Romanian Biotechnological Letters *Vol. 15, No.5, 2010*

ORIGINAL PAPER

Certain bioactive effects of complexes rich in glycosaminoglycans obtained from small sea fish

Received for publication, November 10, 2009
Accepted, September 10, 2010

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Abstract

The extracts rich in glycosaminoglycans (GAGs) were obtained through a proper-patented technology from small sea fish (Sprattus sprattus sprattus, Odontogadus merlangus euxinus and Engraulis encrassicolus ponticus species).

These natural bioactive complexes demonstrate a significant inhibition action against hyaluronidase, collagenase and elastase, the enzymes implicated in the pathology of conjunctive, cartilaginous and bone tissues and favor the in vitro formation of collagen fibrils, these biochemical processes being dependent of the bioactive compound system concentration.

Also, their high content in sulfated glycosaminoglycans (44%), essential amino acids, essential fatty acids and microelements such as Ca, Na, K, Fe, Mg, Se, Ni, Cu, Si, make the bioactive extracts from small see fish useful to prevent the unsettle of the macromolecular structure and keep the functionality of the extracellular matrix from conjunctive, cartilaginous and bone tissue.

Introduction

Our researches have the purpose of obtaining active biological substances from sea organisms for the elaboration of pharmaceuticals with high efficiency and minimum side effects.

Sea organisms arouse a major interest for the extraction of biological active substances with multiple and valuable therapeutically applications all over the world as discussed by Kornprost [1]; Şerban and Roşoiu [2].

The work provides for the elaboration of an original extraction and purification technology of glycosaminoglycans from sea organisms (small sea fish) and their chemical, biological and pharmacological analysis together with in *vitro* and *in vivo* tests, for the purpose of extracts conditioning towards their use as pharmaceutical products (unguents, pastilles) with anti-inflammatory activity, tissue restitution properties, anticlotting and antithrombothic activities, biostimulating, antioxidative and antiproliferative qualities.

There is a global demand towards the development of new therapeutically products based on biocomplexes which will improve the efficiency / toxicity report of some therapies for patients with major illnesses and not merely. The biotechnological researches towards the design of pharmaceutical products are responding to the need of improving and developing new therapeutic strategies. Accordingly, the work is proposing to do a thorough study on biotechnologies of new bioactive complexes, which will be tested *in vitro* and *in vivo* for therapeutically properties.

Material and Methods

The bioactive complexes rich in glycosaminoglycans were obtained from small sea fish (*Sprattus sprattus sprattus*, *Odontogadus merlangus euxinus* and *Engraulis encrassicolus ponticus species*), according to self-developed patented technologies.

The physico-chemical analysis of the glycosaminoglycans rich biocomplex was done with modern methods, as potentiometric titration with phototron (US Pharmacopoiea 30 [3]), horizontal electrophoresis on cellulose-acetate foil, vertical poliacrilamide gel electrophoresis, UV-VIS spectrophotometrie and GC-MS (Homer & al. [4]; Schagger and Jagow [5], Sim & al. [6], Thanawiroon & al. [7], Van de Lest & al. [8], Volpi [9], Zaia & al. [10]).

In this paper *in vitro* models were applied for studying therapeutic applications of glycosaminoglycans complexes. Thus, in the acellular system, were studied:

- Enzymatic activity of hyaluronidase;
- Enzymatic activity of collgenase;
- Enzymatic activity of human fibroblast elastase (metalloproteinase 12);
- Collagen fibrils formation.

Determination of hyaluronidase enzymatic activity

The enzymatic activity of hyaluronidase was evaluated with N-acetyl-glucosamine spectrophotometric method. The hyaluronic acid is cleaved by hyaluronidase in the presence of hyaluronidase, liberating N-acetyl-glucosamine residues, which are measured at 585 nm wavelengths. For the evaluation of N-acetyl-glucosamine quantity formed as consequence of hyaluronidase activity (implicitly of its enzymatic activity) it is necessary to do calibration curves which correlate the N-acetyl-glucosamine quantity with the probes absorbance at 585 nm (the wavelength value where the compound formed by N-acetyl-glucosamine and 4-dimethylaminobenzaldehyde has its maximum of absorbance).

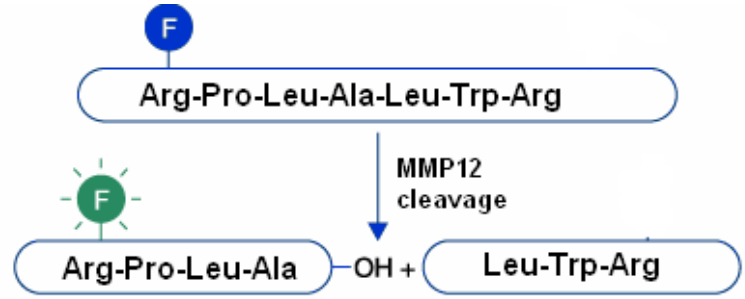
Determination of collagenase enzymatic activity

The residual influence on collagenase activity was determined with a continuously recording spectrophotometric assay, using 2-furanacryloyl-L-leucylglycyl–L-prolyl-L-alanine (FALGPA) and collagenase-specific substrate that is hydrolyzed more rapidly than any other synthetic substrate, but is not cleaved by trypsin, thermolysin or elastase. The procedure involves measurement of decrease in absorbance of FALGPA at its absorption maxim of 345 nm.

$$FALGPA \xrightarrow{Collagenase} FAL + Gly - \Pr o - Ala$$

Determination of human fibroblast elastase (metalloproteinase 12) activity

The peptide 7-methoxicumarin-4-acetyl-Arg-Pro-Leu-Ala-Leu-Trp-Arg-L-α,β-diaminopropionil(2,4dinitrofenil)amide has fluorescent properties which are given by 7-methoxy cumarine cleaved by metalloproteinase 12 at Ala-Leu bound level; this cleavage determines a shift in the fluorescent intensity as fluorescent energy of cumarinic peptide derivate accumulation, measured by fluorimetry.



Determination of collagen fibrils formation

This in vitro experimental method, made in acellular conditions, reproduces the process of collagen fibrils formation morphologically identical with the ones which are synthesized in tissue cell culture. In order to evaluate the effects on collagen matrix, the collagen solution turbidity increase is followed up.

Results and Discussion

The cells of the tissues of human body are incorporated in the ground substance, which enters in any intermediary space and isolates each stationary cell of its neighborhood. The ground substance has to be crossed over by each molecule, which enters or leaves the cell. The variations of the extracellular environment composition deeply influence cellular behavior and in their turn the cells have a strong ability of modifying their intracellular environment. This interdependence is implied in all forms of cell proliferation and inflammation.

The intercellular substance is a complex gel containing water, electrolytes, metabolites, and dissolved gases, microelements in infinitesimal quantities, vitamins, enzymes, carbohydrates, lipids, and proteins. The solution is strongly viscous due to the plenty of macromolecules: acidic mucopolysaccharides with long chain strengthen at microscopic level by a three-dimension network of collagen fibers. An important feature of the intercellular substance is its very high viscosity and cohesion. This feature depends on the chemical integrity of the large molecules. The viscosity can be reduced and structural integrity destroyed by the depolymerizing action of the enzyme called hyaluronidase.

Few years ago, as discussed by Cameron [11] and Lentner [12], a hypothesis that all forms of cellular proliferation and inflammation depend of interaction between the cell and the surrounding environment, has been advanced:

- The cells in the body are incorporated (included) into a strongly viscous environment of ground substance, which physically rejects their inherent tendency to proliferate;
- The proliferation is initiated by the release of hyaluronidase from the cells, which catalyses the hydrolysis of mucopolysaccharides from the immediate environment and allows the cells liberty of division and migration in the alteration limits;
- The proliferation continues as long as the hyaluronidase is set free and stops when the production of hyaluronidase ceases or when the hyaluronidase is inhibited and the environment is allowed to get its normal restriction state.

In the degenerative processes, the acidic mucopolysaccharides (hyaluronic acid and chondroitinsulphates) are intensely depolymerized by hyaluronidase, the matrix-ground substance of the connective and cartilaginous tissues, tissular “binder”, intercellular “cement” being destroyed. Due to the reduction of the molecules dimensions, the viscosity of hyaluronic acid and chondroitinsulphates is reduced. Thus, the penetration of some compounds or foreign bodies into intercellular spaces is possible. The release of hyaluronidase by the bacteria in the infected tissues facilitates their spreading within the organism.

The cellular proliferation process is controlled by the presence in the tissues and in blood of the substance called physiological hyaluronidase inhibitor (PHI). The serum PHI (antihyaluronidase) exists in a normal, well determined concentration in healthy state.

The treatment of degenerative rheumatic diseases consists in (Roşoiu and Lascarache [13]; Roşoiu and Şerban [14]):

1. Surgery: sometimes the total arthroplasty is indicated;
2. Medicinal:
   - Through the strengthening of the ground substance by increasing its resistance to hyaluronidase (through the administration of corticosteroids: estrogens, androgens; thyroxine); or through the administration of AINS-drugs (rather short-term treatment) - synthesis drugs;
   - With drugs that should bring about profactors (precursors) of the aggregates from the ground substance and/or PHI (anti-hyaluronidase).

The physico-chemical analysis of different batches obtained in laboratory revealed that the obtained extracts from small see fish represent a complex of active biological substances composed of glycosaminoglycans (40-60% sulfated form), amino acids 3.5-12%, from which 2-6.5% essential amino acids (valine, leucine, isoleucine, threonine, methionine, lysine, phenylalanine, tryptophan), essentials fatty acids 1-2% (linoleic acid, arachidonic acid). There were identified glicerophosphates, creatinin, mineral salts (calcium, sodium, potassium, iron, magnesium, selenium, nickel, copper, silicon) (Roşoiu & al. [15]).

The hyaluronidase, collagenase and human fibroblasts elastase (MMP12) activities and collagen fibrils formation in acellular systems were investigated in the presence of some extracts (extract A and extract B), obtained through two variant procedures.

Determination of hyaluronidase enzymatic activity

Hyaluronidase acts on hyaluronic acid (and condroitinsulfates), producing depolymerization through β- glycosides bonds cleavage (β → 1.3) to simple structural units and, finally, releaseing of glucuronic acid and N-acetilglucosamine.

Hyaluronic acid is part of the connective tissue (being the main component the basic substance). It is found in cartilage, cornea, in vitreous humor, in sinovial fluid, in the umbilical cord, etc. and fulfill the role of tissue binding, serving as "intercellular cement". In plasma there are some hyaluronidase inhibitors with role in blocking the fundamental substance depolymerization. These inhibitors are called antihyaluronidase (PHI) (Şerban and Roşoiu [16]).

The hyaluronic acid-hyaluronidase system has great importance in connective tissue metabolism and its pathology.

The enzymatic activity of hyaluronidase was determined in the presence of extracts A and B and results are presented in Table 1.

Tabel 1. Hyaluronidase enzimatic activity in the presence of the rich glycosaminoglycans extracts A and B

Substance	Concentration (µg/ml)	Enzymatic activity	% Inhibition
Extract A	**5**	**1.857**	**4.7**
	10	**1.615**	**12.8**
	20	**1.203**	**27.1**
	40	**0.269**	**51.3**
	80	**0.718**	**61.8**
Extract B	**5**	**1.683**	**10.6**
	10	**1.585**	**15.8**
	20	**1.108**	**34.2**
	40	**0.394**	**79.1**
	80	**0.143**	**92.4**
Without active biological compounds	**-**	**1.882**	**-**

Reaction conditions: $T_{incubation} = 37^0C$, $t_{incubation} = 45$ *min,* $\lambda = 585$ *nm,*

Both extracts show an inhibition of the hyaluronidase enzyme activity in a dose-dependent manner. At concentration of 80 mg/ml the extract A produces a complete inhibition of the enzyme (92.4%) while the extract B produces only 61.8% inhibition.

Experimental data show that extracts provide protection against hyaluronidase, the IC_{50} values being 32.4 mg/ml for extract A and 48.3 mg/ml for extract B.

Determination of enzymatic activity of extracellular matrix metalloproteinases

Metalloproteinases from extracellular matrix (MMP) are zinc-dependent endopeptidases able to degrade all types of extracellular matrix proteins and other bioactive molecules. These enzymes are involved in cellular division of surface receptors, in the release of apoptosis ligands and in chemokine activation/inactivation. MMPs are considered as playing a major role on cell proliferation, migration (adhesion/dispersion), differentiation, angiogenesis, apoptosis and host defense.

Metalloproteinases from extracellular matrix play an important role in tissue remodeling associated with various physiological or pathological processes such as tissue repair, cirrhosis, arthritis and metastasis (Parsons & al. [17]).

Metalloproteinases activity was investigated in the presence of extracts A and B, and results are presented in Table 2 and Figures 1 and 2.

Determination of collagenase enzymatic activity (MMP1)

Collagen is the main protein of connective tissue, ligaments, tendons, cartilage and skin in animals and the most abundant protein in mammals, making up about 25% to 35% of the whole body protein content. Collagen constitutes 1% to 2% of muscle tissue, and accounts for 6% of the weight of strong, tendinous muscle. Collagen is degraded *in vivo* and *in vitro* by specific enzymes named collagenases, initially considered of bacterian origin (*Clostridium*) and later identified in animal and human tissues, produced by epithelial and endothelial cells (Şerban and Roşoiu [16]).

Tabel 2. Collagenase enzymatic activity

Substance	Concentration (µg/ml)	Enzymatic activity (Units/mg protein)	% Inhibition
Extract A	40	1.204	26.81
	80	1.121	31.85
Extract B	40	1.034	37.14
	80	0.992	40.01
Without active biological compounds	-	1.645	-

Reaction conditions: T = 25^{0}C, pH 7.5, λ = 345 nm, t = 5 min.

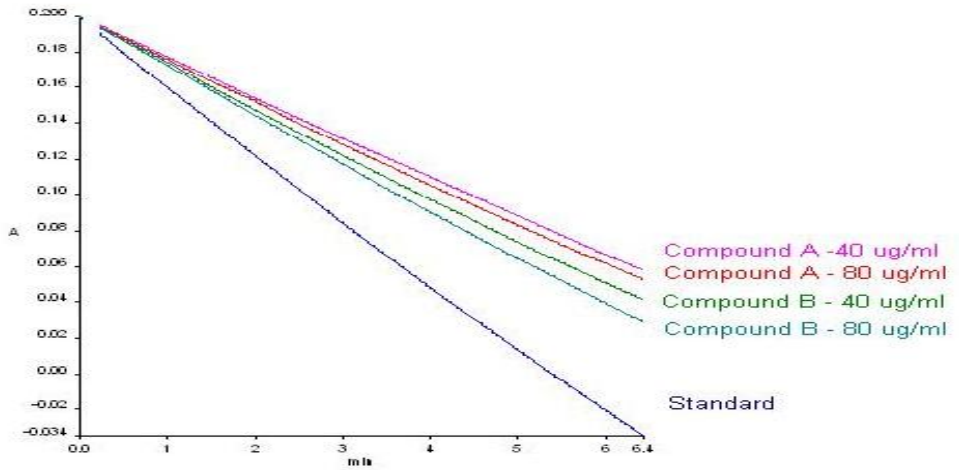


Figure 1. Hydrolyzing in time FALGA by collagenase in the presence of tested compounds.

Determination of human fibroblast elastase (MMP12 activity

Elastins are components of the elastic fibers of arteries and tendons showing elastic and termoelastic properties. They are very resistant to the action of acids and common proteolytic enzymes but are degraded by elastase, a pancreatic specific enzyme (Şerban and Roşoiu [16]).

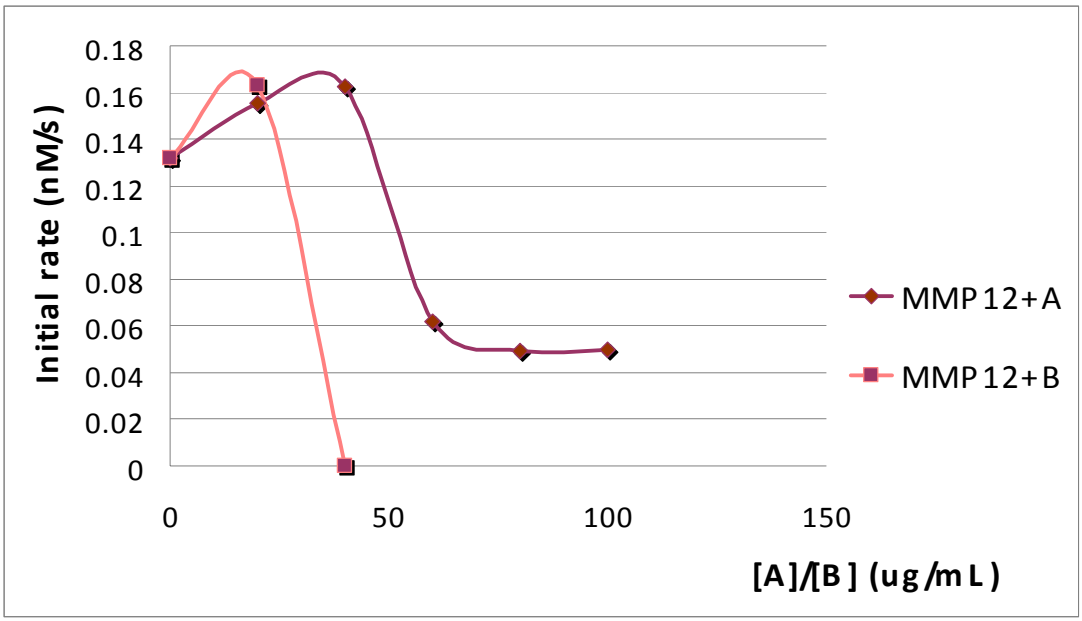


Figure 2. MMP12 enzymatic activity in the presence of extract A and B
(λex = 328 nm, λem = 393 nm, range = 5 - 15 minutes)

As seen from Figure 2, the two glycosaminoglycans extracts obtained from small marine fish in concentrations of 20 ÷ 100 mg/ml induce a significant decrease of elastase activity (MMP12). It was observed also a significant decrease of colagenase activity (MMP1) induced by concentrations of the extracts of 40 ÷ 80 mg/ml.

Determination of collagen fibrils formation

Collagen fibrils formation is a self-assembly process that can be modulated by a variety of macromolecules which include some glycozaminoglycans, proteoglycans and glycoproteins. These macromolecules modulate the kinetics of assembly and the diameter of collagen fibrils.

Perlecan, is a proteoglycan containing heparansulfate and is one of the components that ensure the integrity of basement membrane, being expressed outside of it (*e.g.* in cartilage). Although identified as a heparan sulfate proteoglycan, the perlecan can be partially substituted with chondroitinsulfate. Couchman & al. [18]; Govindraj & al. [19]); Kvist & al. [20], have demonstrated that perlecan is involved in assembling cartilage matrix by increasing collagen fibrils formation. Moreover, they showed that *in vitro,* the enhancement rate of fibrils formation of collagen type II and I is a dependent process of the condroitinsulphate content, whereas heparansulphate had any effect.

The *in vitro* results demonstrate that the bioactive complex rich in glycozaminoglycans is involved by stimulation in collagen fibrils formation. As seen in figures 3-6, this process is dependent on temperature and on the concentration of bioactive complex.

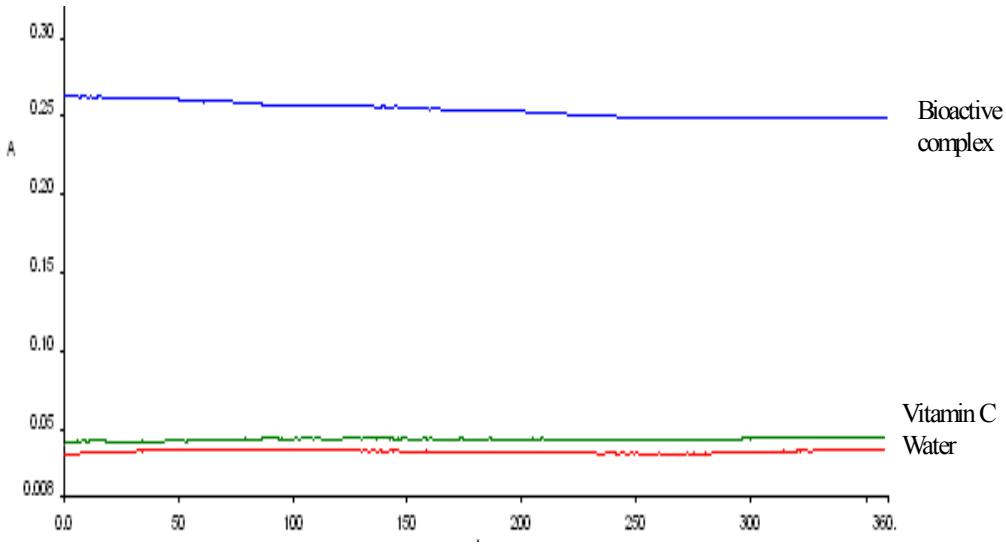


Figure 3. Collagen fibrils formation at 26^{0}C for 360 min

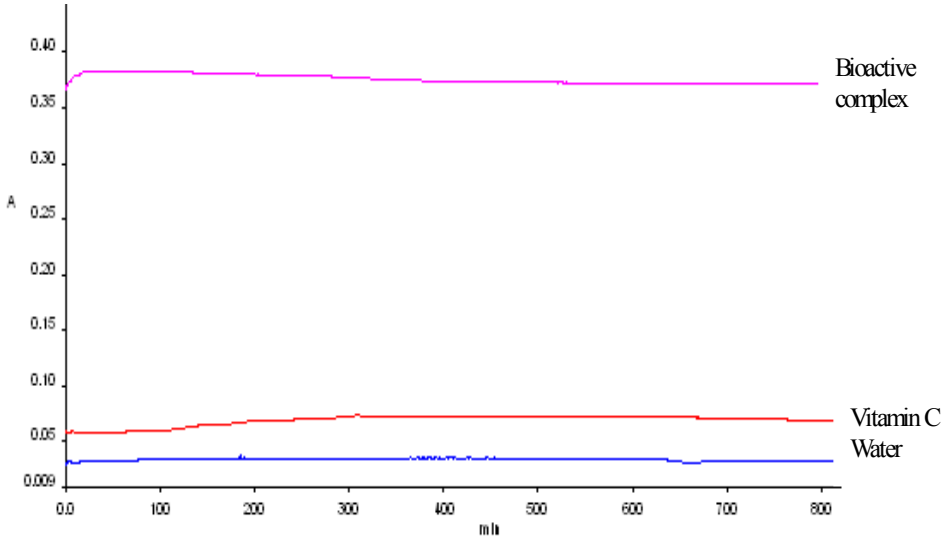


Figure 4. Collagen fibrils formation at 26^{0}C for 800 min

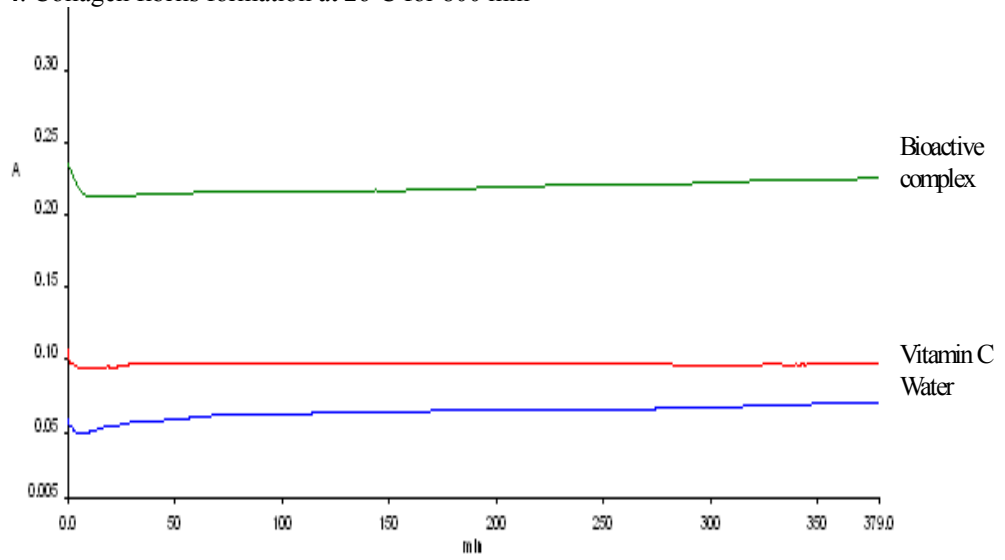


Figure 5. Collagen fibrils formation at 37^{0}C for 360 min

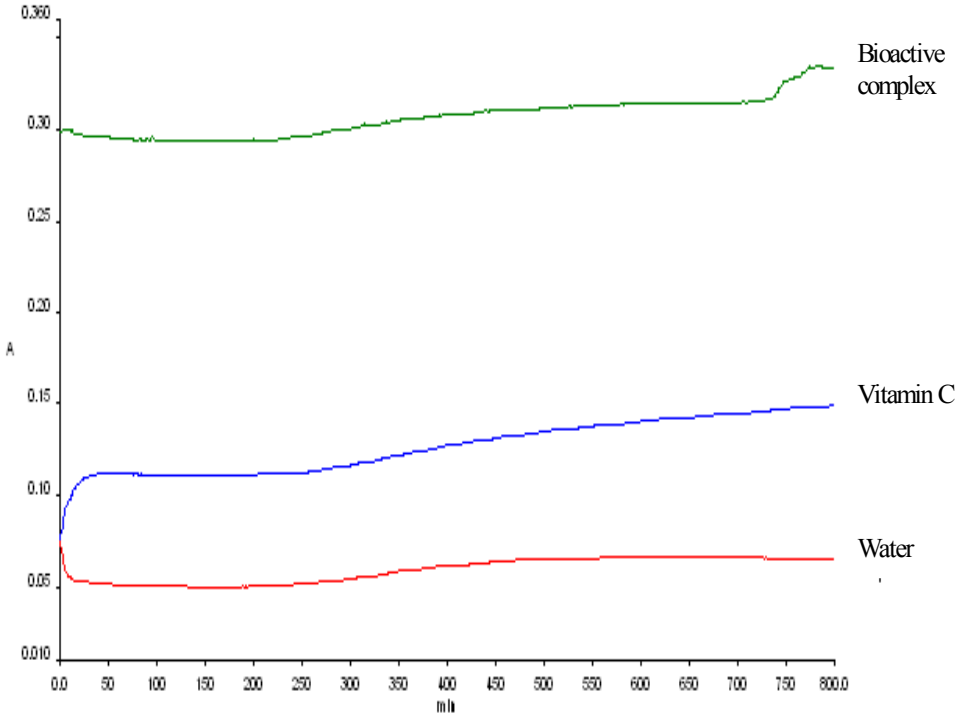


Figure 6. Collagen fibrils formation at 37^0C for 800 min

In the figure 7 is presented the stimulation process of collagen fibrils formation in the presence of bioactive complex A vs. vitamin C.

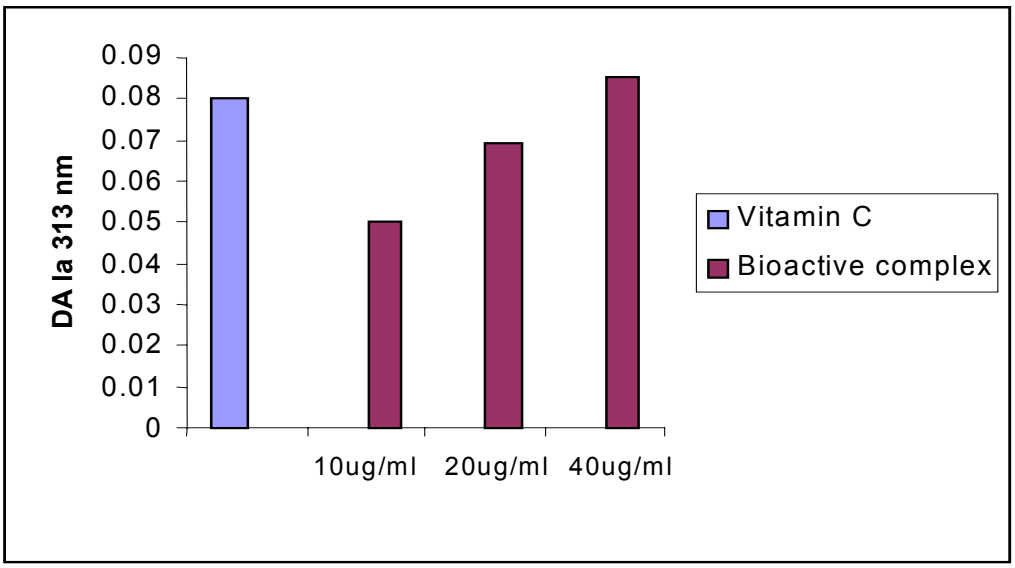


Figure 7. Collagen fibrils formation in the presence of bioactive complex A vs. vitamin C

From the figure 7 is observed that bioactive complex A manifests a comparable positive influence compared to collagen fibrils formation with vitamin C.

Experimental data were statistically processed and the results are presented as mean ± SD of six parallel determinations. Significant statistical difference between the sets of analytical results was calculated with Student t-test (Table 3).

Table 3. The effect of extracts A and B on collagen fibrils formation

Substance	Concentration µg/ml	ΔAbsorbance
Control	-	0.003 ± 0.0006
Positive control - Vitamin C	83	0.127 ± 0.012
	83	0.013 ± 0.006$^{*, a, b}$
Extract A	250	0.043 ± 0.003^{*b}
	333	0.103 ± 0.010**

$^{*}p<0.001$, $^{**}p<0.05$; (vs. control); $^{a}p<0.01$; $^{b}p<0.001$.(vs. Vitamin C)

Statistical analysis of the results obtained in the *in vitro* experiments carried out in order to highlight the ability of extracts to promote collagen fibrils formation and the inhibitory effect on hyaluronidase and collagenase activity enzymes show that (Table 4):

- the significant difference between Vitamin C and extract A can be diminished by increasing the concentration of extract A in the reaction medium and
- there is statistically significant decrease of the activity of these enzymes (depending on the dose applied) in the presence of both extracts.

Table 4. The effect of extracts A and B on the hyaluronidase and collagenase enzymatic activity

Substance	Concentration (µg/ml)	Hyaluronidase activity	Collagenase activity (Units/mg protein)
Control	-	1.887 ± 0.006	1.648 ± 0.007
Extract A	5	1.827 ± 0.020	-
	10	1.614 ± 0.005*	-
	20	1.210 ± 0.011*	-
	40	0.711 ±0.009*	1.205 ± 0.005**
	80	0.266 ± 0.008*	1.122 ± 0.004**
Extract B	5	1.684 ± 0.017	-
	10	1.582 ± 0.012*	-
	20	1.109 ± 0.009*	-
	40	0.392 ± 0.008*	1.033 ± 0.005**
	80	0.150 ± 0.006*	0.992 ± 0.005**

$^{*}p<0.05$, $^{**}p<0.001$ (vs. control)

Conclusions

- Both extracts show a dose dependent inhibition of hyaluronidase.
- The two glycosaminoglycans extracts obtained from small sea fish in concentrations of 20÷100 mg/ml and 40÷80 mg/ml induce a significant decrease of elastase (MMP12) and collagenase (MMP1) enzymatic activity.
- Applying the statistical analysis, we found a significant decrease of hyaluronidase and collagenase activity (depending on the dose applied) in the presence of both extracts.
- The bioactive complexes rich in glycozaminoglycans obtained small sea fish, favor the *in vitro* collagen fibrils formation and this process is dependent on temperature and the concentration of bioactive complex.
- Bioactive extract A manifests a stimulation process on collagen fibrils formation comparable with vitamin C.

Taking into consideration the presented results, the chemical composition (glycozaminoglycans, essential amino acids, essential fatty acids, micro-elements: Ca, Na, K, Fe, Mg, Se, Ni, Cu, Si), of the bioactive complexes obtained from small sea fish and the therapeutic effects highlighted by *in vitro* experiments show that these extracts can be

conditioned and used successfully in the form of medicinal products with valuable therapeutic properties and minimal side effects.

In another paper, which is in phase of elaboration, the authors present the *in vitro* antioxidant activity and *in vivo* anti-inflammatory effect of the bioactive extracts obtained from small sea fish through a patented technology.

Acknowledgments

This work was supported by CNMP PNCDI 2 research program 4, Project 6.1.-043/2007-2010

References

1. J.M. KORNPROBST, Substances naturelles d'origine marine, Editions TEC & DOC, London-Paris-New York, (2005).
2. M. SERBAN, N. ROSOIU, Substante biologic active din organisme marine (Biological active substances from marine organisms), Ed. Romanian Academy, Bucharest, (1992).
3. ***US Pharmacopoeia 30, (2007).
4. K.A. HOMER, L. DENBOW, D. BEIGHTON, Spectrofotometric method for the assay of glycosaminoglycans and glycosaminoglycan-depolimerizing enzymes, Analitical Biochemistry, 214, 435-441, (1993).
5. SCHAGGER, JAGOW, Tricine-SBS-poliacrilamide gel electrophoresis for separation of proteins in the range 1 to 100 kDa, Anal. Biochemistry., 166, (1987).
6. J.S. SIM, G. JUN, T. TOIDA, S.Y. CHO, D.W. CHOI, S.Y. CHANG, R.J. LINHARDT, Y.S. KIM, Quantitative analysis of chondroitin sulfate in raw materials, ophtalmic solution, soft capsules and liquid preparations, J. of Chromatography B, 818, 133-139, (2005).
7. C. THANAWIROON, K.G. RICE, T. TOIDA, R.J. LINHARDT, Liquid Chromatography/Mass Spectrometry Sequencing Approach for Highly Sulfated Heparin-derived Oligosaccharides, J. Biol. Chem., Vol. 279, 4, 2608-2615, (2004).
8. C.H.A. VAN de LEST, E.M.M. VERSTEEG, J.H. VEERKAMP, T.H. Van KUPPEVELT, Quantification and characterization of glycosaminoglycans at the nanogram level by a combined azure-A-silver staining in agarose gel, Analytical Biochemistry, 221, 356-361, (1994).
9. N. VOLPI, Electrophoresis separation of glycosaminoglycans on Nitrocellulose membranes, Analitical Biochemistry, 240, pp.114-118, (1996).
10. J. ZAIA, C.E. COSTELLO, Compositional analysis of glycosaminoglycans by electrospray mass spectrometry, Analytical Chemistry, 73 (2), A-G, (2001).
11. E. CAMERON, Hyaluronidase and cancer, Pergamon Press, New York, (1966).
12. C. LENTNER, Biochemistry, Metabolism of xenobiotics. Inborn Errors of Metabolism, Pharmacogenetics and Ecogenetics, Ciba-Geigy Corporation, West Caldwell, New Jersey, vol.4, p. 182, (1986)
13. N. ROSOIU, Gh. LASCARACHE, ALFLUTOP- a natural extraction drug used for the treatment of degenerative diseases, Rev.Roum.Biochim., 31, 1, 57-64, (1994).
14. N. ROSOIU, M. SERBAN, The hepatic-protective action of two Romanian extraction drugs of small sea fish with prevalently antihyaluronidasic activity, Proc.Rom.Acad. Series B, Chemistry, Life Sciences and Geosciences, 2, 101-106, (2004).
15. N. ROSOIU, R. NITA, L. OLARIU, V. DRUMEA, M.D. ENE, Original bioactive complexes rich in glicosaminoglycans obtained from small fish, Roum. Biotechnol. Lett., 13, 5, 3944-3954, (2008).
16. M. SERBAN, N. ROSOIU, Biochimie Medicală (Medical Biochemistry), Volumul I: Principii de organizare moleculară (Molecular Principles of Structural Organization of Cell), Ed. Muntenia, Constanța, România, (2.003).
17. M.S.L. PARSONS, S.A. WATSON, P.D. BROWN, H.M. COLLINS, R.J.C. STEELE, Matrix metalloproteinases, British Journal of Surgery, 84, 2, 160–166, (2005).
18. J.R. COUCHMAN, R. KAPOOR, M. STHANAM, R.R. WU, Perlecan and Basement Membrane-Chondroitin Sulfate Proteoglycan (Bamacan) Are Two Basement Membrane Chondroitin/Dermatan Sulfate Proteoglycans in the Engelbreth-Holm-Swarm Tumor Matrix, J. Biol. Chem., 271, 9595–9602, (1996).
19. P. GOVINDRAJ, L. WEST, T.J. KOOB, P. NEAME, K. DOEGE, J.R. HASSELL, Isolation and Identification of the Major Heparan Sulfate Proteoglycans in the Developing Bovine Rib Growth Plate, J. Biol. Chem., 277, 19461–19469, (2002).
20. A.J. KVIST, A.E. JOHNSON, M. MORGELIN, E. GUSTAFSSON, E. BENGTSSON, K. LINDBLOM, A. ASZODI, R. FASSLER, T. SASAKI, R. TIMP, A. ASPBERG, Chondroitin Sulfate Perlecan Enhances Collagen Fibril Formation - Implications For Perlecan Chondrodysplasias, J. Biol. Chem., 281, 44, 33127-33139, (2006).