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Functional components of preparations from squid cartilage and their effect on the expression of lymphocyte activation markers

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ABSTRACT

There was shown investigation of the composition and biological activity of hydrolyzed squid cartilage designed to create a functional food or biologically active supplements to food and demonstration of their anti-inflammatory effect with the use of biomarkers. To identify the functional constituents present in the preparations the spectrophotometric and chromatographic analysis were performed. Also there were used methods of capillary electrophoresis, inhibitory analysis and different standard methods. The expression of activation markers on the cell surface was assessed by two-color cytometric analysis. There was shown the presence of uronic acids, hexosamines, free disaccharides, collagen and non-collagenic proteins, amino-acids in soluble condition. Fractional composition of solutions determined by HPLC represents protein-carbohydrate conjugate with prevalence compounds with Mw 30-160 kDa. Among free delta-disaccharides the predominance of non-sulfated ones was established, amount of di-sulfated disaccharides was minor and mono and tri-sulfated disaccharides were completely absent. There was established inhibitory effect concerning serine- and metalloproteinases at digestion collagen and casein. There was determined moderate anti-inflammatory activity and reduction of expression level of early activation antigens of human peripheral blood lymphocytes.

INTRODUCTION

According to "Scientific Concepts of Functional Food in Europe" developed in 1995-1998[1-3] food products can be classified as functional only if there is an opportunity to show their positive effect on one of the key functions of the human body (apart from general nutrition effects) and obtain strong objective evidence to support this relationship. It is recommended to indentify specific markers of these functions sensitive to the modulating effect of dietary functional ingredients, if such positive effects are developed in the use of food (or its active ingredient) in quantities that are safe for the body.

The regulatory effect of food components is shown on various levels: molecular (gene replication, synthesis of various RNA and translation of gene information); cellular (energy and protein synthesis in the mitochondria and ribosome, transport and metabolism of substrates, intermediate and final products on the cell surface and membrane) intracellular (hyaloplasm with nucleus, organelles and inclusions) and pericytial (extracellular matrix where capillaries and nerve endings are localized, intercellular exchange of information and metabolic reactions are carried out); on the levels of individual tissues, organ and body as a whole.

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Undoubtedly there is a correlation between specific dietary substances and molecular-genetic and metabolic determinates identifying normal course of metabolism in cells, certain physiological functions in the organism and, ultimately, human health. Determination of this correlation, at the molecular and genetic level as well will provide a scientific basis for the most reasonable recommendations for the use of relevant nutrients to maintain and promote health and reduce the risk of disease.

At present immune system proved to be crucially important in pathogenesis of chronic destructive diseases, including chronic inflammation of the cartilage[4]. Lymphocytes activation processes are the most important during the course of inflammation. Activated lymphocytes, macrophages, fibroblasts and synoviocytes are able to produce a specific set of proinflammatory cytokines which are very important in the development of systemic manifestations and in maintaining chronic inflammation in the joints.

There are numerous data on the influence of glycosaminoglycans (GAGs), collagen and tissue inhibitors of metalloproteinases (TIMP) on the metabolism and regeneration of cartilage, not only through the use of them as "ready building material" but also because of the capacity to accumulate in foci of inflammation and reduce it[5-9].

The activity of GAGs is determined by the number of functional groups in the molecule providing high hydrophilicity and surface-active properties[10]. The predominate protein of cartilage proteoglycanic complex is collagen which not only performs the mechanical function but is also important in the differentiation and proliferation of cells that determines the use of this protein and its soluble derivatives in osteoporosis and arthritis[11-14]. TIMP are very important in the metabolism of connective-tissue matrix. They suppress the activity of endogenous collagenolytic enzymes which causes inflammation and cartilage depolimeryzation[15-16].

Cytokines are very important in the metabolism of cartilage tissue. Thus, interleukin-1, produced in monocytes and chondrocytes, stimulates the secretion of latent metalloproteinases and plasminogen tissue activator, whereas the polypeptide mediators (insulin-like growth factor and transforming growth factor b) stimulate the biosynthesis of proteoglycans. However to confirm a positive effect it is necessary to estimate not only the final result but also probable mechanism of action.

It is very important to use natural anti-inflammatory drugs that affect mediators of inflammation because the majority of known drugs (corticosteroids, nonsteroid anti-inflammatory drugs and immunosuppressants) have a lot of side effects. At present osteochondral tissue of farm animals is used to produce chondroprotectors[17-18]. At the same time osteochondral tissues which are wastes when cutting fish seem promising to produce these drugs[19-21]. Considerable amount of fish waste allows using it for production of biologically active substances. In addition studying of sharks proved that the concentration of TIMP in marine organisms is much higher[22].

Not only fish but cephalopods particularly squid can be the sources of cartilage. Main cartilage formation of cephalopod is the head capsule of complex form consisting of three separate divisions containing ganglia and statoliths. It is easily separated from the rest of internal organs and tissues and is about 2% of the total mass. Macromolecular composition and histological organization of squid cartilage - the predominance of collagen type 1, low concentrations of hyaluronane and keratan sulfate as well as a weak ability to form aggregates with hyaluronane – allows to consider it as analog to an immature not fully differentiated hyaline cartilage of mammals[23].

The aim of this study was to investigate the composition and biological activity of squid cartilage components, designed to create a functional food or drugs and biologically active substances and to demonstrate their anti-inflammatory effect with the use of biomarkers.

MATERIALS AND METHODS

Pacific squids (*Berryteuthis magister, Ommastrephes bartrami* and *Todarodes pasificus*) was caught by fishing vessels in the Sea of Okhotsk and the Sea of Japan and shipped frozen to the laboratory. After thawing the squid head cartilage was separated from other tissues and used to obtain the preparations.

The preparations from squid cartilage were produced according to the method described in the patent RF № 2250047[24]. The main stages are:

salt extraction and enzymatic hydrolysis with proteolytic enzyme preparations, the subsequent thermal inactivation of enzymes, deposition and removal of insoluble components by filtration. The solution was dried by sublimation or spray method.

All chemicals used were of high purity. 4-dimethylaminobenzaldehyde, glucosamine hydrochloride, glycuronic acid, galactose, hydroxyproline (Sigma, USA), dextran standards with molecular mass (M) of 10, 20, 40, 70, 80, 110, 250, 500 and 2000 kDa (Pharmacia Biotech, Sweden), γ-globulin (160 kDa), bovine serum albumin (67 kDa), ovalbumin (45 kDa), chymotrypsinogen A (24 kDa), cytochrome C (13 kDa), aprotinin (6,5 kDa), bacitracin (1,4 kDa) (ICN, USA).

The enzyme preparation "Krusenzyme" from the hepatopancreas of king crab has been produced in TINRO-center and registered with the Russian Federal Service for consumer protection and human well-being supervision as a technological aid used in the food industry when processing meat and seafood. Certificate of State's registration № RU. 77. 99. 26. 010. E.018573.06.11.

The content of hexosamines amount

The content of hexosamines amount was determined by Elson and Morgan[25] colorimetric method after hydrolysis of samples in 4N HCl during 4 h at 100°C. The method is based on the condensation of amino sugars with alkaline acetylacetone and the formation of chromogens in the presence of Ehrlich's reagent (4-dimethylaminobenzaldehyde). To calculate the content of amino sugars standard solutions of glucosamine hydrochloride were used.

The content of uronic acid

The content of uronic acid was estimated by spectrophotometrical carbazole method of Dische according to the modification proposed by Taylor and Buchanan-Smith[26] (1992). The reaction was conducted in two stages: first, polysaccharides were hydrolyzed by means of concentrated sulfuric acid and then colored compounds were obtained by adding carbazole. Glucuronic acid at a concentration of 20-200 mmol was used as the standard solution.

The content of sulfate ions

The content of sulfate ions was determined by turbidimetric method after 6-hour hydrolysis of samples with 4N HCl, the subsequent deposition of sulfate ions by barium chloride and recording the optical density of the solution at 405 nm by the degree of turbidity produced by barium sulfate formed. Number of sulfate ions was calculated from the calibration graph which was constructed using standard solutions of potassium sulfate.

Total hexose content

Total hexose content was determined by anthrone method as described by Chaplin[27] using galactose as standard.

Determination of amino acid content

Determination of amino acid content was carried out by the amino acid analyzer "Hitachi L-8800" (Japan) under standard conditions. To determine the total amino acids composition the pre-dehydrated with acetone material was subjected to acid hydrolysis in 6N HCl at 110°C for 20 h. Determination of free amino acids was performed after extraction of preparations in 70% ethanol.

Collagen content

Collagen content was calculated by the amount of hydroxyproline using a conversion factor of 12. Hydroxyproline content was determined by the method of Newman and Logan[28], based on the color reaction of hydroxyproline with p-dimethylaminobenzaldehyde as described by Wessner[29]. The calculation was made with the help of calibration graph using standard hydroxyproline solutions.

The content of non-collagen proteins (NCP)

The content of non-collagen proteins (NCP) was calculated using the formula proposed by Smits (cited by Slutsky, 1969)[30]:

$$NCP = \frac{tyr - \left(hyp \times \frac{0,6}{13,6}\right) \times 100}{5,5} \%$$

Where:

Tyr – tyrosine content in the sample, %; Hyp – hydroxyproline content, %; 0,6 – the average content of tyrosine in collagen,%; 5,5 – the average content of tyrosine in non-collagen proteins,%; 13,6 – the average content of hydroxyproline in collagen,%.

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Fractional composition of hydrolyzates

Fractional composition of hydrolyzates was determined by HPLC on a liquid chromatograph Shimadzu LC-10Avp with refractometric detector RID-6A on Shodex Asahipak GS-520H column (with a hydrophilic polymer sorbent - polyvinyl, designed for the separation of water solutions); eluent - distilled water, the speed of the mobile phase - 1 ml/min. The samples were diluted with distilled water to a solids content of 0,5-1% in the solution, filtered through a membrane filter Kurabo 25A (Japan) with a pore size of 0,45 microns.

Refractometric detector was used to determine the percentage of proteins and carbohydrates in the sample. To determine the M of carbohydrate-containing fractions the column was calibrated according to dextran standards with M of 10, 20, 40, 70, 80, 110, 250, 500 and 2000 kDa. The concentration of dextran solution was 1 mg/ml. Under these conditions the column Shodex Asahipak GS-520N had a linear range of the division of compounds with M 180-200 000 Da.

To determine the M of the proteins the column was calibrated according to the following protein standards: gamma-globulin (160 kDa), bovine serum albumin (67 kDa), ovalbumin (45 kDa), chymotrypsinogen A (24 kDa), cytochrome C (13 kDa), aprotinin (6,5kDa), bacitracin (1,4 kDa). Under these conditions Shodex Asahipak GS-520 N column had a linear range separation of proteins with M 10-67 kDa.

Identification and quantitative determination of the carbohydrate components containing chondroitin sulfate was carried out by calibration according to existing standards. Commercial preparations of chondroitin sulfates A (CH A), C (CH C), D (XC D) and A from sturgeon (CHS A-1) and hyaluronane was used as glucosaminoglycan standards.

Molecular distribution of the components in samples was calculated with the help of Multichrom (version 1.6) – firmware designed to collect and process chromatographic data.

Inhibitory effect of preparations from squid cartilage on the activity of proteolytic enzymes

Inhibitory effect of preparations from squid cartilage on the activity of proteolytic enzymes was determined by measuring at regular intervals the enzyme activity and its changes during incubation in the presence of various concentrations of preparations from cartilage. A mixture of enzyme solutions and preparation under study in the ratio 1:0;1; 1: 0.25; 1: 0.5; 1: 1; 1: 5; 1: 8; 1:10 was used. Solutions mixture was kept for 30, 60, 120 min at 4°C. The degree of inhibition was expressed as percent of initial enzyme activity (100%).

Separation of delta-disaccharides

Separation of delta-disaccharides was performed using capillary electrophoresis technique on "Capel-105" (Lumex, Russia) on a quartz capillary (inner diameter – 75 μm, the total capillary length - 60 cm, effective capillary length – 55 cm) at a wavelength of 210 nm and a temperature of 25°C. Before applying the sample capillary was washed with 0,1N NaOH for 1 min, then with working buffer (15 mmol sodium orthophosphate buffer, pH 3,00) for 4 min.

The sample (1 mg/ml) was dissolved in twice-distilled water, passed through a membrane filter with a pore size of 0,45 μm. The sample in the capillary tube was added automatically under the pressure of 30 mbar, the volume of 1,0 ml for 10 s. Separation was performed at 20 kV in the working buffer for 20 min at a flow rate of 1 ml/min in reverse polarity. Under these conditions compounds migrate from cathode (-) to anode (+) according to the electrophoretic mobility and against the electroosmotic flow of buffer.

Peak height and area was calculated with the help of Multichrom (version 1.6) – firmware designed to collect and process chromatographic data. Quantitative evaluation of free disaccharides was expressed in percentage of the amount of sulphated and non-sulphated disaccharides.

The expression of activation markers

The expression of activation markers on the cell surface was assessed by two-color cytometric analysis in the program Cell Quest on a flow cytometer FACS Calibur (Becton Dickinson) using monoclonal antibodies to molecules CD69-PE, CD25-PE (Beckman Coulter) and the corresponding isotypic controls. In order to correctly exclude from the area of analysis all particles which did not conform in size and granularity to live lymphocytes in the histogram of particle distribution according to small-angle lateral light scattering and CD45 were introduced necessary logi-

cal restrictions. At least 10 000 cells were analyzed in each sample. Program Cell Quest was used in all experiments to analyze histograms of cell distribution according to the fluorescence intensity. Average fluorescence of cells in the study population was estimated as well as the percentage of lymphocytes expressing the activation marker.

Peripheral blood with heparin (25 U/ml) obtained from healthy donors diluted 1:1 with complete culture medium: RPMI-1640, 0,01 M HEPES, 200 mmol L-glutamine, 100 mkg/ml gentamicin served as a material for the study of the effect of preparations on the expression of activation antigens. The preparation under study was introduced into the portion of diluted blood in the final concentration of 100 μg/ml and incubated for 2 h in a CO_2 incubator at 37°C and then phytohemagglutinin (PHA) in a final concentration of 10 mg/ml was added and incubation was continued for 24 hours. The control samples (intact cells and cells with PHA) were incubated with complete culture medium.

Statistical analysis

Statistical analysis of the results was performed using an application package «Statistica 6". The following methods were used: check the normal distribution of quantitative attributes for a small number of observations (Shapiro-Wilke W-criterion), paired t-criterion. Selected parameters given in the tables below are marked as follows: the arithmetic mean (M), standard deviation (σ), the median and interquartile scope value Me (LQ-UQ), the volume of the analyzed subgroups (n). Confidence probability level was set equal to 95%.

RESULTS AND DISCUSSION

Previously cranial cartilage component structure of squid *Illex illecebrosus coidentii* of the Mediterranean basin was considered in work of Vynios et al.[31]. When comparing these data with those obtained by us for three species of Pacific squids was found almost complete coincidence of the data (TABLE 1). The main component of squid cartilage - water - its quantity is a little higher than in hyaline cartilage of mammals, accordingly the content of uronic acid and protein (mostly collagen) is lower. At the same time it was noted that hexosamines prevail over uronic acids. Hjerpe and colleagues[32] suggest that this is determined by the presence of chondroitin sulfate in squid, which they called XCE.

Distinctive features of this chondroitin sulfate were a weak ability to form aggregates with hyaluronan and extreme differences in comparison with known analogues with regard to the actions of such extragents as guanidine hydrochloride and sodium dodecyl sulfate.

It should be noted that the data obtained for all three species of Pacific squids differ very little, so the results below apply only to *Todarodes pacificus*, but with a high degree of probability can characterize the other two species.

Methods of enzymatic hydrolysis are often used to isolate the proteoglycans from the cartilage. In this case not only chondroitinazes (specific enzymes to break down the carbohydrate) are used but also proteolytic enzymes. Papain which is able to destroy covalent bonds between the proteins and GAGs[32,33] is used the most frequently. In this study, we didn't aim at isolating and purifying any of the cartilage components. Our goal

TABLE 1 : Chemical composition of squid cranial cartilage, % of initial tissue

Component	Todarodes pacificus	Berryteuthis magister	Ommastrephes bartrami	Illex illecebrosus coidentii*
Water	85,23	84,62	83,87	82,00
Total proteins	11,11	12,08	12,72	7,16 (*collagen*)
Total carbohydrates	2,50	2,62	2,70	-
Hexosamines	1,52	1,41	1,54	1,40
Uronic acids	0,41	0,54	0,52	0,34
Hexoses	0,22	0,26	0,28	0,25
Minerals	1,02	1,10	1,15	-

*Vynios and Tsiganos data, 1989

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TABLE 2 : The composition of enzymatic hydrolyzate from squid cartilage, %

Components	Contents
Uronic acids	1,7±0,04
Hexosamines	2,2±0,02
Sulphate ions	3,3±0,06
Free disaccharides	0,7±0,010
Hexoses	0,3±0,02
Collagen	16,8±0,2
Non-collagenic proteins	45,0±1,1
Free amino-acids	12,5±0,5
Minerals	8,0±0,06
Water	7,1±0,03

was to maintain the diversity of components each of which possesses a functional activity effecting locomotor system and to make the maximum amount of these components soluble. Proteolytic enzymes preparation "Krusenzyme" from the hepatopancreas of king crab characterized by the presence of unusual serine proteinases with very broad substrate specificity capable to hydrolyze native collagen was used for cartilage hydrolysis[34,35]. The activity of composite enzyme preparation was 1200 U/g (according to the modified method of Anson), and the quantity taken for the hydrolysis - 1% to the initial cartilage.

Milled cartilage was extracted in 1% NaCl before proteolysis. During extraction there was a partial degradation of protein-polysaccharide complexes. Not only salt- and water-soluble proteins, but also glycoconjugates as well as free hexosamines transited into solution. After this procedure the availability of protein components to enzymatic degradation considerably increased. At the same time the indicated concentration of sodium chloride did not reduce enzyme activity but stabilized it. The process of hydrolysis was stopped by heating for a short time after that the precipitate was separated and the solution was subjected to drying.

Preparations obtained after proteolysis are multicomponent containing carbohydrates and proteins (TABLE 2).

Non-collagen proteins characterized as shown below by low molecular mass and containing no hydroxyproline prevail over other investigated components. The total content of soluble collagen is significantly lower than non-collagenic proteins. Almost one third of collagen remains in the sediment.

There are a large number of free amino acids in the preparation which, of course, are the products of enzymatic degradation. This feature is characteristic for serine proteinases from crab hepatopancreas[36]. Aspartic, glutamic acid and glycine prevail among the amino acids, the content of proline and hydroxyproline is also high (TABLE 3).

The content of hexosamines and hyaluronan (or hyaluronic acid) in preparations from squid cartilage is almost two times less than in preparations obtained by the same method from the tissues of sharks, stingrays and salmons, and the content of sulfate ions is on the same level[33,37,38] which indicates a higher degree of sulfation. The molar ratio of hexosamine : uronic acid : sulfate ions formed for squid cartilage hydrolyzate - 1: 0,86: 3, that is also an indirect confirmation of this assumption.

It is believed that the solubility of collagen depends on the degree of proline hydroxylation. At the same time high content of hydroxyproline should ensure stabilization of the three chain helix, which is associated with the stereo chemical features of pyrrolidine rings[39]. The degree of proline hydroxylation is expressed as the DH Pro,% = (Hyp x 100) / (Pro + Hyp). In collagen from squid cartilage, this value is 35,3%, a little lower than in collagen from squid skin[40].

Mineral elements, including high amount of sodium and potassium make up a significant part in the preparations (up to 8%) (TABLE 4).

Iron, zinc and copper prevail among trace elements. Toxic metals apart from low concentrations of cadmium are not found.

Chromatographic equipment that allows analyzing protein and carbohydrate components simultaneously was used to study the fractional composition of the enzymatic hydrolysates of squid cartilage and analyze molecular weight distribution of fractions. Fraction composition of water-soluble cartilage components not subjected to enzymatic degradation (extraction was carried out for 24h) was analyzed for comparison.

Fractions which were formed during enzymatic hydrolysis contained proteoglycan components, because the coincidence of peaks was observed both in the refractometric detection and at the absorption registration the in the UV spectrum at 280 nm (figure 1).

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TABLE 3 : The content of amino acids in the enzymatic hydrolyzates from squid cartilage, % of amino acids amount

Asp	Glu	Pro	Hpr	Ser	Ala	Cys	Tyr	Gly	Ile	Leu	Lys	Oly	Met	Phe	Thr	Val	His	Arg
10,1	14,5	8,2	4,2	4,9	5,4	1,4	2,9	8,0	3,6	6,3	6,3	1,0	0,6	3,6	4,5	4,0	2,0	7,2

Note: n = 4, p < 0,05

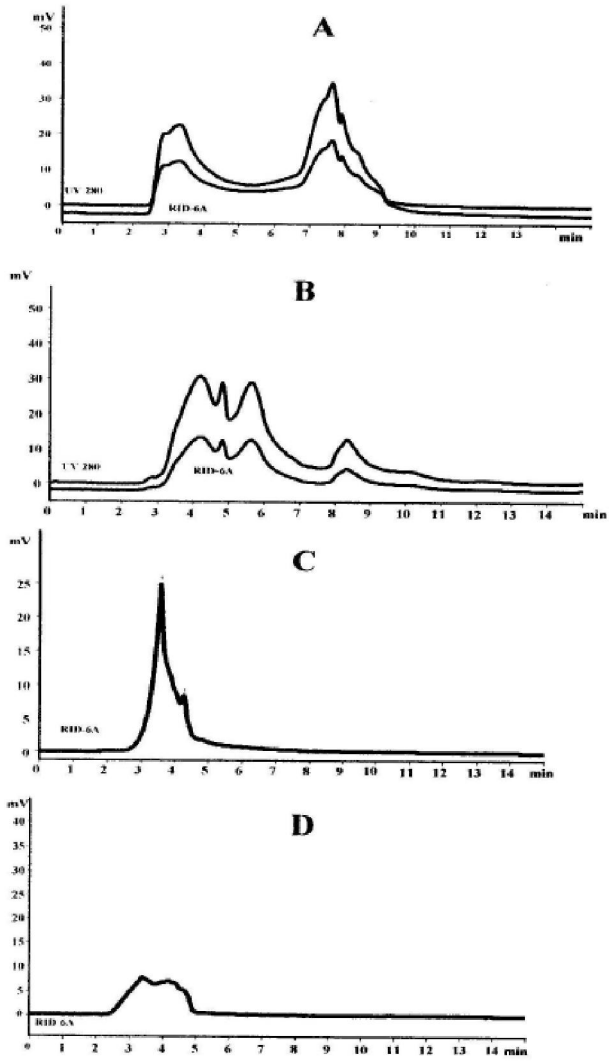


Figure 1 : Chromatograms of an aqueous extract (A), enzymatic hydrolyzate (B) from squid cartilage, standard of chondroitin sulfate A (C) and hyaluronane (D). The separation was performed by HPLC on a column of Shodex Asahipak GS-520 H. Detection was performed simultaneously by refractometric detector RID 6A and under UV 280 nm

For convenience, the components were divided into 3 main groups: fractions containing high (200 kDa and more), middle (30-160 kDa) and low molecular mass components (10 kDa and less). Quantitatively ratio of their distribution was 4: 5: 1.

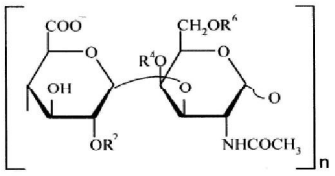
The separation and quantitative determination of carbohydrate components in the samples was compared with the standards, which were represented by commercial preparations from various types of chondroitin sulfates (A, A1, C and D) and hyaluronan. Comparison of samples and standards chromatograms made it possible to assume that high fractions of preparations formed in the enzymatic hydrolysis do contain chondroitin sulfate and hyaluronane (Figure 1).

TABLE 4 : Concentration of mineral substances in squid cartilage preparations

Element	Unit	Quantity
Potassium	Mg/g	6,5
Magnesium	Mg/g	1,5
Calcium	Mg/g	5,2
Sodium	Mg/g	72,2
Manganese	Mg/kg	2,4
Chromium	Mg/kg	5,7
Copper	Mg/kg	20,0
Zinc	Mg/kg	70,0
Iron	Mg/kg	88,0
Nickel	Mg/kg	3,7
Cadmium	*Mg/kg*	*0,3*
Cobalt	*Mg/kg*	*0*
Lead	*Mg/kg*	*0*
Mercury	*Mg/kg*	*0*

Notice n = 3, p < 0,05

Since the output of the peaks belonging to the chondroitin sulfate and hyaluronan occurred in an area marked as high-molecular, it is possible to assume that approximately 40% of the GAGs were



№	Designation	R^2	R^4	R^6
I	Δ Di – 2,4,6- tri S	SO_3^-	SO_3^-	SO_3^-
II	Δ Di – 4,6- di S	H	SO_3^-	SO_3^-
III	Δ Di – 2,4- di S	SO_3^-	SO_3^-	H
IV	Δ Di – 2,6- di S	SO_3^-	H	SO_3^-
V	Δ Di – 2 - mono S	SO_3^-	H	H
VI	Δ Di – 0 S	H	H	H
VII	Δ Di – 0 S	H	H	H

Figure 2 : The structure and composition of the disaccharide units of chondroitin sulfates (I-VI) and hyaluronan (VII), formed after hydrolysis by chondroitinases.

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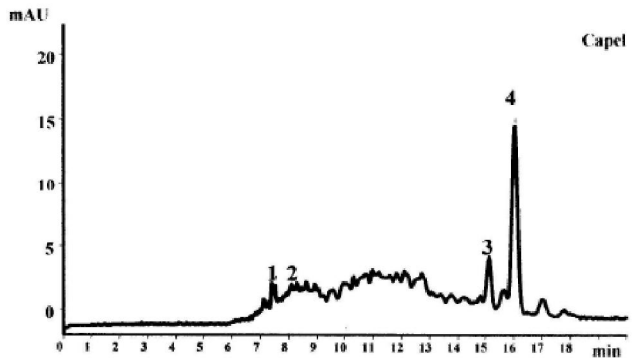

Figure 3 : Electropherograms and composition of free disaccharides (structures I-VII) contained in the enzymatic hydrolysates from squid cartilage.

not subjected to degradation but switched to water-soluble state and 60% were subjected to the change in its structure. As part of the insoluble components of the hydrolyzate, the amount of uronic acids and aminosugars didn't exceed 10% and 28% of the initial amount, respectively.

In the process of enzymatic hydrolysis there was an accumulation of a number of free delta-disaccharides of hyaluronan and chondroitin sulfates, its content reached 1% of the dry weight of the preparation (TABLE 2). GAGs are known to consist of repeating monomers – disaccharides formed by sulfated or non-sulfated residues of d-glucuronic acid (GlA) and N-acetyl-d-galactosamine (GalNAc),which are linked by recurring (β (1 -3)) and (β (1 - 4)) bonds. At the same time differences between individual members of GAGs are expressed in varying amounts and position of sulfate groups, as well as in different combinations of them in polysaccharide chain. The presence of three-, di-, mono- and non-sulfate derivatives depends on the tissue and species. The most significant differences in the number and proportion of variously sulfated disaccharides in GAGs are observed for terrestrial animals and cartilaginous fishes[37,41-43].

It is known that the hydrolysis of proteoglycans occurs under the action of proteolytic enzymes such as papain, this process is often used to break ties of glycosaminoglycans and proteins in order to receive chondroitin sulfates[31,33]. The formation of free disaccharides resulting from non-specific (as opposed to chondroitinase) hydrolysis is probably not direct, but rather mediated result of the weakening bonds of terminal monomers. We used a set of serine proteinases

TABLE 5 : The quantitative composition of free disaccharides contained in enzymatic hydrolysates from squid and shark cartilage

Structure	Preparation from squid		Preparation from shark*	
	Peak number	Content of the amount,%	Peak number	Content of the amount,%
I	-	-	4	13
II	2	3	3	6
III	1	2	2	3
IV	-	-	-	-
V	-	-	4	10
VI	3	17	5	14
VII	4	78	6	54

Sukhoverhova data, 2006

from the king crab hepatopancreas with specificity different from papain and also noted the accumulation of free delta-disaccharides.

To determine the quantitative and qualitative composition of delta-disaccharides we reproduced method for the determination of non-sulfated disaccharides of hyaluronan and chondroitin sulfates, as well as variously sulfated disaccharides of chondroitin sulfates using capillary electrophoresis after enzymatic hydrolysis with chondroitinase ABC[44]. Authors set different patterns of sulfated disaccharides yield and found that, initially, under specific conditions tri-sulfated disaccharides migrated, then di-sulfated, mono-sulfated and non-sulfated. Figure 2 shows the structure and position of sulfate groups of disaccharides obtained after enzymatic hydrolysis by chondroitinase.

Analyzing free delta-disaccharides that make up the preparation from squid cartilage, we got the results that have a high degree of analogy with the results of the above-mentioned work concerning the ability of these components to separate under the conditions of the developed method (figure 3).

Match of all parameters and conditions of electrophoresis, as well as time of factions yield have suggested the presence of disaccharides with similar structures. Of course comparison with standard samples of disaccharides and study of isolated components structure will be the absolute proof.

The predominance of non-sulfated disaccharides (95%) (structures VI and VII) out of which 78% are delta-disaccharides of non-sulfated hyaluronan (VII),

is established when calculating the quantitative distribution of various disaccharides in the obtained preparations. At the same time mono and tri-sulfated delta-disaccharides are completely absent. From di-sulfated delta-disaccharides there were detected only structure II (4,6 - di S) and III (2,4 - di S). Their quantity is 3 and 2%, respectively (TABLE 5).

The preparation from shark cartilage, which was obtained using the same method, has another set of disaccharides[45]. In that case the content of non-sulfated disaccharides – structures VI and VII - 14 and 54%, respectively, mono- and di-sulfated - 10 and 22%. Among di-sulfated structures those with sulfate groups in position 2,6 (IV) predominate. Three-sulfated disaccharides (I) in the composition of free disaccharides in the preparation from squid cartilage were not found, although they are detected in the composition of chondroitin sulfates both in squid and sharks[23].

TABLE 6 : The residual activity of proteolytic enzymes in the presence of preparations from squid cartilage when using different substrates (enzyme: inhibitor 1: 1)

Source of Inhibitor	'Krusenzyme' *Paralithodes camtchatica*	Collagenase *Clostridium histolyticum*
Casein		
Absent	260 u/g	0
Aqueous extract	88,6	-
Hydrolysate	72,8	-
Collagen		
Absent	69 u/mg	220 u/mg
Aqueous extract	22,8	68,2
Hydrolysate	3,5	39,6

It can be assumed, that the degree of sulfation affects the strength of the terminal monomers binding, if so, the binding of non-sulfated disaccharides of hyaluronan is the weakest.

It is known that enzymes - matrix metalloproteinases (stromelysin, collagenase, gelatinase), which are produced by chondrocytes in an inactive form, but when activated can destroy all components of the extracellular matrix – are very important in the metabolism of cartilage[15,16,46]. In addition, metalloproteinases are very important in generalization of processes of tumors invasion and metastasis[47]. Chondroitin sulfates have some inhibitory capability for matrix metalloproteinases and pronounced anti-inflammatory activity. Clinical studies proved the effectiveness of chondroitin sulfates in case of pain syndrome and functional status of joints[48]. But key regulators of inflammation and angiogenesis are tissue inhibitors of metallo-proteinases (TIMP) capable of forming a complex with an inactive form of the enzyme, indicating that the more complex functions than simple inhibition of enzyme activity[49]. The relationship between the expression of the activity of matrix metalloproteinases and their inhibitors can be observed in various physiological and pathological conditions of tissue.

The most common of the well-known inhibitors are the TIMP-1 - glycoprotein with M 30 kDa and TIMP-2 - non-glycosylated protein with M 23 kDa. Both inhibitors are easily extracted from the cartilage[50] and retain the resistance to proteolytic enzymes[47]. Extracts of aquatic organism's cartilage tissue contain a high concentration of TIMP. For example, in shark cartilage the quantity of metalloproteinases and angiogenesis inhibitors is a hundred times higher than in calf cartilage[22,51]. There are no data about similar inhibitory activity in squid cartilage.

In our study, we determined the inhibitory specificity of preparations from squid cartilage. Effect of squid cartilage preparations on the activity of different enzymes was established by the changes of collagenlytic and caseinolytic activity. It should be noted that in the process of cartilage enzymatic degradation the following ratio of source material : enzyme – 100 : 1 (then enzymes was inactivated by heat) was used to obtain preparations and to determine the inhibitory properties of hydrolysates the ratio of enzyme: extract – 10 : 1; 4 : 1; 1 : 1; 1 : 0,5 was used. The highest inhibitory effect was observed at a ratio of 1:1, a further increase in the amount of the inhibitor had no significant effect. Also there was compared effect of aqueous extracts from the cartilage, because it is known on the example of sharks about similar effect of these extracts.

Collagenolytic complex ("Krusenzyme") from the king crab hepatopancreas and collagenase from *Clostridium histolyticum* was used in this part of the work. 'Krusenzyme' contains mainly serine proteinases capable to cleave a wide range of protein substrates including collagen[34]. Substrate specificity of collage-

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TABLE 7 : Change of early activation antigens expression on peripheral blood lymphocytes under the influence of the hydrolyzed squid cartilage products

Lymphocyte activation marker		group	W	M±σ	Mediana (Me) (LQ-UQ) 10,00 -90,00	t-test
CD69$^+$	% of cells expressing the marker	1	0,87	9,0±4,9	7,50 (4,50;9,50)	
		2	0,92	10,5 ±3,1	10,00 (7,00; 15,00)	P_{1-2} 0,406
		3	0, 94	57,7±8,2	56,00 (46,00;68,00)	
		4	0,93	49,0±5,8	49,10 (41,00;55,50)	P_{3-4} 0,014
	average Fluorescence intensity	1	0,87	39,6±4,5	39,00 (35,00; 48,00)	
		2	0,92	29,6 ±6,5	29,50 (21,50; 39,00)	P_{1-2} 0,003
		3	0,87	324,2±55,5	340,00(240,00; 400,00)	
		4	0,93	250,50±29,5	256,00 (210,00;280,00)	P_{3-4} 0,031
CD25$^+$	% of cells Expressing the marker	1	0,92	14,2 ±4,9	15,00 (7,00; 19,50)	
		2	0,96	16,1 ±3,1	17,00 (8,00; 22,00)	P_{1-2} 0,401
		3	0, 89	29,4±5,1	28,00 (24,00;37,00)	
		4	0,93	23,4±6,6	34,00 (14,00; 32,00)	P_{3-4} 0,046
	average Fluorescence intensity	1	0,97	21,6±4,6	22,00 (15,00;28,00)	
		2	0,93	20,6 ±3,3	20,50 (17,00; 26,00)	
		3	0,95	45,1±6,1	46,00 (36,50; 52,00)	
		4	0,93	43,4±6,1	43,00 (35,00;50,00)	P_{3-4} 0,002

Note: 1 - control (intact blood cells), 2 - blood cells incubated with preparation (24), 3 - blood cells stimulated with PHA (24 h), 4 - blood cells, incubated with preparation before stimulation with PHA; W - Shapiro-Wilk criterion (for all values †of the criterion Wp> 0,05 parameters have a normal distribution); t-test - Student's criterion

nase from *Cl. histolyticum* is, in general, similar to the specificity of the typical matrix metalloproteinases, thus, it is able to hydrolyze only collagen. TABLE 6 shows the activity of two enzyme preparations studied and their changes under the influence of various preparations.

The above information shows that protein components with molecular mass below 10 kDa (90% of the total) dominate in an aqueous extract from squid cartilage and after enzymatic hydrolysis, the ratio of fractions is changed in the direction of the prevalence of more tangible components - above 200 kDa (40%) and from 30 to 160 kDa (50%), the proportion of low molecular weight compounds is reduced to 10%. At the same inhibitory activity of the components of the hydrolysis products is significantly higher than that of the aqueous extract. It was found that the products of an aqueous extract of cartilage decrease the cleavage rate of collagen from calf skin by "Krusenzyme" enzymes (mainly serine proteases) and metal-dependent collagenase from *Cl. histoliticum* by 66% and 70% respectively, while the products of enzymatic hydrolysis of cartilage in respect of the same enzymes reduce it by 95 and 82% respectively. As it was shown previously, serine proteinases contained in "Krusenzyme" are able to hydrolyze a wide spectrum of protein substrates of both globular and fibrillar structures[34]. The components of hydrolyzed squid cartilage and its aqueous extract inhibit the digestion of substrates such as collagen and casein by the same enzymes. The observed residual activity in the process of casein decomposition was 28%, and collagen – 6%. Inhibitory ability of the preparation from the degraded shark cartilage (previously obtained in the same way) in relation to microbial collagenase was lower than for the preparation from squid, and the activity of collagen was decreased by 33%[45].

So, it was determined that hydrolysis products of squid cartilage demonstrate anti-collagenolytic and anti-caseinolytic activity. Belonging of the inhibitory activity to the specific component of the investigated preparations hasn't been determined yet, nor is it clear how an increase of inhibitory activity of hydrolyzed cartilage happens. However, the presence of anti-protease ac-

tivity suggests the ability of anti-inflammatory responses. Therefore, the next stage of the works studied the effect of the preparations on the expression of activation membrane molecules of peripheral blood lymphocytes.

The initial stages of the activation processes are expressed in the launch of the cascade mechanisms (usually enzyme), the ultimate aim of which is the expression of corresponding genes. Products of some genes associated with activation, so-called activation markers or "activation" antigens appear at different periods on the surface of lymphocytes[52]. These include the CD69 – early activation marker, involved in the mechanisms of T cells, EK cells, monocytes and platelets activation. CD69 regulates the immune response by modulating the expression of various cytokines[53]. CD69 is not expressed on resting peripheral blood lymphocytes, but appears on the cells within 1-2 h after stimulation. Expression of CD69 requires the synthesis of matrix RNA and is very unstable, since matrix RNA is rapidly degraded under the influence of functional motive AU-rich [54]. Investigation of the role of CD 69 in the pathogenesis of rheumatoid arthritis began in the 90s, when Laffont and collaborators[55] found that high levels of CD69 + T lymphocytes in the synovial fluid and synovial membrane of RA patients correlate with disease activity. In addition Murata and collaborators[56] proved the impossibility of collagen-induced arthritis in CD 69-null mice. These data show an important role of the CD69 molecule in the pathogenesis of arthritis and its use as a possible therapeutic target.

For example, it is shown the decrease of CD 69 expression on NK-cells of mice and neutrophils of patients with rheumatoid arthritis treated with anti-TNFá-therapy[57,58]. Receptors for IL-2 (CD25) appear on the membrane of T lymphocytes in the G1-phase of cell cycle and their expression is necessary for the transition of T-lymphocytes from the G1 to S-phase and subsequent proliferation and is an important regulator of immune system required for the expansion of a clone and survival of activated lymphocytes. Coexpression of CD69 + CD25 and HLADR is significantly expressed in T cells of synovial fluid of patients with rheumatoid arthritis[59].

The study of modulation nature of activation antigens expression showed that incubation of peripheral blood cells for 24 h with squid cartilage preparation at the final concentration of 100 μg/ml is not accompanied by the change in the number of lymphocytes expressing the activation molecules CD69 and CD25. At the same time the density of molecules on the membranes of lymphocytes measured by the average fluorescence intensity is reduced (TABLE 7).

Incubation of peripheral blood with the hydrolyzate from squid cartilage before adding mitogen leads to a decrease in the number of cells expressing the activation molecules CD69 and CD25 ($p = 0,014$ and $p = 0,046$, respectively) and reduction of their density on the membranes. Average fluorescence intensity of SD69 on lymphocytes, treated with preparation and stimulated with PHA, is significantly lower compared to that on cells, stimulated with PHA ($p=0,003$ and $p = 0,002$ respectively).

The experimental data obtained demonstrate the possibility of reducing the expression of CD69 and CD25 molecules on human peripheral blood lymphocytes under the influence of the hydrolyzed products of squid cartilage. Weakening of activation antigens expression may determine changes in the functional activity of cells and their ability to modulation may cause anti-inflammatory effect of biopolymers under study. Modulating the expression of structures on active domain cell surface, that mediate intercellular interactions, it is possible to change the early stages of immune system cells activation and the subsequent stages of the development of their functional response that accompanies the development of inflammation.

Thus, the complex preparation, containing soluble proteins and polysaccharides, as well as low molecular weight components, including free amino acids and delta-disaccharides, was obtained from pacific squid's cartilage using the method of enzymatic hydrolysis. The preparation obtained possesses inhibitory activity concerning serine and metal-dependent proteinases. There was also determined moderate anti-inflammatory activity and reduction of expression level of early activation antigens of human peripheral blood lymphocytes.

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REFERENCES

[1] Scientific concepts of functional foods in Europe, Consensus document, British J.Nutrition., **81**, 1 **(1999)**.

[2] F.Bellisle, A.T.Diplock, G.Hornstra, B.Koletzko, M. Roberfroid, S.Salminen, W.H.M.Saris; British J.Nutrition, **80**, 193 **(1998)**.

[3] P.M.Verschuren; British J.Nutrition, **88**, 125 **(2002)**.

[4] E.Y.Gusev, V.A.Chereshnev, L.Yurchenko; Cytokines and Inflammation, **6**, 9 **(2007)**.

[5] L.Palmieri, A.Conte; Drug Research, **40**, 17 **(1990)**.

[6] H.Kresse, E.Schönherr; J.Cell.Physiol., **189**, 266 **(2001)**.

[7] G.Henry, W.L.Garren; Surg.Clin.N.Am., **83**,1736 **(2003)**.

[8] C.J.Malemud; Front.Biosci., **11**, 1696 **(2006)**.

[9] E.Karousou, M.Ronga, D.Vigetti, A.Passi, N.Maffulli; Clin.Orthop.Rel.Res., **19**, 466-1577 **(2008)**.

[10] H.Habuchi, O.Habuchi, K.Kimata; Glycoconj., **21**, 47 **(2004)**.

[11] T.V.Arshinova, V.I.Rykov, L.Y.Kuchumova, O.Sidorkin; Production method of complex preparation containing mucopolysaccharides and collagen from animal feed, Russian Federation Patent 2082416 **(1997)**.

[12] A.Milan, B.Eggersgluss, K.Braumer, R.Schrieber; Use of tasteless, hydrolyzed collagen and agent containing the same, US Patent 5,948,766 **(1999)**.

[13] M.M.Soloviev, L.G.Vladimirova, E.Chechetkina; Method of producing preparation for the activation of reparative osteogenesis, Russian Federation Patent 2088240 **(1997)**.

[14] H.L.Weiner, D.A.Hafler, D.E.Trentham; Method for treating rheumatoid arthritis with a low dose of collagen type II, US Patent 5,856,446 **(2000)**.

[15] A.Fosang, K.Last, V.Knauper, G.Murphy, P.Neame; FEBS Letters, **380**, 17 **(1996)**.

[16] J.Stracke, A.Fosang, K.Last, F.Mercuri; FEBS Letters, **478**, 52 **(2000)**.

[17] T.V.Arshinova, V.I.Rykov, L.Y.Kuchumova, O.Sidorkin; Method for complex preparation containing mucopolysaccharides and collagen from animal feed, Russian Federation Patent 2082416 **(1997)**.

[18] N.V.Timoshenko, A.I.Snizsar, A.A.Vlesko, A.M.Kobosev, M.I.Bereshpolov, Ju.V.Melnikov; Dietary supplement for farm animals, Russian Federation Patent 2153812 **(1997)**.

[19] E.Dupont, P.Brazeau, Ch.Juneau, R.Bliveau; Shark cartilage extract: process of making, methods of using and compositions US Patent 2,157,695H **(2000)**.

[20] T.N.Pivnenko, L.M.Epstein, Yu.M.Pozdnyakova, N.N.Kovalev, A.K.Alabaster, V.I.Apanasevich, N.N.Besednova; Food bracing health-care product from shark cartilage and method of its receipt, Russian Federation Patent 2161002 **(2000)**.

[21] S.Aoyagi, S.Demichele; Method and product using sturgeon notochord for relief of arthritis symptoms, US Patent 6,423,347 **(2003)**.

[22] A.Lee, R.Langer; Science, **221**, 1185 **(1983)**.

[23] D.H.Vynios, C.P.Tsiganos; Biochimica et Biophysica Acta, **1033**, 139 **(1990)**.

[24] G.Ju.Klychkova, T.N.Pivnenko, L.M.Epstein, N.N.Kovalev, O.Ju.Muzaleva, L.N.Bocharov, Ju.G.Blinov; Food Treatment-and-Prophylactic Product from Cartilage Tissue of Hydrobiontes and Method of its Isolation, Russian Federation Patent 2250047 **(2005)**.

[25] L.A.Elson, W.J.Morgan; Biochem.J., **27**, 1824 **(1933)**.

[26] K.A.Teylor, J.G.Buchanan-Smith; Anal.Biochem., **201**,190 **(1992)**.

[27] M.F.Chaplin; 'Monosacharides, carbohydrate analysis, A practical approach', IRL Press Limited, Oxford, England, **(1986)**.

[28] R.E.Neuman, M.A.Logan; J.Biol.Chem., **184**, 299 **(1950)**.

[29] J.F.Woessner Jr.; Determination of hydroxyproline in connective tissues, 'The methodology of connective tissues research', D.A.Hall, (Ed); Oxford, England: Johnson-Bruvvers Ltd., 235-245 **(1976)**.

[30] L.Slutsky; 'Biochemistry of normal and diseased connective tissue', Publishing House 'Medicine', Leningrad, **(1969)**.

[31] D.H.Vynios, A.Aletras, C.P.Tsiganos, T.Tsegenidis, C.A.Antonopoulos, A.Hjerpe, B.Engfeldt; Comp.Biochem.Physiol., **80B**, 761 **(1985)**.

[32] A.Hjerpe, B.Engfeldt, T.Tsegenidis, C.A.Antonopoulos, D.H.Vynios, C.P.Tsiganos; Biochim. Biophys.Acta., **757**, 85 **(1983)**.

[33] W.Garnjanagoonchorn, L.Wongekalak, A.Engkagul; Chemical Engineering and Processing, **46**, 465 **(2007)**.
[34] I.Yu.Sakharov, F.E.Litvin, O.Mitkevich; Bioorg. Chemistry, **20**, 190 **(1994)**.
[35] G.N.Rudenskaya; Biorgan.Chemistry, **29**, 117 **(2003)**.
[36] T.N.Pivnenko; J.Evol.Bioch.Physiol., **40**, 16 **(2004)**.
[37] K.Sugahara, J.Ohi, T.Harada, P.Dewaard, J.Vliegenthart; J.Biol.Chem., **267**, 6027 **(1992)**.
[38] H.Uchisawa, B.Okusaki, J.Ichita, H.Matsue; Int. Congr.Ser., **1223**, 205 **(2001)**.
[39] J.S.Kim, J.W.Park; J.Food Science, **70**, 511 **(2005)**.
[40] M.Ando, M.Makino, Y.Tsukamasa, Y.Makinodan, M.Miyosh; J.Food Science, **66**, 265 **(2001)**.
[41] R.Pool; Bioch.Journal, **236**, 1 **(1986)**.
[42] E.Ruoslahti; J.Biol.Chem., **264**, 13369 **(1989)**.
[43] M.L.Alves, A.Straus, H.Takahashi, J.M.Michelacci; Brazilian J.Med.Biol.Research, **27**, 2103 **(1994)**.
[44] N.K.Karamanos, S.Axelsson, P.Vanky, G.N.Tzanakakis, A.Hjerpe; J.Chromatogr., A, **696**, 295 **(1995)**.
[45] G.Y.Sukhoverhova; Biochemical characteristic of cartilage tissue of hydrobiontes and technology of biologically active supplements to food, PhD dissertation, TINRO-centre, Vladivostok, Russian Federation, **(2006)**.
[46] N.Solovieva; Bioorg.Chem., **24**, 245 **(1998)**.
[47] A.Ponton, B.Coulombe, D.Skup; Cancer Research, **51**, 2138 **(1991)**.
[48] W.Abdel Fattah, T.Hammad; JANA, **3**, 16 **(2001)**.
[49] J.M.Milner, S.F.Elliot, T.E.Cawston; Int.J.Exper. Pathol., **81**, 14 **(2000)**.
[50] G.Murphy; Biochemistry, **30**, 8097 **(1991)**.
[51] J.H.Liang, K.P.Wong; Advanc.Experim.Med.Biol., **476**, 209 **(2000)**.
[52] M.Reddy, E.Eirikis, H.M.Davis, U.J.Prabhakar; Immunol.Methods, **293**, 127 **(2004)**.
[53] R.De Maria, M.G.R.Cifone, R.Trotta, M.R.Rippo, C.Festuccia, A.Santoni, R.Testi; Exp.Med., **180**, 1999 **(1994)**.
[54] R.Marzio, J.Mauel, S.Betz-Corradin; Immunopharmacol.Immunotoxicol., **21**, 565 **(1999)**.
[55] A.Laffon, R.Garcia-Vicuña, A.Humbria, A.A.Postigo, A.L.Corbi, M.O.de Landazuri, F.Sanchez-Madrid; J.Clin.Invest., **88**, 546 **(1991)**.
[56] K.Murata, M.Inami, A.Hasegawa, S.Kubo, M.Kimura, M.Yamashita, H.Hosokawa, T.Nagao, K.Suzuki, K.Hashimoto; Int.Immunol., **15**, 987 **(2003)**.
[57] T.A.Moore, H.Y.Lau, A.L.Cogen, C.L.Monteleon, T.Standiford; Shock, **20**, 309 **(2003)**.
[58] F.Capsoni, P.Sarzi-Puttini, F.Atzeni, F.Minonzio, P.Bonara, A.Doria, M.Carrabba; Discussion Arthritis Research and Therapy, **7**, R250 **(2005)**.
[59] A.Afeltra, M.Galeazzi, G.D.Sebastiani, G.M.Ferri, D.Caccavo, M.A.Addessi, R.Marcolongo, L.Bonomo; Int.J.Exp.Pathol., **78**, 331 **(1997)**.