motility and Cytoskeleton*, **38**(2), 163 – 171 (1997).
4. S. Butylina, L. K. Shataeva, and M. Nystrom, *Separat. Purific. Technol.*, **53**(1), 64 – 70 (2007).
5. P. L. Stewart, M. Makabi, J. Lang, et al., *BMC Dev. Biol.*, **5**(3), (2005).
6. J. Luan, Z. Liu, S. Zhang, et al., *Acta Biochim. Biophys. Sinica*, **49**(4), 255 – 264 (2007).
7. S. Karlin, J. Mrázek, and A. M. Campbell, *Nucl. Acid Res.*, **24**(12), 4263 – 4272 (1996).
8. M. Laub, J. A. Steppuhn, M. Bluggel, et al., *Eur. J. Biochem.*, **255**(2), 422 – 431 (1998).
9. C. T. Mant, L. H. Kondejewski, and R. S. Hodges, *J. Chromatogr.*, **816**(1), 79 – 88 (1998).
10. S. Melis, J. Marcos, G. Cao, et al., *Industr. Engineering Chem. Res.*, **35**(6), 1912 – 1920 (1996).
11. L. Mazor, *Methods in Organic Analysis* [in Russian], Mir, Moscow (1986).
12. N. I. Chalisova, V. A. Penniyaynen, A. V. Komashnya, et al., *Dokl. Ros. Akad. Nauk.*, **406**(1), 1 – 4 (2006).