Cercetări marine	I.R.C.M.	Nr.16	235 - 253	1983

CHARACTERIZATION OF ANTIHYALURONIDASIC ACTIVITY FROM MARINE ORGANISMS

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ABSTRACT:

Bioactive preparations with an intense antihyaluronidasic activity, given by the chemical compound which enter in their composition (glycogen, hexoses, microelements and free amino acids), have been obtained from four fish species of the Black Sea: Alosa tanaica nordmanni, Odontogadus merlangus euxinus, Engraulis encrasicholus ponticus, Sprattus sprattus, from a cephalopod (Loligo pealei) and a crustacean (Euphausia superba dana), both from the Atlantic Ocean.

The data that we have presented in the previous work (5), revealed some differences of the biochemical composition at bioactive preparations obtained from four small sea fishes, from Loligo pealei and Euphausia superba dana, their antihyaluronidasic activity ranging between 73-93 U.I./ml. The preliminary data reporting on their chemical composition did not enable us, at that stage, to draw a precise conclusion concerning the active principle. The extremely small quantity of chondroitine sulphate and

the negative metachromatic reaction with toluidine blue, entitle us to suppose that the antihyaluronidasic activity found is determined by other chemical compounds.

As a continuation of these researches, in this work, we present a correlation study between the chemical composition and the antihyaluronidasic activity, at the bioactive preparations obtained from Alosa tanaica nordmanni, Odontogadus merlangus euxinus, Engraulis encrassicholus ponticus, Sprattus sprattus and small sea fishes (combination of four fish species in their natural proportions from the captures).

We have not found data concerning the sulphomucopolysaccharides and any other compounds with antihyaluronidasic activity in the species which we have studied in the available literature.

MATERIAL AND METHODS

The work was done with four fish species of the Black Sea: Alosa tanaica nordmanni, Odontogadus merlangus euxinus, Engraulis encrassicholus ponticus, Sprattus sprattus and small sea fish (combination of the four fish species in their natural proportion from the captures). The specimens were of different size, as can be found in their natural conditions.

The bioactive preparations have been obtained according to an original procedure described in the S.R.R. Patent no.82273/1983 (6).

The bioactive preparations with antihyaluronidasic activity are under the form of limpid solutions (1% active residuum) with a pH ranging between 6.0-7.0, of a colour which varies from light yellowish-white to dark brown, as a function of the pigments present in the marine organisms. They contain a mixture formed by free amino acids, peptides, sugars (glycogen, hexoses, hexosamines, pentoses, methylpentoses), microelements (Na, K, Ca, Mg, Fe, Cu, Zn) and phenol in proportion of 0.45-0.55%, as preservative.

Determinations of evaporation residue, pH, phenol, antihyaluronidasic activity, total nitrogen, peptides, free and total amino acids, sugars, glycogen, reaction for identification of pentoses, methylpentoses, hexoses, hexosamines and heptoses, ash,

mineral ions (Na, K,Ca, Mg, Pb, Cu, Cr, Cd, Zn, Fe), chondroitine sulphate, were carried on for chemical characterization of the bioactive products;the metachromatic reaction with toluidine blue was also carried on for identification of mucopolysaccharides.

Chemical determinations were performed by the following methods:

1. Evaporation residue - 1 ml solution was evaporated to dryness to constant weight in dryer at 105°C.
2. pH was determined potentiometrically.
3. Phenol - 0.1 N potassium bromate,potassium bromite and 10% sulphuric acid were added to the solution to be analysed. It was left for 15 minutes, then potassium iodide was added. After keeping for 10 minutes in the dark, it was titrated with 0.1N fixing salt until decolouring (starch indicator). 1 ml of 0.1N $KBrO_3$ corresponds to 0.001568 g phenol.
4. Determination of antihyaluronidasic activity. The principle of the method is based on the determination of hyaluronidase which remains after the action of the inhibitor, by measuring the quantity of hyaluronic acid present in the sample.The hyaluronic acid,in mixture with serum proteins (horse serum was used), precipitates giving a turbidity whose extinction is proportional to the quantity of hyaluronic acid in the sample.Under the established experimental conditions, there is a linear relation between the hyaluronidase quantity and the hydrolysed hyaluronic acid quantity. First, a hyaluronidase - calibrating diagram is drawn, then the proportion of hyaluronidase remained in the samples in which the enzyme was incubated with the tested inhibitor (5).A unit of the inhibitor is defined as the quantity of it which inactivates 50% of 2 NF units of hyaluronidase, that is the quantity of the inhibitor which, under the established experimental conditions, inhibits an NF unit of hyaluronidase.
5. Total nitrogen - was determined by Kjeldahl-Berthelot method.
6. The peptides - were determined by the Biuret reaction.
7. Total and free amino acids- were determined by paper chromatography, on Whatman No.1 paper (5).
8. Total sugars - were determined by the Kleij method.
9. Glycogen - was extracted with CCl_3COOH, then it precipitated with absolute ethyl alcohol.

10. Identification reactions of sugars (pentoses, methylpentoses, hexoses, hexosamines and heptoses)- the cysteine hydrochloride solution gives specific colours with heptoses, hexoses, pentoses, methylpentoses (4).
11. Identification reaction of hexoses- the anthrone was used because with hexoses it gives a blue, specific colour (4).
12. Ash- was determined by calcination of 10 ml solution at 480°C.
13. Mineral elements (Na, K, Ca, Mg, Pb, Cu, Cr, Cd, Fe, Zn) - were determined at Spectrophotometer with atomical absorbtion "Pye unicam" type SB 2900.
14. Chondroitine sulphate (chondroitine sulphuric acid) - was determined by addition, to 0.1 ml of studied solution, of 0.1 ml Rivanol and 5 ml pH 5.5 citrate acetate buffer solution. After 10 minutes, turbidity was determined at λ = 530 nm (5).
15. Metachromatic reaction with toluidine blue for identification of sulphomucopolysaccharides. The solution to study is adjusted to pH 7.5 with 0.5 N NaOH, after which a droplet of it is set on a paper tape, dried and then sprayed with toluidine blue solution (prepared from a mixture of methyl alcohol and crystalline acetic acid). The acid mucopolysaccharides give a blue-violet coloured spot on a light blue background.

RESULTS AND DISCUSSIONS

The analytical study performed during a research period of three years on a large number of products with antihyaluronidasic activity, obtained from the above mentioned species, shows that they are not of mucopolysaccharides nature (the metachromatic reaction with toluidine blue is negative). Though these products have an important inhibitory activity (30-100 U.I./ml), displaying very strong antiinflamatory properties, only extremely small concentrations of chondoitine sulphate were traced out, namely 0.001 - 0.002%, which makes us suppose that the antihyaluronidasic activity was found is due to other chemical compounds.

Though they are not of mucopolysaccharide nature, our products displayed an intensive antihyaluronidasic activity, superior to the sulphomucopolysaccharide products obtained by other authors from two species of molluscs Mytilus galloprovincialis and Mya arenaria, fowl small intestine and cattle duodenum (5).

Table 1

Bioactive preparation with antihyaluronidasic activity from Alosa tanaica nordmanni

1. Active residue: 1.0 g%; 10,000 µg/ml
2. pH: 6.15
3. Antihyaluronidasic activity: 73 UI/ml
4. Ash: 0.34 g%; 3,400 µg/ml
5. Mineral elements:

	Na	K	Ca	Mg	Cu	Pb	Zn	Cd	Cr	Fe
µg/mg ash	id.	id.	10.873	15.438	0.190	0	0.375	0	0	1.225
g%	id.	id.	0.037	0,053	14×10^{-6}	0	29×10^{-6}	0	0	93×10^{-6}
µg/ml	id.	id.	369.656	524.980	0.1444	0	0.285	0	0	0.9310

6. Total sugars: 0.019 g%; 190 µg/ml
7. Glycogen: 0.0088 g%; 88 µg/ml
8. Reducing sugars (hexoses,pentoses, methylpentoses): 0.0035 g%, 35 µg/ml
9. Identification of sugars compounds:
   a. Cysteine hydrochloride reaction:

Wavelength	Time in minutes				
	5	10	15	20	25
Extinction at:					
390 nm	0.409	0.401	0.400	0.448	0.397
424 nm	0.346	0.308	0.308	0.357	0.307

After 4 hours $E_{510\ nm} = 0.427$

Colour of solution is yellow-orange.

Hexoses and methylpentoses are present in solution (4).

   b. The anthrone method for hexoses identification:

The colour of anthrone solution is brown and not blue, as specific for hexoses. Different oses are present mixtured in solution (4).

Table 2

Bioactive preparation with antihyaluronidasic activity from Odontogadus merlangus euxinus

1. Active residue: 1.0 g%; 10,000 µg/ml
2. pH: 7.10
3. Antihyaluronidasic activity: 83 UI/ml
4. Ash: 0.64 g%; 6,400 µg/ml
5. Mineral elements:

	Na	K	Ca	Mg	Cu	Pb	Zn	Cd	Cr	Fe
µg/mg ash	id.	id.	14.11	14.193	0.22	0	0.25	0	0	3.05
g%	id.	id.	0.09	0.091	28×10^{-6}	0	32×10^{-6}	0	0	387×10^{-6}
µg/ml	id.	id.	903.04	908.272	0.2794	0	0.3175	0	0	3.8735

6. Total sugars: 0.075 g%; 750 µg/ml
7. Glycogen: 0.0286 g%; 286 µg/ml
8. Reducing sugars (hexoses and methylpentoses): 0.0024 g%; 24 µg/ml
9. Identification of sugars compounds:
   a. Cysteine hydrochloride reaction:

Wavelength	Time in minutes				
	5	10	15	20	25
Extinction at:					
390 nm	0.422	0.417	0.407	0.412	0.409
424 nm	0.354	0.350	0.324	0.332	0.361

After 4 hours $E_{510\ nm} = 0.572$

Colour of solution is yellow and becomes light brown. Hexoses and methylpentoses are present in solution (4).

   b. The anthrone method for hexoses identification:
   The colour of anthrone solution is brown and not blue, as specific for hexoses. Different oses are present mixtured in solution (4).

Analysing the values from 1st and 2nd tables, we found at Alosa tanaica nordmanni and Odontogadus merlangus euxinus significant differences correlated with the antihyaluronidasic activity, in what concerns the microelements and sugars content. Although in the literature it's known that the greatest microelements quantity is found to the plankton-eater fishes (Alosa tanaica nordmanni), which have a more reduced functionally activity, our values show a greater content in Ca, Mg, Cu, Zn and Fe at Odontogadus merlangus euxinus, colligated with a more intensive antihyaluronidasic activity.

At Odontogadus merlangus euxinus in which case the total sugars, glycogen and reducing sugars (hexoses, pentoses and methylpentoses) content is more increased comparatively with antihyaluronidases of Alosa tanaica nordmanni (respectively 750 µg/ml given 190 µg/ml, 286 µg/ml given 88 µg/ml and 224 µg/ml given 35 µg/ml), it can be observed an increasingly antihyaluronidasic activity (83 U.I./ml given 73 U.I./ml).

At the bioactive preparations obtained from small sea fish, Odontogadus merlangus euxinus, Engraulis encrassicholus ponticus and Sprattus sprattus, the antihyaluronidasic activity was limited between 30 U.I./ml and 100 U.I./ml (Tables 3-8).

The ash content oscillated between 770 µg/ml - 1540 µg/ml and the following chemical elements can be identified: Na, K, Ca, Mg, Fe, Cu and Zn. Pb and Cd were absent from all the samples.

The total nitrogen content was between 980 µg/ml (Engraulis encrassicholus ponticus) and 1400 µg/ml (Odontogadus merlangus euxinus).

At the bioactive preparations obtained from small sea fish Odontogadus merlangus euxinus and Engraulis encrassicholus ponticus we identified small peptides, in quantities oscillating from 1040 µg/ml to 1580 µg/ml. The signales differences in the peptides content and also their absence in some preparations obtained from the same species are due to the month variations of the biochemical composition, to the various conditions of stocking and transportation of the fishes and of the features for every specie. The kind of life, nutrition, age, the mobility degree, influence in a large measure the biochemical composition of fish which are in reciprocity relations with the abiotical conditions

of life medium, the water with the solved salts and biotical ones (nutrition and other organisms).

Table 3

Bioactive preparation with antihyaluronidasic activity from small sea fish (march-april months)

1. Active residue: 1.0 g%; 10,000 µg/ml
2. Phenol: 0.53 g%
3. pH: 6.0
4. Antihyaluronidasic activity: 55.6 UI/ml
5. Ash: 0.077 g%; 770 µg/ml
6. Mineral elements: Na,K, Ca, Mg, Fe, Cu and Zn have been identificated. Pb, Cd, Cr absent.
7. Total nitrogen: 0.131 g%; 1,306 µg/ml
8. Small peptides: 0.496 g%; 4,960 µg/ml
9. Total amino acids: 0.9730 g%; 9,730 µg/ml

1. cystine+cysteine: 0,065 g%
2. lysine: 0.027 g%
3. histidine: 0.056 g%
4. arginine: 0.028 g%
5. serine: 0.005 g%
6. aspartic acid: 0.059 g%
7. glycocoll: 0.009 g%
8. glutamic acid: 0.030 g%
9. alanine: 0.216 g%
10. proline: 0.061 g%
11. γ aminobutyric acid: 0.043 g%
12. tyrosine: 0.016 g%
13. valine: 0.039 g%
14. phenylalanine: 0.233 g%
15. leucine: 0.086 g%

10. Free amino acids: 0.4800 g%; 4800 µg/ml

The same amino acids, as to the 9th point have been identificated, only quantitatively differences have been registered.

11. Total sugars: 0.0235 g%; 235 µg/ml
12. Glycogen: 0.158 g%; 158 µg/ml
13. Reducing sugars (hexoses and pentoses): 0.0077 g%; 77 µg/ml
14. The sugars compounds identification:

a. The cysteine hydrochloride reaction:

Wavelength	Time in minutes 5	10	15	20	25
Extinction at:					
390 nm	0.385	0.309	0.302	0.291	0.297
424 nm	0.309	0.247	0.240	0.235	0.229
Differences	0.076	0.062	0.062	0.056	0.052

	1 ml of distilled water was added: 3	6	9	12	15	18
Extinction at:						
390 nm	0.205	0.202	0.202	0.204	0.199	0.207
400 nm	0.185	0.183	0.181	0.183	0.176	0.182

After 3 hours $E_{510\ nm} = 0.172$

The colour of solution is yellow-brown, stable in time. Only hexoses and pentoses are present. Pentoses are in a greater quantity (4).

Table 4

Bioactive preparation with antihyaluronidasic activity from small sea fish (june month)

1. Active residue: 0.996 g%; 9960 µg/ml
2. pH: 6.3
3. Phenol: 0.55 g%
4. Antihyaluronidasic activity: 100 UI/ml
5. Ash: 0.080 g%; 800 µg/ml
6. Mineral elements: Na,K, Ca, Mg, Fe, Cu and Zn have been identificated. Pb, Cd, Cr - absent.
7. Total nitrogen: 0.129 g%; 1290 µg/ml
8. Small peptides: 0.528 g%; 5280 µg/ml
9. Total amino acids: 0.528 g%, 5280 µg/ml
   1. cystine+cysteine: 0.0849 g%
   2. lysine : 0.0406 g%
   3. histidine : 0.0286 g%
   4. arginine : 0.0318 g%
   5. serine : 0.0316 g%
   6. aspartic acid : 0.1000 g%
   7. glycocoll : 0.0251 g%
   8. glutamic acid : 0.0300 g%
   9. alanine: 0.2310 g%
   10. proline: 0.0346 g%
   11. γaminobutyric acid:0.0121g%
   12. tyrosine: 0.0334 g%
   13. valine : 0.0459 g%
   14. phenylalanine: 0.0805 g%
   15. leucine : 0.0930 g%
10. Free amino acids: 0.3751 g%; 3751 µg/ml
11. Total sugars: 0.0318 g%, 318 µg/ml
12. Glycogen: 0.0178 g%; 178 µg/ml
13. Reducing sugars (hexoses and pentoses): 0.014 g%; 140 µg/ml
14. Identification of sugars compounds:
    a. Cysteine hydrochloride reaction:

Wavelength	Time in minutes				
	5	10	15	20	25
Extinction at:					
390 nm	0.518	0.404	0.388	0.378	0.371
424 nm	0.457	0.392	0.378	0.365	0.362
Differences	0.061	0.012	0.010	0.013	0.009

	1 ml of distillated water is added					
	3	6	9	12	15	18
Extinction at:						
390 nm	0.245	0.242	0.238	0.235	0.228	0.230
400 nm	0.234	0.230	0.226	0.222	0.218	0.216

After 3 hours E_{510} = 0.235.

The colour of solution is yellow-brown, stable in time. Only hexoses and pentoses are present. Pentoses are in a greater proportion. The sample does not content methylpentoses and heptoses (4).

Table 5

Bioactive preparation with antihyaluronidasic activity from Sprattus sprattus (may month)

1. Active residue: 1.0 g%; 10,000 µg/ml
2. pH: 6.0
3. Phenol: 0.50 g%
4. Antihyaluronidasic activity: 30 UI/ml
5. Ash : 0.154 g%; 1540 µg/ml
6. Mineral elements: Na,K, Ca, Mg, Fe, Cu and Zn have been identificated. Pb, Cd, Cr - absent.
7. Total nitrogen: 0.113 g%; 1,130 µg/ml
8. Peptides: absent
9. Total amino acids: 0.995 g%; 9950 µg/ml

1. cystine+cysteine: 0.079 g%	9. alanine: 0.389 g%
2. lysine: 0.040 g%	10. proline: 0.015 g%
3. histidine: 0.001 g%	11. γaminobutyric acid: 0.124 g%
4. arginine : 0.035 g%	12. tyrosine: identificated
5. serine: 0.001 g%	13. valine: 0.032 g%
6. aspartic acid: 0.001 g%	14. phenylalanine: 0.167 g%
7. glycocoll: 0.002 g%	15. leucine: 0.021 g%
8. glutamic acid: 0.048 g%	

10. Free amino acids: 0.955 g%; 9550 µg/ml
The traced out amino acids from the 9th point were exclusively in a free form.
11. Total sugars:0.0419 g%; 419 µg/ml
12. Glycogen: 0.0319 g%; 319 µg/ml
13. Reducing sugars (hexoses and pentoses): 0.010 g%; 100 µg/ml
14. The sugars compounds identification:

a. Cysteine hydrochloride reaction:

Wavelength	Time in minutes				
	5	10	15	20	25
Extinction at:					
390 nm	0.607	0.592	0.579	0.566	0.555
424 nm	0.439	0.454	0.429	0.422	0.416
Differences	0.168	0.138	0.150	0.144	0.139

	1 ml of distillated water is added					
	3	6	9	12	15	18
Extinction at:						
390 nm	0.416	0.409	0.407	0.396	0.397	0.386
400 nm	0.377	0.372	0.370	0.358	0.362	0.348

After 3 hours $E_{510\ nm} = 0.179$.

The colour of solution is yellow-brown, stable in time. Only pentoses and hexoses are present in solution. Pentoses are in a greater proportion. The sample does not content methylpentoses and heptoses (4).

Table 6

Bioactive preparation with antihyaluronidasic activity from Sprattus sprattus (june month)

1. Active residue: 0.934 g%; 9340 µg/ml
2. pH: 6.0
3. Phenol: 0.51 g%
4. Antihyaluronidasic activity: 50 UI/ml
5. Ash: 0.152 g%; 1520 µg/ml
6. Mineral elements: Na,K, Ca, Mg, Fe, Cu and Zn have been identificated.Pb, Cd, Cr - absent.
7. Total nitrogen: 0.106 g%, 1056 µg/ml
8. Peptides: absent
9. Total amino acids: 0.937 g%; 9370 µg/ml

1. cystine+cysteine:0.072 g%	9. alanine: 0.192 g%
2. lysine: 0.142 g%	10. proline: 0.056 g%
3. histidine: 0.054 g%	11. γ aminobutyric acid: 0.053g%
4. arginine : 0.029 g%	12. tyrosine : 0.058 g%
5. serine: 0.012 g%	13. valine: 0.039 g%
6. aspartic acid: 0.010 g%	14. phenylalanine: 0.156 g%
7. glycocoll: 0.012 g%	15. leucine : 0.027 g%
8. glutamic acid: 0.025 g%	

10. Free amino acids: 0.900 g%; 9000 µg/ml
The traced out amino acids from 9th point were only in a free form.
11. Total sugars: 0.0455 g%; 455 µg/ml
12. Glycogen: 0.0317 g%; 317 µg/ml
13. Reducing sugars (hexoses and pentoses): 0.0138 g%; 138 µg/ml
14. The sugars compounds identification:

a. Cysteine hydrochloride reaction:

Wavelength	Time in minutes				
	5	10	15	20	25
Extinction at:					
390 nm	0.530	0.517	0.522	0.510	0.507
424 nm	0.448	0.437	0.437	0.426	0.426
Differences	0.082	0.080	0.085	0.084	0.081

	1 ml of distillated water was added					
	3	6	9	12	15	18
Extinction at:						
390 nm	0.356	0.347	0.343	0.341	0.339	0.333
400 nm	0.335	0.332	0.319	0.317	0.316	0.309

After 3 hours $E_{510\ nm} = 0,112$

The colour of solution is yellow-brown, stable in time. Only hexoses and pentoses are present (4).

Table 7

Bioactive preparation with antihyaluronidasic activity from Odontogadus merlangus euxinus (june month)

1. Active residue: 1.0 g%; 10,000 µg/ml
2. pH: 7.0
3. Phenol: 0.54 g%
4. Antihyaluronidasic activity: 59.5 UI/ml
5. Ash: 0.110 g%; 1,100 µg/ml
6. Mineral elements: Na,K, Ca, Mg, Fe, Cu and Zn have been identificated.Pb, Cd, Cr - absent.
7. Total nitrogen: 0.140 g%; 1,400 µg/ml
8. Peptides: 0.104 g%; 1,040 µg/ml
9. Total amino acids: 0.9445 g%; 9,445 µg/ml

1.	cystine+cysteine: 0.0416 g%	9.	alanine: 0.3750 g%
2.	lysine : 0.0136 g%	10.	proline: 0.0509 g%
3.	histidine: 0.0066 g%	11.	γaminobutyric acid: 0.0031g%
4.	arginine: 0.0047 g%	12.	tyrosine: 0.0159 g%
5.	serine : 0.0292 g%	13.	valine : 0.0784 g%
6.	aspartic acid: 0.0900 g%	14.	phenylalanine: 0.1561 g%
7.	glycocoll: 0.0205 g%	15.	leucine : 0.0299 g%
8.	glutamic acid: 0.300 g%		

10. Free amino acids: 0.8415 g%; 8,415 µg/ml
The same amino acids as to the 9th point have been identificated. Differences appeared only quantitatively.
11. Total sugars: 0.0198 g%; 198 µg/ml
12. Glycogen: 0.0051 g%; 51 µg/ml
13. Reducing sugars (hexoses, pentoses and methylpentoses) : 0.0147 g%; 147 µg/ml
14. Sugars compounds identification:

a. Cysteine hydrochloride reaction:

Wavelength	Time in minutes				
	5	10	15	20	25
Extinction at:					
390 nm	0.351	0.347	0.344	0.339	0.336
424 nm	0.291	0.282	0.278	0.273	0.268
Differences	0.060	0.065	0.066	0.066	0.068

	1 ml of distillated water was added					
	3	6	9	12	15	18
Extinction at:						
390 nm	0.223	0.225	0.223	0.226	0.226	0.222
400 nm	0.195	0.193	0.191	0.194	0.193	0.190

After 3 hours $E_{510\ nm} = 0.214$.

The colour of solution is yellow-brown, stable in time. Hexoses, pentoses and methylpentoses traces are present (4).

Table 8

Bioactive preparation with antihyaluronidasic activity from Engraulis encrassicholus ponticus (june month)

1. Active residue: 0.958 g%; 9580 µg/ml
2. pH: 6.2
3. Phenol: 0.53 g%
4. Antihyaluronidasic activity: 48 UI/ml
5. Ash: 0.146 g%; 1,460 µg/ml
6. Mineral elements: Na,K, Ca, Mg, Fe, Cu and Zn have been identificated.Pb, Cd, Cr - absent.
7. Total nitrogen: 0.098 g%; 980 µg/ml
8. Peptides: 0.184 g%; 1,840 µg/ml
9. Total amino acids: 0.9031 g%; 9,031 µg/ml

1. cystine+cysteine: 0.0740 g%	9. alanine: 0.2966 g%
2. lysine: 0.0149 g%	10. proline: 0.0572 g%
3. histidine: 0.0181 g%	11. γaminobutyric acid: 0.0139g%
4. arginine : 0.0201 g%	12. tyrosine: 0.0164 g%
5. serine: 0.0226 g%	13. valine: 0.0275 g%
6. aspartic acid: 0.0800 g%	14. phenylalanine: 0.1403 g%
7. glycocoll: 0.0239 g%	15. leucine: 0.0476 g%
8. glutamic acid: 0.0500 g%	

10. Free amino acids: 0.7191 g%; 7,191 µg/ml
The same amino acids as to the 9th point have been identificated. Only quantitatively differences have been registered.
11. Total sugars: 0.0684 g%; 684 µg/ml
12. Glycogen: 0.0581 g%; 581 µg/ml
13. Reducing sugars (hexoses and pentoses): 0.0103 g%; 103 µ
14. Sugars compounds identification:

a. Cysteine hydrochloride reaction:

Wavelength	Time in minutes				
	5	10	15	20	25
Extinction at:					
390 nm	0.473	0.457	0.459	0.450	0.445
424 nm	0.363	0.350	0.352	0.348	0.342
Differences	0.110	0.107	0.107	0.102	0.103

	1 ml of distillated water was added					
	3	6	9	12	15	18
Extinction at:						
390 nm	0.311	0.305	0.300	0.302	0.300	0.298
400 nm	0.270	0.266	0.261	0.262	0.260	0.257

After 3 hours E_{510} = 0.331

The colour of solution is yellow-brown, stable in time. Hexoses and pentoses are present; pentoses are in a greater proportion (4).

The speciality literature (1) quotes that at fishes, peptides are made up of glutamic acid, aspartic acid, proline, arginine and lysine, amino acids which we found in great quantities in our products too.

A number of 16 amino acids were traced out in the analysed products: cystine + cysteine, lysine, histidine, arginine, serine, aspartic acid, glycocoll, glutamic acid, alanine, proline, γ aminobutyric acid, tyrosine, valine, phenylalanine and leucine. At Sprattus sprattus we also found some important quantities of lysine and γ aminobutyric acid, at the same specie the traced out amino acids were only in a free state.

After "rigor mortis" state installation at fishes, the tyrosine, lysine, γ aminobutyric acid, valine and arginine content is increasing due to the intensive enzymatical activity of autolysis, of proteic compounds. These amino acids were traced out in a free form, in large quantities, in all our preparations. At fishes, the qualitative and quantitative composition of amino acids is submitted to some important variations due to the biotop, type of nutrition, salinity and season.

From the analysed values, was found that the products with antihyaluronidasic activity being natural watery extracts, they reflect the variations appeared in the biochemical composition of organisms from which they are obtained. So, their biochemical composition and the antihyaluronidasic activity depend on the biochemical composition of the used raw material, namelly small sea fish, being submitted to the same variation regularity.

At fishes, the most accentuated months variations are registered in the sugars case, these ones being intensively used as power sources in the long effort periods. In our products too, can be observed very large variations of total sugars, glycogen and reducing sugars (hexoses, pentoses and methylpentoses) depending on season and specie. Though the antihyaluronidasic activity is due mostly to the glycogen and reducing sugars content, from the Table no.9 values, it can not be observed a significant correlation between activity and quantity of sugars, the antihyaluronidasic activity being also given by other chemical compounds of solution. Using two identification methods of sugars compounds, in

all our products were traced out free sugars, as hexoses and pentoses, only to Alosa tanaica nordmanni and Odontogadus merlangus euxinus in the form of methylpentoses (but in very small quantities), this being a feature of specie.

Table 9

Bioactive preparation with antihyaluronidasic activity from small sea fish (march-april months)

1. Active residue: 0.68 g%; 6,800 µg/ml
2. pH: 6.1
3. Antihyaluronidasic activity: 32 UI/ml
4. Ash: 0.030 g%; 300 µg/ml
5. Total nitrogen: 0.064 g%; 640 µg/ml
6. Peptides: absent
7. Free amino acids: 0.605 g%; 6,050 µg/ml

1. cystine + cysteine: 0.071 g%	7. alanine: 0.285 g%
2. lysine: 0.038 g%	8. tyrosine: 0.028 g%
3. histidine: 0.028 g%	9. valine : 0.007 g%
4. aspartic acid: 0.014 g%	10. phenylalanine: 0.031 g%
5. serine: 0.003 g%	11. leucine: 0.080 g%
6. glutamic acid: 0.019 g%	

The traced amino acids were only in free form.

8. Total sugars: 0.0286 g%; 286 µg/ml
9. Glycogen: 0.003 g%; 30 µg/ml
10. Reducing sugars: 0.0256 g%; 256 µg/ml

Observation: The samples have been kept at chamber temperature (summer time over +30°C) both variants, lightness and darkness, during 4 months and half; they sedimented and lost about 42% from their activity, recording also other alteration of their chemical composition. The samples have been analysed after filtration. After removing the sediment, the active residue was 0.68 g%.

Analysing the values from Tables no.10 and 11,it can be observed that the products which were exposed from some months at the chamber temperature,both in the light and the dark,were settled out and had loose from their activity,thus some modifications being registered in the chemical composition. By sedimenting,peptides disappeared, being after-wards removed by filtration, the amino acids which remain in sollution only in a free form,diminished in quantity. In the same time with the peptides, the next amino acids disappeared from the samples: arginine, glycocoll, proline and γaminobutyric acid.

Table 10

Bioactive preparation with antihyaluronidasic activity from small sea fish (june month)

1. Active residue: 0.82 g%; 8,200 µg/ml
2. pH: 6.4
3. Antihyaluronidasic activity: 50 UI/ml
4. Ash: 0.03 g%; 300 µg/ml
5. Total nitrogen: 0.110 g%; 1,100 µg/ml
6. Peptides: absent
7. Free amino acids: 0.770 g%; 7,700 µg/ml
   1. cystine + cysteine: 0.121 g%
   2. lysine: 0.100 g%
   3. histidine: 0.053 g%
   4. aspartic acid: 0.128 g%
   5. glutamic acid: 0.073 g%
   6. alanine: 0.100 g%
   7. proline: 0.023 g%
   8. tyrosine: 0.044 g%
   9. valine : 0.019 g%
   10. phenylalanine: 0.066g%
   11. leucine: 0.043 g%

   The traced out amino acids were only in free form.
8. Total sugars: 0.0327 g%; 327 µg/ml
9. Glycogen: 0.0159 g%; 159 µg/ml
10. Reducing sugars: 0.0168 g%; 168 µg/ml

Observation: The samples were exposed at the chamber temperature (summer time over +30°C) in both variants, lightness and darkness, analysed after 4 months and half, they showed a decreasing of antihyaluronidasic activity, active residue (by sedimenting) and other alteration in their chemical composition. The samples have been analysed after filtration.

It can be also observed some alteration of the sugars composition. So, the content in the reducing sugars is increased on the basing of the decreasing of the glycogen en content, due to advanced hydrolyses process of glycogen.

In spite of all these chemical alteration that we signaled, the samples kept about 50% of the initial activity, we can also declare as follows: the active principle is not of peptidical nature (in the intensive antihyaluronidasic activity of products, the peptides were not traced out) and the loss of activity was due to the dilution of samples and to a certain extend to the alteration appeared in the sugars composition.

From these data, it results that the analysed samples were distinguished in what concerns their chemical composition

quantitatively and qualitatively in some extent, but insignificant, and their antihyaluronidasic activity oscillating between 30-100 U.I./ml.

Table 11

Correlation of antihyaluronidasic activity with sugars compounds concentration

Product	Antihyaluronidasic activity UI/ml	Total sugars µg/ml	Glycogen µg/ml	Reducing sugars	Pentoses (E_{390nm} - E_{424nm})
AH from s.s.f.(march-april)	55.6	235	158	77	0.076
AH from s.s.f.(june)	100	318	178	140	0.061
AH from Sprattus sprattus (may)	30	419	319	100	0.168
AH from Sprattus sprattus (june)	50	455	317	138	0.082
AH from Odontogadus merlangus euxinus (june)	59.5	198	51	147	0.060
AH from Engraulis encrassicholus ponticus (june)	48	684	581	103	0.110
AH from Alosa tanaica nordmanni	73	190	88	35	0.063
AH from Odontogadus merlangus euxinus	83	750	286	224	0.068

AH = Bioactive preparation with antihyaluronidasic activity
s.s.f. = small sea fish

From the above data, it can be deduced as follows:

- The quantity variations of the chemical composition of products with antihyaluronidasic activity reflect the variations appeared in the biochemical composition of the sea origine raw material that we used.

- The very small quantities of chondroitine sulphate and the negative metachromatic reaction with toluidine blue enabled us to declare that the antihyaluronidasic activity is due to the chemical compounds assembly, which enter in our products composition, as glycogen, hexoses (glucuronate), microelements and free amino acids.

- The term of antihyaluronidase is used for indicating

the inhibitory effect on the enzymatic activity of hydrolysis of the mucopolysaccharide called hyaluronic acid.

The hyaluronic acid is distributed in the fundamental substance of the conjunctive tissue and achieves a role of tissular bond, working as an intercellular "cement".In the case of inflamatory processes in organism,the hyaluronic acid is intensively dipolymerizated by the hyaluronidase.

The hyaluronic acid is a large molecular weight substance composed by D-glucuronic acid and N-acetylglucosamine, which are bound in β-1.3 position; this recurrent dyglucidical unity (named hyalobiuronic acid) is bound in β-1.4 position by another unity formed by glucuronic acid and N-acetylglucosamine.

As an effect of hyaluronidase on its substrate - the hyaluronic acid - the deoxidizing groups of the N-acetylglucosaminic component are released, the enzyme being a glucosaminidase (2).

MATHEWS and coll. (2) found that heparine, chondroitine sulphate, hyaluronic acid, glycogen and starch, are inhibitors of hyaluronidases. More recently GACESA and coll.(3) proved that the natrium glucuronate compound of hyaluronic acid, displays a competitive inhibition by relation with substrate.

Our products content glycogen and free hexoses (glucuronate)in mixture with other chemical compounds:free amino acids, microelements etc. Besides the directly inhibitory action of hyaluronidases by a competitive inhibition mechanism by relation with substrate and inhibition from reaction products ("feed-back" mechanism) it is possible to produce especially "in vivo" the biosynthesis of the hyaluronic acid in its component parts lying in solution, this explaining the strong antiinflamatory effect.

The antiinflamatory effect consists in inhibating the enzyme which becomes "inapt" to depolymerizate the hyaluronic acid. On the other hand, it's possible that on the hyaluronidases action, the reaction can be conversly produced (it's known that the majority of the enzymatic reactions are reversible). So, in the enzyme presence and an assembly of specifical chemical compounds: free amino acids, sugars (glycogen, hexoses), microelements etc., the synthesis of the hyaluronic acid takes place "in vivo" from its components.

It is also possible that the two phenomena, on the hand the inhibition of the enzyme having as effect the cancellation of the dipolymerization action of the hyaluronic acid; on the other hand, on the action of the same enzyme, the hyaluronic acid synthesis from its specific component parts in a "medium" formed by an assembly of specific chemical compounds, to lead to a common final point, the antiinflamatory effect.

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