Studies on the molecular mechanism of fucoidan-mediated galectin-9 upregulation in food allergy

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DOCTORAL DISSERTATION

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食物アレルギーにおけるフコイダンを介したガレクチン-9 発現上昇の分子機構に関する研究

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ABBREVIATIONS

AP-1: activating protein-1

APC: Antigen Presenting Cells

BMDC: Bone marrow Dendritic Cells

CD83: Cluster of Differenciation 83

CpG-DNA: Cytosine phosphatidyl Guanine deoxyribonucleic acid

CpG-ODN: Cytosine phosphatidyl Guanine Oligodesoxynucleotide

CRD: Carbone Recognition Domain

DC: Dendritic Cells

DNA, Desoxyribonucleic Acid

ER: Endoplasmic reticulum

IEC: Intestinal Epithelial Cells

Ig: Immunoglobulin

IgE, Immunoglobulin E

IKK: IkappaB kinase

IL-10: Interleukin-10

INF-y: Interferon-gamma

IRAK: Interleukin-1 Receptor-associated kinase 4

Abbreviations

IRF-7: Interferon Regulatory Factor 7

JNK: c-Jun N-terminal kinase

MHC: Major Histocompatibility

mRNA: messenger Ribo Nucleic Acid

MyD: Myeloid Differentiation

NF-kb: Nuclear factor-kappa B

NLR: Nod-like receptor

OVA, Ovalbumin

PCA: Passive Cutaneous Anaphylaxis

PRR, Pattern Recognition Receptor

RLR: Retinoic acid-inducible gene I (RIG-I)-like receptor

SR-A, Scavenger Receptor –A

STING: Stimulator of interferon genes

Th1: Type 1 lymphocyte

TLR, Toll-like receptor

TRAF: Tumor necrosis factor receptor–associated factor

Treg: T regulatory

TSLP: Thymic Stromal Lymphopoietin

WAO: World Allergy Organization

CHAPTER 1

GENERAL INTRODUCTION

1.1 Contest of study and Problematic

Food allergy is a common Type I allergy disease, and its prevalence is steadily increasing in the world with an estimation of 10% of children in some countries affected [1]**.** This affection represents an economic, psychological, and health burden for the allergic patients and their relatives, compromising deeply the quality of their life [2]. Protective strategies and care developed thanks to a better understanding of the underlying molecular mechanism through various scientific research have allowed a clear improvement in the management of the allergic subject. These include notably the elimination of the triggering food from the diet, progressive desensitization through sequential and controlled administration of the allergen (called allergen-specific immunotherapy), and the use of prophylactic antihistamine and corticosteroid medications [3,4]. Nonetheless; these treatments are relatively difficult and costly to implement because they require constant vigilance and/or medical care. Besides, some of them often result in detrimental side effects in long-term use [5]. Hence, the need for alternative solutions to overcome this disease. In this regard, the research trend is towards the use of anti-allergic compounds derived from natural resources; which are better tolerated by the body and present no side effects. One of those molecules; intensively studied over the past decades is fucoidan.

"Fucoidan" is a general term conferring to fucose-riched sulfated polysaccharide extracted from brown seaweed species [6]. It is a complex class of macro polysaccharides

with a broad range of biological activities such as anti-oxidant, anti-cancer, anti-coagulant, anti-thrombotic, anti-inflammatory, and anti-allergic effects, amongst others [7,8]. The anti-allergic effect of fucoidan was depicted in numerous research studies. For instance, Herath et al. (2020) in their research showed that oral consumption of fucoidan from *Undaria pinnatifida* attenuated allergic airway inflammation in mice by curbing key features of the allergic reaction such as inflammatory cells infiltration, goblet cells hyperplasia, IgE production, mast cells degranulation, etc [8]. Moreover, others studies reported that oral intake of fucoidan favored beneficial probiotic growth, and acted on intestinal microbiota species such as *Firmicute, Lactobacillus, Bifidobacteria, Ruminococcaceae* which actions in the lumen prevent and restore the gut immune system defect, observed in food allergy conditions [9-13]. Conversely, research in our laboratory veiled the anti-allergic potential of a fucoidan solution extracted from *Saccharina japonica* seaweed, an edible plant largely part of the Japanese diet.

Fucoidan extracted from *Saccharina Japonica* species demonstrated a strong anti-allergic effect in a mouse model of OVA-induced food allergy and passive cutaneous anaphylaxis in mice [14-16]. A deeper understanding of its effect revealed that it took place only when the polysaccharide was orally absorbed, pointing out an important role of the intestinal system in its underlying mechanism. Fucoidan induced an upregulation of galectin-9 protein in the blood [14,15], which further removed IgE on mast cells, preventing their degranulation [15]. The increased expression of galectin-9 protein was a paramount mechanism of fucoidan bioactivity and the blocking of galectin-9 protein by intravenous injection of anti-galectin-9 antibody canceled its protective effect on allergic mice [14-16]. Galectin-9 is a lectin protein that is involved in many physiological functions such as inflammation, immune responses, cell migration, and signaling [17].

Biosynthesis, as well as signaling trafficking of molecules of this class, are not fully understood, mostly due to their unconventional secretory pathway which does not involved the endoplasmic reticulum/golgi apparatus system [18]. Galectin-9 underpinning mechanism of upregulation upon fucoidan administration is unknown and remains an important point to clarify.

Questioning how fucoidan from *Saccharina japonica* induced upregulation of galectin-9 is on point as it is reported that fucoidan is poorly or not absorbed by the body [19,20]. Fucoidan is well-known as a ligand of scavenger receptors, notably scavenger receptor A (SR-A) [21,22] and interestingly also reported as an independent ligand of other pattern recognition receptors like TLR2 and TLR4 [23]. A plausible explanation for our question could therefore lie in a fucoidan/receptors signaling pathway in the intestine, leading to an up-regulation of galectin-9 protein. Recently, several emergency scientific reports suggest an implication of Toll-like receptor 9 (TLR9) in galectin-9 secretion [24-29]. However, no investigation has been done.

TLR9 was primarily described as an endosomal receptor that recognizes a specific sequence of DNA bacteria named CpG DNA [30]. However, numerous studies portray a more complex issue. For instance, its cellular localization (intracellular or surface expression), cell trafficking, and expression have been noted to be dependent on the surrounding environment, be species-specific, cell types dependent, and even trigger-type dependent [31,32]. In addition, cell-surface expression of TLR9 has been noted on intestinal epithelial cells (IEC) [33,34]. Research studies results of de Kivit et al. showed that synbiotics intake favored galectin-9 induction, which was potentiated by combination with non-digestible oligosaccharides [24-26]. Later, distinct reports also substantiate their

observation [27-29]. All these reports open new perspectives on the functionality of this receptor, which may expand its amphoteric knowledge.

Fucoidan from *Saccharina japonica* also upregulated galectin-9 protein expression shown *in vivo* [14,15]. But whether it is through the novel TLR9/galectin-9 pathway is unknown. Fucoidan can be internalized by Class A scavenger receptor (SR-A), which was depicted to trigger intracellular signaling events in cooperation with some PRRs such as TLR4 and TLR9 [21]. Besides, Fucoidan treatment recently had been shown to upregulate genes involved in the signaling of several nucleic acid-sensing receptors such as Retinoic acid-inducible gene I (RIG-I)-like receptor (RLR), Toll-like receptor (TLR), Nod-like receptor (NLR), Stimulator of interferon genes (STING) [35] and to mimic DNA in solution [36].

Therefore, based on the information above, and in consideration of all the specificities of fucoidan, we hypothesized that TLR9 may play a role in the upregulation of galectin-9 observed upon fucoidan intake. As expected, fucoidan-induced galectin-9 secretion involved TLR9 activation.

This study is of interest for the treatment of food allergy with fucoidan; and overall sought at understanding the mechanism of galectin-9 upregulation consequent to fucoidan intake.

For a better understanding of this research, this first chapter was dedicated to reviewing key elements of the study. Relevant information on them related to our research is provided to deepen the comprehension. Their interconnections and implications for this research's goal were also addressed.

1.2 Allergy Disease

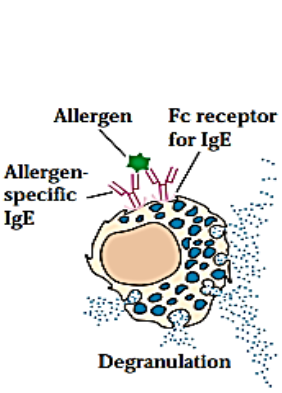
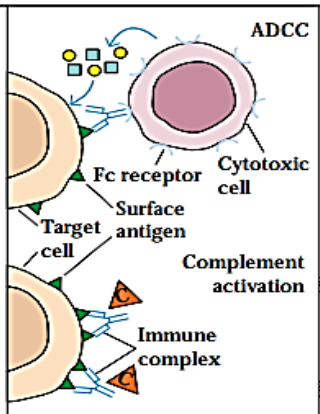
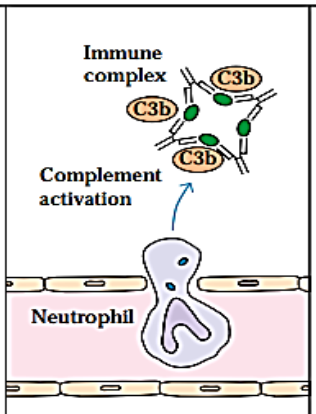
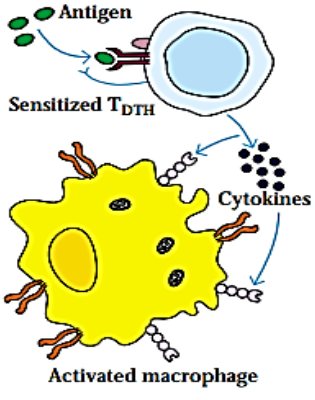
1.2-1 Different types of allergy reactions

Allergy or atopy is a non-communicable disease that reflects a disorder of some individuals' immune system to normally no harmful exogenous substances [37]. Patients with allergy conditions have their immune defense wrongfully overreacting to protein or chemicals-bound protein that can typically be found in pollen, house dust mites, food, drugs, etc., and develop a hypersensitivity. Hence, allergy diseases are also referred to as hypersensitivity reactions. Commonly, patients undergoing an allergic reaction may present some of those symptoms: nausea, vomiting, abdominal pain, itching, sneezing, running nose, difficulty breathing, and in rare cases life-threatening anaphylaxis shock.

Allergic reactions affect a lot of people worldwide with a prevalence rate close to 20% [2]. Primarily observed among the population of westernized countries, it is increasing in developing countries; and that is being linked to intense lifestyle changes (diet, urbanization, improvement of standard sanitization), and impact considerably the quality of life (health, psychological, economic) of the allergic subject and its relatives [3,38]. Notwithstanding great progress made in understanding its pathogenesis, the etiology of allergy disease is not fully understood. In the 2013 report, the World Allergy Organization (WAO) pointed out that several factors, often interconnected, can lead to its occurrence, such as genetic predisposition, health status, a change to a westernized lifestyle, the impact of the environment, the hygiene hypothesis, etc [38]. In addition, allergic reactions share common features, and several allergic reactions type can occur concomitantly in an individual. Nevertheless, they differ in the type of antigens that elicit the reaction, the site of the reaction, the molecular mechanism, and their clinical symptoms [39]. Thus, the

classification of Gell and Coombs subdivided allergic diseases based on their molecular mechanism. Four groups can be distinguished: type I (IgE mediated hypersensitivity), type II (IgM/IgG mediated hypersensitivity), type III (Immune complex-mediated hypersensitivity), and Type IV (T cell-mediated hypersensitivity) [40]. Specificities of each group are detailed in Table 1 [41]. Type I allergy is the most prevalent form of allergic disease and comprises allergic rhinitis, allergic conjunctivitis, asthma, atopic dermatitis, food allergy, and systemic anaphylaxis [37,42]. This study focused on Food allergies.

Table 1: The four types of hypersensitive responses [41]

 Type I	 Type II	 Type III	 Type IV
IgE-Mediated Hypersensitivity	**IgG-Mediated Cytotoxic Hypersensitivity**	**Immune Complex-Mediated Hypersensitivity**	**Cell-Mediated Hypersensitivity**
Ag induces crosslinking of IgE bound to mast cells and basophils with release of vasoactive mediators	**Ab directed against cell surface antigens meditates cell destruction via complement activation or ADCC**	**Ag-Ab complexes deposited in various tissues induce complement activation and an ensuing inflammatory response mediated by massive infiltration of neutrophils**	**Sensitized T_H1 cells release cytokines that activate macrophages or T_C cells which mediate direct cellular damage**
Typical manifestations include systemic anaphylaxis and localized anaphylaxis such as hay fever, asthma, hives, food allergies, and eczema	**Typical manifestations include blood transfusion reactions, erythroblastosis fetalis, and autoimmune hemolytic anemia**	**Typical manifestations include localized Arthus reaction and generalized reactions such as serum sickness, necrotizing vasculitis, glomerulnephritis, rheumatoid arthritis, and systemic lupus erythematosus**	**Typical manifestations include contact dermatitis, tubercular lesions and graft rejection**

1.2-2 Food allergy and its molecular mechanism

Food allergy (FA) is defined as "An adverse health effect arising from a specific immune response that occurs reproducibly on exposure to a given food"[43]. In other words, a food-allergic subject has its immune system wrongfully, constantly overreacting to various compounds commonly found in foods. The most common food allergens identified form the “Big Eight” group, which comprises milk, eggs, tree nuts, peanuts, shellfish, wheat, soy, and fish [44]. IgE-mediated food allergy reaction is an intensive Th2 immune response, characterized by an elevated serum level of immunoglobulin E (IgE) [45]. The reaction is set to occur in the gastrointestinal tract (the intestine) but can also spread to other organs such as the skin and the respiratory tract; leading to a systemic effect, with the possibility of life-threatening anaphylactic shock. The molecular mechanism implicates several coordinated reactions, all subdivide into two critical phases: a sensitization phase and an effector phase referred also to as the "challenge phase" [4]. During the sensitization phase, the uptake allergen is processed by specialized antigen-presenting cells (APC) such as dendritic cells (DC) and presented to the T cell receptor of naïve T cell. With additional costimulatory signals from DC (CD40, B7), naïve T cells differentiate into type 2 helper cells (Th2) with the production of Th2 cytokines IL-4, IL-5, and IL-13. The action of these cytokines (mainly IL-4 and IL-13) on B cells favors an immunoglobulin class-switch recombination from IgG or IgM to IgE, which then binds FcεRI receptors on mast cells. The effector phase is characterized by the occurrence of the allergic symptoms elicited by pro-inflammatory molecules, derived from mast cell degranulation. Mast cell degranulation follows crosslinking of IgE-FcεRI complex with de novo entry of the same antigen [4,46].

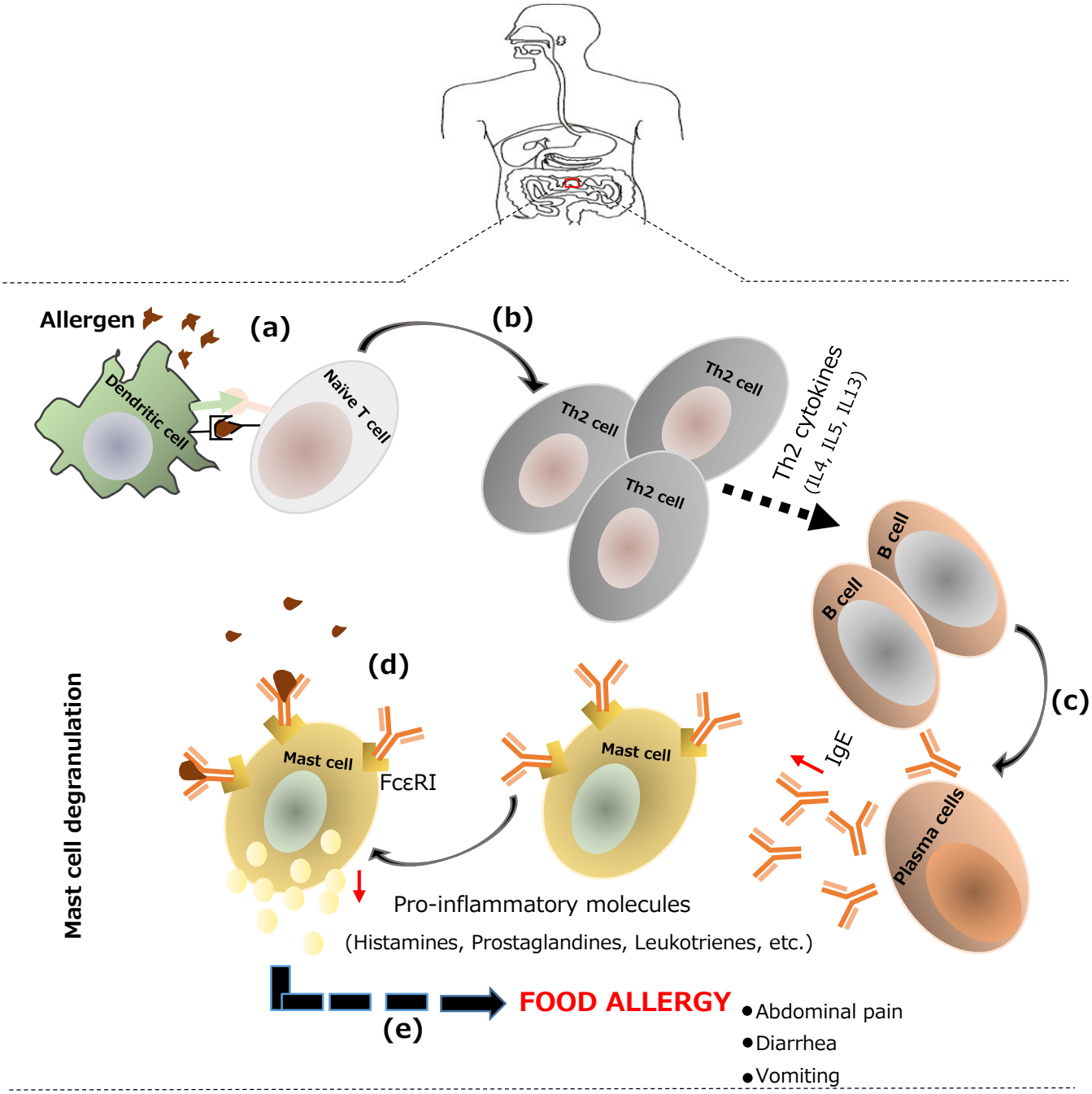


Figure 1.1: Molecular Mechanism of Food allergy

DC uptake the allergen, degrade it inside their cytoplasm and present it to the TCR of naïve T cells by their MHC II **(a)**. Additional costimulatory signals from DC are necessary to complete naïve T cell activation and differentiation into Type 2 Helper cells, producers of Th2 cytokines **(b).** Th2 cytokines acts on B cells, which differentiate into plasma cells with a class switching recombination of IgG/IgM to IgE **(c).** IgE has a high affinity for FcεRI receptor expresses by mast cell and bound to it. Cross-linking of new allergen on IgE/FcεRI complex induces mast cell degranulation, releasing their granules contents, but also newly synthetized inflammatory mediators **(d)**. Those molecules actuate vasodilation, diapedesis of effectors cells to the lamina propria, increase gut permeability, fluid extravasation and all causing the different allergic symptoms observed **(e).**

1.3 Hypersensitivity reaction to foods: outcome of a defect in the gut immune tolerance system

1.3-1 Critical role of intestinal epithelium layer in maintaining oral tolerance to food proteins: Involvement of secretory immunomodulatory molecules

The gastrointestinal tract is constantly exposed to multiple antigens from food, environment, or host cells [32]. However, there is a state of equilibrium, a tolerance of the gut-associated immune system to these different antigenic stimuli to avoid untoward and excessive immune reactions. This account for oral tolerance, which controls the balance between a normal immune response for eliminating an ingested pathogen antigen and maintaining intestinal homeostasis [47,48]. The food allergic reaction is the result of a failure or loss of this system [49].

Oral tolerance is notably assured by the intestinal immune system and its components: the intestinal microbiota, the intestinal mucosa, the epithelium layer, and the immune cells of the lamina propria [48,50]. Because the food allergy reaction is initiated by the interaction of the food allergen, initially present in the intestinal lumen and specialized antigen-presenting cells (DC, macrophages) located in the lamina propria, the integrity and function of the intestinal epithelium layer at the interface between them is therefore critical in the regulation and promotion of food allergy. The IEC layer is made up of several IECs cells subtypes (enterocytes, stem cells, goblet cells, enteroendocrine cells, Paneths cells, tuft cells, and M cells) linked together by tight junctions proteins (occludins, claudins, Zonulin-1,..) [51,52]. That line does represent not only a physical barrier but also a chemical and immunomodulatory line of defense. In fact, some components of the layer are secretory cells. For instance, goblet cells produce mucin, a viscous substance

that prevents allergens from entering the lamina propria, helps to contain and trap commensal gut microorganisms around the epithelial layer and modulates their colonization [51,52]. Moreover, IECs can release proteins with both positive and negative immunomodulatory effects on allergy reactions such as eotaxin-1, CD83, TSLP and galectin-9 [25,53-55]. In addition, they express a myriad of receptors from different families, such as Toll-like receptors, nucleotide oligomerization domain (NOD) receptors (NLRs), retinoic acid-inducible gene I (RIG-1) receptors (RLRs), which distinguish, analyze, and direct a specific response to different stimuli in the surrounding environment [56].

1.3-2 Galectin-9 secretion by IECs and Toll-like receptor 9

Galectin-9 is a lectin protein that possesses a binding function for β-galactoside [57]. It is a single polypeptide chain with two CRDs at its ends linked together: an N-terminal CRD (N-CRD) of 148 amino acids and a C-terminal CRD (C-CRD) of 149 amino acids**,** typical of the so-called ‘‘tandem-repeat type’’ subfamily [58]. In the last few decades, galectin-9 activity in food allergy has attracted much attention. For instance, an increase in serum galectin-9 level was positively correlated with an attenuation of acute allergic symptoms in a mice model of whey-induced food allergy [24]. Similar positive effects of galectin-9 on allergic reaction were also observed in the study of Ikeda M et al. in which sublingual administration of galectin-9 dampened several features of allergic asthma such as AHR, increased eosinophils infiltration, Th2 cytokine production [59].

Analysis of the underpinning modulatory mechanism of galectin-9 revealed that it induces T cell polarization towards Th1 or Treg cell type [24, 60]. In addition, it can

neutralize or remove IgE on mast cells, thus blocking IgE-antigen complex formation, and preventing the release of the granules contents of mast cells [15, 61]. Galectin-9 secretion in the intestine was observed in both *in vivo* and *in vitro* studies. In whey-sensitized mice fed with non-digestible oligosaccharides and a *Bifidobacterium breve* diet, an upregulation of galectin-9 expression was denoted in the ileum and the mesenteric lymph nodes [24]. In addition, other studies observed the release of galectin-9 protein by IECs using *in-vitro* transwell experiments [25-29]. That release followed stimulation of TLR9 in IECs by synthetic unmethylated CpG oligonucleotides (CpG-ODN), models to its natural ligand CpG-DNA; a DNA sequence with Cytosine-phosphate-Guanine dinucleotide (CpG) motif, abundantly found in bacterial and viral DNA [30]. Hypomethylation of CpG-DNA of bacteria is notably at the roof of their immuno-stimulatory activity which induces type I interferon or inflammatory cytokines release [62,63]. TLR9 plays an important role in maintaining gut homeostasis by eliciting and controlling the proper response to the myriad of bacteria that populate the intestinal tract. It also protects against intestinal damage and favors intestinal repair in gut inflammatory conditions. TLR9 as a therapeutic target in food allergy has attracted much attention and from the different research results, appeared to elicit a positive effect (secretion of anti-inflammatory molecules) via its apical activation on IECs [24-29]. The use of the term "apical activation of TLR9" may seem confusing at first, because what was believed about the localization of this receptor until recently was only an endosomal presence after translocation from the ER [64,65]. However, more recent reports have described TLR9 expression at the membrane of several cell types like human tonsils cells, splenic DCs, peritoneal mast cells and IECs [33,34,66-68]. Nevertheless, the factors leading to and implications of such localization versus ER expression on its function are yet obscure and

remain to be fully elucidated. Worth-mentioning, TLR9, like other members of Type I integral transmembrane proteins contain three domains: an ectodomain located outside the membrane, comprising the ligand-biding sites; a transmembrane domain that serves as a membrane anchor and a cytosolic TIR domain for signal transduction [64,65]. Canonical pathways on activation are reported to involve first the recruitment of myeloid differentiation primary response (MyD) 88 adaptor protein to the TIR domain. This initiates a cascade event comprising engagement of further adaptors proteins such as IRAKs, TRAFs, and IKKs leading to activation of transcription factors NF-kb; AP-1, IRF-7 and induction of either proinflammatory molecules or type I IFNs (see Figure 1.3) [62]. In IECs, though, signaling of TLR9 appears to be more complex. IECs unlike immunocytes are polarized by the presence of distinct environments on either side of their compartments. The apical side faces the gut lumen while the basolateral side is in contact with the lamina propria [32]. Each of these sides presented surface expression of TLR9 at similar levels, confirmed by flow cytometry, confocal microscopy imaging and vectorial biotinylation studies [33]. It was notably identified in the study of Lee et al. (2006) that the signal trafficking from TLR9 activation in immunocytes is different from the one induced in IECs. Chloroquine and bafilomycin A1, distinct endosomal inhibitors that act by neutralizing the acidic pH vital for the proper maturation of endosome, and thereby impedes endosomal TLR9 immune responses were ineffective on TLR9-mediated activation of NF-kb or JNK in IECs following basolateral activation [33]. This fact suggests the existence of a signaling pathway different from the aforementioned. Moreover, apical activation versus basolateral activation of TLR9 may serves different features. The first one has been associated with an anti-inflammatory effect while the second one promoted an inflammatory response [22]. The mechanism underpinning

galectin-9 secretion by IECs is poorly known and remains much unclear. Despite, increasing pieces of evidence suggesting an implication of TLR9 in that secretion, no investigation has been done. The secretion of galectin-9 following TLR9 stimulation could be one of the different processes conferring anti-inflammatory properties to the said receptor.

It is clear that much remains unknown about the TLR9 receptor. Its cellular localization, species-specificity, cell type-dependent effect, and even trigger-type dependent outcome are all factors that should be taken into consideration in its study and need deeper investigations. However, none can deny its key role in managing food allergy outcomes due to its expression on IECs and the modulation of IECs´s interaction with the surrounding environment (microbiota, dietary fiber). The novel TLR9/galectin-9 pathway is here also evaluated in this study.

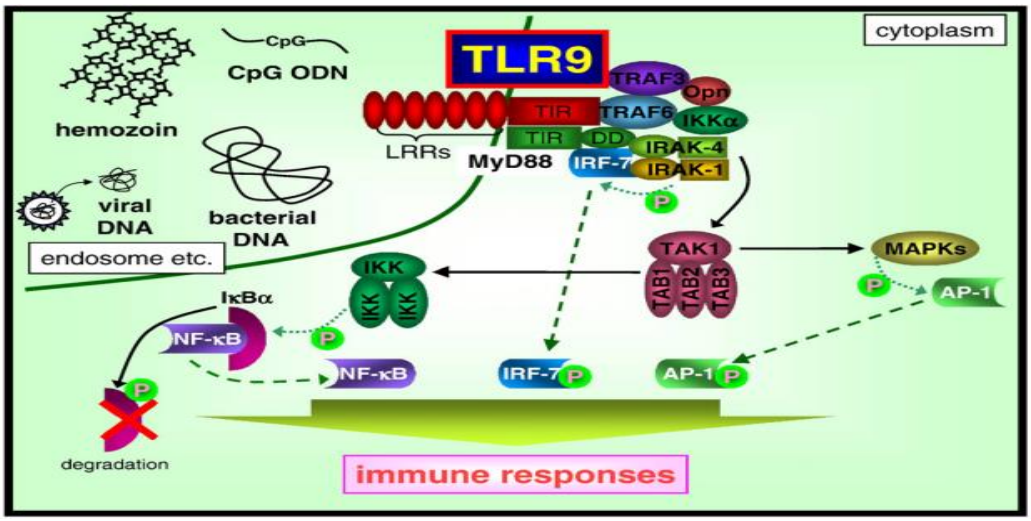


Figure 1.2: Overview of canonical TLR9-mediated signaling pathway expressed on the endosomes.

After activation by its ligand, MyD88 is recruited at the cytosolic TIR domain and initiates a cascade event comprising engagement of further adaptors proteins such as IRAKs, TRAFs, and IKKs leading to activation of transcription factors NF-kb; AP-1 IRF-7 and their consequent immune responses. The illustration is from Kumagai Y et al. (2008) [62]

1.4 Dietary polysaccharides fucoidan

Over the years, the global demand for seaweeds has increased, and their usage has considerably shifted. Initially used mainly for domestic purposes as food and feed, the seaweed biomass has now various applications, ranging from food additives, fertilizers, and biofuels, to nutraceuticals, cosmetics, and pharmaceuticals products [69,70]**.** Derived products from seaweed such as polysaccharides have been intensively studied for their immunologic properties and have revealed pretty interesting activities.

"Fucoidan" is the name given to the group of fucose-rich sulfated polysaccharides extracted from the cell wall of marine algae of the Phaeophyceae class [6]. Several studies have been conducted on its biological properties and revealed diverse bioactivities such as anti-oxidant, anti-inflammatory, anti-cancer, anti-thrombotic, anti-viral, immunomodulatory, anti-allergic effects, etc [7,8]. Thus, growing interest in fucoidan in the medical research field. Fucoidan structure and composition are quite complex and difficult to label due to the heterogeneity and irregularities of the structure among various species of brown seaweed [71,72]. Most commonly, two main types of backbones can be observed: one that comprised repeat units of (1→3)-linked-α-L-fucopyranose residues and another with alternating (1→3) and (1→4) linked-α-L-fucopyranose backbone (Figure 1.3) [21]. The sulfate group is found at C2 or C4 and some components other than fucose may be present such as glucose, galactose, mannose, xylose, arabinose, rhamnose, proteins, glucuronic acids, acetyl groups in variable amounts [71,72]. For instance, fucoidan extracted from *Fucus vesiculosus* was reported to contain 44.1% fucose and 26.3% sulfate arranged in a polymer of α-(1→3) linked fucose with sulfate groups

substituted at the C-4 [72]. Meanwhile, the major sulfated polysaccharide present in *Undaria pinnatifida* fucoidan is characterized to be a galactofucan sulfate, with notably its backbone made up of various sugar linkages such as 1,3-linked fucose, and 1,3-, 1,4-, and 1,6-linked galactose with sulfate ester suggested to be substituted at 2-position of fucosyl residue and 3- or 6-position of galactosyl residue [73]. Moreover, several factors have been noted to impact considerably the chemical compositions and linkages arrangements of fucoidan. Those reported till far are the species (variation among the same specie has also been observed), the geological area of production, the season of harvest, the extraction method applied (hot-water; acetic, alkaline, enzyme-assisted extraction), the temperature and the pressure [74-76]. The anti-allergic activity of fucoidan was examined on different allergic reactions and the results evidenced that fucoidan acted positively at different strategic stages of the allergic reaction mechanism, thus helping to reduce the resulting clinical symptoms. For example, the treatment of atopic dermatitis in the NC/Nga mice model with *U.pinnatifida* fucoidan significantly dampened AD-associated symptoms like tissue erythema, skin scratch, excoriation, and mast cell infiltration [77]. Moreover, fucoidan administration reduced IgE and histamine levels, and also mRNA expression of several inflammatory chemokines (TARC, MDC, RANTES) associated with the severity of AD in a dose-dependent manner [77]. Likewise, a wide number of other studies depicted fucoidan's anti-allergic effect, and highlighted some of its actions such as promoting Th1 over the TH2 immune response; increasing Treg cells counts, impacting B cells class switching to IgE, and favoring induction of anti-allergic regulator molecules like galectin-9 [14,15,78-81].

In previous studies, it was ascertained that dietary intervention of fucoidan from *Saccharina japonica* seaweed favored an upregulation of galectin-9 in the blood, resulting in the attenuation of the food allergic symptoms in mice [14-16]. In a peculiar way, the authors also revealed that the anti-allergic potential took only place upon oral administration of fucoidan [14], emphasizing then the importance of the intestinal system in the induction of galectin-9 protein. In this subsequent study on the anti-allergic properties of *saccharina japonica*'s fucoidan, we aim at highlighting the mechanism of galectin-9 upregulation consequent to fucoidan intake. We hypothesized that TLR9 activation is implicated in the upregulation of galectin-9 in the intestine upon fucoidan consumption and ought to evaluate it. Firstly, in Chapter 2, we did confirm the anti-allergic effect of the fucoidan crude extract *in vivo* by performing a PCA test. In chapter 3, the bioactive fraction, Fraction 1 (F_1), identified in previous studies [14-16], was obtained after chromatographic separation and its content of fucoidan was evaluated by the Dische method. Fraction 1 presented the highest fucoidan content, consistent with some reports [82]. Moreover, a gel filtration analysis was also performed on fraction 1 and revealed its homogeneity and purity, important factors for its subsequent use *in vitro* in analyzing plausible interaction with TLR9. Studies reported that fucoidan because of its physicochemical properties can hardly be degraded by intestinal enzymes and can therefore be present in all its integrity in the lumen of the intestinal tract [19,20]. Likewise, fucoidan has been used for decades in the food industry as a food-grade delivery system, or drug carrier; prompted to its favorable safety and good stability [71]. Therefore, in chapter 3, galectin-9 upregulation observed *in vivo* upon fucoidan's oral administration was evaluated *in vitro* by direct exposure of fucoidan solutions at different concentrations to intestinal epithelium cells line: HT-29 cells. An increasing trend in mRNA expression

of galectin-9 was observed in IECs subjected to fucoidan stimulation, concomitant with a significant upregulation of the protein level. These results showed for the first time *in vitro* that fucoidan interact with IECs (HT-29 cells) to favor galectin-9 secretion. Therefore, fucoidan consumption may also be advised in the preventive treatment of food allergy reactions. The effect of fucoidan on galectin-9 production was notably modulated by the dose and time of exposure. Finally, we investigated *in vitro*, the implications of TLR9 in the induction of galectin-9 protein upon fucoidan. It is often observed that during their activation, PRRs increased both at the transcriptional and translational levels, due to the activity of the receptor. Albeit, some authors mentioned there is no trivial relationship between the concentration of a transcript and that of the derived protein [83]. Here, we analyzed the mRNA expression of TLR9 upon fucoidan exposure on HT-29 cells. Fucoidan stimulation of HT-29 cells tended to increase TLR9 mRNA expression, alike the effect of CPG-ODN (TLR9 ligand) exposed to HT-29 cells, suggesting TLR9 activation by fucoidan. Noteworthy, activation of TLR9 by CpG-ODN favored also galectin-9 protein secretion by HT-29 cells consistent to the result of de Kivit et al. (2013) [25] and combined exposure of fucoidan and CpG-ODN resulted in a synergetic effect on the protein level of galectin-9 secreted by HT-29 cells. Furthermore, using the siRNA silencing technic, we found that knockdown of TLR9 in HT-29 cells tended to reduce galectin-9 protein release following fucoidan stimulation. These results all together demonstrated that TLR9 activation is involved in the secretion of galectin-9 upon fucoidan exposure *in vitro*. Future studies may provide better clues and understanding of the downstream targets and genes involved in this novel TLR9/galectin-9 pathway. Evaluation of IEC-expressed SRs on fucoidan-mediated TLR9 activation is an avenue of research to consider.

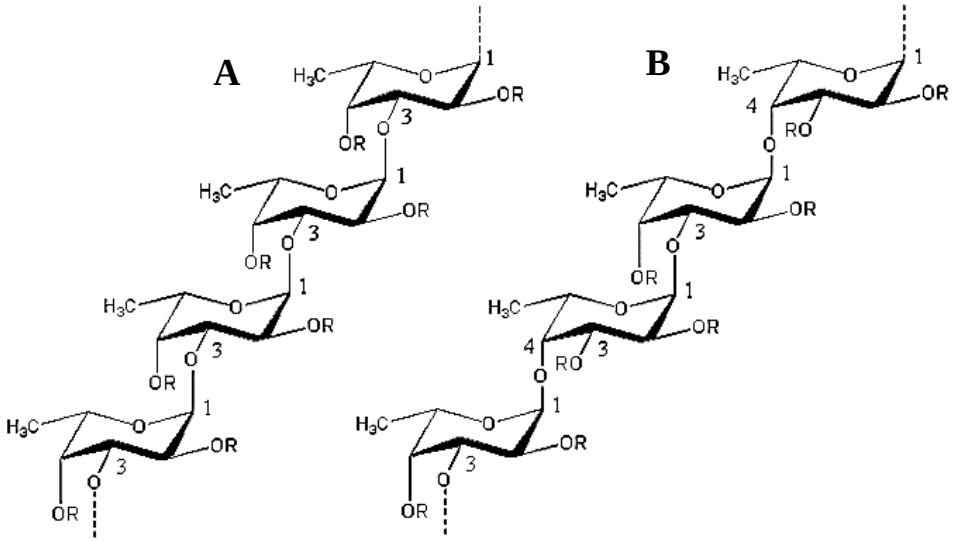


Figure 1.3: The two main structures of fucoidan [21].

(A) Repeat units of (1→3)-linked-α-L-fucopyranose residues (B) Fucoidan backbone made of alternating (1→3) and (1→4) linked-α-L-fucopyranose residue

CHAPTER 2

CONFIRMED ANTI-ALLERGIC BIOACTIVITY OF FUCOIDAN EXTRACTED FROM *Saccharina japonica*

2.1 Introduction

As previously mentioned, food allergy is a condition in which one develops an excessive reaction against food antigens, attesting to a deficiency in the oral tolerance initially provided by the gastrointestinal immune system [49]. To overcome this disease, the research trend is on the utilization of natural bioactive product, possessing anti-allergic activity and having a good safety. Fucoidan from *Saccharina Japonica* have been ascertained to meet all those criteria. *Saccharina japonica* is an edible brown seaweed, which may be found along the coasts of northern Hokkaido, Japan, southern Sakhalin and Primorye on the eastern coast of the mainland of the Russian Far East [84]. The chemical backbone of fucoidan from *Saccharina Japonica* was identified to be made up of repeat units of (1→3)-linked-α-L-fucopyranose residues, with a sulfate group at C-2 or C-4 [15]. As afore-mentioned, most plant polysaccharides have their bioactivity impacted by several factors among which is the applied extraction method [76,85]. Extraction parameters such as pH, time, pressure, chemical solvents, etc can significantly impact the native chemical backbone of fucoidans (glycosidic linkages, sulfate content, type of monosaccharide units, sulfation pattern) and consequently their biological activities and properties [86]. Many studies have notably demonstrated a close relationship between the physicochemical properties of fucoidan and its biological activity [72,87], although this relationship is not fully clarified and well understood.

In this chapter 2, crude fucoidan was extracted from *Saccharina japonica* blades by sodium acetate buffer coupled with ethanol precipitation. The total carbohydrate and protein content of the crude extract were analyzed by phenol-sulfuric acids test and Lowry assay. The anti-allergic effect of the crude extract was assessed in a mouse model of Passive Cutaneous Anaphylaxis reaction (PCA). Results of the experiment showed that fucoidan crude extract possessed anti-allergic activity, consistent with past studies reports. Attenuation of the atopic reaction was effectively observed following oral administration of the crude extract to allergic mice.

2.2 Materials and Methods

2.2-1 Reagents

Bovine Serum Albumin (BSA), acetic acid, and sodium acetate were purchased from Nacalai Tesque, INC (Kyoto, Japan). Mouse anti-2,4,6-trinitrophenyl (TNP) monoclonal IgE was purchased from BD Biosciences (San Jose, CA, USA). 2,4,6-Trinitrochlorobenzene was purchased from Tokyo Chemical Industry (Tokyo, Japan). Other chemicals and reagents were ordinary commercial and guaranteed products.

2.2-2 Extraction of crude fucoidan

Blades of *Saccharina Japonica* seaweed, purchased from Japanese companies were used to extract crude fucoidan according to the protocol established by Tanino et al. (2016) [14]. Practically, 25 g of blades powdered *Saccharina Japonica* were dissolved in sodium acetate buffer (0.1M, pH 4.6) overnight at 4˚C, then centrifuged at 3500 rpm, 4˚C for 10 min. The supernatant was collected and the polysaccharide was obtained after precipitation, by adding two times the volume of 100 % ethanol to it and let stand overnight at 4˚C under gentle stirring. The pellet (polysaccharide) was collected by centrifugation (3500 rpm, 4˚C, 10 min), washed twice with 100% ethanol, centrifuged again at the same condition and finally dissolved in distilled water. Then, the crude extract was lyophilized to obtain purified crude fucoidan. Fucoidan yield was determined as the ratio of the weight of its extract to the weight of dry seaweed flour expressed in percentage. The extraction flowchart is here presented below.

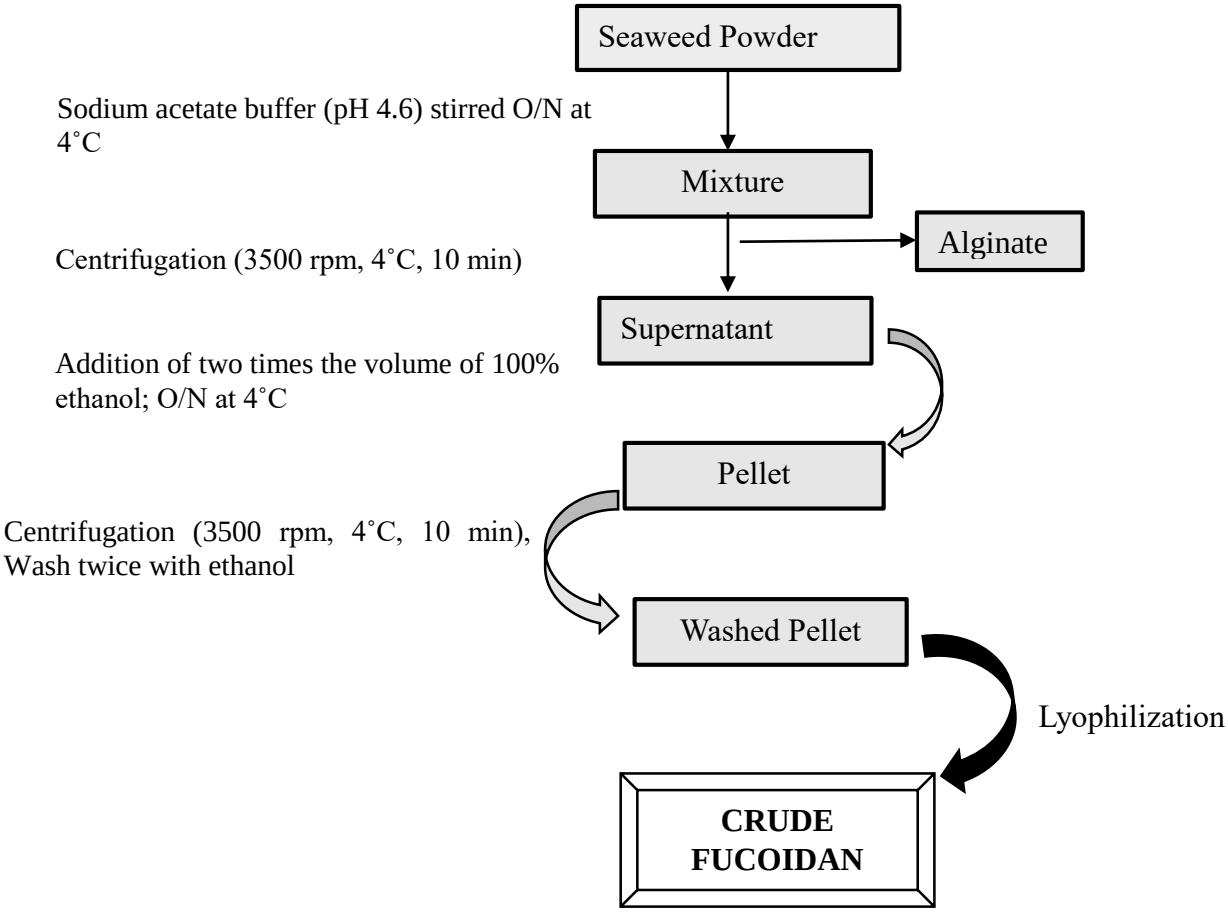


Figure 2.1: Extraction process of crude fucoidan from *Saccharina japonica*

2.2-3 Determination of Total carbohydrate content

Total carbohydrate content was evaluated by phenol-sulfuric acid test [88]. This test is based on the fact that in the presence of concentrated sulfuric acid, sugars contained in polysaccharides are dehydrated into hydroxymethyl furfural compound, which forms a yellow-brown-colored product with phenol and has an absorption maximum at 490 nm UV spectroscopy.

The experiment was conducted as follows: briefly, 2 mg of L-fucose was dissolved in 1mL of distilled water, and distributed in volumes of 0; 50; 100; 150; 200; 250 µL in glass tubes. Volume in all glass tubes was made up to 250 µL with distilled water before adding 500 µL of phenol 5 %, then mixed. 1 mL of concentrated sulfuric acid was added to the different solutions, mixed, and left at room temperature for 20 min. The absorbance of the reaction was read at 490 nm using a microplate reader (SH-9000, Corona electric, Japan) and the values were used to construct a standard curve. Duplicate samples of 250 µL of fucoidan solution (2 mg/mL) were analyzed with the same procedure and the total sugar content was expressed in percentages (%) of weight.

2.2-4 Determination of total protein content

The content of total protein in crude fucoidan was determined the Lowry's method, with some modifications [89]. For the experiment, we collected 1 mL of lowry's solution: solution A and solution B (50:1) and added it to 0.2 mL of samples, vortexed, and left for 15 min at room temperature. 100 µL of a solution of Folin's phenol reagent (1 N) was added subsequently, mixed and all incubated at room temperature for 30 min. The absorbance was measured at 750 nm using a microplate reader (SH-9000, Corona electric, Japan). 2 mg/mL solution of BSA was divided into different samples, which allowed the establishment of a standard curve. Solution A and solution B of lowry's solution were made of 4 mg/mL NaOH added to 20 mg/mL Na_2CO_3 in distilled water, and 10 mg/mL Potassium sodium tartrate added to 5 mg/mL of $CuSO_4$ in distilled water.

2.2-5 Mice

Female BALB/c mice (4 weeks old, 15-20 g) were purchased from Japan SLC (Shizuoka, Japan). All the long, mice were maintained at an air-conditioned animal room temperature of 23 ± 2 ºC and a humidity of 55 ± 10%. Prior to the experiment, mice were acclimatized for 5 days in these conditions with free access to laboratory chow and water *ad libitum*. The animal experiment was approved and performed in accordance with the guidelines of the Animal Experimental Ethics Committee of Kobe University (Registration number: 29-02-02-R3).

2.2-6 Passive Cutaneous anaphylaxis model mice

Mice were randomized into different groups and orally fed with fucoidan samples (200 µg/day) prepared in distilled water for 7 days (see Table 1 below). After the administration period, mice were sensitized by intravenous injection of mouse anti-TNP IgE (BD Biosciences, San Jose, USA) and challenged 30 min later by topical application on the ear of 1.6% Trinitrochlorobenzene in acetone: olive oil (1:1). The increase in ear thickness, resulting from mast cell inflammatory substances' action was assessed as a feature of the ongoing allergic reaction. The difference in the ear thickness 2 h after and before the challenge, measured using a micrometer (Ozaki MFG, Tokyo, Japan) was represented as edema. A crude fucoidan sample extracted by our predecessor, which anti-allergic activity has been confirmed [14] was used as a positive control.

Table 1: PCA experiment plan

	Blank	Ag/IgE	Positive control: Crude fucoidan from Morita [14]	Crude Fucoidan
Anti-TNP IgE (i.v)	-	+	+	+
Antigen(10 µL)	+	+	+	+
Crude Fucoidan (200 µg/day)	-	-	+	+

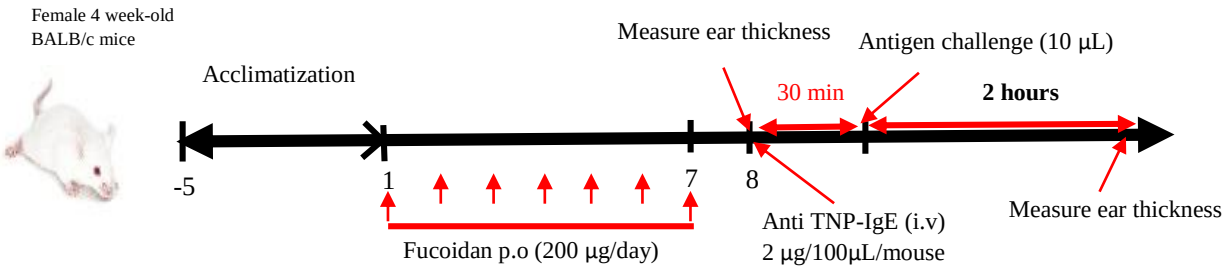


Figure 2.2: PCA Experiment plan

2.2-7 Statistics analysis

Data are expressed as means ±SE. All statistical analyses were performed by Tukey Kramer test. P-values below 0.05 (*P<0.05) and P-values below 0.01 (**P<0.01) were considered significant.

2.3. Results

2.3.1 Crude fucoidan, total carbohydrate and protein content

Crude fucoidan was extracted in an aqueous solution of sodium acetate buffer (0.1M, pH 4.6) and precipitated with a large volume of absolute ethanol. The yield of extraction was 6.52%. The total carbohydrate and protein content of the crude fraction was respectively of 89.53% and 3.78% **(Figure 2.3)**.

A

(a) Blades of *Saccharina japonica*

(b) Blades powdered

(c) Crude fucoidan

B

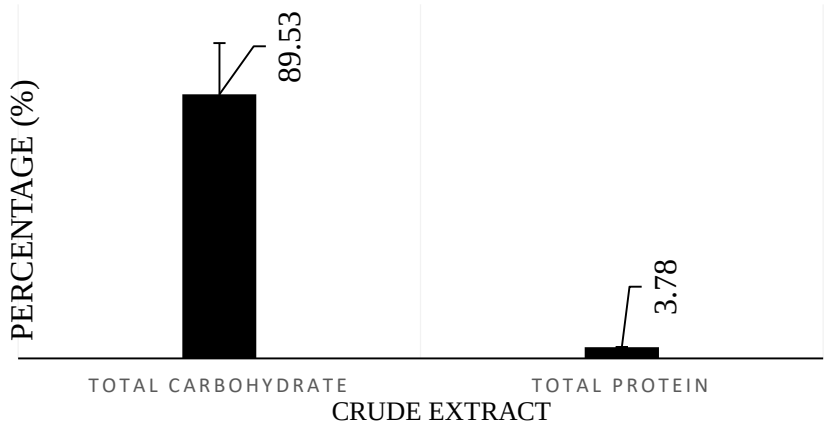


Figure 2.3: Crude fucoidan extracted from *Saccharina japonica.*

Blades of *Saccharina japonica* seaweed were grounded with a blender and the powder used for crude fucoidan extraction by sodium acetate buffer (pH 4.6) coupled to ethanol precipitation. Phenol-sulfuric acid test and Lowry's method were used to determine the sugar and protein content respectively. (A) Products of *Saccharina japonica*. (B) Total carbohydrate and protein content of the crude fucoidan extracted.

2.3.2 Anti-allergic effect elicited by crude fucoidan

To assess the anti-allergic effect of the crude extract, a passive cutaneous anaphylaxis test was performed with female BALB/c mice. Daily administration of fucoidan to the mice did not present any toxicity for them **(Figure 2.4a)**. The sensitized and challenged group of mice had a significant increase in ear edema compared to the control group of mice and fucoidan-fed allergic mice (both the positive control and crude fucoidan) presented a significant decrease in ear edema compared to unfed allergic mice **(Figure 2.4b).** This revealed that oral administration of crude fucoidan extract exerted an inhibition on mast cell degranulation at the site of the allergic reaction (left ear). The crude fucoidan extract possessed an anti-allergic activity.

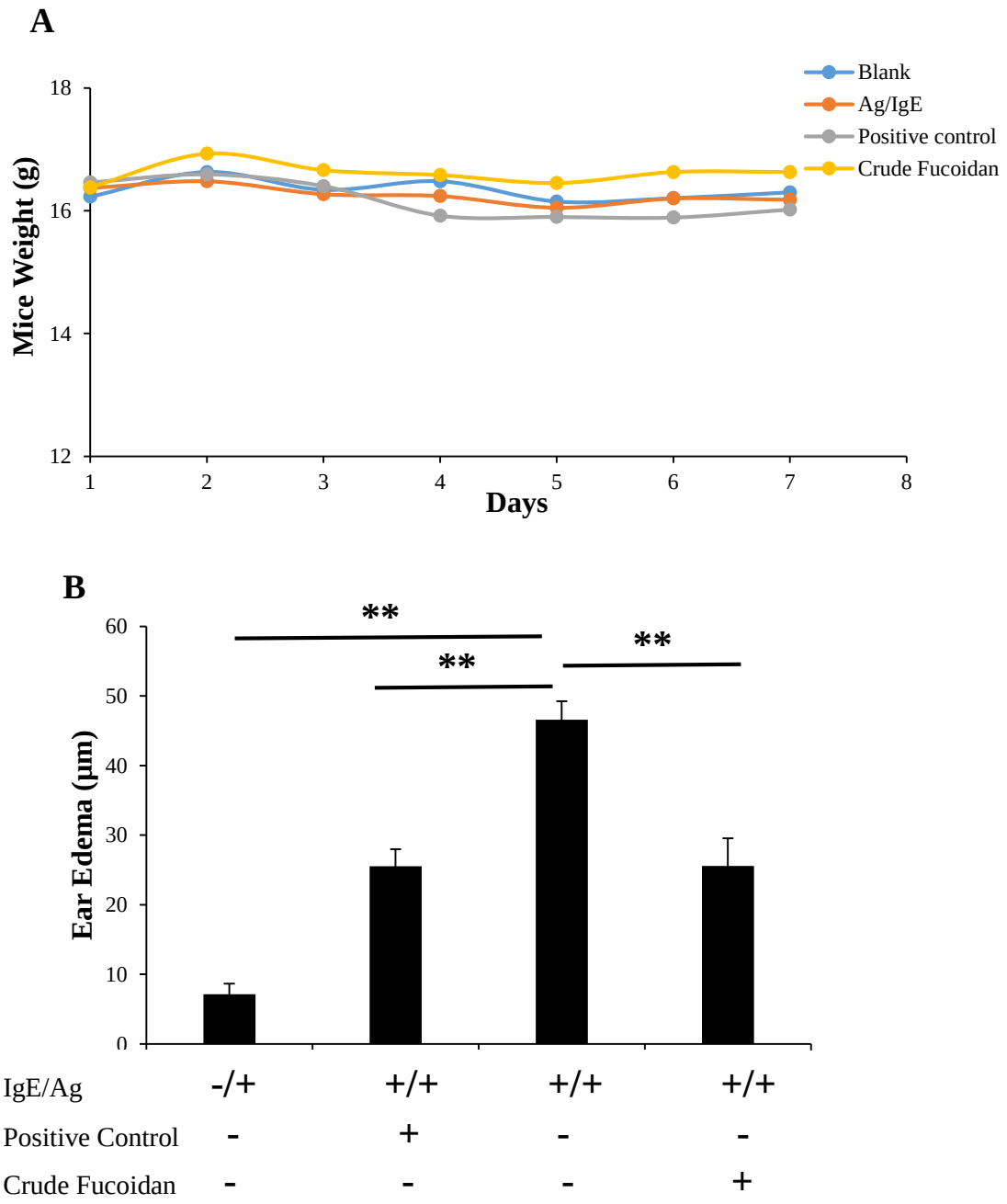


Figure 2.4: Crude fucoidan inhibits IgE-mediated allergic response in PCA mice Female Balb/c mice were orally administrated crude fucoidan (200 µg/day) for 7 days and the anti-allergic effect was analyzed by Passive cutaneous anaphylaxis test. (A) Body Weight changes of mice during the administration period was assessed. (B) Anti-TNP IgE were injected intravenously into the tail of the mice, and the mice were challenge by local application of TNP-BSA on the ear. Data represented by means ± SE (n=3); **P<0.01) by Tukey-Kramer analysis.

2.4. Discussion

PCA reaction is a simple and reliable test used to assess the anti-allergic activity of a compound based on its ability to reduce vascular permeability increase that occurs during an ongoing allergic reaction [90,91]. This test was performed in this study to evaluate the anti-allergic effect of fucoidan crude extract. Fucoidan also called fucan, fucosan or sulfated fucan covers the group of L-fucose riched sulfated polysaccharides extracted from the thallus of brown algae plants [72]. As mentioned earlier, fucoidan is a complex macro-polysaccharide that presents a tremendous heterogeneity of structures and bioactivities which fall under the influence of factors such as the algae source, the habitat, season, age of harvest, and the applied extraction method [71-76] to which one can also add several types of structural modifications [85]. The link between the structure of fucoidan and the resulting biological properties has been intensively studied in the last decades and pieces of evidence suggested a close relationship. For example, in 2013, Ustyuzhanina et al. analyzed the anti-coagulant activity of three structurally different fucoidans (fucoidan from *C.okamuranus*; *F.vesiculosus*, *S.Latissima*) and found that a low degree of sulfation in C.okomuranus was associated with low anticoagulant effect as compared to the others sulfated polysaccharides [92]. The structure-activity relationship was also observed about the anti-allergic effect of fucoidan, where the conformational change of fucoidan due to temperature elevation resulted in the loss of the anti-allergic bioactivity [16]. However, owing to the lack of a standard fucoidan extraction protocol and the heterogeneity and multiplicity of the extraction process that we can observed throughout fucoidan's researches; the structure-relationship of fucoidan is still confusing to establish. This fact strongly hinders the studies investigating their bioactive properties

and the application of fucoidan as therapeutic drug for many immune disorders. Therefore, in the evaluation of the bioactivity of fucoidan, great attention should be paid to the choice of the study plant and especially to the extraction method, which must preserve the target bioactive properties and be reproducible.

In this study, blades of *Saccharina japonica* acquired as previously were grounded into fine particles and used for extraction of fucoidan. The extraction protocol was performed accordingly to what was described by Tanino et al. (2016) in previous studies [14]. Under these conditions, the crude fucoidan yield was 6.52%; with a total carbohydrate and protein content being respectively 89.53% and 3.78% (Figure 2.3). The lower protein content observed is a reflection of the quality grade of the fucoidan extracted, which reflected few co-extracted contaminants and good quality. The PCA test showed that the intake of fucoidan can inhibit allergic reaction. The significant decrease observed in the ear edema of fucoidan-fed mice is a reflection of a reduce mast cell degranulation.

In fact, during an allergic reaction, allergens induce cross-linking of the immunoglobulin (Ig) E antibodies bound to the FcεRI receptor on mast cells and lead to the release of allergic mediators such as histamine and prostaglandins. Histamine is responsible for vasodilation and increases the permeability of vessels near the allergic site [46,91]. Therefore by reducing the ear edema, the crude extract elicited an anti-allergic effect. Moreover, the inhibition of the allergic reaction by the crude fucoidan extract was significantly similar to that of the positive control; which confirms the effectiveness of the extraction method in preserving all the properties of the crude extract necessary for the expression of the anti-allergic effect. It is worth mentioning that differences in extraction methods may result in a loss or modification of the biological activity of the

raw extract [93,94]. This assertion can notably be supported by the recent study of Liu et al. in which different extractions methods (hot water extraction; hydrochloric acid extraction and calcium chloride extraction) applied on blades of *Sargassum fusiforme* resulted in changes of physicochemical characteristics, conformation behaviors and antioxidant activities of *S. fusiforme* fucoidans [75].

CHAPTER 3

ISOLATION, PURITY AND SCREENING OF FUCOIDAN CONTENT IN THE BIOACTIVE FRACTION

3.1 Introduction

Previous studies identified the bioactive fraction in crude fucoidan responsible for the anti-allergic effect. This fraction was isolated by fractionation of the crude extract on anion chromatography column and identified to consist mainly of fucose monosaccharide by thin layer chromatography [16]. However, quantitative analysis of the fucoidan content and evaluation of its physical properties such as structure homogeneity and purity was not yet performed. In chapter 3, we also isolated the bioactive fraction on DEAE-toyopearl resin using increase ionic strength. The fraction of interest: Fraction 1; was eluted at 0 M NaCl in 0.05 M, Tris-Hcl buffer; pH 7.4 and its fucoidan content was analyzed by the DISCHE method. Consistent with what was reported, Fraction 1 presented the highest fucoidan content among the different fractions obtained by separation. As regards to its future use *in vitro* for analyzing the molecular mechanism underlying galectin-9 upregulation upon fucoidan administration, we also assessed the homogeneity and relative purity of Fraction 1 by gel filtration chromatography using S-400HR (MW 20-8000 kDa). Fraction 1 showed homogeneity of structure and good purity. It had then been used for subsequent *in-vitro* experiments.

3.2 Materials and Methods

3.2-1 Reagents

Fucoidan from *Fucus vesiculosus*, Sephacryl S-400 HR, and blue dextran were purchased from Sigma Aldrich (St.Louis, USA). Toyopearl DEAE-650M was purchased from Tosoh Corporation (Tokyo, Japan). NaCl salt was purchased from Wako Pure Chemical Corporation (Osaka, Japan) and L-cysteine was purchased from Nacalai Tesque, INC (Kyoto, Japan). Other chemicals and reagents were ordinary commercial and guaranteed products.

3.2-2 Anion exchange Chromatography

Toyopearl DEAE-650M anion exchange resin was used to separate crude fucoidan into its different fractions. Fucoidan is often coextracted with residual contaminants such as alginate, laminarin, lipids, polyphenols, and proteins that could be eliminated by dialysis. Therefore to reduce the amount of residual contaminants that occurred during the extraction, a dialysis experiment of crude extract was performed before separation on anion exchange resin. Briefly, the crude extract of fucoidan from *Saccharina japonica* was dissolved into distilled water and poured into a dialysis tube with a molecular weight cut-off of 14KDa. The dialysis was performed for 24 h against distilled water at 4°C with a repeating change of water. Then, the crude extract was lyophilized. The lyophilized sample (70 mg) was dissolved in 0.05 M, Tris-Hcl buffer; pH 7.4, filtrated and separated into a column (2.5x20 cm) by adding the elution buffer at different salt concentrations: 0 NaCl; 0.5 M NaCl and 1M NaCl. The elution fractions were gathered together based on the presence of carbohydrates at each salt concentration, dialyzed then lyophilized to obtain the different fractions. The ratio of each fraction after separation was estimated to

the total carbohydrate content in the crude fucoidan applied using a standard curve made with L-fucose.

3.2-3 Fucose content estimation by Dische method

Fucoidan is mainly composed of fucosepyranose residue, which can be qualitatively and quantitatively estimated by the Dische method or cysteine-sulfuric acid assay [95]. The protocol of experiment used in this study is an adaptation of the one described in *Saravana* et al. (2018) [96] with some modifications. Practically, optimal conditions were determined for a standard curve with fucoidan from *Fucus vesiculosus*. For that, 0.5 mL of concentrated sulfuric acid was added to 100 µL of different concentrations of fucoidan standard in test tubes and heated at 80°C for 30 min. After cooling the tubes, 3% of L-cysteine hydrochloride monohydrate (30 µL) was added and all incubated at 4°C for 1 h. The absorbance was read at 396 and 430 nm and subtracted to obtain the absorbance of fucose. Fucoidan content in the different fractions obtained after chromatography was assessed by the same process.

3.2-4 Purity analysis by S-400HR gel filtration Chromatography

The protocol of the experiment is as described: 2 mL of Fraction 1 (10 mg/mL) was dissolved in ultrapure water and added to Sephacryl S-400 HR gel chromatography column (1.5x60 cm) previously packed and checked with blue dextran. The elution was carried out with 0.05 M of sodium phosphate buffer (pH 7) containing 0.15M NaCl and the fractions were collected every 2 mL. The flow rate of the column was 0.4 mL/min. The polysaccharides fractions were identified by phenol sulfuric acids test.

3.3 Results

3.3-1 Highest fucoidan content expressed by Fraction 1 (F1) from crude fucoidan separation by anion exchange chromatography

In order to isolate the bioactive fraction F1, responsible of the anti-allergic effect elicited by crude fucoidan (Confere previous studies of Tanino Y et al. 2016; Morita A et al. 2019; Mizuno M et al. 2020), an chromatography separation at salt concentration selectivity was performed on the crude extract. Toyopearl DEAE-650M anion exchange resin was used and the experiment was conducted following procedures established in past studies (Tanino Y et al. 2016; Morita A et al. 2019; Mizuno M et al. 2020). Fractionation of crude fucoidan gave three fractions: F_1, F_2, F_3 obtained respectively at 0 M Nacl; 0.5 M NaCl; 1M NaCl **(Figure 3.1)**. Fraction 1 (F_1) represented the highest ratio in the crude extract with 44.12% against 28.82% and 18% for fraction 2 and fraction 3. Moreover, analysis of the fucoidan content of each fraction by DISCHE method revealed that fraction 1 (F_1) had the highest fucoidan content **(Figure 3.2)**.

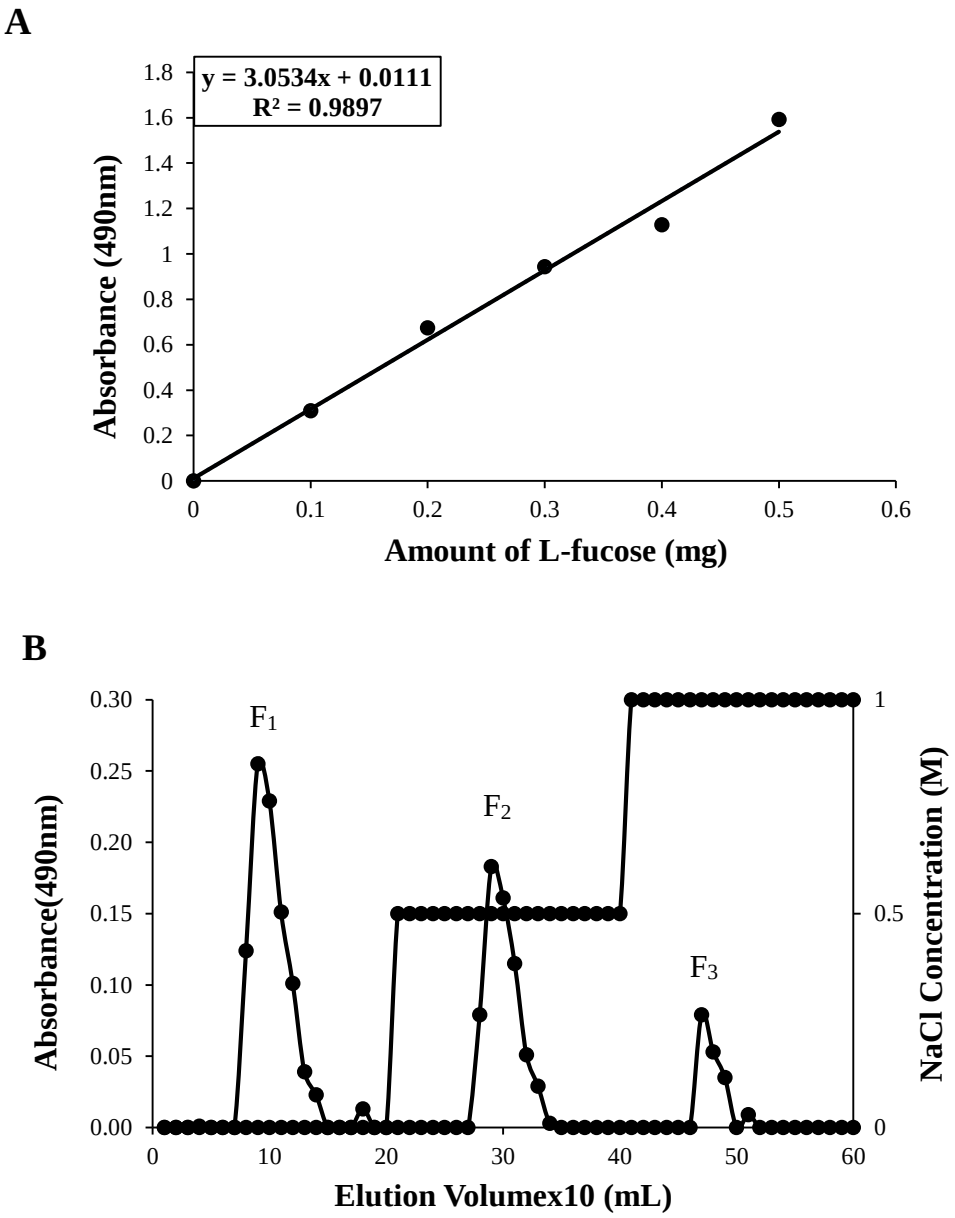


Figure 3.1: Crude fucoidan separated by anion exchange chromatography

Crude fucoidan dissolved in 0.05 M, Tris-Hcl buffer; pH 7.4, was separated on a 2.5x20 cm column filled with Toyopearl DEAE-650M anion exchange resin and equilibrated with the same buffer. Samples were analyzed by phenol sulfuric acid test. Same peak fractions were pooled and dialyzed for 24 hours against distilled water. Fraction 1 separated at 0 M Nacl was selected for further experiments. (A) Standard curve done with L-fucose; (B) Chromatogram profile of crude fucoidan on Toyopearl DEAE-650M anion exchange resin.

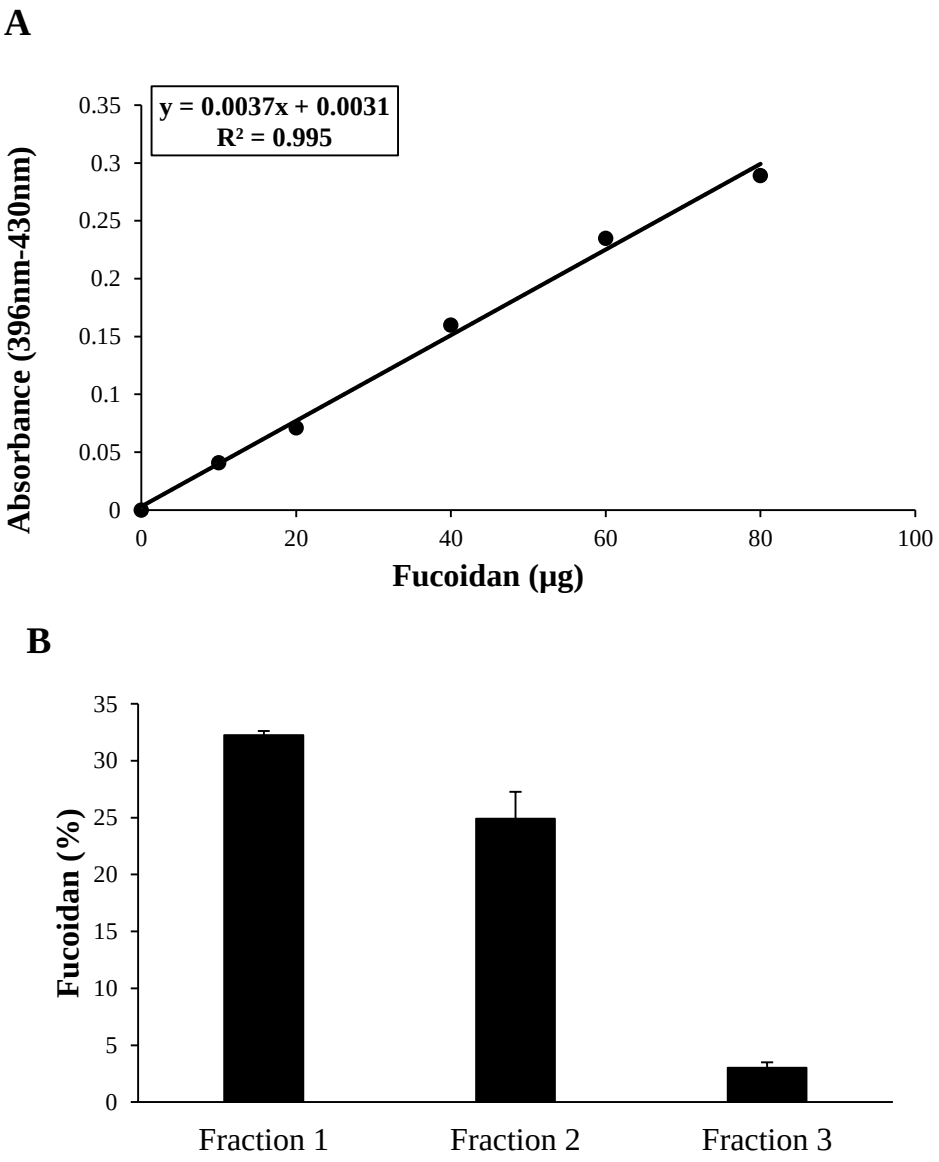


Figure 3.2: Fucoidan content in the different fractions obtained by anion exchange chromatography.

Fraction 1 had the highest fucoidan content. (A) Standard Curve of fucoidan concentration done with fucoidan from *Fucus vesiculosus* (Purity>95%), (B) Fucoidan content analysis by Dische method. Data represented by means ± SE (n=3).

3.3-2 Structure homogeneity and purity of Fraction 1

To assess the homogeneity and purity of fraction 1, fraction 1 was subjected to a gel filtration analysis, using Sephacryl S-400 HR gel. The polysaccharides fractions (2ml/Tubes) were identified by phenol sulfuric acids test. Fraction 1 eluted after the void volume of the column in one strong pick; thus showing a homogeneity of structure and a good purity.

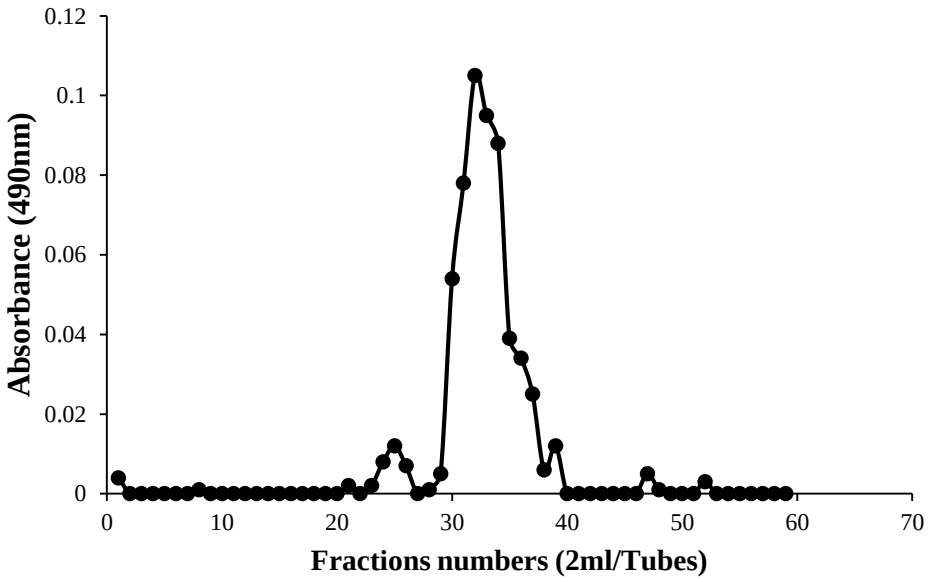


Figure 3.3: Fucoidan fraction 1 (F_1) purity analysis by S-400HR gel filtration

2 ml of F_1 (10mg/ml) dissolved in ultrapure water was separated on Sephacryl S-400 HR gel chromatography column (1.5x60 cm) previously packed and checked with blue dextran. The elution was carried out with 0.05 M of sodium phosphate buffer (pH 7) containing 0.15M NaCl and the fractions were collected every 2ml. Gel filtration of Fraction 1 on Sephacryl S-400HR, Polysaccharide fractions were identified by phenol-sulfuric acids test.

3.4 Discussion

In the preceding studies conducted in our laboratory, it was demonstrated that the anti-allergic effect of the crude fucoidan from *Saccharina japonica* is elicited by the fraction eluted at 0M NaCl (Fraction 1) on anion exchange chromatography [14;16]. The chromatogram of crude fucoidan on DEAE-Toyopearl resin gave us three fractions: F_1; F_2; F_3; eluted respectively at 0M NaCl, 0.5 M NaCl and 1M NaCl salt concentration (Figure 3.1). This result is consistent with previous studies in which three fractions were also observed after the anionic separation of the crude extract [14;16]. Fucoidan content represented by the amount of fucose that can quantitatively and specifically react with L-cysteine was determined by the cysteine-sulfuric acid assay. The use of a standard curve made of fucoidan from *Fucus vesiculosus* (Figure 3.2), with a purity superior to 95% increased the accuracy of this method. We observed that fraction 1 presented the highest fucoidan content, consistent also with the study of Zhao D et al. (2018) [82]. In addition, past report studies ascertained that the anti-allergic effect of crude fucoidan extract was elicited by fraction 1 [14-16], therefore we selected this fraction for subsequent analyses.

Polysaccharides are macromolecules with complex structures. Their study should take into account their structure. Therefore, a gel filtration analysis was done on fraction 1 to assess its homogeneity and purity using a Sephacryl S-400 HR gel chromatography column. This experiment allowed a primary analysis of the polysaccharide polymorphism. As result, the gel filtration of Fraction 1 showed one pick, thus confirming the purity of the sample (Figure 3.3). In the following experiments, Fraction 1 will only be referred to as Fucoidan.

CHAPTER 4

APICAL EXPOSURE OF FUCOIDAN ON IECS UPREGULATED GALECTIN-9 RELEASE IN A DOSE AND TIME DEPENDENT MANNER

4.1 Introduction

It was established that fucoidan induced an increase of galectin-9 in the blood, which was a paramount mechanism to the anti-allergic effect of the fucoidan extract [14,15]. Indeed, the intravenous injection of anti-galectin-9 antibody to fucoidan-fed-allergic mice abrogated fucoidan's suppression of the allergic symptoms in mice [14]. In addition, Tanino Y et al. (2016) observed that the anti-allergy effect took place only when the mice received fucoidan by gastric gavage [14], suggesting an important role played by the intestinal system in galectin-9 induction. A later study revealed that fucoidan intake in mice upregulated the mRNA expression of galectin-9 in the intestine, albeit not significantly [15]. Despite those insights, the molecular mechanism of galectin-9 upregulation remains globally unclear. In this chapter, we aimed at understanding the mechanism of galectin-9 upregulation by investigating *in-vitro* the induction of galectin-9 under fucoidan action. Studies reported that fucoidan, because of its physicochemical properties can hardly be degraded by intestinal enzymes and can therefore be present in all its integrity in the lumen of the intestinal tract with good stability [71]. Therefore, IECs were directly stimulated with fucoidan solutions of different concentrations at different times. Both mRNA expression of galectin-9 and its protein released was evaluated. Moreover, the direct effect of conditioned galectin-9 released on mast cell degranulation *in vitro* was also assessed. We found that apical exposure of fucoidan on IECs upregulated

galectin-9 mRNA and protein release with respect to the dose and time applied. The results from this study showed for the first time a direct upregulation of galectin-9 protein in IECs following fucoidan exposure. Moreover, the inhibition of mast cell degranulation *in vitro* by galectin-9 was confirmed at a 5 µg/mL concentration

4.2 Material and Method

4.2-1 Reagents

Mc Coy's 5A medium and RPMI medium were purchased from Gibco BRL (Grand Island, NY, USA) and Nissui Pharmaceutical (Tokyo, Japan) respectively. MTT was purchased from Wako Pure Chemical Industries (Osaka, Japan). Sepasol-RNA I Super G was purchased from Nacalai Tesque, (Kyoto, Japan). High capacity cDNA reverse transcription kit was purchased from Life Technologies (Carlsbad, USA). Fast Start universal Probe Master (ROX) was purchased from Roche Diagnostics GmbH (Mannhein, Germany). The Taq Man probes (Life Technologies, Carlsbad, USA) used were referenced as: human Galectin-9 Assay ID: Hs00371321_m1 and human GAPDH Assay ID: Hs99999905_m1 (housekeeping gene). Recombinant human galectin-9 protein and pairs of monoclonal anti-human galectin-9 antibodies were all purchased from RαD system (Minneapolis, USA) Transwell insert plates were purchased from Corning (Corning, N.Y., USA). 96 well PCR plate was purchased from Watson Bio Lab (Fukaekasei Co, Japan) and High binding capacity ELISA plate was purchased from Greiner bio-one (Solingen, Germany). Other chemical and reagents were ordinary commercial and guaranteed products.

4.2-2 Cell culture

Rat basophilic leukemia cells (RBL-2H3 cells) and Human IEC line, HT-29 cells were cultured in RPMI 1640 medium supplemented with 10% (v/v) FBS, 100 µg/mL streptomycin, 100 U/mL penicillin, 2mM L-glutamine and in McCoy's 5a medium modified supplemented with 10% (v/v) FBS, 100 µg/mL streptomycin, 100 U/mL penicillin respectively. The cells were maintained in a humidified incubator at 37°C; 5% CO2. At 80% confluence, the cells medium was removed, and the cells were washed with PBS, detached from the flask by adding trypsin and then centrifuged. The collected cells were further appropriately grown in different plates and transwells according to experimental designs.

Hence, RBL-2H3 cells were placed in 12 well plates (Corning, NY, US) for β-hexosaminidase test. HT-29 cells were grown in 96 well plates, 6 well plates and 12 transwell insert filters (Corning, NY, USA) for cytotoxicity test, transcriptional analysis and protein expression experiment of galectin-9 respectively.

4.2-3 Cytotoxicity analysis of Fucoidan Application on Intestinal Epithelial Cells

Cell viability of HT-29 cells exposed to fucoidan as cytotoxicity index was determined by MTT assay as described by Mosmann [97] with little modifications**.** The colorimetric assay of (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) (MTT) analyzes the cell viability by monitoring their mitochondrial activity to metabolize MTT product into formazan, a purple derivative. Practically, HT-29 cells were seeded in 96 well plates (100 µL of 5×10^4 cells/well) and incubated overnight at 37°C, 5% CO_2 to

allow good attachment of the cells to the plate. Different concentrations of fucoidan (0.25%; 0.5%, 1% w/v) prepared in McCoy's 5a medium were added to the cells for 24 hours stimulation at 37°C, 5% CO_2. Then, the samples were removed from the cells and MTT solution was added for 4 hours with the cells put back at the same stimulation condition. Thereafter, its reducing product was dissolved in dimethyl sulfoxide (DMSO) solution for 10 min. The absorbance was read at 570 nm and cell viability was calculated using the formula below.

$$\textbf{\% Cell Viability} = \frac{(\text{Absorbance of test} - \text{Absorbance of Blank}) \times 100}{(\text{Absorbance of cell alone} - \text{Absorbance of Blank})}$$

4.2-4 Solid Plate and Tranwell experiments

HT-29 cells were cultured on 6 well-flat bottom plates. After reaching confluence, the cells were incubated with fucoidan solutions (0.05 %; 0.1 %; 0.2% w/v) for 24 hours. Then, the medium was removed and the cells were washed with PBS. The total RNA of the cells was extracted and q-RTPCR was performed to analyze the mRNA expression of galectin-9 in IECs. HT-29 cells were also cultured on 12 transwell inserts (0.4µm; 12mm diameter insert) and apically exposed to medium or fucoidan solutions (0.05; 0.1; 0.2 % w/v) for 24 hours. The medium in the lower chamber of the transwell referred to as a conditioned basolateral medium was collected to analyze the protein expression of galectin-9 using Enzyme-Linked ImmunoSorbent Assay (ELISA). In addition, a kinetic of galectin-9 released by IECs was performed by incubating the cells with fucoidan solution at 0.1 % w/v for 6, 12, 24 and 48 hours. The conditioned basolateral mediums were later used in a degranulation assay with RBL-2H3 cells. Below is a shematic description of these methods (Figure 4.1).

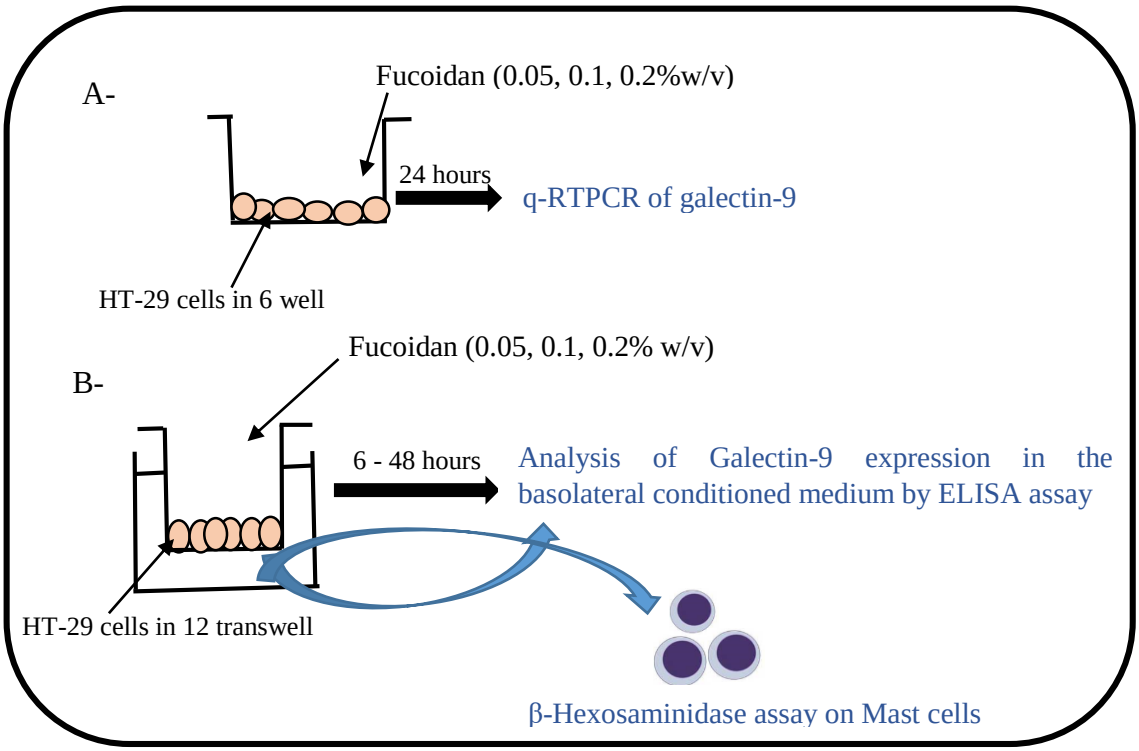


Figure 4.1: Experiments Design.

(A) HT-29 cells in grown 6 well plates were stimulated with fucoidan solutions at different concentrations for 24 hours. Total RNA of the cells was extracted and q-RTPCR performed to evaluate galectin-9 mRNA expression. (B) HT-29 cells grown in 12 transwell plates were stimulated with fucoidan solutions at different concentrations for 24 hours. Conditioned basolateral mediums were collected, galectin-9 protein in it was analyzed by ELISA and the medium later used for β-hexosaminidase assay

4.2-5 RNA preparation and cDNA synthesis

IECs transcriptional response to fucoidan exposure regarding galectin-9 was analyzed by RT-PCR. After treatment with fucoidan, cells were harvested and total RNA extraction was performed using Sepasol-RNA I Super G coupled to chloroform/isopropanol precipitation following the manufacturer's standard protocol. Selective precipitation of RNA in a solution can be achieved by LiCl. This is due to the fact that the ribose sugar of RNA can specifically interact with the positive cations of lithium, allowing the folding

and precipitation of RNA only, and leaving out other compounds in the aqueous solutions [98,99]. Thus, the total RNA extract was further purified with LiCl. Briefly, in precooled Eppendorf tubes, LiCl solution was added to total RNA extract (1:1) for a final concentration of LiCl to be 2.5 M. The solution was chilled at -30°C for 1 hour and then centrifuged at 16000xg for 15 min at 4°C. The supernatant was discarded and the RNA pellet precipitated in 500 µL of ice-cold 75% ethanol, then centrifuged again at 16000xg for 15 min at 4°C. RNA was finally resuspended in RNase-free water and its purity, as well as its concentration, were checked using a NanoDrop 2000 spectrophotometer (Thermo Scientific, U.S.A). cDNA of purified mRNA was synthesized by using High capacity cDNA reverse transcription kit in accordance with the manufacturer's standard protocol. The RT reaction was performed in a T100 thermal cycler (BIO-RAD, Singapore) at 25°C for 10 min, 37°C for 120 min and 85°C for 5 sec, corresponding to the primer annealing, the cDNA elongation and the denaturation steps respectively.

4.2-6 Quantification of gene expression by q-real time PCR

Quantitative real-time polymerase chain reaction was performed on a 7500 Fast-Time PCR System (Life Technologies) using a Fast Start Universal Probe Master (ROX). Conditions of the PCR system were set to 95 ºC for 10 min, 95 ºC for 10 sec, and 60 ºC for 30 sec. The Taq Man probes used were referenced as: human Galectin-9 Assay ID: Hs00371321_m1 and Hs99999905_m1 for human GAPDH (housekeeping gene). Gene expressions were normalized using GAPDH and analyzed with a ΔΔCT quantification method.

4.2-7 Detection of galectin-9 protein by Enzyme Link Immunosorbent Assay (ELISA)

Galectin-9 protein was assessed in the conditioned basolateral media of the transwell by ELISA. Pairs of monoclonal anti-human galectin-9 antibodies from RαD system (Minneapolis, USA) were used in this experiment. High binding capacity ELISA plates (Greiner bio-one, Solingen, Germany) were coated with monoclonal galectin-9 capture antibody (0.75 µg/mL) in PBS and incubated overnight at 4°C. The plate was then washed with PBST (PBS containing 0.05% Tween-20), blocked for 2 h with 1% BSA solution prepared in PBST, and thereafter washed and incubated with the different test samples (basolateral conditioned medium) for 2 h. Following the incubation, and an additional washing step, biotinylated anti-human galectin-9 detection antibody (0.75 µg/mL) was added for 1 h. The plate was incubated with streptavidin-HRP (1:500 dilution) followed by development with tetramethylbenzidine (TMB). The reaction was stopped by the addition of 2M H_2SO_4 and the optical density was measured at 450nm. A standard curve was performed in each experiment using recombinant human galectin-9 protein (RαD system, Minneapolis, USA).

4.2-8 β-Hexosaminidase assay

The degranulation of mast cells and the release of their contents (pro-inflammatory substances) is a key step in the allergic reaction mechanism. Indeed, the different clinical symptoms of the allergic reaction aforementioned are due to the actions of these mediators at the challenged tissue site in the body. RBL-2H3 mast cells line was used to evaluate the inhibitory effect of galectin-9 on allergic reactions. The β-hexosaminidase test was performed as follows: RBL-2H3 cells were sensitized for 2 hours with anti-TNP IgE (200

ng/mL). Unbound IgE antibodies were removed by washing with Siraganian buffer and the cells were incubated at 37°C; 5 % CO_2 with either the conditioned basolateral media from transwell experiments or recombinant galectin-9 protein (3 µg/mL; 5 µg/mL) for 2 hours. After incubation, the cells were challenged with TNP-BSA (20 ng/mL) for 1 hour. The reaction was stopped by incubation of the cells on ice for 10 min. Then, the supernatant of each test sample was added to a 96-well plate together with an equal volume of substrate solution (5 mM p-Nitrophenyl N-acetyl-β-D-glycosaminide prepared in 0.2 M citrate buffer at pH 4.5) and incubated 1 hour at 37°C. 100 µL/well of stop buffer (0.2 M glycine-NaOH) was added and the optical density was read at 405 nm wavelength. The percentage of β-hexosaminidase released into the supernatants was estimated to the percentage of the degranulation group (IgE/Antigen group). Quercetin (50 µM) was also used as a positive control.

4-2.9 Statistics analysis

Data are expressed as means ±SE. All statistical analyses were performed by Tukey Kramer test. P-values below 0.05 (*P<0.05) and P-values below 0.01 (**P<0.01) were considered significant.

4.3 Results

4.3-1 Fucoidan concentrations below 1% is not toxic for the cells

Cytotoxicity evaluation of fucoidan on HT-29 cells assessed by MTT test showed no toxicity at concentrations below 1% w/v **(Figure 4.2)**. Therefore, fucoidan concentrations below 1% w/v were selected in the subsequent experiments.

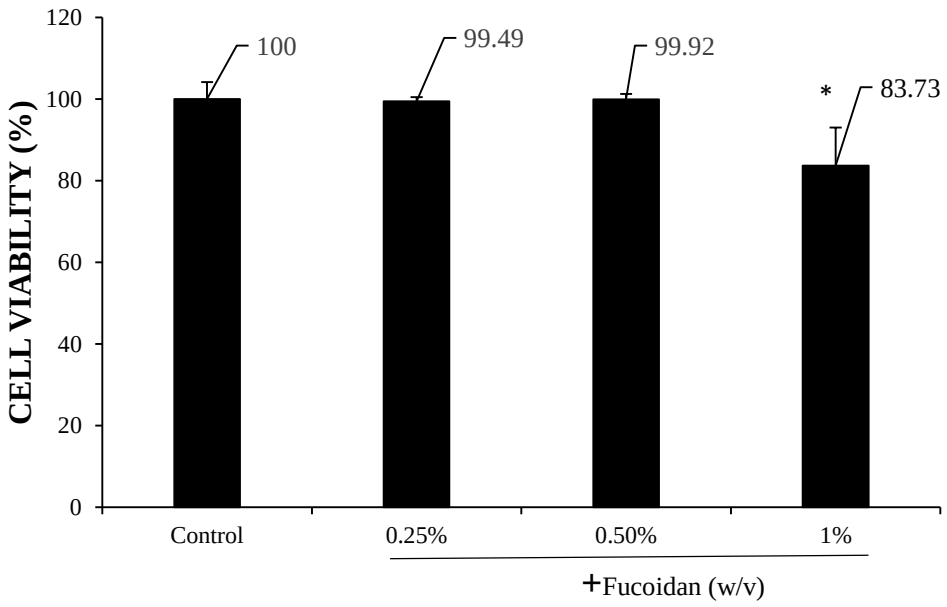


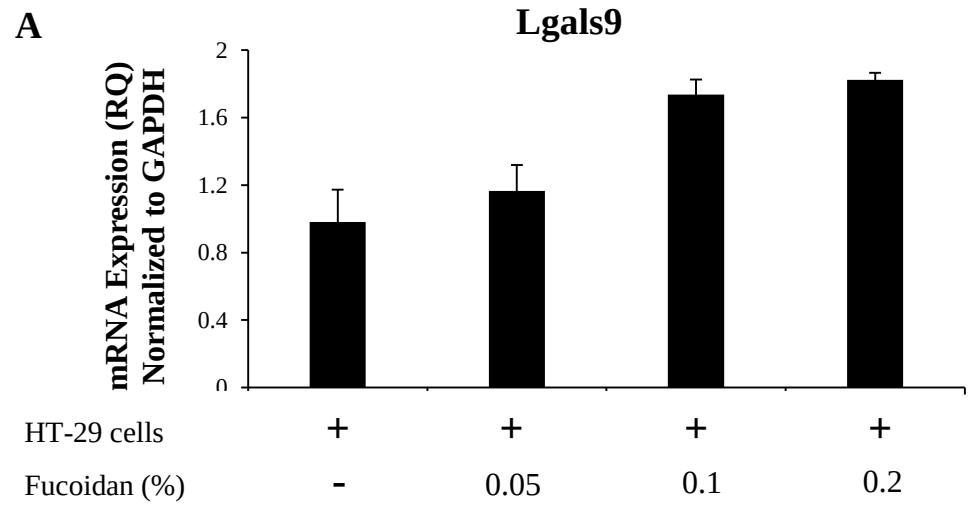
Figure 4.2: Effect of fucoidan on HT-29 cells viability

HT-29 cells ($5x10^4$) seeded in 96 well plate were stimulated with 0.25%, 0.5% and 1% w/v fucoidan for 24 h. Following the incubation period, MTT reagent was added to the cells for 4 hours and the oxidize product dissolved in DMSO. Absorbance was read at 490nm and percentage of cell viability was determined against untreated cells (control). *P<0.05 by Turkey Kramer Test (n=3)

4.3-2 Fucoidan increases gene expression of galectin-9 and favors its protein release by IECs in a dose and time-dependent manner

Past studies reported that fucoidan intake favored up regulation of galectin-9 protein in the blood [14,15], but whether that can be observed in vitro was unclear.

To investigate the induction of galectin-9 *in vitro,* HT-29 cells were apically exposed to different concentrations of fucoidan solution (Figure 2), and the mRNA as well as protein levels was assessed. We found that HT-29 cells stimulation with fucoidan tended to upregulate galectin-9 mRNA (*lgals9*) level **(Figure 4.3a),** albeit not significantly. Galectin-9 protein expression significantly increased in the conditioned medium at the lower chamber of the transwell **(Figure 4.3b).** Repeated experiments in which a standard curve was made with recombinant galectin-9 protein allowed quantification of galectin-9 secreted **(Figure 4.4a & Figure 4.4b).** The results were consistent. In addition, we observed that fucoidan-mediated galectin-9 upregulation increased significantly with increasing the dose or the time of fucoidan applied to HT-29 cells, showing both a dose and a time dependency effect **(Figure 4.5a & Figure 4.5b).**



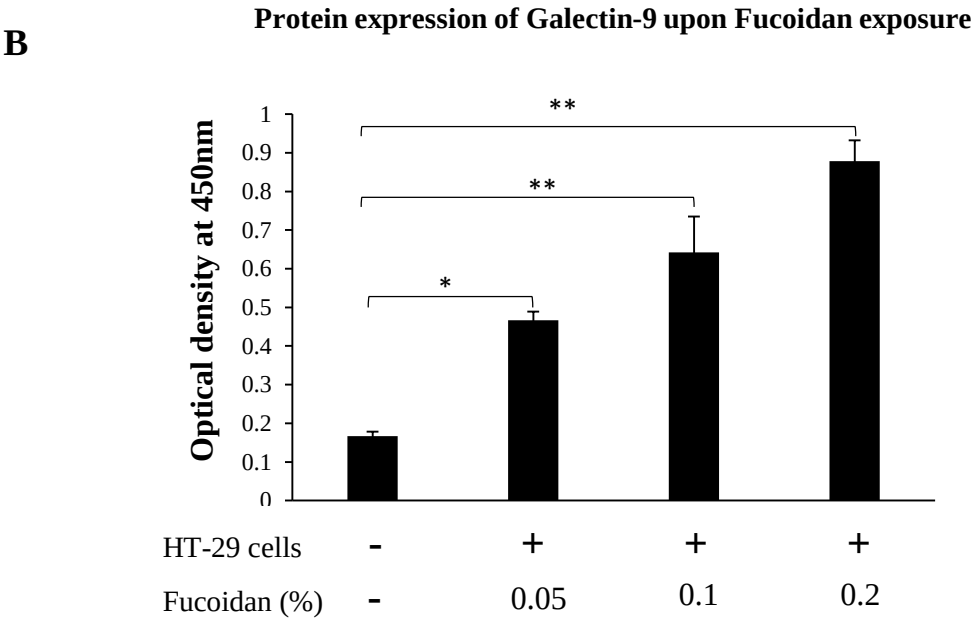


Figure 4.3: mRNA and protein expression of galectin-9 by fucoidan treatment

(A) mRNA gene level of galectin-9 expressed by HT-29 cells after fucoidan treatment (24hours) (B) Optical density measured after 24 hours stimulation by fucoidan on HT-29 cells. *P<0.05; **P<0.01 by Turkey Kramer Test (n=3)

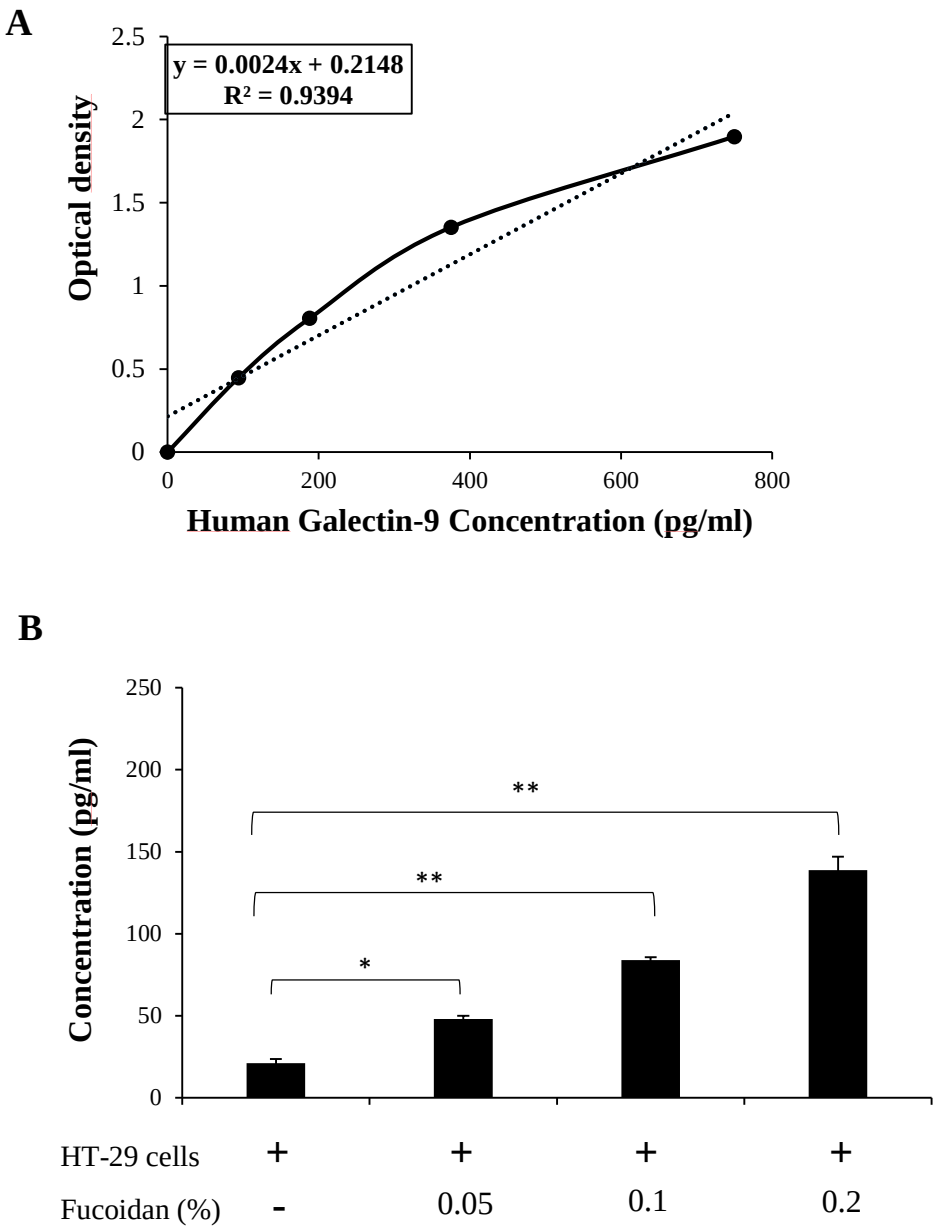


Figure 4.4: Dose response of Galectin-9 secretion by IECs upon fucoidan exposure (24h). HT-29 cells were apically stimulated with different fucoidan solutions. Galectin-9 protein was analyzed by ELISA test. (A) Standard curve of recombinant human galectin-9. (B) Increase galectin-9 release with higher fucoidan concentration applied on HT-29 cells. *P<0.05; **P<0.01 by Turkey Kramer Test (n=3)

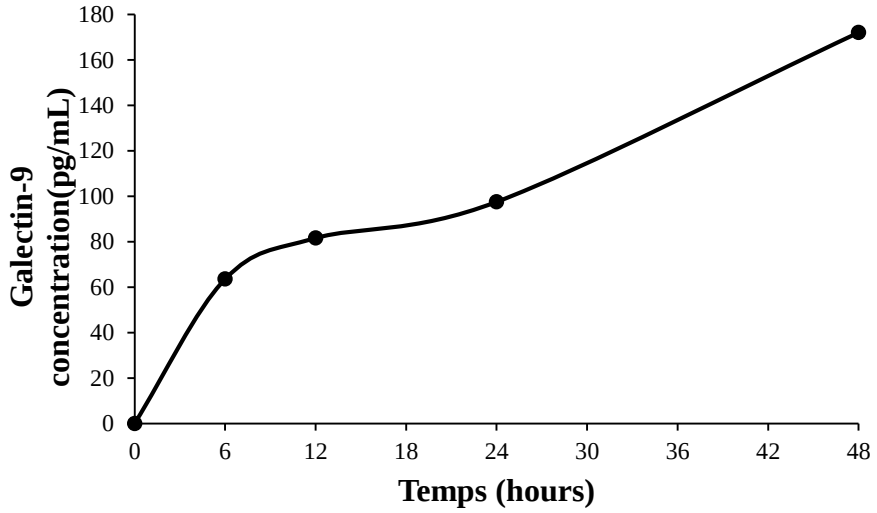


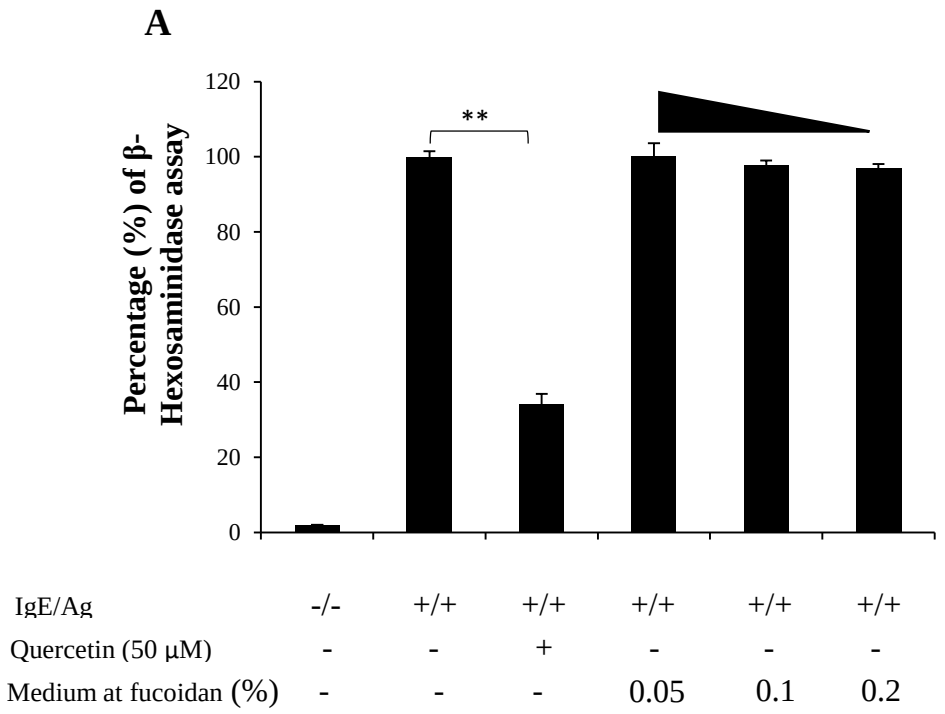
Figure 4.5: Time response of Galectin-9 secretion in fucoidan stimulated-IEC

HT-29 cells in the upper chamber of the transwell were stimulated with fucoidan (1mg/ml) for 6, 12, 24 and 48 hours. Galectin-9 protein secreted in the lower chamber was analyzed by ELISA test. (A) Standard curve of recombinant human galectin-9. (B) Time response of Galectin-9 secretion by HT-29 cells upon Fucoidan exposure. *P<0.05; **P<0.01 by Turkey Kramer Test (n=3)

4.3-3 To what extent galectin-9 released inhibits mast cell degranulation, perspectives of an additional mechanism of fucoidan-induced galectin-9 on allergic reaction resorption

Galectin-9 protein is known to inhibit mast cells degranulation. In order to assess the extent to which galectin-9 released *in-vitro* could dampen mast cells degranulation, the conditioned basolateral mediums were used in a β-hexosaminidase assay. Though a slight decrease in the percentage of hexosaminidase protein was observed with increased galectin-9 released in the conditioned basolateral medium; no significant difference in the

reduction of degranulation was found **(Figure 4.6a).** In addition, a significant reduction of mast cell degranulation was observed with recombinant galectin-9 protein at 5μg/ml **(Figure 4.6b).** Lower concentration of galectin-9 such as those obtained *in vitro* did not exert a significant decrease on mast cell degranulation.



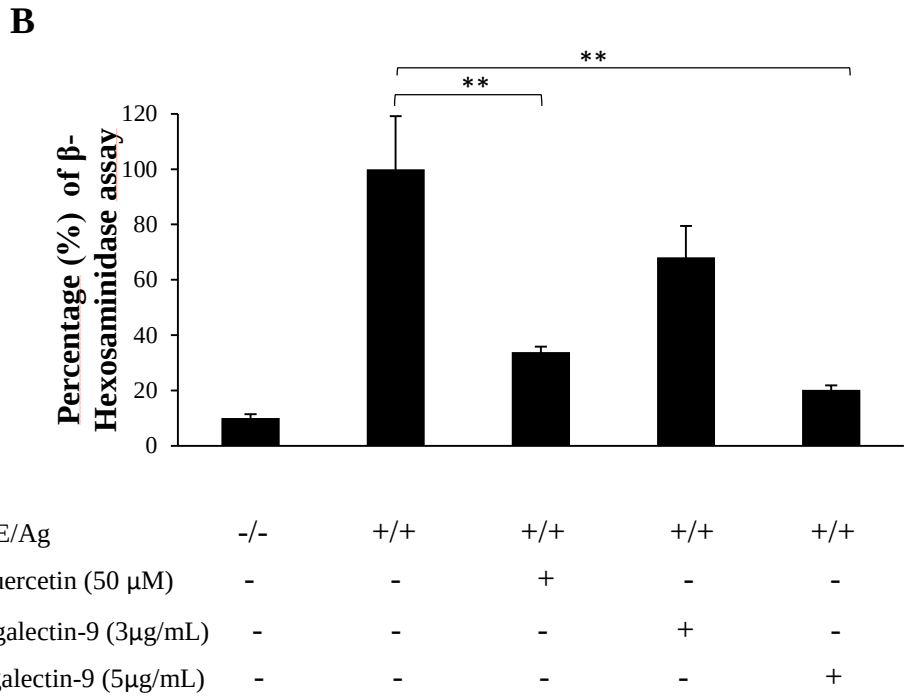


Figure 4.6: Degranulation assay of RBL-2H3 cells

(A) IEC-derived galectin-9 in the conditioned mediums was used to stimulate RBL-2H3 cells. (B) Recombinant galectin-9 protein at 5μg/ml significantly inhibited mast cell degranulation. **P<0.01 by Turkey Kramer Test (n=3)

4.4 Discussion

To investigate the induction of galectin-9 protein *in vitro*, HT-29 cells were selected in this study. HT-29 cell is a human colorectal adenocarcinoma cell line that mimics the epithelial morphology. These cells line have been intensively used as in vitro models in studies on intestinal metabolic functions, food digestion and bioavailability. Indeed, they have been described to present similar characteristics with small intestine enterocytes, such as their structure and the presence of brush border-associated hydrolases [100,101].

Recently, HT-29 cells were shown to secrete galectin-9 protein under the immunomodulatory effect of non-digestible oligosaccharides and of bacterial DNA [24-29]. In this study on investigating the induction of galectin-9 protein upon fucoidan intake, HT-29 cells were selected as model in *in vitro* culture. Some studies reported that fucoidan, because of its physicochemical properties can hardly be degraded by intestinal enzymes, thus be present in all its integrity in the lumen of the intestinal tract with good stability [71]. Therefore, HT-29 cells were apically exposed to different concentrations of fucoidan solution (Figure 4.1). The galectin-9 mRNA (*lgals9*) level tended to increase upon direct exposure to fucoidan (Figure 4.3a), albeit not significantly. IECs are at the cross-borders between dietary foods in the lumen and immune cells present in the lamina propria. This bi-interface of IECs was mimicked here by using transwell plates where IECs were seeded in an apical side of the transwell, and fucoidan solutions were apically applied to them while the lower chamber was filled with medium (basolateral medium). In the conditioned basolateral mediums, galectin-9 protein expression significantly increased with increasing concentration of fucoidan applied to the cells, showing a dose-dependent manner (Figure 4.3b).

Repeated experiments in which a standard curve was made with recombinant galectin-9 protein allowed quantification of galectin-9 secreted (Figure 4.4a & Figure 4.4b). The results were consistent and did confirmed a dose-dependency effect of fucoidan on galectin-9 secretion. Moreover, an increase in the time was noted to favor a higher release of galectin-9 by HT-29 cells (Figure 4.5a & Figure 4.5b), showing also a time dependency effect. The two parameters should therefore be taken into account in fucoidan therapeutic strategies. Interestingly, these results indicate that under the intake of fucoidan *in vivo*, it reached the intestine, where a direct interact with the IECs resulted in up-regulation of

galectin-9 protein. It was thereby ascertained that fucoidan can directly promote galectin-9 induction in IECs.

The anti-allergic action of galectin-9 is exerted by preventing mast cell degranulation. In fact, mast cells have a key role in the mechanism of allergic reactions. They are the ultimate player at the end of the reaction chain which activation and degranulation release several pro-inflammatory substances in the tissues, responsible for the clinical symptoms [4,46]. The extent to which HT-29 cells-derived galectin-9 protein released *in vitro* could inhibit mast cell degranulation was then evaluated using RBL-2H3 cells exposed to the conditioned media. Hexosaminidase protein, one of the several inflammatory molecules released during mast cell degranulation, was used as a marker of degranulation. Though a slight decrease in the percentage of hexosaminidase protein was observed with increasing HT-29 cells-derived galectin-9, no significant difference in the reduction of degranulation was found (Figure 4.6a). The explanation for that fact lies in the lower concentration of galectin-9 released in this assay. Although our results, unequivocally showed the induction of galectin-9 in HT-29 cells by direct stimulation of fucoidan, the lower concentration obtained in the condition of our experiment was insufficient to exert a statistically significant inhibition on mast cell degranulation. Indeed, a subsequent β-hexosaminidase test showed that only recombinant galectin-9 protein at a concentration of 5 µg/mL did exert a significant difference on mast cell degranulation (Figure 4.6b). These latter results raise the question of whether the inhibition of mast cells *in vivo* results from a direct action of galectin-9 on them. In fact, inhibition of mast cell degranulation can also occur through distinct mechanisms. *In vivo,* it is likely that blocking of mast cell degranulation resulted from a cooperative effect of several immune effectors cells such as tolerogenic DC, Treg cells and Th1 cells. Thereby, besides the direct effect of galectin-

9 on inhibiting mast cells granules release through either neutralizing or removing IgE bound-mast cells; DC and T cells present in the intestinal lamina propria may as well be exposed to IEC-derived galectin-9, acquired a tolerogenic phenotype, differentiate towards Th1 or Treg cells and mount the intended immune response (secretion of IL-10; INF-γ). It was notably shown *in vitro* that exposure of galectin-9 protein to activated human peripheral blood mononuclear cells (PBMC) enhanced the frequency of Treg cells [24] and that HT-29 cells-derived galectin-9 promote aldehyde dehydrogenase (ALDH) activity in human moDC cells [26]. ALDH is a vitamin A-converting enzyme highly expressed by tolerogenic DC and important for the induction of Treg in the gut associated lymphoid tissue [102]. Others reports of studies substantiated that observation [60,103].

CHAPTER 5

TLR9 ACTIVATION IS INVOLVED IN FUCOIDAN-MEDIATED GALECTIN-9 UPREGULATION *IN VITRO*

5.1 Introduction

We have ascertained that fucoidan induces galectin-9 upregulation by IECs, which is a paramount mechanism of the anti-allergic effect of fucoidan. Nonetheless, the mechanism of galectin-9 secretion by IECs remains globally unclear. Recently, emerging evidence suggests a role of TLR9 signaling in the release of galectin-9 by IECs [25-29]. It was reported that non-digestible oligosaccharides short-chain galacto- and long-chain fructo-oligosaccharides (GOS/FOS); 2-fucosyllactose; potentiated the release of galectin-9 by IECs [25-29]. Indeed, the authors observed a synergetic effect of these prebiotics with bacteria DNA (CpG-DNA) in potentiating galectin-9 secretion [25-29]. As fucoidan extracted from *Saccharina japonica* also elicited its effect by induction of epithelial galectin-9; we, therefore proposed to analyze the contribution of TLR9 activation in it. No investigation had yet been conducted in this direction and this study is the first one to directly analyze the involvement of TLR9 in the secretion of galectin-9. Fucoidan induction of galectin-9 in this study upon apical stimuli of HT-29 cells, intestinal epithelial cells, was associated with a trendy increase of TLR9 mRNA expression alike the effect of the TLR9 agonist. In addition, the apical stimulation of TLR9 on IECs by its agonist also favored the secretion of the galectin-9 protein, which was even potentiated by a combined exposure with fucoidan. Moreover, knockdown of TLR9 in HT-29 cells resulted in a decreased tendency of fucoidan-induced galectin-9 protein. Taken together,

these results strongly imply that TLR9 is involved in the increase of galectin-9 and its basal secretion upon exposure of fucoidan on IECs.

5.2 Materiel and Method

5.2-1 Reagents

Opti-MEM reduced serum medium and Mc Coy's 5A medium were purchased from Gibco BRL (Grand Island, NY, USA). Lipofectamine^{TM} RNAiMAX Transfection Reagent and TLR9 ligand (Type C CpG oligonucleotide M362, CpG-ODN) were purchased from InvivoGen (San Diego, Calif, USA). TLR9 siRNA (code product: 4392420) and TLR9 probe (human TLR9 Assay ID: Hs00152973_m1) were purchased from Thermo Fisher Scientific (Massachusetts, U.S.A). Other chemicals and reagents were ordinary commercial and guaranteed products.

5.2-2 Effect of GpG-ODN and of cationic lipids-mediated gene delivery on cell viability of IECs

Cell cytotoxicity of CpG-ODN (3 μM, 5 μM, 7 μM) exposed to IECs was assessed by MTT assay as described in Chapter 3. In addition, the influence of transfection complexes on direct cytotoxicity and cell viability was measured by MTT assay. Practically, HT-29 cells ($5x10^4$) were seeded in 96 well plates and incubated at 37°C, 5% CO2. The cells were then transfected with different concentrations of TLR9 siRNA (25 μM; 50 μM, 75 μM, 100 μM) for 48 hours, following which MTT reagent was added to the cells for 4

hours. The formazan product derived from the oxidation reaction of MTT in the mitochondria was dissolved in DMSO and its absorbance was measured at 570 nm.

5.2-3 TLR9 mRNA expression of IECs following fucoidan and TLR9 agonist stimulation

HT-29 cells were cultured on 6 well-flat bottom plates. After reaching confluence, the cells were incubated with either fucoidan solution at 1 mg/mL (0.1% w/v) or TLR9 agonist (CpG-ODN: 7 μM) for 24 hours. Then, the medium was removed and the cells were washed with PBS. The total RNA of the cells was extracted as previously described and q-RTPCR was performed to analyze the mRNA expression of TLR9 in IECs using TLR9 probe (human TLR9 Assay ID: Hs00152973_m1). Gene expressions were normalized using the housekeeping gene human GAPDH (Assay ID: Hs99999905_m1) and analyzed with a ΔΔCT quantification method.

5.2-4 Combined exposure of fucoidan and TLR9 agonist in the induction of galectin-9 protein

The effect of both TLR9 agonist and combined exposure of TLR9 agonist and fucoidan on IECs-derived galectin-9 was assessed by this experiment. HT-29 cells were cultured in 12 transwell plates and apically exposed to either fucoidan (0.1% w/v) and/or TLR9 agonist (CpG-ODN, 7 μM) for 24 hours. The basolateral conditioned mediums were collected and galectin-9 protein in it was analyzed by ELISA test, following procedures described in Chapter 3.

5.2-5 siRNA silencing of TLR9 on IECs and its effect on fucoidan-mediated galectin-9 released by IECs

Silencing of TLR9 in HT-29 cells was performed as previously reported [104] with some modifications. HT-29 cells were transfected using Lipofectamine RNAiMAX reagent and TLR9 siRNA for 48 h. Briefly, the cells were transfected at 40% confluence by smooth addition of siRNA lipocomplexes at final concentrations of 25 nM and 50 nM; prepared in OptiMEM medium following manufacture instructions. The absence of siRNA treatment (mock) was included as a control. Subsequently, the cells were stimulated with CpG-ODN (7 µM) as positive control and effective knockdown of TLR9 was confirmed in cell lysates by q-RTPCR. The same TLR9 silencing procedure was carried out in transwell experiments and the impact of TLR9 knockdown on galectin-9 release upon fucoidan was assessed by quantitative analysis of galectin-9 protein in the conditioned basolateral medium with ELISA.

5.2-6 Statistical analysis

Data are expressed as means ±SE. All statistical analyses were performed by Tukey Kramer test. P-values below 0.05 (*$P<0.05$) and P-values below 0.01 (**$P<0.01$) were considered significant.

5.3 Results

5.3-1 GpG-ODN stimulation of HT-29 cells did not impact their viability

Cytotoxicity of CpG-ODN (M,362), a TLR9 agonist on HT-29 cells was assesed by MTT assay. The results showed that TLR9 agonist stimulation on HT-29 cells does not impact their viability. CpG-ODN is not toxicity to the cells.

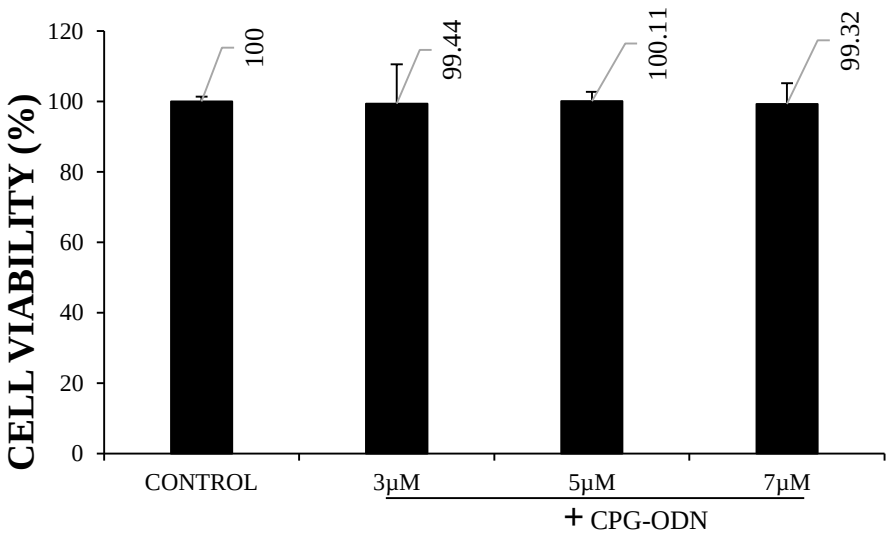


Figure 5.1: Effect of CpG-ODN on IECs cell viability

5.3-2 Synergetic effect of fucoidan and TLR9 agonist (CpG-ODN) in galectin-9 secretion

To evaluate the synergetic effect of fucoidan and prebiotics DNA, mimicking *in vitro* by synthtetic CpG-ODN (M,362); HT-29 cells seeded in 12 transwells plate were stimulated with either fucoidan (0.1% w/v) or CpG-ODN alone, and with combined fucoidan and

CpG-ODN for 24hours. Stimulation of HT-29 cells with CpG-ODN significantly potentiated the release of galectin-9 protein by HT-29 cells, similar to the effect of fucoidan. Combined exposure of fucoidan and CpG-ODN to HT-29 resulted in twice the release of galectin-9 protein. Thus fucoidan and CpG-ODN had a synergetic effect in potentiated IECs-derived galectin-9 protein *in vitro*.

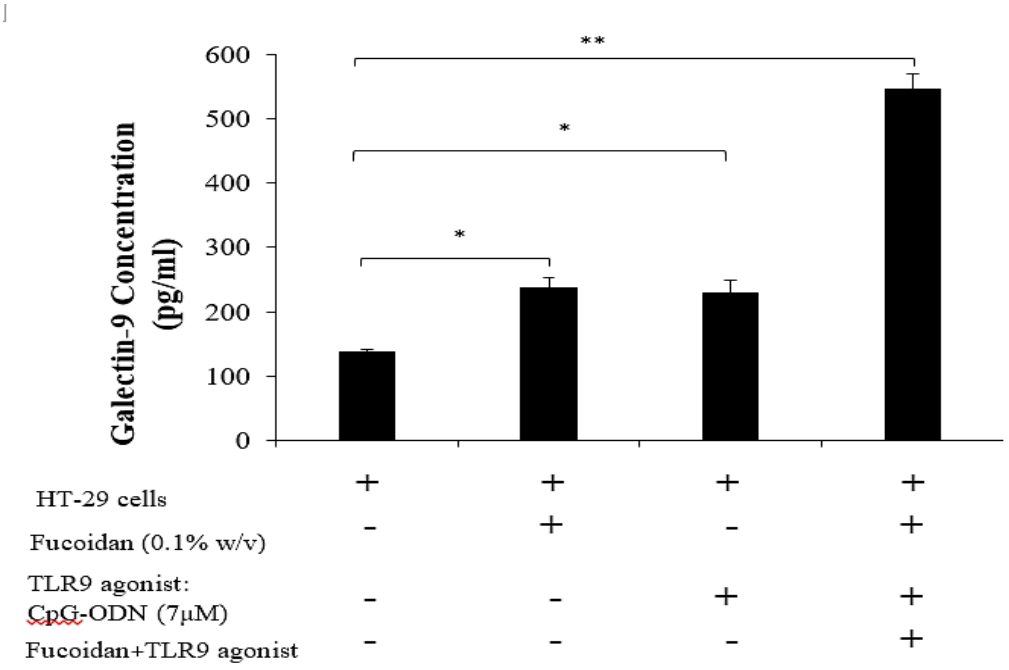


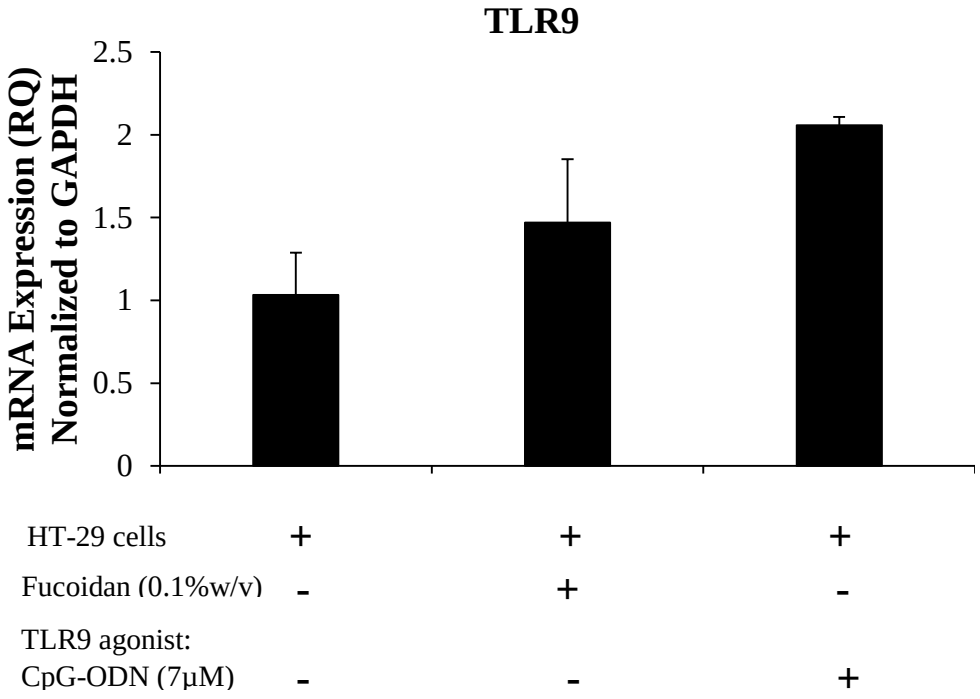
Figure 5.2: Synergetic effect of fucoidan and TLR9 agonist in inducing galectin-9 secretion by IECs

IECs cultured in transwell inserts were apically exposed to Fucoidan and or TLR9 agonist (CpG-ODN) for 24 hours. Galectin-9 protein in the basal medium was analyzed by ELISA test. Combined exposure of fucoidan and TLR9 agonist synergistically potentiated galectin-9 protein release. Data are presented as mean ± SE (n=3) *P<0.05; **P<0.01 by Turkey Kramer Test.

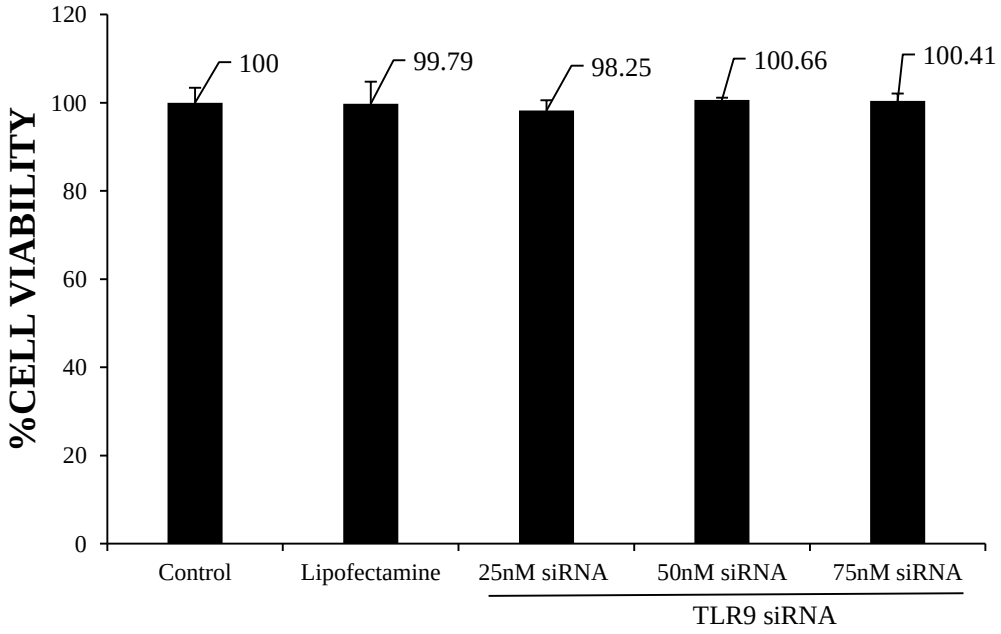
5.3-3 Fucoidan upregulated TLR9 mRNA expression in IECs and knockdown of TLR9 decreased the level of galectin-9 protein

To examine the role of TLR9 in fucoidan-mediated galectin-9 release, we first evaluated the expression of TLR9 in IECs following fucoidan exposure. mRNA expression of TLR9 in IECs tended to increase upon apical fucoidan stimulation. Likewise, stimulation of the cells with CpG-ODN, a TLR9 agonist tended to increase the TLR9 mRNA expression **(Figure 5.3a).** Gene delivery of TLR9 siRNA effect on HT-29 cells was assessed by MTT assay and showed no toxicity to the cells **(Figure 5.3b).** TLR9 knockout in HT-29 cells was established at 40% decrease with TLR9 siRNA (decrease mRNA expression of TLR9 as compared to positive control cells stimulated with TLR9 agonist) **(Figure 5.3c).** To assess the impact of TLR9 knockdown on fucoidan-mediated galectin-9 upregulation, transfected cells with TLR9 siRNA were subsequently stimulated with fucoidan and galectin-9 protein in the conditioned basolateral medium was quantified by ELISA. A decreased tendency of galectin-9 released upon the effect of fucoidan was observed, showing an implication of TLR9 in fucoidan-mediated galectin-9 upregulation on IECs **(Figure 5.3d).**

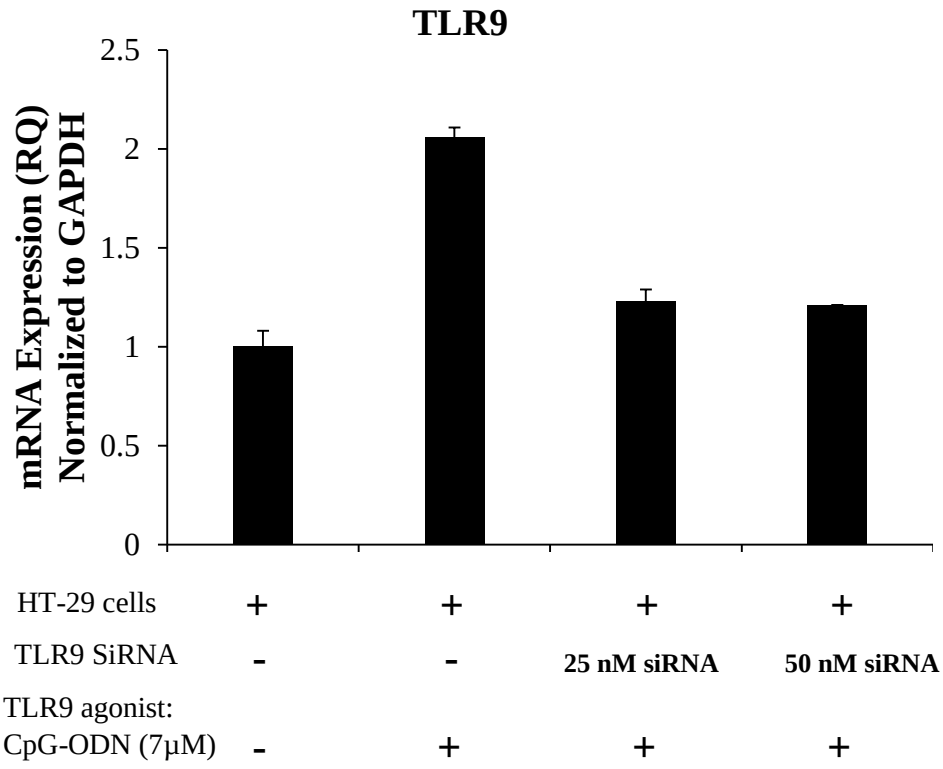
A


TLR9
mRNA Expression (RQ)
Normalized to GAPDH
2.5
2
1.5
1
0.5
0
HT-29 cells + + +
Fucoidan (0.1%w/v) - + -
TLR9 agonist:
CpG-ODN (7µM) - - +

B


%CELL VIABILITY
120
100
80
60
40
20
0
100
99.79
98.25
100.66
100.41
Control
Lipofectamine
25nM siRNA
50nM siRNA
75nM siRNA
TLR9 siRNA

C


TLR9
mRNA Expression (RQ) Normalized to GAPDH
2.5
2
1.5
1
0.5
0
HT-29 cells + + + +
TLR9 SiRNA - - 25 nM siRNA 50 nM siRNA
TLR9 agonist: CpG-ODN (7µM) - + + +

D

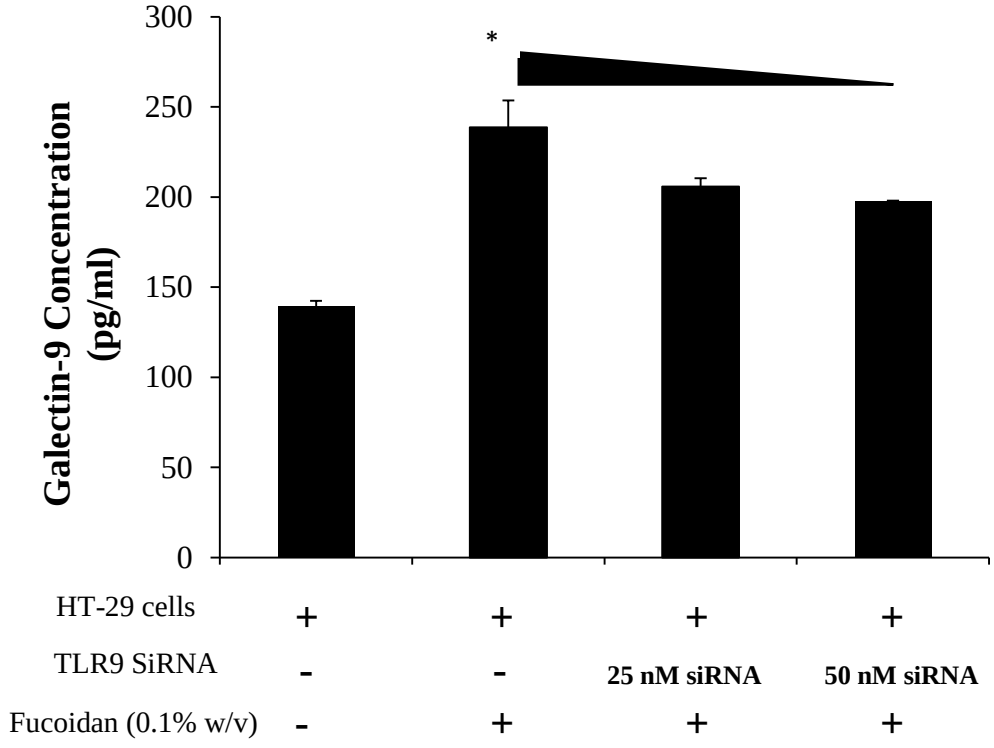

Galectin-9 Concentration (pg/ml)
300
250
200
150
100
50
0
*
HT-29 cells + + + +
TLR9 SiRNA - - 25 nM siRNA 50 nM siRNA
Fucoidan (0.1% w/v) - + + +

Figure 5.3: Fucoidan upregulated TLR9 mRNA expression in IECs and knockdown of TLR9 decreased the level of galectin-9 protein.

(A) mRNA gene level of TLR9 expressed by HT-29 cells after fucoidan and TLR9 agonist stimulation for 24 hours. (B) Effect of cationic lipids-mediated gene delivery on cell viability of IECs. (C) Knockdown of TLR9 in HT-29 was performed with TLR9 siRNA at 25 nM and 50 nM final concentration. (D) Knockdown of TLR9 on HT-29 cells further resulted in a gradual decrease of galectin-9 secretion induced by fucoidan. Data are presented as mean ± SE (n=3) *P<0.05 by Turkey Kramer Test.

5.4 Discussion

The immune-stimulatory potential expressed by microorganisms was linked to the presence in their sequence of palindromic unmethylated Cytosine-phosphate-Guanine dinucleotide (CpG) motif, abundantly found in bacterial and viral DNA [62,63]. In vitro, bacteria DNA trigger is mimicking by the use of synthetic unmethylated CpG oligonucleotides (CpG-ODN), models to its natural ligand CpG-DNA. Type C CpG-ODN, used in this study was notably implicated in several studies to analyze IECs-derived galectin-9 [25-29]. Cytotoxicity evaluation of CpG-ODN on HT-29 cells assessed by MTT test showed no toxicity (Figure 5.1) and apical stimulation of HT-29 cells with CpG-ODN (7 μM) resulted in increased galectin-9 release (Figure 5.2). Fucoidan is a prebiotic; it has been shown to impact gut microbiota and potentiate their activities [9,11]. Also, recently, it was evidenced that galectin-9 secretion by IECs is involved in the immunomodulatory effect of several prebiotics such as short-chain galacto-oligosaccharides (scGOS)/long-chain fructo-oligosaccharides (lcFOS), 2´fucosyllactose; in synergetic action with bacteria DNA [25-29]. Therefore, we evaluated the effect of

combined exposure of fucoidan/CpG-ODN on HT-29 cells-derived galectin-9. The cells, seeded in a transwell plate were apically exposed to either fucoidan solution (0.1% w/v) or CpG-ODN (7 μM) alone or to a combined solution of fucoidan and CpG-ODN for 24 hours. Combined exposure of CpG-ODN and fucoidan induced twice the release of galectin-9 protein, showing a synergetic effect (Figure 5.2). From this result, it can be understood that the upregulation of galectin-9 protein observed *in vivo* was the result of a synergistic effect of fucoidan with probacteria, in addition to the direct induction of galectin-9 by fucoidan. Lastly, to examine the role of TLR9 in fucoidan-mediated galectin-9 release, we first evaluated the expression of TLR9 in HT-29 cells following fucoidan exposure. The mRNA expression of TLR9 in HT-29 cells tended to increase upon apical fucoidan stimulation (Figure 5.3a). Likewise, stimulation of the cells with CpG-ODN, the TLR9 agonist tended to increase the TLR9 mRNA expression (Figure 5.3a). The gene delivery technic did not impact the cells viability (Figure 5.3b) and effective knockdown of TLR9 using TLR9 siRNA was established with 40% decrease in the mRNA expression of TLR9 as compared to positive control cells (cells stimulated with TLR9 agonist) (Figure 5.3c). Lastly, the impact of TLR9 knockdown on fucoidan-mediated galectin-9 upregulation was assessed. Transfected cells with TLR9 siRNA were subsequently stimulated with fucoidan and galectin-9 protein in the conditioned basolateral medium was quantified by ELISA. A decreased tendency of galectin-9 released upon the effect of fucoidan was observed, showing an implication of TLR9 in fucoidan-mediated galectin-9 upregulation on IECs (Figure 5.3d).

CHAPTER 6

GENERAL DISCUSSION AND CONCLUSION

Fucoidan is a fucose-containing sulfated polysaccharide, derived from brown seaweed with many therapeutic activities (anti-cancer, anti-oxidant, anti-coagulant, anti-inflammatory, anti-allergic) [7,8]. Studies on the bioactive properties of this polysaccharide have gained interest in this recent decades, and many reports highlighted its potential as a therapeutic drug in some diseases, such as food allergies. Food allergy is a type 2 immunity disease, characterized by important IgE production and involving mast cell degranulation (release of inflammatory molecules in the surrounding area) [45]. It differs from other allergic diseases in that the site of initiation of the reaction is the gastrointestinal tract [43]. Fucoidan has been reported to dampen the allergic reaction in several ways, acting on the key steps and immune players in the reaction. For instance, it can reduce IgE production from B cells; reestablish the Th1/Th2 immune balance and direct T cell polarization toward a regulatory type (Treg cells) [77-81]. Moreover, some effects on gut microbiota diversity had been mentioned [9-11]. This diversity of action takes root in the physicochemical heterogeneity of fucoidan structure among the brown seaweed specie [71-72], that being related to the extraction method applied (hot water, acetic, alkaline, enzyme-assisted extraction), the geographic area of production, the season of harvest, etc [74-76]. Indeed, the link between the structure of fucoidan and its biological activity is unequivocal [72,75.87]. However, not well defined to date. In past studies, fucoidan extracted from *Saccharina japonica* specie as performed in chapter 1 showed strong anti-allergic effect on OVA-induced food-allergic mice and in a mouse model of passive cutaneous anaphylaxis reaction [14,15]. Consistently, repeating the

extraction method in this study gave us a fucoidan extract that demonstrated good anti-allergic properties. In fact, the increased ear edema, a characteristic feature of the ongoing passive cutaneous anaphylaxis reaction was significantly attenuated in the group of fucoidan-fed allergic mice compared to unfed allergic mice. The result of this study brings substantial proof of the anti-allergic effect elicited by fucoidan extract from *Saccharina japonica*, revealed in preceding studies. The molecular mechanism underpinning the anti-allergic effect of *Saccharina japonica´*s fucoidan was partially investigated; then reported to be linked to the upregulation of galectin-9 protein in the blood, which removed IgE on mast cell receptors. Worth mentioning, the effect was subjected to the administration route; precisely being only effective in oral consumption [14]. This fact suggested an important role played by the intestinal system in its underlying molecular mechanism. However, no subsequent study deeply investigated it, and some aspects of the mechanism, like how fucoidan favored the galectin-9 increase were unclear. This study provided some insights into that question.

Firstly, even though past studies observed an increasing tendency in mRNA expression of galectin-9 protein in the intestine [15] and galectin-9 protein expression in ex-vivo IECs [14], clear evidence on galectin-9 upregulation´s site was still needed. Here, by using intestinal epithelial cell lines (HT-29 cells), we novelty showed that IECs are the secretory cells of galectin-9 protein upon fucoidan intake. In addition, we revealed that galectin-9 released by IECs was dose and time-dependent. The impact of dose on the anti-allergic effect of fucoidan was notably precedently confirmed *in vivo* [14]. As fucoidan was reported to be hardly degraded by intestinal enzymes and can therefore be present in all its integrity in the lumen of the intestinal tract [19,20,74], HT-29 cells used here as

IECs were subjected directly to fucoidan stimulation and we observed the upregulation of galectin-9 protein released. This fact lifts a veil on the molecular mechanism and suggests that when reaching the intestine, fucoidan may directly interact with the intestinal epithelium cells, resulting in the up-regulation of galectin-9 protein.

In recent days, TLR9 receptor´s role in the maintenance of gut homeostasis has emerged. Varga and Lin (2020) mentioned and explained the therapeutic potential of TLR9 in treating several gut inflammatory conditions (protection against intestinal damage, potentiation of intestinal repair) such as ulcerative colitis, chron´s disease, and inflammatory bowel disease [32]. In fact, numerous studies unveiled a dichotomous role of TLR9, one being anti-inflammatory while the other one pro-inflammatory [33,34, 105,106]. The anti-inflammatory effect of TLR9 activation in IECs had been associated with the stimulation of this receptor on the cell membrane [24-29], which led to the secretion of anti-inflammatory molecules. In several studies, combined stimulation of HT-29 cells with TLR9 agonist (CpG-ODN, M362) resulted in increased release of galectin-9 protein [25-29]. In this study, the stimulation of HT-29 cells with CpG-ODN (M 362) as well as fucoidan favored the secretion of the galectin-9 protein, which was even potentiated in a combined exposure. The precise mechanism beyond the synergetic effect is to date unclear but as regards fucoidan, several plausible explanations could be formulated. Some studies mentioned that fucoidan can be internalized by SR-A, which may trigger the intracellular signaling of some PRRs, notably TLR9 [21]. In that line, a recent study showed that fucoidan's treatment upregulated genes involved in the signaling of several nucleic acid-sensing receptors such as RLR, TLR, NLR, and STING [35]. Moreover, it is not excluded that fucoidan may interact directly with TLR9, independently

of an association to SR-A. Yamasaki et al. showed in their study that fucoidan mimics DNA in solution [36] and Makarenkovaa et al. that fucoidan polysaccharides was independent ligands of TLR2 and TLR4 [23].

Long considered to possess only endosomal expression, TLR9 expression is being reconsidered by the results of recent studies reporting also a membrane surface expression. Both surface and intracellular expression of TLR9 has been observed in several cell types like human tonsils cells, splenic DCs, peritoneal mast cells and IECs [33,34,66-68]. In their recent review entitled "Cell surface expression of endosomal Toll-like receptors-A necessity or a superfluous duplication", Mielcarska et al. (2021) gathered different studies that supported the recent observation of endosomal receptors being expressed on the surface and attended to explained it. Yet, the factors leading to and implications of such localization versus ER expression are not clearly defined, and like they said remains to be resolved [31]. The overall expression of TLRs on IECs is significantly different of that of others cells or tissues [107]. IECs express similar expression of TLR9 at both apical surface (facing the lumen of the intestine) and basolateral surface (facing the immune environment of lamina propria), demonstrated by confocal microscopy imaging, flow cytometry, and vectorial biotinylation [33]. We found here that fucoidan-induced galectin-9 upregulation upon apical stimuli of HT-29 cells was associated with a trendy increase of TLR9 mRNA expression like the effect of the TLR9 agonist. In addition, combined exposure of fucoidan and CpG-ODN resulted in a synergetic effect on HT-29 cells-derived galectin-9. Furthermore, gene silencing of TLR9 (40% effective knockdown by TLR9 siRNA) on HT-29 cells decreased of the amount of galectin-9 protein secreted, and tended to downregulate the upregulation observed upon fucoidan exposure. These

results all together strongly imply that TLR9 is involved in fucoidan mediated galectin-9 upregulation. Knowledge of TLR9 is far from complete and the complexity of this receptor, linked to intense variability in its cellular localization (intracellular or surface expression), cell trafficking, cell types dependent, and even trigger-type dependent [44,45] just began to be analyzed.

Conclusion

The results of this study provide a new insight into how fucoidan potentiated galectin-9 increase, observed in mice, and add to the understanding of fucoidan anti-allergic mechanism. A graphical overview is notably provided below in Figure 6. Galectin-9 upregulation appeared to result from both the direct interaction of fucoidan with IECs and a synergetic effect with probiotics DNA (mimicking *in vitro* with the use of CpG-ODN, M362). Subsequent translational studies *in vivo* should be considered.

In addition, this study is a piece of additional evidence to the existence of the TLR9/galectin-9 pathway in IECs, which stands as a promising path for the resorption of allergy disease. Advances in molecular biology technic will sure allow for deeper investigations on TLR9 signalization on IECs leading to galectin-9 upregulation.

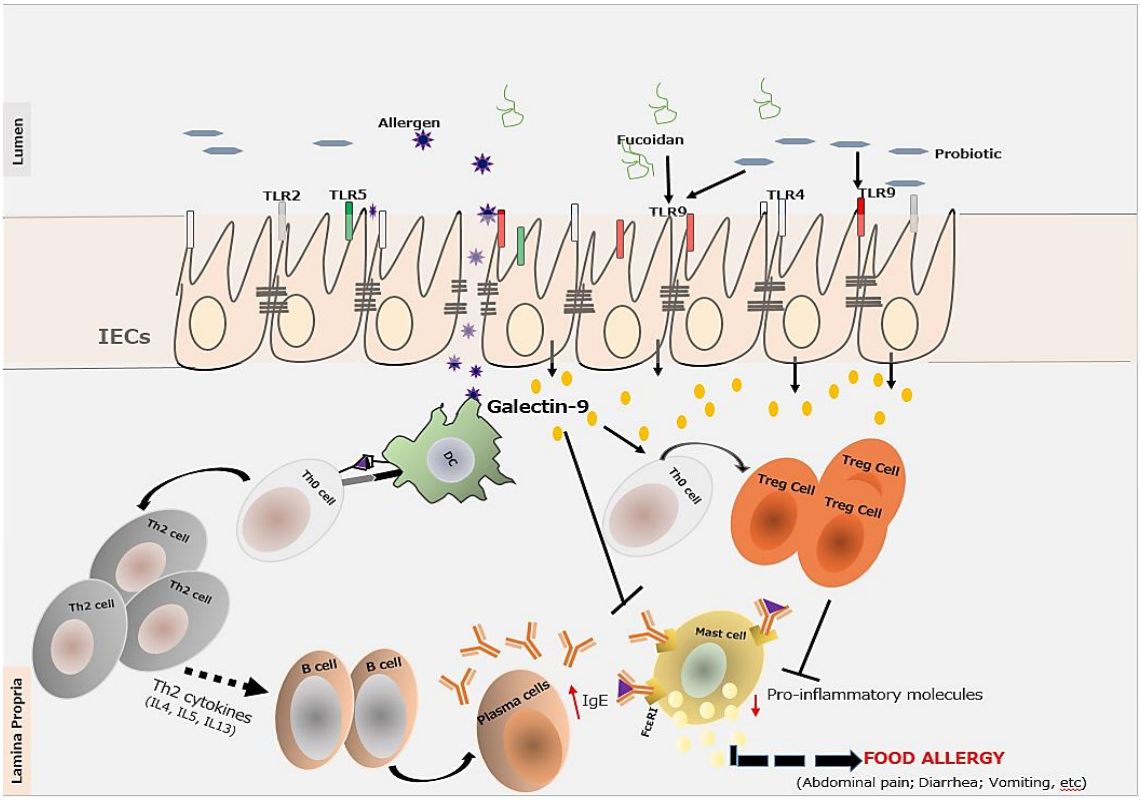


Figure 6: Graphical overview of how fucoidan-mediated galectin-9 upregulation in the intestine and effect of galectin-9 on food allergy resorption

After oral intake, fucoidan reached the intestine where it interacted with TLR9 expressed by IECs and synergized with probiotics to induce galectin-9 release by IECs. Galectin-9 protein prevented mast cell degranulation by either removing IgE bound on their surface or by potentiated T regulatory cells polarization. Thereby, the release of pro-inflammatory molecules is dampened and consequently the food allergy is attenuated.

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