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ORIGINAL RESEARCH PAPER

An efficient process for production and purification of hyaluronic acid from *Streptococcus equi* subsp. *zooepidemicus*

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Abstract Growth of *Streptococcus zooepidemicus* in a 10 l bioreactor with 50 g sucrose/l and 10 g casein hydrolysate/l gave 5–6 g hyaluronic acid/l after 24–28 h. Purification of hyaluronic acid gave a recovery of 65% with the final material having an Mr of $\sim 4 \times 10^6$ Da with less than 0.1% protein.

Keywords Fermentation · Hyaluronic acid · Purification · *Streptococcus zooepidemicus*

Introduction

Hyaluronic acid (HA) is a uniformly repetitive, linear glycosaminoglycan composed of 2,000–25,000 disaccharides of glucuronic acid and *N*-acetylglucosamine linked alternately by β-(1-3)- and β-(1-4)-glycosidic bonds. HA exists as a hydrated gel and is ubiquitous in human and animal tissues (Swann and Kuo 1991). It provides lubrication in the joints and serves as shock absorber. HA also plays important role in embryogenesis, signal transduction and cell motility and is associated with cancer invasiveness and metastasis (Kogan et al. 2007). Its distinctive viscoelastic properties, coupled with its lack of immunogenicity or toxicity, have led to a wide range of applications in the cosmetic and pharmaceutical industries, including skin moisturizers, osteo-arthritis treatment, ophthalmic surgery, adhesion prevention after abdominal surgery and wound healing (Goa and Benfield 1994; Laurent and Fraser 1992).

HA purified from a variety of sources has Mr values ranging from 10^4 to 10^7 Da (Shimada and Matsumura 1975). Many of the clinical applications of HA depend on its molecular size and there are several patents that focus on this aspect (Swann and Kuo 1991). Though HA is usually isolated from animals (Van Brunt 1986), there is an increasing demand for products from non-animal sources. Microbial HA production is now being investigated both for obtaining higher yield and quality, achieved through physiological and genetic manipulations. The most commonly used microbial strain for the production of HA belong to the Group A or C *Streptococcus*. Few studies have optimized the culture conditions for producing high molecular size HA in *Streptococcus zooepidemicus* (Johns et al. 1994; Armstrong and Johns 1997) and the majority of the literature has focused mainly on obtaining a highly pure product suitable for clinical applications. The present report describes the effect of carbon source and media composition on the Mr of HA. A novel and efficient purification process for high molecular size medical grade HA is the highlight of this report.

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Materials and methods

Bacterial strain and media

Streptococcus equi subsp. *zooepidemicus* ATCC 39920 was grown in a medium constituting casein enzyme hydrolysate, 25 g/l; yeast extract, 3.5 g/l; K_2HPO_4, 2 g/l; NaCl, 1.5 g/l; $MgSO_4 \cdot 7H_2O$, 0.4 g/l; and carbon source, 20 g/l; either in a 10 l bioreactor (Bioengineering L-1523). The fermentor was operated at 37°C at 400 rpm for 28 h at 2 vvm aeration. All experiments were carried out at least in triplicates to ensure reproducibility.

Estimation of HA

HA in the fermented broth was routinely estimated by the carbazole assay measuring uronic acid (Bitter and Muir 1962). To avoid interference in the assay by media components, the HA in the cell-free broth was precipitated with 2-propanol (1:1 v/v), re-dissolved in 3% (w/v) sodium acetate and then estimated. The assay detects the glucuronic acid released after the hydrolysis of HA with H_2SO_4.

Molecular size determination

The molecular size of HA was determined by size-exclusion chromatography on HPLC using a Shodex OH-Pak SB805-804HQ columns connected in series. The mobile phase used was 1 M $NaNO_3$ at 1 ml/min. The eluate was monitored using an RI detector. The column was calibrated with pullulan standards (Shodex P-82) of varying molecular weights.

Purification of HA

The highly viscous fermented broth having 5 g HA/l or higher is diluted with equal volume of pyrogen-free water and centrifuged at 17686*g* for 20 min at 4°C for removal of cells. HA from the clarified broth was purified as described in Table 1.

Results and discussion

Distribution of HA of various molecular size during growth

As reported elsewhere (Armstrong and Johns 1997), the production of HA is a growth-associated phenomenon with the low Mr HA ($\sim$5 kDa) appearing in the beginning (data not shown) followed by the accumulation of high Mr HA (>800 kDa) by the end of 22 h of fermentation. As lactose or sucrose in the medium gave HA of higher Mr (>800 kDa) as than glucose, all our subsequent experiments were carried out using sucrose as carbon source.

Table 1 Purification table for a typical batch of hyaluronic acid

Treatment	Volume (ml)	HA yield (mg/ml)	Protein (mg/ml)	Total HA (mg)	Total protein (mg)	% Protein w.r.t. HA
IPA[a]	100	3.4	0.56	340	56	16.5
Silica gel[b]	90	3.2	0.15	288	13.5	4.7
Carbon[c]	90	3.1	0.02	279	1.8	0.6
Diafiltration[d] (5X)	128	1.7	0.001	217.6	0.13	0.06
0.22 μm Filtration[e]	128	1.7	0.001	217.6	0.13	0.06

[a] HA from clarified broth was precipitated with 2-propanol (1:1 v/v) and resuspended in 3% (w/v) sodium acetate

[b] The resuspended HA solution was treated with silica gel at 2% (w/v) in batch mode at ambient temperature at 150 rpm for 2 h. Clarified HA solution was collected by centrifugation (18000*g* for 20 min at 4°C)

[c] HA solution was passed through a charcoal (0.45 μm) filter assembly at 14 ml/min

[d] The carbon-treated HA solution was further purified by ultrafiltration in diafiltration mode after five fold dilution with pyrogen-free water. Diluted HA solution was pumped (15–20 ml/min) into a cross-flow filter holder equipped with 50 kDa cut-off polyether sulphone cassette. The retentate containing HA was concentrated to original volume

[e] The HA solution from diafiltration process was sterilized by passing through a 0.22 μm filter

Effect of sucrose and casein enzyme hydrolyzate concentration on production of high molecular size HA

Increasing the sucrose concentration in the medium from 20 to 50 g/l with concomitant decrease in casein enzyme hydrolyzate from 25 to 10 g/l resulted in dramatic increase in the viscosity of the fermentation broth as a result of higher HA production (Table 2). These conditions favour a lower growth rate and higher production of HA resulting in a yield of over 5 g/l (Fig. 1). Unlike another report wherein lysozyme was added to the culture broth to increase the Mr to 3.8×10^6 Da (Kim et al. 1996), our process yields HA of Mr of about 3.5×10^6–3.9×10^6 Da in 24 h at 37°C, pH 7.0, 400 rpm and 2 vvm aeration without any major modification.

Purification of HA

Production and purification of high Mr HA that meets the specifications set for medical applications by the pharmacopeia has been a challenge and quality rather than quantity has been the focus of strain and process development in patents on HA production. The inherent viscosity of HA which increases with increase in Mr presents a major obstacle during fermentation as well as in purification. Fermentation parameters that balance the production of HA, which is inversely related to the growth, are crucial.

Several previous separation procedures have used multiple solvent precipitations, cationic detergent treatment, diafiltration, anion exchange resin treatment, protease digestion for purification of HA. Table 1 describes a novel purification process for HA that includes silica gel filtration combined with active carbon treatment followed by diafiltration. A single solvent precipitation step is used which drastically reduces the solvent usage unlike other processes (Brown et al. 1994; Han et al. 2004). Removal of protein impurities by 96% is achieved through treatment with silica gel and active carbon rather than detergents which necessitate multiple washes post-treatment (Nimrod et al. 1988; Brown et al. 1994). Ultrafiltration in the diafiltration mode further removes the impurities yielding a product with 0.06% protein with respect to HA. Although diafiltration has been used in other reports (Carlino and Magnette 2002), our process is more efficient involving very low dilution with solvent and yielding a better quality of HA. A final 0.22 μm filtration step renders the

Table 2 Effect of casein enzyme hydrolysate on production of HA

Casein enzyme hydrolysate (g/l)	HA yield (g/l)
2	1.0
3.3	2.5
10	5.1
25	2.4

Sucrose concentration in the medium was maintained at 50 g/l in all these experiments

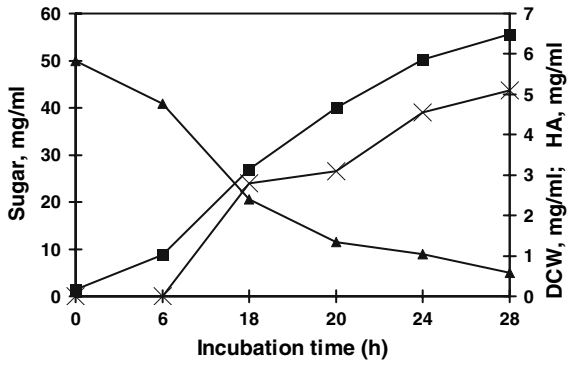

Fig. 1 Media optimization for increased yield of HA in a 10 l batch fermentation process. The sucrose content (▲), HA content (mg/ml) (X) and dry cell weight (■) during the process are indicated

Table 3 Properties of the hyaluronic acid from a typical batch

Test	BP specifications[a]	Sample
Appearance of solution	Clear; $A_{600\ nm} = \leq 0.01$	Clear; $A_{600\ nm} = 0.004$
IR spectra[b]		Complies
pH	5.0–8.5	6.65
Nucleic acids	$A_{260\ nm} = \leq 0.5$	$A_{260\ nm} = 0.033$
Protein	≤0.1%	0.056%
Chlorides	≤0.5%	Complies
Loss on drying	≤20% by weight	18.2%
% Na-hyaluronate[c]		99.2%
Molecular size		3.9×10^6 Da

[a] British Pharmacopeia 2003

[b] The spectrum of the test substance corresponds to the reference spectrum of sodium hyaluronate

[c] Not less than 95% and not more than 105% of sodium hyaluronate calculated on the basis of dried material

product sterile with a yield of 65%. The quality of HA obtained complies with the specifications of British Pharmacopeia (BP 2003) for medical grade HA (Table 3). Thus, the present process described is simple, economical and reproducible giving high yield of medical grade high Mr HA.

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