Disclosed is a new method for extraction and purification of cartilage type proteoglycan, in which an acid is used as an eluting solvent of cartilage. To obtain proteoglycan, the method may for example comprise extracting crude proteoglycan using acetic acid as an eluting solvent of cartilage; filtrating a solution containing the crude proteoglycan to remove drags from the solution; centrifuging the solution obtained by the filtrating; adding ethanol saturated with sodium chloride to a supernatant liquid obtained by the centrifuging; and then centrifuging the supernatant liquid to which the ethanol saturated with sodium chloride has been added, to concentrate the crude proteoglycan as a precipitate. The proteoglycan may be used in food, medicine, cosmetics and so on.
ABSTRACT

Disclosed is a new method for extraction and purification of cartilage type proteoglycan, in which an acid is used as an eluting solvent of cartilage. To obtain proteoglycan, the method may for example comprise extracting crude proteoglycan using acetic acid as an eluting solvent of cartilage; filtrating a solution containing the crude proteoglycan to remove dregs from the solution; centrifuging the solution obtained by the filtrating; adding ethanol saturated with sodium chloride to a supernatant liquid obtained by the centrifuging; and then centrifuging the supernatant liquid to which the ethanol saturated with sodium chloride has been added, to concentrate the crude proteoglycan as a precipitate. The proteoglycan may be used in food, medicine, cosmetics and so on.
SPECIFICATION

TITLE OF THE INVENTION
A METHOD FOR EXTRACTION AND PURIFICATION
OF CARTILAGE TYPE PROTEOGLYCAN

BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

The present invention relates to a new method for extraction and purification of cartilage type proteoglycan.

DESCRIPTION OF THE PRIOR ART

One molecule of cartilage type proteoglycan recognized as a conjugated carbohydrate is characterized to have a structure shown in Fig. 1, which is a biopolymer having the following structural feature. That is, from several to tens of glycosaminoglycan chains (hereinafter shortened to GAG), each of which has a molecular weight of from several tens of thousand to several hundreds of thousand, are bonded to one backbone protein molecule having a molecular weight of from several tens of thousand to several hundreds of thousand which is called as a core protein. GAG can be classified to several kinds such as chondroitin sulfate and dermatan sulfate according to the base structure, and, basically is a long chain hetero acidic polysaccharide composed of repeating structures of a disaccaride with an amino sugar and uronic acid. In this structure, GAG except hyaluronic acid are bonded to a core protein and forms proteoglycan.

In almost all animal organisms, proteoglycan is generally present as one of important components of extracellular matrix which exists among cells (refer to Fig. 2), which is similarly present with collagen and
hyaluronic acid. And, not only it plays an important part of an organism construction, but also forms physical circumference surrounding cells and controls various cell activities such as coupling, multiplying and differentiating. Each component of extracellular matrix or GAG individually has some functions such as retaining and supplying of water, antidote or analgesic. When these components bond each other and form a macro-molecule structure and each component acts reciprocally, a more remarkable effect is displayed.

The cartilage type proteoglycan, which is the object of the present invention, has a huge molecular weight in comparison with collagen, hyaluronic acid or GAG and has a complicated structure. Therefore, even if proteoglycan alone, it has better water retaining and supplying ability than other components in the extracellular matrix, further, can have other functions depending on biological information signal organization of its GAG portion.

In the meanwhile, in the method for extraction and purification of proteoglycan of nowadays, cartilage of cow or whale is used as a starting material, and extracted and purified by a complicated procedure using toxic or harmful agents such as chloroform, methanol or guanidine hydrochloride. And this method is not recognized as an industrial level. Some kinds of proteoglycan are available on the market in a very small amount as a reagent, and the price of them is approximately tens million yen per one gram.

The applicant of the present invention had previously invented a novel mass-producing simplified method for extraction and purification of proteoglycan that can be used as an industrial scale using nasal cartilage of salmon. More specifically, this method comprises a crushing step of
nal cartilage of salmon, a deoiling step, an extraction step by solvent and a dialysis process. By this method, extraction and purification in a large amount at a low price could be accomplished, however, not only chloroform, methanol and guanidine hydrochloride but also other harmful agents such as an inhibitor for a protein decomposing enzyme are used, therefore, the possibility for use as a material for medicine to be taken into human body or additives to health supporting foods or supplements is difficult, and the use is limited to non-drug chemicals or cosmetics. Further, since the market price of above mentioned chemical agents are relatively high, the reducing of extraction and purification cost is limited.

In the meanwhile, since the applicant of this application has presented the low cost proteoglycan, the volition for the development of goods in connection with proteoglycan is enhanced not only in cosmetics industry but also in processed foods industry, health supporting foods or supplements industry and medicines industry. However, for the substantial application of proteoglycan to the processed foods industry, health supporting foods or supplements industry or medicines industry, a special consideration must be cared for the method for purification of proteoglycan. In the conventional method for extraction and purification of proteoglycan, the use of hydrochloric acid salt of guanidine is common. But, for the new application of proteoglycan, it is strongly required not to use the guanidine hydrochloride and other toxic or harmful agents such a chloroform, methanol or hindering agent for protein decomposing enzyme. Still further, the development of more simplified and lower cost method for extraction and purification of proteoglycan had been strongly desired.
The inventor of this invention has conducted an intensive study trying to develop a method for extraction and purification of proteoglycan, in which the toxic or harmful agents are not used, and which is characterized to be more simplified and at a lower cost, and accomplished the present invention.

**BRIEF SUMMARY OF THE INVENTION**

The present invention provides an extraction method of crude proteoglycan, wherein an acid is used as an eluting solvent of cartilage.

The present invention also provides a purification method of crude proteoglycan, which comprises: extracting crude proteoglycan using acetic acid as an eluting solvent of cartilage, filtrating a solution containing the crude proteoglycan to remove dregs from the solution, centrifuging the solution obtained by the filtrating, adding ethanol saturated with sodium chloride to a supernatant liquid obtained by the centrifuging, and then centrifuging the supernatant liquid added the ethanol saturated with sodium chloride to concentrate the crude proteoglycan in the precipitate.

The present invention further provides a purification method of crude proteoglycan, which comprises: extracting crude proteoglycan using acetic acid as an eluting solvent of cartilage, filtrating a solution containing the crude proteoglycan to remove dregs from the solution, centrifuging the solution obtained by the filtrating, adding ethanol saturated with sodium chloride to a supernatant liquid obtained by the centrifuging, centrifuging the supernatant liquid added the ethanol saturated with sodium chloride to concentrate the crude proteoglycan in the
precipitate, dissolving the precipitate containing the crude proteoglycan using acetic acid as an eluting solvent of the crude proteoglycan, and then dialysing.

That is, the important point of the present invention is to use acetic acid, sodium chloride and unmodified ethanol in all steps of extraction and purification of proteoglycan, instead of the toxic or harmful agents such as chloroform, methanol and inhibitors for protein decomposing enzyme. These above mentioned agents, that is, acetic acid, sodium chloride and unmodified ethanol are used in ordinary processed foods. For the purpose to accomplish more simplified method for extraction and purification, a substitution step by urea and a separation and purification step by DEAE-Sephacel* are omitted.

**BRIEF DESCRIPTION OF THE DRAWINGS**

Fig. 1 is a structural model of proteoglycan.

Fig. 2 is a schematic view of extracellular matrix.

Fig. 3 is a graph showing a change of eluting state of crude proteoglycan with the passage of time.

In the drawings, numerical marks have the following meanings: 1: core protein, 2: glycosaminoglycan chain, 3: hyaluronic acid, 4: collagen, 5: proteoglycan.

**DESCRIPTION OF PREFERRED EMBODIMENTS**

The present invention will be illustrated more in detail hereunder.

*Trade-mark
As the starting material of proteoglycan of the present invention, cartilage of cow or whale can be used. However, from the viewpoint of easy purchase and price, the nasal cartilage of salmon is desirably used. Especially, it is desirable to use head parts of white salmon wasted from the process of processed foods such as a canning industry using white salmon which are caught at coastal fishery along the coast of Aomori prefecture of Japan.

As acetic acid to be used in the present invention, any kind of acetic acid, e.g. for foods use or for industrial use, can be used and selected depending on the purpose of the use of proteoglycan. The desirable concentration of the acetic acid eluting solvent is generally 2 to 6%, especially approximately 4% by weight according to the test results mentioned later. However, the concentration is not intended to be limited to this range or amount.

For extracting crude proteoglycan from the cartilage, convenient is to soak the cartilage in the acetic acid eluting solvent for a period sufficient to extract the crude proteoglycan, preferably for about 10 to about 100 hours at a relatively low temperature, e.g., from about 0 to 10°C.

**EXAMPLE**

As the starting material, the wasted head parts of white salmon from the canning process of processed food manufacturing, which are caught at the coastal fishery along the coast of Aomori prefecture, and the head parts are temporary preserved at the temperature of -30°C.
The above mentioned preserved material is defrosted at 4°C for 20 hours, and nasal cartilage part is cut off from the head part using a kitchen knife and the starting material is prepared. From the nasal cartilage of salmon, solid fat is removed using tweezers and rinsed by physiological saline solution. Then pulverized finely by a hand mincing machine and mincemeat of nasal cartilage of salmon is obtained.

A part of the mincemeat is soaked into 4°C business use brewing vinegar diluted to 10w/v (used by diluting to 4% concentration which is the same concentration as that of acetic acid in vinegar; hereinafter, appreciated as "4% acetic acid solvent") for 0, 6, 12, 24, 48, 72, 120 and 168 hours and stirred. The change of eluting state of crude proteoglycan is observed with the passage of time, as the amount of uronic acid by carbazole-sulfuric acid method. The obtained results are shown in Fig. 3. As clearly indicated in Fig. 3, the amount of eluted crude proteoglycan remarkably increases during first 24 hours, and the increasing of eluting amount is not so remarkable after 24 hours. From the obtained results, it is understood that the most effective eluting time of crude proteoglycan with 4% acetic acid solvent is 48 hours.

Based on the above mentioned results, 50g of mincemeat of nasal cartilage of salmon is soaked into 4% acetic acid solvent of 4°C for 48 hours and stirred so as to elute nasal cartilage, and crude proteoglycan is obtained.

Then the eluted solution is filtrated using stainless steel mesh (150 μm) so as to remove dregs that are not eluted. After that, the solution in which crude proteoglycan is contained is separated by a centrifuge (4°C,
10000 r.p.m., for 20 minutes). Ethanol saturated sodium chloride in an amount three times is added to the obtained supernatant liquid, and separated by a centrifuge (4°C, 10000 r.p.m., for 20 minutes) again, then concentrated precipitate containing crude proteoglycan is obtained.

The obtained precipitate containing crude proteoglycan is dissolved again in 4% acetic acid solvent, then the solution is sufficiently dialysed against water by a membrane dialysis tube of cellulose ester of molecular mass cut off of 1000Kda, and proteoglycan is obtained as a high purity aqueous solution.

It is desirable to freeze-dry the obtained liquid state proteoglycan and preserve it in powder state. In this Example, the dialysed inner solution is freeze-dried and 240mg of powder state proteoglycan specimen obtained.

The chemical features of proteoglycan specimen obtained by this process (including the dialysis) are measured by the following method.

The results of chemical analyses are shown in Table 1.

<table>
<thead>
<tr>
<th>Chemical analyses of proteoglycan specimen from nasal cartilage of salmon</th>
</tr>
</thead>
<tbody>
<tr>
<td>molar ratio</td>
</tr>
<tr>
<td>-------------</td>
</tr>
<tr>
<td>1.00</td>
</tr>
</tbody>
</table>

* indicates molar ratio when the amount of hexosamine is settled to 1.00

In Table 1, the amounts of uronic acid and sulfate are indicated by mole ratios when the amount of hexosamine is settled to 1.00, and are 0.99 and 0.67, respectively. It
is understood that these three components are present in almost same amounts. Further, the amount of core protein is 6.99% (w/w), and the ratio of it to uronic acid (core protein/uronic acid) is 0.23 (w/w). This numeric value shows one index to indicate the purity of proteoglycan and is close to 0.2 which is the theoretical value.

Amino acids constituting the protein of this specimen are analyzed, and the results show that the amounts of glycine, serine and glutamic acid are remarkably great. Namely, in all amino acid 1000 residues, the total number of glycine, serine and glutamic acid residues is 386, while the number of hydroxyproline residues is 2. Hydroxyproline is a typical amino acid in collagen protein, and the mingle of collagen in this salmon nasal cartilage proteoglycan can be recognized, but the amount is very small and cannot be said to be significant. Therefore, it can be said that the purity of the obtained salmon nasal cartilage proteoglycan is very high.

Then, for the purpose to obtain information referring to the molecular size of salmon nasal cartilage proteoglycan, high-performance liquid chromatography analysis is carried out using SB805HQ* column (8x300 mm), and the eluting position is confirmed by UV absorbency at 215 nm. This result is compared with that of cow nasal cartilage proteoglycan which is available on

*Trade-mark
the market as the reagent. In a case of salmon nasal cartilage proteoglycan, the elution position (Kav) recognized as a symmetrical peak from SB805HQ column is 0.28, while in a case of cow nasal cartilage proteoglycan is 0.17. These results show that the molecular size of salmon nasal cartilage proteoglycan is smaller than that of cow nasal cartilage proteoglycan.

Further, the core protein part of salmon nasal cartilage proteoglycan is digested by pronase, and remaining GAG specimen is treated by an electrophoresis analysis on a film made of cellulose acetate together with chondroitin sulfate (Ch6S), dermatan sulfate (DS) and hyaluronic acid (HA) which are the standard specimens. According to the results, the single band coincided with chondroitin sulfate (Ch6S) which is standard specimen is indicated, and consequently it becomes clear that most of GAG of salmon nasal cartilage proteoglycan is chondroitin sulfate.

This disaccharide unit isomer is investigated too. After proteoglycan is digested by pronase, further digested by chondroitinase ABC, and generated unsaturated disaccharide is analyzed by high-performance liquid chromatography (Polyamin-II). The obtained results are shown in Table 2. From the results of Table 2, it is clear that the most part of GAG is monosulfated disaccharide unit.

Table 2

<table>
<thead>
<tr>
<th>ΔDi-0S</th>
<th>ΔDi-6S</th>
<th>ΔDi-4S</th>
<th>ΔDi-diSD</th>
<th>ΔDi-triS</th>
</tr>
</thead>
<tbody>
<tr>
<td>15.1</td>
<td>59.4</td>
<td>25.1</td>
<td>0.3</td>
<td>0.1</td>
</tr>
</tbody>
</table>

As mentioned above, the fact that the proteoglycan whose starting material is salmon nasal cartilage is obtained only by using agents listed as the additives to foods [for example, "Explanation of Analytical Method of Additives in Foods, part III, Food Additives Except Chemically Synthetic Compound" edited by Akio Tanimura et al (1992, Kodansha)], or agents used as the material for a food preserving agent or a seasoning ["Encyclopedia of Safety Supply of Food" edited by Kageaki Kurihara et al (1995, Publishing Center of Sangyo Chosakai)], can be said as an epoch making invention. Further, the fact that by the present invention, the processes which take time and troublesome such as substitution by urea or separation and purification by DEAE-Sephacel method are omitted can be said as an epoch making
invention. That is, by the present invention, the object to develop a simplified and low cost method for extraction and purification of proteoglycan can be accomplished.

From the above mentioned results, salmon nasal cartilage proteoglycan obtained by the method of the present invention can be orally taken, and the purity of it is almost same to that of obtained by a conventional method.

Effect of the Invention

Currently, hyaluronic acid can be produced from bacterium safely and in large quantities, and is used for medicine application. In the meanwhile, proteoglycan is known to have an excellent water retaining ability, water supplying ability, antidote function and analgesic function, further is expected to have other functions based on GAG portion. However, proteoglycan obtained by a conventional method for extraction and purification can not be prescribed to human and to inspect it's usefulness to human body. Still further, the separation of conjugated carbohydrate proteoglycan originated from salmon nasal cartilage was not tried until said method is developed and applied. However, by the present invention, It becomes possible to extract and purify proteoglycan which has excellent functions safely and in large quantities. Therefore the needs to proteoglycan becomes more impatient and more wide applications are expected.

Further, since organic solvent such as chloroform, methanol or acetone which are used to remove solid fat from head part of salmon are not used, the treatment of wasted liquid becomes not necessary and consequently the problem of environment does not occur. The procedure of the present invention is simplified and effective, and proteoglycan obtained by said method is safe and can be orally taken. The development of novel applied products becomes possible in the fields of cosmetics, non-drug chemicals, medicines, medical products, processed foods, healthy supplemental foods and artificial internal organs by the development of this invention. Therefore, the present invention largely contributes to the health of human and medical fields.
CLAIMS:

1. A method for extraction of crude proteoglycan, wherein an acid is used as an eluting solvent of cartilage.

2. A method for extraction of crude proteoglycan comprising:
   
   extracting crude proteoglycan using acetic acid as an eluting solvent of cartilage;

   filtrating a solution containing the crude proteoglycan to remove dregs from the solution;

   centrifuging the solution obtained by the filtrating;

   adding ethanol saturated with sodium chloride to a supernatant liquid obtained by the centrifuging; and

   then centrifuging the supernatant liquid to which the ethanol saturated with sodium chloride has been added, to concentrate the crude proteoglycan as a precipitate.

3. A method for improving purity of crude proteoglycan comprising:

   extracting crude proteoglycan using acetic acid as an eluting solvent of cartilage;

   filtrating a solution containing the crude proteoglycan to remove dregs from the solution;

   centrifuging the solution obtained by the filtrating;

   adding ethanol saturated with sodium chloride to a supernatant liquid obtained by the centrifuging;
centrifuging the supernatant liquid to which the ethanol saturated with sodium chloride has been added, to concentrate the crude proteoglycan as a precipitate;

dissolving the precipitate containing the crude proteoglycan using acetic acid as an eluting solvent of the crude proteoglycan; and

then dialysing.

4. The method according to claim 3, in which the dialysis is conducted against water and the proteoglycan is obtained as a high purity aqueous solution; and which further comprises freeze-drying the aqueous solution of the proteoglycan to obtain the proteoglycan in a powder form.

5. The method according to claim 1, wherein the acid is acetic acid.

6. The method of any one of claims 2 to 5, wherein the acetic acid has a concentration of approximately 4%.

7. The method of any one of claims 1 to 6, wherein the cartilage is of cow, whale or salmon.

8. The method of any one of claims 1 to 6, wherein the cartilage is nasal cartilage of salmon.
Fig. 1
Fig. 3

Amount of uronic acid (µg/ml)

Time (h)