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Isolation of the Hyaluronic Acid from the Eggshell Membranes

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Abstract

Hyaluronic acid (HA), polysaccharide of egg shells, is a natural, linear polysaccharide made of repeating disaccharide units of D-glucuronic acid and N-acetyl glucosamine linked by β(1,4) and β(1,3) glucosidic bonds. It is occurring in human body, as an important part of the extracellular matrix of connective tissue. Hyaluronic acid is usable for the treatment of osteoarthritis. It could be used in gynaecology, dermatology, in rhinology where is successfully administered to minimise adverse side effects. Hyaluronic acid could be isolated from different natural materials, e. g. from cock´s combs, egg shells, liver of stingray Aetobatus narinari, or produced by microorganisms. The acid isolation from these materials is carried out by chemical and enzymatic methods. Chemical hydrolysis could be made in ethanol with added HCl, in solution of sodium acetate, etc. Enzymatic hydrolysis could be applied by using of different enzymes, as tryptase, papain, trypsin, pepsin. In our paper, we present the isolation of the hyaluronic acid from the eggshell membranes by enzymatic hydrolysis using pepsin. We tested and found specific conditions of hydrolysis: it progresses for five hours at 40°C with pH=3. The content of HA were tested spectrophotometrically by carbazole method. We determined approximately 5% HA per 1 g of dry matter. Eggshell membranes are a new natural resource, in which naturally occur glykosaminoglykan and proteins essential for maintaining healthy tissues. It is no longer true that the purified hyaluronic acid is usable solely for the treatment of osteoarthritis. They may be applied in gynecology, wound healing, dermatology, in rhinology where they are successfully administered to minimise adverse side effects.

Key words: hyaluronic acid, hydrolysis, pepsin

Introduction

Hyaluronic acid is a non-sulphated glycosaminoglycan composed of repeating units of β-(1,3)-D-glucuronic acid and β-(1,4)-N-acetyl-D- glucosamine in a ratio of 1:1 [1], present in the body of higher organisms [2]. It is characterised by a high molecular weight that depends on the way of isolation and the amount of originating starting material [3, 4, 5]. It is a biodegradable linear polysaccharide, which is an important component of the extracellular matrix of connective tissue and capillary walls. It is found mostly in connection with sodium as sodium hyaluronate. Measurement of particle size, molecular weight and intrinsic viscosity showed that the chains of hyaluronic acid in the solution are expanded into a random structure of a ball. Particle size of hyaluronic acid varies with the pH and salt concentration. Spiral structure of hyaluronic acid depends on the ion of opposite charge (counter-ion), pH, temperature and the degree of hydration [6]. It is a highly hydrophilic compound that binds water to form a viscoelastic solution [7].

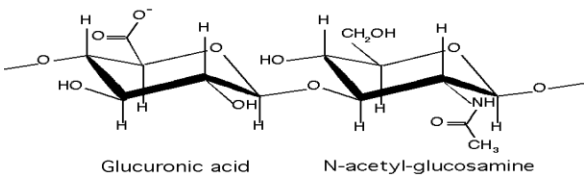

Fig. 1: Structure of hyaluronic acid

It is suitable for biomedical applications. Thanks to its properties it has found application in medical, cosmetic and clinical fields [4, 8].

The main biological functions of hyaluronic acid include supporting connective tissue viscoelasticity and manage their hydration. Its viscous solutions act as a lubricant in the moving parts of the body, e. g. joints [9, 10]. It was demonstrated that hyaluronic acid has also a certain role in the processes of tumours growth [11]. According to recent findings, the hyaluronic acid actively participates in immunological processes as a signal molecule and affects the mobility and the adhesiveness of cells in their proliferation and differentiation. Its insoluble form is used in medicine, for example as a matrix in drugs [12].

The shape and function of a number of anatomical structures in the body of higher animals depends precisely on the high molecular weight of hyaluronic acid, which is present in the extracellular matrix [13]. Long chains of hyaluronic acid effectively regulate immune processes, stimulate angiogenesis and initiate the synthesis of proinflammatory cytokines [2]. It promotes wound healing, has bacteriostatic and antiseptic properties, protects tissues against ingress of substances of high molecular weight, and regulates migration of fygocytes into inflammatory areas [9].

Commercially isolated hyaluronic acid is extracted from rooster combs, human umbilical cord, or by fermenting bacteria Streptococcus equi and Streptococcus zoopidemicus [8, 14, 15, 16, 17]. This polysaccharide was also isolated from other sources, such as the vitreous of the eye,

synovial fluid or skin. ÜNLÜER et al. [4] isolated hyaluronic acid by precipitation of a fisheye hexadecyltrimethylammonium bromide and an aqueous solution of ethanol.

Eggshell membrane is a bilayer insoluble membrane located between the shell and egg white. Membranes have a fibrous structure and exhibit antimicrobial and antibacterial effects. At present, the eggshell membranes are mainly used in cosmetics, medicine and in the pharmaceutical industry [18]. The developed technologies enable effective separation of the membranes from the shell, thus allowing isolation of active compounds from the membrane [19]. Eggshell membranes are rich sources of biologically active substances such as glucosamine, chondroitin sulphate, hyaluronic acid, collagen and glycoprotein's sulphate [19, 20, 21, 22, 23]. Amino acids, arginine, glutamic acid, methionine, valine, cysteine and proline are present to a greater extent in the proteins contained in eggshell membranes [22]. The membranes of eggshells are used as the biomembranes for the immobilization of urease, serve as a carrier matrix for the immobilization of the enzymes D - amine oxidase, catalase, glucose oxidase and tyrosinase [2, 20].

Obtaining hyaluronic acid from the membranes is carried out hydrolytically in two ways:

Enzymatically: hyaluronic acid is isolated from the biological material with the help of enzymes: tryptase [24, 25], papain [26], trypsin [27], pepsin [7, 25].

Chemically: hyaluronate can be isolated by gradually reducing the length of the chain, changing the pH [28], by ultrasound [7], by the action of sodium acetate [29], and ethanol with the addition of HCl [30, 31, 32].

In our work, we focus on testing conditions for enzymatic hydrolysis of hyaluronic acid from eggshell membranes. The basis for our work was selected outcomes by Zhao and Chi [27].

Materials and methods

As a starting material, we used dry eggshell membranes provided by Biomin a.s., Cífer. Hyaluronic acid was hydrolysed from dry, homogenized eggshell membrane by pepsin (EC 3.4.23.1, 800 FIP-U/g, M = 36000 g/mol) in universal Britton and Robinson buffer in the range of pH 2 to 5.

After homogenization, we hydrolysed eggshell membranes with an appropriate amount of buffer and the enzyme at a constant temperature. After the time of hydrolysis, we centrifuged solids and subsequently in hydrolysates we determined the concentration of hyaluronic acid. In this work we tested the course of hydrolysis at pH 2, 2.5, 3, 4 and 5, at 30°C, 37°C, and 40°C.

We also investigated the influence of hydrolysis time for extraction of hyaluronic acid. Hydrolysis was carried out in various periods of time: 3, 5 and 8 hours.

Hyaluronic acid concentration was determined by carbazole method of Rosa et al. [1]. The reaction is based on the action of carbazole reagents, by which is achieved the colour change of the reagent. Then, 2 ml of sulphuric acid containing borate (0.025 mM) was blended into 400 ml

of the studied sample. After 10 min of heating at 100°C followed by cooling, we added 100 ml of carbazole (0.125 %). After re-boiling (15 minutes at 100°C) and cooling, the absorbance was measured at ℨ = 525 nm and the content of HA was expressed in mg of HA per ml of hydrolyzate. As a standard, the sodium hyaluronate, with concentrations of 5-15 ɜg/cm3 was used.

Results and discussion

Extraction of biologically active substances from natural sources is an important process, because suitable conditions of extraction provide a qualitative and quantitative presence of the tested substance.

In the first phase of our work, we tested the effect of pH on the course of hydrolysis of hyaluronic acid from eggshell membranes. We tested the yield of hyaluronic acid at pH 2, 2.5, 3, 4, 5, at 37°C, using pepsin 80 FIP-U/g DM, for 5 hours (Fig. 2).

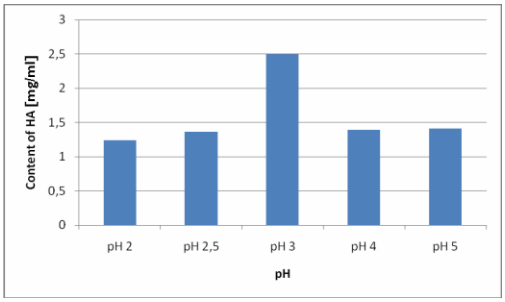

Fig. 2: Content of hyaluronic acid at different pH values of hydrolysis

From Fig 2 can be seen that the best results were obtained when hydrolysis was carried out at pH = 3, which is also consistent with Zhao and Chi [27], who also indicated as the optimal pH = 3. At pH = 2, we obtain by 49. 65% less hyaluronic acid, at pH = 4 by 55. 93% and at pH = 5 by 56.5% less hyaluronic acid.

In our further study we tested the influence of temperature of the hydrolysis on the hyaluronic acid content in the hydrolyzate. Hydrolysis was carried out at 30°C, 37°C and 40°C, other conditions of hydrolysis were the same pH = 3, reaction time of 5 hours, using pepsin 80 FIP-U/g DM (Fig. 3).

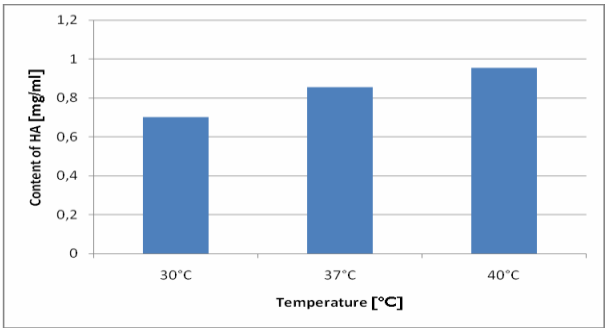

Fig 3: Content of hyaluronic acid at different temperatures of hydrolysis

Fig 3 shows hyaluronic acid content in the hydrolyzate at different temperatures, when the highest values of hyaluronic acid was determined by hydrolysis of pH = 3, hydrolysis time 5 hours, using pepsin 80 FIP-U/g DM at 40°C. Our findings are different from the indications given by Zhao and Chi [27], as in their experiments was proved to be the optimum temperature of 37°C. In our work, at the temperature 37°C we measured by 10.5% less and at 30°C by 26.43% less hyaluronic acid than at 40°C.

In experiments, in which we tested the effect of the amount of added enzyme on the yield of hyaluronic acid in hydrolyzate, we compared the effect of varying the amount of pepsin added to the reaction as follows, the 80 FIP-U/g, 40 FIP-U/g DM at 30°C, 37°C, 40°C, pH = 3, using 40 ml of buffer.

Tab 1: Content of hyaluronic acid by using different amounts of enzyme at different temperatures of hydrolysis

Temperature	**HA amount (mg/ml hydrolyzate)**	
	80 FIP-U/g DM	**40 FIP-U/g DM**
30°C	0.854	0.483
37°C	0.702	0.430
40°C	0.954	0.715

In the Tab 1 is shown a hyaluronic acid content by using various amounts of enzyme at different temperatures of hydrolysis. The highest content of hyaluronic acid was recorded at 40°C, 0.954 mg/ml and the amount of pepsin 80 FIP-U/g. At the same temperature with half the amount of enzyme, we obtained 0.715 mg HA/ml, what express, comparing the higher temperature, the decrease by 25.1%. Our findings differ from the findings of Zhao and Chi [27], who conducted experiments extraction of hyaluronic acid under similar conditions and give optimum hydrolysis temperature 37°C. On the contrary, in our experiments, at this temperature we obtained the smallest amount of hyaluronic acid. By the use of enzyme 80 FIP-U/g DM, we obtained 0.702 mg/ml, by using 40 FIP-U/g DM we received 0.430 mg/ml hyaluronic acid, what represents the decrease by of 26.5 % when using 80 FIP - U/g DM and by 55 % with 40 FIP-U/g DM of the enzyme at 37°C compared to the results obtained at 40°C.

In the next phase of the experiment we tested the amount of added buffer and its impact on the course of hydrolysis. We used different amounts of buffer: 40 ml, 30 ml and 20 ml buffer per 1 g of dry membrane.

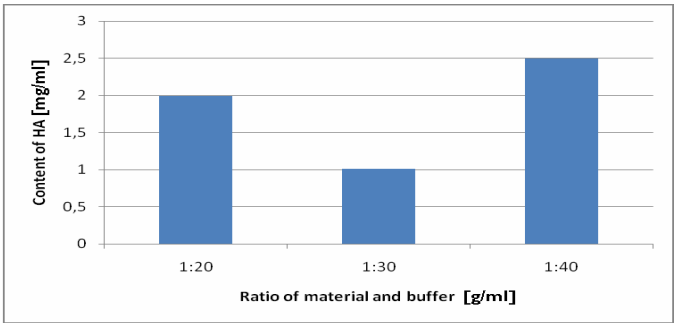

Fig 4: Contents of hyaluronic acid using varying amounts of buffer.

In Fig 4 shows the amount of hyaluronic acid obtained by hydrolysis by using varying amounts of buffer at 40°C, using an enzyme 80 FIP-U/g DM for 5 hours. These results show that the highest content of hyaluronic acid was obtained by using 40 ml buffer per 1 g of dry membrane. By using 20 ml of buffer per 1 g of dry membranes we obtained by 20.15 % less, and by using 30 ml buffer per 1 g of the membrane by 55.32 % less hyaluronic acid in comparison with the use of 40 ml buffer per 1 g of dry membrane.

In the last phase, we tested the effect of hydrolysis time on its course. Under standard conditions, temperature 40°C, pH = 3, and an amount of pepsin 80 FIP-U/g DM, we varied the time of hydrolysis. At 3, 5 and 8 hours, hydrolyzate samples were collected and compared the content of hyaluronic acid in them (Fig 5). After the expiry of the entire period of hydrolysis we were centrifuged the solid and hyaluronic acid was determined by standard procedures.

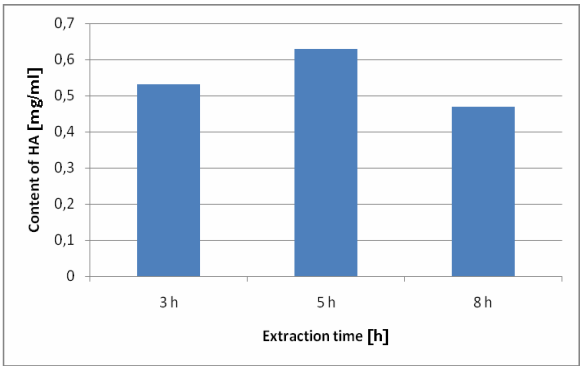

Fig 5: Content of hyaluronic acid during different times of hydrolysis

Fig 5 shows different results in various lengths of time of hydrolysis. The best results were achieved when the hydrolysis lasted for five hours. At 8 hours of hydrolysis hyaluronic acid content decreased.

Conclusion

The paper presents the results of pilot testing of hydrolysis of hyaluronic acid membranes of eggshells membranes as a potential source of biologically active substances.

For extraction of hyaluronic acid, pepsin was used (EC 3.4.23.1, 800 FIP-U/g, M = 36000 g/mol). Extraction was most effective at 40°C, pH = 3 and when hydrolysis was carried out 5 hours in universal buffer Britton and Robinson in an amount of 40 ml per 1g of eggshell membranes.

By pepsin hydrolysis of 80 FIP-U/g DM we obtained approximately 5% of hyaluronic acid from 1 gram of dry membrane, which represents 1.027 mg of hyaluronic acid per 1 g of dry membrane.

Zhao and Chi [27], who published the results of similar experiments, described the optimal hydrolysis conditions at 37°C for 5 hours of hydrolysis at pH 3, using the enzyme 11 000 U/g

DM and 40 ml buffer per 1 g of membranes. They determined average content of hyaluronic acid in eggshell membranes approximately 2.5% per gram of dry matter [27], which is slightly different from the results obtained by our team. It was confirmed that the membranes of eggshells are a potential source of biologically active substances such as hyaluronic acid and enzymatic hydrolysis is an effective method of isolation.

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