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Identification of Small Peptides of Acidic Collagen Extracts from Silver Carp Skin and Their Therapeutic Relevance

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A – research concept and design; **B** – collection and/or assembly of data; **C** – data analysis and interpretation; **D** – writing the article; **E** – critical revision of the article; **F** – final approval of article

Abstract

Background. Low-temperature techniques that prevent protein denaturation are being used to extract collagen from fish skin for cosmetic purposes. These extracts contain collagen with its triple helix structure preserved, as well as a number of other proteins.
Objectives. The aim of the study was to investigate collagen extracts from the skin of silver carp for the presence of small-molecule peptides.
Material and Methods. Liquid chromatography-mass spectrometry (HPLC-MS) was performed to analyze collagen extracts from silver carp skin for the presence of small-molecule peptides.
Results. A large number of different peptides were detected in the silver carp skin collagen extracts analyzed. Among the smaller peptides, the most abundant were those of 7–29 aminoacids originating from the following proteins: collagen Iα1, collagen Iα2, collagen Iα3, collagen VIα3, decorin, lumican, histone H2A, histone H2B and histone H4.
Conclusions. The study demonstrated that, in addition to high-molecular-weight collagen proteins, acidic collagen extracts acquired from the skin of silver carp at temperatures up to 16°C also contain considerable amounts of small 7–29 amino-acid peptides. The application of these peptides could therefore be expected to result in beneficial clinical effects in patients in need of reconstructive treatment (**Adv Clin Exp Med 2016, 25, 2, 227–235**).

Key words: collagen, histones, matrikines, HPLC-MS/MS, silver carp skin peptides.

For a few years now, low-temperature techniques that prevent protein denaturation has been used to extract collagen from fish skin for cosmetic purposes. These extracts contain collagen with its triple helix structure preserved, as well as a number of other proteins. An interesting question concerning composition of those extracts is the presence of small-molecule peptides.

Preparations of collagen are known to have a stimulatory influence on some cellular and tissue-specific processes – effects that are caused only by active compounds of low molecular weight, which are capable of penetrating into the skin and deeper tissues [1]. Reports [2–6] on the beneficial effects of collagen-containing products on wound healing were confirmed in the authors' pilot study on five patients with varicose leg ulcers from the Plastic Surgery Department at the Medical University of Gdańsk (Poland) using collagen extracted from the skin of silver carp [7]. These ulcers are wounds that are very difficult to heal. A few effects were seen: wound closure, accelerated granulation, anti-inflammatory effect, decreased evaporation of water from the wound. The collagen gel also protected the wound edges from the effects of toxic substances produced by the infected wound (proteolytic enzymes, etc.) [7].

Figure 1 shows examples of the treatment effects of collagen from the skin of silver carp – the subject of present article – on difficult-to-heal wounds in leg ulceration. The ulceration remained

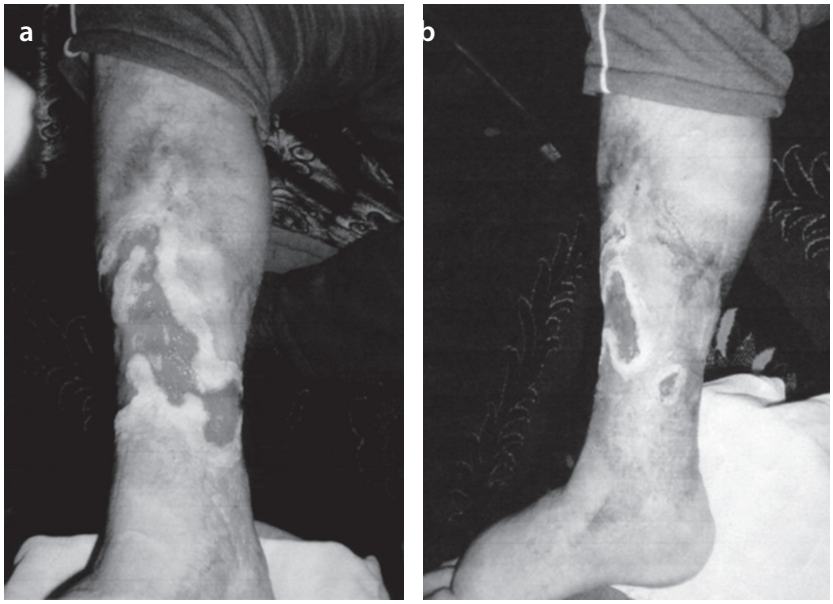

Fig. 1. Leg ulceration with the use of collagen from the skin of silver carp, a: before, b: after 3 months of collagen gel application directly to the wound twice a day [7]

unhealed for one year when other methods were used, but significant effects were achieved after three months of collagen gel applied directly to the wound twice a day.

There are also a few reports that confirm the biological activity not only of collagen extracts derived at low-temperatures without denaturation, but also of gelatin [8, 4]. Clinical studies suggest that the ingestion of 10 g daily of pharmaceutical-grade collagen hydrolysate obtained by hydrolysis of pharmaceutical gelatin reduces pain in patients with osteoarthritis of the knee or hip [8]. The first known description of the beneficial effects of gelatin ingestion in humans is from 1175, when St. Hildegard wrote that eating gelatin improved joint conditions by reducing pain [8]. In cases of individuals affected with osteoporosis, studies of the effects of calcitonin with and without a collagen hydrolysate-rich diet suggested that calcitonin plus collagen hydrolysate more effectively inhibited bone collagen breakdown than calcitonin alone [8]. Ausar et al. found that "orally administered bovine tracheal type-II collagen in the treatment of rheumatoid arthritis... induced clinical benefits in 90% of the patients" [9]. Oral administration of collagen peptide increased bone mineral density in rats and mice [10–12]. Other researchers have found that oral administration of type-II collagen decreases autoimmune response in rheumatoid arthritis and reduces joint inflammation in mice [13, 14]. The influence of collagen hydrolysate intake (including fish collagen hydrolysate) on skin has been demonstrated [15].

A few years ago, the term matrikines was proposed [16] for peptides liberated in the process of partial proteolysis of extracellular matrix macromolecules and capable of regulating cell functions. Many proteins of the intercellular matrix contain matrikines, which influence such phenomena as wound healing, malignant transformation and the atherosclerotic process. Some matrikines exert an inhibitory influence on these processes, while others augment them. Examples of source proteins for known matrikines, along with their stimulatory (+) and inhibitory (–) effects on malignant transformation and growth, are presented in Table 1.

As illustrated in Table 1, the effects of matrikines are selective. Also, the same proteins may be the source of both stimulatory and inhibitory matrikines in certain proportions. Therefore, it is important to bear in mind that extracts from the intercellular matrix may have either a beneficial or a detrimental effect with respect to malignant transformation or other processes, depending on the method of preparation of the extract.

The small leucine-rich proteoglycans decorin, lumican, biglycan and fibromodulin are secreted extracellular matrix molecules that associate with fibrillar collagens and regulate collagen fibrillogenesis [17]. A study on ligament proteoglycans yielded the following results: Proteoglycans represent less than 3% of the dry weight of ligaments, which is consistent with the levels found in other dense fibrous connective tissues; approximately 90% of the total proteoglycans in fresh ligament was decorin; and approximately 23% of the decorin detected in the matrix was degraded [18]. Intact decorin and decorin fragments similar to those observed in the matrix were also found in the medium of ligament cultures; similarly, with versican, biglycan and aggrecan, the co-appearance of intact proteoglycans and of large fragments was observed; also,

Table 1. The origins of known matrikines: Their source proteins and effects on malignant transformation and growth

Matrikines originating from	Tumor progression	Tumor proliferation	Tumor angiogenesis	Tumor metastasis
Collagen IV	–	0/–	–	+/–
Collagen VIII		–	–	
CollagenXV		0	–	–
CollagenXVIII	–	–	–	–
Elastin	+/–	–	+	+
Fibronectin	+/–	0/–	–	–
Laminins				+/–
Perlecan		+	+/–	
Thrombospondin -1	–		–	
Collagen Iα2			+	
Decorin		–	–	–
Lumican				–

(+) – activation of malignant transformation and growth; (–) – inhibition of malignant transformation and growth; (+/–) – activation or inhibition, different effects for different fragments of the source protein; (0) – no effect of a particular fragment on a given process, with concomitant activity towards another malignancy-related process [51–60].

type XII collagen appeared in both intact and degraded forms [11]. In the case of decorin, an electrophoretic technique utilizing antibodies applied to the ligament of 1- to 2-year-old steers revealed after-deglycosylation; in addition to full-length protein 43 kDa, smaller fragments of 32, 30, 21, 18 and 13 kDa were also present, in relative amounts ranging from 2.5% do 10% of the total amount of the full-length protein [18].

This report focuses on decorin, lumican and histone peptides which were detected in the extracts, as these may be particularly important for the therapeutic properties of crude collagen extracted in low-temperature conditions.

Material and Methods

Collagen Extracts

Collagen extracts were prepared as follows: The skin of silver carp (*Hypophthalmichthys molitrix*) was dissected from fat and muscle tissues, and 40 g/L of the skin was immersed in 1% solution of lactic acid for 24 h at 16°C. The gel obtained was filtered through a silk cloth filter to assure the homogeneity of the extract. For the analysis of native small peptides, the extracts were diluted 1 : 10 with 1% solution of lactic acid or acetonitrile and subjected to ultrafiltration through a filter with a molecular weight cut-off of 5 kD. Analyses were then performed for each of the two extracts.

Mass Spectrometry

The HPLC-MS/MS analysis was performed at the Laboratory of Mass Spectrometry at the Polish Academy of Sciences in Warsaw. Peptides were separated by nanoscale reverse phase high-performance liquid chromatography followed by electrospray ionization. Tandem mass spectra were obtained on a LTQ FT mass spectrometer and the ion generator used was a Finnigan Nanospray (both from Thermo Finnigan, Ringoes, NJ, USA). The separation conditions on the C18 nanocolumn were: acetonitrile gradient 0–40% in 0.05% solution of formic acid; separation time 60 min (division into approx. 2000 fractions).

A preliminary interpretation of the results was done with Mascot software (Matrix Science Inc, Boston, MA, USA). Only results with Mascot scores for proteins above 30 were included in the analysis; protein scores are related to the consistency of the measured masses of protein fragments with those obtained with theoretical calculations for a given mass, and therefore only indirectly related to the magnitude of the signal. All the results fell within the range 0–130.

Due to the lack of direct information on the ionic currents from fragments of different peptides, a scoring system was adopted in this study, in which the total numbers of occurrences of particular sequences were calculated in the measurements of longer or shorter peptides – that is, the sum of repetitions of the same peptide in a num-

ber of adjacent chromatographic fractions representing a wider peak was calculated, as well as occurrences of different peptides representing the same protein fragment, but scattered over a period of time in the chromatographic assessment. Additionally, points for samples of a given extract filtered in acetonitrile and lactic acid were added up.

Results

HPLC-MS/MS Analysis

A large number of different peptides were detected in the analyzed silver carp skin collagen extracts. Among the smaller peptides, the most abundant were those of 7–29 aminoacids, originating from the following proteins: collagen Iα1, collagen Iα2, collagen Iα3, collagen VIα3, decorin, lumican, histone H2A, histone H2B and histone H4.

Table 2 presents the results of the assessment of silver carp skin preparations. The extracts were prepared from skin samples obtained from fish originating from three different fish cultures in Hungary and collected in different seasons of the year. Four different extracts from the skin preparations were investigated. One of them was pale and transparent (marked as A). Two of them contained some quantities of melanin, which resulted in their having a dark tint (marked as B and C). The fourth sample (D) was degraded collagen obtained from extract A (a preparation additionally kept at 40°C for a period of 48 h was marked as Adeg for degraded.). Most of the proteins in preparation D were degraded by proteolysis. The degradation was revealed by SDS-polyacrylamide electrophoresis; the data is not presented.

Next to the peptide sequence the score is given reflecting the estimated quantitative differences in peptide content (italic characters were used for peptides found in the degraded sample only). The sequences presented in Fig. 2 represent the places within the proteins identified as the sites of origin of some families of shorter peptides.

Similar results demonstrating the presence of decorin, lumican and histones peptides were obtained in a series of verifying measurements with a slightly modified protocol (data not included).

HPLC/UV-VIS Analysis

Separate measurements (data not included) of the content in the filtrates of substances absorbing at 280 nm, performed with use of an RP 18 analytical column (*i.e.*, of markedly lower resolution) under separation conditions similar to those in the HPLC-MS/MS method, revealed the presence of about 40 highly distinct absorption peaks.

For extracts A, B and C there were over 300% differences in peak magnitudes for 12 peaks. Analogous comparisons done for separately prepared skin extracts from ventral and melanin-containing dorsal parts of the same fish revealed a difference in peak magnitude of over 300% for one peak only. These results show, therefore, that differences in peptide content between extracts A, B and C are not related to fish skin melanin content and the resulting degree of protection against UV, but rather to other environmental factors, fish age or genetic differences.

Discussion

The authors subscribe to the hypothesis that the biological activity of collagen extracts depends on the content of biologically active peptides be-

Decorin (*Danio rerio*)

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  1 MKSACLSLLL VSVCWALPFR QSGFMDFVME DEPASGDGPG PELPTTRKPH
 51 VERLPMMPEG PEVPFCPFRC QCHLRVAQCS DLGLKTVPEK IPLDTTLLDL
101 QNNKITEIKE NDFKGLKGLQ TLILVNNKIT IIHAKAFSSL INLERLYLSK
151 NLLKEVPANI PKSLQELRIH ENQINKIKKS SFAGMANVIV MELGSNPLSS
201 SGVDNGAFAD LKRVSYIRIA DTNLTSIPKG LPSSLFELHL DGNKITKVTA
251 DSLKGLKNLS KLGLSHNEIS VVENGSLANV PHLRELHLEN NALTAVPAGL
301 ADHKYIQVIY LHSNKIAAVG TEDFCPPGYN TKKAMYSGIS LFSNPVPYWE
351 VQPITFRCVF DRSAIQLGNY RKK
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Protein similar to vertebrate lumican (*Danio rerio*)

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  1 FLSRVLGPHT TVPEEREAGR FNLSCAAMFA LGSILLAGLL SLSLAQYDYY
 51 DEYYIPSAPL EGVSSPSCAQ ECECPINFPT AMYCNERNLK FIPIVPTGIK
101 YLYLQNNFIE EIKAGVFDNA TDLRWLVLDN NNITSDKIQA GTIDKLGSLE
151 KLLFSHNKLT KPPGSLSKSL DELKLIGNKL TSFPANTLAG MENLTTVHLS
201 KNKLTTESLT GAFKGLKSLI LLDVSENKLK KLPSGVPASL LMLYADNNDI
251 DSIPNGYLAK LPLLQYLRIS HNKLVDSGVP AGVFNVSSLL ELDLSFNKLK
301 TIPEINESLE HLYLQVNEIN KFELTNICRF SSPVNYSRLR TLRLDGNNIT
351 HSSMPDDTAN CLRQASEIIF E
```

Fig. 2. The distribution of peptides found in two example protein sequences

Table 2. The most abundant peptides from the skin of silver carp

Protein	Found peptide	A	B	C	A deg
Collagen Iα1 (*Danio rerio, Oncorhynchus mykiss*)	FIAQPQEKAPDPFRHFRA LKMCHPDWKSGEYWIDPDQGCNQD IAQPAQEKAPDPFRHF SGLPGPIGPPGPRGRSGEMGP GMPGERGAAGLPGLKGD	15 1 1	18 1	4 1	
Collagen Iα2 (*Danio rerio*)	TSGGYDEYR LRGHPGLQGMPGPNGPSGDSGPAGI MPGPNGPSGDSGAAGIAGPSGPRGPAGPN *PGPVGVKGDSGVKGE*		1 1 1	1	 3
Collagen Iα3 (*Danio rerio*)	GPDPLRGGY FPGPKGT *PGLQGPKGD* *AGKEGQRGARGEKGPAGRPGEAG* *GKTGDRGEAGPAGPAGPSGPAGARGALGPA*		4 1	 1	3 4 8 1
Collagen IVα1 (*Danio rerio*)	*PGLQGIKGD* *PGIPGTKGD* *PKGDRGDQGQPGERGATGEQGPPGIP*			 1	2 2
Protein homologous with collagen VIα3 (*Tetraodon nigroviridis*) (six repetitions in the gene of the sequence: LLDGSDGTRSGFPAMRDF)	LLDGSDGTRSGFPAMRDF PRGKDVVFLLDGSDGTRSGFPAMRDF DRVSVVQYSRD RGGAPVRTGAALQYVRD		16	8 4 1	
Protein similar to collagen VII (*Danio rerio*)	*GEQGEKGPAGPQGPTGRAIGERGPEGP*			1	
Procollagen VIIIα2 (*Mus musculus*)	*GPRGDRGLKGD*				1
Decorin (*Danio rerio*)	ELGSNPLSSSGVDNGAFADLKRVSYIR FSNPVPYWEVQPIT HLDGNKITKVTAD ILVNNKITIIHAKAFSSLINL	 1	3 3 2 2	5 1 2 3	
Protein similar to *vertebrate lumican* (*Danio rerio*)	DLSFNKLKTIPEINESLEHL LDVSENKLKKLPSGVPASLLML	6 6	13 3	16 7	
Histone H2A (*Danio rerio*)	AVLLPKKTEKPAKS ILELAGNAARDNKKTR AVRNDEELNKLLGGVTIAQGGVLPNIQA	6	7 1	10 1 1	
Histone H2B (*Rattus norvegicus*)	SSTAAVLAQRLVPEYNMPEPTKSVPAPK KVLKQVHPDTGISSKAMGIMNS		1 1		
Histone H4 (*Mus musculus*)	YTEHAKRKTVTAMD			2	

longing to the four groups described above. In the extracts obtained in this study a large number of peptides – several hundred – originated from protein cleavage. The most abundant were decorin, lumican, histone 2A and four types of collagen. Reports from other studies confirm that, apart from their well-explored structural function, all four types of proteins, in the form of both intact molecules and smaller peptide derivatives, exhibit properties of signaling and protective molecules related to tissue repair processes.

The results obtained in this study demonstrate a certain discrepancy in the peptide composition of collagen extracts from skin containing different quantities of melanin. The degraded specimen was not found to contain decorin, lumican and his-

tone peptides, although it should be admitted that, considering the greater amount of peptides originating from collagen breakdown in the degraded specimen as compared with non-degraded extracts (Mascot score 100–130 vs. 30–70, respectively), the decorin, lumican and histone peptide signals could have been outweighed by signals for collagen peptides – in this method only the peptide with the strongest signal is selected from each chromatographic fraction by the software analysis system.

As for collagen type VI alpha 3, among the obtained peptides there were some containing six repetitions of the sequence LLDGSDGTRSGFPAMRDF found in this protein, representing classic examples of a matrikine cleaved from the protein in multiples.

This investigation of collagen extracts from silver carp skin demonstrated the presence of large dominant amounts of decorin, lumican and histone peptides, along with a number of single or low-quantity peptides from different types of collagen. Considering the total length of the particular proteins, decorin, lumican and histone peptides accounted for an even greater proportion of those proteins. Although the method of mass spectrometry used in this study does not have the capacity to reveal the presence of all the peptides present due to major sequence-related differences in the degree of their ionization, and also because of certain limitations resulting from the method of chromatographic separation, and therefore all quantitative estimations without an internal standard must be taken as rough approximations, the very fact of the presence of large quantities of decorin, lumican and histone peptides in the analyzed extracts can be recognized as an indicator of their important biological role for homeostasis in the fish skin. The amino acid sequences for the peptides that were detected in the skin of silver carp were largely inconsistent with those described in other reports. There were only two peptides for which a partial overlap was found between the sequences detected in the present study and those reported by other researchers.

Using decorin, lumican and histone for treatment and prevention seems to be a promising perspective. Therefore, the authors of the current study investigated available references and collected information on their effects, which may prove helpful in the course of further clinical investigations. The significant properties of these three proteins and their smaller derivatives are summarized below.

Decorin is a naturally occurring antagonist of scar formation; it promotes adult sensory neuron axon growth across spinal cord scar tisssue [19, 20]. Direct infusion of human recombinant decorin into acute stab injuries of an adult rat spinal cord resulted in major reductions in astrogliosis, macrophage accumulation and deposition of the axon growth inhibitors neurocan, brevican and phosphacan within the spinal cord scar tissue [19]. Decorin has been successfully employed to reduce tissue fibrosis in different disease models in the kidney, lung, and vascular structures. The antifibrotic properties of decorin have been confirmed in a mouse model of pulmonary fibrosis, in which fibrosis was induced by transient overexpression of active TGF-β using adenoviral gene transfer and followed by 21-day overexpresion of decorin, also by adenoviral vector [21]. Decorin is required for the proper fibrotic evolution of myocardial infarction [22] and plays a role in limiting the inflammation process [23]. It is also a natural inhibitor of fibroblast proliferation, its main function probably being to repress the action of transforming growth factor-β (TGF-β), which is involved in wound healing [24–26]. Decorin is a natural anticancer agent; it significantly suppresses the growth of rat breast carcinoma cells and could also inhibit metastases to the lungs [27]. Decorin induces both *in vitro* and *in vivo* tumor cell apoptosis [28]. The antitumoral effect of decorin is mediated inter alia by epidermal growth factor receptor EGFR and TGF-β [27, 29, 30]. Decorin also suppresses tumor cell-mediated angiogenesis both *in vitro* and *in vivo* [31]. As Sulochana et al. wrote, "not only purified decorin but also the 26-residue leucine-rich repeat 5 of decorin core protein functions as angiogenesis inhibitor by inhibiting both vascular endothelial growth factor (VEGF) and basic fibroblast growth factor-induced angiogenesis" [32, 33].

As Chakravarti et al. wrote: "Lumican is a major constituent of the corneal stroma, where it plays a significant role in the acquisition of corneal transparency by regulating collagen fibril diameter and interfibrillar spacing" [34]. It is also present in the dermal extracellular matrix of the skin [34]. Lumican deficiency leads to increased cell proliferation, down-regulation of p53, down-regulation of the CDK inhibitor p21 and increased expression of G1-S cyclins; lumican also plays an important role in Fas-mediated regulation of apoptosis. Lumican-deficient mice show decreased apoptosis of stromal keratocytes [35]. Lumican inhibits melanoma progression. After subcutaneous injections of transfected B16F1 melanoma cells in syngenic mice, lumican expression significantly decreased subcutaneous tumor formation *in vivo* [36]. Lumican core protein expression in growing tumors inhibited the expression of cyclin D1, which is a major cyclin controlling Cdk activation and the regulation of cell cycle progression. These results

suggest that the mechanism of action of lumican on melanoma cells is different from that of decorin [36]. Lumican, as well as leucine-rich repeat peptide from human lumican, inhibits melanoma cell migration [37, 38].

Peptides containing 20–50 amino acids originating from the N-terminal tail of histone H2A possess antimicrobial activity. Examples of such peptides are hipposin from the skin mucus of Atlantic halibut [39, 40], buforin I [41] and buforin II [42], isolated from the stomach tissue of *Bufo bufo gargarizans* (an Asian toad), and parasin from the mucous layer of wounded catfish skin [43, 44]. Parasin I is generated from histone H2A in the skin mucus of catfish by the action of cathepsin D activated by a procathepsin D processing enzyme – matrix metalloproteinase 2 (MMP2) – induced upon epidermal injury [45, 46]. Intact histone H2B possesses antimicrobial and antifungal activity in catfish, rainbow trout and sunshine bass [47]. Histone H2B is a functional polypeptide of the antimicrobial defense in human colon mucosa. It has also been suggested that histones H2A and H2B participate in the host defense of the fetus by being produced in the placenta and then secreted into the amniotic fluid [48]. They also possess hormone-releasing activity [49]. Buforin I also possesses anticoagulant activity [50]. Bone marrow regeneration is associated with a marked increase in the serum levels of a 14-amino acid osteogenic peptide identical to the C-terminus of histone H4 [49].

Given the fact that investigations on matrikine peptides are still not very advanced, the potential role of the peptides contained in fish skin collagen extracts, as well as in other collagen preparations, in such important functions and reactions as regulation of proliferation, angiogenesis, metastasis, apoptosis, wound healing, fibrosis, immunological response and hormonal response deserves serious attention and seems to cast some light on why collagen-containing and collagen-derived products have become so popular over the last few years.

An issue of special interest is the possibility of using fish skin peptides in the form of preparations for oral use. The capacity of peptides to penetrate into tissues is the reason they outperform proteins with the same regulatory actions in practical medical applications. Moreover, it is known that peptides may be absorbed from the digestive tract, which makes them easy to use orally. Although it is frequently stated that proteins such as gelatin taken in oral form are enzymatically digested to their amino acid components in the intestinal tract, gelatin peptides are only digested to a certain degree within the gastrointestinal tract, with a proportion of intact high-molecular-weight proteins reaching the serum subsequent to passing through the intestinal wall at a level of approximately 10% [8].

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