The present invention relates to a method for purifying hyaluronic acid or salts thereof. Specifically, the present invention relates to a method for purifying hyaluronic acid or salts thereof in high purity, which comprises ultrafiltering hyaluronic acid-containing culture broth obtained by fermentation of microorganism to remove hyaluronic acid having a low molecular weight and pigments, and adding charcoal and optionally gamma alumina to remove impurities including proteins, nucleic acids, endotoxins, etc.
Description

METHOD FOR PURIFYING HYALURONIC ACID

[1] [Technical Field]

[2] The present invention relates to a method for purifying hyaluronic acid in high purity, which comprises ultrafiltering hyaluronic acid-containing culture broth obtained by fermentation of microorganism to remove hyaluronic acid having a low molecular weight and pigments, and adding charcoal and optionally gamma alumina to remove impurities including proteins, nucleic acids, endotoxins, etc. The present invention also relates to a method for purifying hyaluronic acid or salts thereof, which comprises the step of removing metal ions.

[3] [Background Art]

[4] Hyaluronic acid is colorless, transparent, highly viscose, and linear polysaccharides having a molecular weight ranging from 50,000 to 10,000,000 Daltons, and has the repeating units of glucuronic acid and N-acetylglucosamine combined in alternative linkage at (1-3) and (1-4) positions. Hyaluronic acid is widely distributed in skin, vitreous body of eye, joint fluid, umbilical cord, rooster comb, etc., or produced by bacteria such as Streptococcus genus, etc.

[5] Hyaluronic acid having a high molecular weight shows lubrication effect and protection effect against bacterial infection, etc., and so has been widely used as additive in cosmetics, protective agent for ocular tissue during ophthalmic operation, therapeutic agent for degenerative arthritis, etc.

[6] There are two methods for obtaining hyaluronic acid: one is to extract hyaluronic acid from the above mentioned tissues of a living body, and the other is to ferment microorganism and recover hyaluronic acid as a fermentation product.

[7] The method for purifying hyaluronic acid from animal tissues such as rooster comb, umbilical cord, etc. (US 4,141,973) is not appropriate for mass production due to high cost because it requires complicated purification steps to remove macromolecular impurities such as chondroitin sulfate, glucosaminoglycan, etc. Also, in this method, it is probable that the pathogenic materials derived from animals are retained in the product.

[8] On the other hand, the production of hyaluronic acid by using a microorganism takes a relatively low production cost, and the hyaluronic acid having a high molecular weight can be obtained in high yield by a simple purification process. It is also
reported that the possibility of retainment of pathogenic materials derived from animals is low in this method.

[10] Highly pure hyaluronic acid is required for hyaluronic acid to be used for commercial use, in particular, for medical use. The following methods are known in the art for the purification of hyaluronic acid produced by microorganisms.

[11] US 4,517,295 discloses a method for purifying hyaluronic acid by adding trichloroacetic acid to a culture broth of *Streptococcus pyogenes* to flocculate the cells, filtering the broth through a 0.2 μm filter to remove the cells, removing the medium-derived contaminants having a low molecular weight by using a membrane having the molecular weight cut off of 30,000 Da, and precipitating hyaluronic acid from the solution by adding an organic solvent. In this method, in order to obtain a highly pure hyaluronic acid, the precipitate is redissolved, and such salts as CETAB (a mixed trimethylammonium bromide), CPC (cetyl pyridium chloride), etc. are used to obtain precipitate. This precipitate is then dissolved, and subjected to a sterilizing filter. The resulting hyaluronic acid shows a protein content of 0.3-0.03% and a nucleic acid content of A$_{260}$ =0.314, which does not meet the protein content standard of 0.1% or less under the European Pharmacopoeia.

[12] US 4,782,046 discloses a method wherein 0.01% of anionic surfactant lauryl sulfate is added to a culture broth of *Streptococcus equi* to separate hyaluronic acid attached to the cell wall, the hyaluronic acid is precipitated by adding nonionic surfactant hexadecyltrimethyl ammonium bromide, the precipitate was dissolved in 2M calcium chloride (CaCl$_2$) solution, and the cells are centrifuged or filtered to obtain supernatant. Alcohol is added to the supernatant to precipitate and recover hyaluronic acid. A complicated process of dissolving precipitate, adding alcohol to precipitate hyaluronic acid, and recovering the precipitate is further carried out at least three times to purify hyaluronic acid.

[13] US 4,784,990 discloses a method wherein the culture broth of *Streptococcus zooepidemicus* is diluted 3-4 fold with 3% acetic acid buffer solution, and the medium is filtered by using 05g/L of diatomaceous-earth as a filter-aid to remove the cells. The same amount of isopropanol is added to the supernatant, the resulting precipitate is dissolved, isopropanol is added again, the resulting precipitate is re-dissolved, lg/L of charcoal is added, and the mixture is filtered to remove charcoal. Isopropanol is added again to the filtrate, and the precipitate is washed with isopropanol and dried. In order to use the hyaluronic acid as a medicine, complicated purification steps should be further carried out, which comprises dissolving said precipitate, adding CPC (Cetyl
pyridium chloride), dissolving the resulting precipitate, adding isopropanol again, dissolving the resulting precipitate, adding CPC, dissolving the resulting precipitate, passing the resulting solution through a magnesium silicate column and a 0.2 \( \mu \text{m} \) filter, adding isopropanol, and finally washing the resulting precipitate with ethanol.

The above-mentioned methods commonly use quarternary ammonium salt to remove impurities. However, the use of quarternary ammonium salt requires a long time in re-dissolving the precipitate, during which a large amount of waste water containing the surfactant quarternary ammonium salt is generated. In particular, it is highly probable that the quarternary ammonium salt is retained in the final product.

Thus, in order to avoid such demerit, the following method using ion exchange resin, without using quarternary ammonium salt, has been developed.

JP-A-63-012293 provides a method of treating hyaluronic acid-containing solution with macroreticular anionic exchange resin having high divinylbenzene content (Dianion HPA-25, HPA-75, IRA-900, IRA-904) to remove pyrogens and proteins. Specifically, the culture broth of \textit{Streptococcus zooepidemicus} is diluted with water, charcoal is added to adsorb impurities, and then removed by filtration. The supernatant is passed through a column filled with macroreticular anionic exchange resin, sodium chloride is added in a concentration of IM, an organic solvent such as ethanol, etc. is added, and the resulting precipitate is washed with ethanol, and dried. However, this method uses 25L of expensive anionic exchange resin per 3L of broth, and so is not economical enough to be applied to commercial production.

Accordingly, the following method using a relatively cheap adsorbent for the removal of impurities has been developed.

KR Patent No. 10-0236766 describes a method wherein ethanol is added to the culture broth of hyaluronic acid-producing strain to precipitate hyaluronic acid, the precipitate is washed with 65% ethanol several times, the precipitated is dissolved, and charcoal is added to adsorb impurities and removed by filtration. Zinc aluminate powder is added to adsorb impurities, and removed by filtration. The resulting solution is passed through a silica gel column, filtered through a 0.2 \( \mu \text{m} \) filter, precipitated by ethanol, and washed with ethanol several times to obtain purified hyaluronic acid.

KR Patent No. 10-0149793 describes a purification method characterized in that hyaluronic acid-containing solution recovered from the broth is treated with hydrophobic polymer (polyethylene, polypropylene, polystyrene), activated alumina is added to selectively remove impurities and hyaluronic acid having a low molecular weight, an organic solvent is added, and the precipitated hyaluronic acid is dried under
Further, a method of using ultrafiltration and filtration through an electrically charged filter together with the adsorbent treatment has been developed to increase the efficiency of removing impurities.

JP 2731545 describes a method wherein the broth is diluted with water, centrifuged to remove the cells, and ultrafiltered to remove the medium. Hyaluronic acid is precipitated by ethanol, and dried under vacuum. The resulting precipitate is dissolved, the hyaluronic acid-containing solution is treated by alumina and silica gel one after the other to remove pyrogens, proteins, nucleic acids, metallic impurities, etc., and an organic solvent is added to precipitate the purified hyaluronic acid.

JP-A-06- 199656 describes a method wherein the hyaluronic acid-containing solution is passed through a membrane filter charged to pH 6-10 to remove pyrogens, and ethanol is added to precipitate the purified hyaluronic acid.

However, the above methods using an adsorbent should include the steps of precipitating by ethanol, washing, drying, and dissolving prior to the adsorbent treatment. Accordingly, the total time to carry out the process becomes longer, with making the process more complicated.

The methods as explained above are not appropriate for the mass production due to their complexity, or do not thoroughly remove such impurities as proteins, pyrogens, nucleic acids, metals, etc. When a quaternary ammonium salt such as CPC, etc. is used, the salt may be retained in the purified hyaluronic acid. The use of adsorbent also requires ethanol precipitation and dissolution process as pretreatment. Therefore, a simple method for purifying hyaluronic acid in high purity has been required.

The present inventors have extensively studied to develop a simple method that can be easily applied to mass production as well as can produce highly pure hyaluronic acid by effectively removing such impurities as proteins, nucleic acids, endotoxins, metal ions, etc. As a result, the inventors found that such purpose can be achieved by a method as explained below, and so completed the present invention.

Thus, the present invention is to provide a method for easily purifying hyaluronic acid from a hyaluronic acid-containing culture broth that is obtained by fermentation of microorganism.
[31] [ Technical Solution ]
[32] The present invention relates to a method for purifying hyaluronic acid or salts thereof, which comprises the steps of

(a) removing cells from a hyaluronic acid-containing broth,

(b) ultrafiltering the cell-free broth,

(c) adding charcoal and optionally gamma alumina to the solution obtained in step (b) to adsorb impurities, and removing the adsorbent, and

(d) adding an organic solvent to the solution obtained in step (c) to precipitate hyaluronic acid, washing the precipitate with an organic solvent, and drying the precipitate.

Specifically, the present invention provides a method for obtaining highly pure hyaluronic acid more conveniently, characterized in that (i) ultrafiltration and (ii) charcoal and optionally gamma alumina treatment are subjected one after the other after removal of the cells.

The present invention also relates to a method for purifying hyaluronic acid or salts thereof, which comprises the step of removing metal ions.

[39]

[ Best Mode ]

[41] The culture broth containing hyaluronic acid in the present invention may be obtained by culturing the hyaluronic acid-producing strains known in the art, for example, strains from Streptococcus genus, preferably Streptococcus equi, Streptococcus zooepidemicus, and Streptococcus pyogen, under a culture condition known for each strain. Specific kinds of strains and culture conditions are known in the art.

The cells may be removed from the hyaluronic acid-containing broth by any method known in the art, for example, centrifugation, organic acid precipitation, filtration, etc. Filtration may be carried out by using filter press, drum filter, depth filter or membrane filter, and diatomaceous earth or perlite is used as a filter aid. Preferably, depth filter is used with diatomaceous earth as a filter aid.

Diatomaceous earth is a porous particle having silicone oxide (SiO$_2$) as the main component, and usually used as a filter aid. If the cells are removed from the hyaluronic acid-containing broth by using diatomaceous earth as a filter aid, the diatomaceous earth particles are piled with the cells, and prevent the blockade of the filter membrane, and so the filtration can be facilitated. Also, the cells may be filtered by the porous structure of the diatomaceous earth, whereby the removal efficiency of
the cells can be improved. As the amount of diatomaceous earth used decreases, the
cells block the pores of the filter and make the filtration time longer. On the other
hand, as the amount of diatomaceous earth used increases, broader filtration area is
needed. Generally, the amount of diatomaceous earth used for the removal of cells
preferably is in the range of 05 to 5%, and the concentration of hyaluronic acid
preferably in the range of 05 to 20g/L.

In the prior arts, organic solvent treatment is conventionally carried out after removal
of the cells. However, the present invention has the technical feature that ultrafiltration
is directly applied without the time-consuming and complicated organic solvent
treatment. The ultrafiltration herein means that the solution is passed through a
membrane having a constant pore size. The substances having a lower molecular
weight pass through the pore, and those having a higher molecular weight remain on
the membrane. Under the situation, water is continuously supplied to remove the
substances having a lower molecular weight. Thus, if ultrafiltration is carried out
directly after the removal of the cells, the hyaluronic acid having a low molecular
weight and the impurities derived from the broth may be removed. The molecular
weight cut off of the ultrafiltration filter that can be preferably used in the present
invention ranges from 5,000 to 300,000Da, more preferably from 10,000 to
100,000Da. The use of filter whose cut off is more than 300,000Da allows hyaluronic
acid to get through the filter to make the yield low. In contrast, the use of filter whose
cut off is less than 5,000Da results in slow filtration and insufficient removal of
substances having a high molecular weight, which all lower the removal efficiency of
impurities. It is preferable that the ultrafiltration is performed until the conductivity
of the hyaluronic acid filtrate reaches 4mS/cm or less.

After the ultrafiltration process, charcoal and optionally gamma alumina are added as
adsorbent. Use of gamma alumina is not indispensable, but it is preferable to add
gamma alumina with charcoal (see Example 3). Charcoal is carbon-based particles
having a high adsorption effect, and shows an excellent ability to remove proteins,
nucleic acids, endotoxins, etc. If gamma alumina is further added, the residual proteins
that are not removed by the single use of charcoal can be removed effectively. This
effect can be confirmed by Example 3. That is, even though charcoal is more
adsorptive than gamma alumina, when gamma alumina is used with charcoal, the ad-
sorptivity of charcoal jumps up (see Table 3). Charcoal is used in an amount of
05-5%, and alumina is preferably used in an amount of 0-3%. Increased amount of
adsorbent is beneficial for the effective removal of impurities. However, excess use of
adsorbent requires more cost, and also decreases the yield due to the adsorption of hyaluronic acid to the adsorbent. If the amount of adsorbent is not sufficient, impurities cannot be effectively removed. It is preferable that the charcoal treatment is carried out under the hyaluronic acid concentration of 05~20 g/L, pH of 4.0~8.0 and the temperature of 0~40 °C. The low concentration of hyaluronic acid results in decreased treatment efficiency, and the high concentration thereof results in high viscosity and poor mixing.

Sodium chloride may be added to the solution, for example, at the concentration of about IM, during the step of adsorbent. Sodium chloride may contribute to the adsorption of impurities to the adsorbent, and may change the shape and density of precipitates in the ethanol precipitation step in the desirable manner. Specifically, if ethanol is added at a low concentration of sodium chloride, hyaluronic acid precipitates in the form of fiber, and so the process further needs homogenization process (e.g., cutting process) after drying. Reversely, if ethanol is added at a high concentration of sodium chloride, hyaluronic acid precipitates in the form of particle, resulting in the homogeneous precipitate having a reduced volume.

As explained above, the present invention purifies hyaluronic acid in the order of removing the cells preferably by diatomaceous earth, ultrafiltrating without the organic solvent precipitation, and adsorbing by adding charcoal preferably with gamma alumina. This method can give desired hyaluronic acid in a higher purity than the case of not carrying out the ultrafiltration or adsorption process, or of reversing the order. For example, as can be seen from Comparative Examples 1, 2, 3, 4, and Tables 1 and 2, the constitutions according to the present invention, i.e., carrying out ultrafiltration and adsorption together and taking the order of ultrafiltration first and adsorption later, exhibit excellent effect in decreasing the amount of impurities such as proteins, endotoxins, nucleic acids, etc. to give hyaluronic acid in high purity.

Also, the present inventors have confirmed according to the present invention that decrease of metal ion content in the purified hyaluronic acid contributes to the prevention of inflammation reaction, particularly in eyes. Generally, hyaluronic acid for medical use requires NF (Non-inflammatory activity). However, the inventors have found that inflammation reaction may occur although hyaluronic acid is purified to meet the standards of protein, nucleic acid and endotoxin under the European Pharmacopoeia, and confirmed that the cause of inflammation lies in the presence of metal ion. Hyaluronic acid is negatively charged very strongly, and so metal cations, especially alkaline earth metal ions, can easily bind thereto. Thus, even the purified
hyaluronic acid may have a large amount of metal ions.

Any method known in the art may be used for decreasing the metal ion content in the hyaluronic acid. Chelating agent, more preferably EDTA, may be used for this purpose. This step of removing metal ions may be additionally applied to any method of purifying hyaluronic acid, and preferably, to the purification method according to the present invention. The step of removing metal ions may be inserted to any step of the above mentioned purification method, but the present inventors have found that the metallic impurities can be easily removed by adding EDTA to the solution after the charcoal treatment. For example, as confirmed by Comparative Example 5 and Table 4, such metallic impurities as barium, calcium, magnesium, etc. are detected in considerable amounts when EDTA is not used, but they are simply and effectively removed when EDTA is used. It is preferable to adjust the concentration of EDTA to 0.01-10mM. Thus, the present inventors confirmed that EDTA treatment conducted after the charcoal and optionally gamma alumina treatment according to the present invention gives hyaluronic acid which does not induce any inflammation reaction and is safe as a medicine.

After the charcoal treatment, an organic solvent is introduced to the solution for the precipitation of hyaluronic acid, whereby the purified hyaluronic acid can be recovered. As the organic solvent, any conventional organic solvent such as ethanol, methanol, acetone, isopropyl alcohol, etc., preferably ethanol, may be used. After the precipitation, an organic solvent washing process may be additionally carried out for further removing impurities and making the drying process easy. Hyaluronic acid precipitated by an organic solvent is dried according to a conventional drying process known in the art, for example, nitrogen or air purging, freeze drying, vacuum drying, etc., to give the purified hyaluronic acid as white solid. The present inventors determined whether the finally obtained hyaluronic acid solid meets the standards of use as a medicine, according to the following method.

As for the endotoxin, the hyaluronic acid sodium salt is dissolved to the concentration of 1mg/ mL, and tested by using the LAL kit of Charles River Endosafe. The endotoxin content standard under the European Pharmacopoeia is 0.05 IU/mg or less. As for the protein, the hyaluronic acid sodium salt is dissolved to the concentration of 10mg/ mL, and quantified by Lowry method. The protein content standard under the European Pharmacopoeia is 0.1% or less. Also, the protein of the sample in the intermediate step of purification is quantified by measuring the absorbance at 280nm. As for the nucleic acid, 0.10g of hyaluronic acid is dissolved to the final volume of 30.0
mL, and quantified by measuring the absorbance at 260nm. The nucleic acid content standard under the European Pharmacopoeia is 0.5 or less. As for the metallic impurities, 0.25g of hyaluronic acid is dissolved in 1 mL of nitric acid, heated, diluted to 10.0 mL with purified water, and quantified by the atomic absorption spectrometry II according to the European Pharmacopoeia (see Sodium Hyaluronate Test Method and Bacterial Endotoxins in the European Pharmacopoeia).

Mode for Invention

The present invention will be more specifically explained by the following examples and comparative examples. However, it should be understood that these examples are intended to illustrate the present invention, but not in any manner to limit the scope of the present invention.

Example 1: Preparation of culture broth containing hyaluronic acid

1.1. Preparation of seed culture medium

A medium having the final concentrations of 20g/L of glucose, 30g/L of yeast extract, 9g/L of sodium glutamate, 2g/L of sodium chloride, 2g/L of magnesium sulfate, and 2g/L of potassium phosphate dibasic was prepared. Only glucose and magnesium sulfate were dissolved in another flask, sterilized, cooled, and mixed with the other components.

1.2. Seed culture 1

To 50 mL of the medium prepared in Step 1.1 was inoculated 1 mL of Streptococcus zooepidemicus (KCTC 0075BP), and the mixture was incubated for about 24 h in a shaking incubator under the conditions of 35 °C and 100rpm.

1.3. Seed culture 2

To 950 mL of the medium prepared in Step 1.1 was inoculated 50 mL of the broth of seed culture 1 prepared in Step 1.2, and the mixture was incubated for about 24 h in a shaking incubator under the conditions of 35 °C and 100rpm.

1.4. Preparation of main culture medium

A medium having the final concentrations of 70g/L of glucose, 30g/L of yeast extract, 9g/L of sodium glutamate, 2g/L of sodium chloride, and 2g/L of magnesium sulfate was prepared. Glucose and magnesium sulfate were dissolved in another flask.
and sterilized. The other components were sterilized in a 15L fermenter, cooled, and mixed with the components in the flask.

1.5. Main culture

To a 15L fermenter including 9L of the medium prepared in Step 1.4 was inoculated 1L of the broth of seed culture 2, and the mixture was incubated under the conditions of 35°C and 200rpm until the concentration of glucose is lowered to lg/L or less. The culture broth was maintained at pH 6.9 to 7.3, and IVVM of air flow during the incubation.

Example 2: Purification of hyaluronic acid

2.1. Removal of cells

To the hyaluronic acid-containing broth prepared in Example 1 was added the same volume of purified water to dilute the solution to have the concentration of hyaluronic acid within the range of 0.1-0.4%. 2% of diatomaceous earth with respect to the diluted solution was added, and the resulting solution was filtered through a depth filter to remove the cells and to obtain the hyaluronic acid-containing supernatant.

2.2. Ultrafiltration

The cell-free hyaluronic acid-containing supernatant was concentrated to 1/2 volume by using a membrane whose molecular weight cut off is 30,000 Da. Ultrafiltration was carried out while constant volume was maintained by adding purified water, and stopped when conductivity of the hyaluronic acid-containing solution was 2mS/cm.

2.3. Adsorption

2% of charcoal and 1% of gamma alumina were added to the solution obtained from the ultrafiltration, and sodium chloride was added to the concentration of IM. The resulting mixture was stirred for about 5h to adsorb such impurities as proteins, nucleic acids, endotoxins, etc., and filtered to remove the adsorbent and obtain a supernatant.

2.4. EDTA treatment

To the supernatant after the adsorption was added EDTA to make the final concentration ImM, and the resulting mixture was stirred for 10 min.
2.5. **Ethanol precipitation**

To the hyaluronic acid-containing supernatant was added the same volume of 95% ethanol to precipitate hyaluronic acid. Thus obtained precipitate was recovered, washed with 80% ethanol five (5) times, and dried under nitrogen gas.

**Comparative Example 1**

The same procedure as Example 2 was carried out to purify hyaluronic acid except that ultrafiltration was omitted.

**Comparative Example 2**

The same procedure as Example 2 was carried out to purify hyaluronic acid except that adsorption was omitted.

The following Table 1 compares the contents of impurities such as proteins, endotoxins, nucleic acids, etc. in the hyaluronic acid purified according to Example 2, Comparative Examples 1 and 2 after dissolving the hyaluronic acid powder in water. As is confirmed by the results of Table 1, carrying out both ultrafiltration and adsorption is very effective for the removal of impurities.

**Table 1**

<table>
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<tr>
<th></th>
<th>Protein (%)</th>
<th>Endotoxin (IU/mg)</th>
<th>Nucleic Acid ($A_{260}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ex. 2</td>
<td>0.01</td>
<td>0~0.03</td>
<td>0.01</td>
</tr>
<tr>
<td>Com. Ex. 1</td>
<td>0.09</td>
<td>0~0.03</td>
<td>0.02</td>
</tr>
<tr>
<td>Com. Ex. 2</td>
<td>1.60</td>
<td>0.4~4.0</td>
<td>0.80</td>
</tr>
</tbody>
</table>

**Comparative Example 3**

The ultrafiltration and adsorption steps were carried out in the same manner as Example 2, but the ethanol precipitation and the subsequent steps were omitted.

**Comparative Example 4**

Hyaluronic acid was purified according to the same procedure as Comparative Example 3 except that ultrafiltration and adsorption were carried out in the reversed order.
The following Table 2 compares the contents of impurities such as proteins, endotoxins, nucleic acids, etc. in the hyaluronic acid purified according to Comparative Examples 3 and 4. As is confirmed by the results of Table 2, it is more effective for reducing the contents of impurities to carry out ultrafiltration first and adsorption later.

Table 2

<table>
<thead>
<tr>
<th></th>
<th>Protein (μg/ml)</th>
<th>Endotoxin (IU/ml)</th>
<th>Protein (A₂₈₀)</th>
<th>Nucleic Acid (A₂₆₀)</th>
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<tbody>
<tr>
<td>Com. Ex. 3</td>
<td>1.2</td>
<td>0–0.03</td>
<td>0.023</td>
<td>0.010</td>
</tr>
<tr>
<td>Com. Ex. 4</td>
<td>9.9</td>
<td>0.12–1.2</td>
<td>0.073</td>
<td>0.160</td>
</tr>
</tbody>
</table>

The experimental results of Tables 1 and 2 show that the combination of ultrafiltration and adsorption is very effective for removing such impurities as proteins, endotoxins, nucleic acids, etc. from the hyaluronic acid-containing broth. Specifically, they show that the contents of impurities may be reduced to meet the standards of the European Pharmacopoeia only when ultrafiltration is carried out first, and adsorption later.

Example 3: Effect for removing impurities depending on the kind and amount of adsorbent

The cells were removed, and then ultrafiltration and adsorption were carried out according to the same procedure as Example 2, except that the kind and amount of adsorbent were changed as shown in the following Table 3. After removal of the adsorbent, the amounts of proteins, endotoxins and nucleic acids in the hyaluronic acid-containing supernatant were measured, as compared in Table 3.
The results of Table 3 shows that (i) charcoal is more effective than gamma alumina in removing such impurities as proteins, endotoxins, nucleic acids, etc.; (ii) 3% concentration of charcoal is more preferable than 0.5% concentration thereof; and (iii) it is more advantageous to use charcoal and gamma alumina together than to use only either of them.

Comparative Example 5: Effect for removing metallic impurities by EDTA treatment

Hyaluronic acid was purified according to the same procedure as Example 2 except that EDTA treatment was not carried out. The following Table 4 compares the concentrations of metallic impurities in the purification product obtained with or without the EDTA treatment. The results of Table 4 show that the EDTA treatment is essential for treating metallic impurities.

Table 4
The purification method according to the present invention can be broadly used for purifying hyaluronic acid from the hyaluronic acid-containing broth obtained by the culture of microorganism. Also, the present invention makes it possible to omit the complicated organic solvent precipitation step in the purification process, whereby hyaluronic acid having a high purity can be obtained by a simple and economical method. Thus obtained hyaluronic acid has a purity that is appropriate as a medicine.
Claims

[1] A method for purifying hyaluronic acid or salts thereof, which comprises the steps of:
(a) removing cells from a hyaluronic acid-containing broth,
(b) ultrafiltering the cell-free broth,
(c) adding charcoal to the solution obtained in step (b) to adsorb impurities, and removing the adsorbent, and
(d) adding an organic solvent to the solution obtained in step (c) to precipitate hyaluronic acid, washing the precipitate with an organic solvent, and drying the precipitate.

[2] The method of Claim 1 wherein the cells are removed by using diatomaceous earth.

[3] The method of Claim 2 wherein diatomaceous earth is used in an amount of 0.5 to 5%.

[4] The method of Claim 1 wherein the molecular weight cut off of the ultrafiltration membrane filter ranges from 5,000 to 300,000Da.

[5] The method of Claim 1 wherein gamma alumina is further used with charcoal in the adsorption step (c).

[6] The method of Claim 1 wherein charcoal is used in an amount of 0.5-5%, and gamma alumina is used in an amount of 0-3%.

[7] A method for purifying hyaluronic acid or salts thereof, which comprises the step of removing metal ions.

[8] The method of Claim 7 wherein a chelating agent is used for removing metal ions.

[9] The method of Claim 8 wherein EDTA is used as chelating agent for removing metal ions.

[10] The method of Claim 1, which further comprises the step of adding EDTA.

[11] The method of Claim 10, which comprises the step of adding EDTA between the steps (c) and (d).

[12] The method of Claim 10 or 11, wherein EDTA is added to a final concentration of 0.01-10mM.

[13] The method of Claim 1, wherein the organic solvent of step (d) is selected from ethanol, methanol, acetone and isopropyl alcohol.

[14] The method of Claim 13, wherein the organic solvent is ethanol.
INTERNATIONAL SEARCH REPORT

PCT/ISA/210 (second sheet) (April 2007)

A. CLASSIFICATION OF SUBJECT MATTER

C08B 37/08(2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 8 C12N 1/20, A61K 31/715, A61K 38/16, C12P 19/04, A61K 31/175, C12N 15/01, C12P 19/26

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Korean Utility Models and Applications for Utility Models since 1975
Japanese Utility Models and Applications for Utility Models since 1975

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKIPASS, WPI, USPTO, PAJ, CAPPLUS(STN), INSPECT 'hyaluronic acid, purification, EDTA, active carbon, organic solvent, filtration membrane, chelating agent, methanol, ethanol, etc'

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No</th>
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<tr>
<td>X</td>
<td>WO 2004/016771 A1 (Han, H Y , et al ) 26 Feb 2004 - see page 5, line 20 - page 10, line 4</td>
<td>1, 4, 13-14</td>
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<tr>
<td>A</td>
<td>US 5902795 A (Toole, B P and Baneqee, S D ) 11 May 1999 - see example</td>
<td>7-9</td>
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<td>A</td>
<td>US 5591716 A (Siebert, J W , et al ) 7 Jan 1997 - see the whole document</td>
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<td>A</td>
<td>US 5563051 A (Ellwood, D C , et al ) 8 Oct 1996 - see the whole document</td>
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28 FEBRUARY 2008 (28 02 2008)

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