Comparative Analysis of Collagen-Containing Waste Biodegradation, Amino Acid, Peptide and Carbohydrate Composition of Hydrolysis Products

Stanislav Sukhikh 1, Svetlana Noskova 1, Svetlana Ivanova 2,3,* Elena Ulrikh 4,5, Alexander Izgaryshev 1,6, Timothy Larichev 7, Oksana Kozlova 6, Alexander Prosekov 8,9 and Olga Babich 1

1 Institute of Living Systems, Immanuel Kant Baltic Federal University, A. Nevskogo Street 14, 236016 Kaliningrad, Russia; stas-asp@mail.ru (S.S.); svykrum@mail.ru (S.N.); a.izgarishev@mail.ru (A.I.); olich.43@mail.ru (O.B.)
2 Natural Nutraceutical Biotesting Laboratory, Kemerovo State University, Krasnaya Street 6, 650043 Kemerovo, Russia
3 Department of General Mathematics and Informatics, Kemerovo State University, Krasnaya Street 6, 650043 Kemerovo, Russia
4 Kuzbass State Agricultural Academy, Markovtseva Street 5, 650056 Kemerovo, Russia; elen.ulrich@mail.ru
5 Institute of Agroengineering and Food Systems, Kaliningrad State Technical University, Soviet Avenue 1, 236022 Kaliningrad, Russia
6 Department of Bionanotechnology, Kemerovo State University, Krasnaya Street 6, 650043 Kemerovo, Russia; ms.okvk@mail.ru
7 Department of Fundamental and Applied Chemistry, Kemerovo State University, Krasnaya Street 6, 650043 Kemerovo, Russia; t.larichev@yandex.ru
8 Laboratory of Biocatalysis, Kemerovo State University, Krasnaya Street 6, 650043 Kemerovo, Russia; a.prosekov@inbox.ru
* Correspondence: pavvm2000@mail.ru; Tel.: +7-384-239-6832

Abstract: This paper aimed to study the biodegradation of collagen-containing waste (pork skin) induced by collagenase and Neutrase 1.5 MG enzymes and compare the amino acid, peptide, and carbohydrate composition of hydrolysis products. It was found that the degree of biodegradation of collagen-containing raw materials (pork skin) reached 78% when using an enzyme preparation (collagenase with a concentration of 250 U/g of the substrate) at pH 7.0, 40 °C, and a 360 min process duration. It was shown that the content of peptides with a molecular weight of 6.5–14.0 kDa in the hydrolysis products (collagenase) of collagen-containing wastes was 13.4 ± 0.40%, while in the products of hydrolysis (Neutrase 1.5 MG) it was 12.8 ± 0.38%. The study found that the hydrolysis products (Neutrase 1.5 MG) of collagen-containing raw materials contain fewer hexoses, free hexosamines, and hyaluronic acid than the hydrolysis products (collagenase) of collagen-containing raw materials. The content of chondroitin sulfates is practically the same in all samples of hydrolysis products. Proteases with collagenolytic activity are widely used in industry. Recently, they have increasingly been used in pharmaceutical, food, and other industries. Collagenases are promising enzymes for the production of chondroprotectors used for the treatment of osteoarthritis.

Keywords: osteoarthritis; collagenase; neutrase; biodegradation; peptides; hexosamines; hyaluronic acid; chondroitin sulfate

1. Introduction

Osteoarthritis (OA) is the most common musculoskeletal disorder [1–3] affecting both large and small joints. OA affects many joints but is usually localized in the joints that bear the load and most often occurs in the knee joints. This disease leads to a progressive change in all structures of the joint tissues [4]. Among all joint diseases, knee OA has a prevalence of about 10% in men and 13% in women over the age of 60 [5,6]. Although aging and OA are closely related, aging is not the only cause of this disease. Preceding joint injuries,
obesity, genetics, gender, and anatomical factors related to the shape and position of the joints are also predisposing factors \cite{7}. This disease entails significant physical limitations, leading to disability \cite{3,8-10}.

Increased cartilage degradation is a symptom of this destructive joint disease. In the cartilage matrix, proteoglycan and collagen exist as basic elements, and damage to the proteoglycan can cause cartilage degeneration \cite{2}, followed by catabolism of collagen fibrils, which increases the loss of structural integrity of the cartilage \cite{11}. Matrix metalloproteinase (MMP)-induced cartilage degeneration is controlled by endogenous tissue inhibitors of metalloproteinase (TIMP), and the imbalance in the TIMP to MMP ratio can lead to permanent matrix destruction in OA \cite{12-14}.

There is currently no cure for osteoarthritis nor method to prevent its progression. The available treatments are only effective in relieving symptoms and can cause severe and fatal side effects in older people \cite{15-19}. Control of OA symptoms includes oral or local administration of non-steroidal anti-inflammatory drugs or intra-articular injections of corticosteroids \cite{20-22}. While autogenous grafts are the most preferred method, especially for significant defects, this treatment is limited by the lack of donors, the risk of inflammation, rejection, etc. Thus, the focus of research in this area is to develop regenerative biomaterials that do not require donors, do not induce an immune response, and can integrate with stem cells \cite{3}.

One of the solutions to this problem can be chondroprotectors based on the hydrolysis products of collagen-containing compounds that are part of animal husbandry waste.

Many products are discarded as waste both in industry and everyday life. Regardless of the worldwide tendency towards waste reduction, the amount of generated waste is increasing every year. Interest in exploring possible means of more efficient use of underutilized resources and industrial waste has been growing. Tanneries and fur factories, as well as slaughterhouses, discard significant quantities of materials containing mainly keratin (wool, bristles, horns, feathers, hooves, etc.) or use them in the production of meal which is used as a protein additive in feeds for domestic animals. This meal was found to be a carrier of prions of some related diseases (mad cow disease, swine fever, etc.). As a result, feeding domestic animals with this meal was strictly prohibited in many countries. Incineration of animal husbandry waste is now seen as a reliable way to stop the proliferation of prions, but it is expensive. This waste is mainly disposed of in controlled waste deposits. However, the latter pose severe environmental and sanitary problems around the world \cite{23-25}.

The connective tissue of cattle slaughter waste contains a large amount of collagen \cite{26}. Collagen, the main structural component of the extracellular matrix of all tissue types, consists of a triple helix containing two identical alpha chains (\(\alpha_1\)). Its high biocompatibility and non-toxicity make it an excellent material for medical use \cite{27-29}.

Due to its properties, it is widely used in many fields of science, including bioengineering, medicine, and tissue engineering \cite{30,31}. Several new collagen-based materials can be expected shortly due to their biocompatibility, lack of immune response, and film-forming properties. Biomedical companies and the cosmetic industry are interested in developing new collagen-based materials since there are still many possibilities for modifying this biopolymer \cite{21}. The processing of such collagen waste into preparations with biofunctional properties is a very profitable area of biotechnology. Bovine collagen is a precursor of ACE-inhibiting peptides with high bioavailability, which may be of practical value in medicine \cite{32,33}.

This paper aimed to study the biodegradation of collagen-containing waste (pork skin) induced by collagenase and Neutrase 1.5 MG enzymes and compare the amino acid, peptide, and carbohydrate composition of hydrolysis products.

2. Results

The biodegradation degree dynamics of protein molecules in samples of collagen-containing raw materials (pork skin) depending on the concentration of collagenase and
Neutrase 1.5 MG enzyme preparations, pH value, and temperature of the medium is shown in Figures 1–3. The hydrolysis degree values of the samples are comparable to each other; however, a higher rate of the process was observed in the samples with catalase.

Table 1 shows generalized results on selecting parameters and substantiating technological modes of biodegradation of collagen-containing waste by collagenase and Neutrase 1.5 MG.

![Graph](a)

**Figure 1.** Study of the biodegradation of protein molecules in samples of collagen-containing raw materials by (a) collagenase or (b) Neutrase 1.5 MG at various concentrations of the enzyme, U/g: 1–150, 2–200, 3–250, 4–300. Values followed by the same letter do not differ significantly (*p* > 0.05) as assessed by post hoc test (Tukey test).
Figure 2. Results of studying the biodegradation of protein molecules in samples of collagen-containing raw materials by (a) collagenase or (b) Neutrase 1.5 MG at different pH values: 1–5.0; 2–6.0; 3–7.0; 4–8.0. Values followed by the same letter do not differ significantly ($p > 0.05$) as assessed by post hoc test (Tukey test).

Table 1. Generalized results on selecting parameters and substantiation of technological modes of biodegradation of collagen-containing waste by enzyme preparations.

| Conditions          | Enzyme Preparations | Collagenase | Neutrase 1.5 MG |
|--------------------|---------------------|-------------|-----------------|
| Enzyme concentration, U/g | 250                  | 250         |
| Duration, h        | 6                   | 7           |
| pH                 | 7.0                 | 6.0         |
| Temperature, °C    | 40                  | 50          |
| Hydrolysis degree, % | 77–78               | 72–74       |
Figure 3. Results of studying the biodegradation of protein molecules of samples of collagen-containing raw materials by (a) collagenase or (b) Neutrase 1.5 MG at different temperatures, °C: 1–30; 2–35; 3–40; 4–45; 5–50; 6–55. Values followed by the same letter do not differ significantly (p > 0.05) as assessed by post hoc test (Tukey test).

Table 1 shows generalized results on selecting parameters and substantiating technological modes of biodegradation of collagen-containing waste by enzyme preparations.

Table 1. Generalized results on selecting parameters and substantiation of technological modes of biodegradation of collagen-containing waste by enzyme preparations.

| Conditions | Enzyme Preparations |
|------------|---------------------|
|            | Collagenase | Neutrase 1.5 MG |
| Enzyme concentration, U/g | 250 | 250 |
| Duration, h | 6 | 7 |
| pH | 7.0 | 6.0 |
| Temperature, °C | 40 | 50 |
| Hydrolysis degree, % | 77–78 | 72–74 |

Next, we analyzed the amino acid, peptide, and protein profiles of samples of collagen-containing waste hydrolysis products obtained using collagenase and Neutrase 1.5 MG.

At the first stage of this task, we analyzed the peptide and protein profile of samples of collagen-containing waste hydrolysis products obtained using the collagenase and Neutrase 1.5 MG enzyme preparations.

The results of studying the peptide and protein profile of samples of collagen-containing waste hydrolysis products obtained using collagenase and Neutrase 1.5 MG are presented in Table 2.
Table 2. Results of studying the peptide and protein profile of samples of collagen-containing waste hydrolysis products obtained using collagenase and Neutrase 1.5 MG.

| The Molecular Weight of the Protein Profile, kDa | The Proportion of Protein and Peptide Fraction in the Hydrolyzate, % |
|-----------------------------------------------|---------------------------------------------------------------|
| 66.0–89.0                                     | I: 0.1 ± 0.003 II: 0.3 ± 0.001                                |
| 57.0–65.0                                     | I: 0.8 ± 0.02 II: 0.6 ± 0.02                                  |
| 45.0–57.0                                     | I: 3.6 ± 0.11 II: 3.8 ± 0.11                                  |
| 36.0–45.0                                     | I: 2.1 ± 0.06 II: 1.9 ± 0.05                                  |
| 29.0–36.0                                     | I: 23.8 ± 0.71 II: 22.4 ± 0.72                               |
| 14.0–29.0                                     | I: 15.7 ± 0.47 II: 14.5 ± 0.46                                |
| 6.5–14.0                                      | I: 13.4 ± 0.40 II: 12.8 ± 0.38                                |
| 3.5–6.5                                       | I: 37.6 ± 0.45 II: 39.2 ± 1.17                                |
| 3.0–3.5                                       | I: 48.3 ± 1.44 II: 46.7 ± 1.40                                |

I—collagen waste hydrolyzate obtained by collagenase; II—collagen-containing waste hydrolyzate obtained by Neutrase 1.5 MG.

Next, we studied the amino acid profile of samples of collagen-containing waste hydrolysis products obtained using collagenase and Neutrase 1.5 MG. The results are presented in Figure 4.

Figure 4. Results of studying the amino acid profile of the pork skin enzymatic hydrolysis products obtained using (a) collagenase or (b) Neutrase 1.5 MG: 1—isoleucine, 2—valine, 3—phenylalanine, 4—lysine, 5—histidine, 6—methionine, 7—cysteine, 8—glycine, 9—methionine, 10—threonine, 11—aspartic acid, 12—cystine, 13—alanine, 14—glutamic acid, 15—leucine, 16—arginine.
The results of the carbohydrate composition analysis of the samples of collagen-containing waste hydrolysis products obtained using collagenase and Neutrase 1.5 MG are presented in Table 3.

**Table 3. Results of the carbohydrate composition analysis of collagen-containing waste hydrolysis products.**

| Indicator       | The Proportion of Carbohydrate Components in the Hydrolyzate, % |
|-----------------|---------------------------------------------------------------|
|                 | I                          | II                          |
| Hexose          | 0.33 ± 0.01                | 0.28 ± 0.01                |
| Hexosamines     | 2.14 ± 0.06                | 2.12 ± 0.06                |
| Free hexosamines| 6.87 ± 0.20                | 6.64 ± 0.20                |
| Hyaluronic acid | 1.61 ± 0.04                | 1.58 ± 0.04                |
| Chondroitin sulfates | 6.32 ± 0.18         | 6.31 ± 0.18                |

I—collagen waste hydrolyzate obtained by collagenase; II—collagen-containing waste hydrolyzate obtained by Neutrase 1.5 MG.

**3. Discussion**

Hydrolysis forms a complex mixture of decomposition products of protein molecules with different molecular weights. The quantitative and qualitative composition of the decomposition products of protein molecules of collagen-containing raw materials depends on the technological parameters of the hydrolysis (biodegradation) process and the properties of the applied enzyme preparation [34]. It is generally accepted that the efficiency of enzymatic biodegradation of collagen-containing raw materials by enzyme preparations depends on the quantitative ratio of enzyme:substrate [35]. In our study (Figure 1a), up to 300 min of enzymatic hydrolysis by collagenase at concentrations of 250 and 300 U/g showed almost the same dynamics. Starting from 300 min, no difference \( p > 0.05 \) in the hydrolysis degree of the samples depending on the preparation concentration was found. An increase in the concentration of collagenase above the U/g of the substrate increases the hydrolysis degree, but it should be noted that the increase in concentration is unreasonable and is associated with additional time and costs [36]. Throughout hydrolysis with Neutrase 1.5 MG (Figure 1b), the samples obtained at a concentration of 150 U/g significantly differed \( p < 0.05 \) in the hydrolysis degree from samples obtained with a higher enzyme preparation concentration. In the interval from 180 to 420 min, the hydrolysis degree of the samples obtained with the addition of Neutrase 1.5 MG 300 U/g was significantly higher than in other samples. After 420 min of hydrolysis, the samples obtained with the addition of Neutrase (200–300 U/g) did not differ significantly from each other.

Throughout the entire hydrolysis process by collagenase, the best indicators for the hydrolysis degree were achieved at pH = 7.0. The samples obtained at pH = 6.0 and 8.0 had no statistically significant differences. The samples obtained by hydrolysis with neutrase at pH = 6.0 significantly exceeded the rest of the samples. It was found that the use of collagenase with a substrate concentration of 250 U/g led to an increase in the hydrolysis degree in a neutral medium, while the maximum activity of Neutrase 1.5 MG was manifested in an acidic medium.

When the duration of hydrolysis by collagenase was more than 360 min, the change in temperature did not significantly affect the hydrolysis degree. At 30 and 35 °C, no significant differences were observed throughout the hydrolysis by Neutrase 1.5 MG.

The advantage of collagenase is confirmed by the potential of using it for enzymatic hydrolysis of collagen-containing raw materials without the additional use of inorganic acids that accelerate the hydrolysis process. The highest degree of biodegradation of collagen-containing raw materials in the samples was achieved using collagenase with a substrate concentration of 250 U/g at 40 °C and a process duration of 6 h, while Neutrase 1.5 MG maximally hydrolyzed the collagen-containing raw materials at a higher temperature. It was found that the hydrolytic activity of Neutrase 1.5 MG is lower than...
that of collagenase. Our data are in good agreement with the results of the study [37]. The following biodegradation tests were carried out in that study: in vitro hydrolytic degradation of collagen membranes in phosphate-buffered saline at 37 °C; enzyme resistance test: samples were immersed in 0.13% porcine trypsin solution and incubated at 37 °C; bacterial collagenase resistance test using collagenase solution from Clostridium histolyticum bacteria. It is a mixture of several enzymes, including collagenase, that breaks down collagen tissue. This preparation contains collagenase, nonspecific proteases, and clostripain and exhibits neutral protease and aminopeptidase activity. The experiment demonstrated that collagenase exhibits the best values for the biodegradation of collagen membranes, as in our studies. The study [38] also investigated the effect of Clostridium histolyticum collagenase on the degradation of collagen-containing wastes. The results show that when peptides are present on the surface of collagen fibrils, the amide bonds break at a much faster rate. The fibrillar structure of collagen leads to more pronounced destruction and dissolution of collagen peptides under the action of collagenase.

The amount of peptide fractions with different molecular weights in enzymatic hydrolysis samples of collagen-containing wastes is significantly comparable (Tables 2 and 3). In both cases, hydrolysis was almost complete with collagen degradation to low-molecular-weight peptides. At the end of the enzymatic hydrolysis process, both collagenase and collagen hydrolyzate samples contained a significant amount of low-molecular-weight proteins with a small number of high-molecular-weight peptides. Obviously, collagenase showed significantly higher activity (Figures 1–3) than neutrase, but no significant difference in the qualitative characteristics of hydrolysis products was found (Tables 1 and 2). Similar results are described in [39–42].

It is known that enzymatic hydrolysis of collagen of secondary collagen-containing raw materials by collagenase leads to the production of certain bivalent products, which then spontaneously react with the formation of more complex, stable cross-links [39]. This involves many different spontaneous, non-specific reactions that correlate with glucose and the products of its oxidation, which leads to the development of glycation end products [40]. Glucose, ribose, and other sugars and sugar oxidation products react with lysine, hydroxylysine, and arginine to form complex products, of which only a few have been characterized to this date [39,42]. In addition to peptides and carbohydrates, a significant amount of glycoprotein and glycosaminoglycan is formed [39]. In humans and animals, glycosaminoglycans are covalently bound to the protein part of proteoglycans and are not found in free form. Enzymatic hydrolysis allows isolating glycosaminoglycans from secondary collagen-containing raw materials that are not covalently bound to proteoglycans [39]. Thus, during the enzymatic hydrolysis of collagen-containing raw materials by collagenase, the biological activity of the obtained low-molecular-weight compounds is significantly higher than the biological activity of low-molecular-weight compounds obtained as a result of the enzymatic hydrolysis by Neutrase 1.5 MG.

The study [43] presents the results of studying protein hydrolysates obtained from shrimp waste, mainly consisting of heads and shells of Penaeus monodon, by enzymatic hydrolysis for 90 min using four microbial proteases (alkalase, neutrase, protamex, and flavurzyme), where the corresponding enzymes were compared to select the best protein profile for studying. The alkalase that showed the best result was used to optimize the hydrolysis conditions. The maximum hydrolysis degree under the action of alkalase was 33.13%. The obtained protein hydrolyzate contained a high content of protein (72.3%) and amino acids (529.93 mg/g), of which essential amino acids and aromatic amino acids were 54.67–55.93% and 38.32–39.27%, respectively. A protein efficiency factor of 2.99 and a chemical rating of 1.05 of the hydrolyzate were adequate enough to be recommended as a high protein fraction. Since alkalase is a proteolytic enzyme, like collagenase, our studies are in good agreement with the results of [39], in which neutrase is also inferior in proteolytic properties to alkalase (collagenase).

Analysis of the obtained results showed that hydrolysis under the action of the studied enzyme products proceeded almost completely, and a significant amount of free
amino acids was formed in the hydrolyzate, and a bound complex of glycosaminoglycans was released. The quantitative content of hexosamines, chondroitin sulfates, hyaluronic acid, and hexose in the samples of collagenase and Neutrase 1.5 MG hydrolysis products was practically identical (Table 3). It was found that samples of hydrolysis products obtained using Neutrase 1.5 MG contained fewer hexoses than samples obtained using collagenase. The mass fraction of hexoses in the samples of collagenase hydrolysis products was 1.17 times higher than in Neutrase 1.5 MG hydrolysis products. Both collagenase and Neutrase 1.5 MG hydrolysis samples contained about 2% hyaluronic acid. Its presence has increased the biological, therapeutic, and prophylactic value of hydrolysates as a possible basis for effective chondroprotectors that contribute to OA prevention and treatment. There are studies proving the reparative capacities and the ability to curb the progression of osteoarthrosis with drugs including hyaluronic acid, which are confirmed by MRI and histological examination of tissues \[44-46\].

The studies \[47,48\] investigated the antioxidant and enzymatic (hydrolytic) activities of enzymes (neutralase, alkalase, papain, savinase, esperase, and collagenase). It was found that by antioxidant and enzymatic activity, enzymes can be arranged in the following order: collagenase > alkalase > savinase > papain > esperase > neutralization. When comparing the same amount of peptide, peptides from secondary collagen-containing raw materials hydrolyzed by collagenase were the most effective for use as chondroprotectors in drugs aimed at preventing and treating OA. Neutrase was the least effective protease \[47\].

The study \[49\] developed and approved a method for the quantitative determination of chondroitin sulfate in raw materials and food additives in the range from about 5% to 100%. Chondroitin sulfate was first selectively hydrolyzed with the enzyme chondroitinase to form non-, mono-, di-, and trisulfated unsaturated disaccharides. The resulting disaccharides were then quantitatively determined using ion-pair liquid chromatography with ultraviolet detection. The amounts of the individual disaccharides were added together to obtain the total amount of chondroitin sulfate in the material. The reproducibility accuracy for the total chondroitin sulfate content ranged from 1.60 to 4.00. The relative standard deviation was 72%, with HorRat values ranging from 0.79 to 2.25. The extraction of chondroitin sulfate from collagen-containing waste was 6.23%, which is consistent with our research data.

The study \[50\] investigated the quantitative content of hexosamines, chondroitin sulfates, hyaluronic acid, and hexoses in samples of hydrolysis products of collagen-containing raw materials obtained by collagenase and neutrase enzymes. It was found that the amount of hexose, free hexosamine, and hyaluronic acid in the hydrolysis products obtained by collagenase exceeded the content of these chondroprotectors obtained during hydrolysis by neutrase. The content of chondroitin sulfates was practically the same in all hydrolysis products, which is consistent with our studies.

The study \[51\] showed that highly mineralized collagen-containing raw material is a source of valuable protein nutraceuticals. They consisted of high-molecular-weight proteins, oligopeptides, and amino acids, of which the anabolic and physiological potential was not used sufficiently. Protein nutraceuticals were obtained by high-temperature hydrolysis of beef raw materials combined with enzymolysis by proteolytic enzyme preparations: Alcalase 2.5 L, Protamex, Protosubtilin G3x. The water-soluble fraction of hydrolysates was studied after its separation and lyophilization for the content of nitrogenous compounds, fats, minerals, formol-titrated nitrogen, and fractional molecular composition. The authors proposed a rational technological scheme for the complex processing of collagen-containing raw materials with the production of protein, fat, and mineral protein supplements. The amino acid composition of the resulting protein supplements was analyzed by various hydrolytic methods. The organoleptic, functional, and technological properties of lyophilized protein compositions were investigated. Amino acid potential, digestibility, and physiological activity of protein nutraceutical-chondroprotectors from collagen-containing raw materials were studied. They were recommended to be used as a part of specialized dietary...
supplements (DS) for osteotropic food in the formulations of specialized and personalized products, as a source of amino acids and active peptides [52].

The world market for enzymes is segmented (by type) into carbohydrases, proteases, lipases, polymerase nucleases, etc. [53]. The protease enzyme is a significant expenditure source, since it is widely used for collagen hydrolysis, DNA replication, transcription, differentiation of cell proliferation, ovulation, fertilization, and many other biological processes [48]. However, enzyme preparations, including proteinases (collagenase), have undeniable advantages over acid and alkaline hydrolysis [53]. The advantages of enzymatic hydrolysis are: (1) environmental friendliness (does not require acids and alkalis); (2) preservation of many essential amino acids; (3) the presence of biologically active peptides in the preparation [53].

4. Materials and Methods

4.1. Objects of Research

The objects of research in this study were secondary collagen-containing raw materials obtained during pork processing. Collagen-containing wastes (pork skin) purchased from LLC “STTs” (Novosibirsk, Russia) were the objects of the research. The skins were acquired after slaughtering fattening pigs of the large white breed. Large skins were used skins of adult boars (castrated males), with an area greater than 120 dm² and a thickness of 2.7–3.7 mm. The maximum weight of the large skins was 6 kg. Collagenase enzyme preparations (obtained from crustaceans) and Neutrase 1.5 MG enzyme preparation (obtained from microorganisms) were purchased from TD Biopreparat, Moscow, Russia, and Pishcheprom-product, Moscow, Russia, respectively. Collagenase is an enzyme that breaks down collagen to release the free amino acid hydroxyproline. An important property of collagenase is its ability to biodegrade the main protein of the extracellular matrix collagen. The enzyme can break down almost all types of collagens and destroy not only the peptide chain of a protein but also numerous bonds within the triple helix of molecules. Neutrase is a neutral protease obtained by the production of a purified genetically unmodified Bacillus amyloliquefaciens strain. Neutrase 1.5 MG is a brown, free-flowing micro-granulate that does not spray.

4.2. Biodegradation of Collagen-Containing Waste by Enzymes

The parameters and technological modes for the biodegradation of collagen-containing waste by enzyme preparations were selected. Hydrolytic cleavage of pork skin collagen was carried out, with the collagen-containing raw materials previously subjected to a degreasing process and treated with running water at a temperature of 45 °C. The collagen-containing raw materials were then weighed on a counterbalance (Novolab, Novosibirsk, Russia) with an accuracy of two decimal places and ground in a laboratory mill (Novolab, Novosibirsk, Russia) until obtaining 1 mm pieces. The raw material was then washed with 0.1 M phosphate buffer for 30 min at a temperature of 60–65 °C.

The degree of hydrolysis and the quantitative content of amine nitrogen in hydrolysis products were considered as parameters characterizing the biodegradation of collagen-containing wastes [33].

The hydrolysis degree (%) of collagen-containing waste was calculated using the formula:

\[ HD = \left( \frac{N_{TN} - N_{AN0}}{N_{TN} - N_{AN}} \right) \times 100 \% \]

where \( N_{TN} \) — total nitrogen content, %; \( N_{AN0} \) — amine nitrogen content in non-hydrolyzed raw materials, %; \( N_{AN} \) — the amine nitrogen content in the hydrolysis products of collagen-containing raw materials for a specific time, %.

The quantitative content of total nitrogen in the hydrolysis products of collagen-containing raw materials was studied by the Dumas method. The quantitative content of amine nitrogen was studied by formol titration. The activity of proteolytic enzyme preparations during the experiment was studied by the Anson method [34].
Next, the effect of active acidity on the activity of enzyme preparations used for the biodegradation of collagen-containing raw materials was studied. For this, the hydrolysis (biodegradation) of collagen-containing waste was carried out at an enzyme concentration of 250 U/g of the substrate, with a process duration of 8 h (480 min), and process temperature of 40 °C. During the experiment, the value of the active acidity of the reaction medium was varied from 5.0 to 8.0. The range of active acidity chosen during the experiment depended on the type of enzyme and its characteristics presented in the passport.

The next stage studied the effect of the process temperature on the activity of enzyme preparations used for the biodegradation of collagen-containing raw materials. The biodegradation was carried out at an enzyme concentration of 250 U/g of substrate, active acidity of 5.0 (for collagenase), and 6.0 (for Neutrase 1.5 MG). The process duration was 8 h. During the experiment, the temperature of the reaction medium was varied. The choice of the temperature range depended on the type of enzyme and its characteristics according to the passport of the enzyme preparation.

All reagents were purchased from Moskhimtorg, Moscow, Russia.

4.3. Analysis of Peptide and Protein Profile

The peptide and protein profile was analyzed using electrophoretic studies. For this, samples of fermented collagen hydrolysates were prepared as follows: 50 µL of the sample was resuspended in 50 µL of the separating gel buffer; 50 µL of the dissociating mixture was then added and incubated for 10 min in a boiling water bath (Novolab, Novosibirsk, Russia); finally, 50 µL of staining solution was added. Two markers were selected for analysis: Lowrange marker (Sigma Aldrich, St. Louis, MO, USA) and Widerangemarker (Sigma Aldrich, St. Louis, MO, USA). Finished samples of fermented hydrolysates were loaded onto a gel and electrophoretic separation of a mixture of proteins was carried out.

A denaturing polyacrylamide gel (12% separating and 4% focusing) with 0.1% sodium dodecyl sulfate was used to separate a mixture of proteins. Electrophoretic separation was carried out on a single electrode buffer with the addition of 0.1% sodium dodecyl sulfate at 15 mA. The gel was stained with 0.2% Coomassie (R) Brilliant Blue R250 (Serva, Heidelberg, Germany), which was preliminarily prepared in glacial acetic acid for 7–10 min and then washed three times with distilled water.

The study results were visualized using a TCP-20M UV transilluminator (Vilber Lourmat, Eberhardzell, Germany). Electrophoretic processing of the obtained results was carried out using a Vitran-Photo gel documenting system (Biocom Company, Moscow, Russia).

All reagents were purchased from Moskhimtorg, Moscow, Russia.

4.4. Analysis of the Amino Acid Composition of Proteins

To study the amino acid composition of proteins, we used a method based on the decomposition of the objects of study under the action of acids and inorganic alkali into free forms of amino acids. Their further separation and quantitative determination of the amino acids was made on a Kapel-105/105M capillary electrophoresis device (Lumeks, St. Petersburg, Russia) using the M-04-38-2009 technique (Feed, compound feed and raw materials for their production; methods for measuring the mass fraction of amino acids by capillary electrophoresis using the Kapel capillary electrophoresis system).

4.5. Analysis of the Carbohydrate Composition of Hydrolysis Products

The carbohydrate composition of the hydrolysis products of collagen-containing wastes obtained by collagenase and Neutrase 1.5 MG was then analyzed. To evaluate the carbohydrate composition of the samples of the hydrolysis product of collagen-containing wastes, parameters such as the mass fraction of chondroitin sulfates, hexose, and hexoamines, as well as the content of hyaluronic acid, were chosen. During the study of samples of the hydrolysis product of collagen-containing waste (pork skin), the content of free disaccharides (hexoamines) was determined.
The mass fraction of chondroitin sulfates in the collagen-containing waste hydrolysis product samples was estimated by the quantitative content of sulfate ions (FS 42-1286-99 Determination of hexoses and sulfate ions: State Pharmacopoeia).

The mass fraction of hexosamines was studied spectrophotometrically (FS 42-1286-99 Determination of hexoses and sulfate ions: State Pharmacopoeia). The mass fraction of hexosamine \( X \) in the hydrolysis products of collagen-containing waste (pork skin) was calculated using the formula:

\[
X = \frac{C \times V \times 10 \times 100\%}{a \times 0.5 \times 5} = \frac{2 \times C \times V}{a \times 0.5} \times 100\%,
\]

where \( V \)—the volume of the neutralized hydrolysis products, mL; \( a \)—mass of hydrolyzate products, mg; \( C \)—the amount of hexosamine in 1 mL, according to the calibration graph built on galactosamine, mL; 0.5—the volume of neutralized hydrolysis products, mL.

The mass fraction of free hexosamines was similarly determined, but preliminary hydrolysis of the samples under study was not carried out.

The mass fraction of hyaluronic acid in the samples of hydrolysis products of collagen-containing raw materials was estimated by the quantitative content of glucosamines as a monosaccharide residue in the structure of natural disaccharides localized in the hyaluronic acid molecule, based on the method described in the pharmacopoeial monograph (FS 42-1785-96, Determination of the amount of amino sugars).

All reagents were purchased from Moskhimtorg, Moscow, Russia.

4.6. Statistical Analysis

Each experiment was repeated three times, with all data expressed as mean ± standard deviation. Data processing was carried out using standard statistical methods. The homogeneity of the sampling effects was checked using the Student’s \( t \)-test. Post hoc analysis (Tukey test) was undertaken to identify samples that were significantly different from each other. The equality of the variances of the extracted samples was checked using the Levene test. The data were subjected to analysis of variance (ANOVA) using Statistica 10.0 (StatSoft Inc., 2007, Tulsa, OK, USA). Differences between means were considered significant when the p-value was less than 5% \( (p < 0.05) \).

5. Conclusions

The study resulted in the selection of parameters and substantiation of technological modes of biodegradation of collagen-containing wastes by the selected enzymes.

Proteases with collagenolytic activity are increasingly used in pharmaceutical, food, and other industries. Microbial collagenases are of current interest because of their availability. Although earlier studies have mainly focused on collagenase from pathogenic and anaerobic microbial sources, raising some safety concerns, newer studies report collagenase production from non-pathogenic and aerobic microbial sources [49]. Collagenases are promising enzymes for the production of chondroprotectors used for the treatment of osteoarthritis.

Author Contributions: S.S., S.I. and O.B. conceived and designed the research; S.S., A.I., S.N. and A.P. analyzed and interpreted the data; S.S., E.U., A.I., T.L. and O.K. contributed reagents, materials, analysis tools or data; S.I., E.U. and O.B. wrote the paper. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Russian Foundation for Basic Research, grant number 20-316-70002, and by the Ministry of Science and Higher Education of the Russian Federation, agreement No. 075-15-2021-694.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.
Conflicts of Interest: The authors declare no conflict of interest.

References

1. Arslan, Y.E.; Derkus, T.S.; Emregul, E.; Emregul, K.C. Fabrication of human hair keratin/jellyfish collagen/eggshell-derived hydroxyapatite osteoinductive biocomposite scaffolds for bone tissue engineering: From waste to regenerative medicine products. Colloids Surf. B 2017, 154, 160–170. [CrossRef] [PubMed]

2. Bhagwat, P.K.; Dande, P.B. Collagen and collagenolytic proteases: A review. Biocatal. Agric. Biotechnol. 2018, 15, 43–55. [CrossRef]

3. Bilek, S.E.; Bayram, S.K. Fruit juice drink production containing hydrolyzed collagen. J. Funct. Foods 2015, 14, 562–569. [CrossRef]

4. Da Costa Reichenbach, B.R.S.; Keller, N.; Narrey, L.; Wandel, S.; Jüni, P.; Trelle, S. Effectiveness of non-steroidal anti-inflammatory drugs for the treatment of pain in knee and hip osteoarthritis: A network meta-analysis. Lancet 2017, 390, e21–e33. [CrossRef]

5. De Luca, P.; Colombini, A.; Carimati, G.; Beggio, M.; de Girolamo, L.; Volpi, P. Intra-Articular Injection of Hydrolyzed Collagen to Treat Symptoms of Knee Osteoarthritis. A Functional In Vitro Investigation and a Pilot Retrospective Clinical Study. J. Clin. Med. 2019, 8, 975. [CrossRef] [PubMed]

6. Felician, F.F.; Xia, C.; Qi, W.; Xu, H. Collagen from marine biological sources and medical applications. Chem. Biodivers. 2018, 15, e1700557. [CrossRef] [PubMed]

7. Felician, F.F.; Yu, R.H.; Li, M.Z.; Li, C.J.; Chen, H.Q.; Ji, Y.; Xu, H.M. The wound healing potential of collagen peptides derived from the jellyfish Rhopilema esculentum. Chin. J. Traumatol. 2019, 22, 12–20. [CrossRef] [PubMed]

8. Ferrario, C.; Rusconi, F.; Pulaj, A.; Macchi, R.; Landini, P.; Paroni, M.; Candia Carnevali, M.D. From food waste to innovative novel functional food ingredients. Innov. Food Sci. Emerg. Technol. 2019, 51, 199–208. [CrossRef]

9. Freire, V.; Bureau, N.J. Injectable corticosteroids: Take precautions and use caution Seminars in musculoskeletal radiology. Thieme 2016, 20, 401–408.

10. Fu, Y.; Young, J.F.; Rasmussen, M.K.; Dalsgaard, T.K.; Lametsch, R.; Aluko, R.E.; Therkildsen, M. Angiotensin I–converting enzyme–inhibitory peptides from bovine collagen: Insights into inhibitory mechanism and transepithelial transport. Food Res. Int. 2016, 89, 373–381. [CrossRef]

11. Goh, K.L.; Hiller, J.; Haston, J.L.; Holmes, D.F.; Kadler, K.E.; Murdoch, A.; Wess, T.J. Analysis of collagen fibril diameter distribution in connective tissues using small-angle X-ray scattering. Biochim. Biophys. BBA-Gen. Subj. 2005, 1722, 183–188. [CrossRef] [PubMed]

12. Hashim, P.; Hashim, M.D. Collagen in food and beverage industries. J. Food Sci. Technol. 2015, 22, 1–8.

13. Jeevithan, E.; Bao, B.; Zhang, J.; Hong, S.; Wu, W. Purification, characterization and antioxidant properties of low molecular weight collagenous polypeptide (37 kDa) prepared from whale shark cartilage (Rhincodon typus). J. Food Sci. Technol. 2015, 52, 6312–6322. [CrossRef] [PubMed]

14. Jhample, S.B.; Bhagwat, P.K.; Dande, P.B. Statistical media optimization for enhanced production of fibrinolytic enzyme from newly isolated Proteus penneri SP-20. Biocatal. Agric. Biotechnol. 2015, 4, 370–379. [CrossRef]

15. Sukhikh, S.; Noskova, S.; Ivanova, S.; Ulrikh, E.; Babich, O. Chondroprotection and molecular mechanism of action of phytonutraceuticals on osteoarthritis. Molecules 2021, 26, 2391. [CrossRef]

16. Asyakina, L.K.; Fatina, N.V.; Igzharysheva, N.V.; Slavyanskiy, A.A.; Neverova, O.A. Geroprotective potential of in vitro bioactive biomaterial: Sea urchin-derived collagen for applications in skin regenerative medicine. Mar. Drugs 2020, 18, 414. [CrossRef]

17. Freire, V.; Bureau, N.J. Injectable corticosteroids: Take precautions and use caution Seminars in musculoskeletal radiology. Thieme 2016, 20, 401–408.

18. Fu, Y.; Young, J.F.; Rasmussen, M.K.; Dalsgaard, T.K.; Lametsch, R.; Aluko, R.E.; Therkildsen, M. Angiotensin I–converting enzyme–inhibitory peptides from bovine collagen: Insights into inhibitory mechanism and transepithelial transport. Food Res. Int. 2016, 89, 373–381. [CrossRef] [PubMed]

19. Goh, K.L.; Hiller, J.; Haston, J.L.; Holmes, D.F.; Kadler, K.E.; Murdoch, A.; Wess, T.J. Analysis of collagen fibril diameter distribution in connective tissues using small-angle X-ray scattering. Biochim. Biophys. BBA-Gen. Subj. 2005, 1722, 183–188. [CrossRef] [PubMed]

20. Jeevithan, E.; Bao, B.; Zhang, J.; Hong, S.; Wu, W. Purification, characterization and antioxidant properties of low molecular weight collagenous polypeptide (37 kDa) prepared from whale shark cartilage (Rhincodon typus). J. Food Sci. Technol. 2015, 52, 6312–6322. [CrossRef] [PubMed]

21. Jhample, S.B.; Bhagwat, P.K.; Dande, P.B. Statistical media optimization for enhanced production of fibrinolytic enzyme from newly isolated Proteus penneri SP-20. Biocatal. Agric. Biotechnol. 2015, 4, 370–379. [CrossRef]

22. Sukhikh, S.; Noskova, S.; Ivanova, S.; Ulrikh, E.; Babich, O. Chondroprotection and molecular mechanism of action of phytonutraceuticals on osteoarthritis. Molecules 2021, 26, 2391. [CrossRef]

23. Asyakina, L.K.; Fatina, N.V.; Igzharysheva, N.V.; Slavyanskiy, A.A.; Neverova, O.A. Geroprotective potential of in vitro bioactive compounds isolated from yarrow (Achillea millefolii L.) cell cultures. Foods Raw Mater. 2021, 9, 126–134. [CrossRef]

24. Babich, O.; Sukhikh, S.; Prosekov, A.; Asyakina, L.; Ivanova, S. Medicinal Plants to Strengthen Immunity during a Pandemic. Pharmaceuticals 2020, 13, 313. [CrossRef]

25. Kozłowska, J.; Sionkowska, A.; Skopinska-Wisniewska, J.; Piechowicz, K. Northern pike (Esox lucius) collagen: Extraction, characterization and potential application. Int. J. Biol. Macromol. 2015, 81, 220–227. [CrossRef]

26. Sukhikh, S.; Babich, O.; Prosekov, A.; Patyukov, N.; Ivanova, S. Future of Chondroprotectors in the Treatment of Degenerative Processes of Connective Tissue. Pharmaceuticals 2020, 13, 220. [CrossRef] [PubMed]

27. Loeser, R.F.; Collins, J.A.; Diekman, B.O. Ageing and the pathogenesis of osteoarthritis. Nat. Rev. Rheumatol. 2016, 12, 412–420. [CrossRef]

28. Mashiko, T.; Takada, H.; Wu, S.H.; Kanayama, K.; Feng, J.; Tashiro, K.; Takato, T. Therapeutic effects of a recombinant human collagen peptide bioscaffold with human adipose-derived stem cells on impaired wound healing after radiotherapy. Tissue Eng. Regen. Med. 2018, 12, 1186–1194. [CrossRef] [PubMed]

29. Mobasher, A.; Bay-Jensen, A.C.; Van Spil, W.E.; Larkin, J.; Levesque, M.C. Osteoarthritis Year in Review 2016: Biomarkers (biochemical markers). Osteoarthr. Cartil. 2017, 25, 199–208. [CrossRef]

30. Nishikimi, A.; Koyama, Y.I.; Ishihara, S.; Kobayashi, S.; Tometsuka, C.; Kusubata, M.; Kuwaba, K.; Hayashida, O.; Hattori, S.; Katagiri, K. Collagen-derived peptides modulate CD4+ T-cell differentiation and suppress allergic responses in mice. Immun. Inflamm. Dis. 2018, 6, 245–255. [CrossRef]

31. Pai, G.K.; Suresh, P.V. Sustainable valorisation of seafood by-products: Recovery of collagen and development of collagen-based novel functional food ingredients. Innov. Food Sci. Emerg. Technol. 2016, 37, 201–215. [CrossRef]

32. Paré, F.; Tardif, G.; Fahmi, H.; Ouhaddi, Y.; Pelletier, J.P.; Martel-Pelletier, J. In vivo protective effect of adipsin-deficiency on spontaneous knee osteoarthritis in aging mice. Aging 2020, 12, 2880. [CrossRef]

33. Richette, P.; Latourte, A.; Frazier, A. Safety and efficacy of paracetamol and NSAIDs in osteoarthritis: Which drug to recommend? Expert Opin. Drug Saf. 2015, 14, 1259–1268. [CrossRef]
