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Short communication

Mechanism for the effect of agitation on the molecular weight of hyaluronic acid produced by *Streptococcus zooepidemicus*

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ABSTRACT

Various views persist about the effect of agitation on hyaluronic acid (HA) molecular weight, and the mechanism remains unclear. In this paper, the effect of agitation on HA molecular weight was investigated. The HA molecular weight increased with impeller speed, due to a mass transfer enhancement; however, higher impeller speed caused a decrease in the HA molecular weight. According to the experimental results, it is proposed for the first time that reactive oxygen species (ROS) under higher impeller speed in the bioreactor, which were generated by NADH oxidase, could cause the reduction in the HA molecular weight. ROS generation increased remarkably during the exponential growth phase when the impeller speed increased from 450 rpm to 1000 rpm, and the NADH oxidase activity increased correspondingly. Finally, it was proved that the addition of salicylic acid could increase the HA molecular weight, but inhibit the efficiency of the HA synthesis.

1. Introduction

Hyaluronic acid (HA) is a uniformly repetitive, linear glycosaminoglycan composed of alternating β-1,4-glucuronic acid and β-1,3-N-acetylglucosamine. Due to its unique structure and physiological functions, HA is used as an additive in high-grade cosmetics and eye drops, medicines for ophthalmic surgery and arthritis (Kim, Park, & Kim, 2006), as well as a food supplement (Alkayali, 2006).

Traditionally, HA is extracted from rooster combs. In recent years, HA produced by fermentation of *Streptococcus zooepidemicus* is receiving increased attention, because the risk of cross-species viral and other adventitious agent infections can be avoided. Many investigations have focused on the optimisation of HA fermentation. Since the HA molecular weight, which reflects its physiological property, determines its application fields and commercial value, the factors influencing the HA molecular weight, such as pH, temperature, impeller speed and aeration rate, have been extensively studied (Armstrong & Johns, 1997; Gao et al., 2003; Kim et al., 1996, 2006). However, controversial views on the effect of impeller speed still exist. Gao et al. (2003) found that high impeller speed caused a decrease in the HA molecular weight. In contrast, Kim et al. (1996) observed that the HA molecular weight increased remarkably when impeller speed increased. In addition, Armstrong and Johns (1997) reported that impeller speed had little effect on the HA molecular weight.

In our previous work, we investigated the role of impeller speed over the time course of HA fermentation. The broth viscosity increased significantly, due to the accumulation of HA during the HA fermentation process, and thus higher impeller speed was required for good mixing and oxygen mass transfer in a stirred bioreactor. On the one hand, vigorous agitation favoured an increase in the HA molecular weight, due to good mass transfer and mixing; on the other hand, high shear stress caused a decrease in the HA molecular weight (Duan, Yang, Zhang, & Tan, 2008). Sahoo, Rao, and Suraishkumar (2006) observed that high shear stress resulted in an increase in reactive oxygen species (ROS) level in a Couette flow bioreactor. Fong Chong, Blank, Mclaughlin, and Nielsen (2005) proposed a possible mechanism in which the generation of ROS might lead to HA depolymerisation under aeration conditions, yet it has not been proved by experiments so far.

Accordingly, we were interested in whether ROS mediate HA degradation during the HA production process by *S. zooepidemicus*. In this paper, the effect of shear stress induced by agitation on the HA molecular weight, ROS generation and NADH oxidase activity were investigated in a stirred bioreactor. Subsequently, a possible mechanism was proposed based on the experimental results. Finally, the effect of oxygen radical scavengers on HA production was discussed, based on experimental results.

2. Materials and methods

2.1. Organism

S. zooepidemicus G1, a mutant of *S. zooepidemicus* ATCC 39920 induced by exposure to ultraviolet (UV) light and N-methyl-N′-nitro-N-nitrosoguanidine (NTG), was used as the source of HA.

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2.2. Inoculum and medium

The isolation of a pure culture was achieved by streaking onto agar plates that contained 2 g/l of glucose, 10 g/l of beef extract, 20 g/l of polypeptone, 5 g/l of yeast extract, 2 g/l of NaCl, 1 g/l of Na_2HPO_4, 0.12 g/l of KH_2PO_4, and 20 g/l of agar. The inoculum was prepared in a shaken 250-ml flask with 30 ml of the medium and incubated at 37 °C for 8 h. The medium consisted of 2 g/l of glucose, 10 g/l of beef extract, 20 g/l of polypeptone, 5 g/l of yeast extract, 2 g/l of NaCl, 1 g/l of Na_2HPO_4, and 0.12 g/l of KH_2PO_4.

2.3. Cultivation conditions

The fed batch culture experiments were performed in a 5-l fermentor (BIOREA-2000, Shanghai, China) with a working volume of 3 l, and the agitation provided by two 6-bladed turbines with 45°-pitched blades and one anchor. The medium composition was as follows: 20 g/l of polypeptone, 10 g/l of yeast extract, 2 g/l of NaCl, 1 g/l of $MgSO_4{\cdot}7H_2O$, and 2.5 g/l of K_2HPO_4, and the fermentor was inoculated with 5% (v/v) inoculum. The pH and temperature were 7 and 37 °C, respectively. The initial concentration of glucose was 40 g/l, and the 50% (w/v) glucose was added to maintain the concentration of glucose at 10 g/l. The dissolved oxygen level was controlled at 50% by mixing the inlet air with oxygen or nitrogen.

2.4. Analytical methods

The cell concentration was measured at 660 nm with a spectrophotometer. The glucose concentration was determined using the Miller method (Miller, 1959), the HA concentration determined using the Bitter-Muir method (Bitter & Muir, 1962), and the weight-average HA molecular weight measured using the Laurent method (Duan et al., 2008).

Reactive oxygen species (ROS) level was measured using 2′,7′-dichlorodihydro-fluorescein diacetate (H_2DCF-DA) (Fan, Cai, & Tan, 2007). Broth samples (1 ml; 1×10^7 cells) were centrifuged and treated with 5 μl of 10 μM H_2DCF-DA at 37 °C for 20 min. The fluorescence intensity was determined with excitation and emission wavelengths at 488 and 530 nm, respectively.

The activity of NADH oxidase was determined at 30 °C, according to the method provided by Fong Chong and Nielsen (2003). One unit of NADH oxidase activity was defined as 1 μmol of NADH oxidised per minute per milligram.

3. Results and discussion

3.1. Effect of impeller speed on HA molecular weight

The effect of impeller speed during HA fermentation was investigated in our previous work (Duan et al., 2008). The results showed that impeller speed had a significant impact on the HA molecular weight (Fig. 1).

The change in impeller speed was from 150 to 1000 rpm, with dissolved oxygen maintained at 50%. The HA molecular weight was only $(1.69 \pm 0.03) \times 10^6$ Da when impeller speed was 150 rpm, and this could be probably explained by the poor mixing and low oxygen mass transfer rate. The HA molecular weight reached its maximum value of $(2.01 \pm 0.05) \times 10^6$ Da at 450 rpm, whereas it decreased at both 700 rpm and 1000 rpm. Gao et al. (2003) considered that on the one hand, high impeller speed may cause the abnormal synthesis of HA and forced HA to shed into the medium prematurely; on the other hand, it may result in the breakage of the HA chains. The latter reason was dismissed in our previous work (Duan et al., 2008). However, the former reason could not be dismissed until now. In this study, it was speculated that the decreased HA molecular weight caused by agitation might be correlated to the generation of ROS.

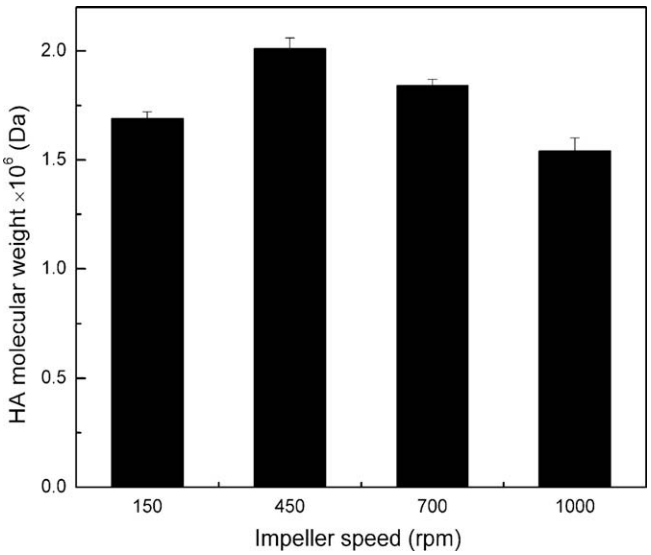

Fig. 1. Effect of impeller speed on HA molecular weight during HA fermentation.

3.2. Effect of impeller speed on the generation of ROS

ROS, which consist of superoxide anion radical ($O_2^{-\cdot}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\cdot}$), are generated during aerobic metabolism when O_2 is not completely reduced to water. It has been suggested that the reduction of high HA molecular weight is attributed to ROS (Grootveld et al., 1991). Subsequently, *in vitro* studies of HA degradation by ROS have been extensively carried out (Praest, Greiling, & Kock, 1997; Presti & Scott, 1994; Soltes et al., 2007; Stern, Kogan, Jedrzejas, & Soltes, 2007). Praest et al. (1997) found that the process of HA depolymerisation was accelerated with increasing concentration of ROS. Sahoo et al. (2006) observed that higher shear stress might cause an increase in ROS level. Similarly, shear stress induced by agitation in the bioreactor might also result in the accumulation of ROS and decrease of the HA molecular weight.

Fig. 2 shows the effect of impeller speed on the generation of ROS during HA fermentation. ROS generation increased remarkably at both 700 rpm and 1000 rpm at the exponential growth phase, where massive synthesis of HA started simultaneously (Duan et al., 2008). ROS reached maximum values at 6 h, and the values at 1000 and 700 rpm were about 18-fold and 9-fold higher than at 450 rpm, respectively. However, they decreased obviously after 6 h. It was indicated that the ROS level changed significantly with the fermentation time, and most ROS were generated during the exponential growth phase. Armstrong and Johns (1997) reported that agitation rate had no effect on the HA molecular weight with an impeller speed of 300 rpm for the first 12 h followed by 1000 rpm for a further 6 h (stationary phase). Our experimental results demonstrated that less ROS were generated during the stationary phase. Thus, it was inferred that the reason why the measured impeller speed had no effect on the HA molecular weight might be that the increase of impeller speed during the stationary phase could not cause the substantial generation of ROS (Armstrong & Johns, 1997).

In this study, it was concluded that both oxygen mass transfer and ROS generation affected the HA molecular weight. At low impeller speed, poor mixing and low oxygen mass transfer occurred, due to high broth viscosity. As impeller speed increased,

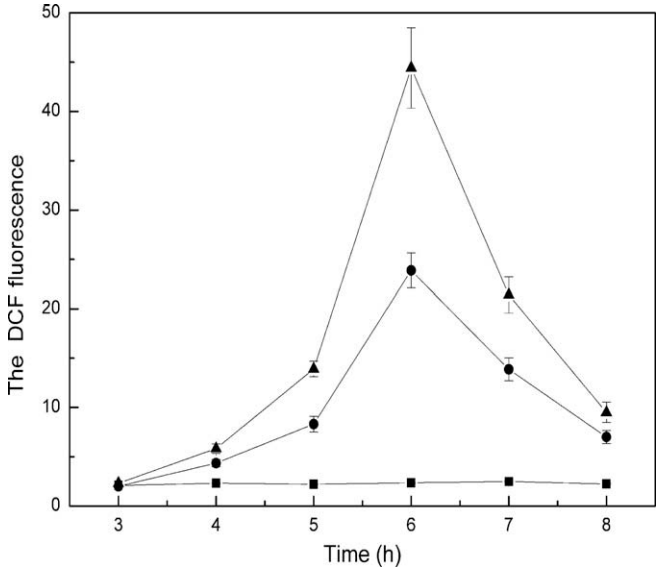

Fig. 2. Effect of impeller speed on the generation of ROS during HA fermentation. The levels of ROS were analyzed using H_2DCF-DA. (■): 450 rpm; (●): 700 rpm; (▲): 1000 rpm.

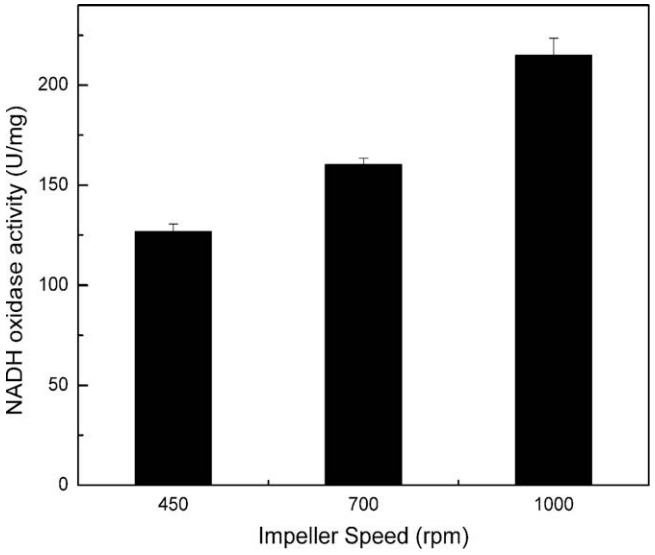

Fig. 3. Effect of impeller speed on NADH oxidase activity during the exponential growth phase (6 h) during HA fermentation.

mixing and mass transfer rate were improved; meanwhile, ROS generation increased correspondingly. Furthermore, the generation of ROS could be the primary reason for the decrease in the HA molecular weight under high impeller speed, and a similar phenomenon was observed by Sahoo et al. (2006). Hence a proper agitation was necessary to obtain a high molecular weight of HA, avoiding either uneven mixing or ROS generation.

3.3. Effect of impeller speed on the activity of NADH oxidase

Having establishing that high impeller speed leads to the degradation of HA molecular weight by the generation of ROS, we were interested in investigating the link between these two events. In *Streptococcus mutans*, it was found that ($O_2^{-\cdot}$) and H_2O_2 were generated by NADH oxidase (Thomas & Pera, 1983). Therefore, it was necessary to determine whether the activity of NADH oxidase was affected by impeller speed in a stirred bioreactor.

The NADH oxidase activities were measured at 6 h during the exponential phase at various impeller speeds, and the results are shown in Fig. 3. The activity of NADH oxidase was about 127 ± 3.8 U/mg when the impeller speed was 450 rpm. In contrast, as it increased up to 1000 rpm, the NADH oxidase activity increased to 215 ± 8.5 U/mg, which was 1.7-fold of that at 450 rpm. It was indicated that the NADH oxidase could be activated by agitation and more ROS were generated, which finally resulted in the depolymerisation of HA. Similar effects of ROS were also obtained by Sahoo et al. (2006).

In the present work, the correlation between high shear stress in a stirred bioreactor, the ROS level and NADH oxidase activity could be used to explain the descending trend in the HA molecular weight with increasing impeller speed. Consequently, an efficient method was needed to inhibit ROS generation or eliminate ROS, in order to avoid the negative effect of agitation on the HA molecular weight.

3.4. Effect of oxygen radical scavenge on HA production

Protection of HA against ROS has been achieved by antioxidants or oxygen radical scavengers. Cazzola, O'Regan, and Corsa (1995) reported that HA molecular weight increased with the addition of oxygen radical scavengers. Mendoza et al. (2007) observed that the higher antioxidant concentration, the lower the degradation of HA. However, little research has been focused on the effect of the addition of salicylic acid on biomass, HA yield, and HA molecular weight. Therefore, salicylic acid was used as an oxygen radical scavenger to eliminate ROS generation during HA fermentation.

Table 1
Effect of salicylic acid concentration during HA fermentation.

Salicylic acid concentration (g/l)	Biomass (gDW/l)	HA yield (gHA/l)	$Y_{HA/X}$ (gHA/gDW)	HA molecular weight ($\times 10^6$ Da)
0	2.60 ± 0.11	3.92 ± 0.20	1.51 ± 0.10	1.59 ± 0.09
0.1	2.64 ± 0.09	3.45 ± 0.24	1.31 ± 0.10	2.12 ± 0.13
0.3	2.53 ± 0.12	2.53 ± 0.18	1.00 ± 0.09	2.05 ± 0.10

The effect of salicylic acid was investigated at an impeller speed of 1000 rpm, and the results are summarised in Table 1. The results showed that the addition of salicylic acid had little effect on biomass because of the aerotolerant characteristics of *S. zooepidemicus* (Fong Chong et al., 2005). There was a decrease in the HA yield when the concentration of salicylic acid increased from 0 to 0.1 g/l, but the HA molecular weight increased remarkably from $(1.59 \pm 0.09) \times 10^6$ Da to $(2.12 \pm 0.13) \times 10^6$ Da. When the concentration of salicylic acid increased to 0.3 g/l, the HA yield decreased by 35.5% whereas the HA molecular weight was still $(2.05 \pm 0.10) \times 10^6$ Da.

The yield coefficient, $Y_{HA/X}$, was also investigated, and the results showed that $Y_{HA/X}$ decreased significantly as the concentration of salicylic acid increased. It was indicated that salicylic acid might inhibit the efficiency of the HA synthesis, despite the fact that it favoured an increase in the HA molecular weight. Therefore, it is necessary to optimise the addition of salicylic acid according to the requirement of the HA yield and molecular weight.

4. Conclusions

The mechanism for the effect of agitation on the HA molecular weight was investigated during HA fermentation by *S. zooepidemicus* in a stirred bioreactor. The results demonstrated that uneven

mixing resulted in a lower HA molecular weight at low impeller speed. However, high shear stress induced by agitation decreased the HA molecular weight. The shear stress could activate NADH oxidase and cause an increase in ROS level, which decreased the HA molecular weight. Moreover, salicylic acid was shown to effectively protect the HA molecular weight from ROS, but suppress the efficiency of the HA synthesis.

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