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Culture Conditions Affect the Molecular Weight Properties of Hyaluronic Acid Produced by *Streptococcus zooepidemicus*

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The effect of five culture variables on the molecular weight properties of hyaluronic acid (HA) produced by *Streptococcus zooepidemicus* was studied in batch culture with a complex medium containing glucose and 10 g of yeast extract per liter. Neither the culture pH (pH 6.3 to 8.0) nor the agitation speed (300 to 1,000 rpm) affected the weight-average molecular weight (M_w) of HA under anaerobic conditions at 37°C when 20 g of glucose per liter was used initially. M_w was in the narrow range of 1.5×10^6 to 2.3×10^6, and polydispersity (P) was between 1.8 and 2.5. When *S. zooepidemicus* was grown at lower temperatures or with aeration, higher-molecular-weight polymer and increased yields were observed. The polydispersity, however, remained unaffected. Anaerobically, the mean M_w (based on three samples taken within 4 h of glucose exhaustion) was $(2.40 \pm 0.10) \times 10^6$ and $(1.90 \pm 0.05) \times 10^6$ at 32 and 40°C respectively. Aeration of the culture at 1 vol/vol/min produced HA with mean M_w of $(2.65 \pm 0.05) \times 10^6$ compared with $(2.10 \pm 0.10) \times 10^6$ under equivalent anaerobic conditions. The initial glucose concentration had the most pronounced effect on polymer characteristics. Increasing this concentration from 20 to 40 g/liter produced HA with mean M_w of $(3.1 \pm 0.1) \times 10^6$ at 1-vol/vol/min aeration. The molecular weight of HA also exhibited time dependency, with smaller chains (M_w, ca. 2.5×10^6) detected early in the culture time course, rising to a maximum (M_w, 3.2×10^6) in the late exponential phase of growth. The mean polydispersity was also greater (2.7 ± 0.1) under these conditions. Replicate experiments performed under conditions resulting in the lowest (40°C, anaerobic) and highest (40 g of glucose per liter, 1-vol/vol/min aeration)-M_w polymer demonstrated excellent experimental reproducibility.

Hyaluronic acid (HA) is a high-molecular-weight linear polysaccharide composed of repeating units of D-glucuronic acid (GlcUA) and *N*-acetylglucosamine (GlcNAc) linked by β(1-3) and β(1-4) glycosidic bonds. The biopolymer is widespread in nature, having been identified in vertebrate soft tissues (e.g., skin, synovial fluid, and vitreous humor of the eye), cultured eukaryotic cell lines, and certain prokaryotes (21). In bacteria, particularly in Lancefield group A and C streptococci, HA is found in the form of a lavish extracellular capsule.

HA isolated and purified from a variety of sources is chemically identical and polydisperse, with a molecular weight typically in the range 10^4 to 10^7 (19). HA in aqueous solution is described as a semirigid wormlike polyelectrolyte (8). The molecule occupies a large hydrodynamic volume, and even at low concentrations (<1 mg/liter) chains are able to interact to form networks. These structural features, which are highly dependent on molecular weight, account for the viscoelastic rheology and ability of the polymer to retain large volumes of water and are important in determining the physiological functions of HA.

The most significant clinical applications of HA are in the areas of ophthalmology, orthopedics and wound healing. Emerging uses include drug delivery, coatings and implants, and therapeutics related to the ability of HA to modify cellular behavior (21). In many of these applications, product performance is dependent on the molecular weight of the biopolymer. Consequently, the molecular weight of HA is a primary criterion in patents describing HA production, which have been reviewed by Swann and Kuo (21). Therefore, it is critical that during the manufacture of HA, the molecular weight distribution, specifically the average molecular weight and polydispersity, be controlled.

Traditionally, most HA has been produced by extraction of animal tissues (22), although in recent years, attention has turned to the microbial route, where strains of group A and C streptococci are used. The bacterial process presents the opportunity to optimize the product yield and quality through genetic engineering and control of culture conditions. Several manufacturers claim to be able to manipulate the fermentation to obtain selected molecular weights (22), although, as one would expect, there is reluctance to identify the critical factors.

Batch culture is the most frequently reported HA culture method, producing a polymer with an average molecular weight typically in the range 1×10^6 to 2.5×10^6. Above this level, the molecular weight is considered high. The culture temperature is usually in the range 30 to 37°C, and the pH is 6.5 to 7.5. Most processes use glucose as the primary carbon and energy source, and commonly quoted concentrations fall in the range of 10 to 60 g/liter. Although these variables can be readily manipulated, little has been reported about the effects of temperature, pH, and glucose concentration on HA molecular weight in streptococcal culture.

The biopolymer can be produced anaerobically or aerobically, and it appears that the latter condition favors a higher molecular weight (2). The effect of agitation is unclear. The need for "vigorous" agitation is described (2), probably to enhance oxygen transfer, yet the polymer chain is reportedly susceptible to mechanical stress (3).

Although there is an abundance of literature about the process, conclusions about the parameters that influence the molecular weight of HA are not easily made for a number of reasons. First, the methods used to measure the molecular

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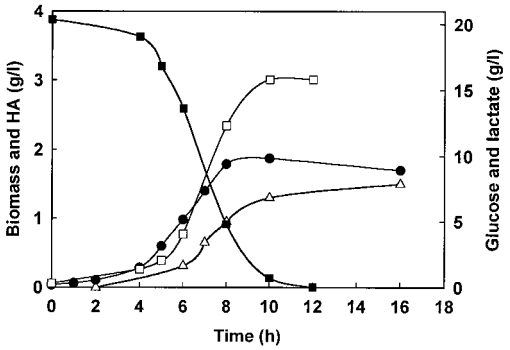

FIG. 1. Time course of a batch culture of *S. zooepidemicus* grown under standard conditions. Symbols: △, HA; ●, biomass; ■, glucose; □, lactate.

weight vary considerably (e.g., laser light scattering, intrinsic viscosity, size exclusion chromatography) and can lead to large inaccuracies if poorly applied. For example, size exclusion chromatography (SEC) has been used frequently for HA characterization but becomes inaccurate for molecular weights greater than 2×10^6 to 2.5×10^6 due to the limiting (small) pore size of available aqueous SEC media (1, 12, 16). Also, the use of non-HA polymer standards for column calibration is inadvisable because of differences in polymer shape and size (16). Despite these shortcomings, one recent report describes microbially derived HA in the range of 3×10^6 to 5×10^6 measured by SEC with pullulan calibration standards (11). A second difficulty is that indicators of measurement precision are often not published. Finally, there are few studies which have systematically considered appropriately wide operating ranges for the culture parameters in question. In this paper, we describe the effect of various culture conditions on the molecular weight and polydispersity of HA produced by a strain of *Streptococcus zooepidemicus* in batch culture.

MATERIALS AND METHODS

Organism. *Streptococcus equi* subsp. *zooepidemicus* ATCC 35246 HA^+ Lac^+ Em^s was obtained from the American Type Culture Collection (Rockville, Md.) as a freeze-dried culture in ampoules and maintained in the dark at 4°C. An original ampoule was cultured, and multiple ampoules were prepared from this stock. Throughout this paper, the bacterium will be referred to as *S. zooepidemicus.*

Inoculum and media. The contents of an ampoule were suspended in sterile reverse osmosis (RO) water, streaked onto sheep blood agar plates, and incubated for 16 h at 37°C (the same temperature was used for all subsequent incubations). Pure mucoid colonies were then transferred into 30 ml of M17-glucose broth and incubated for 3.5 h. The contents were added to 70 ml of VIG broth, and after a 3-h incubation, the culture was added to 250 ml of VIG broth in a 500-ml measuring cylinder. Once an optical density at 530 nm greater than 0.6 had been reached, typically after 3 to 4 h, 200 ml was used to inoculate the fermentor (13%, vol/vol). Lower inoculum volumes tend to result in less consistent fermentation outcomes.

SBA comprised tryptic soy broth (Difco) containing 5% (vol/vol) fresh sheep blood and was solidified with 1.8% (wt/vol) agar. M17-glucose broth contained the following (grams per liter of RO water): glucose, 5.0, polypeptone (BBL), 5.0; phytonpeptone (BBL), 5.0; yeast extract (Difco), 2.5; nutrient broth (BBL), 4.0; ascorbic acid, 0.5; and sodium glycerophosphate, 19.0. VIG medium comprised veal infusion broth (Difco) supplemented with 8 g of glucose per liter. The base medium contained the following (grams per liter of RO water): glucose, 20.0; yeast extract, 10.0; $Na_2HPO_4 \cdot 12H_2O$, 2.5; and $MgSO_4 \cdot 7H_2O$, 0.5. The media were sterilized at 121°C for 20 min. Glucose solutions were autoclaved separately and mixed aseptically with the other components on cooling.

Cultivation. Batch culture experiments were performed in a 2-liter fermentor (LH Fermentation, Stoke Poges, England) with a working volume of 1.5 liters. Agitation was provided by two six-bladed disk turbines. Automatic temperature and pH control were used, with the latter employing a steam-sterilizable glass pH probe (Ingold AG, Urdorf, Switzerland) and sterile 5 M NaOH. Standard fermentation conditions consisting of anaerobic culture, 20 g of glucose per liter (initially), pH 6.7, 37°C, and an impeller speed of 600 rpm are defined to describe the prevailing culture conditions apart from the parameters under study. No provision was made to flush the fermentor headspace with nonoxygen gases prior to anaerobic culture. Replicate experiments were performed under conditions which gave rise to the lowest- and highest-molecular-weight HA to assess experimental reproducibility.

Cell growth. The cell concentration was measured at 530 nm with a spectrophotometer (Hitachi, Tokyo, Japan). Culture samples were diluted in water to give an optical density (OD) of less than 1, and the OD was then multiplied by the dilution factor. Biomass (grams per liter) was determined from the OD by using a calibration curve.

Chemical analysis. The concentrations of glucose and lactate were determined by high-pressure liquid chromatography (HPLC), using the method of Johns and Stuart (10). The HA concentration was measured by HPLC as previously described (1), and streptococcal HA (Sigma H-9390) was used to prepare a standard curve of peak area against concentration. The data have a 95% confidence limit of ±5%. Molecular weight parameters were estimated by two methods due to range limitations. The weight-average molecular weight (M_w), polydispersity (P), and molecular weight distribution of HA were measured by size exclusion chromatography with an exponential Hamielec calibration. For samples with M_w greater than 2.4×10^6, intrinsic viscosity determinations were used, since this material eluted in the void volume of the HPLC-SEC column and could not be resolved. Single-point measurements were performed on diluted samples containing 3 to 10 μg of HA per ml in 0.1 M NaCl at pH 7.2. Intrinsic viscosities greater than 2,960 ml/g were multiplied by a factor of 1.247 to correct to zero shear. The Mark-Houwink constants were $k = 0.016$ and $a = 0.841$. Both methods are detailed elsewhere (1).

RESULTS

Effect of pH. Batch fermentations of *S. zooepidemicus* were performed under standard conditions, and the effect of pH was examined over the range of pH 6.3 to 8.0. This range encompasses pH values at which the HA production rate and yield are optimal (9). The results of a fermentation at pH 6.7 are shown in Fig. 1. Biomass grew rapidly ($\mu_{max} = 0.55 \pm 0.05\ h^{-1}$) to a maximum level at 10 h, which coincided with glucose depletion. HA and lactate were the only extracellular products detected at appreciable levels, which is usual for anaerobic *S. zooepidemicus* fermentations. The rise in HA concentration after glucose exhaustion was primarily due to shedding of the polysaccharide capsule from the cell. The effect of pH on growth and HA production was similar to that observed by Johns et al. (9).

There was no effect of pH on either the M_w (Fig. 2) or the polydispersity of HA produced. Values of M_w were in the range of 1.5×10^6 to 2.3×10^6, and polydispersity was between 1.8 and 2.5. M_w increased marginally during cell growth but was subsequently unchanged with time (except for the data at pH 6.7). A batch was also cultured at pH 5.5, but cell growth and HA production were very poor.

Effect of temperature. The effect of culture temperature was studied over the range from 32 to 40°C, and the results are summarized in Table 1. The values of M_w and polydispersity

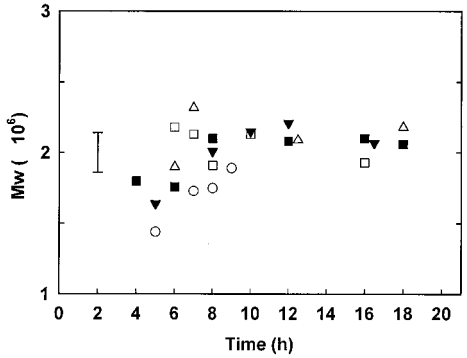

FIG. 2. Effect of culture pH on the M_w of HA produced by *S. zooepidemicus* in batch culture under standard conditions. Symbols: ○, pH 6.3; □, pH 6.7; ■, pH 7.1; △, pH 7.5; ▼, pH 8.0. The error bar represents a 95% confidence bound on an individual measurement.

TABLE 1. Effect of culture temperature on growth and HA production by *S. zooepidemicus*

Temp (°C)	μ_{max} (h^{-1})	Maximum HA concn (g/liter)	$M_{w_{mean}}$, 10^{6a}	$P_{mean}{}^{b}$	*n*
32	0.45 ± 0.15	1.60	2.40 ± 0.10	2.1 ± 0.2	3
35	0.65 ± 0.15	1.60	2.25 ± 0.05	2.1 ± 0.2	3
37	0.55 ± 0.05	1.45	2.10 ± 0.10	2.2 ± 0.1	4
40	0.80 ± 0.30	1.10	1.90 ± 0.05	2.3 ± 0.1	3
40[c]	0.75 ± 0.05	1.20	1.85 ± 0.05	2.3 ± 0.1	3

[a] Mean M_w (±2SE) of *n* samples.
[b] Mean polydispersity (±2SE) of *n* samples.
[c] Replicate experiment.

given represent the mean of measurements taken from a minimum of three samples obtained between 2 h prior to glucose exhaustion and up to 4 h afterwards. No significant change in the M_w was observed during this time interval. Temperature had a significant effect on the M_w of HA produced under standard conditions (Fig. 3). Lower culture temperatures favored higher M_ws, with a mean value of $(2.40 \pm 0.10) \times 10^6$ at 32°C. There was no significant dependence of M_w on the fermentation time during the growth phase, and the culture temperature and fermentation time had no clear effect on polydispersity.

The maximum HA concentration and bacterial specific growth rate were also temperature dependent. Cultures at the lower temperatures produced more of the biopolymer but grew more slowly than those at 40°C (Table 1). The replicated 40°C experiments demonstrated excellent reproducibility.

Effect of the agitation rate. A batch experiment was performed at 37°C and pH 6.7 with an impeller speed of 300 rpm for the first 12 h followed by 1,000 rpm for a further 6 h. Maximum concentrations of biomass (2.2 g/liter) and HA (1.35 g/liter) were attained at 6 and 12 h, respectively. The results of this experiment can be compared with those of the standard culture, for which an impeller speed of 600 rpm and a temperature of 37°C were used (Table 1). There was no significant difference in either the mean M_w ($[2.1 \pm 0.2] \times 10^6$; $n = 3$) or polydispersity (2.3 ± 0.1) of HA produced under the two agitation conditions. Time-related trends were also absent. In particular, the shift in speed from 300 to 1,000 rpm had no effect on the molecular weight characteristics of the polymer.

Effect of aeration. Batch experiments were performed under standard conditions but with aeration rates of 0.2 and 1.0 vol/vol/min. Aeration resulted in no change of the maximum specific growth rate of *S. zooepidemicus* but enhanced HA production (Table 2). At both levels of aeration, acetate was produced in addition to lactate and appeared during the mid-exponential phase, reaching concentrations of 1.4 and 1.7 g/liter for 0.2 and 1.0 vol/vol/min, respectively. In Fig. 4, the resulting HA M_w is compared with that from anaerobic culture under the same conditions. Aeration of cultures increased the M_w of the biopolymer, with average values of $(2.30 \pm 0.15) \times 10^6$ and $(2.65 \pm 0.05) \times 10^6$ for fermentations performed at aeration rates of 0.2 and 1 vol/vol/min, respectively. The HA produced showed no substantial variation in M_w with culture time.

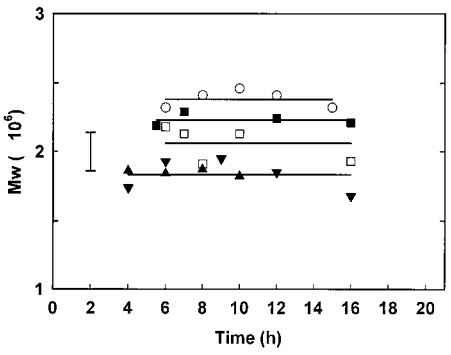

FIG. 3. Effect of culture temperature on the M_w of HA produced by *S. zooepidemicus* in batch culture under standard conditions. Symbols: ○, 32°C; ■, 35°C; □, 37°C; ▼ and ▲, 40°C replicates. The error bar is as in Fig. 2.

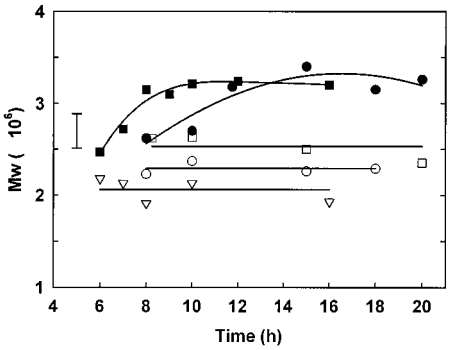

FIG. 4. Effect of aeration and initial glucose concentration on the M_w of HA produced by *S. zooepidemicus* in batch culture at 37°C and pH 6.7 with agitation at 600 rpm. Symbols: ▽, anaerobic, 20 g of glucose per liter; ○, 0.2 vol/vol/min, 20 g of glucose per liter; □, 1.0 vol/vol/min, 20 g of glucose per liter; ●, 0.2 vol/vol/min, 40 g of glucose per liter; ■, 1.0 vol/vol/min, 40 g of glucose per liter. The error bar is as in Fig. 2.

The measurement of polydispersity was less accurate for HA with a M_w greater than 2.5×10^6, because the HPLC size exclusion columns used were unable to resolve the larger molecule fraction satisfactorily. This is a common problem with very high molecular weight polymers (1). However, large changes in polydispersity due to a proportional increase in the number of smaller molecules (e.g., because of polymer degradation) will still be detected. In this regard, no significant shift in polydispersity, as a result of aeration, was observed (Table 2).

Effect of initial glucose concentration. The effect of initial glucose concentration on the M_w in aerated culture under otherwise standard conditions was studied. Experiments were limited to a maximum initial glucose concentration of 60 g/liter, since higher concentrations led to such high apparent viscosities in the broth, due to HA production, that the homogeneity of the bioreactor, especially with regard to pH, could not be maintained, despite increases in agitation rate or aeration.

Initial glucose concentrations of 40 and 60 g/liter had no effect on the maximum specific growth rate of *S. zooepidemicus*, which was identical to that at 20 g of glucose per liter. A feature of high initial glucose concentrations, however, was diauxic growth (Fig. 5). A very substantial increase in the maximum HA concentration was observed (Table 2), although the yield of HA on glucose ($Y_{P/S}$) fell, indicating less efficient conversion of glucose to HA. A higher initial glucose concentration markedly increased the M_w, which also increased with culture time (Fig. 4). In marked contrast to results at an initial glucose concentration of 20 g/liter, increasing the aeration from 0.2 to 1 vol/vol/min did not alter the polymer M_w at 40 g of glucose per liter. Replicated cultures operated at 1 vol/vol/liter and 40 g of glucose per liter demonstrated excellent reproducibility (Table 2).

The combination of high aeration and high initial glucose

TABLE 2. Effect of aeration and initial glucose concentration on growth and HA production by *S. zooepidemicus*

Aeration (vol/vol/min)	Glucose concn (g/liter)	μ_{max} (h^{-1})	Maximum HA concn (g/liter)	$Y_{P/S}$[a] (g/g)	$M_{w_{mean}}$, 10^6	P_{mean}	*n*
	20	0.55 ± 0.05	1.45	0.075	2.10 ± 0.10	2.2 ± 0.1	4
0.2	20	0.65 ± 0.15	1.60	0.080	2.30 ± 0.15	2.4 ± 0.1	2
1.0	20	0.60 ± 0.10	1.65	0.085	2.65 ± 0.05	2.3 ± 0.1	2
0.2	40	0.65 ± 0.10	2.70	0.070	3.1 ± 0.4	2.4 ± 0.1	3
1.0	40	0.60 ± 0.10	2.70	0.070	3.1 ± 0.1	2.7 ± 0.1	2
1.0	40[c]	0.60 ± 0.10	2.70	0.070	3.2 ± 0.1		3
1.0[b]	60	0.65 ± 0.10	4.20	0.070	3.0 ± 0.2	2.7 ± 0.1	2

[a] Yield of HA on glucose.
[b] Culture temperature, 32°C.
[c] Replicate experiment.

concentrations gave rise to increased polydispersity (Table 2). Molecular weight distributions of HA produced under these conditions reveal an increase in the low-molecular-weight shoulder (Fig. 6).

DISCUSSION

The effect of culture variables, examined over a wide operating range, on the M_w and polydispersity of HA produced by *S. zooepidemicus* varied significantly. The inherent variability of biological processes and the viscous nature of the HA-containing broths are both potential obstacles to achieving repeatable data during these studies. Nevertheless, replicate fermentations show that excellent reproduction of data was obtained for all measurements. This permits a high degree of confidence in the observed trends. The culture pH had little influence on the molecular weight properties of HA, although it exerts a considerable influence on the rate of production and yield of the biopolymer over the range tested (9). Consequently, subsequent experiments were performed at pH 6.7, since we have previously found this to be optimal for HA production by the strain of *S. zooepidemicus* used. Furthermore, the molecular weight characteristics of HA were unaffected by different levels of agitation (300 to 1,000 rpm) in the fermentor. This is important because it suggests that HA molecules are quite resilient to impeller-induced shear forces. Consequently, high agitation rates, which improve the HA yield and production rate (9), may be used without damaging the biopolymer molecules.

Lower culture temperatures (32 to 35°C) gave rise to increases in both the M_w and yield of HA, whereas the specific growth rate of *S. zooepidemicus* was highest at 40°C. There have been several reports suggesting that extracellular polysaccharide synthesis is maximal at temperatures below the growth optimum (18), and patent literature describing HA production often specifies culture temperature in the range of 30 to 35°C (6, 20). However, little has been reported about the influence of temperature on the M_w of HA. We suggest that this effect is the result of a decreasing specific growth rate of *S. zooepidemicus* with decreased temperature (Table 1). Competition for substrate and energy resources between catabolic, anabolic, and HA synthesis pathways must exist during active growth of the bacteria. The metabolite UDP-GlcNAc, for example, is a common precursor of both HA polymerization and cell wall synthesis (17). When the bacterial growth rate is low, precursor molecules (particularly glucose and glutamine) and energy may be more available for HA synthesis.

The likelihood that HA yield and M_w are energetically controlled is also suggested by the observation that aeration of cultures led to a higher molecular weight and yield compared with anaerobic fermentation. Under aerobic conditions, some acetate was produced from pyruvate, which leads to the concomitant regeneration of an extra 1 mol of ATP per mol of glucose consumed, compared with lactate production. HA biosynthesis is an energy-intensive process requiring 5 ATP equivalents per disaccharide unit. The higher ATP yield for *S. zooepidemicus* when grown under aerobic conditions may partly

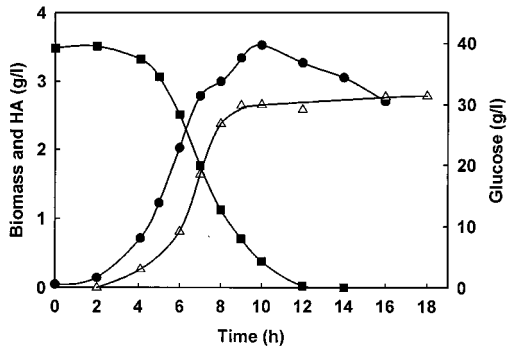

FIG. 5. Time course of a batch culture of *S. zooepidemicus* grown on an initial concentration of 40-g of glucose per liter at 37°C and pH 6.7 with 600 rpm agitation and 1 vol/vol/min aeration. Diauxic growth is evident at t = 7 h. Symbols: △, HA; ●, biomass; ■, glucose.

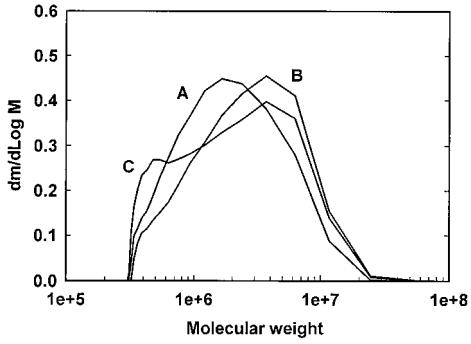

FIG. 6. Molecular weight distributions of HA produced in batch culture at 37°C and pH 6.7 with 600 rpm agitation. Curves: A, 20 g of glucose per liter, anaerobic; B, 20 g of glucose per liter, 0.2 vol/vol/min aeration; C, 40 g of glucose per liter, 1.0 vol/vol/min aeration. dm/dLog M is the change in mass fraction of total polymer with log molecular weight. Samples were taken when the maximal HA concentration had been achieved.

explain the enhanced yield and degree of polymerization of the HA produced.

It has been suggested that aerobic conditions can give rise to degradation of HA by oxygen-derived free radicals (15), which would presumably lead to a drop in M_w and a widening of the molecular weight distribution. We did not observe any evidence for this. In contrast, cultures (20 g of glucose per liter initially) aerated at both 0.2 and 1.0 vol/vol/min produced HA with similar polydispersity to the anaerobic culture and with higher M_w. Only under conditions of both high glucose and aeration was polydispersity increased, yet this increase was not dramatic.

Of the culture conditions examined, the initial glucose concentration had the most pronounced effect on the M_w of HA. We can offer two explanations. First, when the activated sugar monomers UDP-GlcUA and UDP-GlcNAc are present at high concentrations, HA chain elongation is thought to persist (14). The external glucose concentration may be linked to the internal concentrations of these monomers, because they are derived from glucose and their synthesis requires substantial energy consumption. Second, diauxic growth was observed in cultures with high initial glucose concentrations. A dramatic drop in the specific growth rate (μ) was observed 5 to 6 h before glucose exhaustion and the completion of HA synthesis. This lower μ may result in the synthesis of higher-M_w HA during the following period of slower growth, as in the case of the temperature effect previously described. Further work is needed to more conclusively comment on this effect.

Although the M_w was greater at high initial glucose concentrations, the HA yield ($Y_{P/S}$) was inferior. This is probably due to mass transfer limitations resulting from the very high broth viscosity, which prevented good mixing of the bioreactor even at an agitation speed of 1,000 rpm.

A pathway for HA synthesis by streptococci has been proposed, and progress is being made in the genetic characterization of the hyaluronate synthase (4, 7, 13), yet the mechanisms controlling HA chain length and hence M_w are not fully understood. The determining factors appear to fall under two broad categories. First, the bacterial cell may proceed to elongate HA polymer chains without interruption until substrate or energy resources are depleted. While this is taking place, other agents may be involved in chain cleavage, thus determining the molecular weight distribution of the polymer. Van de Rijn (23) described a membrane-bound hyaluronidase-like activity which cleaved attached "cellular" polysaccharide, releasing smaller, discrete sizes of HA ($M_w \sim 2 \times 10^6$) into the fermentation broth. Some degradation of HA by free radicals also remains a possibility. The action of shear forces, however, appears to be less likely.

Second, chain termination may be signalled by transient changes in certain metabolites. A receptor molecule is thought to reside on the protoplast membrane in proximity to the hyaluronate synthase, which serves to bind the growing HA chain. Mausolf (14) suggests that a high ATP concentration (millimolar range) and/or a low UDP-GlcNAc concentration (micromolar range) leads to phosphorylation of the receptor and subsequent release of HA into the medium. It has also been shown that hyaluronate synthase can be shed from the growing streptococci, together with the HA chain (15). It has not been established whether this form of release is related to the previously described mechanism.

Based on knowledge of the mean number-average molecular weight (M_n) of HA and the rate of HA polymerization by hyaluronate synthase (5), the average time interval required to generate a complete HA molecule by a single enzyme can be estimated. By using our data of M_w and polydispersity and assuming negligible postrelease degradation, the chain generation time appears to be shorter than 1 h. This leads us to believe that events of longer duration, such as extracellular nutrient depletion or cell division, are not primarily responsible for determining the chain length of HA.

The fact that the M_w of HA does not dramatically decline over the culture time course (and in fact increases under conditions of high initial glucose concentration), coupled with relatively constant polydispersity, is a significant advantage from a process point of view. It allows the manufacturer to choose an optimum culture time for product yield without compromising molecular size characteristics.

Working with a strain of *S. zooepidemicus*, we have shown that temperature, aeration, and initial glucose concentration are important variables in determining the M_w of HA produced in batch culture. A high initial glucose concentration (40 g/liter) accompanied by moderate aeration (0.2 to 1 vol/vol/min) resulted in the highest-M_w polymer. The mechanisms which control the M_w of HA are not yet well defined. As these are identified, manipulation of culture conditions and strain modification may be more rationally applied to regulate the size characteristics of this clinically important biopolymer.

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