Sulfated Polysaccharides Derived from Dietary Seaweeds Increase the Esterase Activity of a Lymphocyte Tryptase, Granzyme A

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Summary Intake of sulfated polysaccharides, such as fucoidan or α-carrageenan extracted from seaweeds, has been shown to enhance immune responses, resulting in inhibition of tumor growth. However, little is known about the mechanisms by which these sulfated compounds mediate the enhancement. In the present study, we examined the effect of sulfated polysaccharides from seaweeds on esterase activity of a lymphocyte tryptase, granzyme A (GzmA), which is believed to induce the production of cytokines in a variety of cells. Inclusion of fucoidan (from Fucus vesiculosus) or α-carrageenan (from Gigartina aciculata and Gigartina) in the reaction mixture increased the hydrolysis of N-benzoyloxy-L-lysine thiobenzyl ester (BLT) by a recombinant rat GzmA in a concentration-dependent manner. Heparin, a sulfated polysaccharide from animal tissues, also increased the BLT hydrolysis, but the effect was less remarkable than those of the polysaccharides from the seaweeds. Hanes-Woolf analysis revealed that the enhancements in the presence of these sulfated compounds from the seaweeds were attributed to the increases in the affinity of the enzyme toward the substrate but not to those in the turnover rate. Chondroitin sulfate A, a sulfated polysaccharide found in animal and plant tissues, showed no positive effect on the hydrolysis. In the present paper, we propose that the enhancement of immune responses by intake of the sulfated polysaccharides from seaweeds can be partially accounted for by their direct effects on GzmA.

Key Words sulfated polysaccharide, seaweeds, granzyme A, esterase activity

Seaweeds have long been utilized as food materials especially in Asian areas, including Japan. They might have been invaluable sources of nutrients such as minerals for people living in the area. Recently, much attention has been paid to the enhancement of immune responses by intake of seaweeds, and the effects have been mainly attributed to sulfated polysaccharides constituting the cell walls of these organisms (1, 2). For example, orally administered Mekabu fucoidan, a polysaccharide composed predominantly of sulfated α-(1,3)-L-fucose, enhanced the cytolytic activity of natural killer (NK) cells and increased the amount of interferon-γ produced by T cells, which resulted in the increased survival of tumor-bearing mice (1). Also, oral administration of λ-carrageenan [(1,3)-β-D-galactose-2-sulfate-(1,4)-α-D-galactose-2,6-disulfate-polymer], another sulfated polysaccharide from Chondrus ocellatus, showed the similar effects in mice (2). However, in vivo molecule(s) that directly counteract these sulfated compounds to mediate enhancement of immune responses have remained unknown.

Granzyme A (GzmA) is one of the serine proteases termed granzymes, which exhibits a proteolytic activity with trypsin-like specificity (trypptase). This enzyme, together with perforin and other granzymes, is packaged in cytoplasmic granules of cytotoxic T-lymphocytes (CTLs) or NK cells (3). GzmA and granzyme B (GzmB) have been believed to enter viral-infected cells or growing tumors via pores in the target cell membrane formed by perforin and to mediate the apoptotic DNA fragmentation (3). However, studies using GzmA or GzmB single-knockout mice have indicated that GzmB is the major CTL effector molecule for the induction of apoptosis, with GzmA playing only a minor part (4). CTLs possess the constitutive exocytosis pathway of GzmA, resulting in the release of this enzyme into blood (3, 5). In vitro studies have demonstrated that GzmA converts pro-interleukin (IL)-1β to its active form and mediates the production of IL-6 and IL-8 by monocytes, fibroblasts, and epithelial cells (possibly via activation of protease-activated receptors on the cell surface) (3). These findings suggest that GzmA mainly functions under extracellular milieu conditions to mediate activation and/or enhancement of the immune system.

It has been reported that the proteolytic activity of GzmA is increased in the presence of heparin (6), a sulfated polysaccharide in animal tissues [(1,4)-α-L-idu-
ronic acid-2-sulfate-(1,3)-N-acetyl-β-D-galactosamine-4-sulfated polymer]. Heparin and dietary sulfated polysaccharides such as fucoidan share biological activities including anti-coagulant activity (7). By analogy, these sulfated compounds are expected to exhibit a similar effect on the lymphocyte tryptase. In this study we investigated the effect of sulfated polysaccharides from dietary seaweeds on GzmA activity in vitro.

Fucoidan (from Fucus vesiculosus) and λ-carrageenan (from Gigartina aciculare and Gigartina pistillata) were purchased from Sigma Chemicals (St. Louis, MO, USA). For comparison, the effect of heparin (Sigma, grade IA, from porcine intestine) and chondroitin sulfate A (Nacalai Tesque, Inc., Kyoto, Japan) were also examined. Chondroitin sulfate A is a polymer of (1,4)-β-D-glucuronic acid-(1,3)-β-D-N-acetyl-galactosamine 6-sulphate, which is widely distributed in animal and plant tissues. A recombinant rat GzmA produced in yeast (Pichia pastoris) was used in this study (8). The recombinant GzmA was incubated at the final concentration of 1 nM with 100 µM of Nα-benzyloxy-L-lysine thiobenzyl ester (BLT) (Sigma), which has been commonly used as a substrate for this enzyme (3), and 500 µM of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) (Sigma), in buffer A [20 mM HEPES (pH 8.0), 145 mM NaCl, 0.1% (v/v) Triton X-100], in the presence or absence of sulfated polysaccharides. The other reaction conditions were the same as described in Ref. (8).

The effects of various sulfated polysaccharides on the esterase activity of recombinant rat GzmA are illustrated in Fig. 1. Either fucoidan or λ-carrageenan increased the reaction rate of the BLT hydrolysis by GzmA in a concentration-dependent manner. At any concentrations tested, λ-carrageenan significantly increased the rate of the BLT hydrolysis against that in the absence of polysaccharides (p<0.05, unpaired t-test). At the higher concentration (0.25 or 5.0 µg/mL), the effect of fucoidan was statistically significant (p<0.05, unpaired t-test). Although heparin showed a similar effect, it was not statistically significant at any concentration examined. Chondroitin sulfate A showed no positive effect on the reaction at any concentration tested. Under the same experimental conditions, fucoidan and λ-carrageenan showed no apparent effects on the BLT hydrolysis catalyzed by bovine pancreatic trypsin (Sigma) or by human plasma α-thrombin (Calbiochem-Novabiochem Corp., San Diego, CA, USA) (data not shown). We performed Hanes-Woolf analysis to explore the mechanism of the enhancement of the BLT hydrolysis by fucoidan, λ-carrageenan, and heparin. GzmA was incubated at the final concentration of 0.1 nM with 0 to 400 µM of BLT and 1,000 µM of DTNB in buffer A, in the presence or absence of these compounds. As summarized in Table 1, all of the sulfated polysaccharides decreased Michaelis constants

Table 1. Effect of fucoidan, λ-carrageenan, and heparin on the kinetic constants for the BLT hydrolysis catalyzed by GzmA.

<table>
<thead>
<tr>
<th>Additions (µg/mL)</th>
<th>Km (µM)</th>
<th>kcat (s⁻¹)</th>
<th>kcat/Km (M⁻¹s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>230±11.3</td>
<td>18.2±0.340</td>
<td>79,000±3,780</td>
</tr>
<tr>
<td>Fucoidan 0.1</td>
<td>227±5.41</td>
<td>17.8±0.272</td>
<td>78,800±1,650</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>222±7.53</td>
<td>17.9±0.415</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>176±8.36*</td>
<td>17.0±0.330</td>
</tr>
<tr>
<td>λ-Carrageenan 0.1</td>
<td>215±8.94*</td>
<td>17.0±0.496</td>
<td>79,900±814</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>203±4.89*</td>
<td>17.0±0.440</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>167±7.33*</td>
<td>17.8±0.262</td>
</tr>
<tr>
<td>Heparin 0.1</td>
<td>235±4.15</td>
<td>18.6±0.240</td>
<td>79,100±893</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>227±5.36</td>
<td>18.1±0.186</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>199±7.15</td>
<td>18.1±0.502</td>
</tr>
</tbody>
</table>

The kinetic constants were given by Hanes-Woolf analysis. Experimental conditions were as described in the text and in Ref. (8). Values are means±SE of three separate experiments performed in duplicate. *Significantly different from the value in the absence of any sulfated polysaccharides by unpaired t-test (p<0.05).
(Km) in proportion to the amounts of the sulfated compounds included, whereas they showed almost no effect on rate constants (kcat). At any concentration examined, λ-carrageenan significantly decreased Km against the substrate.

The effect of fucoidan (10 μg/mL) was statistically significant (p<0.05, unpaired t-test). No significant decreases in Km were observed when heparin was used. These results indicated that the enhanced reaction rates by the sulfated polysaccharides from the seaweeds can be attributed to their ability to increase the affinity of the enzyme toward the substrate. GzmA has been known to bind strongly to polysaccharides, such as heparin, that are negatively charged by sulfate and carboxyl groups (3). Upon the binding, the positive charge of GzmA can be diminished, which may, in turn, facilitate the binding of the enzyme to BLT and probably to other natural substrates. It is, however, unlikely that the effect of the polymers is due solely to the negative charge, because chondroitin sulfate A had no positive effect on the BLT hydrolysis (Fig. 1). The spatial positioning of the negatively charged groups and the stereostructure of sulfated polysaccharides may also be critical to the enhancement on the substrate recognition of GzmA.

To date, there has been no evidence that the sulfated polysaccharides from dietary seaweeds can be absorbed into body in their intact forms to encounter endogenous molecules including GzmA. However, previous studies have revealed orally administrated sulfated polysaccharides such as chondroitin sulfate (9), heparin (10), and dextran sulfate, a synthetic sulfated polysaccharide (11), are absorbed in their intact forms via gastrointestinal tract. It is, therefore, possible that this is also the case for sulfated polysaccharides from dietary seaweeds. Very little is known about the cellular mechanism(s) by which gut epithelia absorb intact sulfated polysaccharides.

Scavenger receptors expressed in Kupffer cells have been shown to mediate the uptake of high molecular weight fractionated heparin (16,000–24,000 Da) (11). It is interesting to speculate that a portion of high molecular weight dietary polysaccharides are absorbed via known or unknown scavenger receptors expressed on the apical surface of gastrointestinal epithelia. Alternatively, these polymers as well as other macromolecules may be absorbed by energy-dependent pinocytosis which mature gut retains (12). Further studies are required to explore the mechanism by which these sulfated compounds are absorbed into the body.

In the present study, we showed that sulfated polysaccharides derived from dietary seaweeds increased the BLT hydrolysis by GzmA in vitro. It should be clarified whether or not the reactions mediated by this lymphocyte tryptase, such as conversion of pro-IL-1β, are also enhanced by these sulfated polymers in vivo. Nevertheless, the increase in the esterase activity of the GzmA could be included in the enhancement of immune responses by the sulfated compounds.

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REFERENCES