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BIOPHYSICS AND BIOCHEMISTRY

Preparation of Type II Collagen Short Peptides: Temperature Conditions of Cartilage Homogenization and Collagen Hydrolysis

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Physicochemical properties of hyaline cartilage homogenates were studied by the method of microcalorimetry. Collagen hydrolysates were obtained after homogenization of hyaline cartilages under high pressure conditions at the temperatures that denaturate collagen. Thermodynamic parameters of thermal transition of collagen in cartilage suspension were determined. Enthalpy of thermal transition ΔH decreases in comparison with the control. Thermal transition half-width ΔT varies with temperature. More denatured and homogeneous samples were obtained at homogenization temperature 80°C. According to spectral studies, particles in the samples obtained at the temperature of 80°C were smaller. The temperature of 80°C is preferred for homogenizing hyaline cartilages and obtaining collagen type II short peptides.

Key Words: *homogenization of hyaline cartilages; collagen hydrolyzates; collagen type II short peptides; temperature; microcalorimetry*

Collagen hydrolyzates are used in medicine for the prevention and treatment of diseases of the musculoskeletal system. Hydrolysates obtained on the basis of gelatin, denatured collagen, have chondroprotective properties. Moreover, collagen hydrolyzates improve the condition of patients with arthritis and arthrosis at the initial and middle stages of the disease [15]. For preparing hydrolysates, collagen extracted from tissues is subjected to enzymatic hydrolysis. However, the yield of collagen amino acids and peptides from connective tissues is insignificant [11].

Obtaining collagen hydrolyzates directly from the connective tissues is difficult due to their structure. It is known that connective tissues are characterized by high mechanical strength. The extracellular matrix of cartilages contains tightly bound coiled molecules and fibrils of type II collagen. Dense packing of collagen molecules is also achieved by intermolecular bonds in the transverse and longitudinal directions of the fibrils. The spatial structure of collagen fibrils in the cartilage tissues is stabilized by proteoglycans. Various methods of tissue disintegration can destroy the strong network of the extracellular matrix. The procedure of processing raw materials in high-pressure homogenizer makes it possible to obtain particles of minimum size [9]. Therefore, it is possible to break down long collagen fibrils into fragments.

High pressure homogenization is a proven method for the production of collagen short peptides derived from the cartilages [6]. We studied hydrolysis of collagen under the effect of several proteolytic enzymes

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[5-7]. It should be emphasized that collagen proteolysis is difficult due to rigid packing of helical molecules into supercoiled fibrils. For this reason, native collagen is poorly available for enzyme cleavage. Therefore, collagen denaturation is a necessary step for effective enzymatic hydrolysis. It is known that when proteins are denatured, the secondary, tertiary, and quaternary structures are destroyed. For collagen, this step means disintegration of fibrils and unwinding of triple helices with the formation of tangles of polypeptide chains.

It should be noted that denaturation of collagen in cartilage fibrils occurs in a wide temperature range: from 50 to 90°C [1]. Proteoglycans prevent complete denaturation of type II collagen in biological tissues. Therefore, at the first stage of cartilage processing, it is necessary to destroy the proteoglycan network, which can be achieved by mechanical homogenization of the tissues. Determination of the temperature conditions for homogenization will allow influencing collagen proteolysis and the yield of the final product. At ~100°C collagen denatures in such a way that during enzymatic hydrolysis, the molecules were split to amino acids, but not peptides [14]. However, amino acids in the gastrointestinal tract are absorbed more slowly than short peptides [12]. Short peptides consisting of 2-3 amino acid residues are quickly absorbed, enter the cells, and participate in collagen biosynthesis. In addition, short peptides regulate the functions of the neuroendocrine and immune systems.

The purpose of this work is to study physicochemical properties of hyaline cartilage homogenates obtained in a high pressure homogenizer (HPH). Two factors (pressure and temperature) can be combined in the Donor-3 HPH device developed in Pushchino. Note that temperature is an essential factor for the production of short collagen peptides from the cartilage tissue. At the same time, temperature conditions for cartilage homogenization that affect collagen denaturation are poorly studied.

MATERIALS AND METHODS

Hyaline cartilages of cattle trachea were used as the biological material. The cartilages contain approximately 8% collagen and 7% proteoglycans [2]. The cartilages were dispersed in a Braun MQ 520 mixer (electromechanical tissue homogenizer). Then, the cartilage suspension was passed through a Donor-3 HPH homogenizer [9]. The homogenization parameters (pressure, temperature, and time) are presented elsewhere [7]. In our work we studied combined action of high pressure and temperature denaturing collagen. After processing was completed and the samples were cooled to room temperature, the particle sizes in the homogenates were measured. The control experiment was carried out at 20-30°C and atmospheric pressure.

Particle sizes in the samples were determined by photon correlation spectroscopy using Delsa Nano S submicron particle size analyzer (Beckman Coulter). Calorimetric measurements were carried out using a DASM-4 microcalorimeter (Institute of Biological Instrumentation, Russian Academy of Sciences) in the cell with a working volume of 0.5 cm^3. The heating rate was 1 K/min.

Data collection was performed using the SCAL software program (Institute of Protein Research, Russian Academy of Sciences). Based on the analysis of the calorimetric curves, the following thermodynamic characteristics of thermal transition were determined: the temperature of the peak maximum (T_m), half-width of the thermal transition (ΔT), and enthalpy of the thermal transition (ΔH). Numerical values of the thermodynamic parameters were obtained as described previously [8]. Calculations were performed using Origin 6.0 software (Microcal Software, Inc.).

RESULTS

Figure 1 shows calorimetric curves of the homogenized samples. In the control sample, thermal transitions of collagen molecules and collagen fibrils from native to disordered state occurred in the range from 25 to 80°C. The minor peak with maximum T_m=44.3°C corresponded to denaturation of collagen molecules that are weakly bound to each other. The main peak with the maximum at 60.7°C reflects melting of densely packed fibrils [1]. Thermal transitions of collagen

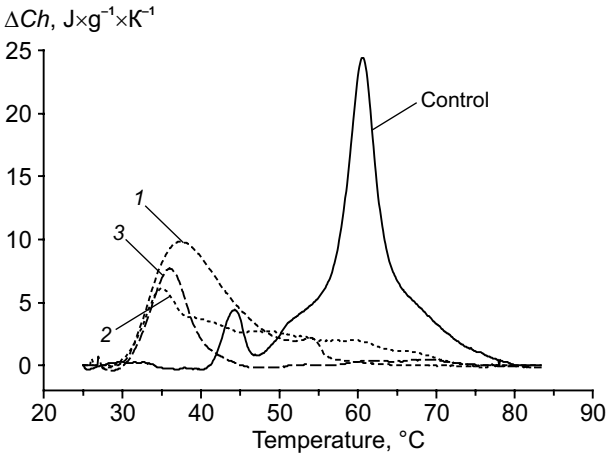

Fig. 1. Temperature dependences of excess specific heat capacity of collagen in hyaline cartilage samples at high pressures and temperatures of homogenization in comparison with the control. *1*) Sample after homogenization at 70°C; *2*) sample homogenized at 70°C, cooled to 20°C, and then thermostated at 70°C and atmospheric pressure; *3*) sample after homogenization at 80°C.

fibrils with different degrees of binding between collagen molecules and, respectively, different thermal stability (T_m was 55 and 68°C) were also recorded. Interestingly, these transition temperatures were close to those obtained in a previous study [3], where proteolysis with trypsin and chymotrypsin was used to destroy the proteoglycan network in the cartilage. The main temperature peak (59.8°C) coincides with that for the control sample of hyaline cartilage in our work.

Mechanical homogenization of hyaline cartilages at 20-30°C and atmospheric pressure destroys the proteoglycan network. Fibers consisting of collagen fibrils and proteoglycans were also destroyed. It is known that proteoglycans stabilize the fibrillar structure of collagen in the cartilage [1]. After removal of the proteoglycans, thermal transitions were recorded in the samples corresponding to the melting of collagen structures [1]. Analysis of the homogenate of the control sample allowed us to determine the maximum temperatures that denature collagen. On the calorimetric curves these temperatures were in the range of 68-80°C. The temperatures above the temperature of the thermostable peak T_m=68°C were chosen. Therefore, the following temperature conditions were used for the homogenization of the hyaline cartilages: 70 and 80°C. The enthalpy ΔH of the control sample is 69.1 J/g. This value is close to the data on the enthalpy of fibrillar collagens of types I and II [1].

Homogenization at 70°C (sample No. 1) changed the ratio of the transition peaks compared to the control. The main peak had maximum T_m=37.4°C, minor peak had T_m=54°C. Moreover, the temperature of the maxima of the main and minor peaks decreases by 7°C in comparison with the control (curve *1*). There are no additional transition peaks on the calorimetric curves. The main peak appears to be related to melting of the polypeptide chains of collagen molecules. During homogenization, long collagen molecules break down to fragments, while after cooling the samples to room temperature and below, the polypeptide chains randomly bind together. The minor transition peak is related to fibrillar collagen. However, the fibrils in sample No. 1 were less thermostable than in the control. Homogenization at 70°C led to an increase in ΔT parameter up to 11.1°C. In the control, ΔT of the main transition was 4.8°C, and ΔT of the minor transition was 2.9°C. The wide main transition peak indicates, that the peak obtained at 70°C was non-uniform. The homogenate contained collagen aggregates and tangles of polypeptide chains. Under these homogenization conditions, heterogeneous samples with the enthalpy equal to 42.4 J/g were obtained.

We carried out additional thermostating of sample No. 1 at 70°C and atmospheric pressure over 1 h (sample No. 2). The temperature of the main peak of the transition dropped to 35°C. The minor thermostable peak with a maximum T_m=54°C remained after thermostating. At the same time, other minor transition peaks appeared (curve *2*). Their formation was due to additional collagen denaturation at 70°C. After warming of the samples, new random bonds were formed between the collagen polypeptide chains. In this way, collagen aggregates and polypeptide tangles can form. The enthalpy of thermal transition is reduced to 34.6 J/g. In this experiment, incubation of sample No. 1 at 70°C and atmospheric pressure was carried out to break the thermostable bonds between collagen molecules in fibrils. However, additional processing of the homogenate at this temperature did not lead to effective denaturation. Therefore, in further experiments we used homogenization temperature of 80°C and high pressure. According to published data [10], high pressure suppresses protein aggregation. Thus, homogenization under high pressure conditions has an advantage over homogenization under atmospheric pressure.

After homogenization of the cartilages at 80°C, the temperature of the main peak of transition of fibrillar collagen (sample No. 3) decreased to 36°C. In addition, the increase of homogenization temperature to 80°C shifted the transition temperature of the main peak by 1.4°C to the low-temperature region (curve *3*). In this case, the half-width of the transition (ΔT=5.7°C) slightly increased in comparison with the control. The enthalpy ΔH decreased by ~4 times in comparison with the control (to 16.9 J/g). After increasing the temperature from 70 to 80°C, the enthalpy decreased by more than 2 times. There were no additional thermostable transition peaks on the calorimetric curves. These results attest to more complete denaturation of collagen at homogenization temperature of 80°C.

It should be noted that enthalpy ΔH is used to estimate the total number of non-covalent bonds in macromolecules [13]. ΔT, *i.e.* half-width of the thermal transition, can be used to analyze the homogeneity of the obtained samples. In previous study of one of the authors of this work, the minimum ΔT of fibrils *in vitro* was determined [8]. Fibrils were formed from a homogeneous collagen solution at physiological temperature. Minimum ΔT value correlated with minimum fibril thickness, which coincided with the size of fibrils *in vivo* [4].

Comparative analysis of particle sizes in the samples is shown in Figure 2. Sample No. 1 was obtained at homogenization temperature of 70°C. The sample contained particles with the mean diameter of 1500 nm. Sample No. 3 was obtained at homogenization temperature of 80°C. The sample contained

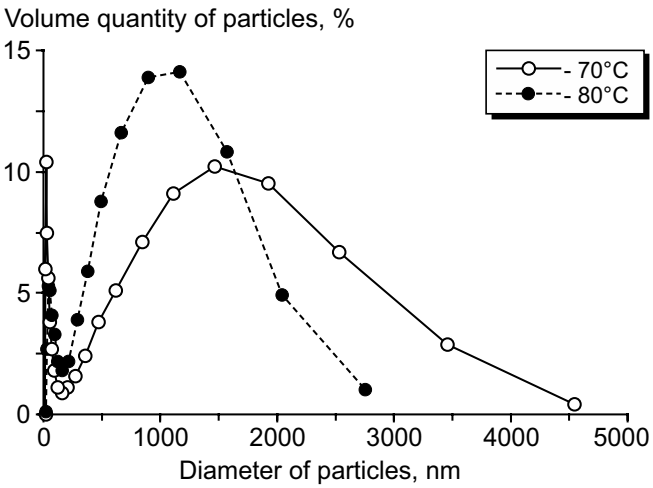

Fig. 2. Particle size distribution in hyaline cartilage samples homogenized at high pressure and different temperatures.

smaller particles with the mean diameter of 1000 nm. Photon correlation spectroscopy allows determining not only the size of the particles, but also their distribution in volume. Figure 2 shows that sample No. 3 is characterized by a narrower distribution of particle diameters. Hence, more homogeneous samples were obtained at 80°C and less homogeneous at 70°C. The data of photon correlation spectroscopy agree with the results obtained by the microcalorimetry method.

The novelty of our work lies in the fact that the thermodynamic parameters of collagen fibril disordering after cartilage homogenization at 70 and 80°C were determined. The values of the parameters ΔT and ΔH decreased by 2 times with increasing temperature: the number of intermolecular bonds decreased; the structure of collagen fibrils was more disordered. There were no thermostable peaks of thermal transitions on the calorimetric curves. The distribution of particles in the homogenate formed at 80°C was narrower. The use of scanning microcalorimetry and photon correlation spectroscopy allows comparing homogeneity of dispersed hyaline cartilages. For each type of cartilage raw material, it is possible to determine the optimal homogenization temperature for obtaining short collagen peptides directly from cartilage tissues.

The homogeneous sample of dispersed hyaline cartilage was obtained at 80°C. More complete denaturation of collagen also occurred at 80°C. The particles in the homogenate at 80°C were 1.5 times smaller than at 70°C. Collagen molecules in the homogenate at 80°C were more accessible for enzyme digestion. Homogenization at 70 and 80°C yielded samples with different physicochemical properties. Thus, we determined the optimal temperature for cartilage homogenization to obtain short peptides of collagen type II — 80°C.

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