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Sequential optimization strategy for hyaluronic acid extraction from eggshell and its partial characterization

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ABSTRACT

The optimization of hyaluronic acid (HA) extraction from eggshell was studied by using a sequential experimentation approach. Using the results of factorial experiments, optimization experiments were carried out by response surface methodology. The experimental data as well as the validation tests showed the optimum performance when the extraction pH and time were bounded in the ranges of 3.4–3.5 and 3–3.4 days, respectively, at 9 °C. The purified HA was characterized by FTIR spectrum, harmonic mean size and average molecular weight. The study depicted the potential of eggshell as an alternative source for HA extraction to use in industry and medicine.

1. Introduction

Hyaluronic acid or hyaluronan (HA) is a linear, unbranched polysaccharide formed by repeating disaccharide units consisting of *N*-acetyl-D-glucosamine and D-glucuronic acid. The chain lengths of HA can reach up to 25,000 disaccharide units [1]. HA is usually found in human and animal tissues. It plays an important role both mechanical and transport purposes in the body in which it gives elasticity to the joints and rigidity to vertebrate disks. HA is also one of the major components in the synovial fluids of particular joints and the vitreous body of the eye [2,3]. The macromolecule has a significant influence on cellular behaviors such as growth, differentiation and migration [4]. HA has also an interesting and distinctive viscoelastic property, which is coupled with its lack of immunogenicity or toxicity, leading to a wide range of applications in the cosmetic and pharmaceutical industries such as skin moisturizers, adhesion prevention after abdominal surgery, wound healing osteoarthritis treatment and ophthalmic surgery [5].

HA is produced commercially from rooster combs as well as fermentation of group C *Streptococcus* as part of its outer capsule [6]. The HA content of rooster combs reaches about 0.75% (wet weight) with an average molecular weight in order of million Daltons [7,8]. Several repeated and complex purification procedures are needed to produce HA from the source of rooster comb, making its production tediously and costly. Besides, rooster comb-based HA products can be problematic to those who are allergic to avian products. Alternatively, HA can be produced through the fermentation process in which it is removed from the capsule surrounding *Streptococcus zooepidemicus* [9,10]. Fermentation-origin HA typically includes significant levels of toxins that cause inflammation when used in compositions for treating mammals. Therefore, it is important to develop another alternative source of HA that reduces the risk of pathogen contamination in HA production process.

Recently, the eggshell has attracted increasing attentions as a valuable source of HA due to the results obtained by few studies, which show the new source contains at least 0.5% (w) HA [8]. Eggshells of commercial hens are not efficiently utilized and are presently disposed as waste. Eggshell is about 10–11% of egg weight with an average weight of 5–6 g per eggshell [11]. For a city with one million people, with considering consumption of 1 egg per person, about 5 tons eggshells per day are discarded as waste. For example, in Tabriz city located in northwest of Iran with more than one million population and several egg processing factories, a lot of eggshells are discarded as waste. Eggshells, therefore, are widely available both at a low cost and in a large amount.

In this work, extraction performance of HA from the economical source of eggshell was investigated by the parameters of extraction strategy, temperature and extraction time using a full factorial

Abbreviations: HA, hyaluronic acid; FTIR, Fourier transform infrared spectroscopy; ES, extraction strategy; CCD, central composite design.

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experimental design. Extraction optimization was then carried out using information obtained from the factorial experiments by response surface methodology. The extracted HA was partially characterized after purification to evaluate its potential applications in industry and medicine. The objective of the study was to assess the possibility of use of the eggshell wastes as an alternative source for HA production.

2. Materials and methods

2.1. Materials

Commercial eggs were broken in half to obtain eggshells. The eggshells containing membranes were washed by dilute sodium chloride solution (0.1 M) and deionized water, respectively, and were dried at room temperature (25 °C). In order to facilitate suspension of HA in the solution, the particle size of source was reduced (30 mesh size) by using planetary mill (Pulverisette-Fritsch) prior to commencing the extraction process. All reagents used in this study were analytical grade (Sigma–Aldrich and Merck Inc.). The standard HA was hyaluronic acid sodium from human umbilical cord (H1879, FW: 66 kDa, Sigma–Aldrich).

2.2. Experimental set-up and procedure

The milled eggshells were treated by acetic acid to extract HA at different experimental conditions. At first, a 350 mL water-jacketed contactor was used with an internal diameter (id) of 64 mm equipped by a chilled water circulator (Model: UC5000, Sahand Azar Co.) and an on/off pH controller (Model 3611, Jenco Instruments Inc.). The contactor was placed on a magnetic stirrer (IKA) for the extraction process and the HA was extracted at 200 rpm. To control pH of the extraction in the desired value, acetic acid solution (8 M) was added to the contactor by a peristaltic pump (Roller Pump RP-1000, Eyela). At start of each run, 15 mL acetic acid (4 M) was added to 15 g milled eggshells and the experiments were carried out according to the experimental design. The extracted HA was then precipitated and purified as described elsewhere with some modification [9]. Briefly, the crude HA solution was precipitated with equal volume of isopropanol. The precipitate separated by centrifugation at 18,000 × *g* for 20 min at 4 °C, was suspended in 1 L of 3% sodium acetate using a mechanical homogenizer. Purification step was then followed with silica gel at 2% final concentration as well as activated charcoal for separation of impurities such as proteins. The salt solution of HA was separated from the silica gel and activated charcoal by centrifugation at 20,000 × *g* for 20 min at 4 °C. Finally, the purified solution was filtered through 0.45 μm and later through 0.2 μm membrane aseptically and was lyophilized to obtain purified product. The purified HA salt was then used for the characterization step.

2.3. Experimental design

To carry out the experiments of the HA extraction, at first, a three-factor randomized full factorial design with three replications was used. Extraction strategy (ES), temperature (*T*) and extraction time (*t*) were considered as three main factors. Two levels for the extraction strategy (without pH control, with pH control at 3.5), three levels for temperature (4, 9 and 14 °C) and four levels for extraction time (1, 2, 4 and 6 days) were selected. In the case of without pH control strategy, the eggshells and acetic acid 1 (w/v) were agitated and the extraction process was let continue until the initial pH of 2 reached to 5.5. The acidic hyaluronate suspension was then centrifuged at 10,000 × *g* and 4 °C for 20 min. To maximize the HA extraction, the eggshells were contacted with multiple aliquots of the fresh acid. The clarified acidic hyaluronate solutions (by centrifugation) were then combined and analyzed to evaluate the extraction performance. In the case of with pH control, the pH value of extraction process was controlled near 3.5 with adding the concentrate acetic acid under the on/off pH control system. The experiments were carried out randomly based on the experimental design and the extraction performance was monitored in term of the extracted HA. The *F*-test analysis of variance with a 95% confidence interval was then used to evaluate statistically the effect of main factors and their interactions on the values obtained for the extracted HA as a typical output of the system.

A central composite design (CCD) with five replicates of center point was then used to optimize the extracted HA based on the results revealed from the factorial experiments. The *F*-test analysis of variance with the 95% confidence interval was also used to evaluate statistically the regression significance. The analysis of variance and estimation of the parameters was carried out by using MINITAB 14 software. Validation experiments at the obtained optimum condition were carried out by higher experimental scales using higher amounts of milled eggshells (up to 100 g) as well as water-jacketed contactors with volumes of 900 mL (id = 88 mm) and 1500 mL (id = 104 mm).

2.4. Analytical methods

HA in the taken samples was routinely estimated by the carbazole assay measuring uronic acid [12]. The assay detects the glucuronic acid released after the hydrolysis of HA with H_2SO_4. The absorbance of samples was determined by UV–vis spectrophotometer (Bio Quest, CE2501). Protein and nucleic acid content of the purified HA solution were also determined by measuring absorbance at 280 nm and 260 nm spectrophotometrically. Temperature and pH values were measured online as described in Section 2.2.

FTIR spectra of samples were obtained by using a FTIR spectrophotometer (Unicam, 4600). Size distribution of polysaccharides was measured using a Nanoparticle sizing instrument (liquid refractive index = 1.33) equipped with the intelligent evaluation software LaPass (Analysette 22 Nano Tec, Fritsch, GmbH). Intrinsic viscosity of samples was measured by an Ubbelohde viscometer. The relation of average molecular weight of samples with intrinsic viscosity was calculated according to the Mark–Houwink equation, as described elsewhere [13].

In the statistical data analysis, main factors and interactions with a *P*-value less than 0.05 were considered as important and effective parameters on the output variable of the system.

3. Results and discussion

3.1. Effect of strategy, temperature and time on HA extraction performance

To evaluate the influence of operating parameters on the performance of HA extraction process, the first stage of experiments was carried out by using theparameters of ES, *T* and *t* according to the chosen experimental design, as shown in Fig. 1. As illustrated, the extracted HA value (mg/g eggshells) showed a considerable increase (about three times) when the pH control strategy was used in the extraction process regardless of temperature and extraction time. This magnitude of increase in the extraction performance was not observed when the other main factors of *T* and *t* changed based on the experimental design. Temperature changes did not show a noticeable influence on the extracted HA in the ES of without pH control, while the extraction performance fluctuated slightly with increasing *T* with a maximum value of 5.3 mg HA/g eggshell at 9 °C in the ES of pH control. The extraction performance for the both strategies showed a similar