

# COMPARISON OF ENZYMATIC HYDROLYSIS OF POLYSACCHARIDES FROM EGGSHELLS MEMBRANES

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**Abstract:** AHyaluronic acid (HA) is part of the extracellular matrix of connective, epithelial and neural tissues, as well as the synovial fluid, skin, and cartilage. It is composed of repeating disaccharide units of D-glucuronic acid and N-acetyl glucosamine. Hyaluronic acid is used in abdominal surgery, ophthalmology, dermatology, rhinology; it is usable for the osteoarthritis treatment. The membranes of eggshell are a natural source of hyaluronic acid, collagen, glycosaminoglycan and collagenous proteins. In paper, we tested the possibility of extraction hyaluronic acid from the eggshell membranes by enzymatic hydrolysis. We identified optimal conditions of hydrolysis with trypsin at reaction temperature of 37 °C and pH 8; with pepsin at 40 °C and pH 3, as well as with papain at 60 °C and pH 7.5. The content of hyaluronic acid in samples was determined spectrophotometrically using the carbazole method. The experimental results showed a yield of ~ 4 -4.5 % hyaluronic acid per 1 g of dry eggshell membranes.

**Key words:** enzymatic hydrolysis, papain, pepsin, trypsin, hyaluronic acid

## 1. Introduction

Hyaluronic acid is a biodegradable linear polysaccharide, which is a component of connective tissue and capillary walls present in the body of higher organisms (ILLIÁS *et al.*, 2011). It is non-sulphated glycosaminoglycan. Hyaluronic acid is composed of alternating residues of  $\beta$ -(1, 3)-D-glucuronic acid and  $\beta$ -(1, 4)-N-acetyl-D-glucosamine via 1-3 and 1-4 bonds. The characteristic high molecular weight of its depended on natural material and the way of isolation (ROIG *et al.*, 2013; ÜNLÜER *et al.*, 2013; ŠLEZINGROVÁ *et al.*, 2012). It is a very hydrophilic compound that binds water and creates a viscoelastic solution (SLÍVA and MINARIK, 2009).

Hyaluronic acid, thanks to its properties, has found application in medicine, cosmetics and clinical praxis (ÜNLÜER *et al.*, 2013; COLLINS and BIRKINSHAW, 2013, KANCHANA *et al.*, 2013). The main biological function includes hydration connective tissue and supporting their viscoelasticity, it acts as a lubricant in the knees, hips (POLÁKOVÁ, 2012; PODŠKUBKA *et al.*, 2006). Hyaluronic acid has a certain role in the processes of tumours growth (LUKÁČOVÁ and LUKÁČ, 2008). As a signal molecule it participates in immunological processes. Long chains of hyaluronic acid effectively regulate immune processes, stimulate angiogenesis and initiate the synthesis of proinflammatory cytokines (ILLIÁS *et al.*, 2011). Its insoluble form can be used in medicine as a matrix in drugs (BENEŠOVÁ *et al.*, 2006).

It promotes wound healing, has bacteriostatic and antiseptic properties, protects tissues wound healing, has bacteriostatic and antiseptic properties, protects tissues against ingress of substances of high molecular weight, and regulates migration of fagocytes into inflammatory areas (POLÁKOVÁ, 2012).

Hyaluronic acid is extracted from rooster combs, fish tissues, human umbilical cord, or produced by fermenting *Streptococcus equi* and *S. zoopidemicus* (COLLINS and BIRKINSHAW, 2013; SADHASIVAM *et al.*, 2013; MURADO *et al.*, 2012; SARANRAJ *et al.*, 2011; PRICE *et al.*, 2007; GIRISH and KEMPARAJU, 2007). This polysaccharide was also isolated from the synovial fluid, skin or vitreous fluid of the eye (ÜNLÜER *et al.*, 2013).

Eggshell membranes have a fibrous structure. They are rich sources of biologically active substances such as hyaluronic acid, collagen, glucosamine, chondroitin sulphate, and glycoprotein's sulphate (KHANMOHAMMADI M. *et al.*, 2014; RUFF *et al.*, 2012; D'SOUZA *et al.*, 2013; RUFF *et al.*, 2009; ZHAO and CHI, 2009; ZHAO and CHI, 2008; NAKANO *et al.*, 2003). Extraction hyaluronic acid from the biological material is carried out enzymatically; hyaluronic acid is isolated from the tissues with enzymes, e.g. alcalase (PANAGOS *et al.*, 2014), tryptase (NAGYERY *et al.*, 2011; ZHAO and CHI, 2008), papain (SADHASIVAM *et al.*, 2013; PI *et al.*, 2011; VOLPI and MACCARI, 2003), trypsin (ZHAO *et al.*, 2008), pepsin (SLÍVA and MINÁRIK, 2009; ZHAO and CHI, 2009) or chemically; hyaluronate can be isolated changing the pH (TOMMERAAS and MELANDER, 2008) by ultrasound (SLÍVA and MINÁRIK, 2009), by sodium acetate (VÁZQUEZ *et al.*, 2013), and ethanol with the addition of hydrochloric acid (NIMPTSCH *et al.*, 2010; YU and GAO, 2007).

In our paper, we present the isolation of the hyaluronic acid from the eggshell membranes by enzymatic hydrolysis using pepsin, papain, and trypsin. The basis for our work was results published by ZHAO and CHI (2008) and PI *et al.* (2011).

## 2. Materials and Methods

Dry eggshell membranes, provided by Biomin a.s., Cífer, were used as a source of hyaluronate. Homogenized eggshell membranes were hydrolysed by pepsin (EC 3.4.23.1, 0.8 FIP-U/mg, AppliChem), trypsin (EC 3.4.21.4, 334 U/mg, Amresco) and papain (EC 3.4.22.2, 31523 USP U/mg, AppliChem) in universal Britton and Robinson buffer. We investigated the influence of pH, temperature and time of hydrolysis for extraction of hyaluronic acid, and ratio of material to fluid (of solid to liquid).

A centrifuged liquid after enzymatic hydrolysis was subsequently determined for the concentration of hyaluronic acid by carbazole method of ROSA *et al.* (2007). The method is based on the colour development due to the action of organic components, carbazole reagent, after the hydrolysis of hyaluronic acid with H<sub>2</sub>SO<sub>4</sub>. The absorbance was measured at  $\lambda = 525$  nm and the content of hyaluronic acid were expressed in mg of hyaluronic acid per gram of dry matters of eggshell membranes. Sodium hyaluronate was used as the standard.

### 3. Results and Discussion

The aim of work was extraction of hyaluronic acid from homogenized dry membranes of eggshell by enzymatic hydrolysis, using pepsin, trypsin and papain. We investigated the effect of pH, temperature, amount of enzymes, ratio of eggshell membranes and liquid and time of hydrolysis.

In the beginning, we tested the influence of pH level on the hydrolysis. Enzymatic hydrolyses were at 37 °C using enzymes for 5 hours in ratio of solid to liquid 1 : 40, amount of enzymes 100 mg/g DM of pepsin, and 1 mg/g DM of trypsin and papain. We tested conditions of enzymatic hydrolyse the eggshell membranes at pH 2; 2.5; 3; 4; 5, using pepsin (80 FIP-U/g DM); at pH 7; 7.5; 8; 8.5 using trypsin (33 U/g DM); and at pH 3.5; 4; 4.5; 5; 5.5; 6; 6.5; 7 and 7.5, using papain (3152 USP U/g DM).

The best results of pepsin hydrolysis were showed at pH 3; this finding consistent with work of ZHAO and CHI (2008), in which was the indicated pH 3 as the optimal for hydrolysis using pepsin. The content of hyaluronic acid was 34.91 mg/g DM. For hydrolysis by trypsin ZHAO and CHI (2008) indicated as the optimal pH 8.5, our results were different. The most hyaluronic acid we isolated by trypsin at pH 8. Amount of hyaluronic acid in trypsin hydrolysate at pH 8 were 40.13 mg/g DM, it was more, than in experiments of ZHAO and CHI (2008) at pH 8.5. At pH 7.5, we obtain the optimal condition for isolation of hyaluronic acid by papain; these results correspond with the best extracting condition at pH 7.3 in accordance PI *et al.* (2011).

In following experiments we proceed with investigation of the effect of reaction temperature on hydrolysis and yield of the hyaluronic acid in the hydrolysate. Hydrolysis was carried out 5 hours for all enzymes; amount of enzymes and ratio of solid to liquid were the same as in the previous experiment. Other conditions of hydrolysis using pepsin were pH 3, temperature at 30 °C, 37 °C, 40 °C and 50 °C, using trypsin were pH 8, temperature at 30 °C, 37 °C, and 40 °C, using papain were pH 7.5 and temperature at 40 °C, 50 °C, 60 °C and 70 °C (Fig. 1).

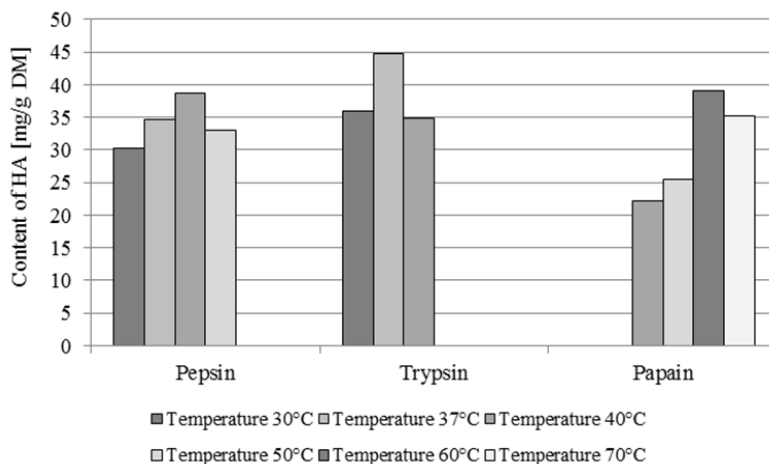


Fig. 1. Content of hyaluronic acid at different temperatures of hydrolysis by enzymes pepsin, trypsin and papain.

The content of hyaluronic acid in the hydrolysates at different reaction temperature shows Fig. 1. We obtained the optimal temperature 40 °C for hydrolysis by pepsin, at temperature 30 °C was the content of hyaluronic acid by 21 % less, and at 37 °C by 10.5 % less than at 40 °C. This results show different optimal temperature as in work by ZHAO and CHI (2008), who published optimal temperature 37 °C, using pepsin. The optimal temperature of trypsin was obtained at 37 °C, in opposite of ZHAO and CHI (2008) work, who obtained the optimal temperature for trypsin hydrolysis 50 °C. In our experiments content of hyaluronic acid at 40 °C decreased about 23 % in comparison with content at 37 °C.

The yielding rate of hyaluronic acid was 39.02 mg/g DM in hydrolysate at 60 °C using papain, this finding corresponded with VOPII and MACCARI (2003) work. The amount of hyaluronic acid at 50 °C, optimal temperature used by PI *et al.* (2011), was 34.5 % less. At the temperature 70 °C the yield of hyaluronic acid decreased about 9.5 %.

In further experiments, we observed the influence of the enzyme amount on the hydrolysis and content of hyaluronic acid in hydrolysate. We tested the effect of added enzymes in conditions as follows: pepsin the 80 FIP-U/g, 40 FIP-U/g at 40 °C, pH 3; trypsin the 17 U/g, 33 U/g, 50 U/g at 37 °C, pH 8; and papain the 1576 USP U/g, 3152 USP U/g, 9457 USP U/g, 15762 USP U/g at 60 °C, pH 7.5.

Table 1. Effect of amounts of enzyme on content of hyaluronic acid.

Enzyme and its activity		Hyaluronic acid [mg/g DM]
Pepsin	80 FIP-U/g	38.79
	40 FIP-U/g	29.70
Trypsin	17 U/g	38.45
	33 U/g	40.13
	50 U/g	44.83
Papain	1576 USP U/g	34.76
	3152 USP U/g	39.02
	9457 USP U/g	38.45
	15762 USP U/g	34.42

Table 1 shown yield of hyaluronic acid at optimal pH and temperature of hydrolysis by using various amounts of pepsin, trypsin and papain. The best result was proved after the addition of pepsin in amount 80 FIP-U/g, 38.79 mg/g. At the same conditions, with amount of pepsin 40 FIP-U/g was determined 29.07 mg hyaluronic acid per 1 g eggshell membranes. The yield rate, comparing with the double quantity of pepsin, was decreased by 25.1 %.

The best results of hydrolysis by trypsin were obtained at 50 U/g DM in other conditions: pH 8 at 37 °C. The content of hyaluronic acid was 44.82 mg/g DM. By the use of papain 3152 USP U/g DM, we received 39.02 mg/g DM. The content of hyaluronic acid by using 1576 USP U/g DM of papain was decreased of 11 %,

and using the amount of enzyme 15762 USP U/g DM was decreased by 12 % at the same temperature, pH and ratio of solid to liquid.

In following phase of the experiments, we followed the influence of amount of buffer added on hydrolysis, ratio of material-fluid (solid-liquid). We used amounts of universal Britton and Robinson buffer as follows: 20 mL, 30 mL and 40 mL per gram of the eggshell membranes at the best conditions from previous experiments.

The highest content of hyaluronic acid was obtained by using ratio of material-fluid 1 : 40, i.e. addition 40 mL buffer per gram of dry eggshell membranes. By the ratio of solid to liquid 1 : 20 we obtained by 20 % less hyaluronic acid in average, and by using ratio of solid to liquid 1 : 30 was yield of hyaluronic acid by 45 % less in comparison with the use of ratio of solid to liquid 1 : 40.

In the next, we tested the impact of hydrolysis time on yield of hyaluronic acid. The conditions of experiments were under optimal pH, temperature, ratio of solid to liquid, and amount of enzymes. The hydrolysis time was varied: three, five and eight hours. After the time of hydrolysis we compared the content of hyaluronic acid in samples by standard procedures (Fig. 2).

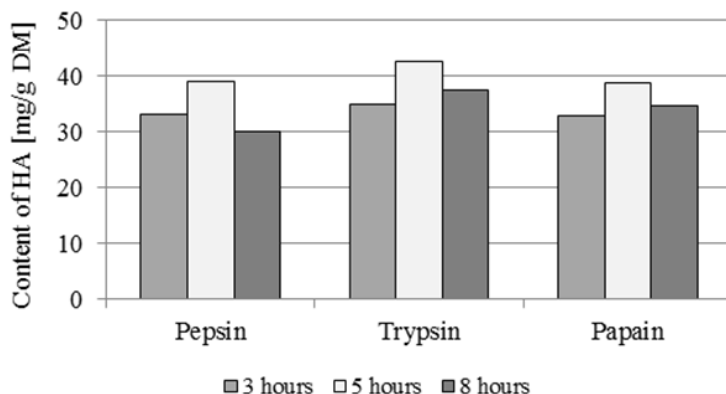


Fig. 2. Effect of hydrolysis time on content of hyaluronic acid.

Fig. 2 shows results in various time of hydrolysis. The highest yield of hyaluronic acid we received at time of hydrolysis 5 hours, in the times of hydrolysis 3 and 8 hours was the content by 11 – 23 % less.

The best extracting conditions of hyaluronic acid from eggshell membranes were followed: time of hydrolysis 5 hours, ratio of solid to liquid 1 : 40 generally. Other optimal conditions for hydrolysis were: amount of pepsin 80 FIP-U/g DM, pH 3 at temperature 40 °C; pH 8, temperature 37 °C, and amount of enzyme 50 U/g DM using trypsin; and pH 7.5, temperature 60 °C, and amount of enzyme 3152 USP U/g DM using papain.

In the final experiment, we compared effectiveness of hydrolysis by different enzymes at the standard, optimized parameters. Fig. 3 shows the highest yield of hyaluronic acid using trypsin, 42.82 mg/g DM. The content of hyaluronic acid in hydrolysate by other enzymes was about the same, 13 % for papain or 13.5 %

for pepsin less. In generally the yielding rate of hyaluronic acid was higher in all hydrolysis, minimal 38.79 mg/g DM, than reported ZHAO and CHI (2008), by trypsin 13.29 mg/g DM, by pepsin 24.49 mg/g DM or and PI *et al.* (2011) 12.2 mg/g DM by papain.

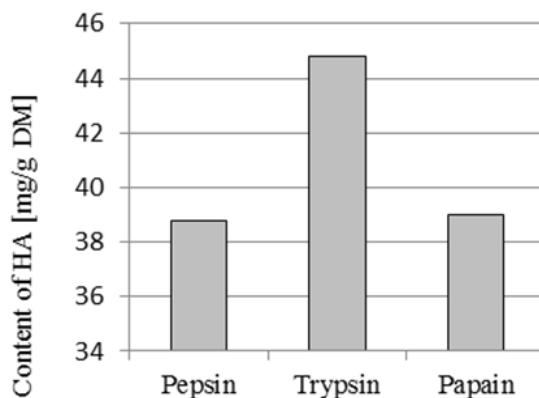


Fig. 3. Comparison of content of hyaluronic acid using pepsin, trypsin and papain.

#### 4. Conclusions

The paper presents the results of extraction of the hyaluronic acid from the membranes of eggshell using various enzymes.

For isolation of hyaluronic acid, enzymes pepsin, trypsin, and papain were used. Extraction in all cases was most effective in ratio of solid (eggshell membranes) to liquid (buffer) 1 : 40; time of hydrolysis 5 hours. The best hydrolysing conditions of hyaluronic acid were as followed: amount of pepsin 80 FIP-U/g DM, reaction temperature 40 °C, pH 3. Under the condition, the hyaluronic acid yielding rate reached 38.79 mg/g DM eggshell membranes. Hydrolyse of eggshell membranes by papain shows the highest amount of hyaluronic acid under conditions: amount of papain 3152 USP U/g DM, reaction temperature 60 °C and pH 7.5. Content of hyaluronic acid under these conditions was 39.02 mg/g DM. The optimal parameters of trypsin were obtained and shown as follows: amount of trypsin 50 U/g DM, reaction temperature 37 °C, pH 8. Therefore, the yield of hyaluronic acid was found to be 44.82 mg/g dry eggshell membranes. This indicated that effect of the yielding rate with trypsin was better than pepsin or papain.

In our work we obtained approximately 4 – 4.5 % of hyaluronic acid per gram of eggshell membranes by enzymes hydrolysis. This result are slightly different from the results obtained ZHAO and CHI (2008). They proved content of hyaluronic acid in one gram membranes of eggshell about 2.5 %. Alike PI *et al.* (2011) in their work describe yield of hyaluronic acid less, 1.2 %. It was shown that enzymatic hydrolysis of the eggshells membranes offers an opportunity to obtain biological active substances such as hyaluronic acid.

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