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A Simple Quality Evaluation Method for Proteoglycan after Addition to Beverages

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Abstract: Over the past 10 years, many products utilizing the functionality of salmon cartilage proteoglycan have come on the market, and consumer awareness of proteoglycan has increased. During this period, the biggest issue has been how to evaluate the amount and quality of proteoglycan in the cartilage extract blended in the products. In this study, we propose an immunological method that can easily evaluate the amount and quality of proteoglycan in the proteoglycan-containing compositions. By the present method, it is possible to evaluate not only the retention of the functional domains of the core protein of proteoglycan, but also that of chondroitin sulfate chains linked to the core protein. Furthermore, the binding activity of proteoglycan to hyaluronan can be evaluated if hyaluronan is used as a probe instead of an antibody. This method is expected to be useful for proteoglycan quality evaluation during the manufacturing process and product storage.

Key words: proteoglycan, aggrecan, domain, chondroitin sulfate, hyaluronan, cartilage extract

The major proteoglycan (PG) of cartilage is aggrecan which has a high-molecular-weight and complex structure carrying multiple chondroitin sulfate (CS) chains on a core protein.[1] Aggrecan has functional domains on a core protein including the globular domain 1 (G1 domain) having a hyaluronan (HA) binding site, the glycosaminoglycan attachment domain, and the globular domain 3 (G3 domain) having epidermal growth factor (EGF)-like module(s),[1)2)] and these domains are conserved among species. Salmon aggrecan also has all functional domains like the mammalian aggrecan[3] (Fig. S1; see J. Appl. Glycosci. Web site). Hereafter, in this paper, the term "PG" in cartilage extracts refers to aggrecan.

There is extensive research on new functions and uses of salmon cartilage extracts containing PG. For example, the suppression of photo-aging in UV-induced skin aging model mice[4] as well as inhibition of the angiogenesis in *in vitro* experiments[5] by cartilage extracts have been reported although there were differences in the extract-preparation method. Cartilage extracts having such functions are expected to be useful materials for functional foods and cosmetics, and various products containing PG are already commercially available.

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Abbreviations: PG, proteoglycan; CS, chondroitin sulfate; G1 domain, globular domain 1; HA, hyaluronan; G3 domain, globular domain 3; EGF, epidermal growth factor; HABP, hyaluronan binding protein.

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PG does not always maintain its complete structure depending on the extraction method.[6)7)] The structure of the core protein of PG is susceptible to degradation or deactivation depending on the conditions such as pH value, temperature, or proteases activity when blended into foods, beverages, and cosmetics, and the subsequent storage conditions, whereas the CS structure is relatively stable. The effectiveness of PG may depend on the complete structure of high molecular weight, or on the specific functional domain structure of PG even if it is degraded. It is important that PG in the products maintains the initial amount, structure, and function until the products are used by the consumer. Therefore, a simple method to detect both structure and amount of blended PG in products without any purification was eagerly sought. In this study, in view of this background, we demonstrated a method that can easily assess the amount and structural condition of PG in the PG-containing composition by immunologically measuring multiple domains or the module. By this method, the retention and type of CS binding to the core protein are known in addition to the structural condition of the functional domains of the core protein.

In these experiments we used the same hot water extract from salmon cartilage as was used in a previous report.[7] Mouse monoclonal antibody, 12/21/1-C-6, recognizing the G1 domain of rat PG, was from the Developmental Studies Hybridoma Bank of the University of Iowa, USA. Anti-rabbit polyclonal antibody against the synthetic peptide ([2277]DGHPMQFENWRPNQPDN[2293]) in the G3 domain of human PG was from Affinity BioRe-agents (Golden, CO, USA). Anti-EGF-like module of salmon PG antibody was generated by immunizing rabbits with synthetic peptide

([1069]RDLCEPNQCGTGTCSVQDGI[1088]) with amino-terminal for conjugation with bovine thyroglobulin (Immuno-Biological Laboratories Co., Ltd., Gunma, Japan) to obtain three lots of good antibodies. Antibodies CS-56 and 3-B-3, recognizing CS or CS stubs generated by chondroitin ABC lyase (from *Proteus. vulgaris*, EC 4.2.2.4), respectively, were from Seikagaku Biobusiness Co. (Tokyo, Japan). Peroxidase-conjugated goat anti-rabbit immunoglobulins, peroxidase-conjugated rabbit anti-mouse immunoglobulins, and peroxidase-labeled streptavidin were from Dako Japan Inc. (Tokyo, Japan). Chondroitin ABC lyase and biotinylated HA-binding protein (HABP) were from Seikagaku Biobusiness Co. (Tokyo, Japan). HA (from *Streptococcus. zooepidemicus*, average M_r of $1-1.4 \times 10^6$) was from Food Chemifa Co., Ltd. (Tokyo, Japan).

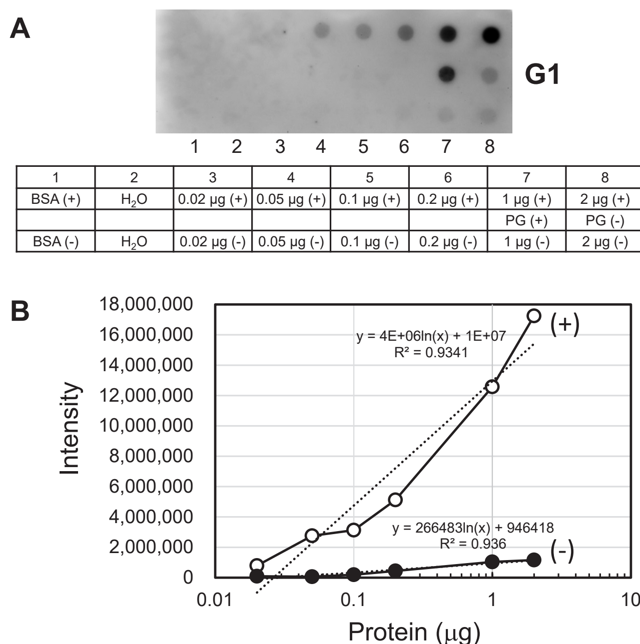
Immunological analysis was performed on PG-containing compositions blotted onto a PVDF membrane (Millipore Corporation, Billerica, MA, USA) according to the method of Towbin[8] using the ECL system (GE Healthcare Japan Corporation, Tokyo, Japan) for detection. Hot water extract from salmon cartilage with or without reduced-alkylation[9] or the extract-added beverages without reduced-alkylation, were blotted onto a PVDF membrane (Millipore Corporation) using a slot blotter (Scie-Plas Ltd., Cambridge, UK), and probed with antibodies recognizing the G1 domain, the G3 domain, the EGF-like module, or CS chains. When using the antibody 3-B-3 which recognizes unsaturated chondroitin 6-sulfate disaccharide structure covalently linked to a core protein, samples were treated with chondroitin ABC lyase for 2 h before blotting on the PVDF menbrane. In the HA binding experiment, HA was used as a non-immunological probe and detected by the ECL system. Biotinylated HABP and peroxidase-labeled streptavidin were used to detect HA bound to PG via the G1 domain. Chemiluminescent signals were analyzed by ChemiDoc XRS+ system (BioRad Laboratories, Inc., Hercules, CA, USA). Protein content was determined by the method of Bradford.[10]

As a quantitative simulation of PG in beverage, hot water extract from salmon cartilage was added to the centrifugal supernatant of various commercially available beverages to 12.5 μg/mL (for detection of G1) or 2 μg/mL (for HA binding experiment) and each mixture was analyzed immediately or after allowing to stand for a week at 4 °C, by the present method without reduced-alkylation, probed with the antibody recognizing the G1 domain or with HA. Information about beverages used in this study (pH values and ingredients) are shown in Table S1 (see J. Appl. Glycosci. Web site).

Even without reduced-alkylation, standard curves sufficient for detection were obtained in the range of 0.02 to 2 μg of hot water extract from salmon cartilage in the case of the antibodies to detect the EGF-like module, the CS linked to core protein (3-B-3), and the G3 domain (Figs. S2, S4, and S5; see J. Appl. Glycosci. Web site). Detection of domains were difficult in the case of an antibody for the G1 domain and CS-56 in this range of protein amount (Figs. 1 and S3; see J. Appl. Glycosci. Web site), however, a good standard curve was obtained for the G1 domain in 0.2 to 20 μg of hot water extract from salmon cartilage (Figs. 2A and B). Therefore, we decided to perform dot blot analysis without reduced-alkylation as our basic quantitative method for PG.

Domains of PG in the beverages to which the hot water extract was added could also be detected adequately by this method. An example of the result of G1 domain is shown in Figs. 2A and C. The reason why the signal of the G1 domain in some samples was lower than that of distilled water (control), even in the analysis immediately after adding the hot water extract, is not clear from the present experiment alone. However, the method proposed here would be useful for exploring the reason by changing conditions of beverages to which the PG is exposed. A possible explanation for this observation from the information in Table S1 (see J. Appl. Glycosci. Web site) is high pH in the case of ionized alkaline water. In the beverage samples allowed to stand at 4 °C for a week after addition of the extracts, reactivity with the antibody against G1 domain seemed to be lower than those analyzed immediately after adding the extract. It is also unclear whether this is an effect due to the coexisting compounds in beverages from the present data alone. In the case of unsweetened Japanese green tea (with matcha powder) and unsweetened apple juice, the signal was observed in white spots (Fig. 2A). Common components in the two beverages are considered plant-derived compounds like polyphenols. Polyphenols interact with proteins non-covalently or covalently and these interactions can interfere with immunological reactions.[11,12]

In order to examine whether the detected G1 domains retained ability to bind to HA, the G1 domains were analyzed non-immunologically using HA as a probe for the same beverages containing hot water extract from cartilage. The standard curve of the extract did not show linearity above 0.2 μg (Fig. 3B). All beverages in which the G1 domain was detected other than protein-containing drinks showed binding ability to HA, and no significant decrease in binding ability was observed even 1 week after addition (Figs. 3A



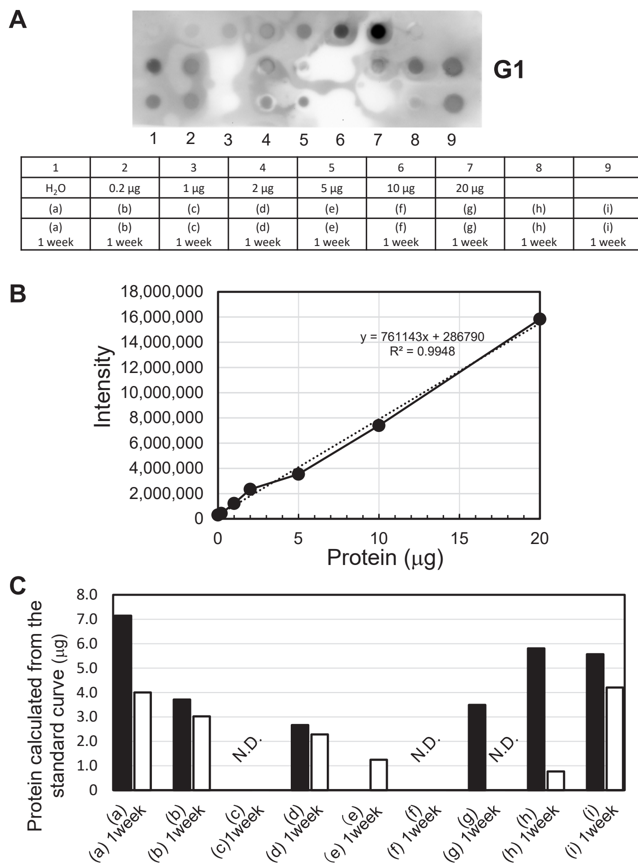
1	2	3	4	5	6	7	8
BSA (+)	H_2O	0.02 μg (+)	0.05 μg (+)	0.1 μg (+)	0.2 μg (+)	1 μg (+)	2 μg (+)
						PG (+)	PG (-)
BSA (-)	H_2O	0.02 μg (-)	0.05 μg (-)	0.1 μg (-)	0.2 μg (-)	1 μg (-)	2 μg (-)

Fig. 1. Effect of reduced-alkylation of PG-containing extract on the reactivity to the antibody against the G1 domain.

A, Dot blot image of the G1 domain detected immunologically (upper). Spot positions of hot water extract from salmon cartilage with known protein amount (lower). B, Standard curves derived from the dot blot analysis. BSA, 0.2 μg of bovine serum albumin; PG, 0.2 μg of purified salmon PG; (+), with reduced-alkylation; (–), without reduced-alkylation.

and C). A possible consideration for this is that it was difficult to observe the differences because the amount of protein applied was 0.2 μg, which is in the concentration range that did not show linearity on the protein amount-signal standard curve. In the case of the water and the sports drink, signals of G1 were significantly reduced 1 week after the addition of cartilage extract, whereas the binding ability to HA compared well with those of immediately after addition. This may indicate that even if part of the G1 domain is lost and the reactivity with the anti-G1 antibody is reduced, the HA binding ability is not affected. An explanation of this may be that if is due to the difference between the site recognized by the anti-G1 antibody and the site binding to HA. As in the case of immunological detection using an antibody against the G1 domain as a probe, in green tea and apple juice white spots were observed in non-immunological detection with HA. Interactions between polysaccharides and polyphenols, and formation of ternary complexes of protein-polysaccharide-polyphenol are also reported.[13] There is a possibility that interactions with hyaluronan involving phenolic compounds like catechin in tea or pectin in apple juice interfered with the non-immunological analysis using hyaluronan as a probe. In addition, a protein supplemental drink became slightly white although the appearance of the white spots was a little different from those in the tea and apple juice (Fig. 3A). The protein supplemental drink does not contain polyphenols but does contain animal proteins that may interfere with the binding between the G1 domain and HA. A similar result was obtained when HA with different molecular weight (M_r = 80,000) was used for the probe (data not shown).

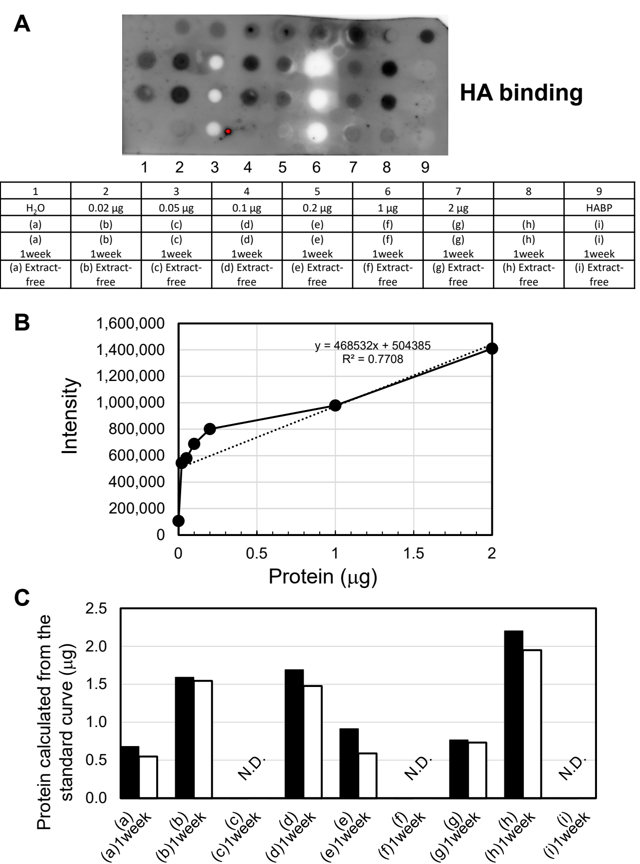
PG-added beverages, comestibles, and cosmetics generally contain various components such as carbohydrates, proteins, lipids, food dyes, flavorings, and preservatives. In the conventional attempts to quantify PG in PG-added compositions by a colorimetric quantification detecting CS chains or measurement of turbidity, correct results could not be obtained because coexisting molecules such as HA or pectin (polysaccharides), or glucose (monosaccharide) affected the analyses. In the present method based on immunological measurement, the amount and structural condition of PG can be evaluated as a water-soluble mixture of PG with various components,



1	2	3	4	5	6	7	8	9
H_2O	0.2 μg	1 μg	2 μg	5 μg	10 μg	20 μg		
(a)	(b)	(c)	(d)	(e)	(f)	(g)	(h)	(i)
(a) 1 week	(b) 1 week	(c) 1 week	(d) 1 week	(e) 1 week	(f) 1 week	(g) 1 week	(h) 1 week	(i) 1 week

Fig. 2. Quantification of the G1 domain of PG in the cartilage extract added to beverages by immunological analysis without reduced-alkylation.

A, Dot blot image of the G1 domain detected immunologically (upper). Spot positions of beverages to which were added hot water extract from salmon cartilage and each spot contains 5 μg of the extract (lower). From No. 1 to 7 in the top row is the hot water extract from salmon cartilage with known protein amount. B, A standard curve derived from the dot blot analysis. C, Quantification of the G1 domain. (a) distilled water; (b) ionized alkaline water; (c) unsweetened Japanese green tea (with matcha powder); (d) sweetened carbonated water beverage; (e) lactobacillus drink: (f) unsweetened apple juice; (g) collagen supplemental drink; (h) sports drink; (i) protein supplemental drink. N.D., not determined.



1	2	3	4	5	6	7	8	9
H_2O	0.02 μg	0.05 μg	0.1 μg	0.2 μg	1 μg	2 μg		HABP
(a)	(b)	(c)	(d)	(e)	(f)	(g)	(h)	(i)
(a) 1week	(b) 1week	(c) 1week	(d) 1week	(e) 1week	(f) 1week	(g) 1week	(h) 1week	(i) 1week
(a) Extract-free	(b) Extract-free	(c) Extract-free	(d) Extract-free	(e) Extract-free	(f) Extract-free	(g) Extract-free	(h) Extract-free	(i) Extract-free

Fig. 3. Evaluation of HA binding activity of G1 domain.

A, Detection of HA binding activity of the G1 domain by non-immunological analysis using HA as a probe (upper). Spot positions of beverages to which were added hot water extract from salmon cartilage and each spot contains 0.2 μg of the extract (lower). From No. 1 to 7 in the top row is the hot water extract from salmon cartilage with known protein amount. B, A standard curve derived from the HA binding analysis. C, Quantification of HA binging activity. (a) distilled water; (b) ionized alkaline water; (c) unsweetened Japanese green tea (with matcha powder); (d) sweetened carbonated water beverage; (e) lactobacillus drink: (f) unsweetened apple juice; (g) collagen supplemental drink; (h) sports drink; (i) protein supplemental drink. Extract-free, beverages without hot water extract from salmon cartilage. N.D., not determined.

without performing complicated purification steps. The target of the quality evaluation method may be a composition containing PG in which the entire structure is retained or a composition containing fragmented PG by degradation. Cartilage extracts, which are the functional ingredient of PG-containing products on the market, are obtained by different extraction procedures such as by hot water or by acetic acid. To evaluate the quality of PG in the cartilage extracts, the standard curve by purified PG should be used. For the quality evaluation of PG in the product, it is important to use the standard curve of the same preparation and the same lot as the extract blended in the products.

For efficient detection of the globular domains of PG by the immunological measurement, reduced-alkylation before the measurement is required to irreversibly dissociate disulfide bonds of protein. In the present method, the reduced-alkylation can be skipped when appropriate amounts of samples are applied and, that will be advantageous for versatility. To detect the CS attachment domain, an antibody that recognizes the stub of CS, linked to the core protein, generated by chondroitin ABC lyase is used, and this enzymatic treatment is simpler than reduced-alkylation.

When a PG-added composition is an aqueous solution such as a beverage or a lotion, it can basically be analyzed by the present method as is. In fact, a commercially available cosmetic solution containing PG was well analyzed without any treatment although it contains alcohols (data not shown). In the case of an aqueous solution containing insoluble materials such as tea leaves or solids in fruit juice, the insoluble materials can be removed by filtration, *etc*. When the PG-added composition is a solid, the highly water-soluble PG is dissolved in an aqueous solvent in the absence of protease inhibitor and then can be analyzed immunologically (data not shown). Since polyphenols in plant-derived extracts contained in beverages may interfere with immunological reactions and HA binding assay and make the dotted signal whiteout, it is preferable to analyze after pretreatment with polyvinylpolypyrrolidone and so on. This is a future issue.

Although the present method is for analyzing core protein, by detecting the CS attachment domain using antibodies recognizing CS, it is possible to evaluate the presence or types of CS linked to the core protein of PG without performing sugar chain analysis. Furthermore, by using HA as a probe instead of an antibody and detecting the HA bound to PG through biotinylated HABP, it is possible to evaluate whether the retained G1 domain of PG actually contributes to HA binding. By measuring all of the detectable domains such as the G1 domain, the CS attachment domain, the EGF-like module, and the G3 domain, it will be possible to comprehensively evaluate the structural condition and ratio of each functional domain of PG. The present method is considered to be invaluable for checking the amount of PG in the cartilage extract and for simple quality checks in the manufacturing process and during storage of products containing PG.

CONFLICTS OF INTEREST

The authors declare no conflict of interests.

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