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## Original paper

# Influence of Temperature on the Anti-allergic Activity of Fucoidan Extracted from *Saccharina japonica*

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It has been ascertained in our laboratory that fucoidan, a polysaccharide contained in *Saccharina japonica*, shows anti-allergic activity through galectin 9 secretion in blood. A crude fucoidan fraction was chromatographically fractionated into three fractions using a Toyopearl-DEAE 650 M column in stepwise elution with 0, 0.5 and 1.0 M NaCl in 0.05 M Tris-HCl buffer (pH 7.4). Each fraction was assessed using the passive cutaneous anaphylaxis (PCA) reaction. The non-absorbed fraction of the three fractions suppressed PCA, whereas the fractions eluted with 0.5 M and 1.0 M NaCl did not. Moreover, it was discovered that heat treatment of fucoidan at 50 °C for 10 min abolished its anti-allergic activity in the PCA reaction. Using DEAE chromatography, it was demonstrated that heat-treatment of the crude fucoidan fraction decreased the non-absorbed fraction, which possessed the anti-allergic activity, and increased the two fractions eluted with 0.5 M and 1.0 M NaCl. This clearly revealed the importance of temperature in maintaining the anti-allergic activity of fucoidan.

Keywords: Anti-allergic activity, fucoidan, polysaccharide, passive cutaneous anaphylaxis, *Saccharina japonica*

## Introduction

Allergic disorders are one of the major health problems in developed countries (Masoli *et al.*, 2004; Asher *et al.*, 2006). Allergy is defined as an excessive immune response against normally harmless substances such as food, pollen or metals. Allergy is classified into four groups, from type I to type IV, on the basis of mediators, antigen, and effector mechanism. In particular, type I allergy patients are increasing world-wide (Galli *et al.*, 2008). Although there are many steps in the development of type I allergy, antigen-specific immunoglobulin E (IgE) production, with subsequent fixation of IgE to FcεRI receptors on mast cells, is central to the initiation and propagation of immediate hypersensitivity reactions (Stone *et al.*, 2010). Therefore, the passive cutaneous anaphylaxis (PCA)

reaction is often used as an animal model for the inflammatory reaction in type I allergy.

Foods have gained attention in the amelioration of allergy. Actually, it has been reported that type I allergy is suppressed by many food factors such as flavonoids (Wu *et al.*, 2006; Kim *et al.*, 2009), polyunsaturated fatty acids (Wang *et al.*, 2015), *Lactobacillus* species (Murosaki *et al.*, 1998; Shida *et al.*, 2002; Lee *et al.*, 2013), and polysaccharides (Ramberg *et al.*, 2010). Fucoidan, which is one of the polysaccharides in sea algae, possesses various activities, such as antitumor (Synytsya *et al.*, 2010) and anticoagulant (Athukorala *et al.*, 2006; Ren *et al.*, 2013) activities, through the modulation of immune responses. Moreover, fucoidan has been reported to have anti-allergic effects (Vo *et al.*, 2015; Oomizu *et al.*, 2006; Yanase *et*

**Abbreviations:** DEAE, diethylaminoethyl; IgE, immunoglobulin E; PCA, passive cutaneous anaphylaxis; TLC, thin-layer chromatography; TNP, 2,4,6-trinitrophenyl;

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*al.*, 2009). Fucoidan is a sulfated polysaccharide found in brown sea algae. Although the structure of fucoidan varies between different species of brown sea algae, it is mainly composed of fucose and sulfated fucose (Li *et al.*, 2008). The structure of fucoidan mainly consists of  $\alpha$ -1,3 linked fucose and is branched at C-2 or C-4 (Li *et al.*, 2008). Besides these monosaccharides, fucoidans from other brown sea algae also contain specific sugars such as glucose, galactose, xylose and uronic acid (Li *et al.*, 2008). Tanino *et al.* (2016) reported that fucoidan from *S. japonica* mitigated type I allergic symptoms.

Kombu is an ingredient used to make Kombu broth. Kombu is usually soaked in cold water and then simmered to extract the umami components. However, it was not clear whether the anti-allergic activity of fucoidan is affected by heat treatment. In this study, we demonstrated that temperature is very important in maintaining the anti-allergic activity of fucoidan.

## Materials and Methods

**Materials** Mouse anti-2,4,6-trinitrophenyl (TNP) monoclonal IgE was purchased from BD Biosciences (San Jose, USA). 2,4,6-Trinitrochlorobenzene was purchased from Tokyo Chemical Industry (Tokyo, Japan). Anion-exchange chromatography was carried out using Toyopearl-DEAE 650 M (Tosoh, Tokyo, Japan).

**DEAE chromatography of fucoidan** Fucoidan was extracted from *S. japonica*. Lyophilized *S. japonica* was stirred in a 20 times volume of 0.1 M sodium acetate buffer (pH 4.6) overnight at 4 °C. After centrifugation (3 500 rpm, 10 min), the supernatant was evaporated to half of the original volume and the concentrate was then added to twice the volume of ethanol to precipitate the polysaccharides. After centrifugation again, the precipitate was dissolved in distilled water and lyophilized. The lyophilized powder was separated by a Toyopearl-DEAE 650 M column (2.0 × 15 cm) with stepwise elution using 0.05 M Tris-HCl buffer (pH 7.4) containing 0, 0.5 and 1.0 M NaCl. Each fraction was collected, lyophilized after dialysis against distilled water, and weighed. Alternatively, the sugar content of each fraction was measured by the phenol-H<sub>2</sub>SO<sub>4</sub> method (Dubois *et al.*, 1956). A standard curve was calculated using fucose as the main component sugar of fucoidan. Crude fucoidan (500 mg) was dissolved in 0.05 M NaCl solution (50 mL) and treated at 10, 30 and 50 °C for 10 min in a water bath.

**Monosaccharide analysis** Thin-layer chromatography (TLC) was utilized to determine the sugar components following hydrolysis by 1 % trifluoroacetic acid degradation at 100 °C for 1 h, as previously described with slight modifications (Huang *et al.*, 2016). TLC was developed using a solvent system consisting of n-butyl alcohol/acetic acid/water (6:3:1, v/v). The monosaccharides were visualized on the plate after dipping into sulfate acid/methanol (1:1, v/v) and heating. D-Galactose, L-fucose, and D-glucuronic acid were used as standard

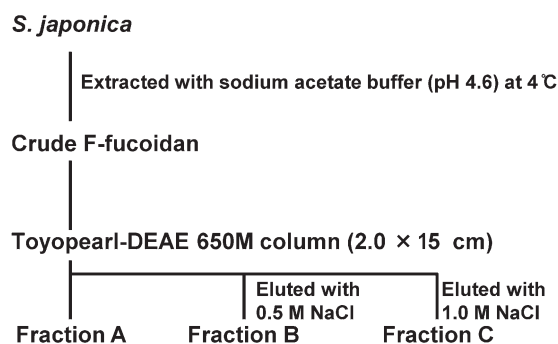


Fig. 1. Protocol of separation of crude fucoidan.

monosaccharides.

**Mice** Female BALB/c mice (4 weeks old) were purchased from Japan SLC (Shizuoka, Japan). Mice (n = 3) were housed in an air-conditioned animal room at a temperature of 23 ± 2 °C and 55 ± 10 % humidity. Mice were acclimatized for almost 1 week, during which time food and water were provided *ad libitum*. This study was approved by the Institutional Animal Care and Use Committee and carried out according to the Kobe University Animal Experimentation Regulations (permission number: 25-06-04).

**Passive cutaneous anaphylaxis reaction** Mice were intravenously sensitized with anti-TNP IgE and then challenged by application of 1.6 % 2,4,6-trinitrochlorobenzene in acetone:olive oil (1:1) as the antigen to the ear 30 min after sensitization. Ear thickness was measured using a micrometer (Ozaki MFG., Tokyo, Japan) before and 2 h after antigen challenge, and the difference in ear thickness was defined as edema. Before the PCA reaction, mice were orally administered the test samples dissolved in distilled water at appropriate concentrations every day for 4 d.

**Statistical analysis** Data were expressed as the mean ± standard error. Statistical analysis was performed using the Tukey-Kramer test. *p* < 0.05 was considered statistically significant.

## Results and Discussion

**Identification of fractions possessing anti-allergic activity** As shown in Fig. 1, crude fucoidan was subjected to DEAE chromatography in a stepwise manner using 0, 0.5 and 1.0 M NaCl in 0.05 M Tris-HCl buffer (pH 7.4). Crude fucoidan was fractionized into three fractions, Fractions A to C, with increasing NaCl concentration (Fig. 2). This chromatographic method was consistent with that reported by Zhao *et al.* (2018). The weight ratio of Fractions A, B and C in the crude fraction was approximately 30, 23 and 30 %, respectively. It has been reported that 200 µg of crude fucoidan shows anti-allergic activity in the PCA reaction (Tanino *et al.*, 2016). To confirm which fractions possessed anti-allergic activity, PCA was carried out at doses of 200, 60, 46 and 60 µg of crude fucoidan, Fractions A, B and C, respectively. As shown in Fig. 3, of the 3

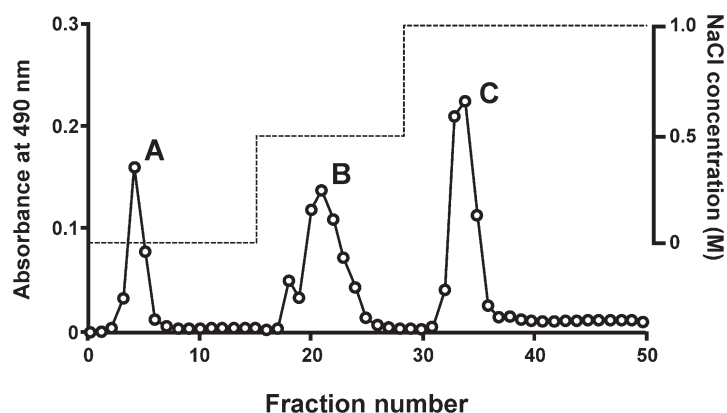


Fig. 2. DEAE-chromatogram of crude fucoidan.

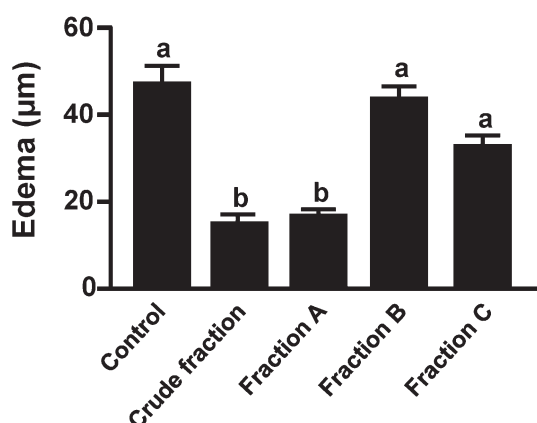


Fig. 3. Anti-allergic activity of each fraction in crude fucoidan. Female BALB/c mice (5 weeks old) were orally administered crude fucoidan, Fraction A, B and C (200, 60, 46, 60 μg/day/mouse, respectively) for 4 d and applied for PCA reaction. Values represent the means  $\pm$  SE (n=3). Items with different letter were significantly different ( $p < 0.05$ ).

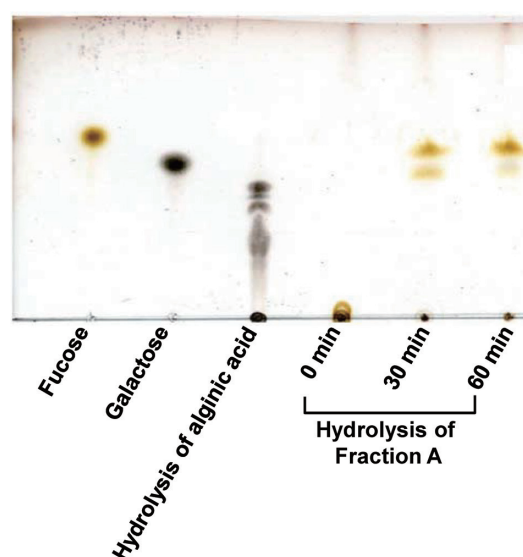


Fig. 4. TLC analysis of sugar components in Fraction A from *Laminaria japonica*.

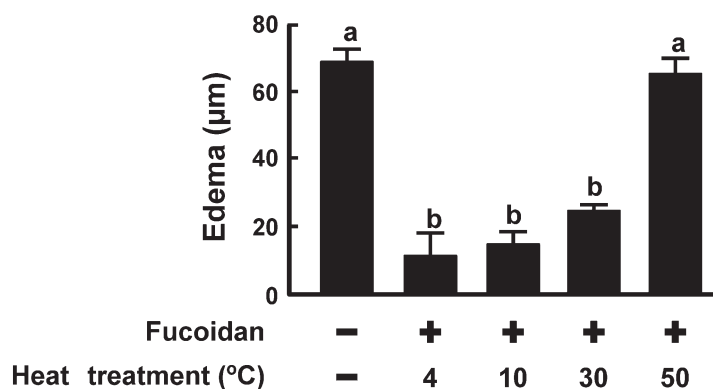
fractions, edema induced by antigen sensitization was significantly decreased only by oral administration of Fraction A, to almost the same level as crude fucoidan administration. However, the other two fractions (Fractions B and C) produced no drastic changes. These results indicated that Fraction A contained the active compounds possessing the anti-allergic activity in crude fucoidan from *S. japonica*.

**Sugar analysis of Fraction A by thin-layer chromatography**  
The sugar components of Fraction A were determined by TLC. As shown in Fig. 4, relative to the TLC spots corresponding to authentic fucose and galactose, the hydrolysate of Fraction A for 30 and 60 min showed prominent spots corresponding to fucose as the hydrolysis time elapsed. Fraction A did not contain any of the sugar components found in alginic acid. In consideration of these results, it was predicted that Fraction A was fucoidan, which mainly consisted of fucose. It was reported that the non-absorbed fraction in DEAE-SephroseA-25 chromatography had the greatest fucose content and was proposed to be the source of the anti-oxidant activity

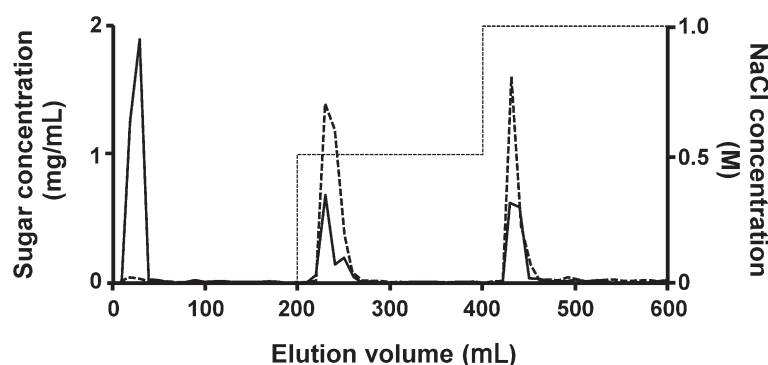
of fucoidan (Zhao *et al.*, 2018).

**Influence of heating temperature on anti-allergic activity**  
Seaweeds (Kombu) are often simmered to extract the umami components. Therefore, it is highly likely that Kombu is treated at more than 50 °C. However, few reports have described the influence of temperature on the anti-allergic activity of fucoidan. To confirm the stability of its anti-allergic activity under high temperature, crude fucoidan was heated at 10, 30 and 50 °C for 10 min and thereafter applied to PCA. Oral administration of crude fucoidan treated at 50 °C did not prevent ear edema ( $67.3 \pm 3.3$  μm) and produced almost the same edema level as the IgE/antigen group ( $68.2 \pm 3.2$  μm) (Fig. 5). However, heat treatment at less than 30 °C maintained the same level of edema suppression ( $14.1 \pm 4.3$  μm at 10 °C and  $24.0 \pm 2.0$  μm at 30 °C) as crude fucoidan ( $11.3 \pm 6.4$  μm).

To detect component differences between non-heated and heated crude fucoidan, these fucoidans were applied to DEAE chromatography. As shown in Fig. 6, heat-treatment of the crude fucoidan fraction almost abolished the non-absorbed



**Fig. 5.** Effect of heat treatment of crude fucoidan on PCA reaction. Crude fucoidan was heated at 10, 30 and 50 °C for 10 min and kept at room temperature to cool down. Values represent the means  $\pm$  SE (n=3). \*  $p < 0.05$



**Fig. 6.** DEAE-chromatogram of non-heated and heated crude fucoidan. Solid and dotted lines express chromatography of non-heated and heated crude fucoidan, respectively.

**Table 1.** Weight change of each fraction by heat treatment

	Non-heat treatment (mg)	Heat treatment (mg)
Fraction A	33.3	0.8
Fraction B	11.3	30.8
Fraction C	13.6	24.8
Total	57.6	56.4

fraction and increased the amounts of the two fractions eluted with 0.5 M and 1.0 M NaCl. The contents of Fractions A, B and C in the non-heated fucoidan were estimated to be 33.3, 11.3 and 13.6 mg (converted into equivalent fucose), respectively. On the other hand, those of Fractions A, B and C from heated fucoidan were 0.8, 30.8 and 24.8 mg, respectively (Table 1). As shown in Fig. 3, the non-absorbed fraction contained the active compounds possessing anti-allergic activity. Therefore, it is clear that the elimination of the anti-allergic activity by heat treatment is attributable to the degradation of the non-absorbed fraction in crude fucoidan.

Regarding the thermal stability of fucoidan, cooking was reported to lower the anti-oxidative potential of fucoidan in the DPPH assay, but not that of laminarin or  $\beta$ -glucan from seaweed

(Moroney *et al.*, 2015). Moreover, Yuan and Macquarrie (2015) reported that the anti-oxidant activity of fucoidan was highest when fucoidan was extracted from brown seaweed by microwave-assisted extraction at 90 °C rather than 150 °C. Thus, the thermal stability of fucoidan has been evaluated in regards to anti-oxidant activity at high temperature. However, this is the first report to demonstrate that the anti-allergic activity of fucoidan is not stable at a temperature of 50 °C. Taking these results into consideration, attention should be paid to the cooking temperature of fucoidan in order to maintain its functional activities such as anti-allergic activity.

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