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Detection of keratan sulfate by immunological methods in commercial chondroitin sulfate preparations

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ABSTRACT

Chondroitin sulfate (CS), a well known nutraceutical, and keratan sulfate (KS) are glycosaminoglycans involved in the structure of cartilage proteoglycan, aggrecan. Since CS is extracted from cartilage, there may be a possibility that purified CS is contaminated with small amount of KS. A total of 15 samples, including four samples of CS as laboratory reagents, one sample of CS as a food additive and ten samples of dietary supplements containing CS were examined to detect KS in these samples by using immunodiffusion and enzyme-linked immunosorbent assay (ELISA) with anti-KS monoclonal antibody (IgM). With the exception of three samples of CS as laboratory reagents, all samples were found to contain varying amounts of KS. It was concluded that both the immunodiffusion, a quick one-step method, and ELISA for quantification, are reliable methods to detect KS contamination in CS products.

1. Introduction

Chondroitin sulfate (CS) is an acidic polysaccharide (or glycosaminoglycan, GAG) comprised of repeating disaccharide units of N-acetylgalactosamine and glucuronic acid (Lamari & Karamanos, 2006; Nakano, Betti, & Pietrasik, 2010; Rodén, 1980; Wight, Heinegård, & Hascall, 1991). The disaccharide unit is usually sulfated at either the C-4 or C-6 position of N-acetylgalactosamine. Keratan sulfate (KS) is also a GAG comprised of repeating disaccharide units of N-acetylglucosamine and galactose. Both CS and KS are involved in the structure of aggrecan, which is a major proteoglycan abundant in cartilage accounting for 5–10% of the tissue wet weight (Heinegård & Oldberg, 1989). In bovine cartilage aggrecan, approximately 100 CS chains with molecular mass of 10–25 kDa and 30–60 KS chains with molecular mass of 4–8 kDa are covalently attached to the core protein as GAG side chains (Hascall, 1977). In the extracellular matrix of articular cartilage, aggrecans with highly hydrophilic polyanionic CS chains interact with GAG hyaluronic acid to form large aggregates, which is believed to contribute to the resilience of load-bearing tissue (Mow, Ratcliffe, & Poole, 1992). Cartilage tissues are also known to contain dermatan sulfate proteoglycans, decorin and biglycan (Rosenberg et al., 1985) and KS proteoglycan, fibromodulin (Heinegård et al., 1986) as minor constituents accounting for 1–2% of the total mass of cartilage proteoglycans (Heinegård & Oldberg, 1989). KS in fibromodulin (KS-I) is distinguished from most of KS in aggrecan (KS-II) by the structure of linkage to the core protein, in that KS-II is O-glycosidically linked to serine or threonine, whereas KS-I is N-glycosidically linked to asparagine (Barry et al., 1995; Plaas, Neame, Nivens, & Reiss, 1990).

CS extracted and isolated from other GAGs present in cartilage has a wide range of applications in food, cosmetic and pharmaceutical industries. Oral administration of CS was reported to be beneficial in maintaining healthy articular cartilage or treatment of osteoarthritis (Uebelhart et al., 2004; Volpi, 2009), and thus dietary supplements containing CS has been popular products seen in health food market.

Several groups of researchers (Adebowale, Cox, Liang, & Eddington, 2000; Ji, Roman, Zhou, & Hildreth, 2007; Sakai, Otake, Toida, & Goda, 2007; Sim et al., 2005, 2007; Volpi & Maccari, 2008) have determined the content of CS or its disaccharide composition in commercially available samples of dietary supplements. Volpi (2009), in a review of the quality of commercial CS preparations, reported presence of hyaluronic acid as a contaminant in dietary supplement samples. This author, however, did not discuss the possibility of contamination with KS (derived from aggrecan and fibromodulin) or dermatan sulfate (from decorin and biglycan) in CS preparations. There is unlikely any report of dermatan sulfate contamination in CS preparations. However, Møllar, Møllar-Pedersen, Damsgaard, and Poulsen (1995) reported KS contamination in a commercial preparation of CS from shark cartilage (see below for the method of detection). More recently,

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Table 1
Results of immunodiffusion analysis.

Sample no.	Product	Source	Immunoreaction	Keratanase treatment
1	CS (laboratory reagent)	Porcine rib cartilage	–	nd[a]
2	CS (laboratory reagent)	Sturgeon cartilage	–	nd
3	CS (laboratory reagent)	Shark cartilage	–	nd
4	CS (laboratory reagent)	Bovine tracheal cartilage	+	–
5	CS (food additive)	No information on the label	+	–
6	CS/glcosamine-sulfate	Bovine cartilage	+	–
7	CS/glcosamine-sulfate	Bovine tracheal cartilage	+	–
8	CS/glcosamine-sulfate	Bovine cartilage	+	–
9	CS/glcosamine-HCl	Bovine, porcine and avian cartilages	+	–
10	CS/glcosamine-HCl	No information on the label	+	–
11	CS/glcosamine-sulfate	Bovine cartilage	+	–
12	CS/glcosamine-sulfate	Bovine cartilage	+	–
13	CS/glcosamine-sulfate	Bovine cartilage	+	–
14	CS/glcosamine-sulfate	Bovine cartilage	+	–
15	CS/glcosamine-HCl	Bovine cartilage	+	–
16	Standard KS	Human costal cartilage	+	–
17	Standard C4S	Sturgeon notochord	–	nd
18	Standard C6S	Cranial cartilage of sturgeon	–	nd
19	Glcosamine-HCl	Shrimp/crab exoskeleton	–	nd
20	Glcosamine-sulfate	Shrimp/crab exoskeleton	–	nd

+: Positive result with precipitin line formation.
–: Negative result with no visible precipitin line formation with 40 μg of CS sample/well or with approximately 40 μg of dietary supplement sample containing average 12 μg SGAG per well.
[a] Not determined.
See text for other details.

Pomin, Piquet, Pereira, and Mourão (2012) analyzed samples of CS formulations for oral administration prepared from shark and bovine cartilages, and reported presence of significant amounts of KS in samples prepared from shark cartilage but not from bovine cartilage. These authors suggested bovine tissues as a preferable source of CS. Since both CS and KS chains are covalently attached to the same core protein of cartilage aggrecan (see above), there may be a possibility of the presence of KS as a contaminant in CS preparations, if purification processes are not efficient. To detect KS, Pomin et al. (2012) used several techniques including agarose gel electrophoresis, strong anion exchange HPLC, digestion with specific GAG lyases including keratanase and chondroitin AC lyase, and nuclear magnetic resonance spectroscopy.

An alternative method to detect KS is the use of antibody specific to this GAG. It turned out that immunodiffusion is a very applicable method to detect KS in CS preparation with small amounts of sample due to its simplicity, specificity, and straightforward application (Nakano, Pietrasik, Ozimek, & Betti, 2012; Srichamroen, Nakano, Pietrasik, Ozimek, & Betti, 2013). Møllar et al. (1995) used an enzyme-linked immunosorbent assay (ELISA) with anti-KS monoclonal antibody (5D4) to detect KS in a low grade commercial CS (>120 kDa) from shark cartilage. As far as we know, however, there has been no published information available on the application of immunological method to detect KS in commercial samples of dietary supplements containing CS.

KS free CS as a research chemical is needed as a standard CS for either quantitative or qualitative analysis of this GAG. Dietary supplement containing KS free CS is also needed to determine the effect of oral administration of CS on the serum concentration of KS, a metabolite of cartilage proteoglycan, which may be affected in patients suffering from osteoarthritis (Thonar et al., 1985).

Analysis of KS may be important for the quality control in CS producing factories, and thus development of a simple sensitive method to detect KS in CS preparation is needed. This study was undertaken to determine whether KS can be detected by using immunodiffusion and ELISA methods in commercially available samples of CS and dietary supplements containing CS.

2. Materials and methods

2.1. Materials

Samples of CS as laboratory reagents (Table 1) included chondroitin sulfate A from porcine rib cartilage and bovine tracheal cartilage, both obtained from Sigma–Aldrich Canada Ltd., Mississauga, ON, Canada, and chondroitin sulfate A from sturgeon notochord and chondroitin sulfate C from shark cartilage, both obtained from Seikagaku Corporation, Tokyo, Japan. A sample of CS as a food additive (Table 1) was obtained from a supplier in the United States. All CS samples were in the form of powder each in a sealed bottle.

A total of 10 samples of dietary supplements containing CS (each from different supplier) (Table 1) were obtained from health food stores in Edmonton, Canada. Most of CS in these samples was derived from bovine cartilage as shown in the table. They were in the form of capsuled powder (five samples) or gel (one sample) and tablets (four samples). Samples of dietary supplements including glucosamine-HCl and glucosamine-sulfate both in the form tablets were obtained from health food stores in Edmonton, Canada. All tablets were powdered using a motor and pestle.

Standard GAGs including chondroitin 4-sulfate (C4S) from sturgeon notochord, chondroitin 6-sulfate (C6S) from sturgeon cranial cartilage, KS from bovine nasal cartilage and hyaluronic acid from human umbilical cords were gifts from Drs. M. B. Mathews and J. A. Cifonelli, University of Chicago, USA. Ascites fluid containing anti-KS monoclonal antibody, AH12 is an IgM raised against bovine fibrocartilage CS proteoglycan containing KS (Nakano, Imai, Koga, Dodd, & Scott, 1993). By using an ELISA inhibition assay, Nakano et al. (1993) reported a much higher antigenicity in KS-peptide prepared from bovine fibrocartilage CS proteoglycan compared to bovine corneal KS (KS-I) [concentrations for 50% inhibition or IC_{50} being >70 times lower in KS-peptide than in corneal KS under the experimental condition used by Nakano et al. (1993)]. AH12 has been used for determinations of KS by immunodiffusion (Nakano et al., 1993, 2012; Srichamroen et al., 2013), immunohistochemical localization (Nakano et al., 1993; Nakano, Sim, Imai, & Koga, 1996; Nakano, Imai, Koga, & Sim, 1996; Sunwoo, Nakano, Hudson, & Sim, 1998) and ELISA (Nakano & Sim, 1995; Nakano & Scott, 1996).