OPTIMIZING THE STAINING OF SDS-PAGE GELS AND HIGHLIGHTING THE MAIN PROTEIN CHAINS OF COLLAGEN AFTER GAMMA IRRADIATION IN DIFFERENT DOSE AND TEMPERATURE CONDITIONS

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Due to its intrinsic biocompatibility, collagen is of particular interest in bio-medical applications. Regardless of the type of collagen-based product and its intended use, it must be sterilized, and the sterilization method can alter its properties. Our study focused on optimizing the staining of the SDS-PAGE gel for collagen samples, profile characterization of type I collagen hydrogels prepared by a local manufacturer, and characterization of band profile changes after gamma irradiation. The optimized SDS-PAGE method can be used for the identification of protein fractions on irradiated collagen hydrogel samples, and for the study of fragmentation of collagen upon irradiation

Keywords: Collagen, SDS-PAGE, Flamingo Fluorescent dye, Coomassie staining

1. Introduction

Type I collagen is the most abundant protein produced by the human body and, as the primary structure, it is formed with the main contribution of three amino acids: glycine (33%), proline and hydroxyproline (22%), in a triple helix formed by 3 α chains. Each alpha chain is composed of ~1014 amino acids, with a molecular weight of around 100 kDa. These chains are twisted in a left-handed helix, with 3 amino acids per turn - in its secondary structure. The chains are further twisted on themselves, in a triple helix that forms a rigid structure (tertiary structure). The super-helix represents the basic structure of fibrillar collagen (as a quaternary structure); it is a very stable structure due to the intramolecular hydrogen bridges that form between glycine residues in adjacent chains. Thus packed, the collagen molecule has ~300 kDa

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molecular weight,280 nm in length and 1.4 nm in diameter [1], [2].

Fibrillar collagen strengthens and supports numerous body structures, including bones, cartilage, tendons, skin, and the sclera of the eye.

Due to its intrinsic biocompatibility and to recently growing demand, collagen is of particular interest in medical applications. Collagen hydrogels are intensively studied for medical devices applications, such as in dentistry [3], [4]. Whether it is collagen dressings [3] [5] or surgical scaffolds [6], collagen needs to be sterilized.

All of the current sterilization techniques alter the characteristics of fibrillar collagen, whether it is found in a colloidal solution, a hydrogel, or a solid state. The SDS-PAGE method shows great potential in terms of setting up collagen extraction as well as preserving its characteristics during sterilization (i.e., keeping molecular integrity and triple helix structure).

In this study, we proposed:

1. finding a method for staining the SDS-PAGE gel - optimal for type I collagen, which allows the highlighting of all its peptide fractions;
2. identification of the main collagen macromolecular chains when they are found in the form of hydrogel (profile characterization);
3. characterization of band profile changes after gamma irradiation, at two irradiation doses and temperatures.

In order to achieve our goals, we conducted two studies where we investigated various staining techniques and defined the typical band profile of collagen. We subsequently carried out two further experiments where we examined the alterations in band profile that result from irradiation.

2. Materials and methods

2.1. Collagen samples

Collagen samples in gel form, with a concentration of 0.92%, were prepared in-house by Deltarom SRL and kept in the freezer (-20° C) until the time of analysis. The collagen hydrogel was obtained as follows: a tendon quantity of 22.13 g was subjected to enzymatic digestion (enzyme concentration 0.63%), using acetic acid $pH = 2.3$ as a reaction medium, at a temperature of 20º C. The hydrogel has the following characteristics: soluble protein: 5.94% relative to the dry substance, Hyp% content: 11.58%, collagen content 92.68%, free amino acids 7.16% (Gly equivalents).

2.1.1. Collagen extraction

The Achilles tendon of cattle was used to extract collagen type I. According to the approach outlined in the literature, collagen was extracted using a non-specific enzymatic digestion process [7]. The tendon was cleansed with distilled water, then divided into pieces of 1 cm, frozen, and ground. The frozen tendon underwent 24 hours of swelling in acetic acid (0.4 M). The swollen tendon was acid hydrolysed by adding acetic acid solution (0.4 M) for 18 to 20 hours while being stirred at 80 rpm in a reactor. The biomass was subsequently exposed to one-step enzymatic digestion using a pepsin solution (0.63%) made in 0.02M HCl (1:10,000NF XII/2010 USP; origin: porcine mucosa). At a temperature of 20° C, the mixture was constantly swirled for around 24 hours. Neutralization of extracted collagen was achieved by dialysis at room temperature against distilled water. The collagen in wet form obtained and characterized by SC DELTAROM SRL was stored at -80ºC until gamma irradiation.

The moisture contents

Dry matter was determined by a thermogravimetric method, using a Kern Moisture Analyzer DBS thermobalance. The drying was done at 105º C, in triplicate.

Hydroxyproline content

In the hydrolysis flask, 2g of sample was weighted. 30 ml of sulfuric acid solution [8] were added and incubated in an oven set at 105 ºC for 16 hours. The hydrolysate was filtered at room temperature, and the filtrate was collected according to Kolar (1990) and Sirbu et al. (1993) [8], [9]. A total of 2 ml of diluted (10-fold) hydrolyzed sample was combined with 1 ml of 1.41% oxidative chloramine T solution produced in an aqueous buffer solution comprising 15 g NaOH, 90 g sodium acetate trihydrate, 30 g citric acid monohydrate, and 290 ml of 1-propanol per liter. After shaking the reaction tubes for 20 minutes, 1 ml of chromophore reagent (10 g of 4-dimethylaminobenzaldehyde with 35 ml of 60% sulfuric acid and 65 ml of 2-propanol) was added and incubated at 60°C for 15 minutes. The absorbance at 558 nm was measured after the sample had been incubated.

Soluble Protein content

In order to evaluate the soluble protein concentration, 1 g of collagen was solubilized in 10 ml of PBS (phosphate buffer saline, *pH*=7.4). The sample was centrifuged at 10000 rpm for 10 min at 4º C, and the supernatant was used for the assessment. The Bradford test was used to assess the protein content [10]. The calibration curve was created using bovine serum albumin (BSA) solutions ranging from 1.4 to 0.1 mg/ml (R^2 = 0.9983). The absorbance was measured at λ= 595 nm

using a FlexStation3 UV-Vis spectrophotometer (Molecular Devices, San Jose, CA, SUA).

Free aminoacides content

Following protein precipitation with trichloroacetic acid (TCA), total free amino acids were measured. The samples were centrifuged for 15 minutes at 10,000 rpm (Thermo Scientific, Waltham, MA, USA), and 10 µL of the supernatant was added to 300 µL of Cd-ninhydrin reagent. The samples were maintained at 84 °C for 10 minutes and the absorbance was measured at = 507 nm. The calibration curve (glycineR2 = 0.9940) was measured for concentrations ranging from 10-0.625 mg/ml. The Cd-ninhydrin reagent was made in the following manner: 1 ml of 1 g/ml CdCl2 was added to 90 ml of 100% ethanol: glacial acetic acid 8:1 (v:v) solution containing 0.8 g ninhydrin. The resulting solution was diluted 1:1.5 with distilled water [11].

2.3. Gamma Irradiated collagen hydrogel samples

The collagen hydrogel samples were subjected to gamma irradiation treatment in the Co60 irradiation facility SVST Co-60/B of IRASM Department (IFIN-HH). The irradiation experiments had the target absorbed doses of >15 kGy and >25 kGy. The delivered doses were (17.7+0.8) kGy and (26.6+1.2) kGy. The irradiation experiments (>15 kGy and >25 kGy) on the collagen hydrogel samples have been performed at two different temperature: -70 ºC (frozen) and at room temperature, then the samples have been analyzed by SDS-PAGE.

2.4. SDS-Page analysis

Collagen controls

Two reference collagen samples were included in the tests as a point of comparison: Sigma-Aldrich products Collagen solution from bovine skin (C4243) – bovine collagen, type I, purified from the skin, and, respectively, Bovine Collagen Solution (804592) – bovine collagen purified with pepsin from the hoof growth area, predominantly type I (with 5% type III). Both standards are presented in the form of a solution with a concentration of 3 mg/ml (0.3% w:v) and *pH*=2, intended for cell culture (formation of growth matrices), sterilized by filtration.

Additionally, we included 2 gelatine samples, namely Sigma G1890 - from pig skin (marked with A in our experiments) and Sigma C9382 - gelatine *type I*, from bovine skin (marked with B) as a comparative example.

Gamma Irradiated collagen hydrogel samples

The collagen hydrogel samples were subjected to irradiation treatment, at two doses, in two temperature conditions, then analyzed by SDS-PAGE.

Preparation of the sample for testing

In order to bring the native and irradiated collagen from Deltarom to a concentration around 0.3%, it was weighed and diluted with two parts deionized water (w:v). The diluted collagen was heated up to 80º C, 3–4 minutes for dissolution and homogenization, resulting in a transparent solution. The temperature was set to achieve homogeneity rather than denaturation.

Gelatine was weighed and dissolved warm (45º C) in 100 parts of deionized water (w:v). Afterwards, hot denaturation was performed, with 1 part Laemmli buffer to 3 parts collagen solution (25 µL + 75 µg), w:v, by heating for 5 minutes at 95º C. The *pH* was not adjusted before denaturation.

The denatured samples were loaded into wells (approx. 50-55 µl/well) and migrated at 100-110 V, in TRIS-glycine buffer (Bio Rad #1610734), for approx. 90 min., until the blue front was almost of the gel exit limit.

The samples irradiated at room temperature showed large syneresis, probably due to crosslinking of collagen. For these samples, only the undiluted solid (cross-linked) fraction was used, in a ratio of 3:1 (w:v) with Laemmli buffer. The syneresis water was removed. When heated, the cross-linked sample fragment contracted, the collagen being only partially dissolved.

Collagen hydrogel samples migration in 10% polyacrylamide gel

For the SDS-PAGE method, we used denaturing polyacrylamide (PAA) gels with a concentration of 10%. The reagents used were in the form of a ready-to-use kit (the TGX FastCast™ Acrylamide Solutions range from Bio-Rad). Some gels were prepared in the Stain-Free version - a patented formula by Bio-Rad, which uses a trihalo compound incorporated in the gel; the compound binds to tryptophan residues in proteins and can be visualized later by activation with UV light (through the special tray). The method is fast, compared to other staining methods, but it requires the presence of tryptophan in the protein bands - to be highlighted. To allow the passage of UV light, the gel must be detached from the glass plate on which it has migrated, as with the other staining methods.

If not specified otherwise, after migration, the gels were fixed for 1-3 hours (or overnight - as the case may be) in a solution of 50:10:40 methanol: acetic acid: water (a partial cross-linking of the acrylamide takes place, preventing the mobilization of proteins, therefore the diffusion of the bands).

Flamingo staining was performed overnight, in the dark, in a freshly prepared solution, 1x dilution, of Flamingo™ Fluorescent Protein Gel Stain dye (10x solution) (Bio Rad #1610490).

Coomassie staining was performed in Coomassie R250 dye solution (Bio-Rad #1610436), for 1 hour at ~60º C, or by heating 2 times until boiling, in a microwave oven, both variants followed by decolorization in successive baths of methanol:acetic acid:water (50:10:40), until the background is removed.

The visualization of colored gels was carried out on a Gel Doc EZ Gel Documentation System (Bio-Rad), with viewing trays dedicated to the device and the dye, in the light:
- white – for Coomassie staining
- UV – for Flamingo coloring (280-400; max 300 nm)
- respectively 302 nm – for StainFree Gels [1], [12]
As a weight marker, the Spectra™ Multicolor Broad Range Protein Ladder (Thermo #26634) was used.

PAA gels visualization

After migration in PAA 10%, with incorporated Stain Free dye (BioRad #1610183) the gels were visualized immediately (without staining) - using the tray specific for stain-free staining. In the next step, the gel was fixed in a solution of methanol: acetic acid: water. After fixation, the PAA gel was stained overnight with Flamingo Stain (Bio Rad #1610490), then visualized in UV light (purple tray). Afterwards, the gel was recolored with Coomassie R250 - hot (bringing to a boil 2 times, in the microwave oven), followed by bleaching for ~24h.

On Coomassie staining, the calibration curve was performed according to the expected bands from the Ladder and the weight of the main bands from the collagen samples was calculated.
- Irradiation doses: 17.7 and 26.6 kGy (According to Treatment Certificate no. 28 / 14.06.2023)
- Irradiation temperatures: -70º C and room temperature (inside the irradiation enclosure, the temperature rises slightly)
Unless otherwise specified, all weights (percentage of the band from the total protein content of the well) were estimated on Coomassie-stained gels.

3. Results and discussion

Assessment of Physico-Chemical Parameters

The steps taken to obtain some varieties of collagen hydrogel had two goals: (i) obtaining specifically formulated and conditioned samples for the evaluation of the change in their properties and characteristics as a result of gamma irradiation, and (ii) establishing the optimal parameters for irradiating collagenous forms in a wet state.

To highlight the degree of degradation of the protein structure, the content of soluble proteins and free amino acids was evaluated. The content of soluble proteins was determined, after which the protein mass was precipitated with 6.1 N trichloroacetic acid, and the free amino acids were determined from the supernatant. The results are presented in **Table 1**.

Collagen contains a specific amino acid, hydroxyproline, not found in detectable amounts in other proteins in the animal body. This amino acid provides thermal stability to collagen molecules due to the formation of hydrogen bonds and the presence of a hydroxyl group (–OH), limiting the rotation of the peptide chain [1]. Through the total hydrolysis of the collagen hydrogel, a hydroxyproline content of about 11.58% was obtained, which corresponds to a collagen content of 92.68% (Table 1).

Although collagen basically remains insoluble in water, reducing the *pH* of the solution can improve solubility. Native collagen has an isoelectric point (pI) around neutral *pH*, which implies it has limited water solubility under physiological conditions, thus limiting its use [13]–[15]. Insoluble collagen is converted to soluble gelatine by acid or alkaline processing, but whereas products made from native collagen possess significant strength, this strength is lost when soluble collagen is used [16]. For this purpose, the concentration of soluble proteins was evaluated, which in general for collagen have molecular masses between 1 kDa and 10 kDa, and provides information on the degree of native collagen hydrolysis [17].

Table 1.

Physico-chemical parameters

	Dry matter (%)	Total soluble protein (% related to dry matter)	Total free aminoacids (% related to dry matter)	Hyp content (%)	Collagen content (%)
Collagen sample	0.92±0.11%	5.94±0.86	6.42±1.05	11.58±1.28%	92.68±1.28%

Optimizing the amount of sample per well and the time of discoloration

The first experiment aimed primarily at optimizing the amount of protein sample (collagen) to load in each well. As a benchmark, we started from the concentration of the reference collagen solution (bovine: Sigma C4243 and 8045923), namely 3 mg/ml, i.e., 0.3%.

Thus, we tested 2 dilutions of the Deltarom collagen sample (of 0.92%): 3 times, up to ~0.3% and, respectively, 9 times, up to ~0.1%. The dilutions were carried out with deionized water, by gradually heating up to ~80º C, with gentle stirring; the collagen hydrogel has completely dissolved.

The denaturation and migration protocol were the ones described in *Materials and Methods* (general conditions). For comparison, we also included the two different samples of gelatine, at a concentration of 0.3%.

After staining with Coomassie R250 (24h - at room temperature), the gel was bleached (also at room temperature) moderately (~24h), respectively

accentuated (~48h), with visualization after each bleaching time. The gel was not fixed in a mixture of methanol: acetic acid: water. The results are presented in **Fig. 1**.

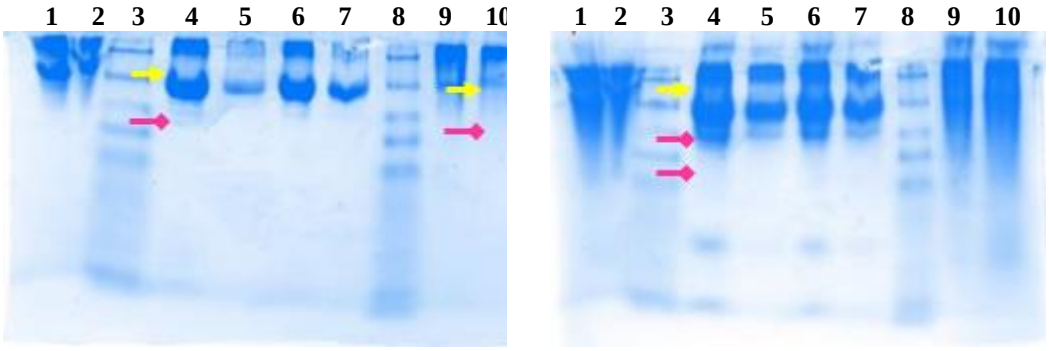


Fig. 1. Protein fractions of different types of collagens and gelatine. Coomassie R250 staining. The stacking gel has not been removed.

Staining with Coomassie R250 at room temperature and pronounced discoloration (~48h - left), vs. moderate (~24h - right). In the moderately discolored one (right), both α chains can be seen (two pink rhombuses); in the accentuated discolored one (left), the lighter chain is no longer evident. On the other hand, through pronounced discoloration, distinct bands can be seen in gelatine (wells 9 and 10 - left), corresponding to the same main chains as in collagen.

1 - Collagen Sigma C4243 (bovine skin) - reference
2 - Collagen Sigma 8045923 (bovine) – reference
3 & 8 - Ladder Thermo 26634
4 & 6 - Deltarom collagen& 1/3 dilution
5 &7 - Deltarom collagen, dilution 1/9
9 - Sigma Gelatine G1890 (A - from pigskin)
10 - Sigma Gelatine C9382 (B – bovine, type I)

For a concentration similar to that of the reference collagen (0.3 mg/ml), Deltarom collagen (in hydrogel form - concentration 0.92%) was diluted with water (3 times) up to a concentration of ~0.3 mg/ml. The gelatine was prepared in the same concentration. To facilitate the visualization of distinct bands, the actual sample volume loaded into the well was reduced from 75 µL to 50-55 µl/well.

Both the reference collagen (Sigma) and the native collagen from Deltarom show the two major characteristic bands, from 260-300 kDa (β chain - arrow, yellow) and 135-150 kDa (chains α1 and α2 - rhombus, pink). These bands are also

visible in gelatin samples - after pronounced discoloration. The PAA gel did not have enough resolution to distinguish between close chains.

Gelatine is much more fragmented than collagen, with numerous protein fractions, of very similar sizes - almost in a gradient. However, when the discoloration is accentuated, the two majority bands are revealed. With Coomassie staining, the bleaching time can influence the presence or appearance of the band. For this reason, the discoloration must be done gradually. Also, for optimal discoloration, the concentration of all migrated proteins must be similar.

In addition, dyeing at high temperature (reaching a boiling point) will also be attempted - for better fixation of the dye and fixation of the gel (to prevent the diffusion of the bands during bleaching).

Optimization of staining with Coomassie R250

The samples were the same as those from the previous experiment, but loaded in a different order. Again, the gel was not fixed in the methanol : acetic acid : water mixture.

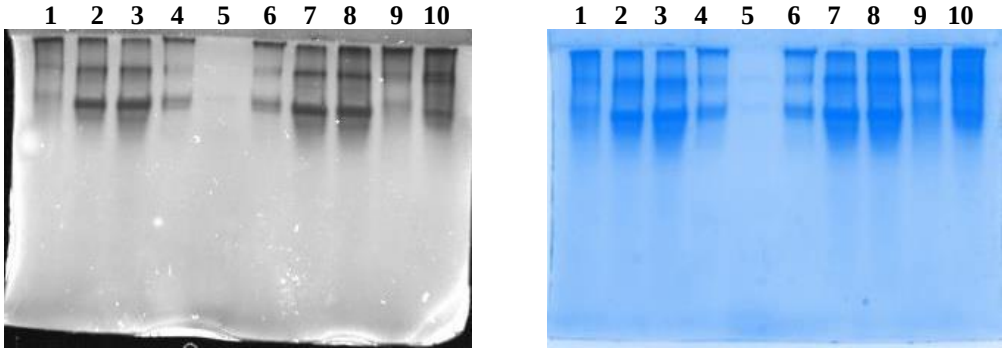
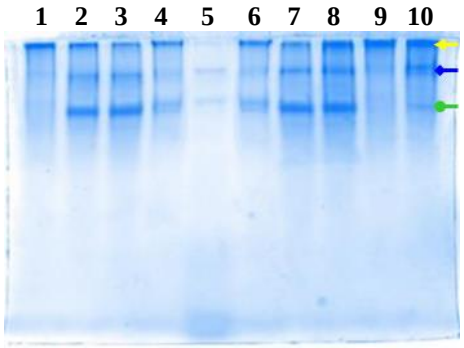


Fig. 2. Protein fractions of different types of collagens and gelatine.

Coomassie R250 staining and visualization in visible - white light (right), respectively in UV - Stain-Free tray (left). Moderate fading - ~24h (top), respectively accentuated - ~48h (bottom).
The main chains can be seen: the trimer (arrow, yellow), the dimer α1, α2 (diamond, blue – 245, 260 kDa), the monomer α1, α2 (circle, green, ~135 kDa).
1& 9 - Gelatine Sigma C9382 (B – bovine, type I)
2& 8 - Collagen Sigma C4243 (bovine skin) – reference
3& 7 - Collagen Sigma 8045923 (bovine) – reference
4& 6 - Collagen Deltarom (gel, 0.3%)
5 - Ladder Thermo 26634
10 - Sigma Gelatine G1890 (A - from pigskin)



Compared to the previous experiment, over the denatured sample, we added glycerol (85%) up to a final concentration of about 10%, before loading - so that the sample remains at the bottom of the well. The gel was visualized after staining with Coomassie R250 (cold) in white light, as well as in UV with 5 min activation – on the StainFree tray, after discoloration ~24h. Afterwards, the discoloration continued for ~24 hours and was visualized again, in white light. The results are presented in **Fig. 2.**

Since the Coomassie dye also generates fluorescence, the bands for gels with mild discoloration stand out more in UV light than in visible light, where there is a strong backdrop. The shortcoming, in this case, is that the ladder is made up of proteins that do not stain enough and, therefore, its bands are very little distinguishable. A possible solution would be to replace the prestained ladder with one of the protein fractions.

Through prolonged bleaching, weak bands tend to disappear (e.g. well 1 - Gelatine Sigma C9382), but the background is removed more effectively and thus, very close bands are clearly highlighted.

A significant amount of the band intensity on Thermo's pre-colored ladder is lost after repeated defrosting.

Identification of protein fractions on irradiated collagen hydrogel samples

After irradiation in frozen state, at both doses, the collagen hydrogel keeps its consistency unchanged; organoleptically, it cannot be distinguished from the non-irradiated gel.

Fig. 3. The appearance of Deltarom collagen hydrogel, in the 5 experimental variants - after gamma irradiation at an average dose of 17.7 (2 and 4) and 26.6 (3 and 5), compared to the non-irradiated control (1).

It can be observed the reticulated fraction of collagen - when irradiated at room temperature: in the upper part of the tube (4) - at a dose of 17.7 kGy, respectively at the bottom of the tube (5) - at a dose of 26.6 kGy

At room temperature, at both doses, the collagen hydrogel reticulates, forming a solid, elastic fraction with resistance to pressure and a watery fraction, respectively syneresis water - **Fig. 3**. We also note that at the low dose (17.7 kGy), the reticulated fraction floats, while at the high dose (26.6 kGy), it settles at the bottom of the tube, probably as a result of the increase in density through the contraction and overpacking of the collagen fibrils, limiting thus space for water (between the meshes of the net).

Protein electrophoresis results are shown in **Fig. 4**. The results are in accordance with the literature [18]. In the case of collagen irradiated in frozen state, between the two dimer bands (from 260 and 245 kDa), only the heavier band (260 kDa – Fig. 4 - diamond arrow, green) is maintained.

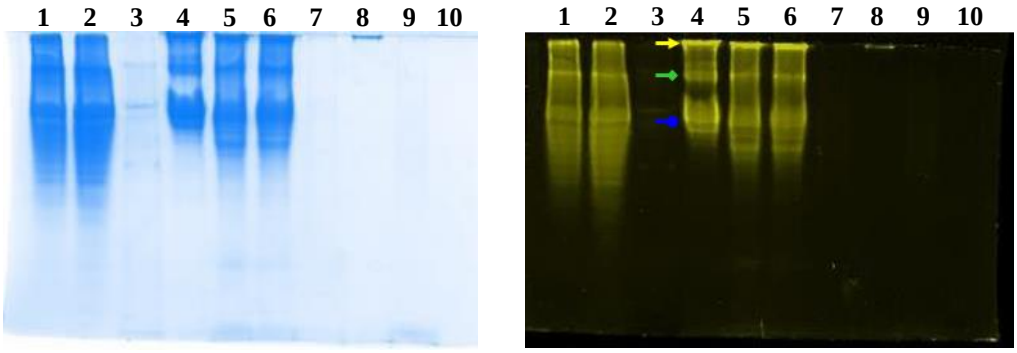


Fig. 4. Protein fractions of Deltarom collagen after irradiation, at room temperature (7-10), respectively in frozen state (5, 6), at a dose of 17.7 kGy (5, 7, 9), respectively 26.6 kGy (6, 8, 10).

Staining with Coomassie R250, visualization in white light (left). Staining with Flamingo, visualization in UV light (right). The partial fragmentation during irradiation of frozen samples (5, 6) compared to non-irradiated (4) is evident, with the maintenance of the main chains: trimer (arrow, yellow), dimer α1, α2 (diamond, green, 260 and 245 kDa), monomer α1, α2 (circle, blue, ~135 kDa).
1 - Collagen Sigma C4243 (bovine skin) - reference
2 - Collagen Sigma 8045923 (bovine) – reference
3 - Ladder Thermo 26634
4 - Collagen Deltarom (gel, 0.3%)
5 - Deltarom collagen (gel, 0.3%), irradiated at 17.7 kGy, frozen
6 - Deltarom collagen (gel, 0.3%), irradiated at 26.6 kGy, frozen
7 - Deltarom collagen, irradiated at 17.7 kGy, at room temperature – cross-linked fraction – diluted x3
8 - Deltarom collagen, irradiated at 17.7 kGy, at room temperature - syneresis water
9 - Deltarom collagen, irradiated at 26.6 kGy, at room temperature – cross-linked fraction – diluted x3
10 - Deltarom collagen, irradiated at 26.6 kGy, at room temperature - syneresis water

For collagen irradiated at room temperature, the protein concentration was too low to produce a visible band - both in the sample from the syneresis water and in the sample from the cross-linked fraction (the part that dissolved from this fraction). For the cross-linked fraction, skip the initial dilution step (to reach the concentration of 0.3 mg/ml), and work directly with the cross-linked sample in the denaturation mixture (Laemmli buffer).

The syneresis water cannot be concentrated and will not be further analyzed.

Fragmentation of collagen upon irradiation

Under identical circumstances, the experiment is a replication of the previous one with two modifications. Firstly, the analysis of syneresis water was

eliminated and secondly, the cross-linked fraction was directly subjected to denaturation with Laemmli buffer 4x (3p collagen + 1p Laemmli).

The results are presented in **Figs. 5 - 8** and **Table 2**.

The native collagen (Deltarom), shows 3 major bands: one at ~300 kDa, one at ~260 kDa and one at ~135 kDa - the majority band in intensity, representing ~65% of the total fractions of its protein (in Coomassie staining), summing up the α1 and α2 chains.

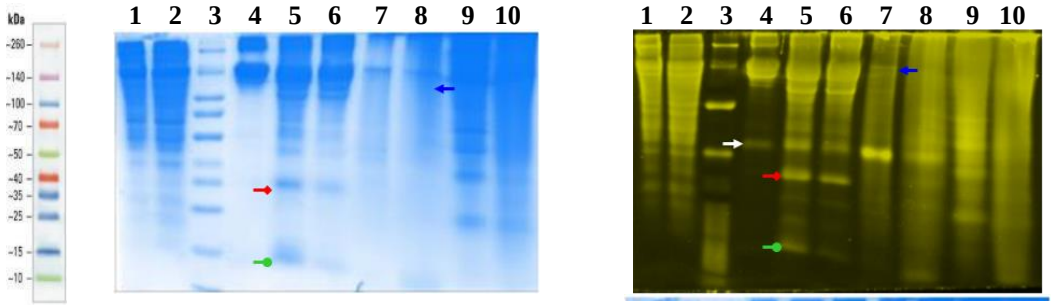
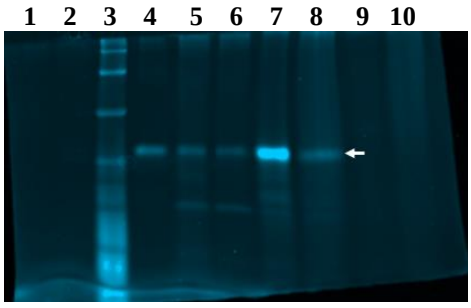


Fig. 5. Deltarom collagen irradiated at 17.7 kGy (5, 7), respectively 26.6 kGy (6, 8) - at room temperature (7, 8), versus frozen at -70 °C (5, 6). Ladder Thermo 26634 (3).

Coomassie staining (left, top), Flamingo (right, top), respectively Free-Stain (left, bottom).

For comparison: gelatin A - Sigma G1890 (9) and B - Sigma C9382 (10).



Analysis of the bands, that the software program automatically detected on the Deltarom collagen section (non-irradiated) - well 4.

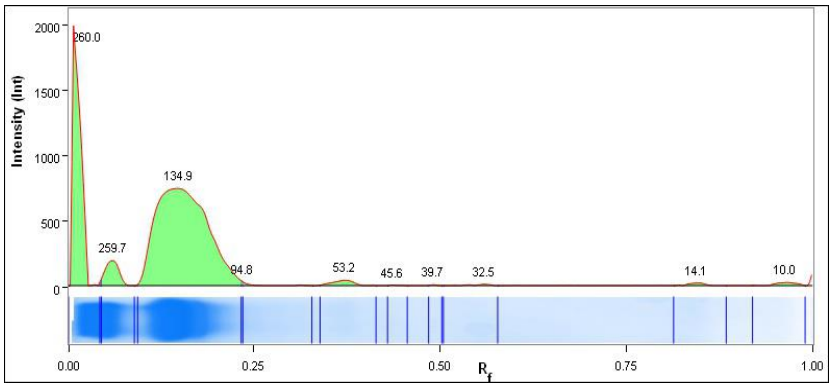


Fig. 6. The protein fractions of native bovine collagen, from Deltarom (well 4).

The most important bands, quantitatively, are those from: >260 kDa (trimer), 259.7 kDa (dimer), 134.9 kDa (monomer) - an agglomeration of 4 bands that stands out as a single band, respectively 53 kDa.

Table 2.

Band analysis of native collagen from Deltarom

Band No.	Mol. Wt. (KDa)	Relative Front	Adj. Volume (Int)	Volume (Int)	Band %	Lane %
1	> 260	0.011038	926667	3952053	27,027,176	26,935,121
2	259.7	0.06181	147546	4603914	4,303,328	4,288,670
3	134.9	0.15011	2239587	14138586	65,319,810	65,097,328
4	94.8	0.236203	9513	7344099	0.277456	0.276511
5	53	0.373068	47817	5831784	1,394,631	1,389,881
6	45	0.437086	1953	2126187	0.056961	0.056767
7	39	0.492274	1953	1467333	0.056961	0.056767
8	32	0.560706	8694	5582871	0.253569	0.252706
9	14	0.845475	17451	5301072	0.508976	0.507242
10	10	0.96468	27468	5058270	0.801132	0.798403

In the Flamingo color, the band at 75.9 kDa (white arrow) is highlighted - probably coming from the collagen of another type (II or III), remaining in the process.

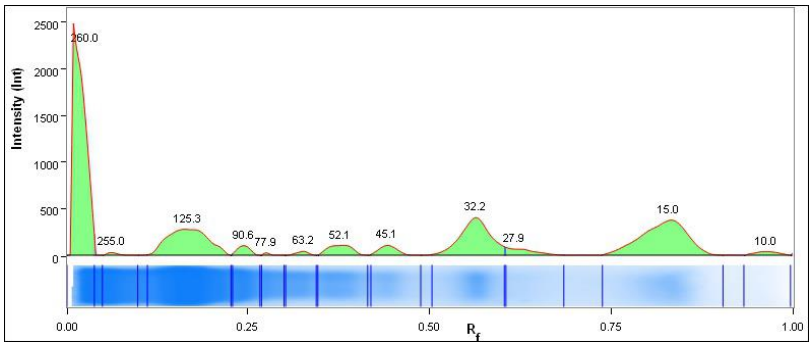


Fig. 7. Protein fractions of bovine collagen, of Deltarom, after irradiation at 17.7 kGy, frozen (well 5).

With the irradiated collagen, at both doses and temperatures, it is evident that the ~72-76 kDa band is stable (white arrow). In the case of native collagen, this band is evident only in the fluorescent colors (Flamingo and Stain-free), in the Flamingo color it represents 26.9% of the total intensity of the migration section (of the total protein fractions).

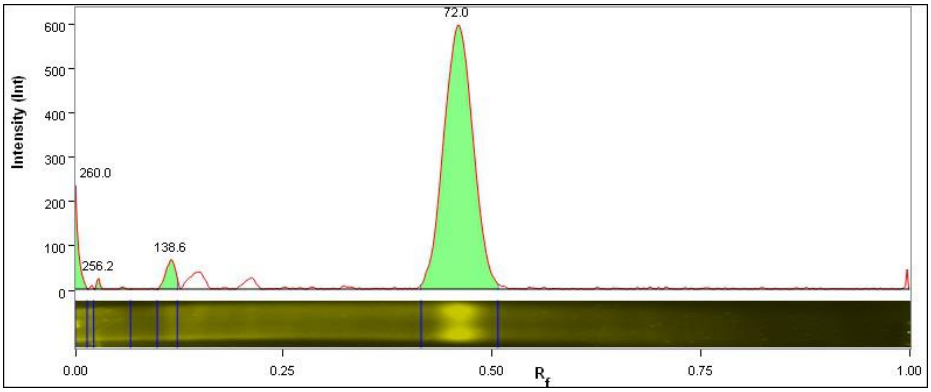


Fig. 8. Protein fractions of bovine collagen, of Deltarom, after irradiation at 17.7 kGy, at room temperature (well 7) - Flamingo staining.

The band is also present in the non-irradiated collagen - visible only in fluorescent staining (Flamingo), but with a much lower weight (29.6%) in the total protein content, compared to the irradiated ones: 35.6% at 17.7 kGy, 45.7% at 26.6

kGy - in frozen state, respectively 84.8% at 17.7 kGy (and 22.4% kGy at 26.6 kGy) - at room temperature.

Also, the main band (dimer α1, α2) at 135 kDa remains visible in the irradiated samples - in Coomassie and Flamingo staining, although in a much smaller amount (blue arrow); again, the heavier band tends to be unstable and disappear upon irradiation.

The band at ~31-32 kDa (red diamond) is present only in samples irradiated in frozen state, which qualifies it as a marker of irradiation at low temperatures. Its weight decreases with the dose, from 14.7% - at the low dose (17.7 kGy), to 11.9% - at the high dose (26.6 kGy). The band is absent in the non-irradiated collagen, as well as in the one irradiated at room temperature.

Similarly, the band at ~15 kDa (circle, green) can be considered a marker of irradiation at low temperatures, being present only in the samples irradiated on dry ice in frozen state- at both doses, with a weight decreasing with the dose, namely 21.1% - at 17.7 kGy, respectively 0.6% kGy – at 26.6 kGy.

Overall, the collagen that was exposed to radiation on dry ice, in a frozen condition, is more fragmented than the collagen that was not exposed to radiation; its general profile resembles standard collagen (Sigma) – which is most likely enzymatically fractured, more than the non-irradiated collagen, even if the primary bands are preserved.

Since with Stain-Free staining, collagen reveals only two bands, we conclude that this staining is not suitable for collagen, nor for gelatine, most likely due to the absence of tryptophan from the component peptides. We note, however, that the band-irradiation marker (white arrow), from ~72 kDa, is visible in stain-free staining (including the samples irradiated at room temperature - high dose). We also note that the gelatine samples are completely invisible in this coloration.

The band is absent in non-irradiated collagen, which is why it can be designated as an irradiation marker.

SDS-PAGE analysis reveals that radiation causes effects at both doses, but they are significantly less pronounced at low temperatures (dry ice) than they are at room temperature.

The main component protein chains of collagen are preserved, but their weight decreases, as a result of the generation of a series of protein fractions of lower weights following the irradiation on dry ice in frozen state. However, the bands are distinct, meaning that the spectrum of molecular weights is not continuous - as with irradiation at room temperature.

Among the bands newly generated after irradiation, we identified, in those irradiated at low temperature, two distinct bands that can be considered a marker of irradiation.

It is also desirable to color the gel successively, first with Flamingo fluorescent dye, then with Coomassie, to highlight the different bands - depending on the amino acid composition of the respective fractions.

4. Conclusions

The collagen hydrogel with a dry matter of 0.92% sterilized at different irradiation doses in frozen state did not crosslink or form syneresis. However, SDS-PAGE method revealed a specific irradiation band at ~15 kDa, which can be used as a marker to identify the effectiveness of sterilization. So, the optimized SDS-Page method was successfully used for identification of protein fractions on irradiated collagen hydrogel samples and study of fragmentation of collagen upon irradiation. The technique can be introduced as routine quality control for the extraction and of collagen and used in validation studies for collagen hydrogels

Using two types of staining (Flamingo fluorescent dye and Coomassie) a more complex pattern of bands is revealed.

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