Determination of chondroitin sulfate from different sources of cartilage

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Abstract

Cartilage is produced as a by-product from slaughter house and fishery industries in Thailand. The enzymatic extraction of chondroitin sulfate followed the method of Nakano et al. [T. Nakano, H.H. Sunwoo, X. Li, M.A. Price, S.S. Jeong, Study of sulfated glycosaminoglycans from porcine skeletal muscle epimysium including analysis of iduronosyl and glucoronosyl residues in galactosaminoglycan fractions, J. Agric. Food Chem. 44 (1996) 1424–1434] and the sulfate GAGs assay [W.R. Farndale, D.J. Buttle, A.J. Barrett, Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue, Biochim. Biophys. Acta 883 (1986) 173–177] was employed to determine the content of chondroitin sulfate in cartilage of shark fin, ray, crocodile and chicken keel. Identification of types of chondroitin sulfate through FTIR spectroscopy KBr pellet technique was carried out. The results indicated that chicken keel, crocodile hyoid and sternum cartilage are the most promising potential sources of chondroitin sulfate. The value ranges from 11.55 to 14.84 g/100 g of dried cartilage, calculated as chondroitin-4-sulfate. Identification of dried chondroitin sulfate extracts from investigated cartilages show the existence of both chondroitin-4-sulfate and chondroitin-6-sulfate.

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1. Introduction

Large amount of cartilage is produced as a by-product from slaughter house and fishery industries. Cartilage matrix is composed of glycosaminoglycans (GAGs) which are mainly chondroitin-4-sulfate and chondroitin-6-sulfate, present in the form of proteoglycans [3]. Chondroitin-4-sulfate and chondroitin-6-sulfate are composed of N-acetyl-d-galactosamine and d-glucuronic acid sulfated at positions 4 and 6, respectively. Papain digestion of cartilage can liberate GAGs that are attached to protein [4]. After removing protein from the mixture, chondroitin sulfate can be separated and purified. Chondroitin sulfate has been widely used as a treatment for osteoarthritis [5]. Clinical studies confirmed the therapeutic effects of orally taken chondroitin sulfate on osteoarthritis patients with improvement of joint function and pain reduction [6–8]. Oral administration of chondroitin sulfate (C4S and C6S) of bovine origin at 800 mg/day for 3 months with intermittent schedule, twice a year, has been shown to produce therapeutic effect of the drug [9]. The known sources of chondroitin sulfate used in nutritional supplements are the cartilaginous rings of bovine trachea, pork ears and snout and shark cartilage. Other available sources of chondroitin sulfate have been investigated by many researchers [5,10–14], also different methods for extraction and analysis of chondroitin sulfate have been reported [15–19].

This study was aimed to investigate the possibility of extracting chondroitin sulfate from cartilage from available sources in Thailand. The enzymatic extraction of chondroitin sulfate followed the method of Nakano et al. [1] and the sulfate GAGs assay [2] was employed to determine the content of chondroitin sulfate in cartilage of shark fin, ray, crocodile and chicken keel to evaluate their feasible potential source of chondroitin sulfate.

2. Materials and methods

Papain (EC 3.4.22.2) was provided free of charge by East Asiatic Co. Ltd., chondroitin-4-sulfate from bovine trachea and chondroitin-6-sulfate from shark cartilage were purchased from Fluka and used as standards. 1,9-Dimethylmethylene blue was purchased from Aldrich. Crocodile cartilage (trachea, hyoid, sternum, etc.) was obtained from countries that have allowed trade of crocodile products.

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sternum, rib), shark fin, ray cartilage and chicken keel were obtained from local sources.

2.1. Preparation of cartilage for chondroitin sulfate extraction

Carcasses of approximately 4 years old crocodile were sampled from slaughter house and cartilage (trachea, hyoid, sternum and rib) were separated, kept on ice for not more than 6 h then kept frozen at −18 °C until used. Shark cartilage, by-products from shark fin soup restaurant, was collected and cleaned manually to remove debris. Ray cartilage was separated from whole fish of ray (Dasyatis zugei) bought from local fish market. The chicken keel cartilage was cut from fresh carcasses bought from local market. Cartilage from all samples were boiled in hot water (90–95 °C) for 10 min to help removing meat and connective tissue. The remaining cartilage were cleaned in portable water and kept frozen at −18 °C until used.

2.2. Enzymatic extraction of chondroitin sulfate from cartilage

Chondroitin sulfate was extracted by a method modified from Nakano et al. [1]. Cartilage, approximately 10 g, were chopped and ground in Waring Blender then hydrolyzed by adding papain at the level of 4 mg/g of cartilage, in 100 ml solution of 0.1 M sodium phosphate buffer pH 7.0 containing 0.005 M ethylenediaminetetra acetic acid, 0.005 M cysteine hydrochloride and 0.02% sodium azide. Enzyme hydrolysis was carried out at 65 °C for 48 h, where the clear solution was obtained. Trichloroacetic acid was added to obtain the final concentration of 7% (w/v). The mixture was kept overnight at 4 °C then centrifuged at 132,000 × g for 30 min, 4 °C to remove precipitated protein. The resulted supernatant was dialyzed in chilled distilled water for 24 h. At this point the solution containing liberated GAGs was purified for chondroitin sulfate according to the procedure outlined in Fig. 1.

Dried extract of chondroitin sulfate was sampled for moisture content determination [20].

2.3. Determination of chondroitin sulfate by sulfate GAGs assay

Dried chondroitin sulfate was dissolved in de-ionized water to make up an 8–12% (w/v) solution and used as a sample for sulfate GAGs assay. The amount of chondroitin sulfate was determined by sulfate GAGs assay according to Farndale et al. [2] using either chondroitin-4-sulfate or chondroitin-6-sulfate as a standard. 1,9-Dimethylmethylene blue was used as metachromatic dye to react with sulfate glycosaminoglycan, and the absorbance at 525 nm was measured using spectrophotometer (Spectronic 22).

2.4. Identification of types of chondroitin sulfate

FTIR BRUKER Equinox 55 Fourier Transform Infrared Spectrometer using potassium bromide (KBr) pellet technique was employed to identify the types of chondroitin sulfate in the sample. Standard chondroitin-4-sulfate and chondroitin-6-sulfate were analyzed and used as standard spectra. Dried chondroitin sulfate sample, approximately 2 ml, was mixed with dried potassium bromide powder (100–200 mg), then pressed into thin disc under hydraulic press and used as a sample for FTIR measurement. The spectrum was obtained at mid-infrared region.

3. Results and discussion

3.1. Composition and yield of chondroitin sulfate from cartilage

Proximate analysis of cartilage samples showed that cartilage from different sources consisted of high protein, ash and carbohydrate (Table 1). The high ash content of crocodile rib cartilage is attributed to the hard cartilage matrices. Enzymatic extraction of cartilage by papain yielded a clear solution after 48 h at 65 °C and most of the proteins were precipitated by TCA. The GAGs in the solution were purified and dried then used as samples for sulfate GAGs assay. Percentage yield and moisture content of dried extracts from various sources are shown in Table 2. Crocodile hyoid gave the highest yield of dried extract per dry weight of cartilage.

3.2. Determination of chondroitin sulfate by sulfate GAGs assay

The sulfate GAGs assay is based on changes in the absorption spectrum of the 1,9-dimethylmethylene blue (DMMB) when
Table 1
Composition of cartilage from various sources

<table>
<thead>
<tr>
<th>Source</th>
<th>Composition (%)</th>
<th>Moisture</th>
<th>Protein&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Fat</th>
<th>Ash</th>
<th>Carbohydrate&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shark fin</td>
<td></td>
<td>74.44</td>
<td>12.30&lt;sup&gt;c&lt;/sup&gt; (47.58)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.17 (0.66)</td>
<td>10.30 (40.30)</td>
<td>2.79 (11.77)</td>
</tr>
<tr>
<td>Crocodile</td>
<td>Hyoid</td>
<td>73.14</td>
<td>13.19 (49.08)</td>
<td>0.79 (2.95)</td>
<td>2.47 (9.20)</td>
<td>10.41 (38.77)</td>
</tr>
<tr>
<td></td>
<td>Rib</td>
<td>53.58</td>
<td>11.85 (25.53)</td>
<td>0.26 (0.56)</td>
<td>23.07 (46.20)</td>
<td>11.76 (27.71)</td>
</tr>
<tr>
<td></td>
<td>Sternum</td>
<td>72.42</td>
<td>15.22 (55.03)</td>
<td>0.54 (1.95)</td>
<td>3.73 (13.55)</td>
<td>8.09 (29.47)</td>
</tr>
<tr>
<td></td>
<td>Trachea</td>
<td>70.28</td>
<td>12.54 (42.19)</td>
<td>0.03 (0.09)</td>
<td>2.02 (6.80)</td>
<td>15.13 (32.95)</td>
</tr>
<tr>
<td></td>
<td>Ray</td>
<td>65.28</td>
<td>13.80 (39.75)</td>
<td>0.19 (0.56)</td>
<td>17.12 (48.92)</td>
<td>3.61 (10.77)</td>
</tr>
</tbody>
</table>

<sup>a</sup> % protein = % total nitrogen \times 6.25.
<sup>b</sup> Carbohydrate calculated by difference.
<sup>c</sup> Values expressed as a percentage of wet weight of cartilage.
<sup>d</sup> Values expressed as a percentage of dry weight of cartilage.

bound to GAGs. Calibration curves using chondroitin-4-sulfate or chondroitin-6-sulfate as standards showed linear relation between absorption at 525 nm of the chondroitin sulfate-DMMB complexes versus concentration of chondroitin sulfate in the solution, with $R^2$ of 0.9912 and 0.9940, respectively, as shown in Fig. 2. The differences in sulfation position of chondroitin-4-sulfate and chondroitin-6-sulfate may affect the absorption at 525 nm of the complex as can be seen by the different slope values of the two lines. The values of chondroitin sulfate in cartilage samples determined by using calibration curve of chondroitin-4-sulfate standard are higher than those obtained using chondroitin-6-sulfate standard (Table 3). Since the proportion of chondroitin-4-sulfate is higher than chondroitin-6-sulfate in most cartilage samples in this study except in shark cartilage (as shown by FTIR spectroscopy discussed in Section 3.3), we, therefore, report the value of sulfate GAGs assay using calibration curve of chondroitin-4-sulfate standard. Cartilages from crocodile hyoid and chicken keel were found to be rich in chondroitin sulfate, containing 14.84 and 14.08% by weight of dried cartilage, respectively (Table 3). The value obtained for chondroitin sulfate content of chicken keel cartilage in this experiment is close to the value (16.8%) reported by Luo et al. [5]. Chondroitin sulfate content of other cartilage samples are as follows: crocodile sternum cartilage 11.55% (13.9% reported by [13]) crocodile trachea cartilage 9.51%, shark fin cartilage 9.6%, crocodile rib cartilage 5.56%, and ray cartilage 5.27% (Table 3). The differences in value of chondroitin sulfate content involve the sources of cartilage: species, locations, as shown in this study. The different techniques used to extract and analyze chondroitin sulfate also attribute to the differences in values reported from the same source of cartilage. According to this study chicken keel, crocodile hyoid and sternum cartilage are the most promising potential sources of chondroitin sulfate. Crocodile trachea and shark fin cartilage are also feasible sources for isolation of chondroitin sulfate. The results on percentage yield of dried chondroitin sulfate extract (Table 2) are higher than the amount of chondroitin sulfate determined by sulfate GAGs assay (Table 3) indicated that other compounds such as proteins and minerals still remain in the extract. Purification steps should therefore be added if high purity of chondroitin sulfate is required.

Table 2
Yield and moisture content of dried chondroitin sulfate (CS) extracted from various sources of cartilage

<table>
<thead>
<tr>
<th>Source</th>
<th>Moisture content (%)</th>
<th>Percentage yield of dried CS extract&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shark fin</td>
<td>10.13</td>
<td>15.05 ± 0.73</td>
</tr>
<tr>
<td>Crocodile</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hyoid</td>
<td>9.47</td>
<td>27.37 ± 1.69</td>
</tr>
<tr>
<td>Rib</td>
<td>9.71</td>
<td>9.05 ± 0.99</td>
</tr>
<tr>
<td>Sternum</td>
<td>8.86</td>
<td>20.09 ± 1.05</td>
</tr>
<tr>
<td>Trachea</td>
<td>8.65</td>
<td>14.72 ± 1.95</td>
</tr>
<tr>
<td>Ray</td>
<td>10.75</td>
<td>7.49 ± 1.27</td>
</tr>
</tbody>
</table>

<sup>a</sup> Per dry weight of cartilage, values expressed as means ± S.D. (n = 3).

Fig. 2. Calibration curves using different chondroitin sulfate standards: chondroitin-4-sulfate (a) and chondroitin-6-sulfate (b).
Fig. 3. FTIR spectrum of chondroitin sulfate (a) FTIR spectrum of standard chondroitin-4-sulfate (b) FTIR spectrum of standard chondroitin-6-sulfate (c) FTIR spectrum of dried chondroitin sulfate extract from hyoid cartilage of crocodile (d) FTIR spectrum of dried chondroitin sulfate extract from rib cartilage of crocodile (e) FTIR spectrum of dried chondroitin sulfate extract from sternum cartilage of crocodile (f) FTIR spectrum of dried chondroitin sulfate extract from trachea cartilage of crocodile (g) FTIR spectrum of dried chondroitin sulfate extract from shark fin cartilage (h) FTIR spectrum of dried chondroitin sulfate extract from ray cartilage (i) FTIR spectrum of dried chondroitin sulfate extract from chicken keel cartilage. The figure on the bottom right is an enlargement of the spectrum indicated by the small rectangular area in the main figure.
3.3 Identification of types of chondroitin sulfate

The types of chondroitin sulfate were identified by FTIR spectroscopy potassium bromide pellet technique using chondroitin-4-sulfate and chondroitin-6-sulfate as standards (Fig. 3a and b). The spectra of the dried chondroitin sulfate samples extracted from various sources of cartilage are shown in Fig. 3c–i. The peaks in resulted spectra were identified for functional groups using data in Infrared Spectroscopy Atlas Working Committee [21]. Comparison of spectra of standard chondroitin-
4-sulfate and chondroitin-6-sulfate (Fig. 3a and b) revealed the presence of peaks at wave number 857 and 826 cm\(^{-1}\), respectively, therefore, the peak at approximately 857 cm\(^{-1}\) was used to identify chondroitin-4-sulfate and the peak at 826 cm\(^{-1}\) was used to identify chondroitin-6-sulfate. These results are in agreement with Uchisawa et al. [22] who reported peaks of chondroitin-4-sulfate and chondroitin-6-sulfate at wave number 854.5 and 823.7 cm\(^{-1}\), respectively. The spectra of all cartilage extracts exhibited peaks at both 856 and 824 cm\(^{-1}\) indicating that these cartilage samples consisted of both chondroitin-4-
sulfate and chondroitin-6-sulfate (Fig. 3c–i). The spectrum of shark fin cartilage extract (Fig. 3g) showed a more distinct peak at 857 cm⁻¹, indicating that they consisted of different proportions of chondroitin-4-sulfate and chondroitin-6-sulfate. The peak area correlates to some extent, to the quantity of chondroitin sulfate. However, in these experiments where FTIR-KBr pellet technique was employed, many factors affected the quantitative results, so the quantitative analysis of chondroitin sulfate in these experiments were reported based on sulfate GAGs assay as shown in Table 3.

4. Conclusion

It has been shown in this study that chicken keel, crocodile hyoid and sternum cartilage are the most promising potential sources of chondroitin sulfate. The value ranges from 11.55 to 14.84 g/100 g of dried cartilage, calculated as chondroitin-4-sulfate. Lower value is found in shark cartilage and crocodile trachea cartilage, ranges from 9.5 to 9.6 g/100 g of dried cartilage, which indicated their feasible sources for isolation of chondroitin sulfate.

Acknowledgement

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References


