Doctoral Dissertation (Shinshu University)

Immunomodulatory activity of phosphorylated buckwheat major allergen and its enzymatic hydrolysate

（リン酸化ソバ主要アレルゲンとその酵素消化物の免疫調節作用）

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Abbreviations

CD, Circular Dichroism

DP-Fag e 2, Digested phosphorylated Fag e 2

IgA, Immunoglobulin A

IgE, Immunoglobulin E

IgG, Immunoglobulin G

IL, Interleukin

OIT, Oral immunotherapy

P-Fag e 1, Phosphorylated Fag e 1

P-Fag e 2, Phosphorylated Fag e 2

SEM, standard error of the mean

SIT, Specific immunotherapy

Tfh, T follicular helper

Th1, T-helper type 1

Th2, T-helper type 2

Treg, Regulatory T cell

Abstract

The potential therapeutic effects of phosphorylated buckwheat allergens on IgE-mediated allergic reactions are sought in this dissertation. This dissertation is divided into four chapters. The background information, related literature, and objectives are presented in Chapter 1. The current medical response towards food-allergic patients is to control the symptoms of allergy without treating it. Recently, a novel approach known as "Specific immunotherapy" (SIT) is a common alternative. Immune tolerance to allergens is established by changes in memory allergen-specific T and B cell responses, joined by changes in both mast and basophil cells activation threshold levels that would lead to preventing the occurrence of allergic symptoms. One method of SIT is Oral immunotherapy (OIT), it involves the oral administration of small doses of an allergen over time, with a gradual increase in the dose. Despite its success in desensitization, OIT could carry safety risks, such as the risk of an allergic response in the initial dose setting, and the need to use epinephrine to mediate anaphylactic reactions. To override the side effects of OIT, some therapies work on modifying the allergen proteins used to decrease the potential of an allergic reaction and still induce tolerance. Such modifications include glycosylation of proteins, or phosphorylation *via* dry heating with pyrophosphate. Phosphorylation of proteins can change the conformational structure, which results in its binding capability with IgE. If phosphorylation occurred on the epitope site of antigen, it could block the binding site from the IgE.

Buckwheat plant has an IgE mediated allergenicity, its major allergens are Fag e 1 and Fag e 2. Fag e 1 is a 22 kDa globulin found in common buckwheat (*Fagopyrum esculentum*) and it is one of the major allergens causing severe allergic symptoms, Fag e 2, a member of 2S albumin family, reported as being resistant to pepsin digestion and appears to be the causative for an immediate hypersensitivity reaction of buckwheat allergy including anaphylaxis.

Chapter 2 deals with the expression of recombinant Fag e 1 using the *Pichia* expression system and preparing an allergen-specific hypoallergenic agent by the controlled dry-heating phosphorylation of Fag e 1 (P-Fag e 1). Then, investigated if P-Fag e 1 can be useful as an immunomodulator in Fag e 1-sensitized mice. The results showed a reduction in both histamine release and both total and specific IgE in P-Fag e 1 treated mice. Combined with increased total IgA, increase T-follicular helper (Tfh) cells, and a decrease of IL-4 from spleen and Peyer's patches of P-Fag e 1 treated mice. Suppress of IgE production in the Fag e 1 treated group might be because of the enrichment of the Tfh cells and IgA production. Therefore, it could suggest that P-Fag e 1 is an allergen-specific immunomodulator in mice allergic to Fag e 1.

In Chapter 3, the effects of phosphorylation on the digestibility of Fag e 2 was investigated. Since it was proven that both Fag e 1 and Fag e 2 will change their immunomodulatory activities upon phosphorylation, the purpose was to test whether digested P-Fag e 2 (DP-Fag e 2) can attenuate allergic reactions in Fag e 2-sensitized mice. Recombinant Fag e 2, obtained using the *Pichia* expression system, was phosphorylated *via* dry-heating in the presence of pyrophosphate. Phosphorylation enhanced the peptic digestibility of Fag e 2. Mice fed DP-Fag e 2 for 6 weeks after Fag e 2 sensitization exhibited reduced allergic symptom scores compared to those of sham-treated mice. Decreased total and specific IgE, decreased specific IgG1, and increased total IgA observed in the serum of the DP-Fag e 2-fed group. These results show that P-Fag e 2 is readily digested in the stomach and induces the attenuation of the IgE-mediated allergic reaction.

Lastly, Chapter 4 provides a summary and conclusion of the two studies. Results suggested that that phosphorylated buckwheat major allergen and its enzymatic hydrolysate can attenuate the allergic reactions in a mouse model of buckwheat allergy. These findings would contribute to the development of safer and more effective immunotherapy for buckwheat allergy.

Chapter I

General Introduction

1. Background and perspectives

Buckwheat (*Fagopyrum esculentum*) is a pseudocereal from the Polygonaceae plant family. Buckwheat can be cultivated in unfavorable conditions for rice, and it is used in Asia and Europe[1)]. Good nutritional qualities of buckwheat include protein, lipids, dietary fibers, and minerals. The protein content is rich in albumin and globulin, but low in gluten and prolamin content[2)]. Health benefits also included lowering cholesterol levels, also buckwheat rutin was proven to have excellent antioxidant activities and was related to reduced high blood sugar[3,4)].

IgE–mediated food allergic reactions show the onset of symptoms immediately after the ingestion of allergic substances. Initially, sensitization to the food stimulates the production of specific IgE antibodies binding to both mast and basophil cells. Later upon re-exposure to a similar food, clinical signs occur as the allergens in food bond to specific IgE and caused a release of histamine and other mediators[5)].

Buckwheat allergy was early described in 1909, since then it was recognized as one of the most life-threatening food allergens[6)]. The prevalence of buckwheat allergy is generally low, but it is linked to the onset of severe anaphylaxis[7,8)]. The two major allergens in buckwheat are the 22 kDa globulin protein, and the 16 kDa albumin protein referred to as Fag e 1 and Fag e 2[9)].

Specific immunotherapy (SIT) has become a tool for prevention and treatment for many allergic diseases[10)]. The benefits of SIT include reduction of disease severity leading to decreased drug usage and prevention of future sensitizations during long-term treatments. It also improves the safety and efficacy of future treatments[11)]. Modification of allergic proteins is considered as an immunotherapeutic treatment. Such as Maillard-type glycosylation or phosphorylation[12)]. The process of allergen phosphorylation has an advantage of both masking the epitope site, and enhancement of immunomodulation by the promotion of type-I IFN secretion[13)].

The digestibility of allergens is known for its relationship with its allergenicity, as allergens are grouped according to their digestibility to either digestion – resistant class I allergens, or labile class II allergens. Gastric digestion decreases the ability of food proteins to bind IgE, which leads to higher allergen dose required to elicit food allergy[14]. Storage proteins and structural proteins have more stability towards digestion[15]. To alter such stability, it was found that the processing of allergens by Maillard-type reaction or phosphorylation can change the structural properties of such allergens, making them more easily digested[16].

2. Literature review

2.1. Buckwheat

Common buckwheat *Fagopyrum esculentum* is one of the globally cultivated buckwheat types of the 9 agricultural valued types worldwide. According to Food and agriculture organization data (FAOSTAT) between 1994 and 2018, buckwheat had been produced mainly by Asia (49%), followed by Europe (45.5%), both North and south America only produced the second-lowest continent (5.2%), and least production was in Africa (0.3%). The triangular seeds are covered by a dark brown or black hull and mainly consumed in a dehulled form. The carbohydrate percentage is 73.3%, with starch as the main component[17]. Buckwheat is rich in albumin and globulin, with poor amounts of gluten and prolamin. Being rich in lysine and arginine, these properties make buckwheat suitable for celiac diet[2]. Buckwheat contains a set of bioactive compounds that are of high interest in the food industry (**Table 1**)[18]. It is suggested that health benefits associated with buckwheat consumption include anticancer, hypocholesterolemia, and general health activities as shown in **Table 2**[19]. Buckwheat can be further used in bakery products (**Figure 1**) and non-bakery products (**Figure 2)**[18].

Table 1. Bioactive compounds of buckwheat. Adopted from Giménez-Bastida et al.[18].

Category	Compounds/distribution	Concentration	Reference
Phenolic compounds	Rutin/groats	80.94 mg/g in TB and 0.20 mg/g in CB	Steadman *et al.* [2001] Fabjan *et al.* [2003] Kim *et al.* [2004] Kalinova *et al.* [2006] Wijngaard & Arendt [2006] Zielińska & Zieliński [2009]
	Quercetin/groats	0.001 mg/g DW	
	Quercetin/hull	0.009-0.029 mg/g DW	
	Quercitrin/seeds	0.1-0.5 mg/g DW	
Inositol	Phytic acid/ bran without hull	35-38 mg/g	Steadman *et al.* [2001] Zielińska & Zieliński, [2009]
	D-*chiro*-inositol/groats		Yao *et al.* [2008]
Vitamins	Thiamine (vitamin B1)/seeds	2.2-3.3 μg/g DW	Bonafaccia *et al.* [2003] Wijngaard & Arendt [2006] Zielińska & Zieliński [2009]
	Riboflavin (vitamin B2)/seeds	10.6 μg/g DW	
	Niacin (vitamin B3)/seeds	18 μg/g	
	Pantothenic acid (vitamin B5)/seeds	11 μg/g	
	Pyridoxine (vitamin B6)/seeds	1.5 μg/g	
	Vitamin C/seeds	50 μg/kg DW	
	Vitamin C/sprouts	250 μg/kg DW	
Tripeptides	Glutatione/groats	1.1 μmol/g DW	Zielińska & Zieliński [2009] Gabrovska *et al.* [2002]
Lipophilic LMWA	Tocopherols (vitamin E)/groats	14.3-54.6 μg/g	Wijngaard & Arendt [2006] Kalinova *et al.* [2006]
	Carotenoids/seeds	2.1 μg/g DW	Zielińska & Zieliński [2009] Wijngaard & Arendt [2006]
Lipophilic LMWA: phytosterols	β-sitosterol/dehulled groats	0.7 mg/g DW	Normen *et al.* [2002]
	β-sitosterol/buckwheat flour	0.86 mg/g DW	
	Campesterol/groats	0.09 mg/g DW	
	Campesterol/ buckwheat flour	0.11 mg/g DW	
	Stigmasterol/groats	n.d.	
	Stigmasterol/ buckwheat flour	0.02 mg/g DW	
Lipophilic LMWA: hormones	Melatonin/groats	470 pg/g DW	Zielińska & Zieliński [2009]
Isoprenoid	Squalene/leaves	98.57 μg/g DW*	Kalinova *et al.* [2006]
Tannins	Condensed/bran	5.9 - 8.6 mg/g DW	Steadman *et al.* [2001] Zielińska & Zieliński [2009]
	Non-condensed/bran	2.3 - 3.8 mg/g DW	
	Proanthocyanidins/buckwheat flour	1.59 mg/g DW	
	Proanthocyanidins/hull	1.38 mg/g DW	

Low molecular weight antioxidants (LMWA); dry weight (DW); Tartary buckwheat (TB); Common buckwheat (CB); * According to Kalinova *et al.* [2006] this is the maximum concentration detected, but certain differences appear among different varieties of buckwheat.

Table 2. Health benefits related to buckwheat consumption. Adopted from Giménez-Bastida et al.[19].

Study focus	Model	Buckwheat dose	Duration	Summery	Reference
Anti-Cancer	Female Sprague−Dawley rats	38.1% diet.	61 days	Reduced incidence of mammary tumors.	Kayashita et al, 1999
	Male Sprague−Dawley rats	277 g/ kg	124 days	Reduced incidence of colon adenocarcinoma.	Liu, et al. 2001
	Female BALB/C mice (n = 50) infected with H22 hepatic carcinoma cells	6.25 mg/ kg	8 days	Reduced ascites formation	Feng, et al. 2015
Hypocholesterolemia	Healthy female humans (n=62)	4 buckwheat cookies/day	4 weeks	Reduced (myeloperoxidase, serum TCh, serum HDL)	Wieslander, et al. 2011
	Healthy male humans (n=12)	100 g / day	4 weeks	Reduced (HDL)	Bijlani, et al. 1985
Health benefits	32 female, 29 male human diabetic patients	2 tablets pressed buckwheat herb 3 times a day	3 months	Improvement of retinopathy parameters	AArchimowicz – Cyrylowska, et al. 1996
	Human patients with chronic venous insufficiency (n=77)	Buckwheat tea equivalent to 270 mg rutin 3 times per day	12 weeks	Leg edema protection	Ihme, et al. 1996

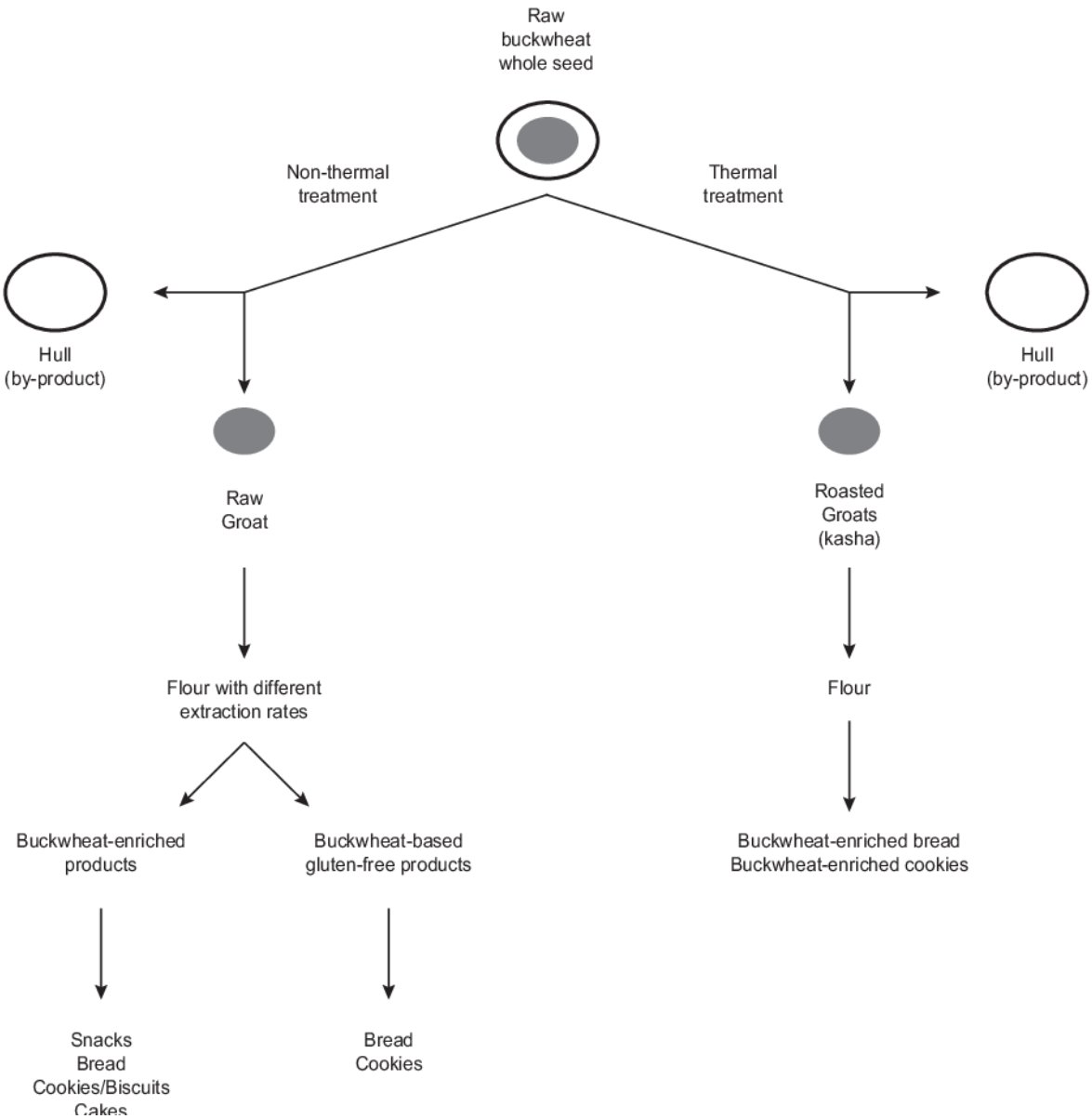


Figure 1. Simplified diagram of the buckwheat derived bakery products. Adopted from Giménez-Bastida et al.[18].

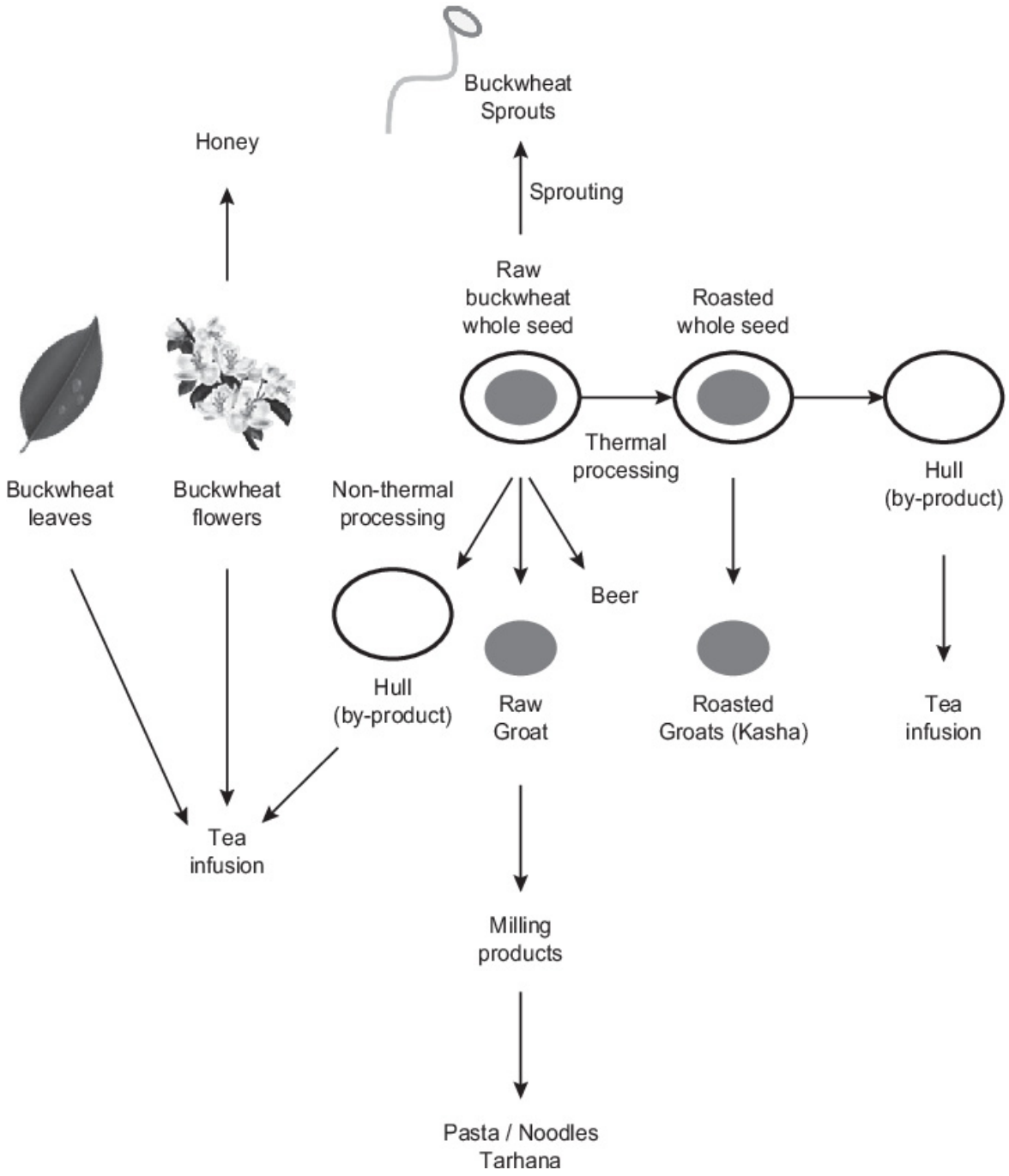


Figure 2. Simplified diagram of the buckwheat derived non-bakery products. Adopted from Giménez-Bastida et al.[18].

2.2. IgE-mediated allergy

The immune adverse response to food protein is called food allergy. Allergens are generally small protein components (10-70 kDa), soluble in water, with some resistance to heat or acid denaturation. This resistance keeps them intact following processing, storage, cooking, and even digestion. IgE–mediated food allergic reactions characterized by immediate onset of symptoms post-ingestion. Initially, sensitization to the food stimulates the production of specific IgE antibodies that binds to mast and basophil cells. Later upon re-exposure to the same food, clinical reactivity occurs because the allergens in food had bonded to specific IgE and caused the release of histamine and other mediators[5)]. In more detail, the interaction of specific IgE antibody with its high-affinity receptor (FcεRI) on the surface of mast cells and basophils, besides the interaction with a low-affinity receptor (FcεRII) on the surface of monocyte, lymphocytes, and platelets. The binding of IgE with FcεRI leads to a release of mediators from mast cells and basophils causing acute allergic symptoms, such symptoms normalize after allergic food has been removed from the diet (**Figure 3**). describes the pathogenesis of IgE mediated allergy, the sensitization phase allergen is taken up by the antigen-presenting cell, leading to Th2 activation that activates B cell into IgE secreting plasma cell. In the elicitation phase upon re-exposure, cross-link FcεRI bond IgE on mast cells, and basophils lead to mediator release and food allergic symptoms[20,21)].

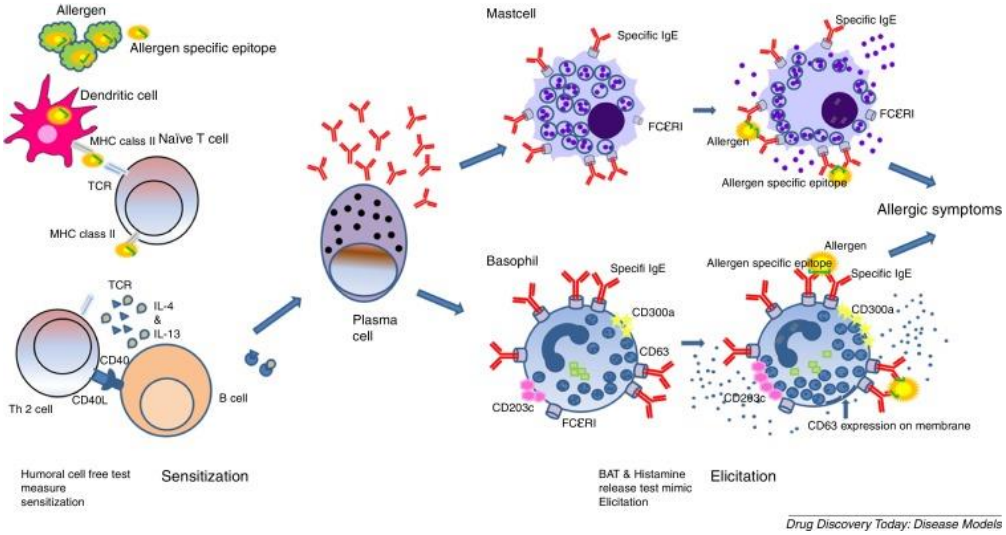


Figure 3. Pathogenesis of IgE-mediated allergy. Adopted from Broekman et al.[21]. IL: Interleukin, TCR: T-cell receptor, Th: T helper, MHC: Major histocompatibility complex.

2.3. Buckwheat allergy

Some Allergens in buckwheat can cause allergy in humans *via* IgE mediated mechanism[22]. The allergic reaction occurs after ingesting buckwheat[23]. Some cases of buckwheat allergy were reported to occur as a domestic exposure *via* pillows stuffed with the husk of buckwheat plant[24]. Recent studies have shown cases of occupational buckwheat allergy such as buckwheat husk pillow factories and repacking plants[25]. The prevalence of buckwheat allergy is considered low (0.22% in Japan, and 0.11% in Korea), as compared to other allergens[26,27]. Nevertheless, complications associated with allergy are severe as anaphylaxis, with even a slight amount of exposure enough to cause life-threatening reactions[8]. Major allergens included 9 kDa, 16 kDa (Fag e2), 19 kDa, and 22 kDa proteins. Allergens of 22 kDa is a globulin protein, and the 16 kDa is an albumin protein and they were referred to as Fag e 1 and Fag e 2, respectively. The clinical signs show a frequency for urticaria (89.5%), dyspnea (84.2), facial angioedema (52.6%), and gastrointestinal signs of (47.4%)[9].

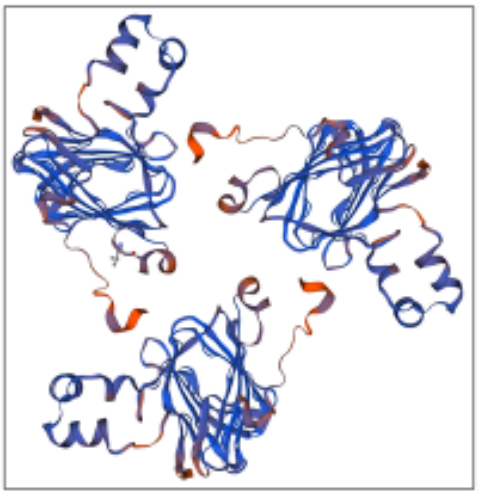
2.4. Fag e 1

The 22 kDa protein of buckwheat named Fag e 1 is a major allergen, belonging to a globulin protein (legumin-like ß subunit). Fag e 1 has 192 amino acids described in **Figure 4A**, and the 3D model obtained by (SWISS model online tool) in **Figure 4B**. The allergenicity of Fag e 1 had been demonstrated with high IgE-binding frequency almost with all buckwheat allergic patients' serum [9,28]. Within Fag e 1 allergen, 8 IgE binding epitope sites were recognized, as described in (**Figure 4C)**. Within these epitopes, six individual amino acids were identified as critical for IgE binding, and from them, arginine occupied 3 positions, such positive charged amino acid can influence IgE binding capacity[29].

A

GLEQAFCNLK FKQNVNRPSR ADVFNPRAGR INTVDSNNLP	40
ILEFIQLSAQ HVVLYKNAIL GPRWNLNAHS ALYVTRGEGR	80
VQVVGDEGRS VFDDNVQRGQ ILVVPQGFAV VLKAGNEGLE	120
WVELKNDDNA ITSPIAGKTS VLRAIPVEVL ANSYDISTKE	160
AFRLKNGRQE VEVFRPFQSR DEKERERFSI V	191

B



C

IgE epitope	Amino acid sequence	Position
1	QNVNRPSR	13–20
2	NNLPILEF	37–44
3	WNLNAH	64–69
4	EGRSVF	87–92
5	KAGNEG	113–118
6	IAGKTSVLRA	135–144
7	KEAFRL	159–164
8	SRDEKERERF	179–188

Figure 4. Structure of buckwheat allergen, Fag e 1. A) The amino acid sequence of buckwheat allergen Fag e 1, **B)** Showing the 3D module structure of the protein, **C)** amino acid sequence of the eight IgE epitope sites in Fag e 1 allergen.

2.5. Fag e 2

The 16 kDa buckwheat allergen Fag e 2 comprises of 149 amino acids (**Figure 5)** and belongs to the 2S albumin protein family. These family group proteins are synthesized as one large precursor of 18-21 kDa, in the lumen of the endoplasmic reticulum, the formation of four intra-chain disulfide bonds occur, these bonds involve eight conserved cysteine residues. Lastly, the final protein is composed of large and small subunits[30]. Two disulfide bonds sustain large and small subunits of the protein, and the other two disulfide bonds are present within the structure of the large subunit. The conserved 8 cysteine motif should have third and fourth cysteine residues consecutive inside the polypeptide of the large subunit, and the fifth and sixth residues separated by only one amino acid residue. In this structure, there are interchain disulfide bonds between cysteine 1-5 and 2-3, on the other hand, cysteine residue 4-7 and 6-8 forms the intrachain bridges [31](**Figure 6**). The conserved motif of 8 cysteine residues that forms disulfide bonds contribute to the digestibility resistance seen in Fag e 2 allergens. Fag e 2 was found to be responsible for the development of an immediate hypersensitivity reaction in buckwheat allergic patients[9,32,33]. One portion of the amino acid sequence EGVRDLKE was identified as the epitope region for Fag e 2. The stability of Fag e 2 under pepsin digestion and the epitope formation are both related to disulfide bonds of the conserved 8 cysteine motif, especially on location Cys65 [34].

Fag e 2 as a protein of the 2S globulin has a compact three-dimensional structure of five alpha-helices labeled (Ia, Ib, II, III, and IV) and one C-terminal loop. This loop is stabilized by four disulfide bonds. For the alpha-helices III and IV, they are connected *via* a segment called the hypervariable region, which is the most important antigenic region[35]. **Figure 7** shows a typical 2S albumin protein structure. It was confirmed that the pattern of 8 cysteine residues is necessary for the maintain ace of tertiary protein structure[36].

A

MKLFIILATA TLLIAATQAT YPRDEGFDLG ETQMSSKCMR	40
QVKMNEPHLK KCNRYIAMDI LDDKYAEALS RVEGEGCKSE	80
ESCMRGCCVA MKEMDDECVC EWMKMMVENQ KGRIGERLIK	120
EGVRDLKELP SKCGLSELEC GSRGNRYFV	149

B

Figure 5. Structure of buckwheat allergen, Fag e 2. A) The amino acid sequence of buckwheat allergen Fag e 2 and its epitope site is underlined, B) Showing the 3D module structure of the protein.

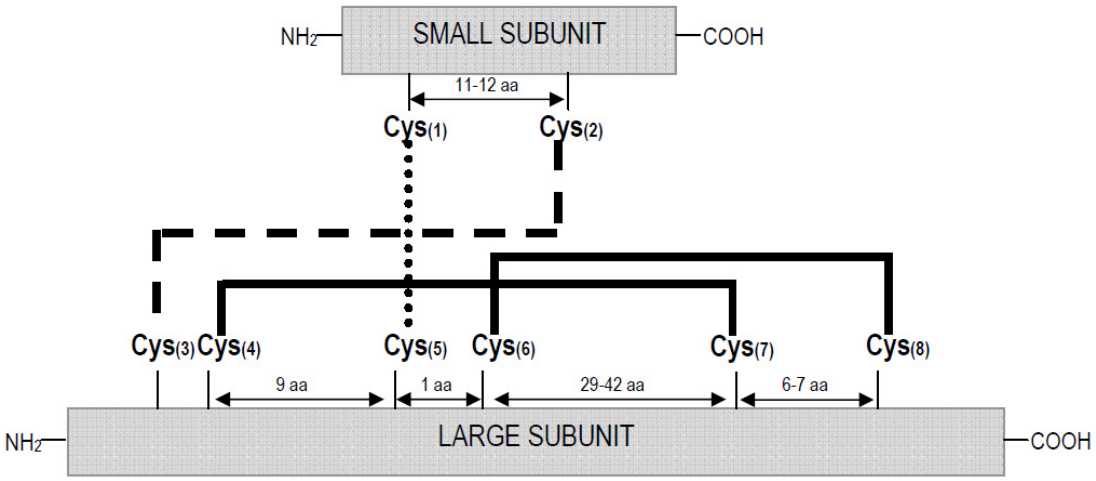


Figure 6. Disulfide bond pattern between the 8-conserved cysteine residues of Fag e 2. Adopted from Moreno et al.[37].

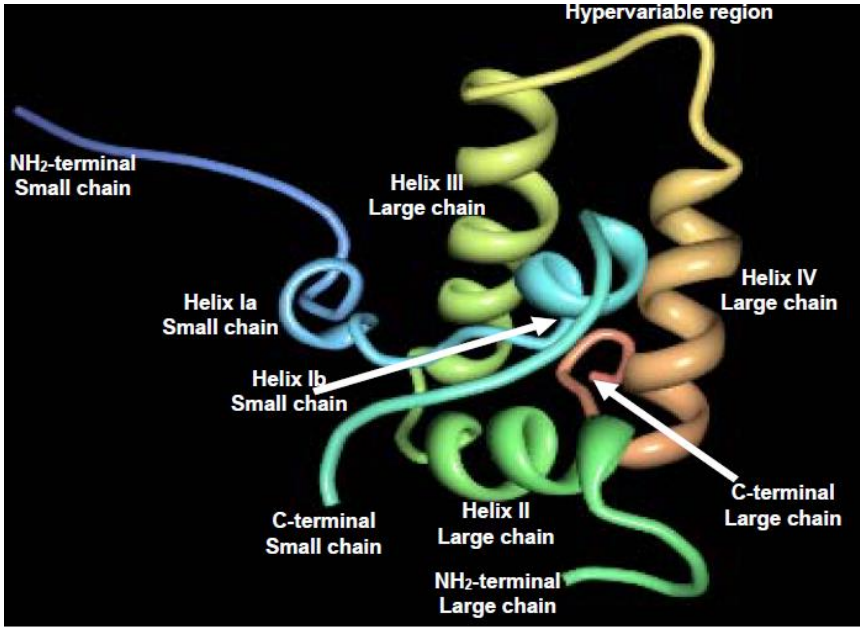


Figure 7. Three-dimensional structure of Ric c 3 allergenic protein. Adopted from Pantoja-Uceda et al.[35].

2.6. Specific immunotherapy (SIT)

SIT was introduced over a century ago[38]. This treatment works by incremental delivery of specific allergen to suppress the clinical symptoms of allergy. This therapy also induces long-term remission upon allergen re-exposure and reduces the chances of new sensitization[39,40]. For now, SIT is the sole treatment for allergic diseases that can provide long-term protection after treatment, against allergen-induced complaints[41,42]. Immunotherapy affects a variety of immune cells, leading to the improvement of allergic clinical parameters. It includes skewing from T-helper type 2 (Th2) to T-helper type 1 (Th1) response, decreased IgE response, increase regulatory T cell (Treg) and mediators, and class–switching into blocking antibodies to reduce the number of both mast cells and eosinophils[43] (**Figure 8)**.

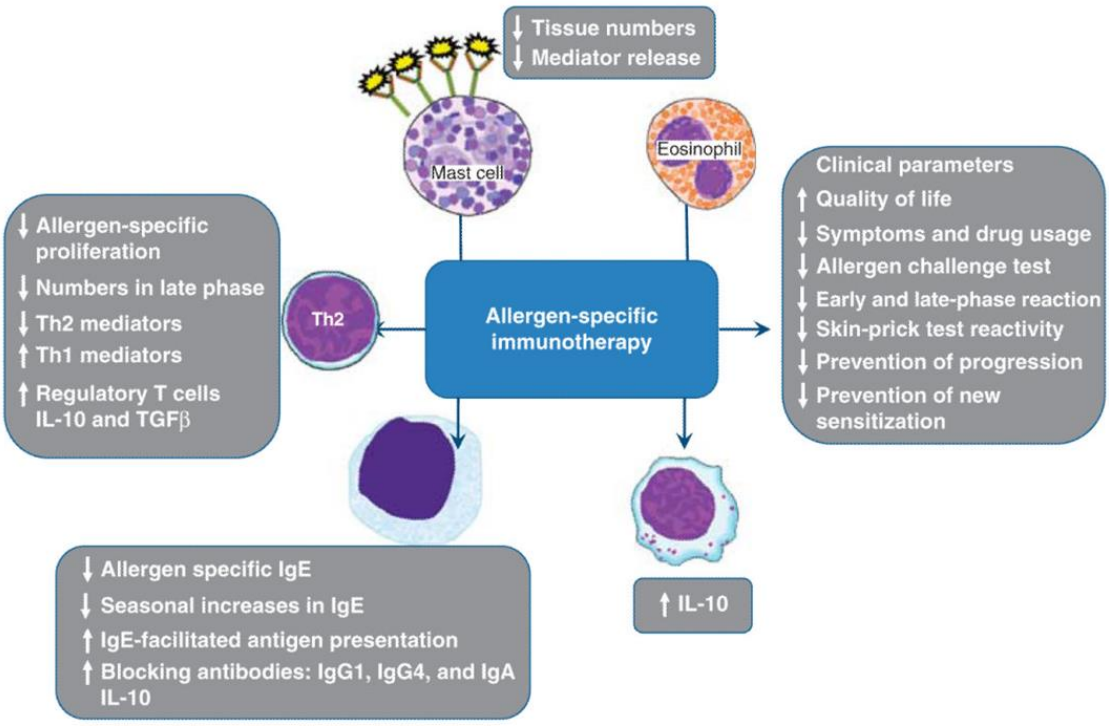


Figure 8. Immunological mechanisms of allergen-specific immunotherapy. Adopted from Lloyd et al.[43]. IL: Interleukin, TGFβ: Transforming growth factor β, Th: T-helper.

The molecular and cellular mechanisms in SIT involve very early desensitization effects, modulating both T and B cell and related antibody isotype responses, and inhibition of migration and release of mediators from mast cells, basophils, and eosinophils[44]. The establishment of T-cell tolerance is associated with the generation of allergen-specific Treg cells[45,46], followed by a decrease in both Th1 and Th2 cells[47]. Treg cells and their cytokines act on suppression of Th2 type immune responses in several major ways. They act on B cells by inducing both IgG and IgA and suppression of IgE. Treg effects act on vascular endothelium to suppress Th2 cell homing to tissues. Mast cells, basophils, and eosinophils are downregulated by Treg suppressive effects, besides suppression of epithelial cell activation[44] (**Figure 9**).

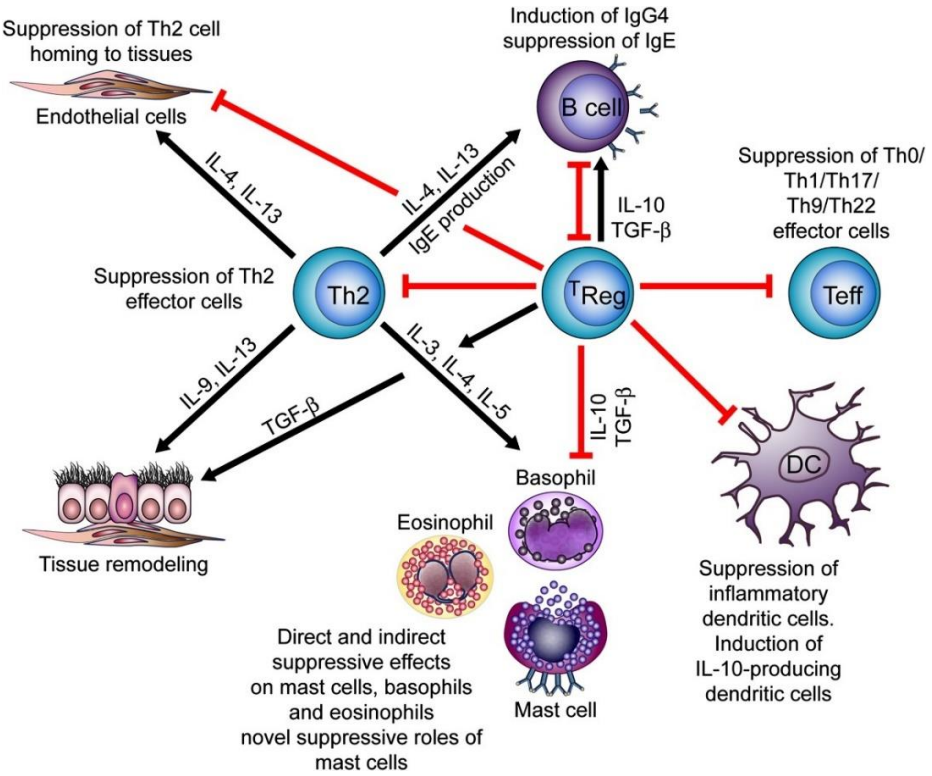


Figure 9. The role of Treg contributes to the control of allergen-specific immune responses. Red arrows indicate suppression effect. Adopted from Akdis et al. [48]. DC: Dendritic cell, IL: Interleukin, TGF-β: Transforming growth factor β, Th: T-helper.

The T cell subsets of Th1, Th2, Treg, Th17, and Tfh are differentiated by different transcription factors, and these cells are characterized by the secretion of different cytokines, expressing different chemokines, and are localized in different sites (**Figure 10**)[49]. T follicular helper (Tfh) helps B cells make antibodies, by affinity maturation and isotype switching. Tfh cells produce IL-21 which inhibits class switching to IgE[50]. Tfh provides IL-21 mediated help signals that are important for B cell survival, and they are distinguished from other T cells by the pattern of their cytokine production. Tfh cells have an important role in the synthesis of both IgG and IgA in B cells[51,52].

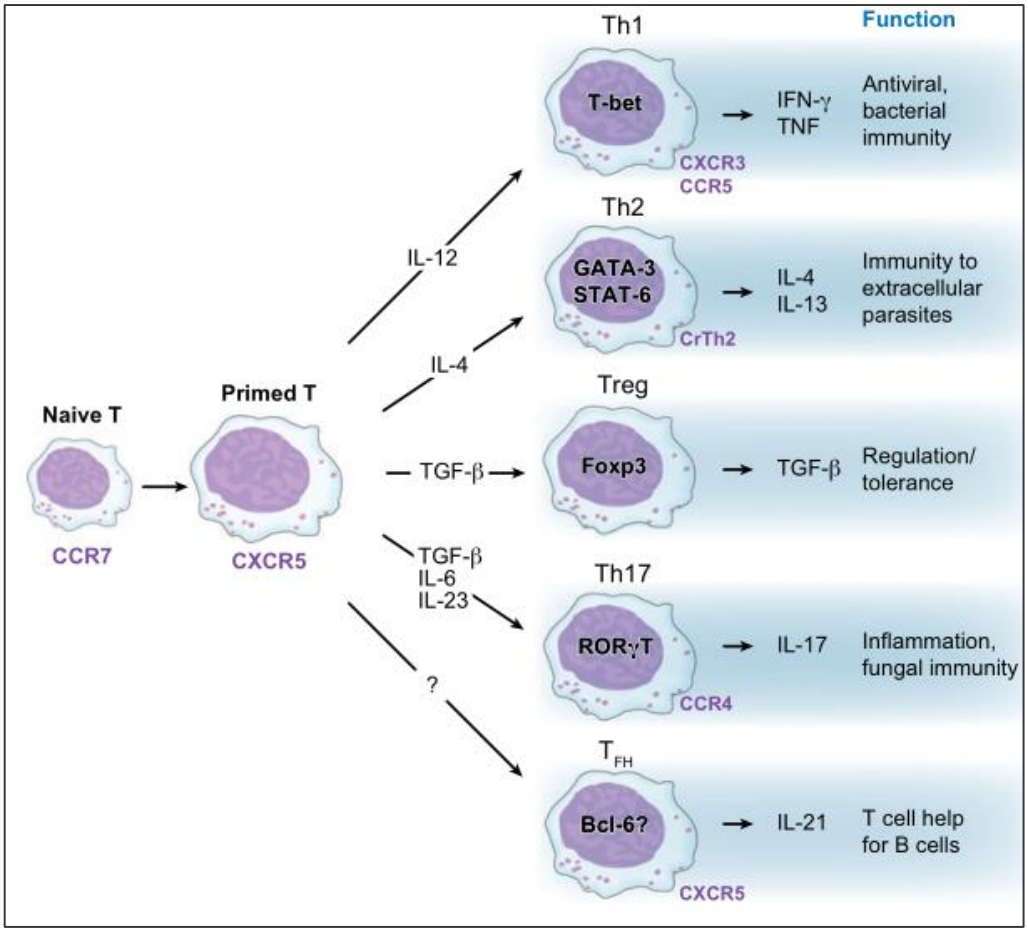


Figure 10. Origins of Tfh cells and the relationship to other subsets of T cells. Adopted from Steinman[53].

2.7. Immune modulation *via* phosphorylation

It was believed that protein phosphorylation was only nutritive additive value, recently different research shown that such phosphoproteins could have immune functions, such as the reduction of β-lactoglobulin immunogenicity by phosphorylation[54]. Phosphorylation is considered one of the most prominent post-transitional modifications, that is based on the formation of covalent bonds by the addition of a phosphate group to a protein. The phosphorylation of proteins is a recognized method for improving functional properties of food proteins, and it can be naturally seen in enzymes and receptors of both prokaryotic and eukaryotic cells, where it triggers conformational changes resulting in inactivation or deactivation of such molecules[55,56]. There are different phosphorylation techniques, such as conjugation of glucose-6-phosphate *via* the Millard reaction, or by dry heating in phosphate buffer[57,58]. Others reported that Drying from phosphate-buffered solutions can result in the phosphorylation of primary and secondary alcohol groups of saccharides, hydroxylated amino acids, proteins, and glycoproteins[59]. One of the recommended methods is to use dry heat phosphorylation, at a low pH of 5.5 or lower, phosphorus can be attached to hydroxyl oxygen and amino nitrogen, the formed phosphoprotein stability at pH between 2.0 to 10.0 and under a temperature of 120ºC favors its usage in food systems[60,61]. Protein phosphorylation was shown improvement of some physical properties such as water solubility, foaming properties, gel-foaming, and emulsification[55].

3. Objectives

The purpose of this study was to investigate the immunomodulatory activities of selected phosphorylated buckwheat allergens, and the enzymatic hydrolysate of these allergens. Buckwheat allergens Fag e 1 and Fag e 2 were phosphorylated, then some of their immune and functional properties were studied. This study hypothesized that phosphorylation caused immunomodulatory activities on both allergen and hydrolysate.

CHAPTER II

Effects of phosphorylation of buckwheat allergen on its allergic reactions in a murine model of buckwheat allergy

1. Abstract

Buckwheat (*Fagopyrum esculentum*) is a pseudocereal plant with high nutritive value, a good source of high quality, easily digestible, gluten-free food. Besides, that buckwheat extracts show anti-mutagenic properties, antioxidants, and improves diabetes symptoms. Fag e 1 and Fag e 2 are both major allergens in buckwheat, and Fag e 1 is a globulin protein with a size of 22 kDa and 192 amino acids. I used the *Pichia* expression system to successfully get an expression yield of 20mg/L-culture medium of recombinant Fag e 1. Followed using pyrophosphate in the dry heating method to phosphorylate Fag e 1, to obtain P-Fag e 1. Finally, I established Fag e 1 sensitized murine model to assess if P-Fag e 1 can be good specific immunotherapy and found that it succeeded to diminish IgE responses, reduce IL-4, and skew Th2 response by Tfh cells, leading to excessive production of IgA. P-Fag e 1 could be a good candidate for a modified allergen for specific immunotherapy.

2. Introduction

Buckwheat (*Fagopyrum esculentum*) ascends from the Polygonaceae family. It is not a cereal or grass despite the grain-like use of the crop, as it is a pseudocereal. The crop originated from Asia, later it was introduced to Europe, followed by spread to Canada, the United States of America, Latin America, and even to the high-altitude mountains of Nepal[62]. The seeds of buckwheat are a good source of high quality, easily digestible, gluten-free food. Besides, that buckwheat extracts show anti-mutagenic properties, antioxidant, and improves diabetes symptoms[63,64]. Buckwheat allergy was early described in 1909, since then it was recognized as one of the most life-threatening food allergens[6]. A small amount of buckwheat scrubbed on lips or licked by tongue could induce allergic symptoms[8]. The major allergenic proteins in buckwheat have been identified to be 9, 16, 19, and 22 kDa in size based on their mobility on SDS-polyacrylamide gel electrophoresis, and the allergens in buckwheat with the 22 kDa size globulin protein referred to as (Fag e 1) with a 192 amino acids[9,29]. Fag e 1 allergen had a high IgE-binding frequency with almost all buckwheat allergic patients serum[9,28]. A total of 8 IgE binding epitope sites are identified in Fag e 1 allergen, within these epitopes, the critical IgE binding positions were identified in six individual amino acids[29].

One of the recognized long-term treatments of food allergy is by SIT, if treatment is orally it referred to as oral immunotherapy (OIT)[10]. Using SIT as an alternative treatment of food allergy barriers great benefits, it includes reduction of disease severity leading to decreased drug usage as well as prevention of future sensitizations during long-term treatments. It also improves the safety and efficacy of future treatments[11]. SIT is accepted for the prevention and treatment of different allergic diseases, as the allergen is given to allergic patients to modulate their immune response and abolish their symptoms. Briefly explained, SIT works to shift from T helper cell type -2 (Th2)

immune responses to a T helper cell type -1 (Th1) immune response. Such shifting associates with the production of T regulatory (Treg) cells which produce the cytokine interleukin 10 (IL-10) reducing the levels of allergen-specific IgE antibodies, increasing allergen-specific IgG antibodies, and reducing the release of pro-inflammatory cytokines from mast cells, T-cells, and eosinophils [65,66]. The goal of SIT is to reach immune tolerance, which can be explained by isotype switching. Mature B cells will secret immunoglobulins according to co-stimulatory signals, if B cells secreted IgG instead of IgE, they will lead to inhibition of the binding between allergen and corresponding IgE on the surface of the mast and basophil cells[67,68]. Besides, the Tfh subset of T cells also supports SIT by the production of IL-21 cytokines, and enhances the production of IgA leading to a reduced state of allergenicity[49,52].

Allergens used in SIT can be further improved to reduce their allergenicity by chemical modification of allergens[12]. One method of antigen modification is the allergic protein conjugation with polysaccharides, in previous studies, this shown significant effects on shifting splenic T helper cells towards Th1 response[69,70]. Fermentation by *Rhizopus oligosporus* was applied to buckwheat and reported that 22 kDa protein was completely degraded[71]. Another way of modification is by phosphorylation of the allergic protein. In a previous study on oral administration of phosphorylated Fag e 2 (P-Fag e 2) in a Fag e 2 sensitized mice, results shown a suppression in Th2 allergic responses[72].

In this study, to obtain Fag e 1 allergic protein, I used the *Pichia* expression system. First, Fag e 1 was cloned, inserted into a *Pichia* expression vector, pGAP with a GDP promotor, and transformed this vector into the *Pichia pastoris* strain X-33 to successfully get an expression yield of 20mg/L-culture medium of recombinant Fag e 1. Next, I used pyrophosphate in the dry heating method to phosphorylate Fag e 1, to obtain P-Fag e 1. To test the hypoallergenic effects of P-Fag

e 1, this study used a Fag e 1 sensitized murine model to measure the ability to diminish IgE responses, suggesting the involvement of Tfh mechanisms.

3. Materials and methods

3.1. Materials

Common buckwheat (*Fagopyrum esculentum* Moench), cultivar "Shinano No.1", was obtained from the Education and Research Centre of Alpine Field Science (AFC) in Shinshu University (Minamiminowa, Nagano, Japan). *Pichia pastoris* X-33 was purchased from Invitrogen (Carlsbad, CA, USA). Anti-mouse IgE and IgA antibodies used in these experiments were purchased from Pierce (Rockford, IL, USA). All the other reagents were biochemical grade and purchased from commercial suppliers.

3.2. Large-scale production of Fag e 1

The first step was to establish the *Pichia pastoris* expression system. The complementary DNA (cDNA) of Fag e 1 was obtained from the seeds of *F. esculentum*, cloned, and inserted into a pUC18 vector after digestion with *EcoR* I and *Sal* I. Then, it was digested with *Xho* I and *Xba* I and ligated into a *Pichia* expression vector, pGAPZα-A (Invitrogen), at a site that was downstream of the GAP promoter. The vector was linearized with *Bln* I and transformed into *P. pastoris* X-33 by electroporation (**Figure 11**).

Fag e 1 expressing *Pichia pastoris* were inoculated into YPD medium (1% yeast extract, 2% peptone, and 2% glucose) and incubated at 30°C with shaking at 250 rpm for 3 days to increase the cellular population. Then, transformed yeasts were inoculated at a ratio of 1:100 into an expression medium containing glucose (1.34% yeast nitrogen base, 0.005% asparagine, and 0.5% glucose). After a 1-day induction period at 25°C, the culture was centrifuged at 10,000 × g for 10 min to collect the supernatant containing Fag e 1. Culture supernatant was purified with size

exclusion chromatography using a Sephacryl S-100 column (GE Healthcare, Little Chalfont, UK) and lyophilized to obtain powdered samples (**Figure 12**).

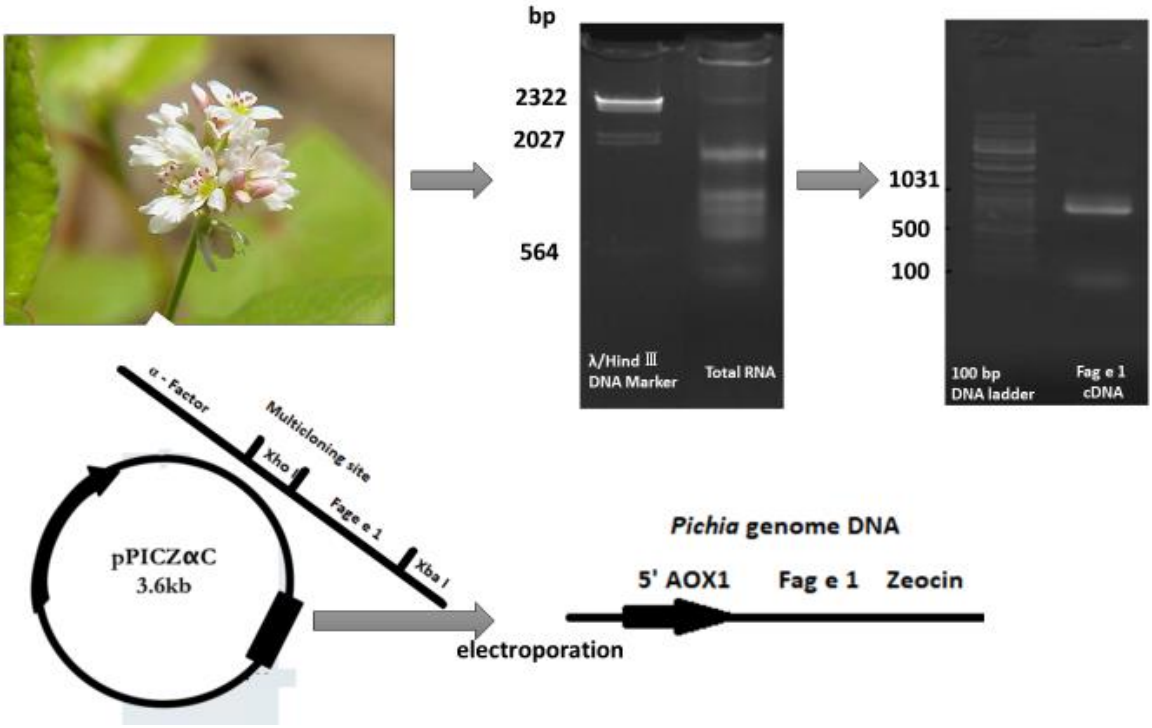


Figure 11. Cloning of cDNA and transforming into *Pichia* genome for expression of Fag e 1.

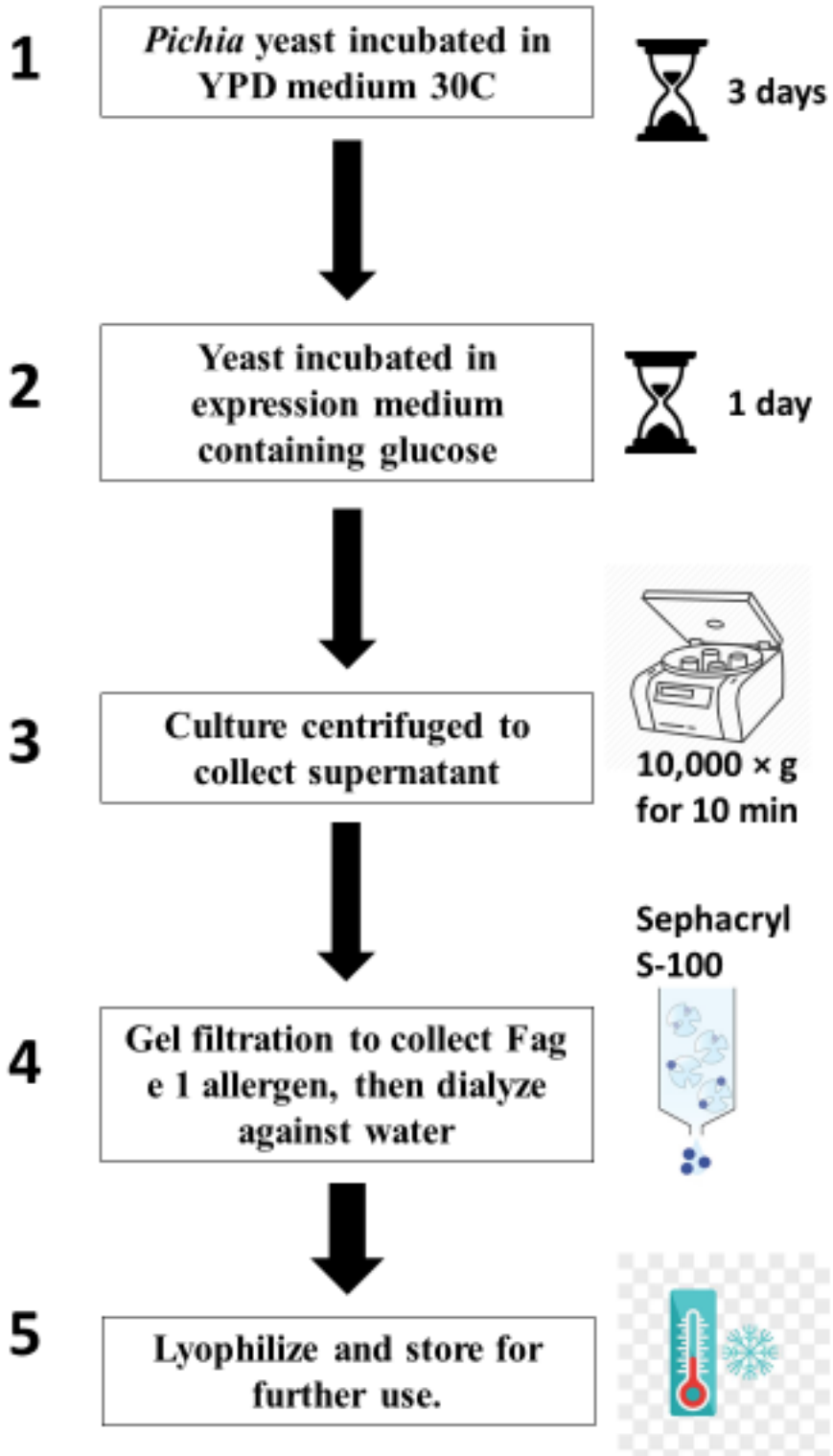


Figure 12. Major steps of expression and purification of Fag e 1.

3.3. Phosphorylation of Fag e 1

Phosphorylation of Fag e 1 was performed by following the protocol given by Enomoto et al.[73]. Briefly, freeze-dried Fag e 1 was dissolved in 0.1 M sodium pyrophosphate at a concentration of 1 mg/mL and lyophilized. Then, the resulting dry powder was incubated at 85°C for 7 days. After incubation, the dry-heated powder was dissolved in distilled water, dialyzed for 3 days using a dialysis tube with 6-8 kDa MW-cutoff membrane (Spectrumlabs, Rancho Dominguez, CA, USA), and finally, lyophilized (**Figure 13**). The phosphorus content of P-Fag e 1 was determined according to the method described by Chen et al.[74]. Briefly, these samples were mixed with 4 mM potassium persulfate and then, incubated for 20 min at 120°C. Further, the cooled samples were mixed with 100 mM ascorbic acid and then, with 35 mM ammonium molybdate-antimony potassium tartrate. The processed samples were measured at 880 nm by using a UV spectrophotometer (Shimadzu). The resulting lyophilized powder was used as P-Fag e 1 for further experiments.

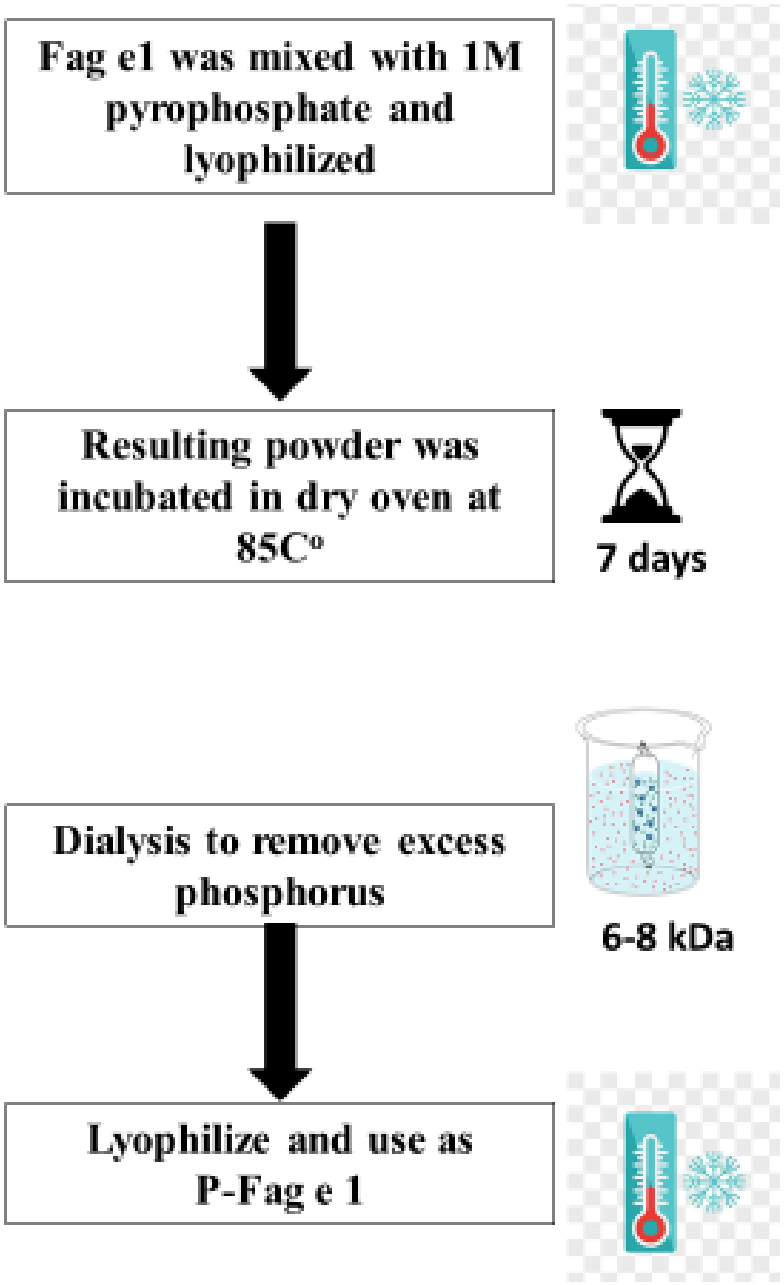


Figure 13. Steps of Fag e 1 phosphorylation.

3.4. Animal experiments

Five-week-old female BALB/c mice were purchased from Charles River, Ltd. (Tokyo, Japan). They allowed acclimatizing to the environment for one week before the experiment period. The mice were given free access to food and water (MF; Oriental Yeast Co., Tokyo, Japan) under the condition with temperature (23 ± 2°C), humidity (50 ± 10%), and light cycle (12 h/12 h). All the experiments conformed to the guidelines for Animal Experimentation at Shinshu University (permission number: 270075).

3.5. A murine model for Fag e 1 sensitization

Eighteen BALB/c female mice were divided into three groups (six mice per group) labeled Control group, Fag e 1 group, and Fag e 1/ P-Fag e 1 treated group. All groups except control were sensitized with 50 μg of Fag e 1 by intraperitoneal injection, and the immunized mice were given a booster dose 14 days after the initial immunization. The level of specific IgE was found to be elevated on day 21. The mice in the control group and Fag e 1 group were fed with a normal diet, and the mice in the P-Fag e 1-treated group were fed with P-Fag e 1 (30 mg of sample/100 g of body weight) for 6 weeks. Next, the mice were challenged by oral administration of 30 mg of Fag e 1. Then, they were euthanized by CO_2, and their serum, spleen, and Peyer's patches were collected for studying the immune response and T follicular helper (Tfh) cell population (**Figure 14**).

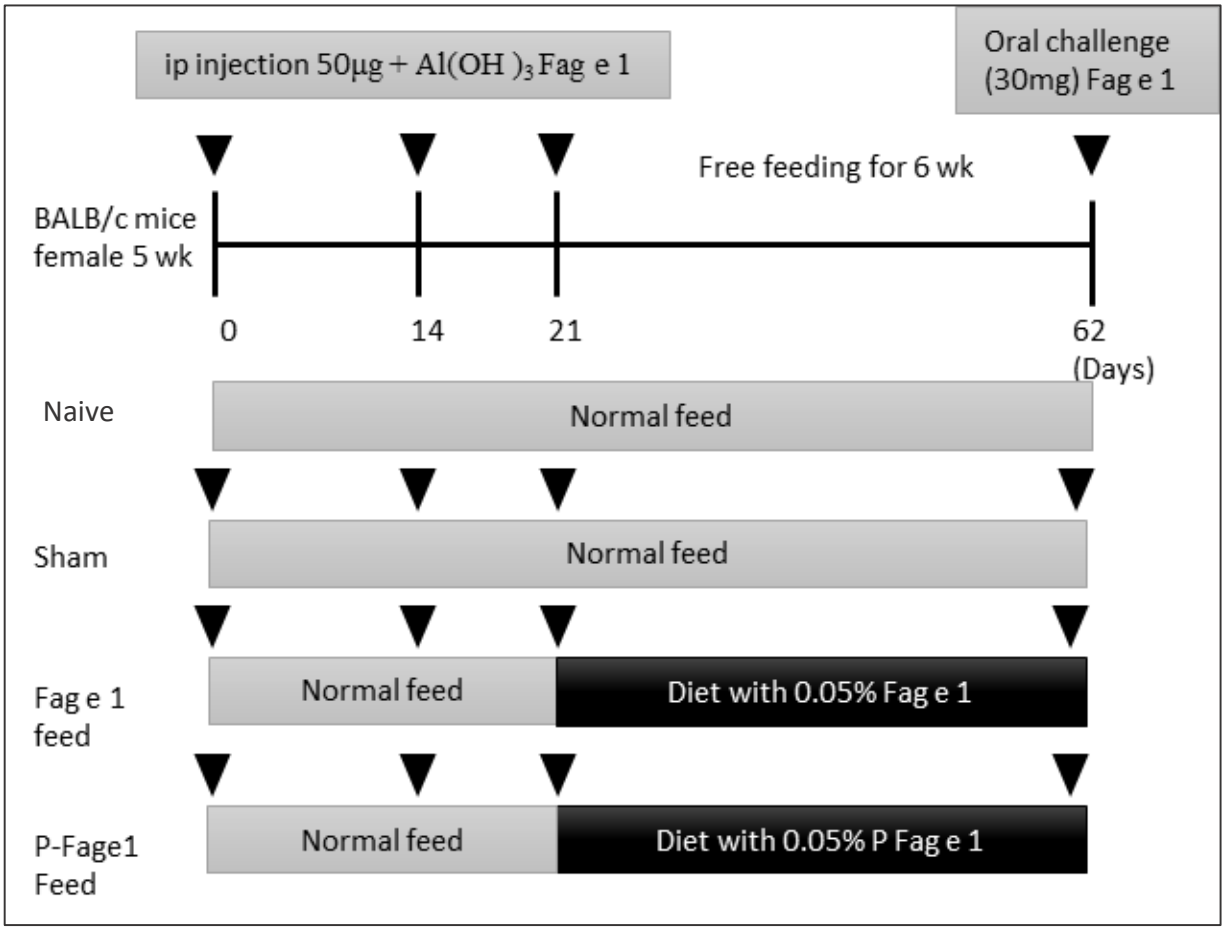


Figure 14. Scheme of Fag e 1 in vivo experiments. For each group, (n=6). Naive group was not sensitized and provided a normal diet. Sham group was sensitized and aporvied a normal diet. Fag e 1 and P-Fag e 1 geed were fed a diet containing 0.05% of Fag e 1 and P-Fag e 1, respectively.

3.6. ELISA measurements for the serum levels of total and Fag e 1-specific IgE and IgA

The serum of the immunized mice was used to determine the levels of total IgE, total IgA, histamine, and Fag e 1-specific IgE using sandwich ELISA or indirect ELISA. In brief, the wells of a 96 well plate were coated with anti-mouse IgE (1:500), IgA (1:1000), and histamine (1:1000) antibodies and incubated overnight. After removing the antibody samples, the wells were washed using a washing buffer and incubated with mice serum (1:500). Then, an HRP-labeled antibody (1:1000) was added to each well. In the case of Fag e 1-specific IgE, the wells were coated with Fag e 1 (100 μg/mL) and then, mice serum (1:50) and HRP-labeled secondary antibody (1:1000) were added, respectively. Finally, *o*-phenylenediamine substrate and stop solution were added to each well. The optical density was determined using a microplate reader at 450 nm.

3.7. Measurement of cytokine levels in culture supernatants

The harvested spleen and Peyer's patch cells were incubated with 50 μg/mL with Fag e 1 (Fag e 1 and Fag e 1/P-Fag e 1 groups) or PBS (non-sensitized control group) at 37°C in a humidified atmosphere with 5% CO_2 for 72 h (**Figure 15**). Cytokine levels in culture supernatants were determined by ELISA, which uses the following HRP-conjugated antibodies and hydrogen peroxide with *o*-phenylenediamine as the substrate: rat anti-mouse IL-4 and IFN-γ antibodies, biotinylated rat anti-mouse IL-4 and IFN-γ antibodies, and streptavidin-HRP conjugate (all purchased from Peprotech, Rocky Hill, NJ, USA).

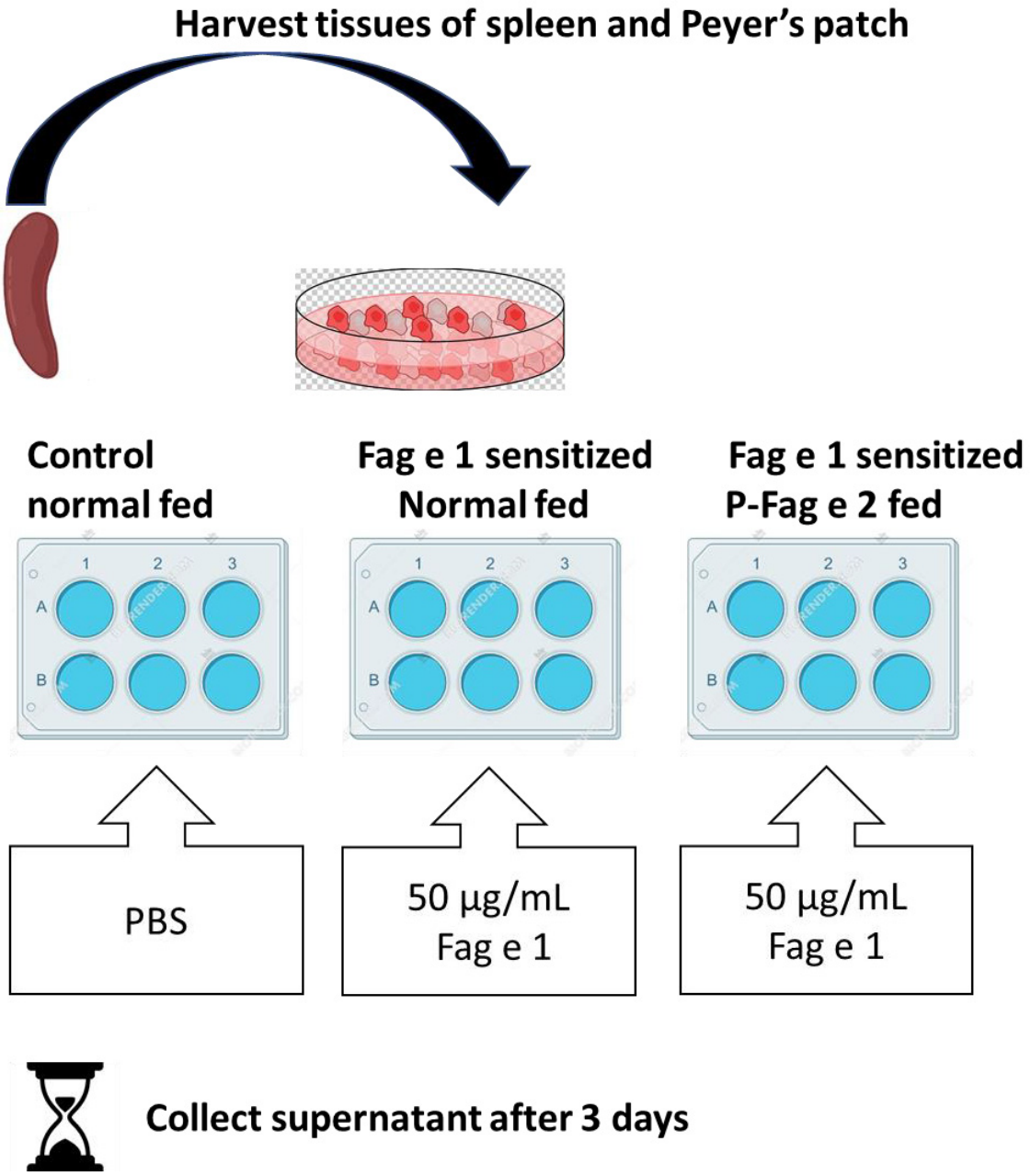


Figure 15. Outlines of ex-vivo experiments.

3.8. Flow cytometry analysis

Tfh cell population in the Peyer's patches of mice was evaluated using flow cytometry. The Tfh cells were detected by staining with FITC-labeled anti-CD4 and PE-labeled anti-CXCR5

antibodies (1:100) (BD Biosciences, Bedford, MA, USA). The Tfh population was analyzed by using a FACSCalibur flow cytometer with CellQuest software (BD Biosciences).

3.9. Statistical analysis

All the results were expressed as the mean ± standard error of the mean (SEM). The data were also subjected to analysis of variance and Tukey's multiple comparison tests. *p*-values less than 0.05 were considered as statistically significant.

4. Results

4.1. Expression of Fag e 1 using the *Pichia* expression system

This study established a method for producing buckwheat allergen Fag e 1 by using the *Pichia pastoris* expression system. Gel filtration chromatography was used to purify recombinant Fag e 1, followed by running on SDS-PAGE. The Fag e 1 protein with a molecular mass of 22 kDa was confirmed. This observation suggested that the *Pichia* expression system successfully expressed recombinant Fag e 1 (**Figure 16**). Measurements of purified Fag e 1 in yeast expression culture show a yield of 20 ± 1.5 (n=3) mg/L, making this expression system a good choice for collecting enough samples for further experiments. Then, I phosphorylated recombinant Fag e 1 by dry heating in the presence of pyrophosphate to confirm successful phosphorylation with a phosphorus content of 0.38%.

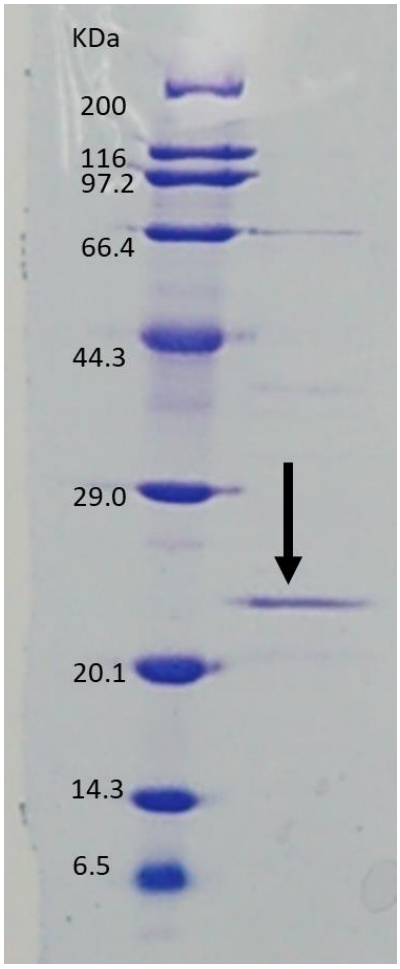


Figure 16. SDS-PAGE profile of Fag e 1 secreted using the *Pichia* expression system.

4.2. Effects of P-Fag e 1 effects on Fag e 1-sensitized murine model

Next, I investigated the effects of P-Fag e 1 on the allergenicity using mice models that were immunized with Fag e 1 (twice with the Fag e 1 allergen). The mice were fed on a diet with P-Fag e 1 for 6 weeks and then, they were challenged by oral administration of 30 mg of Fag e 1. The results show that the serum of the mice in the P-Fag e 1-treated group had reduced histamine secretion than the Fag e 1 group (**Table 3**). Furthermore, the total IgE and Fag e 1-specific IgE were significantly decreased in P-Fag e 1 treated group when compared with the Fag e 1 group. Besides, the level of total IgA was increased in the P-Fag e 1 treated group.

Table 3. Changes in total and specific IgE, histamine, and total IgA levels in serum.

Sample	Total IgE	Specific IgE	Histamine (nM)	Total IgA (ng/ml)
Control[†]	0.26 ± 0.03	0.01 ± 0.00	15.8 ± 1.3	2.54 ± 0.19
Fag e 1[¶]	0.86 ± 0.07	0.25 ± 0.02	30.2 ± 3.7	1.65 ± 0.08
Fag e 1/P-Fag e 1[§]	0.50 ± 0.05[*]	0.14 ± 0.01[*]	21.7 ± 1.4[*]	2.38 ± 0.32[*]

[†]Test serum was obtained from normal mice without any treatment. [¶] Serum from the sensitized mouse with Fag e 1. [§] Serum from P-Fag e 1-treatment mouse followed by Fag e 1-sensitization. Each value is the mean ± standard error of three replications. [*] There was a significant difference ($p<0.05$).

Furthermore, this study investigated cytokine products that could be produced because of Tfh cells in both spleen and peyer's patches in the murine model sensitized with Fag e 1. The results showed that the production of IL-4 (Th2-associated cytokine) in both the spleen and Peyer's patches was significantly decreased in the P-Fag e 1 treated group, compared with the Fag e 1 group (**Table 4**). Nevertheless, the changes in the IFN-γ production in the spleen and Peyer's

patches were not statistically significant. Therefore, the results suggested that the phosphorylated form of Fag e 1 might be able to safely and effectively diminish the IgE-mediated allergic reaction against buckwheat allergens.

Table 4. Changes in IL-4 and IFN-γ levels in spleen and Peyer's patch cells.

	IL-4 (pg/ml)		IFN-γ (pg/ml)	
Sample	Spleen	Peyer's patch	Spleen	Peyer's patch
Control[†]	120.6 ± 7.6	80.6 ± 2.5	2206.3 ± 525.7	733.0 ± 105.2
Fag e 1[¶]	389.3 ± 32.9	337.0 ± 40.3	2524.5 ± 274.4	496.3 ± 47.3
Fag e 1/P-Fag e 1[§]	262.1 ± 28.9[*]	218.4 ± 47.4[*]	2032.5 ± 330.1	630.5 ± 59.7

[†]Test serum was obtained from normal mice without any treatment. [¶] Serum from the sensitized mouse with Fag e 1. [§] Serum from P-Fag e 1-treatment mouse followed by Fag e 1-sensitization. Each value is the mean ± standard error of three replications. [*] There was a significant difference (p<0.05).

4.3. Differentiation of Tfh cells in response to oral administration of P-Fag e 1

In this Fag e 1-sensitized murine model, cells from Peyer's patches were isolated, stained with specific antibodies against markers on Tfh cells, and subjected to flow cytometry for the analysis of Tfh population. Results show an increase in the Tfh cell population from the P-Fag e 1 fed mice group compared to the Fag e 1 fed mice group (**Figure 17**).

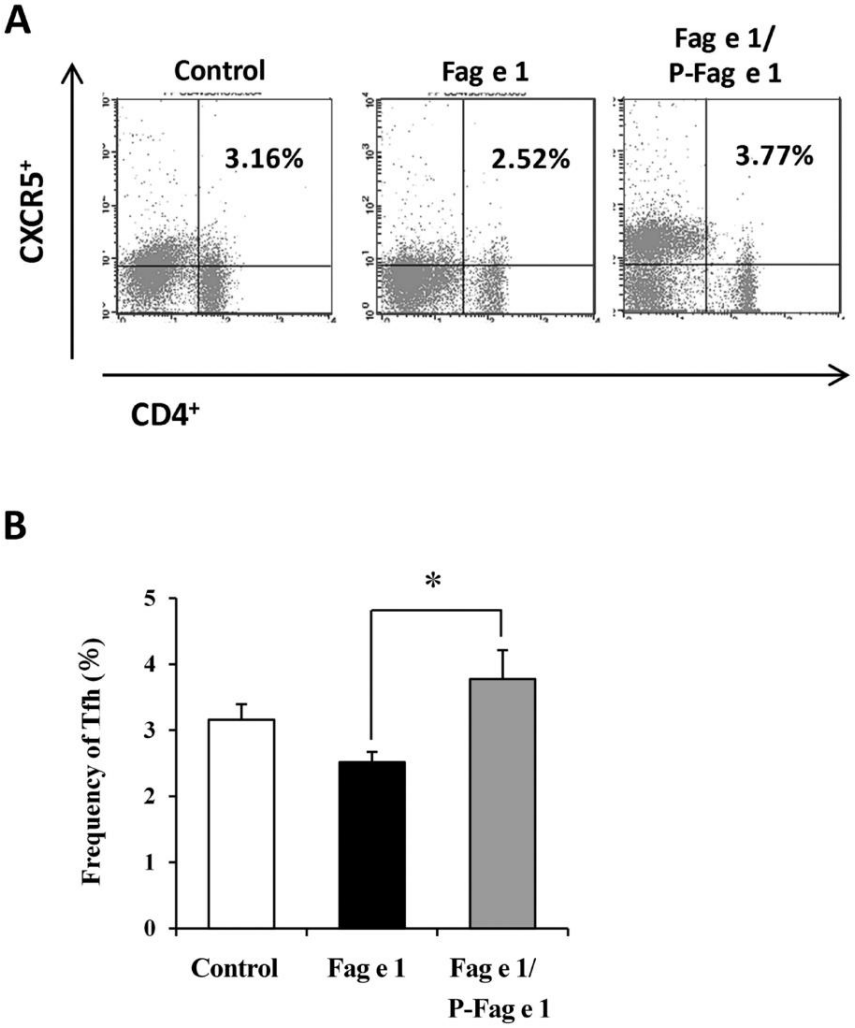


Figure 17. Effects of oral administration of P-Fag e 1 on the population of Tfh cells among Peyer's patch cells of Fag e 1-sensitized mice. Tfh cell populations in Peyer's patch of Fag e 1-sensitized mice. Tfh cells were isolated from Peyer's patch of Fag e 1-sensitized mice. Total cells obtained from Peyer's patches were stained with FITC- labeled anti-CD4 and PE-labeled anti-CXCR5, and subjected to flow cytometry. The Tfh population was analyzed using a FACSCalibur flow cytometer. **A.** Representative flow cytometry dot plots. **B.** The percentage of Tfh cells in the Peyer's patches cells collected from mice. The data are representative of six mice and are presented as the mean ± SEM. $*p < 0.05$.

5. Discussion

Fag e 1 is a major allergen in buckwheat plant, belongs to a globulin protein family, and is reported to cause severe allergic symptoms[75]. In this study, I tried to apply the idea of specific immunotherapy on a Fag e 1-sensitized murine model. This study used recombinant Fag e 1 prepared by using the *Pichia pastoris* expression system and phosphorylated it to be further used as SIT allergen.

Decreased levels of total and specific IgE and decreased histamine levels could indicate decreased allergic symptoms since there is a strong relationship between increased IgE levels and increased allergic symptoms[21]. These results are found in alignment with previous work on using P-Fag e 2 as an allergen in SIT for mice sensitized by Fag e 2 allergens in both studies, there was an indication that allergic symptoms dropped. Total IgA levels increase for P-Fag e 1 fed mice show isotype switching, similar to the P-Fag e 2 SIT study, as the increased in IgA could be related to Tfh cells[72].

Th2 immune response promotes IgE production *via* IL-4 and IL-5 release. Furthermore, IL-4 will further induce naïve CD4+ cells to differentiate into Th2 cells. I investigated the release of Th2 cytokine IL-4 from Th2 cells in both Fag e 1 fed, and P-Fag e 1 fed mice, and results indicate that treatment with P-Fag e 2 reduced Th2 response.

The increase in total IgA levels could be related to Tfh cell activity, Cao et al. and King et al. show that Tfh cells, which are a subset of T cells, not only enhance B cell differentiation and function but also increase IgA production[49,52]. Tfh cells can also impair the immune response of Th2 cells[49]. Thus, combining the two, Tfh could influence the reduction of total IgE, and increase IgA production.

Li et al. have revealed that these amino acid residues of ovalbumin and lysozyme could be phosphorylated by dry-heating with pyrophosphate[76]. It has been reported that some of the IgE-binding regions in Fag e 1 protein contain serine and threonine residues[29]. Thus, phosphorylation of Fag e 1 might be effective in reducing its potential allergenicity, and IgE-binding capacity. On the other hand, Ntshepisa et al. found that casein phosphopeptide attenuated IgE-modulated allergic reaction in ovalbumin-sensitized mice and the immunomodulatory activity can be enhanced by additional phosphorylation[13]. In conclusion, this study demonstrated that the oral administration of P-Fag e 1 attenuated the Th2-type allergic response in Fag e 1-sensitized mice. The reduction of allergic response is due to the Tfh cell activation. These findings could facilitate the development of a hypoallergenic agent for buckwheat allergy combined with the usage of the *Pichia* expression system for large scale production of P-Fag e 1.

CHAPTER III

Effects of enzymatic hydrolysate obtained from phosphorylated Fag e 2 on allergic reactions in a murine model of buckwheat allergy

1. Abstract

Fag e 2 is a 16 kDa major buckwheat allergen that is highly resistant to pepsin digestion and poses a major risk of anaphylaxis. In this study, I investigated the effects of phosphorylation on the digestibility of Fag e 2 and assessed whether digested P-Fag e 2 (DP-Fag e 2) can attenuate allergic reactions in Fag e 2-sensitized mice. Recombinant Fag e 2, obtained using the *Pichia* expression system, was phosphorylated *via* dry heating in the presence of pyrophosphate. The peptic digestibility of Fag e 2 was enhanced by phosphorylation. Thus, I studied some of the structural changes that occurred to P-Fag e 2 compared to native form that could affect digestibility, Circular Dichroism data, and total sulfhydryl groups have shown a degree of conformational changes. Fag e 2-sensitized murine model was used, Fag e 2 sensitization exhibited reduced allergic symptom scores compared to those of sham-treated mice. Furthermore, decreased total and specific IgE, decreased specific IgG_1, and increased total IgA were observed in the serum of the DP-Fag e 2-fed group. These results suggest that P-Fag e 2 is easily digested in the stomach because of conformational changes, and it induces the attenuation of the IgE-mediated allergic reaction.

2. Introduction

Buckwheat (*Fagopyrum esculentum*) is one of the most important buckwheat species cultivated worldwide, the food-related product is from the common buckwheat *Fagopyrum esculentum* is one of the globally cultivated buckwheat types of the 9 agricultural valued types worldwide. The triangular seeds are covered by a dark brown or black hull and mainly consumed in a dehulled form. The carbohydrate percentage is 73.3%, with starch as the main component[17]. Buckwheat contains a set of bioactive compounds that are of high interest to for food industry products[18].

Chapter 2 explained that Fag e 1 is a major allergen in buckwheat allergy, indeed Fag e 2 was also considered a major allergen with 16 kDa molecular weight, and 149 amino acids. One of the most important properties of Fag e 2 is its resistance to pepsin digestion as it is a member of the 2S protein family. Members of this family including Fag e 2 are characterized by having 8 cysteine residues that form disulfide bonds assisting to the resistance to digestibility[9,32,33]. The epitope region of Fag e 2 was identified with the amino acid sequence EGVRDLKE. The stability of Fag e 2 under pepsin digestion is related to the disulfide bonds of the conserved 8 cysteine motif, especially on location Cys65 [34].

Structure and function are very related in Fag e 2 resistance to pepsin, the 2S family proteins are synthesized as one large precursor of 18-21 kDa, in the lumen of the endoplasmic reticulum, the 8 cysteine conserved residues are involved in the formation of four intra-chain disulfide bonds that gives a final protein composed of large and small subunits[30]. In the final structure, two of the formed four disulfide bonds bond the large and small subunits, while the other two disulfide bonds are embedded inside the structure of the larger subunit. The original residues of cystines have third and fourth residues inside the polypeptide of the large subunit, while fifth and sixth residues have

one non-cystine residue separating them. The two interchain disulfide bonds form between cysteine 1-5 and 2-3, on the other hand, intrachain disulfide bonds connecting larger and smaller subunits are formed between residues 4-7, and 6-8 (**Figure 6)** in Chapter 1[31)]. The conserved motif of 8 cysteine residues that forms disulfide bonds contribute to the digestibility resistance seen in Fag e 2 allergen. Fag e 2 was found to be responsible for the development of an immediate hypersensitivity reaction in buckwheat allergic patients[9,32,33)]. Based on family group data, Fag e 2 has a compact three-dimensional structure of five alpha-helices labeled (Ia, Ib, II, III, and IV) and one C-terminal loop. This loop is stabilized by four disulfide bonds. For the alpha-helices III and IV, they are connected *via* a segment called (the hypervariable region) that is the most important antigenic region[35)]. It was confirmed that the pattern of 8 cysteine residues is necessary for the maintain ace of tertiary protein structure[36)].

SIT is a promising treatment for food allergy; however, the use of crude allergen extracts can result in serious side effects[77,78)]. In a previous study, the allergenicity of Fag e 2 was attenuated by phosphorylation, and the oral administration of P-Fag e 2 exhibited decreases in both allergic scores and Fag e 2-specific IgE levels with attenuation of the Th2-dominated reaction in Fag e 2-sensitized mice[72)]. Chapter 2 of this theses described that phosphorylation of buckwheat allergen Fag e 1 resulted in the attenuation of allergic reactions in Fag e 1-sensitized mice[79)]. Thus, phosphorylated hypoallergenic proteins appear to be good candidates for immunotherapeutic food allergy treatments. On the contrary, enzymatic digestion can also alter allergenicity by cutting the epitope region. The gastric digestion of bovine milk proteins reduced allergenicity; the digestibility and antigenicity of β-lactoglobulin were affected by several factors, such as heat, pH, and applied shear[80,81)].

Therefore, this study hypothesized that the combination of phosphorylation and enzymatic digestion would provide a safer and more effective method. This work showed that pepsin digestibility for P-Fag e 2 was increased compared to the intact form of Fag e 2. Moreover, I investigated some structural changes of P-Fag e 2 by analyzing the Circular Dichroism (CD) spectrum and total sulfhydryl groups. Subsequently, I assessed whether the digests of P-Fag e 2 attenuate allergic reactions in the Fag e 2-sensitized murine model. The results of this study show that P-Fag e 2 was more digestible than Fag e 2. Furthermore, conformational changes occurred in P-Fag e 2 that could aid in its digestibility. In the Fag e 2-sensitized murine model, different parameters have shown that digested P-Fag e 2 (DP-Fag e 2) peptides have hypoallergic activities on Fag e 2-sensitized murine model.

3. Materials and methods

3.1. Preparation of Fag e 2 and P-Fag e 2

Fag e 2 and P-Fag e 2 were prepared according to a previously described method, similar to Fag e 1 and P-Fag e 1 preparation methods illustrated in **Figure 12** and **Figure 13** in Chapter 2 of this thesis with slight changes in expression stage[72,73,79]. *Pichia pastoris* were inoculated into YPD medium (1% yeast extract, 2% peptone, and 2% glucose) and incubated at 30°C with shaking at 250 rpm for 3 days to increase the cellular population. Then, transformed yeasts were inoculated at a ratio of 1:100 into an expression medium containing (1.34% yeast nitrogen base, 0.005% asparagine, and 1.25% methanol). Incubated for 2 days at 25°C, with a daily supplement of 0.125% methanol. Next, the culture medium was centrifuged at 7,500 × g for 20 min to collect the supernatant containing Fag e 2. Culture supernatant was purified with size exclusion chromatography using a Sephacryl S-100 column (GE Healthcare, Little Chalfont, UK), a re-concentrated supernatant of 50 mg/ ml in 50 mM ammonium bicarbonate buffer pH 7.5 was run through the column in the settings of a flow rate 1 ml/ min, injection volume 1 ml, column volume 120 ml, pressure 0.15 Pa, fraction size 4 ml. and lyophilized to obtain powdered samples (**Figure 18**).

For phosphorylation, Fag e 2 was dissolved in 0.1 M sodium pyrophosphate at a concentration of 1 mg/mL and then lyophilized. The resulting powder was incubated at 85°C for 7 days and then dissolved in distilled water and dialyzed against distilled water for 3 days using a 6–8 kDa cut-off membrane to remove free pyrophosphate. Finally, the solution was lyophilized and stored at −20°C until further use. Phosphorus contents were determined according to the method described by Chen et al.[74]. Absorbance was measured at 880 nm using a UV spectrophotometer (Shimadzu, Kyoto, Japan).

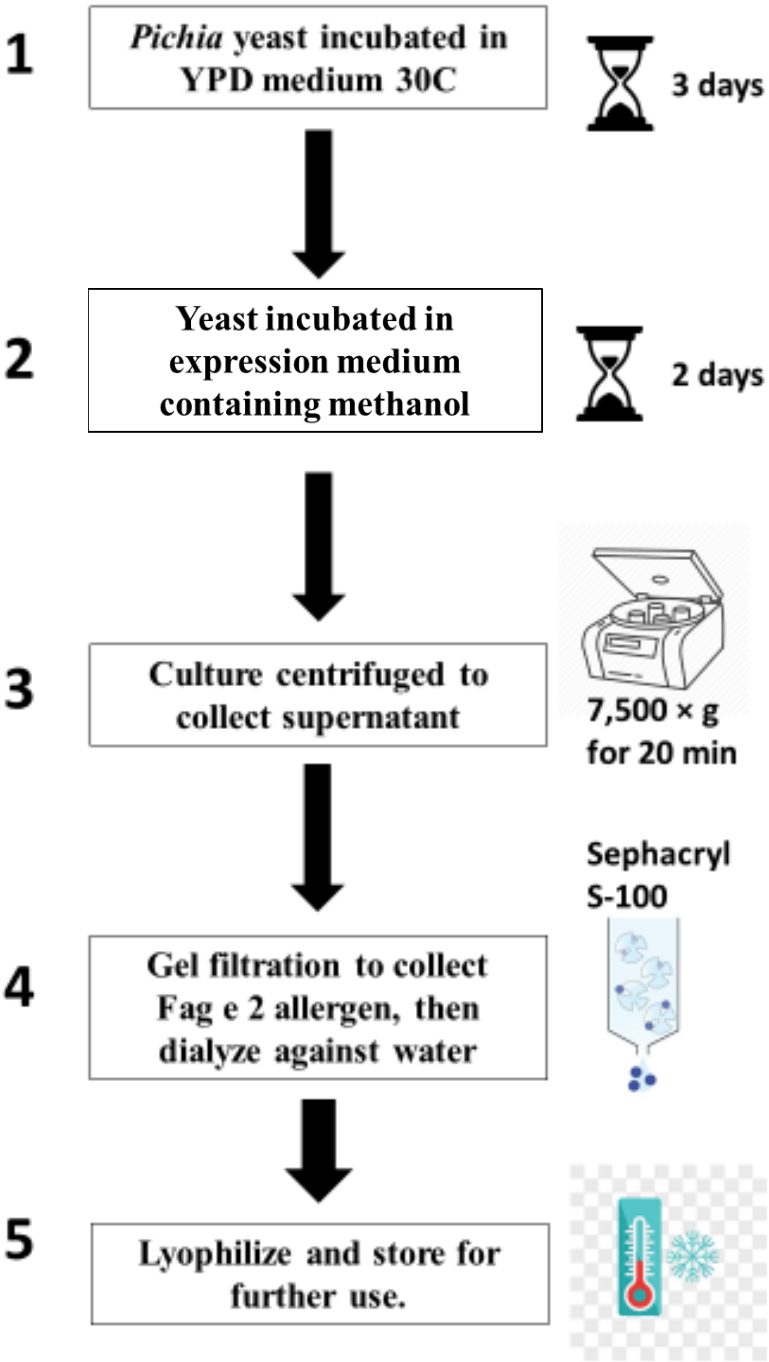


Figure 18. ***Pichia*** **expression system of Fag e 2**

3.2. In vitro digestion

In vitro digestion was performed according to a method described by Pinto et al., with slight modifications[82,83]. The freeze-dried powder of each sample was dissolved in distilled water, and the pH was adjusted to 2.5 using 0.1 M HCl. Porcine gastric mucosa pepsin (Wako, Tokyo, Japan) was then added to achieve a final activity of 50 or 500 units/mg of protein. Digestion was performed at 37 °C for 120 min and stopped by adjusting pH to 7.5 using 1 M sodium bicarbonate.

3.3. SDS-PAGE

SDS-PAGE was conducted according to a method published by Laemmli[84] using 15% (w/w) acrylamide separating gel, with a 5% (w/w) stacking gel containing 1% (w/v) SDS. Digested samples were diluted 2-fold with Tris-glycine buffer (pH 8.8) containing 1% SDS and 1% β-mercaptoethanol and heated at 95°C for 5 min before being loaded onto the gel. After electrophoresis, gels were stained for protein with 0.025% (w/v) Coomassie brilliant blue R-250 solution. For sugar stain, periodic acid/Schiff reagents were used prepared in the lab and for silver staining, EzStain Silver (ATTO, JAPAN) was used.

3.4. CD Spectra

CD measurements have been made in a (JASCO J-725, Japan) spectropolarimeter at 25 ± 0.1 °C. 0.2 mg/ml sample was used in 1 cm cuvette for the far and near-UV CD measurements. UV titration curves were recorded at 190 and 250 nm, respectively, as the greatest changes occurred at those wavelengths.

3.5. Sulfhydryl thiol groups

Free total sulfhydryl groups were measured using the DTNB method according to Ellman's procedure[85]. A 1:1 mixture of protein solution and Tris-glycine buffer (pH 8.0) containing 5% SDS were incubated at 40 °C for 30 min then, 50 µL solution of 4 mg DTNB in 1 mL of 100 mM Tris-glycine buffer pH 8.0 was added and the mixture was incubated for 20 min followed by reading absorbance at 412 nm. Using a molar extinction coefficient of 13600 M^{-1} cm^{-1} at 412 nm of 2-nitro5-mercaptobenzoic acid, total sulfhydryl groups was calculated as moles of sulfhydryl groups per mole of protein.

3.6. Murine model

BALB/c (female, 5 weeks of age) mice were purchased from Charles River, Ltd. (Tokyo, Japan). Mice were acclimatized for 1 week before the study and were provided with free access to water and a commercial diet (MF; Oriental Yeast Co., Tokyo, Japan). Environmental conditions were maintained at 25 ± 1ºC and 55 ± 10% humidity. The light-dark cycle was set to a 12-h interval. All experiments conformed to the guidelines for Animal Experimentation at Shinshu University (permission No. 270075).

Digested Fag e 2 (D-Fag e 2) and DP-Fag e 2 were prepared by digestion with pepsin (50 and 500 units/mg of protein, respectively) at 37°C for 120 min. Mice were divided into 4 treatment groups (6 mice per group) plus one naive group. The sham, Fag e 2-treated, D-Fag e 2-treated, and DP-Fag e 2-treated groups were all injected intraperitoneally with 50 µg Fag e 2 mixed with alum adjuvant at a total volume of 200 µL on days 0, 14, and 21. The naïve group was injected with an equal volume of PBS. On day 21, elevated specific IgE levels in serum were confirmed by ELISA. The naïve and sham groups were provided with commercial feed; other groups were provided with homemade feed containing 50 mg Fag e 2, D-Fag e 2, or DP-Fag e 2 per 100 g MF pellet for 6

weeks. On day 62, mice were orally challenged with 30 mg Fag e 2 (**Figure 19**). All mice were euthanized by CO_2 asphyxiation. Serum samples and spleens were collected for further analysis.

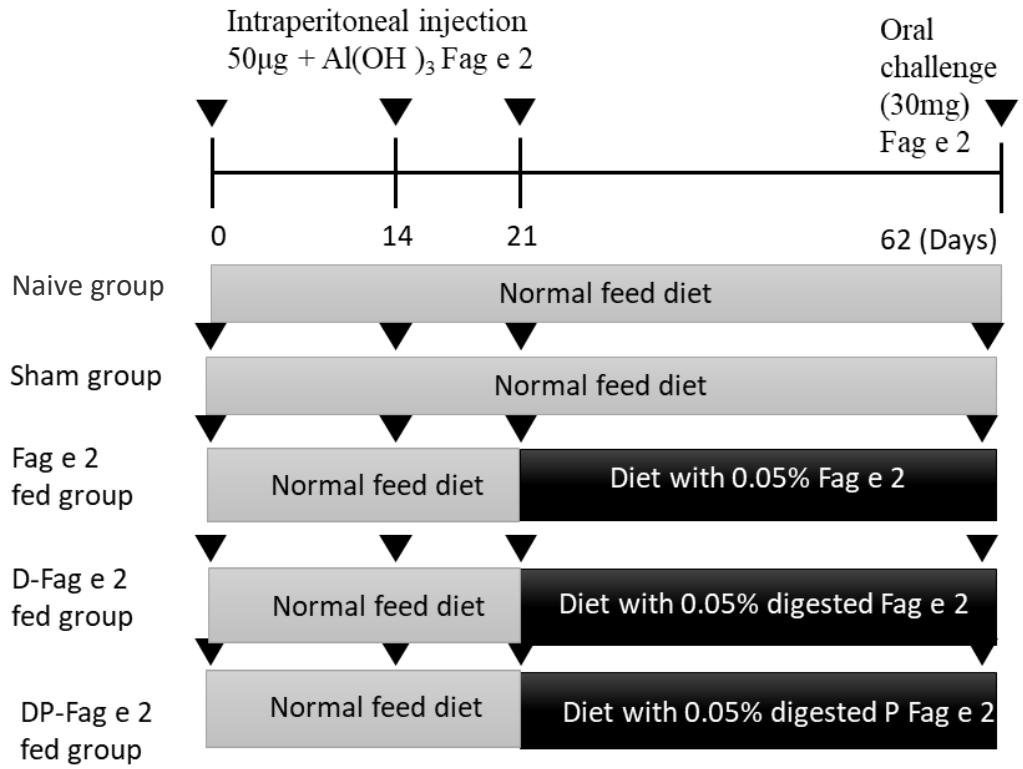


Figure 19. Scheme describing in vivo experimental design. Mice were divided into 5 groups (n = 6 mice/group): naive, no sensitization and normal diet; sham, Fag e 2 sensitization and normal diet; Fag e 2, Fag e 2 sensitization and diet containing 0.05% (w/w) Fag e 2; D-Fag e 2, Fag e 2 sensitization and diet containing 0.05% (w/w) digested Fag e 2 (D-Fag e 2); and DP-Fag e 2, Fag e 2 sensitization and diet containing 0.05% (w/w) digested P-Fag e 2 (DP-Fag e 2).

3.7. Hypersensitivity assessment

Allergic symptoms were measured at 40 min post-challenge and scored as follows: 0, no symptoms; 1, scratching and rubbing around the nose and head; 2, puffiness around the eyes and mouth, diarrhea, pilar ericti, reduced activity, and/or increased respiratory rate with reduced activity; 3, wheezing and labored respiration along with mouth and tail cyanosis; and no activity after prodding or tremor and convulsions[86].

3.8. Measurement of immunoglobulins in serum

The levels of total IgE and IgA in serum were measured *via* sandwich ELISA using HRP-labeled antibodies and hydrogen peroxide with *o*-phenylenediamine as the substrate. The anti-mouse IgE and anti-mouse IgA antibodies and HRP-conjugated anti-mouse IgE and IgA antibodies used in this experiment were purchased from Pierce (Rockford, IL, USA). For the determination of specific IgE, IgG_1, and IgA, the coating antigen was replaced with recombinant Fag e 2 (100 μg/mL).

3.9. Flow cytometry

Flow cytometry was used to assess changes in the number of regulatory T (Treg) cells. After DP-Fag e 2 was orally administered to Fag e 2-sensitized mice for 6 weeks, spleen cells were collected and stained with FITC-labeled anti-CD4 (1:1000, BD Biosciences, Tokyo, Japan) and PE-labeled anti-CD25 (1:100, BD Biosciences). For intracellular staining, the cells were washed, fixed, and permeabilized, and then Foxp3 was stained using Alexa-Flour 647-labeled anti-Foxp3 (1:100, BD Biosciences) monoclonal antibody with a mouse Foxp3 buffer set (BD Biosciences).

All stains and labels were obtained from BD Biosciences (Tokyo, Japan). Cells were analyzed using a BD Accuri C6 Plus Flow Cytometer (BD Biosciences) using CellQuest software.

3.10. Statistical analysis

Statistical analyses were performed using Statcel 4 software (OMS Publishing, Tokyo, Japan). Results are expressed as mean ± SEM. The data were also subjected to analysis of variance and Tukey-Kramer test. Differences between the groups were considered to be significant at $p < 0.05$.

4. Results

4.1. Purified recombinant Fag e 2 samples

The supernatant of *Pichia yeast* expression was further purified. **Figure 20A** shows that initially collected supernatant contains one smeared band above 45 kDa apparent on both PAS and silver stain and one band with around 16 kDa molecular weight representing Fag e 2 that only appears in the silver stain. Chromatography of Sephacryl S-100 column purification shows the collection of two fractions. The first fraction (F1) contains the glycoprotein, and a second fraction (F2) contains Fag e 2 (**Figure 20B**). Reflecting these fractions on SDS-PAGE stained with both PAS stain and silver stain reveals the successful separation of fractions **Figure 20C.**

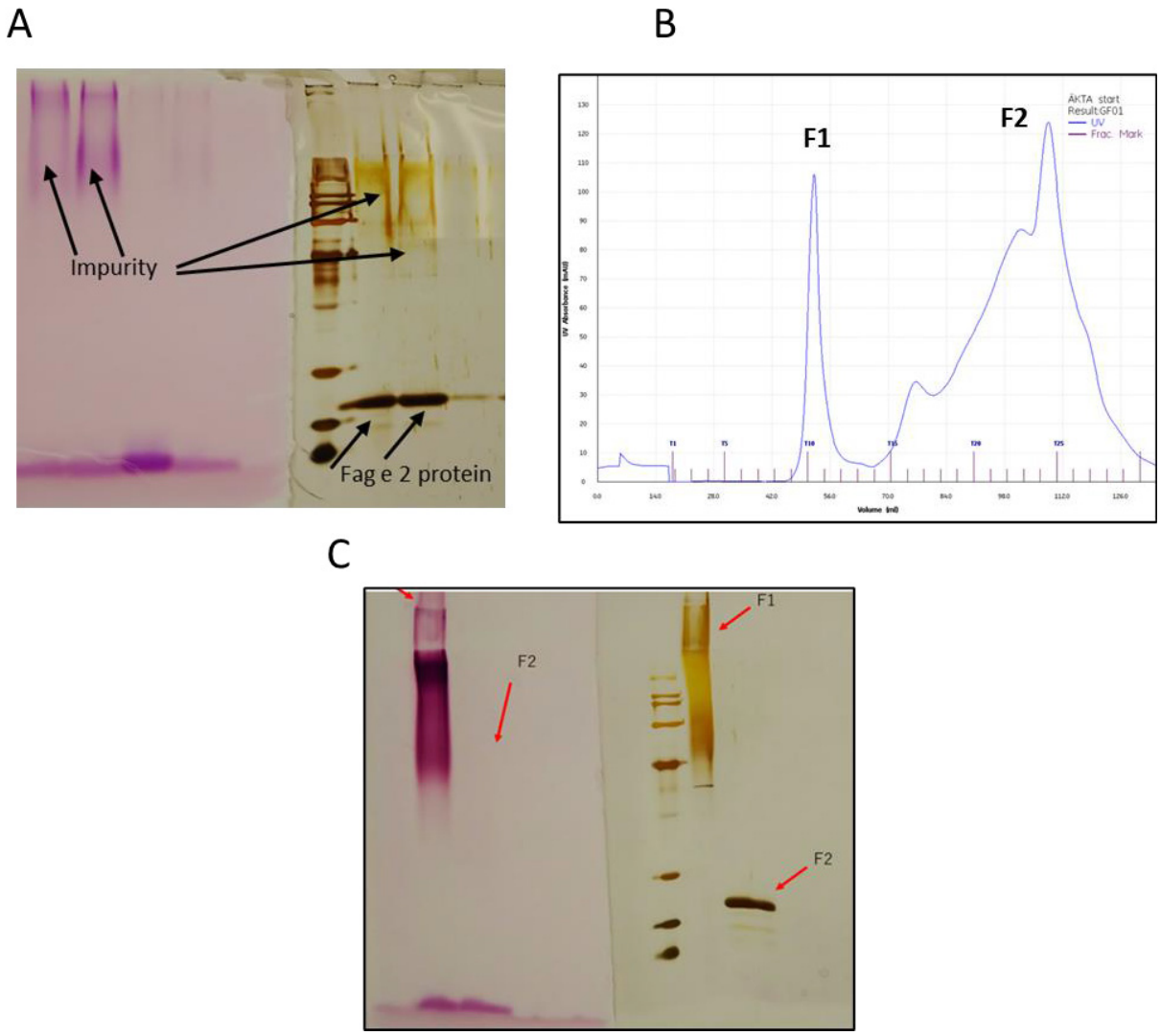


Figure 20. SDS-PAGE and chromatography for Fag e 2 purification. A) SDS-PAGE patterns of intact culture supernatant stained. PAS staining for sugar (left), and silver staining for protein (right) PAS staining for sugar (left), and silver staining for protein (right). B) Chromatogram for separation of proteins. C) SDS-PAGE patterns of Fraction 1 (F1) and Fraction 2 (F2) obtained from chromatography.

4.2. Fag e 2 and P-Fag e 2 responses to pepsin digestibility

This study first investigated changes in the peptic digestibility of Fag e 2 caused by phosphorylation. The phosphorus content of P-Fag e 2 was 0.42%, similar to that reported previously[72]. Fag e 2 or P-Fag e 2 were incubated with pepsin at time intervals of 0, 5, 15, 30, 60, and 120 min. As shown in **Figure 21A**, the Fag e 2 protein band was detected between the 14.3 kDa and 20.1 kDa markers, indicating that the sample was intact. No significant changes were observed in band intensity at time intervals of 5, 15, and 30 min, while decreased intensities were observed at longer time intervals of 60 and 120 min. On the contrary, the P-Fag e 2 bands disappeared, and smeared bands below 6.5 kDa appeared with increasing incubation time (**Figure 21B**). These findings suggest that the phosphorylation of Fag e 2 increases its digestibility. To further understand the peptic digestion pattern of Fag e 2, it was digested with pepsin of higher activity. The intact Fag e 2 band disappeared at 30 min and smeared bands below 6.5 kDa appeared at longer time intervals of 60 and 120 min, suggesting that Fag e 2 is digestible *via* treatment with high activity pepsin **(Figure 21C**).

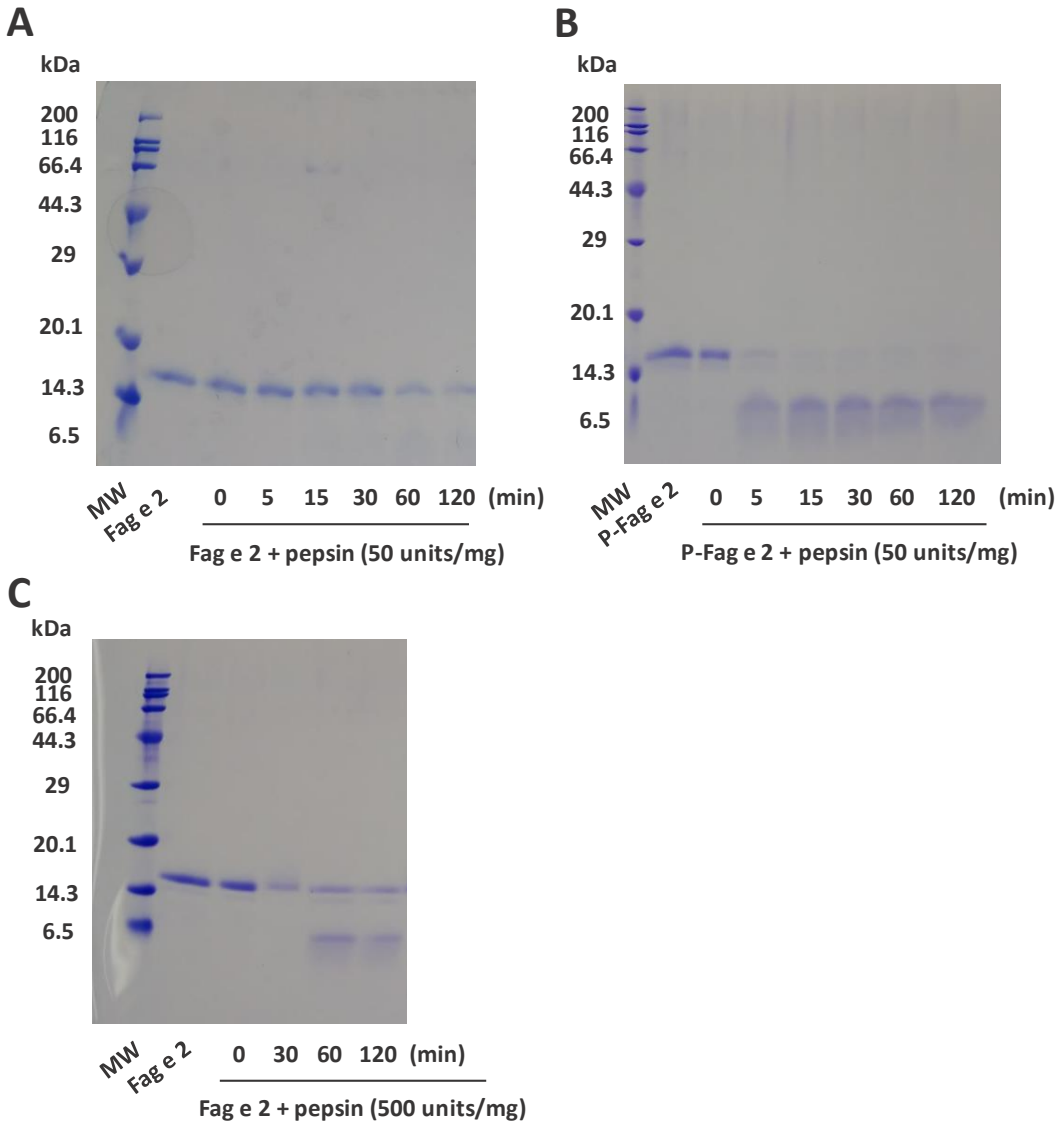


Figure 21. Pepsin digestion profiles of Fag e 2 and P-Fag e 2. The pepsin digestibility of Fag e 2 and P-Fag e 2 were determined. (A) Fag e 2 and (B) P-Fag e 2 were digested at 37 °C for 0, 5, 15, 30, 60, and 120 min. (C) Fag e 2 was digested at 37°C for 0, 30, 60, and 120 min. The obtained samples were subjected to 15% SDS-PAGE.

4.3. Effects of phosphorylation on Fag e 2 structure

To confirm conformational changes on P-Fag e 2, I studied changes in the CD spectrum and total sulfhydryl group. CD spectra of native Fag e 2 and P-Fag e 2 were presented with a broad negative minimum between 190-240 nm (**Figure 22**). Phosphorylation of Fag e 2 protein resulted in a distortion of its random coil structure, compared to intact and dry-heated Fag e 2 protein. This suggests a conformational change in the secondary structure of P-Fag e 2. Total sulfhydryl was measured using the DTNB method and revealed a significant difference between 33.75 and 43.35 mMol/mMol for Fag e 2 and P-Fag e 2, respectively (**Figure 23**). This suggested that more sulfhydryl groups were exposed due to conformational changes in the structure of Fag e 2 upon phosphorylation.

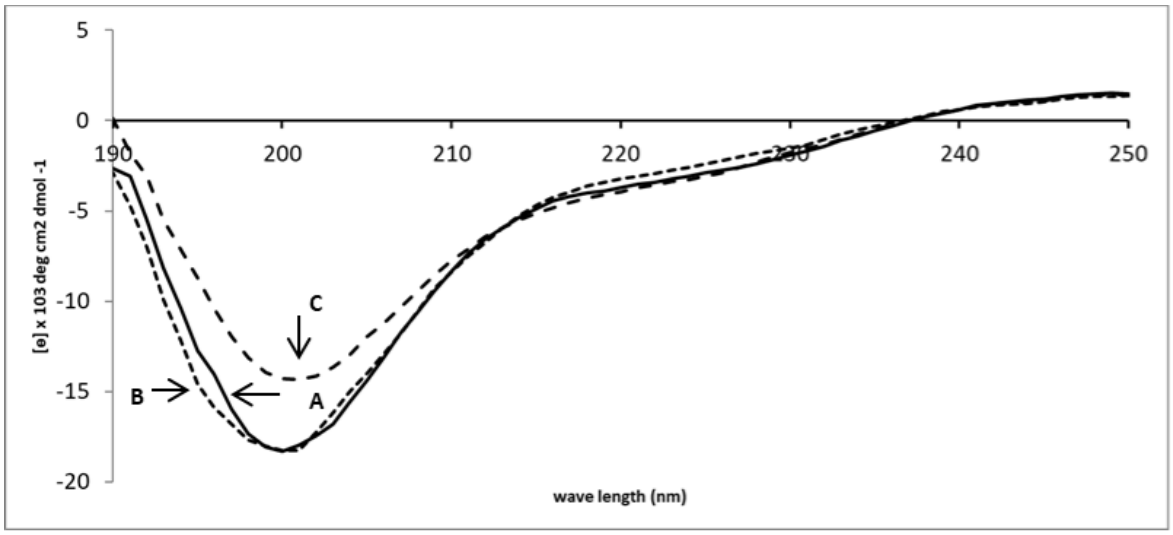


Figure 22. CD spectrum of Fag e 2, dry-heated Fag e 2, and P-Fag e 2. The CD spectrum of Fag e 2 (A), dry-heated Fag e 2 (B), and P-Fag e 2 (C) solution at a protein concentration of 0.2 mg/mL was recorded on the spectropolarimeter over the wave length of 190-250 nm.

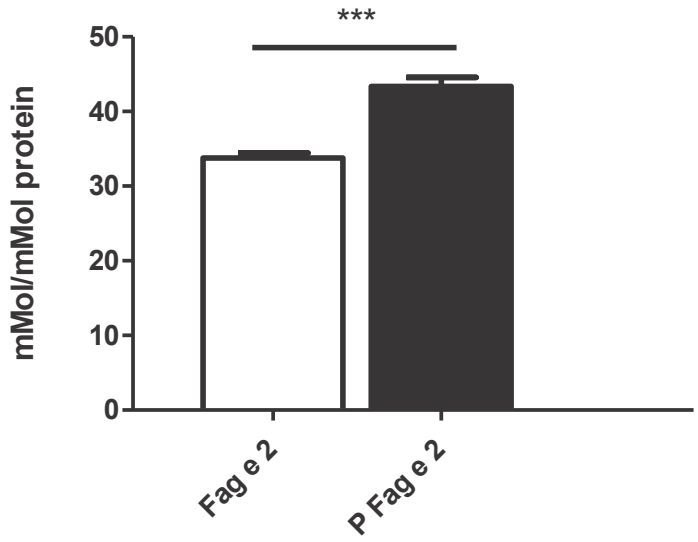


Figure 23. Total sulfhydryl groups of Fag e 2 and P-Fag e 2. Data are representative of three replicates and presented as the mean ± standard deviation. *$p < 0.05$ compared to Fag e 2.

4.4. Immunomodulatory effects of DP-Fag e 2 in a murine model

This study evaluated the effect of DP-Fag e 2 on the allergic response in the murine model of buckwheat allergy. Except for the non-sensitized naïve group, all mice were given the Fag e 2 antigen plus aluminum hydroxide, a known T-helper 2 (Th2) skewing adjuvant 3 times *via* intraperitoneal injection. After sensitization with Fag e 2, mice were fed a normal diet or a diet containing 0.05% (w/w) Fag e 2, D-Fag e 2, or DP-Fag e 2. Allergic symptom scores were measured 40 min after challenge with 30 mg Fag e 2.

Allergic symptom scores were evaluated 40 min after challenge with 30 mg Fag e 2. The sham group exhibited the highest value among all groups. On the contrary, a significant decrease in the allergic symptom score was observed in the DP-Fag e 2-fed group (**Figure 24**).

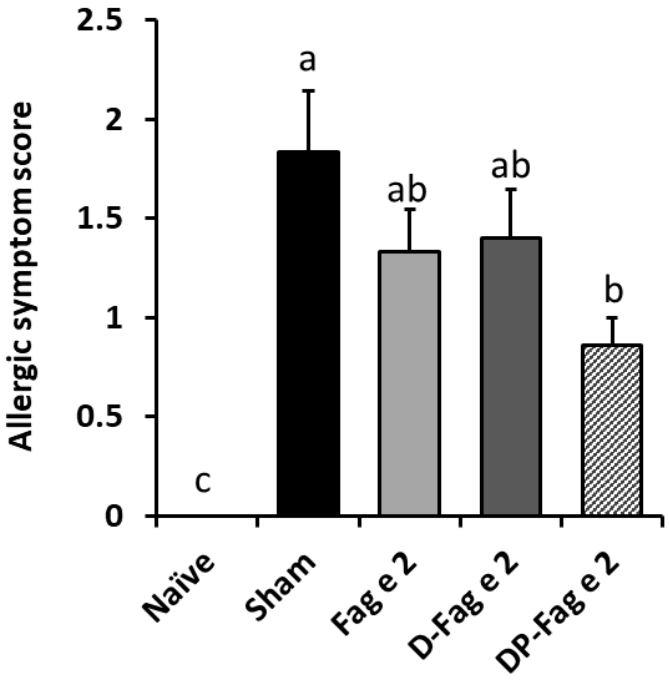


Figure 24. Allergic symptom scores for orally administered Fag e 2, D-Fag e 2, and DP-Fag e 2 in Fag e 2-sensitized mice. Allergic symptom scores were evaluated 40 min after challenge with 30 mg Fag e 2. Data are presented as the mean ± SEM of individual mice. Different letters above bars indicate a significant difference between groups ($p < 0.05$).

I then investigated changes in the serum immunoglobulin profile caused by the oral administration of DP-Fag e 2. The sham group exhibited a higher total IgE level compared to that of the naive group, and a significant reduction in the total IgE level was observed in the DP-Fag e 2 group (**Figure 25A**). D-Fag e 2 treatment also reduced the total IgE level, but the decrease was not significant ($P > 0.05$). Similar reductions were observed in the Fag e 2-specific IgE and IgG_1 levels (**Figures 25B and 25C**). Lower titers were observed for total IgA in both the sham and Fag e 2 groups compared to that in the naïve group; however, the total IgA levels of the D-Fag e 2 and DP-Fag e 2 groups were higher than that of the sham group (**Figure 25D**). The DP-Fag e 2 group exhibited the highest Fag e 2-specific IgA level among all groups (**Figure 25E**).

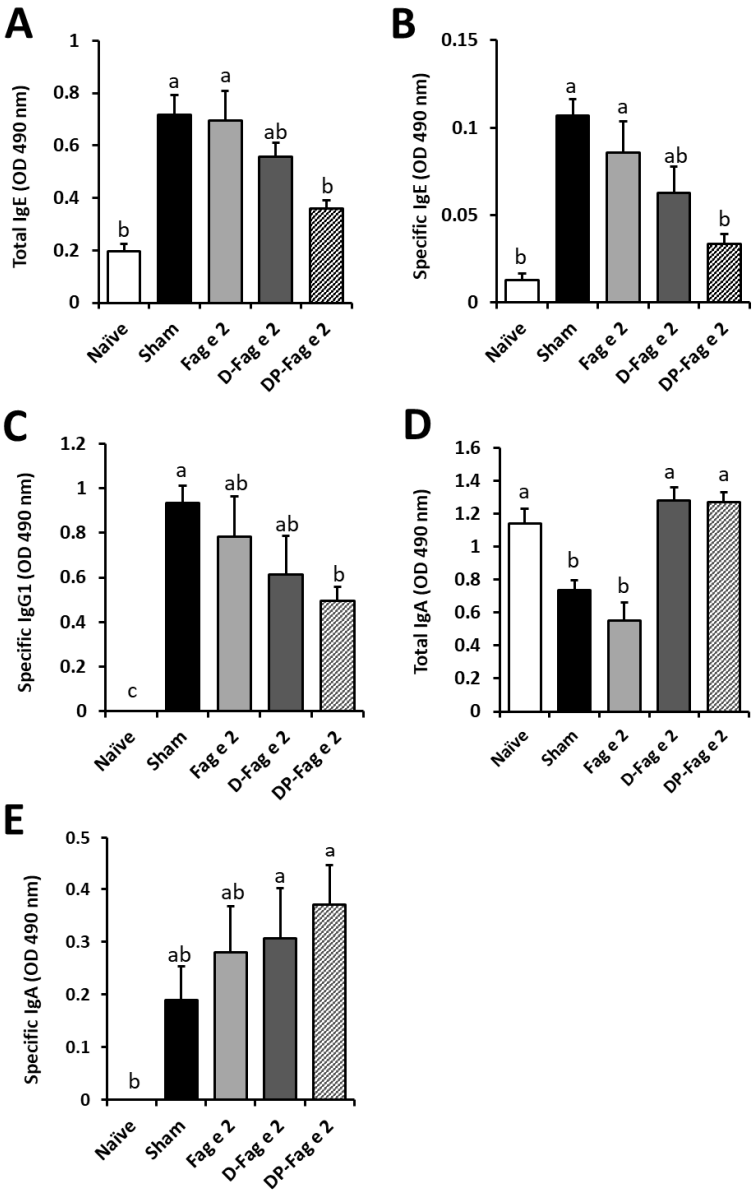


Figure 25. **Immune response effects of orally administered Fag e 2, D-Fag e 2, and DP-Fag e 2 on Fag e 2-sensitized mice.** A) Total IgE, B) Fag e 2-specific IgE, C) Fag e 2-specific IgG_1, D) total IgA, and E) Fag e 2-specific IgA in serum. Data are presented as the mean ± SEM of individual mice. Different letters above bars indicate a significant difference between groups ($p < 0.05$)

4.5. Measurement of the Treg population among splenocytes

I further studied the effects of orally administered DP-Fag e 2 on the differentiation of splenocytes into Tregs. Mouse splenocytes were stained for CD4, CD25, and Foxp3, and analyzed *via* flow cytometry. The population of Tregs in the sham group was lower than that in the naïve group and was similar to those in the Fag e 2 and D-Fag e 2 groups (**Figure 26**). In contrast, oral administration of DP-Fag e 2 increased the Treg population compared to that in the sham group; however, the increase was not statistically significant.

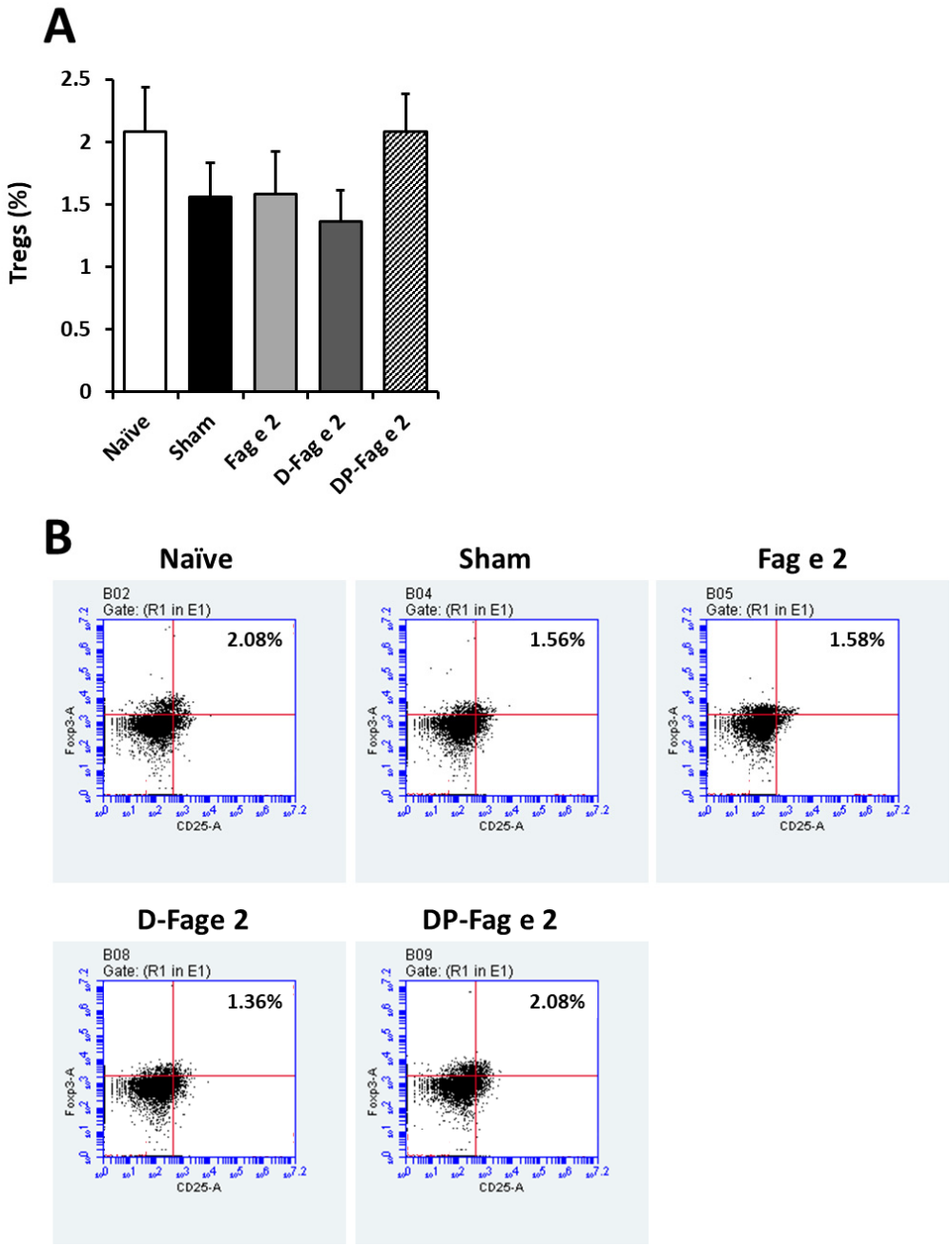


Figure 26. Effects of oral administration of DP-Fag e 2 on the population of regulatory T (Treg) cells among splenocytes of Fag e 2-sensitized mice. Splenocytes harvested from mice were analyzed for the Treg population by flow cytometry. A) Percentage of Tregs among the splenocytes collected from mice B) Dots represent cells stained with fluorescent antibodies. Data are presented as the mean ± SEM of individual mice.

5. Discussion

This study found that P-Fag e 2 was more susceptible to pepsin digestion than intact Fag e 2 (which is highly resistant to pepsin digestion)[87)]. The additional positive charge provided by phosphorylation could change the Fag e 2 protein conformation to expose more pepsin cleavage sites. As the structural properties of proteins in plant foods correlate with their digestibility[88]. In 2S albumin such as Fag e 2, secondary and tertiary structure have a compact structural scaffold containing five α-helices and a C-terminal loop folded in a right-handed superhelix being stabilized by four conserved disulfide bonds. The hypervariable region is exposed and short-chain connects two of α-helices, the most antigenic region of the 2S albumin[35]. The analysis of CD spectra shows a conformational change in the secondary structure of P-Fag e 2. It was previously demonstrated that the conformation of the protein might play a key role in resistance to proteolysis; During pepsin digestion, β-conformations is preferentially hydrolyzed and followed by the digestion of α-helix [89]. In this study, CD spectra results also suggest that conformational change occurred in P-Fag e 2, but not in Fag e 2. It was reported that the 2S protein structure is stabilized by four disulfide bonds [35]. Fag e 2 is a member of the 2S protein family and it contains four disulfide bonds located as (Cys30-Cys65), (Cys66-Cys111), (Cys16-Cys76), and (Cys78-Cys118). Mutational analysis of the recombinant Protein Fag e 2, results that 70% of cysteine residue mutants have weaker IgE binding of patient serum compared with wild type Fag e 2 and this is a decrease in resistance to pepsin digestibility [34]. Such change in sulfhydryl groups could indicate a change in disulfide bonds and explain the decrease in pepsin digestibility observed in this current study, and also explains the weaker immunogenicity reported previously [72]. Measurement of the total sulfhydryl using the DTNB method showed that sulfhydryl groups were exposed due to conformational changes in the structure of Fag e 2 upon phosphorylation, suggesting an alteration of disulfide bonds. It has been

reported that a buried, rigid disulfide maintains the conformation of β-lactoglobulin, and controlling specific disulfide bonds in protein can lower the allergenicity of milk[90].

In this study, a higher concentration of pepsin managed to digest intact Fag e 2, and in another research Tanaka et al. reported that Fag e 2 was not digested after 12 min hydrolysis, and other studies reported hydrolysis of buckwheat allergens at pH 2 with Fag e 2 being digested in 10 min[32,87]. The results of this study agree with these variations, as in vitro assay digestibility is influenced by the conditions of the assay[32]. Because of this variation in the digestibility of Fag e 2, this study used the same conditions for pepsin digestion when compared the digestibility of Fag e 2 and P-Fag e 2.

I then investigated the immunomodulatory effects of DP-Fag e 2 in Fag e 2-sensitized mice. Since desensitization treatments typically use intact allergens, I compared the effects of DP-Fag e 2 to those of the intact and digested forms of Fag e 2. Allergic symptom scores corresponded to immunoglobulin profiles in DP-Fag e 2-fed mice, which displayed the lowest post-oral challenge clinical signs; these results correlate with the reduced levels of total and specific IgE in these mice since IgE is the key player in food allergy and increased IgE levels could lead to increased allergic symptoms[21]. IgG_1 levels significantly decreased in the DP-Fag e 2-fed group; this could indicate a T helper1(Th1) type immune response involving the secretion of IFN-γ cytokines, which inhibit the secretion of both IgE and IgG_1[91]. On the contrary, total and specific IgA levels increased in the DP-Fag e 2-fed mice. Yel et al. reported that IgA-deficient patients are more susceptible to allergic disorders, and the study by Fageras et al. indicated that sensitized children with high salivary IgA levels are less likely to develop allergic symptoms[92,93]. In my previous work, oral administration of P-Fag e 1 significantly increased the serum IgA level compared to that observed in the sham group. Similar results were obtained upon P-Fag e 2 administration[72,79]. In general, it

was reported that the antibody response to small antigens is weaker when compared to the larger one[94]. Smaller peptides could pose less allergenic binding activities to the antibody. This indicates that the phosphorylation of allergen proteins might enhance IgA production; the enzymatic digests of allergen proteins exhibit the same activity.

Tregs play a critical role in allergen-specific immune therapy, and the immune response of effector T cells, such as Th1, Th2, and Th17, is suppressed by Tregs[95,96]. The DP-Fag e 2 group exhibited a higher percentage of Tregs in the spleen compared to the sham, Fag e 2, and D-Fag e 2 groups, suggesting that DP-Fag e 2 treatment might increase the Treg population and induce immunotolerance by inducing the production of immunosuppressive cytokines, such as IL-10 and TGF-β[97,98]. Antigen-carrying dendritic cells activate T and B cells, depending on the carried antigen, to either elevate or downregulate the immune response *via* the formation of Tregs[99]. Digested proteins, such as partially hydrolyzed whey protein, have immunosuppressive functions, such as increasing IgA levels and activating Treg differentiation in spleen and Peyer's patches[100]. Through a similar mechanism, DP-Fag e 2 could generate Tregs from naïve $CD4^+$ T cells in the presence of T-cell receptor stimulation, TGF-β, and retinoic acid produced by $CD103^+$ dendritic cells at the intestinal mucosa[101]. In previous work, results indicated that P-Fag e 2 was recognized by dendritic cells and induced IL-6 production, which led to Tfh induction. Further investigation is required to reveal the mechanism by which dendritic cells recognize P-Fag e 2 digests[10].

These results suggest that Fag e 2 phosphorylation could increase its peptic digestibility. Since protein susceptibility to enzymatic degradation is an important step in assessing its allergenicity, a correlation exists between resistance to digestion by pepsin and allergenic potential[102]. The hydrolysate of P-Fag e 2 reduced IgE-mediated allergic reactions in Fag e 2-sensitized mice. Further studies are needed to elucidate phosphorylation-induced conformational

changes in Fag e 2, as well as the mechanism underlying the immune hypoallergic response to DP-Fag e 2 allergens. The findings discussed in this paper will contribute to the development of hypoallergenic agents for buckwheat-specific immune therapy.

Chapter IV

Conclusions

This study focused on the major allergens for buckwheat allergy Fag e 1, and Fag e 2. The *Pichia* expression system was a useful tool to get Fag e 1 samples for further studies on allergenicity, as it is expected that the yeast synthesized protein show similar structure and function of the authentic Fag e 1 allergen, especially that similar approach was applied previously for Fag e 2 allergen expression.

Fag e 1, as a major buckwheat allergen, was found to be phosphorylated, and the formed P-Fag e 1 exhibited hypoallergic behavior. Using mice model as OIT, Fag e 1 shown to lower allergic responses such as decrease total and specific IgE, decrease histamine release and could affect the production of pro-inflammatory cytokines, increase both IgA and Tfh cells. The mechanism of P-Fag e 1 hypoallergic response could be related to the masking of epitope site. Some allergic epitopes of Fag e 1 are known of having serine and threonine, these amino acids are candidate phosphorylation sites, thus it could alter the epitope binding site. More studies should be done on the structure of Fag e 1, and P-Fag e 1.

Fag e 2 was previously successfully expressed in the *Pichia* expression system and obtained good phosphorylation hypoallergic behavior. Since Fag e 2 is known to be resistant to peptic digestion, this study hypotheses that the process of phosphorylation altered the conformational structure of Fag e 2, making it more susceptible to digestion, and expected that D-Fag e 2 peptides will have hypoallergic activities on Fag e 2-sensitized murine model. P-Fag e 2 had more digestibility in the pepsin environment than Fag e 2. This study also noted that when using high pepsin concentration to digest Fag e 2, the pattern of digestion on SDS-PAGE was different between Fag e 2 and DP-Fag e 2. Next, I found that CD spectrum data confirms changes in

structure. Since Fag e 2 is stabilized by four disulfide bonds, increased sulfhydryl groups indicate alteration of disulfide bonds. For the hypo-allergenicity of DP-Fag e 2, results show decreased total and specific IgE, and increased IgA and Treg, with a reduced the clinical signs. All these results confirm that DP-Fag e 2 is a good hypoallergic agent for Fag e 2-sensitized allergenicity.

Phosphorylation of Fag e 1 and Fag e 1 reduced allergenicity, possibly by epitope masking, or conformational changes that lead to change in digestibility. With the increasing levels of food allergic people, it is no longer enough to treat symptoms, and with such specific immune therapy comes unwanted side-effects that could be demolished if a hypo allergens such as P-Fag e 1 and P-Fag e 2 could be developed. I aim to focus more on understanding the mechanisms that initiate such immune responses.

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