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The Nanobiotechnology of Obtaining of Collagen Gels from Marin Fish Skin and Yours Reological Properties for using Like New Materials in Dental Medicine

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***Abstract*—**This paper aims at presenting the biotechnology used to obtain collagen-based gels from shark (Squalus acanthias) and brill skin, marine fish growing in the Black Sea. Due to the structure of its micro-fibres, collagen can be considered a nanomaterial; in order to use collagen-based matrixes as biomaterial, rheological studies must be performed first, to state whether they are stable or not. For the triple-helix structure to remain stable within these gels at room or human body temperature, they must be stabilized by reticulation.

***Keywords*—**Collagen, biotechnology, reticulation.

I. Introduction

TYPE I fibrillar collagen is a triple-helix structure protein present in the skin and in cartilaginous tissues. The acknowledged disadvantages related to obtaining collagen by extraction from fish skin consist mainly in the persistence of pigments and fish odour, and the fact that the denaturation temperature values are lower – usually below 30° C – which is a disadvantage concerning their use as bio-nanomaterials. Nonetheless, fish-derived collagen can be widely used due to its indisputable advantages:

- it is easier to extract and has shown better performance compared to animal-derived collagen;
- it entails a relatively low risk of containing unknown germs;
- the denatured collagen-based matrixes permit a better tissue regeneration compared to the native collagen-based ones [1], and the reticulum matrixes derived from fish collagen are likely to contain partially denatured structures due to the lower denaturation temperature.

Although fish-derived collagen does not form high-viscosity gels, it is extremely convenient for some applications, such as micro-packaging or obtaining photosensitive coatings. Films and porous matrixes can be obtained from collagen-based gels, just as from mammal-derived ones. They can be successfully used in dental medicine for treating oral diseases – they form bio-absorbable membranes and matrixes and they can incorporate different active ingredients which can be subsequently released in order to obtain the intended therapeutic effects.

Depending on the treatments applied, we can distinguish between four categories of bio-nanotechnologies used for obtaining collagen from marine fish:

- alkaline and acid treatments [2];
- acid treatment [3, 4-6];
- enzyme treatment [5, 6];
- combined treatments.

This paper aims at presenting the biotechnology used to obtain collagen-based gels from shark (*Squalus acanthias*) and brill skin, marine fish growing in the Black Sea. Due to the structure of its micro-fibres, collagen can be considered a nanomaterial – in order to use collagen-based matrixes as biomaterial, rheological studies must be performed first, to state whether they are stable or not. For the triple-helix structure to remain stable within these gels at room or human body temperature, they must be stabilized by reticulation.

II. Materials and Methods

Reticulation has been performed by using glutaric aldehyde at 4-6^{o}C for different concentrations of collagen derived from the two species of fish.

The rheological behaviour has been determined by using a Haake VT 550 rheo-viscometer with the following layout: S_3 (MV1) sensors system, d_1 and d_2 measuring ranges, shearing speed range, $\dot{\gamma}$, between 1.17 and 1872 s^{-1}. The measurements were initially performed at a 25 ± 0.1^{0}C temperature, with a thermostatic time of 20 minutes, and then – using ice – at 20 ± 0.1^{0}C. In order to establish whether the dispersed systems show a time-dependant rheological behaviour, measurements were performed when increasing and decreasing the shearing speed values, which allowed the tracing of the hysteresis loops for the film-forming systems with time-dependant rheological behaviour. The position of the two rheograms in Figs. 1 and 2 – meant to represent the hysteresis loop – proves that the gels obtained from shark skin (Fig. 1) and brill skin (Fig. 2) have a

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time-dependant rheological behaviour, namely a strongly tyxotropic one, without structure recuperation at low shearing speed values.

A. The Biotechnology used to Obtain Collagen from Black Sea Shark Skin

What we identified as the main problem regarding the shark (*Squalus acanthias*) skins was not just that of discoloration and removal of the odour, as in the case of other fishes, but especially the total removal of the epidermis, which contains placoid scales shaped like basal plates provided with a spike directed backwards and solid particles which do not dissolve during the collagen extraction process and impurify it; another problem identified is that of finding a solution for extracting collagen that should not hinder the integrity of the epidermis, if such a solution is possible. Added to the above is the necessity of working at low temperature, due to the low denaturation temperature of collagen extracted from fish skin generally, as compared to that extracted from terrestrial mammals.

The first operation consisted of skinning the shark, followed by removal of muscle remains, then intensively washing it using tap and distilled water, and immediate storage in the freezer.

Below are the treatments to which the skin was submitted:

TABLE I
COLLAGEN EXTRACTION FROM SHARK SKIN BY MEANS OF ALKALINE-ACID TREATMENTS

The alkaline-acid extraction of collagen from shark skin	Treating with 0.5M acetic acid
The first treatment applied was the one frequently employed as pre-treatment for bovine skins, as applied by Yoshimura, Terashima, Hozan and Shirai [9] for the alkaline-acid extraction of collagen from shark skin: 20ml 0.5M concentrated solution of sodium hydroxide and 15% sodium sulphate, with a pH of 11-12, was inserted over the moist skin after dabbing it with filter paper. A high moist skin/solution ratio was used, of 1/21.4, in order to submerge the skin completely. Treatment was performed at room temperature (23^{o}C). After 3 days and a half the skin became a little more elastic and seemed somewhat soaked. The skin was kept in contact with the solution for a week, daily observation was performed, but its aspect did not undergo any significant changes. It was only the solution that got slightly coloured, becoming pale beige. The skin was taken out of the solution and was washed with distilled water, which leads to its stiffening. A new volume of solution was inserted over the washed, squeezed and filter paper-dabbed skin. Another week later, the skin virtually disappears completely, the solution is coloured in an intense hue of beige and its viscosity is reduced, while in the lower layer small deposits are formed of particles previously existing in the epidermis. Low viscosity is an indication of the fact that there is no collagen in the solution, but rather its hydrolytic compounds, so the method cannot be applied for obtaining fibrillar or non-denatured collagen; it can only be used to obtain its hydrolytic compounds. In addition, the extract is impurified by the pigments existing in the skin. The conclusion which can be drawn is that this treatment is too strong for obtaining fibrillar or non-denatured collagen from shark skin and that new methods should be identified that could enable either the preliminary removal of the	As room temperature enabled – in the case of bovine skin – removal of the epidermis, this pre-treatment was also applied to shark skin. Considering the fact that the denaturation temperature of collagen extracted from fish skin is lower than that obtained from bovine skin, the 0.5M acetic acid pre-treatment was applied both at room temperature (23-26^{o}C), and in the frdige (5-7^{o}C), without stirring, using variable skin/acid solution ratios, between 1/7.5 and 1/20, aiming – each time – at submerging the skin entirely. Shark skin quickly becomes soaked in the 0.5M acetic acid solution, significantly increasing its volume and weight. Thus, at 27^{o}C it gains – in about 1 hour – circa 74% in weight, it thickens and gets a jelly-like aspect. Horny epidermis can be cleaned (it was cleaned) and the solution will be clear and of low viscosity, so under these circumstances collagen was not extracted completely (losses are negligible). After cleaning, the skin was washed twice in tap water and twice in distilled water to remove any remains of horny layer, then it was finely chopped and divided into two batches in view of extracting collagen: one batch was still treated with acetic acid of the same concentration, while the other was treated with pH 3.5 hydrochloric acid, in both cases the ratio between soaked and filter paper-dabbed collagen and solution being 1/7. The samples were stirred energetically for 1 hour and 40 minutes, and then they were placed into the fridge. In the meanwhile viscosity increased, appearing to be higher than in the case of the hydrochloric acid solution. On the second day they were removed, left to reach room temperature and forced through fine muslin to remove the jelly-like component, whose suspension also contains visible bits of skin. Colourless filtrates were centrifuged for 30 minutes, then placed into the fridge, while a new amount of acid was inserted over the jelly-like component to continue extraction. Filtrates had low viscosity, which indicates the fact that collagen was denatured.

epidermis, or the extraction of collagen without dissolving the epidermis.	

TABLE II
COLLAGEN EXTRACTION FROM SHARK SKIN BY MEANS OF ALKALINE-ACID TREATMENTS

Pre-treatment using 0.25M sodium acetate and 0.25M acetic acid solution	
Instead, the 24°C, the ease of cleaning being comparable to that in the case of soaking in acetic acid under the same circumstances. The supernatant is colourless, meaning no pigments are extracted. The extent of cleanliness and the aspect of cleaned skin are shown in the picture in Fig. 2, compared to the one soaked in 0.5M acetic acid.	Removal of non-collagen materials, which are soluble in a hydrous environment and are attached to skin fibrils by means of non-specific interactions, can be performed by extraction employing salt solutions, sodium chloride in particular. At the same time, they extract newly synthesized collagen molecules which are not yet incorporated into the fibrils by covalent bonds. Pre-treatment using 10% sodium chloride solution for 24h at room temperature is also specified for the process of extracting collagen from squid skin, but for the purpose of partially separating chromophores (dark brown cells containing pigments), followed by decolouring by means of hydrogen peroxide. The skin, unfrozen and dabbed with filter paper, was replaced into a 10% sodium chloride solution with a pH of 7, at room temperature (cca. 27°C), the skin/collagen ratio being 1/5.After 24h, the solution becomes coloured in beige and gives off an intense fish odour, while the skin is soft and does not appear soaked. Washing was first performed using tap water jet, then the skin was squeezed, washed with distilled water, squeezed again, left in distilled water for 10 minutes, checked for chloride ions, well squeezed, dabbed with filter paper and then weighed. The gain in weight is, indeed, very low, of only cca. 5.2%. The skin thus treated was then divided into two batches to undergo further treatment in view of extracting collagen.

TABLE III
COLLAGEN EXTRACTION FROM SHARK SKIN BY MEANS OF ACID TREATMENTS

Cold-treatment with 0.5M acetic acid	**Treating with pH 3.5 hydrochloric acid**
0.5M acetic acid solution at 6°C was added over the filter paper-dabbed skin, at a skin/acid ratio of 1/5.After 24 hours the skin becomes significantly soaked, its weight more than doubling (it increases about 2.1 times). The epidermis starts coming loose already, although with some difficulty. After 48 hours it will clean well, but the skin seems thinned, and the portions from the abdominal area will tear upon cleaning, which indicates that the soaking time was too long for them. The supernatant, which is colourless and perfectly clear, has a rather high viscosity, which means that a part of the collagen has already been extracted. The collagen was immediately separated by decanting, and then stored in the fridge. A new amount of cold acetic acid was inserted over the cleaned and chopped skin, using the same ratio, and it was left in the fridge for extraction (Fig. 1).	The other batch of skin was inserted into a pH 3.5 hydrochloric acid solution, using a wet skin/solution ratio of 1/5. After 24 hours it seems unchanged and, indeed, its weight increased during this while by only 9%. The skin was removed from the hydrochloric acid, it was washed entirely using tap and distilled water until all chlorine ions had disappeared, then the other treatment was applied, involving cold 0.5M acetic acid solution (skin/acid ratio = 1/7) and storage in the fridge. After 24 hours the skin increased its volume considerably, it started peeling off, although with difficulty and incompletely, which is why it was left for another 24 h in acetic acid, just as in the case of direct treatment with acetic acid. After 48 hours the epidermis was cleaned, the clean skin was chopped into small pieces and then inserted into 0.5M acetic acid solution. Both the vessel containing skin and acetic acid and the colourless and relatively viscous supernatant were placed into the fridge. After 5 days the viscous supernatant was separated from the undissolved skin by decanting, and the two samples were mixed.

Fig. 1 Partly cleaned skin soaked in 0.5M acetic acid solution

Fig. 2 Partly cleaned skin soaked in 0.25M sodium acetate and 0.25M acetic acid solution

III. Brill Skin

Given the experience gained in extracting collagen from shark skin, for brill only the sodium chloride pre-treatment was applied, followed by the extraction of collagen using 0.5M acetic acid solution.

The skin was peeled off the brill, as can be noticed in Fig. 3, then the muscle remains were cleaned off the skin chunks, the skin was washed using tap and distilled water, squeezed as thoroughly as possible, and placed in the freezer for conservation. In the case of brill, removing the epidermis prior to extracting collagen is out of the question, due to the bony scales which are very well rooted in the skin. Therefore, the skin was cut into very small pieces, to enable the removal of the scales.

TABLE IV
Collagen Extraction from Brill Skin by Means of Acid Treatments

Pre-treatment	Collagen extraction using 0.5M acetic acid solution
As far as the pre-treatment is concerned, it was applied for 24 hours at room temperature, stirring for 2 hours prior to removal, in two variants: - using 0.4M (2.34%) sodium chloride, as identified in specialised literature [13]; - using 10% sodium chloride, just as for shark skin. During the stirring stage, the colour of the salt solution became more intense, particularly so for the more diluted solution. At the same time, the skin in the 0.4M solution seemed slightly degraded (ragged) on the muscle side and the smell of the solution was stronger, while the one in the 10% solution was firm, and the solution was somewhat less smelling; both chunks of skin were soft. The chunks of skin were removed from the salt solutions, were washed with tap water, well squeezed, then washed with distilled water, left in it for 24 hours, washed again with distilled water and an inspection was performed to check the absence of chlorine ions.	After being squeezed and dabbed with filter paper, the chunks of skin were inserted into cold 0.5M acetic acid solution, the first bony scaled skin/solution ratio being of ½.For about 3 hours they became well soaked, virtually absorbing all the acetic acid solution. After adding acetic acid to reach a ratio of ¼-1/4.3, they were left for another 31 hours in contact with the acid (for a total of 34 hours). After 24 more hours a very viscous mixture was obtained, from which the scales could be removed manually, but incurring serious gel losses. To reduce the viscosity of the mixture and thus enable an easier removal of the scales, more acetic acid was added, reaching a ratio of 1/5.8. The denaturation temperature of collagen extracted from brill skin could not be found in the specialised literature. By visually observing the disappearance of gel during continuous manual stirring, this temperature can be deemed to be between 22 ^{o}C and 23^{o}C.

The collagen thus obtained also includes, of course, remains of epidermis (coloured in a light grey), which is impossible to separate without a centrifugal device that can enable operation at low temperature, in order to avoid collagen denaturation (the centrifugal device in the laboratory only allows work at room temperature) – see Fig. 3.

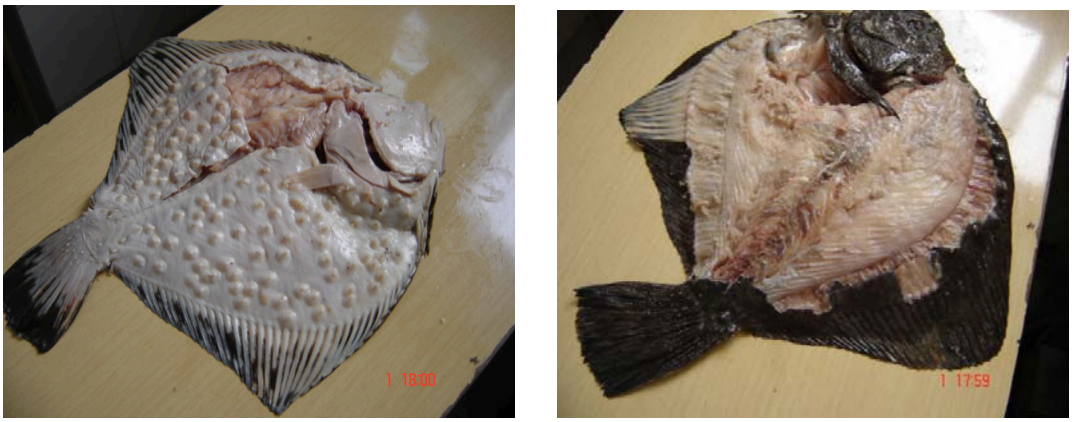

Fig. 3 Brill: a – unskinned; b – skinned

IV. Anguilla Skin

Anguilla can be skinned much more easily than the other two species of fish; two full strips can be obtained from each anguilla, virtually containing no muscles, as can be noticed in Fig. 4. The skin was washed well with tap water, then with distilled water, then it was squeezed and placed into the freezer. During the washing and squeezing procedures water got coloured, which means that a part of the pigments in the epidermis were removed.

TABLE V
Collagen Extraction from Anguilla Skin by Means of Alkaline and Acid Treatments

	10% sodium chloride solution at room temperature, for about 65h	**0.5M acetic acid solution**
A first, preliminary attempt consisted of introducing a small amount of skin into 0.5M acetic acid (1/10 ratio) and storing it in the fridge, in order to establish whether this treatment allowed the removal of the epidermis, which was intensely coloured. After about 65 hours the skin was considerably soaked, the epidermis peeled off, but the one on the abdomen would partly tear upon cleaning, so the contact time with the acetic acid would have to be reduced.	The treatment performed in view of collagen extraction consisted of placing the skin in 10% sodium chloride solution at room temperature for about 65 hours in order to extract non-collagenic proteins and fat, while the salt solution became slightly coloured – in a yellowish beige – and also somewhat opalescent. After washing intensively using a tap water jet and after squeezing well, the skin was washed with distilled water until achieving full removal of chlorine ions (examination performed using a silver nitrate solution), then it was squeezed again and dabbed with filter paper. Following all these operations, the weight of the skin only increased by 3.4%, which indicates a very low level of soaking.	The skin thus treated was inserted into 0.5M acetic acid solution (1/5 ratio). For the next two hours it increased its volume very much and remained unsubmerged, and therefore more acetic acid solution was added, reaching a 1/6 skin/solution ratio.After 2 hours the epidermis would not peel off, only some of the scales. In 20 hours the skin soaked considerably, increasing its weight 2.52 times, while the epidermis peeled off quite easily. After cleaning the epidermis, which causes the loss of quite a large amount of gel from its opposite side, the skin was washed in the acetic acid solution in which the soaking was performed, in order to remove any remains of epidermis, then it was dabbed with filter paper, chopped and inserted into 100 ml of 0.5M acetic acid solution (skin/solution ratio 1/3.25). After 18 hours the mixture is very viscous and another 100 ml of solution was inserted – while stirring (skin/solution ratio 1/6.5).

The denaturation temperature for collagen extracted from anguilla skin was not identified in specialised literature, either, which is why the same manner was used as for brill skin to estimate that temperature, namely by visually observing the

disappearance of the gel, and hence that temperature is between 25 and 26°C.

V. The Rheological Behaviour of Gels

As shown in the introduction, the collagen extracted from fish skin has a 10-25°C lower denaturation temperature than that of the collagen extracted from terrestrial mammals, which creates difficulties for the extraction process and implies special equipment – a thermostat-controlled room or chamber – in order to perform the extraction and the rheological measurements in the case of most species, for temperatures about 20°C below room temperature. Besides, gels obtained from fish skin have a considerably more reduced viscosity.

Fig. 4 Anguillas (a) and anguilla skin from which the epidermis was partially removed (b)

In order that the triple-helix structure remains stable within these gels at room or human body temperature, they must be stabilized by reticulation.

Reticulation has been performed by using glutaric aldehyde at 4-6°C for different concentrations of collagen derived from all three species of fish.

The rheological behaviour was determined by using a Haake VT 550 rheo-viscometer with the following layout:

- S_3 (MV1) sensors system, d_1 and d_2 measuring ranges, shearing speed range, $\dot{\gamma}$, between 1.17 and
- 1872 s^{-1}. The measurements were initially performed at a 25 ± 0.1^0C temperature, with a thermostatic time of 20 minutes, and then – using ice – at 20 ± 0.1^0C.

In order to establish whether the dispersed systems show a time-dependant rheological behaviour or not, measurements were performed when increasing and decreasing the shearing speed values, which allowed the tracing of the hysteresis loops for the film-forming systems with time-dependant rheological behaviour. For systems which are more resistant to shearing, a second and even third cycle of shearing speed increase-decrease was performed. At the same time, measurements were performed in the strict order of shearing speed variation (increase or decrease), moving from one measuring field to the other, given that the two fields have interposing values (for d_1 the shearing speeds vary between 11.69 and 1170 s^{-1}, while for d_2 they vary between 1.17 and 1872 s^{-1}). If only the d_2 field – which has the largest shearing speed interval – had been used, then the differences between these speeds would have been too large at high shearing speeds.

The viscosity values taken into account are those resulted from the values obtained when increasing shearing speed, as the systems are damaged to a less extent under shearing stress.

In some cases, calculations were also made based on values obtained when decreasing shearing speed, or even when it was increased for a second time, but that was only to emphasize the time-dependent rheological behaviour, where applicable.

Figs. 5, 6 and 7 show the hysteresis loops for gels obtained from shark, brill and anguilla skin, respectively.

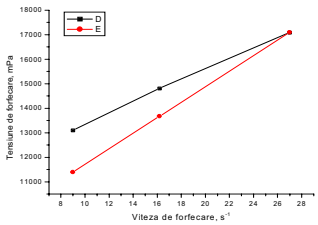

Fig. 5 Rheograms obtained for shark when: D – increasing; E – decreasing shearing speeds

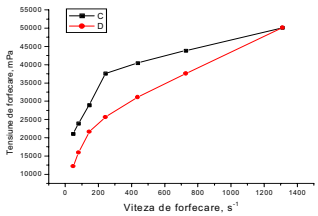

Fig. 6 Hysteresis loop obtained for gel extracted from brill skin when: C – increasing; D – decreasing shearing speeds

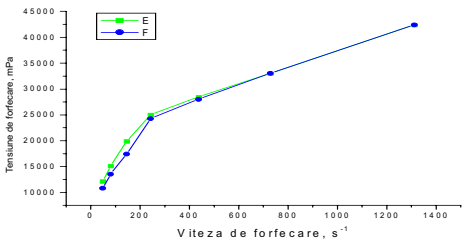

Fig. 7 The hysteresis loop for anguilla during the second cycle of shearing speed increase-decrease: E – increase; F – decrease

VI. Conclusion

To obtain good performance and type I non-denatured fibrillar collagen, the extraction of collagen from the skin of marine fish species employed, namely shark, brill and anguilla, must imply the following operations:

- removal of non-collagen substances by pre-treatment with sodium chloride solution, preferably 10%, at room temperature for 24 hours; simultaneously, it is degreased and deodorized;
- removal of salt by exhaustively washing it with tap water and distilled water until all chlorine ions have disappeared, at the same time as fat and most of the smell;
- treatment of pre-treated skin with 0.5M acetic acid solution for 24-48 hours to remove the epidermis, in the case of shark and anguilla skin, and the placoid scales, in the case of brill skin;
- extracting collagen from the cleaned and chopped skin, at 4-6^{o}C, by using 0.5M acetic acid solution, to prevent degradation;
- filtering the extracted collagen in order to separate it from skin remains either at the same temperature or by centrifugation at the temperature of the extraction process, if possible;
- storing the collagen in appropriate conditions, possibly preserving it by adding sodium nitride.

The conclusions we have reached confirm the fact that the gels based on collagen extracted from shark and brill skin have an ideal plastic behaviour, which allows their use for creating different pharmaceutical formulations.

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