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Amino acid composition in determination of collagen origin and assessment of physical factors effects

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ABSTRACT

The amino acid composition of collagen is a characteristic feature of this protein. Collagen, irrespective of its origin, contains 19 amino acids, including hydroxyproline which does not occur in other proteins. Its atypical amino acid composition is characterized by high content of proline and glycine, as well as the absence of cysteine.

This paper shows the comparison of qualitative composition of amino acids of fish skin (FS) collagen, bovine Achilles tendon (BAT) collagen, and bone collagen. Results demonstrate that FS collagen as well as BAT collagen contains no cysteine and significantly different amount of hydroxyproline. In BAT collagen hydroxyproline content is 30% higher than hydroxyproline content of FS collagen. In bone collagen the amount of hydroxyproline is two times more than in FS collagen.

Furthermore, it is shown that sensitivity to radiation of individual amino acids varies and depends on the absorbed dose of ionizing radiation. The changes observed in the amino acid composition become very intense for the doses of 500 kGy and 1000 kGy.

1. Introduction

The knowledge of collagen chemistry involves primarily the knowledge of elementary composition, especially of amino acid composition, and the alignment of individual amino acids, e.g. the amino acid sequence in the protein molecule. The collagen triple helix is composed of three chains of procollagen interconnected by covalent bonds formed by concentrically located glycine. The spiral structure is formed mainly by alanine, phenylalanine, asparagine, glutamine, histidine, leucine, methionine and tyrosine. Due to the large size of the side chains, valine and isoleucine cannot participate in formation of a stable structure of the alpha-helix. The regular structure of the helix is disturbed by serine, threonine, proline, and hydroxyproline. The first two amino acids act so due to the additional hydrogen bonds formed by their hydroxyl groups [1–5]. In case of proline, the nitrogen atom is embedded in a heterocyclic ring what eliminates the possibility of rotation around the carbon-nitrogen bond and prevents the formation of intramolecular hydrogen bonds. As the result of the proline presence, the chain may be inflected or even create loops.

Protein content can be determined by the Kjeldahl method involving the mineralization of the test material with concentrated sulfuric (VI) acid, the alkalinization of the solution, and finally the distillation and titration of ammonia produced by the nitrogen connections contained in the protein. Thus, the analysis is composed of three stages: sample mineralization, steam distillation, and titration. In addition to the protein nitrogen bonds, ammonium ions and other compounds containing amino or imino groups may also be determined by this method. However, nitrates (V), nitrites (III), and nitrogen heterocyclic aromatic rings are not marked [6]. The Kjeldahl method is an indirect reference method which determines the content of nitrogen and then recalculates it into protein using corresponding conversion factors.

In food products the determination applies to so-called total nitrogen (which includes protein nitrogen and nitrogen derived from the products of protein rebuilding) and nitrogen from other organic compounds. The average nitrogen content of proteins is approximately 16%, which is why the so-called crude protein conversion factor is 6.25 (100:16 = 6.25). Since the protein foods differ in qualitative and quantitative composition of proteins, the amount of nitrogen contained in them may also vary. For certain food products, different conversion factors are used, for example: egg protein—6.67 milk protein—6.38 meat protein—6.25 rye wheat, and oat protein—5.70. The multipliers are given next to the protein content, e.g. $N = 6.38$ for milk. Indirect methods may be used only when

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the test product contains no other nitrogen compounds or contains very few of them [7,8].

Gamma irradiation from Cobalt 60 sources has been used to terminally sterilize bone allografts for many years. Gamma radiation adversely affects the mechanical and biological properties of bone allografts by degrading the collagen in bone matrix. Specifically, gamma rays split polypeptide chains. In wet specimens irradiation causes release of free radicals via radiolysis of water molecules that induces cross-linking reactions in collagen molecules [9].

Gamma irradiation is widely used as a mean of terminal sterilization for surgical implants and medical devices to ensure product safety. This technique offers advantages such as speed of processing and simplified handling of the material before and after sterilization [10,11].

No toxic chemicals are introduced and conditions are relatively mild.

Even though it is a convenient and cost method, it can have adverse effects on bioactive and biodegradable biomaterials. Gamma irradiation has been known to cleave chemical bonds of polymers including peptide bonds of proteins [12]. Damage to collagen by gamma irradiation includes molecular fragmentation via peptide chain scission [9]. The irradiation fragments collagen molecules leading to a loss of collagen network connectivity and therefore loss of toughness. Irradiation resulted in a 62% loss of work-to-fracture [9]. There was significantly less micro-damage formed during fracture propagation in the irradiated bone. Altogether, this new data strongly indicates that a large loss of overall collagen connectivity due to collagen fragmentation resulting from γ-irradiation sterilization leads to inferior cortical bone toughness [13].

Because in modern medicine ionising radiation is widely used to diagnose (medical imaging techniques such as X-ray imaging, computed tomography (CT), single photon emission computed tomography (SPECT) or positron emission tomography (PET)) and treat diseases (radiotherapy) the first purpose of this study was to investigate the effect of gamma irradiation on amino acid composition of collagen, the dominant protein of living organisms, by the example of bone collagen. The second aim of this paper was to compare the amino acid compositions of collagens (paying special attention to hydroxyproline, proline and glycine content) derived from different sources, namely, a bovine collagen, a fish collagen and a bone collagen. The possible convergence of these compositions would suggest that the fish collagen could be a potential substitute for commonly used bovine collagen or bone collagen.

2. Materials and methods

The material used in the study was the collagen type I obtained from fish skin of Salmo salar by applying the acidic hydration method/3-HELISA/. The purified skin had been hydrated in the lactic acid solution of 0.1–1.5% concentration. The process of hydration was performed in glass containers at temperature between 15 °C and 20 °C, for 24–48 h. As a result of repeated filtration with the use of natural silk of increasing density, separation of the cell elements, pigments, and remaining acid solution from pure collagen occurred. They were obtained from the manufacturer, the patent owner. The control material was type I collagen derived from a bovine Achilles tendon (BAT) purchased from Sigma-Aldrich.

The bovine femur was also studied. The bones were collected from 23-year-old bulls within 3–4 h after their slaughter. Then the bones were mechanically cleaned of the soft tissue residues and washed with water. After cleansing, the middle part of the bone was excised and other elements were discarded.

The amino acid composition of FS collagen, BAT collagen, and bone collagen was determined with an amino acid automatic analyzer applying the High Performance Liquid Chromatography (HPLC) to the samples after their prior acid hydrolysis. The determination of protein content was performed by the Kjeldahl method. The study was carried out at the Chemical Laboratory of the Department of Animal Nutrition and Feed Management at the University of Life Sciences in Poznań. For this purpose, an automatic amino acid analyzer AAA T-339 of Mikrotechnika Praha Co. was used. In order to determine the differences in amino acid content after irradiation, amino acid composition of the bone collagen was analyzed. The determination was performed after a 24-h acid hydrolysis of the bone material in a 6 M solution of HCl at 380 K.

To determine hydroxyproline content in each collagen, the Stegmanns spectrophotometric method modified by Hurych and Chvapil [14,15] was used. The measurements were made by using the Cecil Biotest spectrophotometer at the Department of General Chemistry of the Poznań Medical University.

As a physical factor affecting the amino acid composition, gamma radiation was used. Irradiation was carried out in dry air, at room temperature, using a ^{60}Co source. The applied doses were 50, 500, and 1000 kGy, and the dose power was 1.5 kGy/h.

3. Results and discussion

3.1. Determination of amino acid composition of FS collagen, BAT collagen and bone collagen

The results of determination of protein content by means of the Kjeldahl method, hydroxyproline determination with the use of the Stegmann method [15], and the results of amino acid composition tests for FS collagen and BAT collagen obtained with an automatic amino acid analyzer are summarized in Table 1.

The relative error for the determination of amino acid content was 5%.

Both FS collagen and BAT collagen show high and relatively invariant content of glycine (21.83 g per 100 g) of protein for FS collagen and 19.22 g per 100 g of protein for BAT collagen). The FS collagen contains as much proline (13.55 g per 100 g) as BAT collagen (13.92 g per 100 g). One more characteristic feature of amino acid composition of collagen is the polar amino acid content, i.e. asparagine and glutamic acid as well as arginine and lysine. While comparing the quantitative composition of amino acids in FS collagen and BAT collagen, we observe no cysteine and significant differences in the amount of hydroxyproline [16]. Statistical analysis performed by the Welch test showed that content of hydroxyproline in FS collagen is significantly different ($p = 0.0063$) compared to hydroxyproline content in BAT collagen. Collagen contains more glycine than most of proteins but does not contain any cysteine or tryptophan (except for collagen III) [17,18]. The content of methionine is three times higher for FS collagen than for BAT collagen. BAT collagen contains two times more histidine as well as of tyrosine. Tyrosine has an aromatic ring as a side chain, and the hydroxyl group attached invokes the relatively high chemical reactivity of this amino acid.

The determination of protein content by the Kjeldahl method as well as the analysis of amino acid composition allowed to assess the differences in content of total protein and specific amino acids in FS collagen and BAT collagen. Special attention was paid to hydroxyproline, the amino acid characteristic of collagen; according to the literature its content determines thermal stability of collagen. The presence of hydroxyproline as a specific amino acid of collagen is practically used in quantitative determination of collagen.

The determination of amino acids was carried out to assess the differences in amino acid content of FS, BAT, and bone collagen. The smallest amino acid, i.e. glycine, makes up 27% of all amino acids, compared to 25% of hydroxyproline and proline taken together.

Table 1
Amino acid composition of FS collagen and BAT collagen.

	Material		
	FS collagen	BAT collagen	Bone collagen
Total protein content (%)	65.66	79.40	20.51
Amino acid (g per 100 g of protein)			
Asparagine	6.64	7.49	7.12
Threonine	2.84	2.19	2.22
Serine	3.44	3.39	2.99
Glutamic acid	10.61	11.53	11.92
Proline	13.55	13.92	11.95
Cysteine	–	–	0.39
Glycine	21.83	19.22	22.82
Alanine	10.54	9.51	10.12
Valine	2.27	2.45	2.84
Methionine	2.25	0.71	1.07
Isoleucine	1.57	1.87	1.54
Leucine	2.95	3.67	3.26
Tyrosine	1.32	2.02	1.24
Phenylalanine	3.03	4.38	2.28
Histidine	1.21	2.25	1.37
Lysine	3.38	3.11	3.44
Arginine	7.56	7.30	7.72
Hydroxyproline	5.68	8.15	10.79

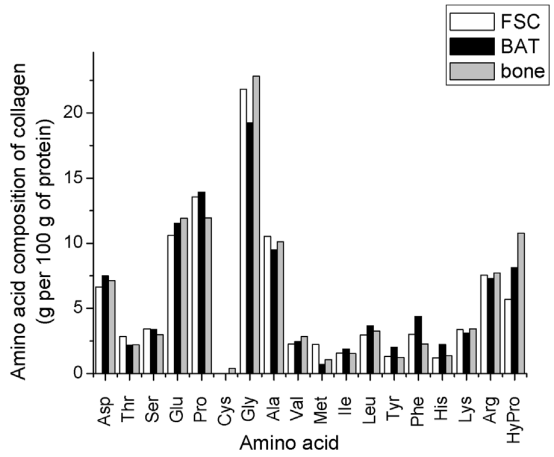

Fig. 1. Amino acid composition of FS collagen, BAT collagen and bone collagen (g per 100 g of protein).

Thus, on the average, glycine makes every fourth amino acid in collagen and so makes proline or its hydroxiderivative, what is typical of collagen. This determines a regular structure of the collagen chain and allows for the formation of stabilizing bonds between chains.

When we compare the qualitative composition of amino acids of FS collagen and BAT collagen to bone collagen, we observe no cysteine in FS collagen or BAT collagen and significant differences in the amount of hydroxyproline. The amount of hydroxyproline in bone collagen is 10.79 g per 100 g which is two times more than in FS collagen. A significant difference is also apparent in the amount of methionine (Fig. 1). The amino acid composition of collagen is a characteristic feature of this protein and it also determines its structure.

Whatever its origin, collagen contains 19 amino acids; two of them, hydroxyproline and hydroxylysine, are practically absent in other proteins. Hydroxyproline content in BAT collagen is 30% higher than hydroxyproline content of FS collagen, and it amounts to 8.15 g per 100 g of protein. In case of FS collagen, it is 5.68 g per 100 g of protein. Hydroxyproline is characteristic protein of collagen and affects its stability.

Amino acids such as glycine, proline, and hydroxyproline have a spatial configuration, and the angles between bonds as well as distances between atoms in various amino acids are slightly different [18]. The amino acid composition of collagen in terrestrial mammals is well known and uniform to some extent, contrary to the composition of collagen of different origin. We note greater derogation for fish and invertebrates [18–20]. The lower value of hydroxyproline is "rewarded" by an increase in serine and threonine content [21].

3.2. Determination of amino acid composition of collagen derived from non-irradiated and irradiated bovine bones

Determination of amino acid was performed for collagen derived from non-irradiated bovine bones and from the bones irradiated with doses of 50, 500, and 1000 kGy. The results of the analysis of amino acid are shown in Table 2 and Fig. 2. They refer to the weight of protein actually recovered during the analysis.

An integral part of the procedure of determining the amino acid composition was the assessment of the total protein content (using the Kjeldahl method) the actual recovery of proteins, and the ammonia peak area relationship to the dose of absorbed ionizing radiation. The results are shown in Table 3, and the actual recovery of protein relationship to the dose is presented in Fig. 3.

The amino acid composition of collagen in the non-irradiated bones is consistent with the results obtained in assays of amino

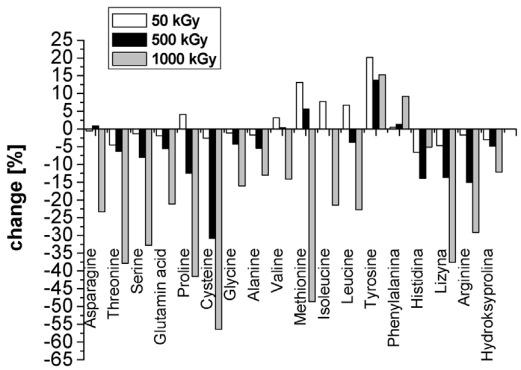

Fig. 2. Irradiation-related changes in amino acid content of bone collagen.

Table 2
Amino acid composition of bone collagen with respect to actual recovery of protein (g per 100 g of protein) and changes in percentage of amino acids caused by irradiation.

Amino acid	Dose of absorbed ionizing radiation						
	0 kGy	50 kGy		500 kGy		1000 kGy	
	g/100 g of protein	g/100 g of protein	%	g/100 g of protein	%	g/100 g of protein	%
Asparagine	7.12	7.08	−0.56	7.18	0.84	5.46	−23.31
Threonine	2.22	2.12	−4.50	2.08	−6.31	1.38	−37.84
Serine	2.99	2.95	−1.34	2.75	−8.03	2.01	−32.78
Glutamic acid	11.92	11.69	−1.93	11.26	−5.54	9.40	−21.14
Proline	11.95	12.44	4.10	10.46	−12.47	6.99	−41.51
Cysteine	0.39	0.38	−2.56	0.27	−30.77	0.17	−56.41
Glycine	22.82	22.56	−1.14	21.86	−4.21	19.16	−16.04
Alanine	10.12	9.95	−1.68	9.57	−5.43	8.80	−13.04
Valine	2.84	2.93	3.17	2.85	0.35	2.44	−14.08
Methionine	1.07	1.21	13.08	1.13	5.61	0.55	−48.60
Isoleucine	1.54	1.66	7.79	1.54	0	1.21	−21.43
Leucine	3.26	3.48	6.75	3.14	−3.68	2.52	−22.70
Tyrosine	1.24	1.49	20.16	1.41	13.71	1.43	15.32
Phenylalanine	2.28	2.29	0.44	2.31	1.32	2.49	−9.21
Histidine	1.37	1.28	−6.57	1.18	−13.87	1.30	−5.11
Lysine	3.44	3.28	−4.65	2.97	−13.66	2.15	−37.5
Arginine	7.72	7.59	−1.68	6.56	−15.02	5.47	−29.14
Hydroxyproline	10.79	10.47	−2.96	10.27	−4.82	9.48	−12.14

Table 3
Dependence of total protein content and NH_3 peak area on absorbed dose.

	0 kGy	50 kGy	500 kGy	1000 kGy	
Total protein content (%)		20.51	19.73	19.82	19.79
Real recycling of protein (%)		94.27	95.68	88.53	72.54
Protein content in the sample weigth (mg)		28.2	29.4	30.1	29.1
NH_3 peak area to protein mass in the sample	cm^2/mg	25.3	28.6	52.3	71.0
weight.	%	100	113	207	280

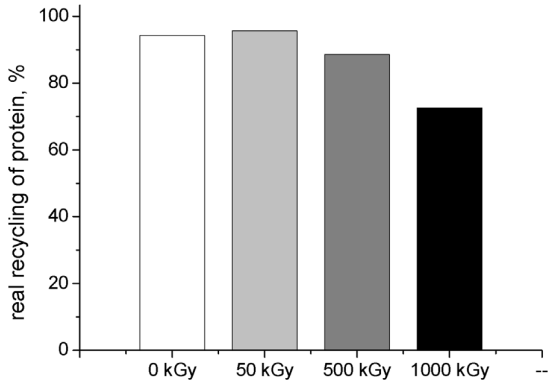

Fig. 3. Effect of radiation dose on the amount of protein recycling.

acid composition of type I collagen derived from skin and tendons [17,22,23]. Sensitivity to radiation of individual amino acids varies and depends on the absorbed dose. The changes observed in the amino acid composition become very intense for the doses of 500 and 1000 kGy.

Comparing the obtained results with those found in literature is difficult due to different conditions of irradiation, the use of collagen from various sources, the spectrum of amino acids determined, and, above all, the doses of ionizing radiation. The amino acid composition of bone irradiated with 50 kGy is similar to the composition determined by Davidson and Cooper [24] and similar to the collagen composition of bovine Achilles tendon irradiated under the same conditions [22]. For the doses of 500 kGy, the amino acid composition results resemble those obtained for the bovine Achilles tendon collagen [25]. The amino acid composition of collagen treated with the doses of 1000 kGy is comparable to this obtained by Cassel [26–28]. In his work on dry collagen from the kangaroo tail tendon irradiated in the air (2200 kGy dose), glycine, proline, threonine, and isoleucine are listed as the amino acids most sensitive to radiation. As far as the bone collagen is concerned, methionine, proline, threonine, and serine are the amino acids most vulnerable to radiation.

An increase in certain amino acids such as tyrosine (for the doses of 1000, 500, and 50 kGy) and methionine (for the doses of 500 and 50 kGy) should be emphasized. For the dose of 50 kGy, an increase in content of the amino acids such as proline, valine, isoleucine, and leucine was observed. An increase in glycine and phenylalanine for radiation doses of 50 and 500 kGy was also observed in the case of collagen derived from bovine Achilles tendon [22].

Tyrosine is an exception as irradiation results in an increase of its content. In case of this amino acid, an increase in its content recorded for the dose of 50 kGy was higher than for the doses of 500 kGy and 1000 kGy.

One of the effects of bone radiolysis, related to a total protein content decrease due to irradiation, is an increase in the ammonia content in the sample. The ratio of the peak associated with NH_3 to the protein mass in the sample is a measure of the content of ammonia (Fig. 4).

Exceeding the dose of 50 kGy reduces the protein content of the sample and causes a dramatic increase in the ammonia content.

4. Conclusions

The presence of hydroxyproline makes collagen more thermally stable, thereby stabilizing the collagen structure. Based on the amino acid analysis it can be assumed that FS collagen is less stable than both BAT and bone collagen.

The comparison of amino acid composition of the bones support the hypothesis that there are two ranges of dose: low and high. It

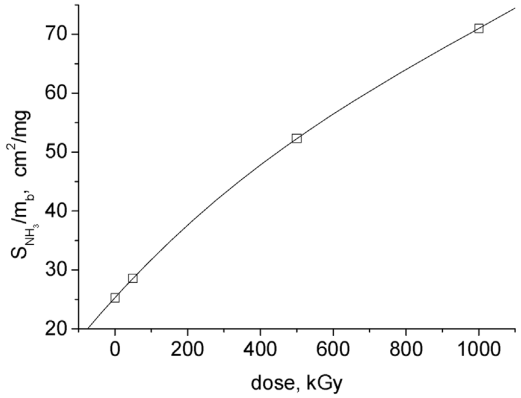

Fig. 4. Relationship between radiation dose and the ratio of S_{NH3} peak area to protein mass m_b in the sample weight.

was found that the doses exceeding 100 kGy cause changes in the content of bound water and structural water of the bones.

The study of amino acid composition of irradiated bone indicates much greater loss of amino acids after absorbing a dose of 500 kGy or 1000 kGy than after absorbing 50 kGy.

For the dose of 500 kGy, both the process of degradation and either a process of secondary cross-linking or the reaction of binding of the radiolysis products with hydroxyapatite (HA) were observed. For the dose over 500 kGy, the processes of degradation start dominating.

It is observed the increase in the dose of absorbed radiation results in reduction of the real recycling of protein and simultaneously in radical increase of ammonia peak area.

The ratio of the peak area of ammonia to the mass of protein in the sample weight increases with the dose of radiation.

The most radiosensitive amino acid in the used range of doses is cysteine and methionine.

References

[1] D.M. Shoulders, R.T. Rainess, Collagen structure and stability, Annu. Rev. Biochem. 78 (2009) 929–958.
[2] G. Höbne, W. Hemminger, H.-J. Flammersheim, Differential Scaning Calorimetry, Springer-Verlag, Berlin-Heidelberg, 1996.
[3] M. Fois, A. Lamure, M.J. Fauran, C. Lacabanne, TSC study of the dielectric relaxations of human-bone collagen, J. Polym. Sci. Part B-Polym. Phys. 38 (2000) 987.
[4] N. Jiravanichanun, N. Nishino, K. Okuyama, Conformation of alloHyp in the Y position in the host-guest peptide with the Pro-Pro-Gly sequence: implication of the destabilization of (Pro-alloHyp-Gly)$_{10}$, Biopolymers 81 (2006) 225–233.
[5] R.Z. Kramer, J. Bella, P. Mayville, B. Brodsky, H.M. Berman, Sequence dependent conformational variations of collagen triple-helical structure, Nat. Struct. Biol. 6 (1999) 454–457.
[6] L.M.L. Nollet, F. Toldra, Handbook of Mscle Foods Analysis, CRC Press, 2009.
[7] C. López, J. Sánchez, L. Hermida, A. Zulueta, G. Márquez, Cysteine mediated multimerization of a recombinant dengue E fragment fused to the P64k protein following immobilized metal ion affinity chromatography, Protein Expr. Purif. 34 (2004) 176–182.
[8] S. Nomura, A. Hiltner, J.B. Lando, E. Baer, Interaction of water with native collagen, Biopolymers 16 (1977) 231–246.
[9] H. Nguyen, D.A. Morgan, M.R. Forwood, Sterilization of allograft bone: effects of gamma irradiation on allograft biology and biomechanics, Cell Tissue Bank. 8 (2007) 93–105.
[10] R.M. Brinston, B.K. Wilson, Converting to gamma-radiation sterilization: an overview for medical device manufacturers, Med. Device. Technol. 4 (1993) 18–22.
[11] B. Han, Z. Yang, M. Nimni, Effects of gamma irradiation on osteoinduction associated with demineralized bone matrix, J. Orthop. Res. 26 (2008) 75–82.
[12] D.T. Cheung, N. Perelman, D. Tong, M.E. Nimni, The effect of gamma-irradiation on collagen molecules isolated alpha-chains, and crosslinked native fibers, J. Biomed. Mater. Res. 24 (1990) 581–589.
[13] B. Burton, A. Gaspar, D. Josey, J. Tupy, M.D. Grynpas, T.L. Willett, Bone embrittlement and collagen modifications due to high-dose gamma-irradiation sterilization, Bone 61 (2014) 71–81.
[14] L. Kubisz, E. Marzec, F. Jaroszyk, Changes in the electrical conductivity of the γ-irradiated BAT collagen, Pol. J. Med. Phys. Eng. 8 (2002) 157–164.
[15] J. Hurych, M. Chvapil, Methods of hydroxyproline determination (in Czech), Kozarstvi 12 (1962) 317–323.
[16] L. Kubisz, Influence of the ionising radiation on electrical properties and thermal of collagen of the animal bone, Poznań, Unpublished results.
[17] G. Ninan, J. Joseph, Z.A. Aliyamveettil, A comparative study on the physical, chemical and functional properties of carp skin and mammalian gelatins, J. Food Sci. Technol. 51 (2014) 2085–2091.
[18] G. Reich, Collagen The Methods, Results and Directions of the Examination, Publishing Company. The Computing Science and Technology, Warsaw, 1970.
[19] N. Ramanathan, V. Mohanaradhakrishnan, Y. Nayudamma, Biochim. Biophys. Acta 102 (1965) 533.
[20] J.C.W. Chien, D.E.P. Chang, Dynamic mechanical and rheo-optical studies of collagen and gelatin, Biopolymers 11 (1972) 2015.
[21] K.A. Piez, J. Gross, The amino acid composition of some fish collagens: the relation between composition and structure, J. Biol. Chem. 235 (1960).
[22] L. Kubisz, Examination of the impact of gamma rays on thermal denaturation of BAT collagen, Poznań, Unpublished results.
[23] K. Pietrucha, Effect of irradiation on collagen solutions in relation to biomedical applications, Polym. Med. 19 (1989) 3–18.
[24] R.J. Davidson, D.R. Cooper, The effect of γ-irradiation on soluble collagen, Biochem. J. 107 (1968) 29–34.
[25] E. Marzec, L. Kubisz, Electric properties of non-irradiated and gamma-irradiated bone, J. Non-Cryst. Solids 305 (2002) 333–338.
[26] J.H. Cassel, Effects of gamma radiation of collagen, J. Am. Leather Chem. Assoc. 54 (1959) 432–449.
[27] N. Sewald, H.D. Jakubke, Peptides: Chemistry and Biology, Wiley-VCH Verlag GmbH & Co. KGaA, 2002.
[28] P.D. Yurchenco, R.P. Mecham, D.E. Birk, Extracellular Matrix Assembly and Structure, Elsevier, 1994, ISBN: 978-0-12-775170-2.