Efficient Recovery of Hyaluronic Acid from Highly Viscous Culture Broth

Jagadeeswara Reddy Kanala*, Kalpana Panati**, and Venkata R Narala***

An efficient method for the separation and recovery of Hyaluronic Acid (HA) from highly viscous broth was developed. In this strategy, the separation of HA from viscous culture broth and purification by optimizing different parameters, viz., temperature, pH, treatment with activated charcoal, were included. In addition, concentrating the HA solution using ultrafiltration facilitated the reduction of the volume of isopropanol that is required for the recovery of HA. The viscosity of culture broth was greatly reduced by lowering its pH to 2.0 rather at neutral pH. The energy demand for the separation of HA from culture broth using centrifugation was considerably reduced by acidification of the culture broth. Moreover, the amount of alcohol required for the precipitation of HA was also reduced by concentrating purified HA solution using ultrafiltration with hollow-fiber membrane cartridge. Clinical grade HA can be produced by this process, and additionally, this strategy might be worthwhile for industrial scale purification.

Keywords: Streptococcus zooepidemicus, Capsule, Hyaluronic acid, Ultrafiltration, Acidification

Introduction

Hyaluronic Acid (HA), a naturally occurring biopolymer, is composed of repeating units of D-glucuronic acid and N-acetylglucosamine linked together via alternating β-1, 4 and β-1, 3 glycosidic bonds. High concentration of HA has been found in rooster combs, human umbilical cord and mucoid capsule of certain gram positive bacteria such as Streptococcus species (Laurent, 1998). Because of its wide range of applications such as in the treatment of osteoarthritis, cosmetic surgery, ophthalmic surgery and wound healing (Laurent and Fraser, 1992; and Goa and Benfield, 1994), the production and recovery process of HA has attained great importance. Isolation and purification of HA from biological tissues is not feasible because the use of animal-derived biomolecules for human therapeutics is being met with growing resistance, and also in view of the risk of viral infection (O’Regan et al., 1994). Hence, the extraction from rooster combs is gradually being replaced by microbial production of HA.

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Several purification procedures were employed previously for isolation and purification of HA which involved complicated treatment processes, in turn, increasing the cost of production and difficulty in removing exothermic material, proteins, nucleic acids completely, etc. (Brown et al., 1994; Johns et al., 1994; Armstrong and Johns, 1997; Han et al., 2004; and Rangaswamy and Jain, 2008). HA is secreted by the bacterial cell as a capsule and released into the medium during bacterial growth. Because of high molecular mass (10^4 to 10^7 daltons) of HA, the viscosity of culture medium is increased upon its production (Shimada and Matsumura, 1975). For efficient recovery of HA, the negative surface charge on the capsulated cell has to be reduced to favor the sedimentation of the cells during the separation process.

Hence, it is a matter of prime concern to develop a suitable method for purification of HA in large-scale from microbial origin that pave the way for separating HA with high purity. In this study, a specific method to get pure clinical grade HA with less production cost has been established.

Materials and Methods

Bacterial Strain and Fermentation Conditions

Streptococcus equi subspecies zooepidemicus (MTCC 3523) was obtained from the Institute of Microbial Technology (Chandigarh, India). Batch cultures of S. zooepidemicus were carried out in a 10 L fermentor (Scigenics, India) containing 4 L of modified Van de Rijn and Kessler (1980) medium which contains carbon source 20 g/L, yeast extract 15 g/L. 1% inoculum and 1% yeast extract were added to the medium (pH 7.2) and the bacterial growth was allowed at 36 °C with 400 rpm for 28 h with the aeration rate of 0.6 vvm.

Estimation of HA, Proteins and Nucleic Acids

HA produced by S. zooepidemicus in fermented broth was estimated by carbazole assay (Bitter and Muir, 1962) in various steps of purification. To avoid the interference by the components present in the culture medium, the HA was precipitated with isopropyl alcohol (1:2 v/v), and redissolved in 0.15 mM sodium chloride and then estimated. The concentration of protein present in samples during purification of HA was determined by Bradford assay (Bradford, 1976) method. Similarly, the concentration of nucleic acids was estimated by measuring the absorbance at 260 nm in UV visible spectrophotometer (Labomed Inc., USA).

Measurement of Viscosity and Molecular Weight

The viscosity of fermented and cell free culture broth was measured by Brookfield viscometer (Brookfield engineering, Middleboro, MA, USA) during purification of HA at 25 °C. Molecular weight was determined by measuring the intrinsic viscosity (η) using Cannon-Ubbelhodes viscometer (Cannon Instrument Co., USA) followed by substituting the intrinsic viscosity in Mark Houwink equation (Martin, 1951; and Laurent et al., 1960). The intrinsic viscosity (η) was calculated using British Pharmacopoeia (2003) method.

Isolation and Purification of HA

The pH and the concentration of HA present in the fermented culture broth were 6.0-6.5 and 5.2-5.8 g/L respectively. 0.1% trichloroacetic acid was used for acidification of the fermented culture. The pH was reduced to 6.0, 4.0 and 2.0 and the cells were separated by centrifugation for 60 min, 30 min, and 15 min at 7,000 rpm respectively. Proteins and nucleic acids were removed from cell free broth by treating with different percentages of activated charcoal (0.2 to 1.0%) and stirred for 30 min, followed by centrifugation at 7,000 rpm for 10 min. The pH of the resultant processed HA solution (supernatant) was brought back to neutral and then diluted to five folds with pyrogen-free water. Diluted HA solution was sterilized by passing through 0.22 μm filter (Sartorius, India). Sterilized HA solution was further purified and concentrated by ultrafiltration in diafiltration (Sartorius) mode after twofold dilution with pyrogen-free water using 300 kDa cutoff membrane and the retentate was concentrated to half of its original volume. Finally, concentrated HA was precipitated with isopropyl alcohol (1:3 v/v) and vacuum dried (Biotron, Korea). The concentrations of HA, proteins and nucleic acids were estimated at each step in the purification process.

Results and Discussion

A batch fermentation of *S. zooepidemicus*, carried out at optimized conditions, yielded highly viscous broth containing 5.2-5.8 g/L of HA (Figure 1). By using the new strategy, which was developed in the present study, more than 5.6 g/L of clinical grade HA was produced.
with an MW of $4.2 \times 10^6$ Da (data not shown). The sugar concentration in the culture broth was restricted to 20 g/L to avoid diauxic growth in culture (Armstrong and Johns, 1997). To maintain constant carbon and nitrogen source, 2% glucose and 1% yeast extract were fed into the fermentor at a flow rate of 1 mL/min after 9 h of growth. Similarly, feeding the glucose during the fermentation increased the yield of HA, as observed in a previous study (Rangaswamy and Jain, 2008).

**Effect of pH on Culture Broth**

As we hypothesized, the HA containing culture broth viscosity was affected by pH (Do et al., 2001). The culture broth viscosity was reduced by adding trichloroacetic acid (0.1%) to fermented broth, which also terminates growth by killing the bacteria (Do et al., 2001). By lowering the pH of culture broth, the aggregation of cells occurred more rapidly. Aggregated cells could be easily settled down, compared to dispersed cells by centrifugation (data not shown). In this study, by reducing the pH of the culture broth from 6.0 to 2.0 cells, impurities were removed efficiently about 63% by centrifugation for 15 min at 7,000 rpm at room temperature (Table 1). The results suggest that reduced viscosity can be achieved by lowering the pH. Trichloroacetic acid treatment has the advantage of facilitating subsequent separation of the HA and this method could be applied in industries for large-scale preparation of HA.

<table>
<thead>
<tr>
<th>Time (min) Required for Centrifugation</th>
<th>Before Centrifugation</th>
<th>After Centrifugation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HA (g/L)</td>
<td>Protein (g/L)</td>
</tr>
<tr>
<td></td>
<td>260 nm Diluted</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>5.61</td>
<td>0.226</td>
</tr>
<tr>
<td>4</td>
<td>5.58</td>
<td>0.224</td>
</tr>
<tr>
<td>6</td>
<td>5.59</td>
<td>0.227</td>
</tr>
</tbody>
</table>

Note: Values in the table indicate the average of three independent experimental results.

As shown in Table 2, we found that 0.4% of charcoal treatment yielded high purity HA and efficiently removed impurities like proteins and nucleic acids. Using this process, we achieved 99.6% purity of HA with better purity, compared to the previous studies (Brown et al., 1994; and Rangaswamy and Jain, 2008). Sterilization and removal of remaining impurities from the clarified HA solution were achieved by neutralizing the pH to 7.0 and then was made fivefold dilutions with pyrogen-free water and passed through a $0.22 \mu m$ filter. Sterilized HA solution was concentrated by ultrafiltration in diafiltration mode with 300 kDa membrane after two-fold dilution with pyrogen-free water. The retentate containing HA was concentrated to half of the original volume with the recovery of 76% and purity.
of 99.6% (Table 2). Final step of precipitation after concentrating the solution reduced the alcohol consumption which is more economical compared to the previous reports (Brown *et al*., 1994; and Han *et al*., 2004). The HA obtained by these process meets the specification of British pharmacopeia (2003) (data not shown).

### Conclusion

A simple and efficient method for the separation and recovery of HA from highly viscous culture broth was developed. The centrifugal separation of cells from culture broth at low pH became much more efficient compared with the operation at neutral pH. The amount of solvent used for precipitation was reduced significantly by concentrating the cell-free culture broth by ultracentrifugation. This purification method efficiently removed exothermic materials, proteins, nucleic acids and endotoxins and minimized organic solvent usage for precipitation of HA. HA purified by this method has high recovery of 76% and purity of 99.6% and meets the specification of British pharmacopoeia (2003).

### References


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