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A simple method for the production of low molecular weight hyaluronan by *in situ* degradation in fermentation broth

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Abstract: Fermentation of hyaluronan (HA) by *Streptococcus zooepidemicus* was carried out in a 10-L fermentor. When the medium pH was controlled at 7.0 and the temperature was maintained at 38°C for 12 h followed by 35°C for 8 h, the yield of HA was 4.83 g/L with a molecular weight of 1,890 kDa. After the cells were removed by centrifugation from the fermentation broth, HA was slowly degraded to low molecular weight HA by hyaluronidase at a suitable temperature without a decrease in HA concentration. If the time and temperature for enzymatic degradation were controlled, the desired low molecular weight HA could be obtained by *in situ* degradation in the fermentation broth. The method does not require the addition of exogenous hyaluronidase, and is a simple way to produce low molecular weight HA.

Keywords: biopolymer; hyaluronan; low molecular weight; enzymatic degradation; hyaluronidase

1 Introduction

Hyaluronan (HA) has a wide range of applications in the fields of medicine and cosmetics, including osteoarthritis treatment, ophthalmic surgery, plastic surgery, drug delivery, skin moisturizers, and wound healing (1,2). Depending on its molecular weight, HA may be used in different applications. HA with high molecular weight (> 2,000 kDa) is usually applied in the pharmaceutical field while HA with low molecular weight (< 1,000 kDa) is generally applied in the cosmetics and food industries (3). Generally, the molecular weight of HA extracted from rooster comb can be as high as 5,000-6,000 kDa, and is between 500 and 2,000 kDa from microbial fermentation (4). In recent years, the demand for low molecular weight HA has increased because of its widespread use in cosmetics.

At present, low molecular weight HA is primarily obtained by hydrolyzing macromolecular HA by physical, chemical, and enzymatic degradation. Physical degradation usually involves heating, mechanical shearing, γ-ray irradiation, ultrasonication and/or ultraviolet treatment. Chemical degradation agents are divided into those that function by acidic or alkaline hydrolysis and those that function by oxidant hydrolysis. Enzymatic degradation is mainly through the action of hyaluronidase (5). Generally, enzymatic degradation has advantages of high efficiency, specificity for substrates within a narrow range of molecular weights and mild reaction conditions. However, application of commercial hyaluronidase extracted from bovine testes (6) or recombinant leech hyaluronidase (7) for enzymatic production of low molecular weight HA has some disadvantages, such as a high price and a requirement for complex manipulation, which have limited its industrial applications (8).

Microbial fermentation has gradually become the main method of HA production (9,10). In the HA fermentation process, we observed that after the HA concentration reached a maximum value, if the cultivation time was extended longer, the concentration and molecular weight of HA both decreased because of the hyaluronidase originating from the bacterial cells. Other studies have found the same phenomenon (11-13). This supports the idea that preparation of low molecular weight HA can be achieved by hyaluronidase hydrolysis in fermentation broth. This method would not require

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additional hyaluronidase and thus, would avoid the need for costly enzyme in the production of low molecular weight HA.

2 Experimental

2.1 Microorganism and media

Streptococcus zooepidemicus GDMCC 60146 (stored at Guangdong Microbial Culture Center, Guangdong, China) was used in this study. It was obtained from the wild strain GIM 1.437 after UV and γ-ray mutation.

Fresh slants were cultured at 37°C for 12 h and used for inoculation. Seed culture medium contained 2.0 g/L glucose, 5.0 g/L beef extract, 5.0 g/L peptone, 2.0 g/L yeast extract, 2.5 g/L K_2HPO_4, and 1.5 g/L $MgSO_4 \cdot 7H_2O$ at pH 7.0. The fermentation medium contained 13.8 g/L glucose, 11.2 g/L beef extract, 10.4 g/L peptone, 4 g/L yeast extract, 4.5 g/L K_2HPO_4, 2.5 g/L $MgSO_4 \cdot 7H_2O$, and 0.5 g/L antifoaming agent.

2.2 Batch fermentation in a 10-L fermentor

A total of 120 mL of the seed culture was inoculated into a 10-L aeration-agitation type fermentor (Model FUS-10L, Shanghai Guoqiang Bioengineering Equipment Co., Ltd., Shanghai, China) containing 6.0 L of fermentation medium. Agitation was supplied by two four-bladed disk turbines at a speed of 300-500 r/min, and the aeration rate was 0.6-1.0 v/v·min. Dissolved oxygen saturation (DO) was maintained at ≥50% by adjusting the agitation speed or aeration rate. The pH was automatically controlled at 7.0 by adding a 2 mol/L NaOH solution. The temperature was maintained at 38°C for 12 h, and then was maintained at 35°C until the end of fermentation.

2.3 Determination of the concentration and molecular weight of HA

The fermentation broth was diluted with an equal volume of 0.1% (w/v) sodium dodecyl sulfate (SDS) for 10 min to free the capsular HA and then centrifuged at 10,000×*g* for 10 min to pellet the HA-free cells. The supernatant was used to estimate the concentration and molecular weight of HA. The pellet was washed with 0.85% NaCl solution twice and weighed after drying at 105°C.

HA in the supernatant was precipitated with five volumes of ethanol and incubated at 4°C for 1 h. The precipitate was collected by centrifugation at 5000×*g* for 10 min, and then re-dissolved in an equal volume of distilled water. These steps were repeated three times. The concentration of HA was measured using the modified carbazole method (14). The molecular weight of HA was determined according to the viscosity method developed by Laurent (15).

2.4 Measurement of hyaluronidase activity

Hyaluronidase activity was measured spectrophotometrically using a turbidity reduction assay (16). One unit of enzyme activity was defined as the amount of enzyme that reduced the absorbance by 0.1 at 600 nm in 30 min at 37°C, pH 7.0.

3 Results and discussion

3.1 Effects of temperature on HA fermentation

After *Streptococcus zooepidemicus* GDMCC 60146 was cultured in the fermentation medium at 38°C for 12 h, the temperature was lowered to 33°C, 34°C, 35°C, 36°C and 37°C. After cultivation continued for 8 h, the results of the concentration and molecular weight determination of HA are shown in Figure 1.

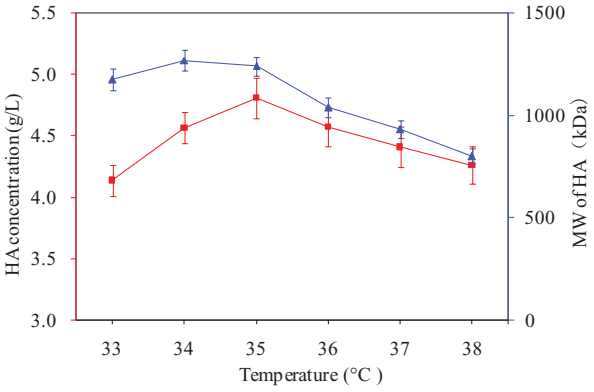

Figure 1: Changes in the concentration and molecular weight of hyaluronan when the temperature was lowered in the second stage of the fermentation. ■ HA concentration, ▲ molecular weight of HA. HA = hyaluronan; MW = molecular weight.

As shown in Figure 1, a high concentration and molecular weight of HA was obtained when the temperature of fermentation in the second stage was lowered to 35°C. If the fermentation temperature was kept constant at 38°C, the molecular weight of HA was generally less than 1,000 kDa, much lower than that obtained by varying the temperature of the fermentation. To obtain a high yield of HA with a relatively high molecular weight, isothermal fermentation temperature was preferred for the fermentation.

3.2 Effects of pH on HA fermentation

During the fermentation of HA, the medium pH drops quickly due to the synthesis of lactic acid, which is related to oxygen deprivation because of fast cell growth (13). Low pH is disadvantageous to the cell growth and HA synthesis. 2 mol/L NaOH was added to control the medium pH to 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0. The results of the concentration and molecular weight determination of HA under varying pH are shown in Figure 2.

As shown in Figure 2, low pH was not suitable to increase the concentration and molecular weight of HA, and high pH is not suitable to increase the concentration of HA, but suitable to increase the molecular weight of HA. The highest concentration and molecular weight of HA were obtained when the medium pH was controlled at 7.0. To obtain a high molecular weight HA, the optimal medium pH should be controlled at 7.0 throughout the entire fermentation process.

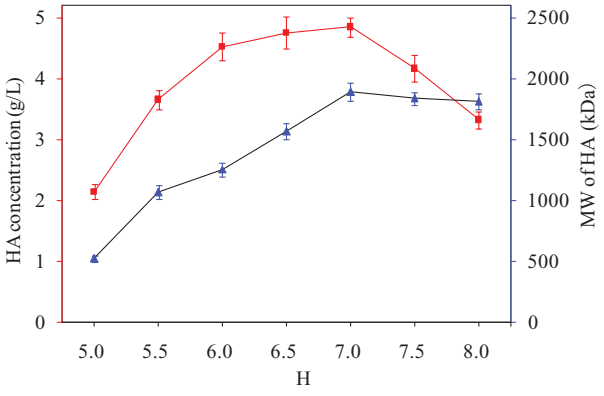

Figure 2: Changes in the concentration and molecular weight of hyaluronan when the medium pH was controlled at different pH values. ■ HA concentration, ▲ molecular weight of HA. HA = hyaluronan; MW = molecular weight.

3.3 Batch fermentation of HA in a 10-L fermentor

The results of batch fermentation of HA in a 10-L fermentor by GDMCC 60146 are shown in Figure 3. As illustrated in Figure 3, the cells started exponential growth after about 6 h of the lag phase and the biomass reached the highest value of 7.88 g/L at 18 h. After 20 h, the biomass began to decrease a little. The synthesis of HA was almost simultaneous with cell growth and its concentration reached the maximum at 20 h, about 2 h later than the time the maximum biomass appeared. Under a constant pH of 7.0 and isothermal conditions, 4.83 g/L of HA was obtained with an average molecular weight of 1,890 kDa in the batch fermentation carried out in 10-L fermentor.

Enzymatic measurement of the cell lysates and the culture supernatants revealed that 23.2 U/mL of the total activity was associated with the cell pellet, and 61.5 U/mL of the total activity was in the culture supernatant accounting for 27.4% and 72.6% of the total activity, respectively. The enzyme distribution was different from Allen's research (17), in which all of the hyaluronidase activity from *Streptococcus suis* was in the culture supernatant. These differences may be explained by the fact that different strains vary in the way they secrete enzymes.

3.4 Production of low molecular weight HA by hyaluronidase degradation

HA in the fermentation broth can be hydrolyzed by hyaluronidase from cells and the supernatant. The

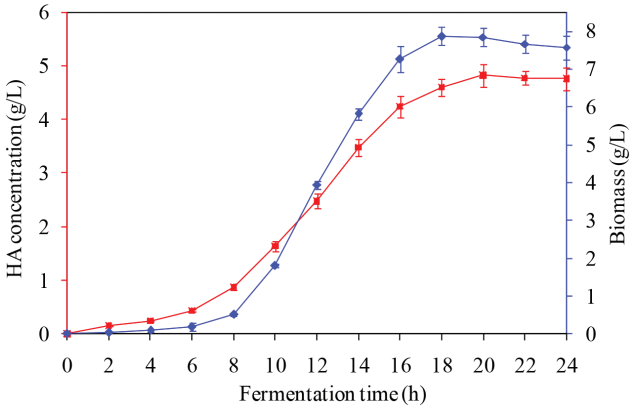

Figure 3: Time course of hyaluronan concentration and biomass in the batch fermentation of HA by *S. zooepidemicus* GDMCC 60146. ■ HA concentration, ◆ biomass. HA = hyaluronan.

concentration and molecular weight of HA in the fermentation broth containing cells and supernatant changed over time as shown in Figure 4. Both the concentration and molecular weight of HA in the fermentation broth containing cells decreased rapidly after incubation at 37°C. In contrast, after removing the cells by centrifugation, the hydrolysis rate of HA was much slower than that without centrifugation. Within 72 h, the concentration of HA in the supernatant did not significantly decrease, but the molecular weight of HA gradually decreased to a low of 125 kDa.

Although the hyaluronidase carried by the cells was only about one third of the total enzyme activity, the removal of cells not only removed some enzymes, but also stopped the regeneration of the enzyme when the cells were incubated in the nutrient containing fermentation broth at 37°C. The degradation of HA involves two steps. First, it is degraded into short-chain HA and then in the second step, it is degraded into monosaccharide units. Under the conditions of low enzyme activity and limited time, HA was not completely degraded, resulting in low molecular weight HA.

After centrifugation, the pH of the supernatant was about 7.0, and the activity of hyaluronidase was about 60 U/mL. Under this condition, temperature is the key factor that affects the rate of enzymatic degradation. As shown in Figure 5, with the decrease of temperature, the rate of HA degradation decreased accordingly. This suggested that choice of an appropriate low temperature for enzymatic degradation will allow the preparation of low molecular weight HA to be more controllable.

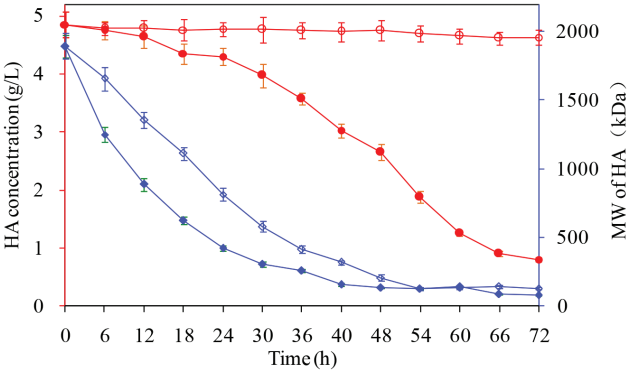

Figure 4: Changes in the concentration and molecular weight of hyaluronan. ● HA concentration in the fermentation broth with cells, ○ HA concentration in the fermentation broth without cells, ♦ molecular weight of HA in the fermentation broth with cells, ◊ molecular weight of HA in the fermentation broth without cells. HA = hyaluronan; MW = molecular weight.

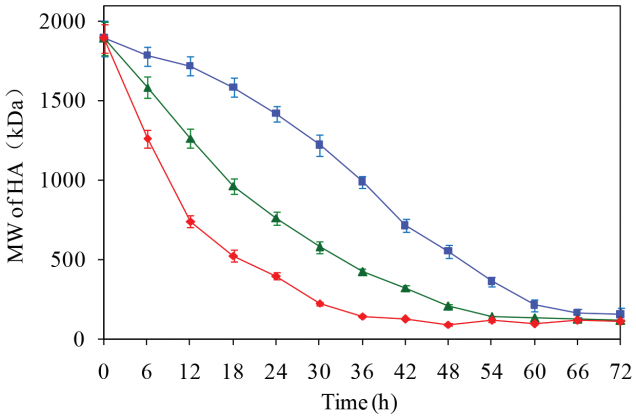

Figure 5: Changes in the molecular weight of hyaluronan in the fermentation supernatant incubated at different temperatures. ■ 34°C, ▲ 37°C, ♦ 40°C. HA = hyaluronan; MW = molecular weight.

4 Conclusions

In the batch fermentation, using a constant pH of 7.0, a temperature of 38°C for 12 h followed by a temperature of 35°C for 8 h, a high yield of HA with a relatively high molecular weight was obtained. After the cells were removed by centrifugation from the fermentation broth, the hyaluronidase activity in the supernatant was about 60 U/mL, and HA was slowly degraded to low molecular weight HA by hyaluronidase at a suitable temperature without a significant decrease of HA concentration. If the time and temperature for enzymatic degradation are controlled, the desired low molecular weight HA can be obtained by *in situ* degradation. The method does not require the exogenous addition of hyaluronidase and is a simple way to produce low molecular weight HA.

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