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Production of crude chondroitin sulfate from duck trachea

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Abstract

This work was aimed to study the preparation and hydrolyzation of cartilage from duck trachea to obtain crude chondrotin sulfate(CS). Duck trachea is comprised of 66.19 ± 0.96% protein and 3.46 ± 0.15% CS (dry basis). HPLC chromatograms revealed that CS of duck trachea contained higher content of ΔDi-4S than ΔDi-6S with trace amount of ΔDi-diSs. To obtain cartilage part of the trachea, use of alkaline (0.05-0.1MNaOH at 4°C and RT for 1-6 h), enzyme (0.1-0.5% alcalase for 1-6 h) and heat (boiling for 1-120 min) treatments were studied. Heating by boiling in water caused the least CS loss among all studied treatments. The cartilage prepared by heat treatment was subjected to papain digestion (0.0625-1.0% for 1-10 h) and the yield, type and size of obtained CS were evaluated. The CS samples from 0.0625-0.25% papain hydrolysis for 1-10 h were similar in size (15-40 kDa). Up to 80% of CS was extracted by 0.25% papain digestion for 10 h. The freeze-dried hydrolysate from trachea cartilage contained 10.6% CS which was concentrated to 39% after protein precipitation.

Introduction

Chondroitin sulfate (CS) is one of the sulfated mucopolysaccharides (MPS), which are largely responsible for the high elasticity and resilience of cartilage tissue. It is comprised of repeating disaccharide units of sulfated D-glucuronic acid and N-acetyl-D-galactosamine attached covalently to core proteins in a form of proteoglycan (Nakano, 2000). CS has been known to play biologically important roles in a human body including a control of pericellular ions (Tanaka, 1978), protection of connective tissues (Bayliss *et al.*, 1999), management of osteoarthritis (Deal and Moskowitz, 1999; Hauselmann, 2001), prevention of cornea (Mac-Rae *et al.*, 1983), and anti-coagulative activity (Bjornsson *et al.*, 1982; Nishino and Nagumo, 1991). Since the discovery of these various functional activities, CS has been used as a functional material in medicine, cosmetics and in functional food. CS from shark cartilage is the most world wide popular product. Due to the increasing high cost of shark cartilage the other economical source such as swine and bovine cartilage are used in commercial production of CS. However, BSE outbreak and religious restriction on the consumption of pork have brought about needs for other alternative source. Many research works have been done in effort to extract MPS or CS from several sources such as sea cucumber, Ciona intestinalis, fish scale, chicken keel, egg shell and cartilages of skate, squid and crocodile (Gu *et al.*, 1999; Nakano *et al.*, 2001; Park *et al.*, 2001; Luo *et al.*, 2002; Sumi *et al.*, 2002; Jo *et al.*, 2004; Choi *et al.*, 2005; Garnjanagoonchorn *et al.*, 2006; Shin *et al.*, 2006). A non-animal CS has also been reported to be produced from microbial source (Schiraldi *et al.*, 2010). Thailand is one of top ten world exporting countries for poultry meat products. Thai agricultural statistic showed the rapid growing of the poultry meat industry, among which broiler and duck are the major products. Tons of tracheas of broiler and duck are discarded as waste from the processing line. Our previous study (Jarernviriyaparp and Vittayanont, 2009) showed that duck trachea possessed higher proportion of cartilage and CS content than that of broiler. This study therefore, was conducted to investigate the proper methods for preparation and extraction of crude CS from duck trachea cartilage as well as its composition and some basic properties.

Materials and Methods

Duck tracheas were obtained from processing plant of CPF Food Products Co., Ltd Thailand. They were washed thoroughly with running tap water and stored at -20°C until used. Alcalase 2.4L (E.C.3.4.21.62), Papain (EC 3.4.22.2), Chondroitinase ABC (EC 4.2.2.4) from *Proteus vulgaris*, unsaturated CS disaccharides (ΔUA-[1→3]-GalNAc: ΔDi-0S, ΔUA-[1→3]-GalNAc-6S: ΔDi-6S, ΔUA-[1→3]-GalNAc-4S: ΔDi-4S and 15-40 kDa chondroitin sulfate from shark cartilage were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were of analytical grade.

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Preparation of trachea cartilage by alkaline, enzyme and heat treatments

To prepare cartilage samples. Duck tracheas were cut into 1 cm. long then treated with (1) 10 volumes (v/w) of boiling water (97±2°C) for 2, 10, 30, 60 and 120 min before manually removing of the surrounding tissue, (2) 5 volumes (v/w) of 0.05M and 0.10 M NaOH at 4°C and room temperature (30±2°C) and (3) 0.1-0.5 % (of protein). Alcalase solution at 55°C pH 8 for 1-6 h. CS content in treated trachea was analyzed and its loss was calculated based on initial content of the trachea sample (raw material).

Preparation of crude CS from duck trachea cartilage by hydrolysis with papain

To study an optimal extraction condition, duck trachea cartilage was prepared by heat treatment (boiling in water for 1 h), ground and hydrolyzed by 0.0625-1.0% Papain for 1-10 h at 65°C, pH 7.0. The obtained hydrolysates were heated to 80°C for 10 min to inactivate enzyme activity and then centrifuged (Beckman Coulter RC-5B Plus, Beckman Coulter, Inc., CA, USA) at 7500×g for 30 min at 4°C to separate solid part before freeze-dried. The extracts were subjected to analyze for their CS type and content (yield) and molecular size by methods described in analyses section.

For preparation of concentrated crude chondroitin product, the liquid part of the extracts from centrifugation was added to 10% Trichloroacetic acid (TCA) to precipitate of proteins before dialysed against de-ionized water and freeze-dried.

Analyses

Proximate analysis

Proximate compositions (moisture, crude protein, fat and ash content) of duck trachea sample and hydrolysate products were analyzed following the AOAC (1999). Hydroxyproline content was determined using method of Kolar (1990). Muscle meat, ligaments and tendon were removed manually and the cartilage was weighed to determine the weight percentage of the cartilage in the trachea samples.

Analysis of type and content of chondroitin sulfate (CS)

For raw material (duck trachea sample), cartilage was first separated by soaking in hot water (75◦C) for 3 min then the surrounding meat and connective tissue were removed. The obtained cartilage was chopped and CS was extracted to obtain the initial content by method described in Ganjanagoonchorn *et al.* (2006). In brief, chopped cartilage was hydrolyzed by papain (4 mg/g of cartilage) at 65°C in 10x volume of 0.1M sodium phosphate buffer pH 7.0 containing 0.005M EDTA, 0.005M cysteine hydrochloride and 0.02% sodium azide for 48 h. Proteins in the hydrolysates were removed by adding TCA to the final concentration of 7% (w/v) followed by centrifugation. The obtained supernatant was dialyzed in distilled water and CS was purified by addition of 1.5% (w/v) cetylpyridinium chloride (CPC). The CS-CPC complex was collected by centrifugation. The precipitate was washed with 0.4M NaCl and centrifuged. The CS in the CPC complex were then isolated by using 2.5M NaCl solution then 1M Potassium thiocyanate was added to form cetylpyridinium thiocyanate precipitates. After filtration the partial purified CS solution was dialyzed against distilled water and lyophilized. For crude CS products, the freeze dried samples were first re-dissolved with distilled water and then followed all steps as mentioned earlier.

To determine the content and type of CS in all samples, the enzymatic depolymerization by chondroitinase ABC was performed following method used by Sim *et al.* (2007). A hundred µl of CS sample solutions (5 mg/ml) were mixed with 850 µl of Tris–acetate buffer. Then the samples were depolymerized with 50 µl of chondroitinase ABC (1 mU/µl) overnight at 37°C. After heating for 5 min and filtering through 0.2 µm filters, disaccharide composition in the depolymerized mixtures (100 µl) were analyzed with a Hypersil SAX column (4.6 x 250 mm, 5 µm) from Thermo Hypersil-Keystone (Bellefonte, PA, USA). After injecting the samples, the column was washed with water (pH 3.5) for 4.155 min corresponding to one column volume (CV). Then, a linear gradient of 0–1.0 M NaCl (pH 3.5) for 41.55 min (10 CV) was used with flow rate of 1.0 ml/min. and the profile was monitored at 232 nm.

Electrophoresis of CS samples

The molecular size of CS was analyzed by Polyacrylamide gel electrophoresis according to Cowman *et al.* (1984) and Sim *et al.* (2007) with some modifications. Freeze-dried samples were dissolved in Tris/Borate/EDTA (TBE) buffer pH 8.3 containing 2 M sucrose and 0.2% bromophenol blue. Fifty to hundred micrograms of CS samples were loaded to 12% polyacrylamide gel and were electrophoresed using Miniprotean III unit at a constant current of 15 mA in TBE buffer. The gels were stained for an hour with 0.5% Azur A in 1.0 % acetic acid then de-stained in 6:3:1 of H_2O: methanol: acetic acid for 90 min.

Results and Discussion

Chemical composition of duck trachea

Proximate analysis results (Table 1) indicate that protein is the major component of duck trachea (66.19±0.96%) with high content of collagen (as indicated by hydroxylproline content), followed by carbohydrate, ash and fat. The avian trachea is composed of stacked cartilage rings which tightly linked together by fibrous connective tissue. Type I and II collagen are protein compositions of connective tissue and cartilage, therefore collagen is found in a high proportion of total protein of duck trachea.

Since cartilage is a major source of chondroitin sulfate (CS), the proportion of cartilage in the trachea was determined along with the content and type of CS. The results showed that duck trachea contained approximately 70-75% weight of cartilage. The total CS content (dry basis) of duck trachea samples was 3.464±0.154% (Table 2) or about 4.7% of the cartilage part (as calculated from 72.5% cartilage in trachea). The CS contents (% dry basis) of other cartilage sources have been reported such as 11.55% in crocodile sternum cartilage, 9.51% in crocodile trachea cartilage, 9.6 % in shark fin cartilage, 5.56% in crocodile rib cartilage, 5.27% in ray cartilage 10-11% in bovine trachea cartilage and 30.08% in chicken keel cartilage (Lagocka *et al*., 1997; Lou *et al*., 2002; Garnjanagoonchorn *et al.*, 2006). The differences in CS content depend on the source of cartilage. Moreover, the extraction or preparation and analytical techniques for CS analysis could cause variation of the obtained data (Garnjanagoonchorn *et al.*, 2006; Walker, 2006).

The types of CS in duck trachea were identified by their molecular compositions using SAX-HPLC. The chromatogram of the duck trachea CS composition is shown in Figure 1b in comparison with those of standard disaccharide of chondroitin-0-sulfate (ΔDi-0S), chondroitin-4-sulfate (ΔDi-4S) and chondroitin-6-sulfate (ΔDi-6S) (Figure 1a). The duck trachea contained chondroitin 4-sulfate and chondroitin 6-sulfate as the major component with trace amount of chondroitin disulfates (ΔDi-diSs). Chondroitin 4-sulfate and chondroitin 6-sulfate are the most abundant GAG in animal body and occur both in skeletal and soft connective tissue. The sulfation pattern of chondroitin disaccharides from normal human cartilage varies with age, topography of the joint surface, and the zone of cartilage examined (Bayliss *et al.*, 1999). Sim *et al.* (2007) reported that CS from land animal source had ΔDi-4S/ΔDi-6S+ΔDi-diSs ratio higher than one, while CS from shark cartilage which is a marine animal had ΔDi-4S/ΔDi-6S+ΔDi-diSs ratio less than one. This value is quite unique and has been proposed to be used as an identifying characteristic of CS from marine animal source. Since we found that CS from duck tracheas contained only trace amount of ΔDi-diSs the ratio of ΔDi-4S/ΔDi-6S was thus calculated and used. Results in Table 2 showed that the ΔDi-4S/ΔDi-6S ratio (2.37) of duck trachea sample was higher than one which is the characteristic of CS from land animal source.

Table 1. Proximate composition and Hydroxyproline (HyP) content of duck trachea

Sample	Composition (%)[a]				
	Protein[b]	Fat	Ash	Carbohydrate[c]	HyP
Duck trachea	66.19±0.96	9.27±0.05	11.48±0.03	13.06	4.11±0.07

[a] Based on dry weight of raw material. Values are expressed as means ± S.D. (n = 3)
[b] Factor N = 6.25
[c] Calculated by difference

Table 2. Type and content of chondroitin sulfate in duck trachea

Sample	Content (%)*				ΔDi-4S/ ΔDi-6S
	ΔDi-0S	ΔDi-6S	ΔDi-4S	Total CS	
Duck trachea	0.040±0.007	1.016±0.048	2.408±0.112	3.464±0.154	2.370

*Based on dry weight of raw material. Values are expressed as means ± S.D. (n = 3).

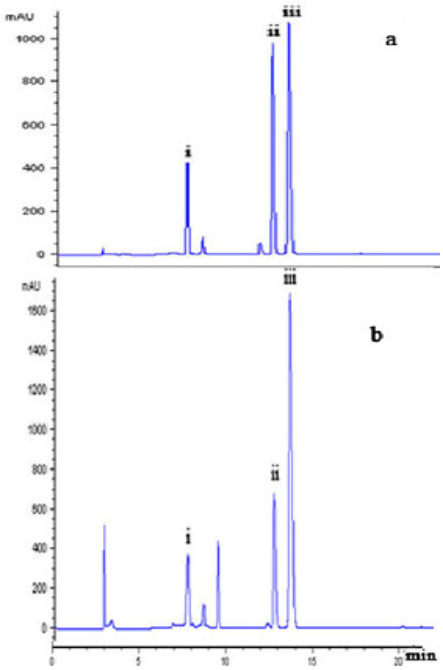


Figure 1. Chromatogram of disaccharides produced by enzymatic depolymerization of chondroitin sulfate from duck trachea (b) as compared to that of standard CS (a) where (i): ΔDi-0S, (ii): ΔDi-6S, and (iii): ΔDi-4S.

Effect of alkaline, enzyme and heat treatment on CS of duck trachea cartilage

As CS deposit mainly in cartilage part therefore a removal process of outer tissue from the trachea is needed to obtain high concentration and purity of CS. Figure 2a-c showed that alkaline treatment caused higher loss of CS (up to 50%) than did the enzyme and heat treatments, respectively. The increase of treatment time and temperature, and the

Table 3. ΔDi-4S/ΔDi-6S ratio of CS from duck trachea treated with alkali, enzyme and heat

Treatment	Treatment time	ΔDi-4S /ΔDi-6S
Raw material	-	2.41^{ab}
0.05 M NaOH, 4^{o}C	1h	*2.55^{a}*
	3h	*2.30^{b}*
	6h	0.75^{d}
0.05 M NaOH, RT	1h	0.94^{c}
	3h	0.92^{c}
	6h	0.91^{c}
0.1 M NaOH, 4^{o}C	1h	0.76^{d}
	3h	0.80^{d}
	6h	0.79^{d}
0.1 M NaOH, RT	1h	0.79^{d}
	3h	0.81^{d}
	6h	0.81^{d}
0.1% Alcalase	1h	2.15^{bcd}
	2h	2.02^{bcd}
	3h	2.44^{b}
	6h	1.73^{d}
0.3% Alcalase	1h	1.69^{d}
	2h	1.80^{cd}
	3h	1.80^{cd}
	6h	1.72^{d}
0.5% Alcalase	1h	2.48^{a}
	2h	2.30^{b}
	3h	2.08^{bcd}
	6h	2.25^{bc}
Heat in boiling water	2 min	2.84^{a}
	10 min	2.78^{a}
	30 min	2.59^{b}
	60 min	2.77^{a}
	120 min	2.81^{a}

* Values are expressed as means ± S.D. (n = 3)
a, b, c The different superscript within the column denoted significant difference (p < 0.5)

Table 4. Major composition of freeze dried hydrolysate from trachea cartilage compared with raw material (whole trachea or cartilage part)

Samples	Composition* (% dry weight)				
	Protein**	Fat	Ash	Hydroxy-proline	CS
Trachea	73.12±0.10	5.87±0.45	11.36±0.69	5.70±0.95	4.28
Cartilage	62.01±0.74	1.16±0.22	12.94±0.21	5.54±0.14	7.93
Hydrolysate (1 h)	75.002±1.98	1.74±0.05	4.79±0.17	7.93±0.12	9.71
Hydrolysate (10 h)	70.22±1.12	0.55±0.02	5.58±0.31	7.78±0.07	10.63

* Values are expressed as means ± SD (n = 3)
** Factor N = 6.25

concentration of alkali or enzyme enhanced the CS loss from the trachea samples. Alkali solution has widely used for solubilization of proteins however proteoglycan in cartilage is also susceptible to alkaline condition. Herbage *et al.* (1977) reported that more than 90% of proteoglycan could be eliminated from bovine articular cartilage by soaking in 0.2 M NaOH at 4^{o}C for 24 h. The β-elimination reaction by dilute alkali is responsible for the release of free CS from core protein by cleavage of the xylose-serine bond (Hardingham, 2007). In this study, the enzyme (alcalase) treatment of tracheas was aimed to eliminate only proteineous tissue surrounding the cartilage. However the increasing loss of CS over the treatment time (Figure 2b) was observed indicating the enzyme activity throughout the thin structure of duck trachea. On the other hand, heating in boiling water for 10 min resulted in negligible loss of CS (less than 1%) and around 10% CS loss was observed

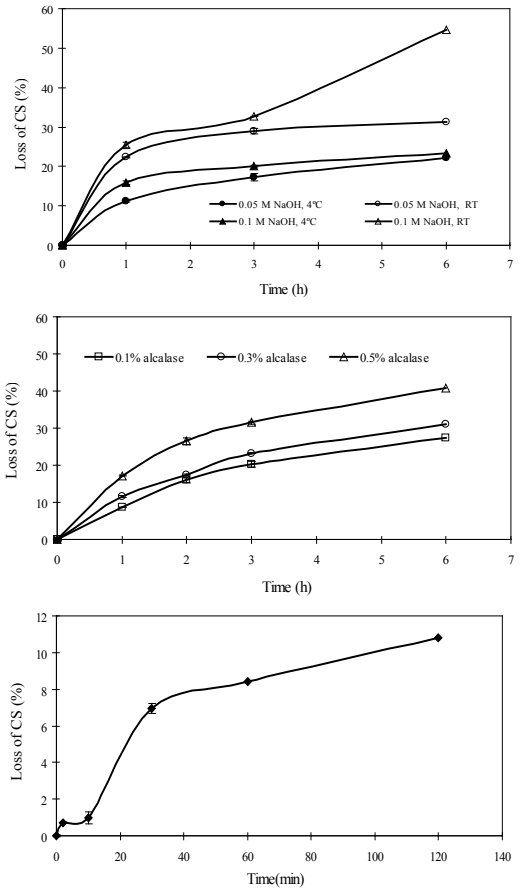


Figure 2. Loss of chondroitin sulfate (A) after soaking in 0.05 M and 0.1 M NaOH at 4oC and room temperature (RT), (B) after treatment with 0.1-0.5 % alcalase at 55oC, pH 8 and (C) after boiling for 2, 10, 30, 60 and 120 min

after 2 h (Figure 2c).

It should be noted that as low as 0.05M NaOH treatment at room temperature reduced the ratio of ΔDi-4S/ΔDi-6S of the trachea CS from 2.4 to less than one (Table 3). This was caused by the decrease of ΔDi-4S when the alkali concentration and soaking time and temperature increased. Gu *et al.* (1999) reported the possible loss of sulfated MPS extracted from *Ciona intestinalis* in 0.1 N NaOH at 100^{o}C which might be caused by the degradation of MPS polymers to monosaccharides and sulfates. There were reports showed that the ratio of ΔDi-4S/ΔDi-6S+ΔDi-diSs directly correlated to the source of CS (marine or land animal). CS from marine source especially shark cartilage, the most popular and expensive among all commercial CS products, has high amount of CS-6 and poly-sulfated CS resulting in a low value (less than 1) of ΔDi-4S/ΔDi-6S+ΔDi-diSs ratio while higher than 1 value was found in CS from land animal (Sim *et al*., 2005). Our result therefore, indicates a caution for adopting such ratio as authentic identification of shark CS in products using alkali treated material or alkaline condition for

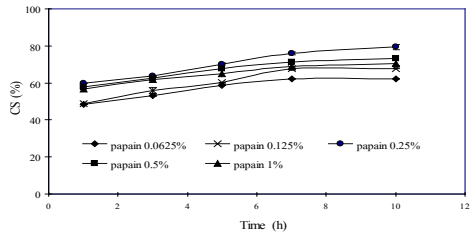
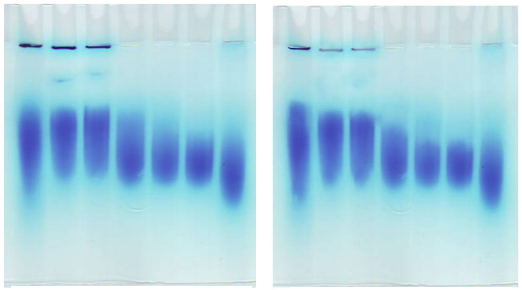


Figure 3. Extraction yield of CS from duck trachea cartilage by hydrolysis with 0.0625-1.0 % enzyme papain for 1-10 h at 65°C



(a) (b)

Figure 4. PAGE patterns of CS hydrolyzed from duck trachea cartilage using 0.0625, 0.125, 0.25, 0.5 and 1.0 % papain (lane 1-5) for 1 h (a) and for 10 h (b) compared to shark cartilage CS of 15-40 kDa (Std) and commercial CS product (C)

CS preparation.

Extraction yield, CS molecular size and chemical composition of hydrolysate product from duck trachea cartilage

To extract CS from the cartilage, hydrolysis with different concentration of papain at various times was performed. The CS yield and size obtained from different hydrolysis conditions were monitored. Figure 3 shows that over 50% of initial CS of duck trachea cartilage was hydrolyzed in the first hour and almost 80% was free from the cartilage matrix in 10 h of hydrolysis by 0.25% papain. Papain exhibited the highest hydrolysis efficacy at 0.25% concentration when compared to those of 0.5 1.0 0.125 and 0.0625%, respectively. The increasing of papain concentration over 0.25% did not promote the extracting rate of CS from the cartilage indicating limit access to the intact matrix form of substrates. After 3 h of hydrolysis, higher yield was observed in sample using 0.25% than those of 0.5 and 1.0%, respectively suggesting change in substrate preference of the enzyme. At high papain concentration, the enzyme in the system may have turned to hydrolyze proteoglycan aggregates earlier cleaved from the cartilage to the smaller size of aggrecans fragments and CS. In Figure 4, the molecular size of CS hydrolyzed by 0.0625-1.0% papain for 1 and 10 h was in the range of 14-40 kDa compared to that of standard CS. Aggregates or clusters of CS were noticeable in the well bottom of standard and samples from 0.0625 and 0.125% papain digestion. However, the vertical band width of CS obtained from the hydrolysis on PAGE was reduced when the papain concentration increased over 0.25% indicating the loss of high molecular size CS fractions. The enzymatic extractions of sulfated MPS including CS from different sources such as shark cartilage (Jo *et al*., 2005), bovine trachea (Lagocka *et al*., 1997), skate cartilage (Lignot *et al*., 2003; Jo *et al*., 2004), chicken keel (Luo *et al*., 2002; Shin *et al*., 2006), sea cucumber (Park *et al*., 2001) and fish scale (Sumi *et al*., 2002) have been reported. The protease activity and hydrolysis efficiency were different based on the type and preparation of raw material, type and concentration of enzyme as well as hydrolysis conditions. Papain and Alcalase have been reported as the most frequently used enzyme. The molecular weight and the molecular composition of CS fraction are affected by the extraction method. Heinegard and Hascall (1974) compared the activity of papain and trypsin on the digestion of proteoglycans and reported that papain cleaves at many sites and results in free single CS chains with four to six attached amino acids and many free peptides. In contrast, trypsin cleaves at fewer sites and generates clusters or fragments with two to ten CS chains attached to peptide. It has been shown that low molecular weight CS as a superior kinetic profile than high molecular weight. Its bioavailability or the absorption has been reported to be affected by the chain length of the molecule. Adebowale *et al.* (2000) studied CS permeability using Caco-2 cell monolayers to examine the intestinal transport of several marketed sources of CS compared to the reference standard CS (16,900 Da) which has been shown to be efficacious and bioavailable in many European and US trials. They found that the molecular weight of CS as a direct influence on its permeability across the gastrointestinal tract, where higher permeability is observed for CS with lower molecular weight.

Weight yield and major chemical composition of the freeze-dried hydrolysate prepared from heat-treated cartilage were determined (Table 4). The total weight of 33% and 46% of freeze-dried products were obtained from 1 and 10 h hydrolysis, respectively. The major composition of the products was protein (70.2-75.0%) with high amount of HyP (7.78-7.93%), suggesting collagen as the major protein constituent. The CS content (9.7-10.6%) was more than twice increased from that of raw material (4.2%) and can be concentrated up to 39% by TCA precipitation of proteins (data not shown).

Conclusion

Duck trachea is a potential source of chondroitin sulfate (CS). Alkaline treatment used for preparation of the cartilage part caused high loss of CS and change in molecular composition. The major compositions of CS extracted from duck trachea cartilage are active types (CS-4 and CS-6) with molecular size fall within the commercial range. Further concentration of CS by precipitation of proteins or glycosaminoglycan or by specific chromatography could enhance the purity of CS for the use in pharmaceutical grade products.

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