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Contributions at the Biomaterials Base Development. The Collagen Hydrolyse from Sheep Skin, for Biomedical Applications

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Abstract

Collagens represent a large fibrous proteins family, with similar structure, founded in animals with protozoa exception. Because the biggest part from the mammal's resistance structure is made by collagen, the spectrum of collagen categories is large. Different types of collagen are necessary to give the biological features characteristics for different types of conjunctive tissues in the body. At least 15 types of collagen were isolated, with helixes of different length and unhelicoidal areas different in nature and dimension. In sheep, pig and cow skin and also with humans we identified a few types of collagen (type I, which is prevailing, type II, III and IV). The most important quantitative to produce the collagenic materials, in order to use them as biomaterials, is animal skin. About the biocompatibility of collagenic sheep biomaterials for humans, it seems that this one is better than the one of cow biomaterials. There are produces raw stuff for biomaterials like: collagen gels, collagen solutions and collagen hydrolyzed. This paper is about the hydrolyze process of gelatin sheep skin in acid and alkaline environments, on the whole pH area, at different temperatures and treatment durations, analyzing: the time of total hydrolyze in different work conditions, the relative viscosity [η_{rel}] and the average molecular mass of collagen hydrolyzated.

Keywords: biomaterials, collagen, medical bioengineering

Introduction

Collagen is a biomaterial with a wide utilization, because it is a natural product, with a low immunogenicity; that is why it is a normal constituent of the body, being preferred just like any foreign material. Collagen can be processed in different states, like: sheets, tubes, sponges, powders, veils, injectable solutions and dispersions, with applications in the medical practice [1,2]. Moreover, scientists are trying to produce it in order to use it for drugs created in different forms in ophthalmology, wounds, dressings for burns, and tumour treatment.

The most important quantitative source of raw material to produce collagen materials in order to use them as biomaterials is the animal skin. During the technological processes of

skin processing results, together with the final products, a large amount of refuses, which sometimes are over 50% from the weight of the crude skin used in fabrication [3]. Taking into consideration that collagen materials used as biomaterials represent a use of higher quality than the value of the tanned and finished leather, we can use for this purpose even green skins in their integrity. The animal skin is made mainly from derma (in a ratio of 95 – 98%), which has a collagen content of 60 –65%, reported to dry substance [3].

Any raw material used to produce biomaterials based on collagen must be homogenous. That is why, in order to obtain sponges, injectable solutions, films and so on, the homogenous raw material is prepared in the collagen material's most adequate form, like: collagen pastes, collagen solutions or hydrolysed collagen.

The hydrolysed collagen

Keeping the collagen at high temperatures, in water environment, with or without the addition of acids, bases or liotrope salts, its hydrolyse is made up to the peptides stage, and, by a treatment intensification, up to aminoacids [1,4,5].

The gelatine is at the superior limit of hydrolysed collagen, and it can be obtained by extraction or by the helix – ball transition of the collagen molecule. The helix – ball transition appears when we start from eucollagen, using liotrope agents, or in a thermal way [6,7,8]. In this way appears the disorder of triple helix and the helixes separation, manifested by a diminishing of solution's viscosity. The helix – ball transition under temperature influence is made between 26^0 and 35^0 C. The gelatine obtained by extraction has industrial importance [9,10], in practice many procedures being used.

The neutral hydrolyse, without chemical agents, leads usually to hydrolysed with a big molecular mass. The chemical hydrolyse of collagen is influenced by the following factors: the nature of the agent that catalyses the reaction, its concentration, the temperature and the treatment duration. The acids make a more intense hydrolyse than the bases, but also generate less modifications at the aminoacides level than the alkaline hydrolyse.

The collagen hydrolyse can also appear in the presence of some proteolysis enzymes. The enzymatic hydrolyse is influenced by the following factors: the enzyme's nature, its concentration, the optimal temperature and pH for enzyme's action, and the treatment duration.

The hydrolysed collagen is made from fragments of α-polypeptides chains of the collagen molecule. The average molecular mass vary from a few hundred up to 90.000 – 95.000, depending on the intensity of the hydrolyse process. They are poly-disperse solutions, with colloidal nature, these characteristics being more evident when the average molecular mass is bigger. The hydrolysed collagen acts in solutions as poly-electrolytes. These are very reactive substances, amphotere, with the isoelectric point very close to the proteins isoelectric point.

Due to the hydrolysed collagen chemical modification, their electrical load is modified; in this way they become available for multiple utilizations. The hydrolysed collagen solutions have properties of surface active agents, generated by their chemical structure, their average molecular mass, the environment pH, the presence of neutral salts, the changing of their reactivity [5,6] and so on.

We have identified many types of collagen in sheep, bovines, swine and also in human (type I, II, III, V).

In the current paper we have studied the process of hydrolyse of gelatine sheep skin, in acid and alkaline environment, on the whole pH scale, at different temperatures and treatment durations, observing: the duration of the total hydrolyse in different working conditions, the

dynamic viscosity (η), the relative viscosity (η_{rel}), the intrinsic viscosity [η] and the average molecular mass.

The relative viscosity represents the ratio between the hydrolysed solution dynamic viscosity (η) and the solvent dynamic viscosity (η_0).

To determine the average molecular mass we used the Mark – Houwink relation: $[\eta] = KM^{\alpha}$

The values used for K and α in order to calculate the molecular mass were: $K = 1.71 \cdot 10^{-3}$ and $\alpha = 0.5$.

Material and Methods

The technology for obtaining hydrolysed collagen from gelatine skin residues contains the same operations like the neutral, acid or alkaline hydrolyse, excepting the acids or bases additions from the acid or alkaline hydrolyse. The acid hydrolyse is usually made with HCl, and the alkaline hydrolyse is made with NaOH.

The scheme of a technological process of obtaining hydrolysed collagen from gelatine skin residues, using the acid hydrolyse, contains the following operations: the gelatine skin is broken in small pieces, of 5 x 5 cm, is washed with water, it is fully decalcified in order to eliminate the alkalinity and, especially, the CaOH – which is present in the skin from the liming operation; after that an acid increase is applied, until to a level of pH = 3.0 – 3.5, the next stage is the hydrolyse, in a pot of reaction at the desired temperature and duration, than there follows a decantation and the hydrolysed solution is neutralised, it is filtered, and it is finally dried by atomisation or it is used as it is obtained [3,4].

The study of collagen hydrolyse was made on the skin of grown-up sheep, from the category 60 dm^2, the Turcana breed; we have cut a sample of 50 cm length and 50 cm width, on the line of vertebral spine, starting from the skin's tail (25 cm in each part of the vertebral spine). The skins of Turcana breed are thicker, with a more compact structure, and the sample was cut from the most uniform area (from a histological point of view). 20 samples of sheep skin were processed as gelatine skin (unhairing, liming, fully decalcefiated and washed) [6]. The samples of gelatine skin were cut in pieces of 3 x 3 cm, and then they were mixed up, in order to obtain a homogenous material for the hydrolyse tests.

Results and Discussions

The chemical composition of the gelatine skin sheep, reported at 0% humidity, was as it follows:

Dermal substance (%)	91,85
Fat substances (%)	6,96
Cinders (%)	1,19
Nitrogen related to the gelatine skin without fat and umidity (%)	17,11

In the gelatine skin, together with the collagen other components also remained, which have a very small influence on the hydrolyse parameters; the hydrolysed still remains a composite, mostly collagen, which can be purified according to utilization needs, to keep only the collagen. We mention that during the hydrolyse, the fat substances pass into fat acids, which become solid at the hydrolysed surface, when the operation is finished, after the cooling process.

The hydrolyses were made in a reaction pot made from stainless steel, continuosly stirred. The meidum used was, in all the tests, of 200% reported to the weight of gelatine skin with 70% humidity.

We studied the influence of temperature, pH and hydrolyse duration.

We made hydrolyses at the following temperatures: 100^0, 120^0 and 135^0 C.

At the temperatures of 120^0 and 135^0C we have studied the influence of pH on the hydrolyse of gelatine sheep skin, making hydrolyses at the next pH values: 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13. The acid environment was made by adding HCl, and the alkaline environment was made by adding NaOH.

Each test of hydrolyse was made in 30, 45, 60, 90, 120 and 150 minutes.

We have determined for the solutions resulted from hydrolyse the dry residual, the dynamic viscosity, and we have calculated the intrinsic viscosity and the molecular weight. The dynamic viscosity was made using an Oswald viscometer, at the temperature of 20^0 C, the solutions being previously transformed to a pH = 6.5.

The hydrolyses made at the temperature of 100^0 C, between 30 and 150 minutes, have been eliminated, because the hydrolyse efficiency was small and the molecular mass, in all cases, was above 50.000.

The percentage variation of collagen passed in solution from the whole quantity of gelatine skin used in hydrolyse, at the temperatures of 120^0 and 135^0 C, depending on the time, to a pH = 6.5 –7.0, is given in **Table 1**.

Table 1. The percentage variation of collagen content to report at drying substance in the collagen hydrolyzed made at the different time and temperature, to a pH = 6,5 – 7,0.

Temperature	Duration , min					
(^{0}C)	30	45	60	90	120	150
120	67,8	76,0	89,5	95,7	99,6	100,0
135	77,5	88,3	92,7	97,8	100,0	100,0

We notice that, while the temperature increases until at 120^0 and 135^0 C and the hydrolyse duration also increases, the efficiency of collagen passed in solution increases until at 100 %.

We consider that the temperature of 120^0 C and the time of 90 – 120 minutes, at pH = 6.5 – 7.0 are the optimal hydrolyse parameters, according to the quantity of the collagen passed in solution.

At the temperatures of 120^0 and 135^0 C we studied the sheep gelatine skin hydrolyse duration as a function of environment pH (ph = 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13), at durations of 30, 45, 60, 90 120 and 150 minutes. The percentage variation of the collagen passed in solution as a function of these parameters is given in **Table 2**.

Table 2. The percentaje variation of drying substances content in the collagen hydrolyzed, depending on time and pH, at the temperature of 120^0 C

pH	Duration, min.					
	30	45	60	90	120	150
2	90,1	96,7	100,0	100,0	100,0	100,0

3	84,5	89,8	99,1	99,1	99,8	100,0
4	78,0	85,5	91,6	95,3	99,6	100,0
5	67,4	73,0	88,4	4,5	99,5	100,0
6	66,6	73,3	88,2	92,5	99,8	100,0
7	65,5	74,2	90,0	92,7	99,8	100,0
8	70,0	85,1	93,3	92,8	99,8	100,0
9	77,7	86,3	90,2	96,4	100,0	100,0
10	80,0	90,0	89,7	96,1	100,0	100,0
11	87,6	92,0	96,6	96,1	100,0	100,0
12	90,2	96,5	100,0	100,0	100,0	100,0
13	90,3	98,7	100,0	100,0	100,0	100,0

From the data presented in **Tables 2** and **3** we notice that, when the environment pH becomes more acid or more alkaline, the collagen hydrolyse is more intense, and its passing in solution is more accentuated. The collagen hydrolyse is faster and more intense at values of alkaline pH than at values of acid pH.

Table 3. The percentaje variation of drying substances content in the collagen hydrolyzed, depending on time and pH, at the temperature of 135^0 C

pH	Duration, min.					
	30	45	60	90	120	150
2	90,0	95,1	100,0	100,0	100,0	100,0
3	86,4	94,2	100,0	100,0	100,0	100,0
4	78,6	86,6	97,8	100,0	100,0	100,0
5	72,2	85,0	96,4	99,1	100,0	100,0
6	72,3	81,6	95,0	98,7	100,0	100,0
7	76,4	84,1	95,0	98,8	100,0	100,0
8	76,7	87,8	97,8	99,6	100,0	100,0
9	84,2	88,6	99,6	100,0	100,0	100,0
10	86,4	92,1	100,0	100,0	100,0	100,0
11	90,2	95,2	100,0	100,0	100,0	100,0
12	92,1	100,0	100,0	100,0	100,0	100,0
13	96,1	100,0	100,0	100,0	100,0	100,0

The dynamic viscosity of collagen hydrolysed solution is strongly influenced by the hydrolyse duration, the temperature and the reaction environment pH.

The curves in **Figures 1** and **2**, which represent the dynamic viscosity variation of solutions obtained by hydrolyse at 120^0 and 135^0 C, as a function of the reaction environment pH and the action duration, highlight the next phenomena:

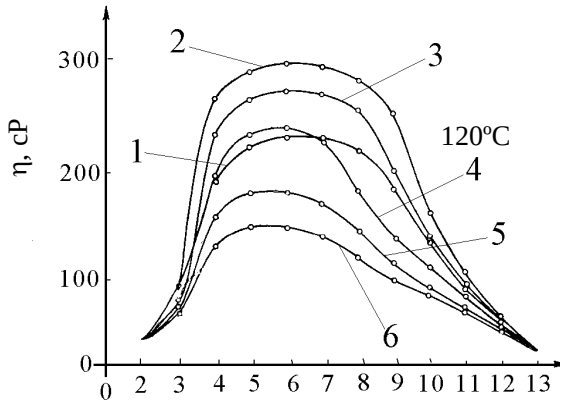

Figure 1. The dynamic viscosity variation of solutions obtained by hydrolyse at 120^0 C, as a function at the pH and the action duration. Action duration, min : 1 – 30; 2 – 45; 3 – 60; 4 – 90; 5 – 120; 6 – 150.

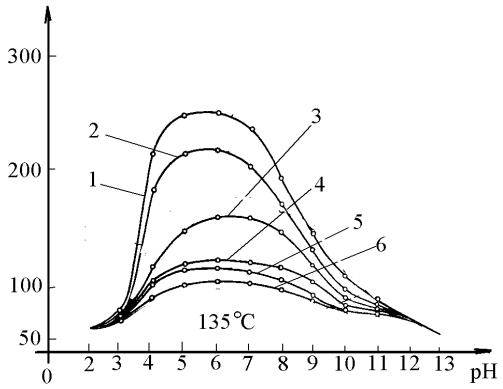

Figure 2. The dynamic viscosity variation of solutions obtained by hydrolyse at 135^0 C, as a function at the pH and the action duration. Action duration, min: 1 – 30; 2 – 45; 3 – 60; 4– 90; 5 – 120; 6 – 150.

After 30 and 45 minutes, the hydrolysed solution dynamic viscosity increases because their concentration grows, and then decreases, as long as the hydrolyse action advances, and also the molecular mass of hydrolyse product becomes smaller.

The dynamic viscosity of collagen hydrolysed solutions decreases as long as the temperature of hydrolyse grows.

The dynamic viscosity is bigger to an acid pH than to an alkaline pH, because the collagen hydrolyse is stronger in an alkaline environment.

The dynamic viscosity at pH = 2 is equal with 60 – 65 cP, then it grows slowly until at pH = 3, after that it grows strongly, until at different maximal values, between pH = 4 and pH = 8, depending by the treatment duration and the temperature. Finally the viscosity decreases slowly, until at pH = 13.

The curves of collagen hydrolysed solutions intrinsic viscosity, obtained at different Ph values and temperatures of 120^0 and 135^0 C have the same general shape as the dynamic viscosity (**Figures 3, 4**).

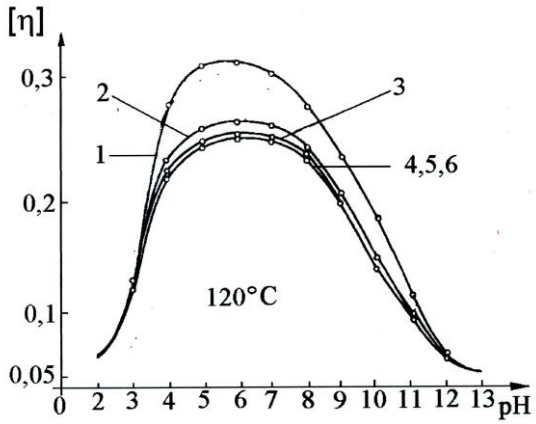

Figure 3. The intrinsic viscosity variation of collagen hydrolyzed solutions obtained at the temperature of 120^0 C, as function at the pH and the action duration.
Action duration, min: 1 – 30; 2 – 45; 3 – 60; 4 – 90; 5 – 120; 6 – 150.

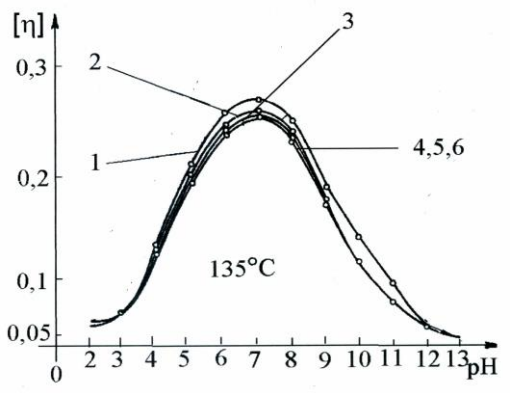

Figure 4. The intrinsic viscosity variation of collagen hydrolyzed solutions obtained at the temperature of 135^0 C, as function at the pH and the action duration.
Action duration, min:1 – 30; 2 – 45; 3 – 60; 4 – 90; 5 – 120; 6 – 150.

The averaged molecular mass of collagen hydrolysed solutions obtained at the temperatures of 120^0 and 135^0 C, the pH values between 2 and 13 and durations of 30, 45, 60, 90, 120 and 150 minutes, was calculated using the Mark – Houwink relation, and has values between 32.000 and 1.300 (**Figures 5, 6**).

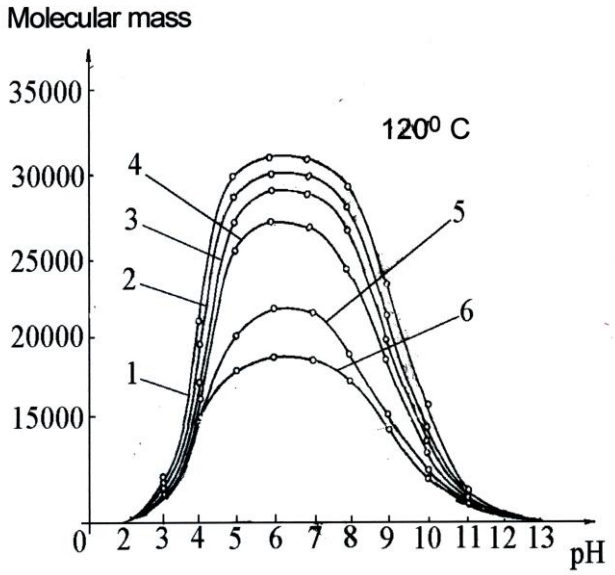

Figure 5. The average molecular mass variation of collagen hydrolyzed solutions obtained at the temperature of 120^0 C, as a function at the pH and the action duration.
Action duration, min: 1 – 30; 2 – 45; 3– 60; 4 – 90; 5 – 120; 6 – 150.

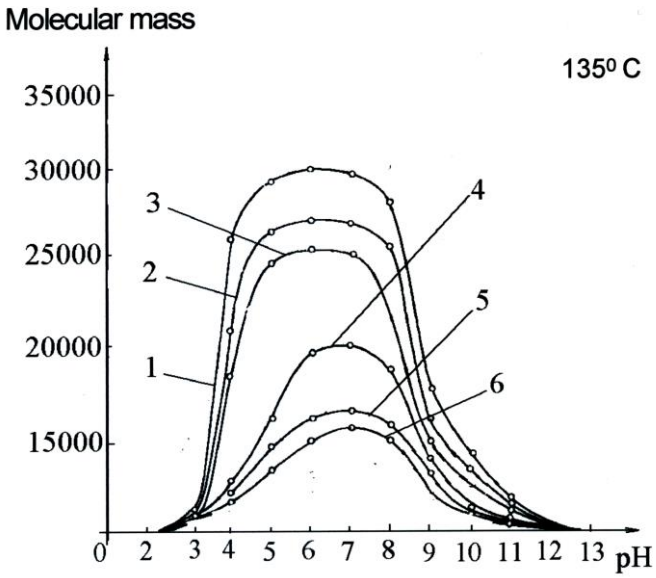

Figure 6. The average molecular mass variation of collagen hydrolyzed solutions obtained at the temperature of 135^0 C, as a function at the pH and the action duration.
Action duration, min: 1 – 30; 2 – 45; 3 – 60; 4 – 90; 5 – 120; 6 – 150.

From the presented data it follows that the collagen from sheep skins is less resistant to hydrolyse than the collagen from bovine skins.

Conclusions

1. Making the hydrolyse of gelatine skin sheep at the temperature of 100^0 C, we notice that after 5 –6 hours, only an amount of 30 – 35% collagen passes in solution, having an averaged molecular mass over 50.000.
2. At the temperature of 120^0 and 135^0 C and the pH = 6.5 – 7.0, the collagen hydrolyse is almost total after 1.5 – 2 hours.
3. The hydrolyse environment pH has a certain influence on the hydrolyse speed and on the characteristics of the obtained collagen hydrolysed solutions. The collagen hydrolyse is faster and more intense in an alkaline pH than in an acid pH.
4. As long as the hydrolyse duration is longer, and the temperature is higher, the viscosity of solutions and the averaged molecular mass of the hydrolysed collagen are smaller.
5. The averaged molecular mass of collagen hydrolysed in the studied working conditions varies between 30.000 – 32.000 and 1.300.

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