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HYALURONIDASE INHIBITORY ACTIVITY OF CHONDROITIN SULFATE AND GLUCOSAMINE SULFATE

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ABSTRACT

The hyaluronic acid, glucosamine, chondroitin are now used as health food by many persons in many countries including Korea. Hyaluronic acid (HA) is an important component of articular cartilage. Hyaluronidases are a family of enzymes that degrade hyaluronic acid. The inhibitory effects of HAase by chondroitin sulfate and glucosamine sulfate were assayed using a Morgan microplate assay. Methanol extract of the chondroitin sulfate was evaluated 36.0% on 800 mg/L and that of the ethanol extract was 35.1% at same concentration. Overall, compounds including glucosamine sulfate and chondroitin sulfate were slightly higher in HAase inhibitory activity than those of pure chondroitin sulfate. Methanol extract of the glucosamine sulfate with chondroitin sulfate was evaluated 41.7% on 800 mg/L and that of the ethanol extract was 40.8% at same concentration. There was significant difference between pure chondroitin sulfate groups and compounds including glucosamine sulfate and chondroitin sulfate groups ($p < 0.05$). The results of this study suggest that the stem extract of chondroitin sulfate has significant HAase inhibitory effect. Chondroitin sulfate and glucosamine sulfate may be useful compounds for studies connected with the hyaluronidase inhibition.

KEYWORDS: Chondroitin sulfate, Glucosamine sulfate, HAase inhibition, Hyaluronidase.

INTRODUCTION

Hyaluronic acid (HA) or hyaluronan was first obtained by Karl Meyer and John Palmer in 1934 from the vitreous body in a cow's eye.[1] HA is a polymer of a repeating disaccharide unit, N-acetyl hyaluronic acid, linked via the hexosaminidic bonds in β-(1→4) linkages.[2]

HA is an important component of articular cartilage, where it is present as a coat around each cell (chondrocyte). HA has been FDA-approved to treat osteoarthritis of the knee via intra-articular injection.

HA can be degraded by a family of enzymes called hyaluronidases. In humans, there are at least seven types of hyaluronidase-like enzymes, several of which are tumor suppressors.

Hyaluronidase (HAase) is an enzyme that depolymerizes the polysaccharide HA in the extracellular matrix of connective tissue. HAase is divided into mammalian HAase (Mammalian type, EC 3.2.1.35, hyaluronoglucosaminidase), leech HAase (EC 3.2.1.36, hyaluronoglucuronidase), and bacterial HAase (Bacteriological type, EC 4.2.2.1, hyaluronate).[3] In particular, mammalian HAase exists in testicles, skin, liver, and placenta fluids in the human body to hydrolyze (1-(1,4) glucosamine bonds, which are components of HA, to produce tetrasaccharides, or chondroline, which are active fluids and cartilage in the joints of our body. Two hyaluronidases found in vertebrates, Hyal1 and Hyal2, have long been considered the primary degradative enzymes for HA.[4] HA must be internalized by cells for degradation by Hyal1 in lysosomes. Hyal2 has often been cited as the principal cell-surface degradative enzyme for HA in most tissues.[5-6]

Chondroitin sulfate is a sulfated glycosaminoglycan (GAG) composed of a chain of alternating sugars (N-acetylgalactosamine and glucuronic acid). It is usually found attached to proteins as part of a proteoglycan.[7] Chondroitin sulfate is an important structural component of cartilage[8] and it has become a widely used dietary supplement for treatment of osteoarthritis, although large clinical trials failed to demonstrate any symptomatic benefit of chondroitin. The effect of chondroitin sulfate in people with osteoarthritis is likely the result of a number of reactions including its anti-inflammatory activity, the stimulation of the synthesis of proteoglycans and hyaluronic acid, and the decrease in catabolic activity of chondrocytes.

Glucosamine is a water-soluble amino monosaccharide available in two forms (glucosamine sulphate and

glucosamine hydrochloride), which is a normal constituent of glycosaminoglycans (GAGs) in cartilage matrix and in the synovial fluid and consequently present in high quantities in articular cartilage.[9]

The objective of the current study was to evaluate chondroitin sulfate and glucosamine sulfate inhibitory effects on hyaluronidase activity of the aqueous, ethanol, and methanol extracts using the microplate method.

MATERIALS AND METHODS

Sample extract

A sufficient amount of chondroitin sulfate is prepared to use the 96 well plate. If 106 capsules, including 10% spare, are required for one experiment, 316 capsules are required for three repetitions. The three bottles (360 capsule per bottle) of chondroitin sulfate were purchased from Doctor's Best (Tustin, California, USA). This capsule contains chondroitin and other ingredients (chloride, sodium, potassium, glucosamine sulfate, and methylsulfonylmethane). For comparison with pure chondroitin and capsules, pure chondroitin sulfate A sodium salt from bovine trachea was purchased from Sigma-Aldrich (Merck, CAS No. 39455-18-0).

Water, ethanol, and methanol were used as the extraction solvent. The capsules of chondroitin sulfate was ground with hot distilled water or 80% ethanol or methanol and a grinding mixer to get rid of gelatin. An aliquot was further mixed with 100 mM Tris-HCl buffer (pH 7.4). The mixture of boiling group was further stirred with a magnetic bar at 100℃ for 60 minutes. The sample was treated with ultrasound at room temperature for 60 minutes. The ultrasound extraction was carried out using an ultrasonic bath (5510, Branson, USA). The mixture was shaken vigorously for one hour at room temperature. Extracted sample was filtered. The sample was evaporated to remove solvent under reduced pressure and controlled temperature by using rotary vacuum evaporator (N-1001S-W, Eyela, Tokyo, Japan). To get dry powder, samples placed in a low temperature vacuum chamber.

Hyaluronidase inhibition assay

The inhibitory effect of HAase by chondroitin sulfate was assayed using a Morgan microplate assay. HAase (Type I-S from bovine testis, Sigma-Aldrich Co., England) is dissolved in 0.1 M acetate buffer (pH 3.5) and mixed with extracts of chondroitin sulfate. The resulting solution was applied to a microplate. A negative control (0.1 M acetate buffer) to serve as a reagent blank was also applied to another wells with enzyme. FBHAL-04 BE-100 (Amsbio, Abingdon, U.K.) was used as a positive control. The plate was put in water bath for 20 minutes at 30°C. 12.5 mM CaCl2 was added to the plate and incubated for 20 minutes at 37°C.

HA (6 mg/ml) which was dissolved in a 0.1 M acetate buffer was added to HAase complex solution and incubated for 40 minutes at 37°C. 0.4 N NaOH and 0.4 M potassium tetraborate were added to terminate the enzymatic reaction for 3 minutes at 100°C. After cooling the mixture until room temperature, 180 µl DMAB solution (0.04 g/5 ml p-dimethyaminobenzaldehyde, 100% 3.5 ml acetic acid) were added to each well and incubated for 20 minutes at 37°C. 50uL of stop solution (10 N 5.0 ml HCl) was added to each well including blank control well and mixed well. The optical density (O.D.) was determined at 450nm using a microplate reader immediately. The color change was measured spectrophotometrically at a wavelength of 540 nm.

HAase assay was validated by demonstrating that pure tannic acid (0.07 mg/ml Sigma-Aldrich Co., England) as a positive control, a known HAase inhibitor,[10-11] give 76-80% enzyme inhibition.[12] I used the FBHAL-04 BE-100 (Amsbio, Abingdon, U.K.) as a positive control. All experiments were done in triplicate.

HAase inhibition was calculated on the ratio of the area under curve (AUC) of HA peak in an inhibitor sample to that of negative control sample as follows:

HAase inhibition (%) = [1- (AUC inhibitor / AUC control)] x 100

AUC inhibitor: AUC of the HA peak with inhibitor

AUC control: AUC of the HA peak of control sample without inhibitor

Control and repeat tests were analyzed by a one sample t test with values above the 95% confidence interval considered significant ($P < 0.05$). The difference in group mean values among in vivo treated groups were analyzed by one way analysis of variance followed by Student Newman Keuls (SNK) multiple comparisons test.[13] In some cases the paired t-test was used for comparisons.

RESULTS

In this study, the inhibitory effects of extracts against HAase inhibition were investigated. Tables 1 was shown the inhibitory effect for pure chondroitin sulfate of three solvent treated groups. It was observed that inhibition percentage values go on increasing with enhancements in concentration of research chondroitin sulfate extracts in the assay mixture. Methanol extract of the chondroitin sulfate was evaluated 36.0% on 800 mg/L and that of the ethanol extract was 35.1% at same concentration. Water extract was 28.0% at same concentration.

Overall, compounds including glucosamine sulfate and chondroitin sulfate were slightly higher in HAase inhibitory activity than those of pure chondroitin sulfate (Table 2). Methanol extract of the glucosamine sulfate with chondroitin sulfate was evaluated 41.7% on 800 mg/L and that of the ethanol extract was 40.8% at same concentration. Water extract was 33.3% at same concentration.

Although methanol extract of the chondroitin sulfate was slightly higher in HAase inhibitory activity than those of water and ethanol, there was no significant difference

among four concentrations (50, 100, 200, and 400 mg/L) ($p > 0.05$) (Table 3). However, there was significant difference among 800 mg/L ($p < 0.001$). There was significant difference among three solvents (water, ethanol, and methanol) on compounds including glucosamine sulfate and chondroitin sulfate groups ($p < 0.05$). There was significant difference between pure chondroitin sulfate groups and compounds including glucosamine sulfate and chondroitin sulfate groups ($p < 0.05$) (Table 4).

In order to verify the popularized potential in practice, we prepared chondroitin sulfate as HAase inhibitor and compared its HAase inhibitory activity with FBHAL-04 BE-100 (Fig. 1). The result showed that HAase inhibitory rate of chondroitin sulfate was 68.7% in the concentration of 800 mg/L, and relative inhibitory rate of glucosamine sulfate and chondroitin sulfate was 74.2% in the concentration of 800 mg/L.

Table 1: The percentages of HAase inhibition activity at various concentrations of the pure chondroitin sulfate.

Concentration (mg/L)	Solvent		
	Water	Ethanol	Methanol
50	3.77±2.07	5.42±1.83	5.75±2.26
100	6.20±0.85	7.38±0.86	7.70±1.61
200	12.35±0.31	12.22±1.60	13.34±3.22
400	20.06±1.91	22.22±2.11	23.93±3.91
800	28.03±2.82	35.07±1.66	36.03±1.67

Data represent the mean ± SD from three replicates.

Table 2. The percentages of HAase inhibition activity at various concentrations of the commercial product containing chondroitin sulfate and glucosamine sulfate with chondroitin sulfate.

Concentration (mg/L)	Solvent		
	Water	Ethanol	Methanol
50	7.83±1.68	11.11±2.14	12.91±2.94
100	12.26±1.20	14.50±2.42	15.94±3.35
200	17.17±1.45	18.20±3.53	21.78±3.94
400	25.74±1.94	31.31±3.92	32.73±3.07
800	33.34±1.05	40.75±2.69	41.71±2.69

Data represent the mean ± SD from three replicates.

Table 3. Compare *F*-test of difference in means of chondroitin sulfate per concentration and glucosamine sulfate with chondroitin sulfate per concentration.

Concentration (mg/L)	Solvent	
	Chondroitin sulfate among three solvents	Glucosamine sulfate and chondroitin sulfate among three solvents
50	1.647	6.163**
100	2.329	2.950*
200	0.429	2.916*
400	2.333	6.751**
800	21.123***	20.194***

*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

Table 4: Compare *F*-test of difference in means of chondroitin sulfate and glucosamine sulfate with chondroitin sulfate at same concentration.

Concentration (mg/L)	Solvent		
	Water	Ethanol	Methanol
50	8.719**	12.154**	13.988**
100	63.849***	28.050***	18.381**
200	39.975***	8.914**	10.317**
400	15.636**	15.622**	11.752**
800	11.642**	12.122**	12.057**

**, $p<0.01$; ***, $p<0.001$.

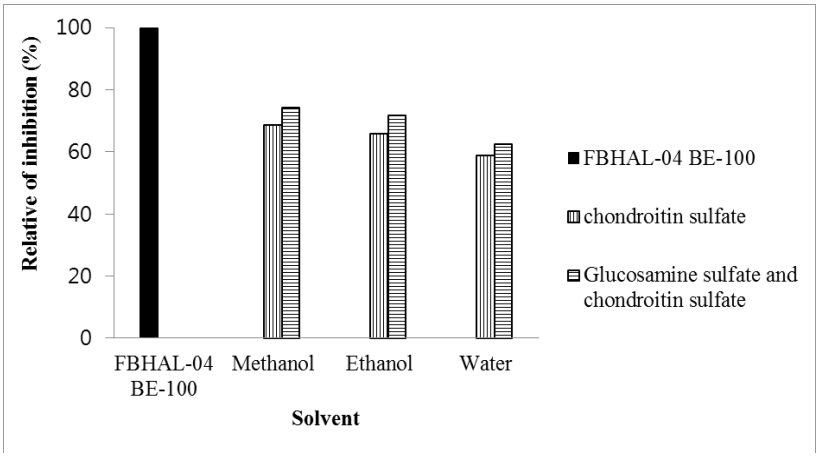

Figure 1. The rate of HAase inhibition activity of FBHAL-04 BE-100 (positive control) and relative inhibitory rate for chondroitin sulfate.

DISCUSSION

Hyaluronic acid is a linear glycosaminoglycan polymer abundantly present in the extracellular matrix of adipose tissue. The extracellular matrix (ECM) plays pivotal roles in cell self-renewal, fate, death, and signaling to regulate diverse functions including migration, proliferation, differentiation, tissue patterning, inflammation, and angiogenesis, among other homeostatic and pathological processes.[14]

Hyaluronic acid (HA) and chondroitin sulfate (CS) are valuable bioactive polysaccharides that have been highly used in biomedical and pharmaceutical applications. Recently, great attention has been given to the use of biomass, including animal wastes and by-products, as a potential source for the isolation of both HA and CS.[15] An important role of CS is to increase the expression of TGF-β1 on the extracellular matrix[16] and to promote the synthesis of HA and collagen type II.[17-18] The mechanism of action of HA and CS seems to be complementary, developing a synergistic effect.[19] Stellavato et al.[20] reported two concentrations of CSb (0.6% and 2% w/v) and HA (0.6 and 1% w/v) significantly reduced both IL-6 and IL-8 gene expression. The HA/CSb formulation also effectively reduced the harmful effect of TNF-α on bladder cells.

Hyaluronidase hydrolyzes glycosaminoglycans including hyaluronan (HA) in the extracellular matrix during tissue remodeling. The balance between synthesis of HA and its degradation by hyaluronidase is a key feature of normal connective tissue.[21] During injury, repair, and regeneration, the balance between synthesis and degradation of matrix components including collagen, glycosaminoglycans, and proteoglycans can be affected adversely.[22]

Glycosaminoglycans are a diverse class of linear polysaccharides that are composed of a repeating disaccharide unit and negative charged polysaccharides. GAGs possess a variety of biologic activities including the ability to bind growth factors and cytokines and promote water retention. Many synthetic and naturally occurring compounds act as HAase inhibitors. HAase inhibition of foliage and central stalk of *Equisetum arvens* was 24.3% at 4.0 mg/ml and that of rhizomatous stem and root was 27.3% at same concentration.[23] The glycosaminoglycans present in ECM include heparin, heparin sulfate, chondroitin sulfate A and B, and hyaluronic acid (HA). HAases also appear to be inhibited by chondroitin and other ingredients (chloride, sodium, potassium, glucosamine sulfate, and methylsulfonylmethane) through a mixed inhibition mechanism in this study.

CONCLUSIONS

An inhibitor of hyaluronidase would be useful for the prevention of infection. Chondroitin sulfate and glucosamine sulfate may be useful compounds for studies connected with the hyaluronidase inhibition.

REFERENCES

1. Necas J, Bartosikova L, Brauner P, Kolar J. Hyaluronic acid (hyaluronan): a review. Veterinarni Medicina, 2008; 53(8): 397–411.
2. Boyer PD. The Enzymes, 3rd ed., Volume 5, Academic Press: London, UK; New York, NY, USA, 1971.
3. Hwang SG, Yang A, Kim SJ, Kim MK, Kim SS, Oh HJ, Lee JD, Lee EJ, Nam KW, Han MD. Screening of hyaluronidase inhibitor in Korean medicinal plants. Korean Journal of Life Science, 24: 498-505.
4. Cowman, MK. Chapter One - Hyaluronan and Hyaluronan Fragments. Advances in Carbohydrate Chemistry and Biochemistry, 2017; 74: 1-59.
5. Feost GI, Csoka T, Stern R. The hyaluronidases: a chemical, biological and clinical overview. Trands Glycosci Glycotech, 1996; 8: 419-34.
6. Menzel EJ, Farr C. Hyaluronidase and its substrate hyaluronan: biochemistry, biological activities and therapeutic uses. Cancer Lett, 1998; 131: 3-11.
7. McAtee CO, Barycki JJ, Simpson MA. Simpson MA, Heldin P (eds.). Chapter One – Emerging Roles for Hyaluronidase in Cancer Metastasis and

Therapy. Advances in Cancer Research, Hyaluronan Signaling and Turnover, Academic Press, 2014; 123: 1-34.
8. Klecker C, Nair LS. Vishwakarma, Ajaykumar; Karp, Jeffrey M. (eds.), Chapter 13 – Matrix Chemistry, 2017; 195-213.
9. Colletti A, Cicero AFG. Nutraceutical approach to chronic osteoarthritis: from molecular research to clinical evidence. International Journal of Molecular Sciences, 2021; 22, 12920. https://doi.org/10.3390/ijms222312920.
10. Girish KS, Mohanakumari HP, Nagaraju S, Kemparaju BK. Hyaluronidase and protease activities from Indian snake venoms: neutralization by *Mimosa pudica* root extract. Fitoterapia, 2004; 75: 378-80.
11. Girish KS, Kemparaju K. The magic glue hyaluronan its eraser hyaluronidase: A biological overview. Science, 2007; 80: 1921-43.
12. Sumantran VN, Kulkarni A, Boddul S, Chinchwade T, Harsulkar A, Patwardhan B, Chopra A, Wagh UV. 2007. Chondroprotective potential of root extracts of *Withania somnifera* in osteoarthritis. J Biosci, 2007; 32: 299-307.
13. Zar JH. Biostatistical analysis (2nd ed.). Pretice-Hall, Inc. New Jersey, 1984.
14. Iozzo RV, Gubbiotti MA, Extracellular matrix: the driving force of mammalian diseases, Matrix Biol., 2018; 1-9: 71-2.
15. Abdallah MM, Fernándeza N, Matiasa AA, Bronzeabc MR. Hyaluronic acid and Chondroitin sulfate from marine and terrestrial sources: Extraction and purification methods. Carbohydrate Polymers, 2020; 243, https://doi.org/10.1016/j.carbpol.2020.116441.
16. Campo GM, Avenoso A, Campo S, D'Ascola A, Traina P, Samà D, et al. Purified human plasma glycosaminoglycans reduced NF-kappaB activation, pro-inflammatory cytokine production and apoptosis in LPS-treated chondrocytes. Innate Immunol, 2008; 14: 233–46.
17. Nishimoto S, Takagi M, Wakitani S, Nihira T, Yoshida T. Effect of chondroitin sulfate and hyaluronic acid on gene expression in a three-dimensional culture of chondrocytes. J Biosci Bioeng, 2005; 100: 123–6.
18. Gouttenoire J, Valcourt U, Ronzière MC, Aubert-Foucher E, Mallein-Gerin F, Herbage D. Modulation of collagen synthesis in normal and osteoarthritic cartilage. Biorheology, 2004; 41: 535-42.
19. Galluccio F, Barskova T, Cerinic MM. Short-term effect of the combination of hyaluronic acid, chondroitin sulfate, and keratin matrix on early symptomatic knee osteoarthritis. Eur J Rheumatol, 2015; 2(3): 106-8.
20. Stellavato A, Pirozzi AVA, Diana P, Reale S, Vassallo V, Fusco A, Donnarumma G, Rosa MD, Schiraldi C. Hyaluronic acid and chondroitin sulfate, alone or in combination, efficiently counteract induced bladder cell damage and inflammation. PLOS ONE, 2019; 14(6): e0218475- June 2019.
21. Sugimoto K, Iizawa T, Harada H, Yamada K, Katsumata M, Takahashi M. Cartilage degradation independent of MMP/aggrecanases. Osteoarthritis Cartilage, 2004; 12: 1006–14.
22. Bralley E, Greenspan P, Hargrove JL, Hartle DK. Inhibition of hyaluronidase activity by *Vitis rotundifolia* (Muscadine) berry seeds and skins. Pharmaceutical Biology, 2007; 45(9): 667-73.
23. Huh MK, Han MD. Inhibitory effect of hyaluronidase and DPPH radical scavenging activity using extraction of *Equisetum arvens*. European Journal of Advanced Research in Biological and Life Sciences, 2015; 3(2): 47-51.