Doctoral Dissertation

(Shinshu University)

Improvement of anti-viral and anti-allergenic activities of milk casein phosphopeptide by chemical modifications

化学修飾による牛乳カゼインホスホペプチドの抗ウィルス性及び抗アレルギー性の改善

September 2019

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ABBREVIATIONS

- **ACE** – angiotensin-converting enzyme
- **APC** – antigen-presenting cells
- **CPP III** – casein phosphopeptide III
- **CRFK** – crandell reese feline kidney
- **DC's** – dendritic cells
- **D-CPP III** – dephosphorylated casein phosphopeptide III
- **DMSO** – dimethyl sulfoxide
- **DW** – distilled water
- **ELISA** – enzyme-linked immunosorbent assay
- **FCV** – feline calicivirus
- **FOSHU** – food for specified health use
- **FoxP3** – forkhead box P3
- **gDNA** – genomic deoxyribonucleic acid
- **GUSB** – β-glucuronidase
- **HSA** – human serum albumin
- **HuNov** – human norovirus
- **IFN** – interferon
- **IFN-γ** – interferon-gamma
- **IgA** – immunoglobulin A
- **IgE** – Immunoglobulin E
- **IgG** – immunoglobulin G
- **IgG2a** – immunoglobulin E
- **IgM** – immunoglobulin M
- **IL** – interleukin
- **IL-4** – interleukin 4
- **IL-6** – interleukin 6
- **ISG** – interferon stimulatory gene
- **JAK** – janus kinase
- **MWCO** – molecular weight cut-off
- **N-CPP III** – native casein phosphopeptide III
- **P-CPP III** – phosphorylated casein phosphopeptide III
- **PepT 1** – peptide transporter 1
- **PP** – payer's patch
- **qPCR** – quantitative polymerase chain reaction

- **SerP** – phosphorylated serine
- **SP** – spleen
- **STAT** – signal transducer and activator of transcription
- **Tfh** – T follicular helper
- **Th1** – T helper type 1
- **Th2** – T helper type 2
- **TLRs** – toll-like receptors
- **TNF-α** – tumor necrosis factor-alpha
- **Treg** – regulatory T
- **α-La** – α-lactoglobulin
- **β-La** – β-lactoglobulin

Summary

The effects of anti-viral and anti-allergenic activities of milk casein phosphopeptides on phosphorylation and dephosphorylation were investigated in this doctoral dissertation.

Chapter 1 describes the introduction, background literature, and objectives. Studies on functional foods in Japan has been fast-tracked and already given birth to Food for Specified Health Uses (FOSHU). Among the FOSHU, Casein phosphopeptide (CPP) has been reported to be one of the potent bioactive agents from milk. CPP is a phosphoserine-rich enzymatic hydrolysis having the basic structure of three phosphoseryl residues followed by two glutamic acid, SerP-SerP-SerP-Glu-Glu. Since the bioactivity of CPP has been related to negative electrostatic charge of phosphate groups attached to its amino acid chain, we hypothesized that modification of the peptide by addition of extra phosphates through phosphorylation can enhance its antiviral and antiallergic activities.

In Chapter 2, the effects of additional phosphorylation and dephosphorylation on antiviral activity of the milk casein-derived peptides rich in phosphate groups called casein phosphopeptide III (CPP III) in vitro. Feline calicivirus (FCV) strain F9, a typical norovirus surrogate, and Crandell Rees feline kidney (CRFK) cells were used as the target virus and host cells, respectively. Higher cell viability was observed in the host cells treated with phosphorylated CPP-III (P-CPP). The expression of anti-viral cytokines such as IFN-α and IFN-β was significantly induced by the treatment with P-CPP, compared to that with native CPP-III (N-CPP). In contrast, dephosphorylation of CPP-III resulted in a decrease in the anti-FCV effect. These results suggest that the anti-viral activity of CPP was enhanced by the introduction of additional phosphates and conversely weakened by

their elimination.

Chapter 3 deals with the anti-allergenic effects of P-CPP and dephosphorylated CPP III (D-CPP) in OVA-sensitized mice. Female BALB/c mice were intraperitoneally sensitized with OVA twice at intervals of 14 days and then orally fed N-CPP, P-CPP, and D-CPP for 6 weeks. Next, the mice were orally challenged with 50 mg of OVA. Oral administration of P-CPP significantly suppressed total and OVA-specific IgE levels in the serum. The treatment with P-CPP exhibited low levels of OVA-specific IgG1 and increased OVA-specific IgG2a. The treatment with P-CPP also suppressed IL-4 production, while D-CPP showed similar a level compared to that of the control. N-CPP and P-CPP increased the regulatory T cells population in spleen compared with D-CPP. Furthermore, the population of the T follicular helper cells in the spleen was increased by P-CPP treatment. These results suggest that additional phosphorylation of CPP can enhance the attenuation of OVA-specific IgE-modulated allergic reactions in OVA-sensitized mice.

Finally, Chapter 4 provides a summary and conclusion of the studies. In this study, it was demonstrated that P-CPP showed anti-viral activity against FCV infection and anti-allergenic activity in OVA-sensitized mice, and these activities were dependent on the phosphate groups. These findings suggest that highly phosphorylated CPP III holds potential as an accessible and cheaper alternative to the food-based nutraceutical ingredients and supplements used to boost the immune system against viral infections and to attenuate the allergenic response. This information will contribute to the development of safe and effective anti-viral and anti-allergenic agents using natural dietary compounds in conjunction with modification techniques to enhance their functionality.

Chapter I

General Introductions

1. Background and Perspectives

Bovine milk, which has a long tradition in human nutrition [1] and also a common and abundant food worldwide, is reported to be an important source of bioactive peptides within its protein fraction [2–5]. If manipulated well milk peptides have the potential of becoming an accessible and cheaper alternative of food-based nutraceutical ingredients and supplements to help boost the immune system against viral infections [6]. Milk makes an important contribution to human nutrition and health due to the enrichment in functional proteins. Encrypted within the milk protein CPPs are a unique kind of peptides yielded after tryptic hydrolysis of calcium-sensitive caseins that are rich in clusters of phosphorylated seryl residues [7]. They are reported to have multiple bioactive functionalities including improvement of mineral absorption mainly calcium, immunomodulatory, antioxidative, antithrombotic, ACE inhibitory activities, enhancement of the intestinal IgA and proliferation of interleukin-6 (IL-6) [8] [9] [7]. Besides, caseins and their fragments have previously reported functioning as antiviral and immune regulatory factors by regulating innate immune response through both up-regulation and down-regulation.

CPPs are phosphorylated casein derived peptides [9] that can be released via in vitro or in vivo enzymatic digestion of α_{s1}, α_{s2} and β-casein [2]. They mostly have a

common structural motif; a sequence of three phosphoseryl followed by two glutamic acid residues [10] [11]. The bioactivities they exhibit are mainly attributable to the presence of phosphate groups attached to some sites on its amino acid chain through phosphorylation. Phosphorylation is a covalent modification that adds a phosphate group to a protein or organic molecule; it emerged as one of the most prominent types of post-translational modification during evolution [12]. It occurs in both prokaryotic and eukaryotic organisms triggering conformational changes in many enzymes and receptors causing their activation and deactivation.

There is currently an increase in viral diseases and allergic reactions caused by different edible proteins. Both allergy and viral infections have now become a global pandemic. With all these ever-increasing health problems, the development of novel food-based bioactive compounds that are safe and effective in controlling the development of allergic reactions, viral adulteration of foods and human infections will be of interest to functional food research scientists and the food industry as a whole. In this regard increasing attention is being focused on physiologically active peptides derived from milk proteins [13,14].

2. Literature review

2.1 Casein phosphopeptide (CPP III)

Casein phosphopeptide (CPP) is derived from milk casein, which is a major constituent of milk protein [15], by tryptic digestion [9]. It is reported to be around 2.8-3.5 kDa [16] and consists of major casein phosphopeptides such as bovine αs_2-casein (1–32) and bovine β-casein (1–28) [17] [18]. Findings from other authors showed that major casein phosphopeptides also correspond to the reported molecular weight range with 2.6 kDa for α_{s2}-casein (2-21)-4P [19], 2.7kDa for α_{s1}-casein (59-79)-5P [19],[20], and 3.125kDa for β-casein (1-25)-4P [21].

A lot of research has been conducted on the enhancement of calcium absorption by CPPs via their phosphoserine rich region. It has also been reported to enhance intestinal IgA levels in mice [17], stimulates the release of IL-6 cytokine in the human epithelial intestinal cell line [16]. Most peptides derived from casein have some bioactivities. The schematic presentation in **Figure 1** shows the main bioactivity of peptides formed by the enzyme digestion of milk proteins [22].

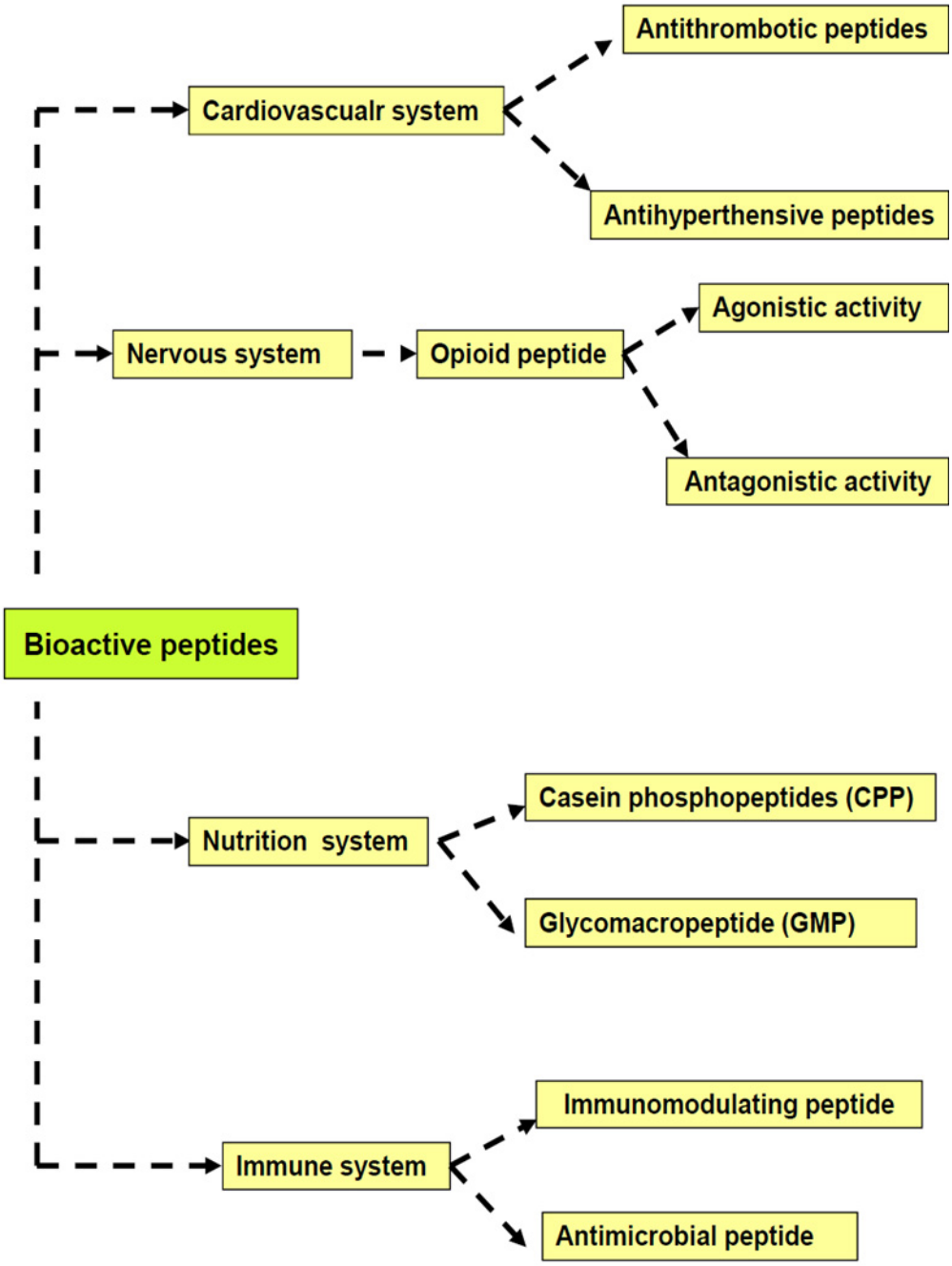


Figure 1. Schematic representation showing the main bioactive peptides formed by enzymatic digestion of milk proteins. Adapted from [22].

2.2 Phosphorylation

This is one of the posttranslational modifications of proteins that can regulate milk protein structure and function [23]. It is the addition of a phosphoryl group (HPO_3) to a molecule. It has been known since the 19^{th} century that phosphates could be bound to proteins through phosphorylation to form phosphoproteins [24]. Recently there are some suggested roles of phosphate groups in physiological and immune functions. For instance, the functional properties of bovine serum albumin including heat stability and calcium phosphate solubilizing ability were improved by attachment of phosphate groups through phosphorylation [25]. Additionally, the reduction of a major allergen in whey protein isolate β-Lactoglobulin's (β-La) immunogenicity by phosphorylation was demonstrated [26]. The phosphoproteins were first discovered in milk caseins and egg yolk phosvitin [27]. This modification method has proven to be a useful tool for improving the functional properties of food proteins.

Recently some researchers have reported several phosphorylation techniques. For example, Aoki *et al*. reported the phosphorylation of OVA and β-La by conjugation of glucose-6-phosphate through the Maillard reaction [28][29]. It was also reported by Tarelli *et al*. that saccharides and proteins with hydroxyl groups can be phosphorylated by dry heating in the presence of a phosphate buffer [30]. Improvement of water solubility,

emulsifying activity, foaming properties, and gel-forming properties of food proteins by phosphorylation has been observed [31].

2.3 Dephosphorylation

This is the opposite of phosphorylation; it is the process of removing phosphate groups from a molecule. Their role has been investigated in the regulation of enzymes [32]. Dephosphorylation has also been reported to reduce the coagulation and gel-forming ability of β-casein leading to reduced net negative charge [33]. Two methods, which are alkaline (chemical dephosphorylation) [34] and enzymatic dephosphorylation, have been studied. Chemical dephosphorylation occurs because of alkaline hydrolysis, releasing phosphate and leaving behind serine residue. However, the enzymatic method is more preferred by food processors than the chemical method [35]. This is probably because as compared to the chemical method the use of enzyme releases the phosphate preserving the serine residue on protein. Also, since enzymes are specific and it is possible to control their reactions, their use in modifying food proteins is deemed more systematic.

2.4 Norovirus

Norovirus is among the enteric viruses that fall in a group of non-enveloped, single-stranded RNA viruses with an icosahedral symmetry classified into the genus

Norovirus of the family *caliciviridae*. It is extremely contagious and humans are the only known reservoir for human norovirus. Transmission occurs via fecal-oral and vomit-oral pathways by four general routes: (i) direct person-to-person, (ii) foodborne, (iii) waterborne and (iv) through environmental fomites; since humans are the only known source of all human infections, all transmission is ultimately person-to-person. Its illness is contracted through contaminated food and water and direct person-to-person transmission [36]. Viral agents are estimated to cause between 30% and 40% of foodborne illnesses [37]. Their ability to persist in the environment and foods, coupled with low infectious doses allows even a small amount of contamination to cause serious problems [38]. Norovirus infections are the most frequent cause of non-bacterial gastroenteritis across all ages in the world. About 70% of viral gastroenteritis cases are believed to be caused by norovirus according to recent epidemiologic studies [39],[40]. The lack of a robust cell culture system for human norovirus [41], and the absence of an animal model are fundamental technological barrier hampering many areas of research [42] and the inability to differentiate infections from non-infectious virus particles [43] has hindered the studies of norovirus leading to use of surrogate viruses like feline calicivirus (FCV) [44]. The structure and Geno-group classification of norovirus is as depicted in **Figure 2 and figure 3** respectively.

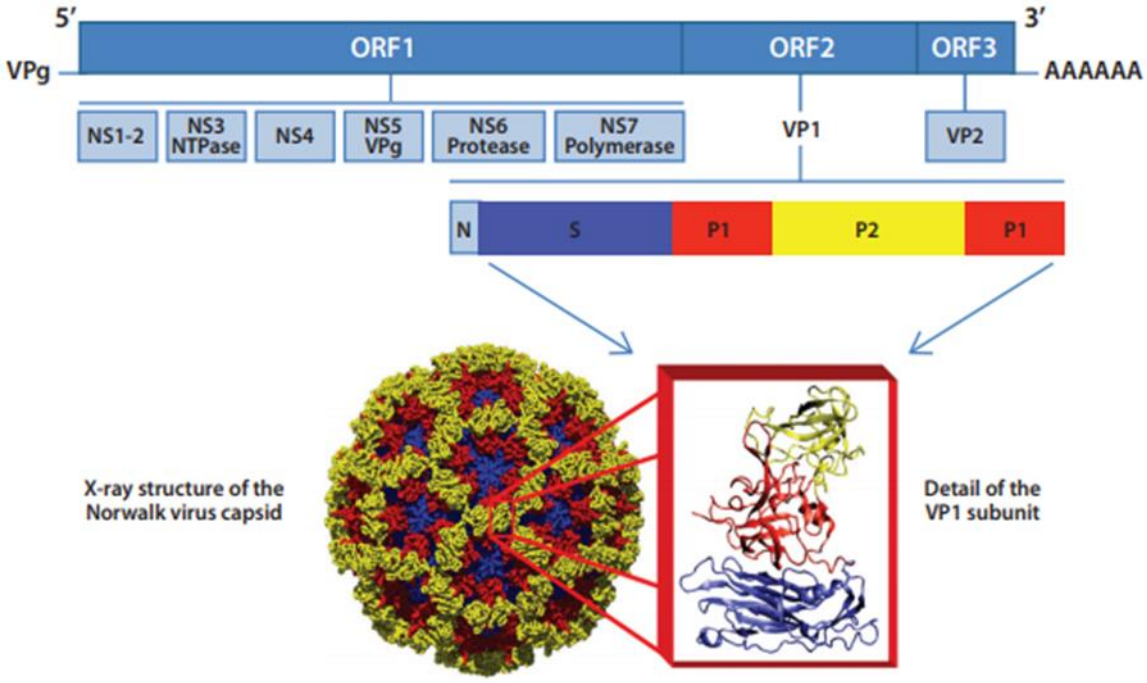


Figure 2. Human norovirus genome organization and virus-encoded nonstructural and structural proteins Adapted from Global burden report [45].

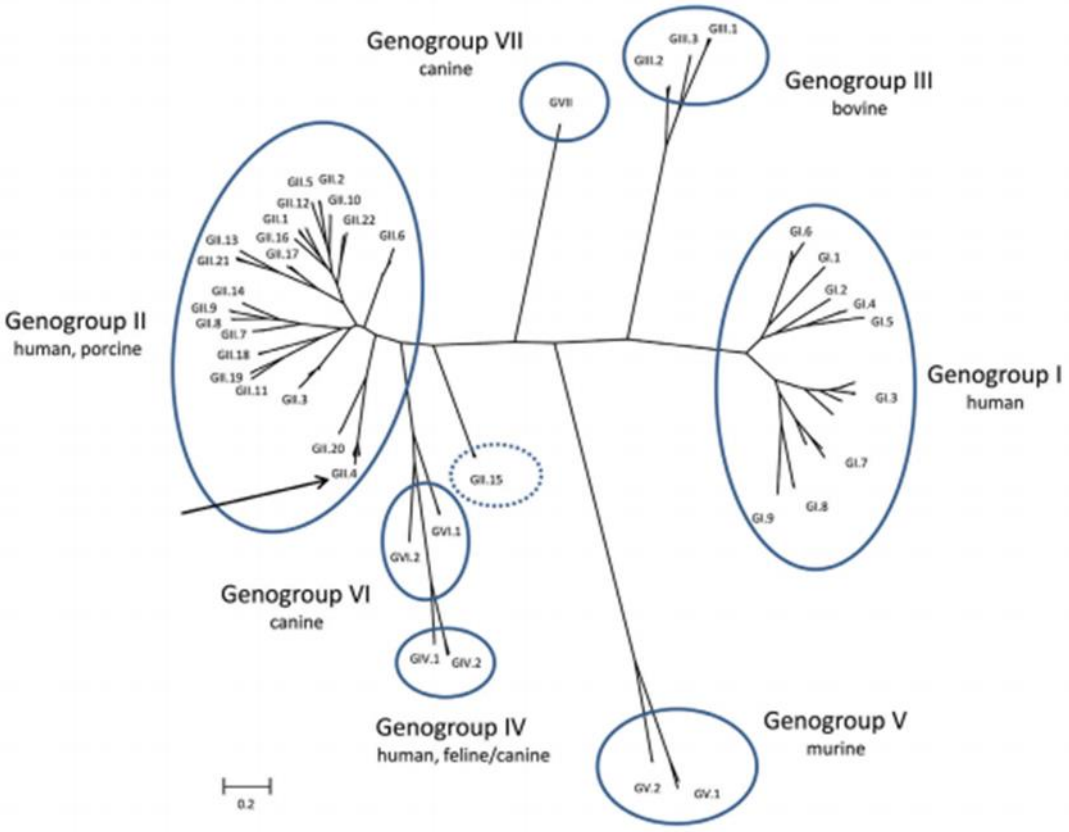


Figure 3. Classification of noroviruses into seven Geno groups (GI to GVII) based on amino acid sequence diversity in the complete VP1 capsid protein. Adapted from [46].

2.5 Feline Calicivirus (FCV)

FCV is reported to be sharing several biochemical properties, similar genomic organization, physicochemical characteristics and primary sequences with norovirus [47]. They are grouped in the same family of *Caliciviridae* [48]. However, unlike norovirus FCV can be cultured in tissue culture [49], can be grown in established cell lines [50], and produce a syncytial form of cytopathic effect [48]. Because of these similarities, it has been used in many types of research as a surrogate for norovirus.

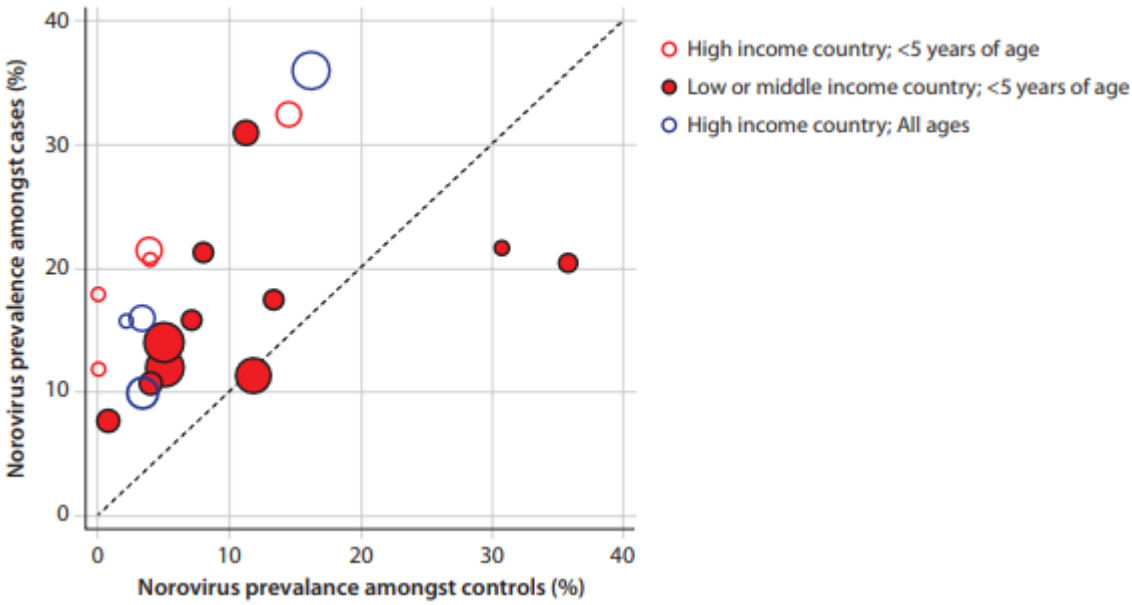
2.6 Crandell Reese Feline Kidney (CRFK)

CRFK is a cat cell line. It is derived from the kidney tissue of a normal domestic cat (*Felis catus*). The cell line was initiated by Crandell *et.al.* in June 1964 at the United States Air Force Base Epidemiological laboratory, Lackland, Texas [51]. It is reported to be epithelioid and having a uniform rate of proliferation [52]. They have been used extensively for viral infectivity assays and study of the biology of various retroviruses and derived vectors [53].

2.7 Norovirus statistics in the world

Infection by norovirus can suddenly trigger the onset of severe vomiting and diarrhea. Signs and symptoms include nausea, abdominal pain/cramps, watery/loose

diarrhea, Low-grade fever, and muscle pains. The virus is extremely contagious and commonly spread through contaminated food and water. Closed and crowded areas like cruise ships, nursing homes, hospitals, schools and military barracks affected by norovirus more frequently. This virus according to the Center for Disease Control and Prevention's global burden report (2015) is reported to cause 685 million cases of acute gastroenteritis worldwide and is estimated to cost 60 billion USD worldwide in health care costs and lost productivity [45]. Previous observations proved that the impact of Norovirus is more prevalent in low-income countries as compared to high-income countries. **Figure 4** alludes to that.

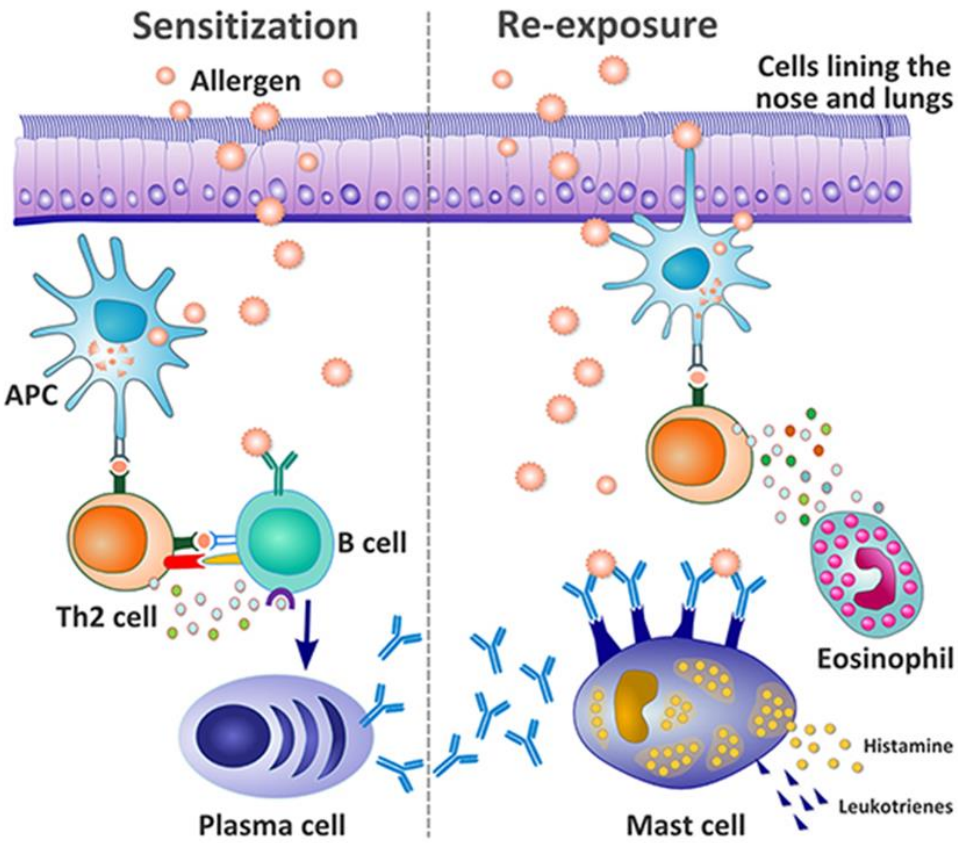


Based on a literature review of published studies, 1998 to 2013. * Size of the circle proportional to a number of subjects in the study.

Figure 4. Norovirus prevalence amongst diarrhea/gastroenteritis cases and healthy controls. Adapted from [54]**.**

2.8 Antigen-specific IgE in pathogenesis of type-1 allergic reactions

In allergy-prone people, initial encounters with an allergy-triggering substance, or allergen, prompt changes in the immune system that eventually may lead to allergy symptoms. This stage is called sensitization. When an allergy-prone person inhales pollen, for example, immune cells in the lining of the nose or lungs engulf the pollen allergen and process it into small fragments. These cells, called antigen-presenting cells (APCs), display the allergen fragments on their surfaces. During sensitization, APCs pick up the allergen and presents part of it to a Th2 cell, which helps a B cell become a plasma cell. Plasma cells produce allergen-specific antibodies called IgE, which binds to mast cells. When allergen returns, mast cells release histamine and other chemicals. In addition, Th2 cells release many different chemicals that attract inflammatory cells such as eosinophils. This result in allergy symptoms such as sneezing, mucus production, swelling, itching, runny nose, coughing, re-exposure to an allergen also causes mast cells and Th2 cells to release chemical messengers that attract and activate other inflammatory cells, such as eosinophils and basophils, which release more chemicals and cause allergy symptoms to worsen and last longer. Nasal steroids are anti-inflammatory medications that help decrease the inflammatory cell responses to an allergen. [Adapted from NIAID] This is pictorially depicted in **Figure 5**.



Adapted from NIAID

Figure 5. Development of an allergic response

2.9 Preventative measures to allergic diseases

The use of antigen-specific immune therapy is the only known radical treatment of allergic diseases and has been used in the USA and European countries. This is whereby antigen extract at a lower dose than the onset threshold is repeatedly injected with a gradual increase in their dosages to induce antigen-specific immune tolerance [55] [56]. Immune oral tolerance is mediated by regulatory T (Treg) cells. Treg does not only suppress Th2 immune response to reduce IgE production in humans but also induce IgG4 immune-suppressive antibodies [57] [58]. The effectiveness of specific immune therapy is evident, but the current use of crude antigen extract presents some risks and severe side effects, which include systemic anaphylaxis. Also, its efficacy widely varies among different subjects [59]. This has hampered the use of specific immune therapy in many countries. These countries now tend to rely on symptomatic drugs to treat allergy diseases. These drugs show improvements only in allergy symptoms but non-curative. Their therapeutic targets include pro-inflammatory mediators secreted by mast cells, IgE and IL-5 [60][61][62]. Recently type-I allergy is diagnosed using crude allergen extract to allow the identification of the allergen-containing molecules responsible for the specific type-I allergic symptoms. The use of recombinant allergens produced by molecular biology techniques has made it simple [63]. The symptomatic drug only suppresses the

allergic symptoms without a permanent cure; this leaves the patient with continued side effects and medical costs. Thus, the need to discover safer and more effective anti-allergic target molecules will be a welcome development.

2.10 Anti-allergic target molecules from functional food

Medical costs are currently escalating and becoming unbearable. As such, preventative medicine is an alternative solution. This is whereby diseases are prevented by promoting mental and physical health bearing in mind their causes and mechanisms of their pathogenesis. Improvement of food lifestyle is the most recommended avenue to live a disease-free life as the analogy "You are what you eat". Avoiding high fat and high-calorie foods, which are commonly the cause of lifestyle-related diseases. Functional foods or foods, which beyond basic nutrition has a potentially positive effect on health. Some of the discovered and reported functional foods include Green tea-derived epigallocatechin gallate which is reported to suppress type I allergic reaction and improves the clinical score of cedar pollinosis in humans [64]. Several researchers studied some strains of Lactobacillus that improve Th1/Th2 balance in food allergy model mice and cedar pollinosis in humans [65][66,67]. These discoveries contribute not only to the improvement of patient`s quality of life but rather to the development of anti-allergic

drugs.

2.11 Possible underlying mechanisms of P-CPP III immunomodulatory effects

Few studies have elucidated the underlying mechanisms by which protein and peptides exert their immunomodulatory activities. This is very important in giving clarity as to how proteins and peptides induce their immune effects and how they can be applied in drug development. Receptor binding and signaling whereby peptides directly stimulate receptors in cell surfaces is one way they can induce their bioactivities. It is reported that most protein and peptides affect Toll-like Receptors (TLRs) (**Figure 6A**). This is a family of pathogen recognition receptors [68], and they are expressed in most immune cells [69] and epithelial cells [70]. For example, in cow`s milk hydrolysates whey hydrolysates were found to affect several TLRs including TLR 2, 3, 4, 5, 7, 8 and 9 [71]. Also depending on the physicochemical properties of specific peptides including size, hydrophobicity and charge the kinetic of peptide uptake is determined. Peptides can be taken up the cell through fluid-phase endocytosis (**Figure 6B**). In this case, according to Knipp et al. using Caco-2 cells the hydrophobic interaction between the peptide and cell membrane was involved in the internalization of the peptide [72]. The other method reported is the peptide transporter (PepT1) (**Figure 6C**). This is di/tripeptide transporter which is

normally expressed in the small intestine and poorly expressed in the colon, but in cases of inflammation such as inflammatory bowel disease, it is upregulated in the colon [73]. It mediates the uptake of di- and tripeptides derived from dietary protein breakdown into epithelial cells [74].

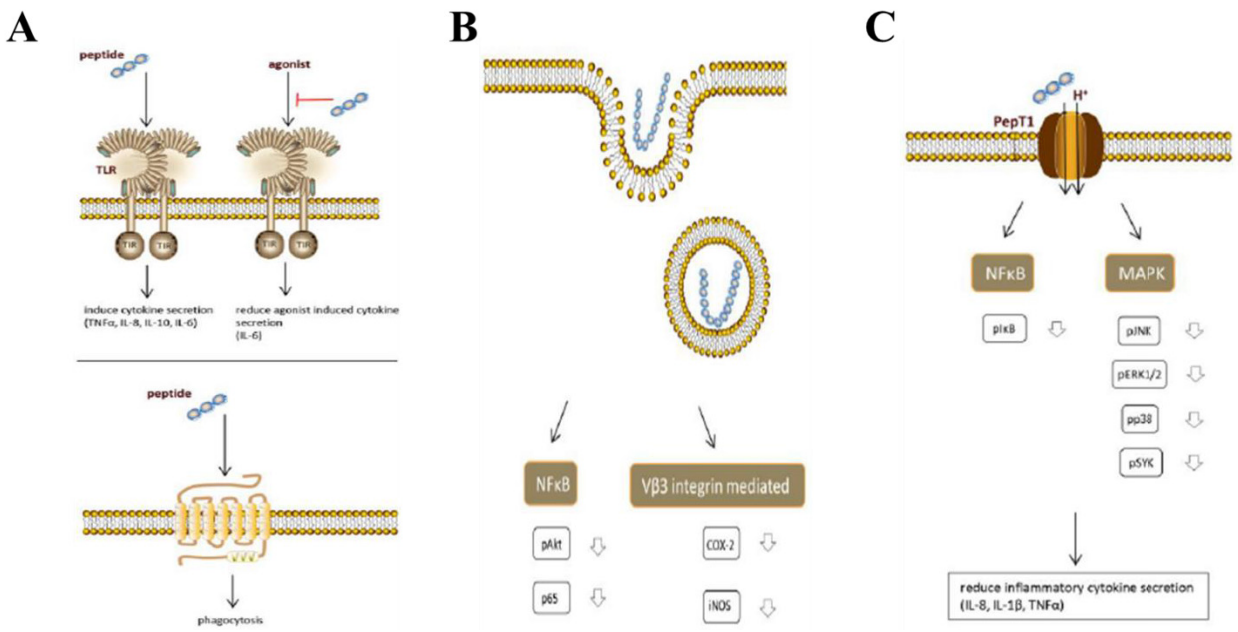


Figure 6. Possible reported mechanisms via which peptides can exert their immunomodulatory effects in the cell. (**A**) Receptor signaling, (**B**) Endocytosis, (**C**) PepT1 transporter. Adapted from [75].

3. Objectives

In this study, I aimed to improve the functional and bioactive properties of CPP III, to enhance its anti-viral effect against human norovirus and its anti-allergic effects through inhibiting the production of IgE and switching the balance from Th2 to Th1 immune response

Since it is reported that chemical modification of food proteins can enhance their bioactive activities, I put in use phosphorylation method in this study. Phosphorylation of the molecular surface of proteins has been demonstrated to be a useful method of improving their functional properties.

CHAPTER II

Role of phosphate groups on the antiviral activity of casein phosphopeptide (CPP III) against feline calicivirus (FCV) as a surrogate for norovirus

1. Abstract

Current research on the gastrointestinal digestion of milk-casein strongly suggests the existence of novel bioactive peptides with antiviral activities that are attributable to their immunostimulatory effects. In the present study, we investigated the antiviral activity of casein peptides rich in phosphate groups, such as CPP-III. We prepared two types of CPPs with different phosphorylation levels to clarify the role of the phosphate groups. Further phosphorylation of CPP-III was conducted by dry heating with sodium pyrophosphate, whereas dephosphorylation was performed enzymatically using alkaline phosphatase and alkaline treatment. FCV strain F9, a typical norovirus surrogate, and Crandell Rees feline kidney cells were used as the target virus and host cells, respectively. Antiviral activity was determined based on the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and quantitative polymerase chain reaction quantification of antiviral cytokine mRNA expression. Higher cell viability was observed in the host cells treated with phosphorylated CPP-III, and a significant up-regulation of type 1 interferon expression was induced compared to that treated with native CPP-III. However, the dephosphorylation of CPP-III resulted in a decrease in the anti-FCV effect. The CPP effect was enhanced by the introduction of additional phosphates and conversely weakened by their elimination. Therefore, CPP-III phosphorylation represents an

emerging approach for the production of food-grade antiviral agents.

2. Introduction

Human Norovirus (HuNov) is the leading cause of recurrent acute nonbacterial gastroenteritis and is the most common cause of foodborne disease outbreaks worldwide [76],[77]. In the USA, it is categorized as one of the top five highest-ranking pathogens concerning the total cost of foodborne illness [78]. Nonetheless, Dey *et al*. noted that mortalities as a result of norovirus infection are higher in developing countries as compared to developed countries [79]. Quinlan further observed that increased rates of foodborne infections are more prevalent among minority racial/ethnic populations [80]. The number of estimated cases of norovirus outbreaks and the economic cost related to foodborne illnesses continues to escalate globally causing a regression in public health and world economies [81],[82]. Although the illnesses caused by norovirus are not always terminal the infection can have a considerable impact on the health of young children, elderly people and those that are immunocompromised [83]. Despite all these, to date, there is no effective vaccine to prevent human norovirus infection and no specific antiviral therapies available to either treat or prevent it. It has also been observed by Ryu *et al*. and Mormann *et al.* that conventional intervention methods used to inactivate norovirus, such as treatment with disinfecting agents (e.g. ethanol, hypochlorite, quaternary ammonium formulations), freezing, cooling and mild heat treatment have shown a lack of efficacy

against human norovirus when they are applied to foods and in food preparation processes [84,85]. Thus, the development of novel food-based bioactive compounds that can safely and effectively control viral adulteration of foods and human infections will be of interest to functional food research scientists and the food industry as a whole. In this regard increasing attention is being focused on physiologically active peptides derived from milk proteins [13,14].

Figure 7 indicates the proposed schematic model of the anti-viral effect of CPP III. By enhancing phosphorylation of CPP III we expect to increase its ability to bind to viral target cells receptors or to the virus itself. This will prevent virus attachment to host cells. It is also expected to induce type-I interferons thereby inhibiting virus replication. CPP III will be sensed as a ligand attaching to viral target receptors on host cells hence preventing viral attachment. The induction of type-I interferons is expected to trigger interferon response in both infected cell and neighboring uninfected cells. Cellular genes that are capable of destroying viral mRNA are activated to inhibit the translation of viral proteins. It has been stated by Dianzani that activation of the interferon system can be operated *in vitro* and *in vivo* also by several non-viral substances of various nature, such as nucleic acids, polysaccharides, aromatic amines, etc [86].

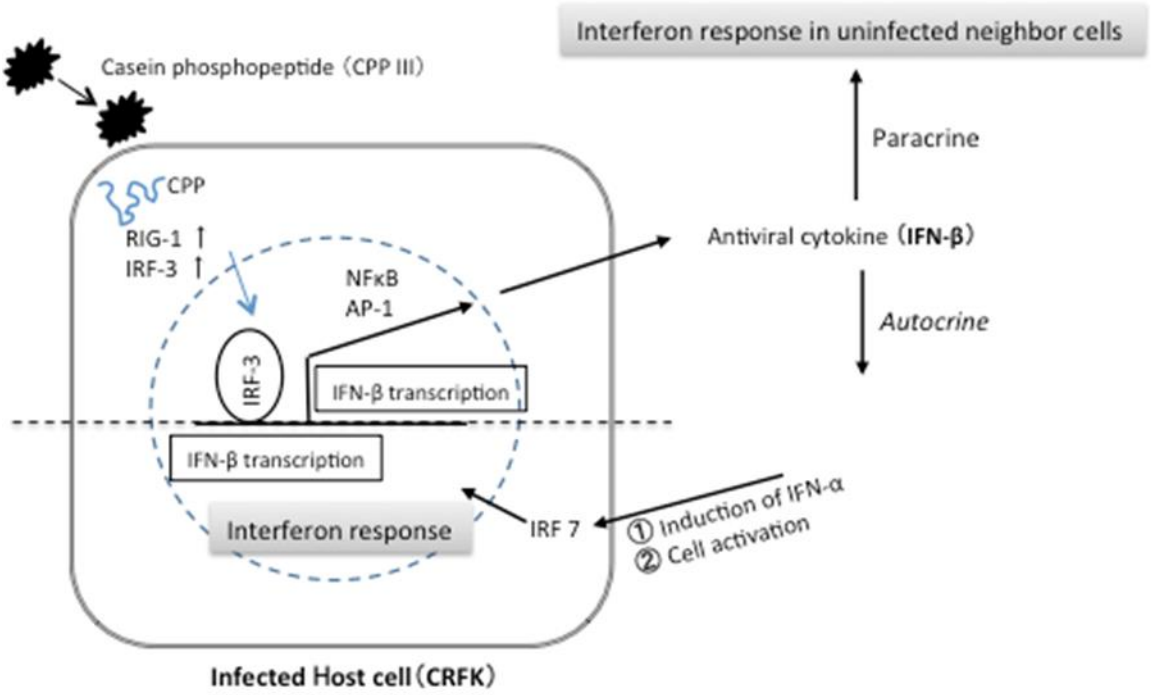


Figure 7. Schematic presentation showing the proposed mechanism of type 1 IFN induction by CPP and the resulting IFN response on infected and uninfected host cells.

3. Materials and Methods

3.1 Materials

CRFK cells were obtained from the Health Science Research Resources Bank (HSRRB, Osaka, Japan). FCV strain F9 was purchased from the American Type Culture Collection (Manassas, VA, USA). CPP III was courtesy of Meiji Co. Ltd (Tokyo, Japan). All other reagents were of biochemical and analytical grade.

3.2 Preparation of phosphorylated CPP III

Phosphorylated CPP III was prepared based on the method of Li *et al* [87]. It was dissolved in 0.1M sodium pyrophosphate buffer of pH 4.0 at a concentration of 1 mg/mL. The solution was then lyophilized, and the resulting powder was dry heated at 85^{o}C for 1-5 days. After dry heating, the powder was dissolved and dialyzed against deionized water for 2 days using membranes with a molecular weight cut off (MWCO) 1000 Da to remove free phosphates. It was then lyophilized to prepare enhanced phosphorylated CPP III (P-CPP III).

3.3 Preparation of dephosphorylated CPPIII

Alkaline dephosphorylation

Alkaline dephosphorylation was conducted following a method of Xu *et.al* [34]

with a slight modification. CPP III was dissolved in Sodium Hydroxide of 0.1, 0.2, 0.3 and 0.4 M at a concentration of 25 mg/mL and incubated at different times, 0, 12, 24 and 72 hours. After pre-determined incubation time, the pH of the solution was adjusted to 7.0 and dialyzed (MWCO 1000 Da) against deionized water for 2 days and lyophilized to prepare dephosphorylated CPP III (D-CPP).

Enzymatic dephosphorylation

For enzymatic dephosphorylation, the method of Darewicz *et.al* [88] was followed. Briefly, CPP III was incubated at 37°C for different time lengths with bovine alkaline phosphatase (10 U/mg of peptide) in a 50 mM Tris-HCl buffer of pH 8.0. Heating at 80 °C for 15 minutes terminated the reaction, and the solution was dialyzed against distilled water for 2 days followed by freeze-drying. CPP III was also treated under identical conditions but without bovine alkaline phosphatase for comparison.

3.4 Determination of phosphorylation and dephosphorylation levels

The levels of phosphorylation and dephosphorylation on samples was determined using the molybdenum blue assay [89]. Briefly, Aliquots (50 µL) drawn from sample 10 mg/mL were mixed with acidic buffer containing (2 g potassium peroxodisulfate in 47.5 mL distilled water (DW) and 2.5 mL concentrated sulphuric acid) and digested for 20 minutes at 120 °C and cooled to room temperature. Samples were then mixed with a

buffer containing hexaammonium heptamolybdate 1.2 g plus Antimonyl potassium tartrate 48 mg in 60 mL DW and 32 mL concentrated Sulphuric acid, 36 mg of ascorbic acid in 500µL DW [90,91]. The absorbance was read at 880 nm. The absorbance of the samples was compared to a standard curve derived from the stock solution of potassium dihydrogen phosphate 0.2-5.0 µg/mL in DW.

3.5 Cell viability and antiviral effect

Cell viability was assessed using the colorimetric MTT metabolic activity assay, which is based on the cleavage of a tetrazolium salt by mitochondrial dehydrogenases in viable cells [92],[93]. The assay was performed according to the method of Mosmann [94]. CRFK cells ($5x10^4$ cells/well) were cultured in a 96 well plate at 37 °C with 5 % CO_2 and exposed to varying concentrations (10 and 100 µg/mL) of Native (N-CPP), P-CPP and D-CPP for 48 hrs before being infected by FCV and incubated for 24 hrs. Cells treated with medium only served as a negative control group. MTT (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in PBS at 5 mg/mL and filtered using a 0.2 µm-syringe filter. MTT stock solution was added to each well of 96 well cell culture plate (10 µL of MTT stock solution/ 100 µL of medium), the plate was incubated at 37 °C for 4 hrs. The formazan crystals incorporated into the viable cells were solubilized with 200 µL of

dimethyl sulfoxide (DMSO). The absorbance intensity was measured at 570 nm wavelength on a multi detection plate reader (DS Pharma Biomedical Co., Ltd, Osaka, Japan) within 1 hr after the addition of DMSO. The relative cell viability (%) was expressed as a percentage relative to the untreated control cells.

3.6 Experimental layout

CRFK cells were seeded in 25 mm cell culture dishes. To achieve a monolayer, cells were cultured and maintained at 37 °C in an incubator with a humidified atmosphere of 5 % CO_2 for 24 hrs until confluency. 100 µg/mL of samples was used to pretreat the cells for 48 hrs before FCV infection, while other cells were only pretreated with samples without viral infection. Cells treated with the only medium acted as positive control while those treated with only FCV acted as a negative control **(Table I)**.

Table 1 Experimental groups for mRNA expression of antiviral cytokines showing pretreated groups with or without viral treatment.

Sample name	Sample concentration	Treatment time	FCV	Infection time
	(μ g/mL)	(hrs)		(hrs)
Control	-	-	-	-
N-CPP	100	48	-	-
P-CPP	100	48	-	-
D-CPP	100	48	-	-
FCV	-	48	5.75 $logTCID_{50}$	24
N-CPP + FCV	100	48	5.75 $logTCID_{50}$	24
P-CPP + FCV	100	48	5.75 $logTCID_{50}$	24
D-CPP + FCV	100	48	5.75 $logTCID_{50}$	24

3.7 RNA extraction and cDNA synthesis

RNA extraction was performed on each sample using the TRI REAGENT® according to the maker`s method. Briefly, 1000 µL of TRI reagent (Molecular Research Center, Ohio, USA) was added to the cells on culture dishes. The cell lysate was passed several through a pipette to mix and transferred to an Eppendorf tube and incubated for 5 minutes at room temperature. Then 200 µL of chloroform was added and the samples were shaken and maintained at room temperature for 10 minutes. The mixtures were centrifuged at 15,000 g for 20 minutes. Then the supernatant was transferred to a fresh Eppendorf tube, 700 µL of isopropyl alcohol (Wako) was added, and the tubes were tilted to mix and for RNA precipitation. The samples were then centrifuged at 15,000 g for 10 minutes. To clean the precipitate, the top liquid was aspirated and 1000 µL of 75% ethanol was added to the tube before vortexing gently. RNA was centrifuged at 15,000 g for 10 minutes, and the supernatant was carefully aspirated. Finally, the precipitate was air-dried for 2 minutes and dissolved in 20 µL of DEPC water. The RNA concentration was measured on Nanodrop Lite Spectrophotometer (Thermo Scientific, USA) at a test wavelength of 260/280 nm. Its quality and yield were assessed by electrophoresis on a denaturing agarose gel.

For cDNA synthesis, a Rever Tra Ace qPCR RT Master mix kit with gDNA

remover (TOYOBO CO., LTD, Japan) was used following the maker`s protocol. Briefly, the RNA solution was heated for 5 minutes at 65 °C to denature the RNA. 500 ng of RNA template was mixed with nuclease-free water to a total volume of 6 µL, 2 µL of 4X DN master mix (with gDNA remover) was added and solution incubated at 37 °C for 5 minutes to remove genomic DNA. 5X DN master mix was added and solution subjected to PCR condition as shown in **Table II** for reverse transcription**.** The resulting cDNA was stored at -30 °C until use.

Table II PCR conditions for cDNA synthesis

	Step	**Temp ℃**	**Time (sec)**
Cycling	1	37	15
	2	50	5
	3	98	4
Holding	4	4	∞

3.8 Gene expression by qPCR

Real Time-Quantitative Polymerase Chain Reaction (RT-qPCR) [95] was performed for the relative quantification of antiviral cytokine gene expression analysis. Results were read by Kapa SYBR fast qPCR kit. The gene expression of IFN-α and IFN-β were examined at the mRNA level with housekeeping gene β-glucuronidase (GUSB) as a control according to Kessler *et.al.* [96].

A mixture with Kapa SYBR fast qPCR kit (Kapa Biosystems, USA), SP water, forward and reverse primer, and cDNA to a final volume of 20 µL was prepared for the expression of IFN-α and IFN-β. The amplification by Real-time PCR was run on Takara TP850 thermal cycler, Dice Real-time system (Takara, Shiga, Japan) following PCR conditions in **Table III**.

Table III Real-time PCR conditions for antiviral cytokine mRNA expression.

	Step	Temp ℃	Time (sec)	Note #
Holding	1	95	20	
Cycling	1	95	15	
	2	60	45	***40 Cycles***

3.9 Statistical analysis

Data were analyzed using Student's *t*-test. Data were reported as the mean±SD (*n*=3). $P < 0.05$ was considered statistically significant.

4. Results and Discussion

Previous studies have investigated the effect of CPP III on the enhancement of immunoglobulins such as IgA, IgG, and IgM, mRNA expression of IL-6 and tumor necrosis factor-alpha (TNF- α) [9] [18]. Its enhancement of mineral absorption in the lower intestine especially that of calcium, effects on oral health and dentistry has also been widely studied [8,97,98]. However, little is known about the effect of the phosphorylation level of CPP III and its anti-viral activity. In this study, I demonstrated *in vitro* the anti-viral and protective effects of enhanced phosphorylated and dephosphorylated CPP III against FCV infection in CRFK cells through the anti-viral cytokine gene up-regulation.

4.1 Phosphorylation of CPP III

The preparation of phosphorylated CPP III was conducted by dry heating for 1 to 5 days at 85°C in the presence of a pyrophosphate buffer of pH 4.0 as shown in **Figure 8**. The phosphorylation level shown to be time-dependent as the longer heating time of 5 days added more phosphate groups to the CPP III as compared to shorter heating times. About 10 μg/mg of protein was added to the CPP III at 5 days compared to ~4 μg/mg of protein of lower heating time (**Figure 9)**. This was also observed in the previous studies

by Li *et.al* 2009 and 2010 where they reported more attachment of phosphate groups after 5 days of dry heating [99] [100]. The amino acids, which have a high possibility to get phosphorylated, are reported to be serine, threonine, and tyrosine. The phosphorylation takes place between the nucleophilic (-OH) groups of the amino acids and the terminal phosphate group of the donor. After the phosphorylation of serine, threonine, and tyrosine amino acids form phosphoserine, phosphothreonine, and phosphotyrosine, respectively. Because of the intrinsic charge of phosphate groups, when it is attached to these amino acids it gives an overall negatively charged residues. Previous studies have pointed out that β-casein was phosphorylated at Ser^{30}, Ser^{32}, Ser^{33}, and Ser^{34} [101], while Li *et al* reported phosphorylation sites at Ser^{50} and Thr^{56} [23]. It has also been shown that phosphorylation of caseins may differ within and among species [102]. In previously conducted studies phosphorylation has been reported to improve the heat stability, emulsifying properties and the gelling properties of food proteins [100] and the negative charges of the attached phosphate groups are thought to be behind the improved functional effects. Many other proteins including β-La, α-Lactoglobulin, and human serum albumin have demonstrated to enhance the anti-viral effect against HIV-1 and HIV-2 when additional negative charges were added by chemical modification in their lysine residues [49]. The phosphate groups are important in many aspects, nutritionally because

they bind large amounts of Ca^{2+} and Zn^{2+} and other polyvalent cations [103].

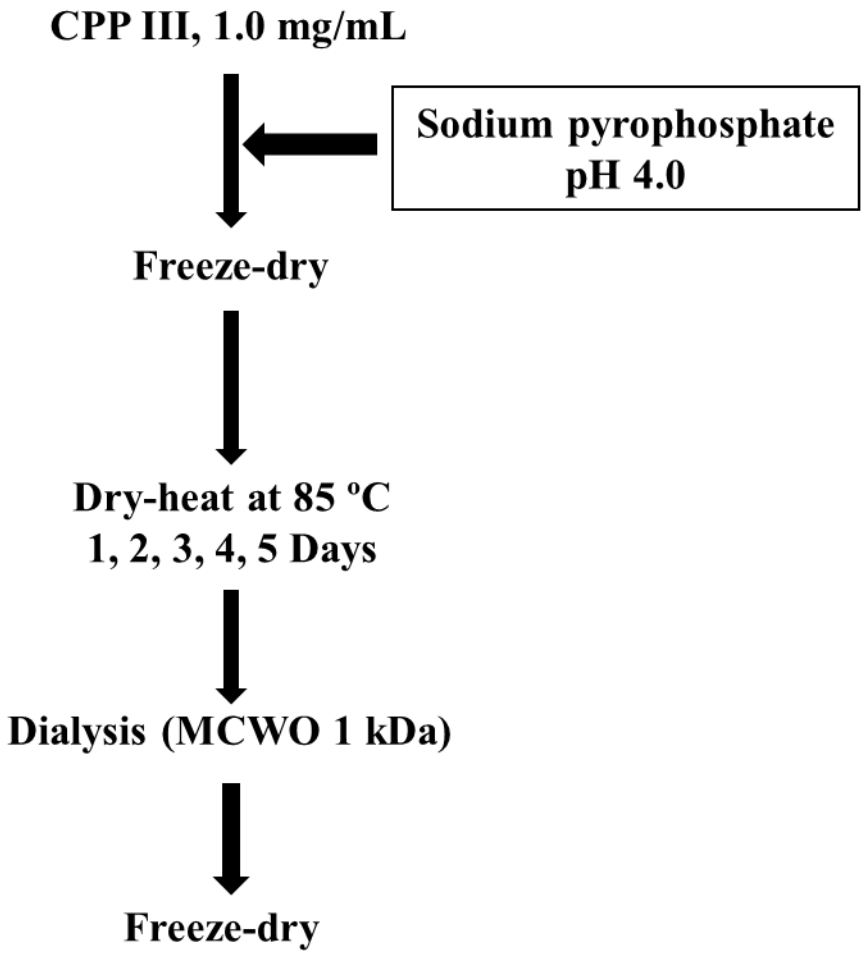


Figure 8. Procedure for phosphorylation of CPP III by dry heating in the presence of a pyrophosphate buffer at different heating days.

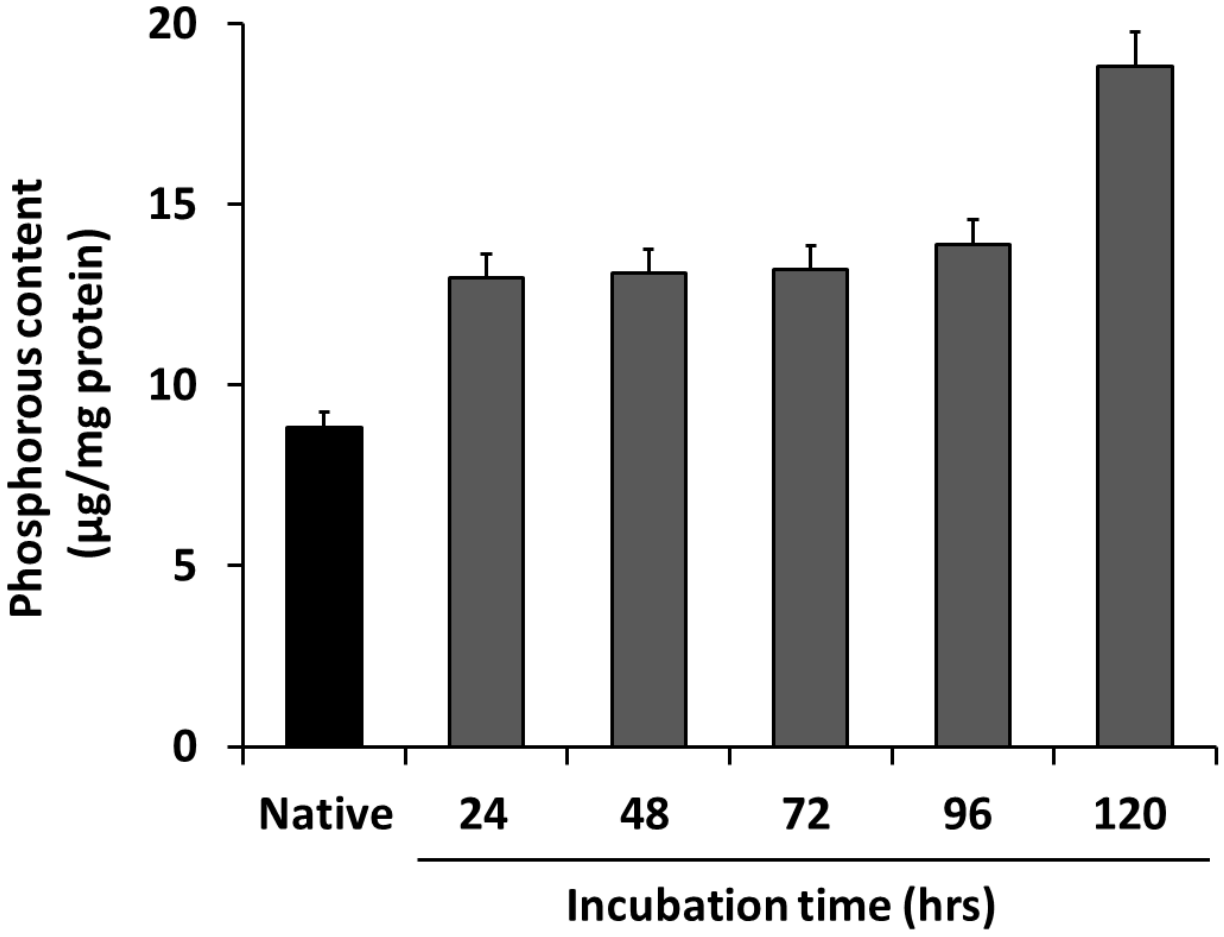


Figure 9. Effect of different dry heating time on phosphorylation rate of CPP III.

4.2 Alkaline and enzymatic dephosphorylation of CPP III

Dephosphorylation can be achieved both chemically and enzymatically [104]. Alkaline and enzymatic dephosphorylation methods were carried out using sodium hydroxide (NaOH) and bovine alkaline phosphatase, respectively. The procedures followed are shown in **Figure 10A** for alkaline dephosphorylation and **Figure 10B** for enzyme dephosphorylation.

The effect of alkaline on phosphoproteins has long been recognized by Plimmer *et al* (1906). They observed casein to be readily dephosphorylated by alkali at room temperature. The dephosphorylation of CPP III by alkaline was dependent on the (i) incubation time **(Figures 10C and 10D)**, and (ii) concentration of sodium hydroxide **(Figure 10E).** CPP III that was incubated without enzyme treatment shown no change on phosphate level **(Figure 10F)**. It was observed that the longer incubation time of 72 hours liberated more phosphate groups than shorter incubation times. Also, the dephosphorylation rate was dependent on the molar concentration of the alkali. The higher molar concentration of 0.4 M removed many phosphate groups from CPP III than lower concentrations. The extent of dephosphorylation dependency on incubation time and alkaline concentration were also similarly observed by Jiang *et al* [105]. Sundararajan *et al* also reported the more dephosphorylation extent by higher molar concentration of

cation. The cation in the alkali is thought to play a major role in dephosphorylation.

Different enzymes specifically phosphatases have been used to remove phosphate groups from phosphoproteins a method we term enzyme dephosphorylation. In this study, the higher incubation time of CPP III with bovine alkaline phosphatase proved to be effective in removing more phosphate groups than shorter incubation times. Bingham *et al* also observed this trend, where they reported higher dephosphorylation by potato acid phosphatase after long incubation time [104]. Cassiano *et al.* mentioned that the removal of some or most of the negatively charged phosphate groups from caseins causes a modification in the net casein charge and consequently, the flexibility and properties of the newly modified casein structure altered [106]. Therefore, this shows the possibility that dephosphorylation alters the structure of CPP III.

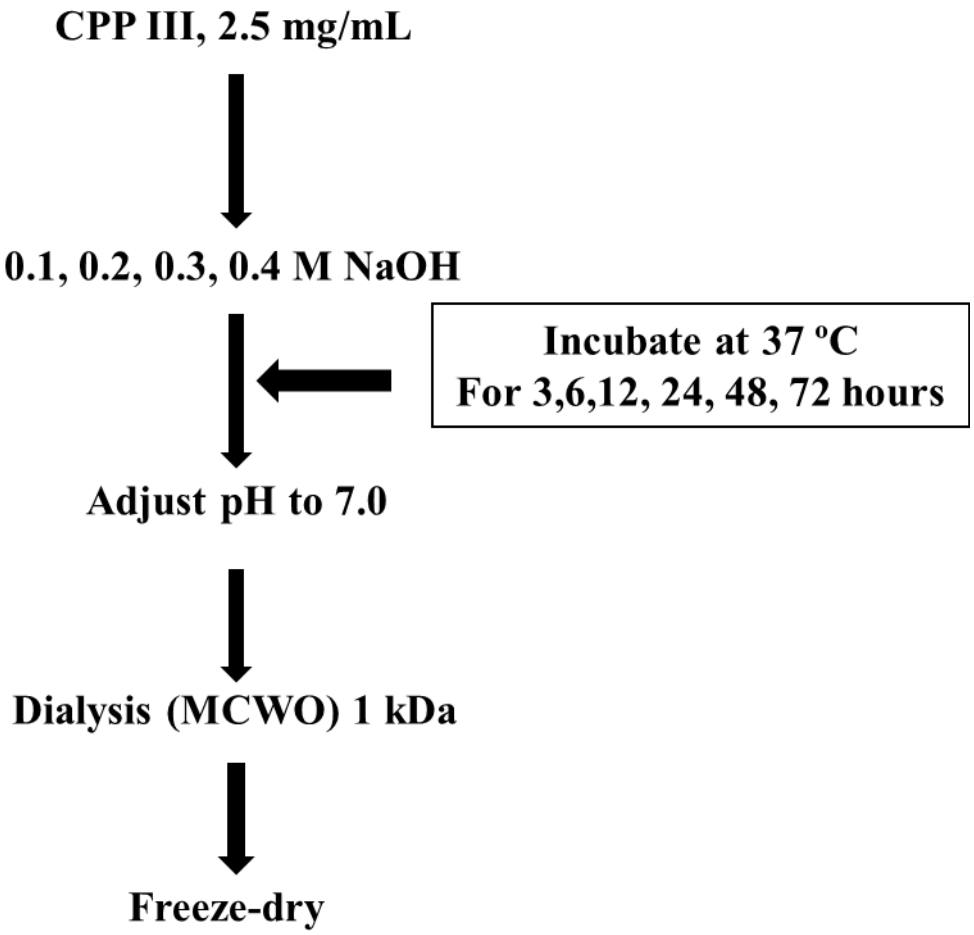


Figure 10A. Dephosphorylation of CPP III by concentrations of sodium hydroxide and different incubation time.

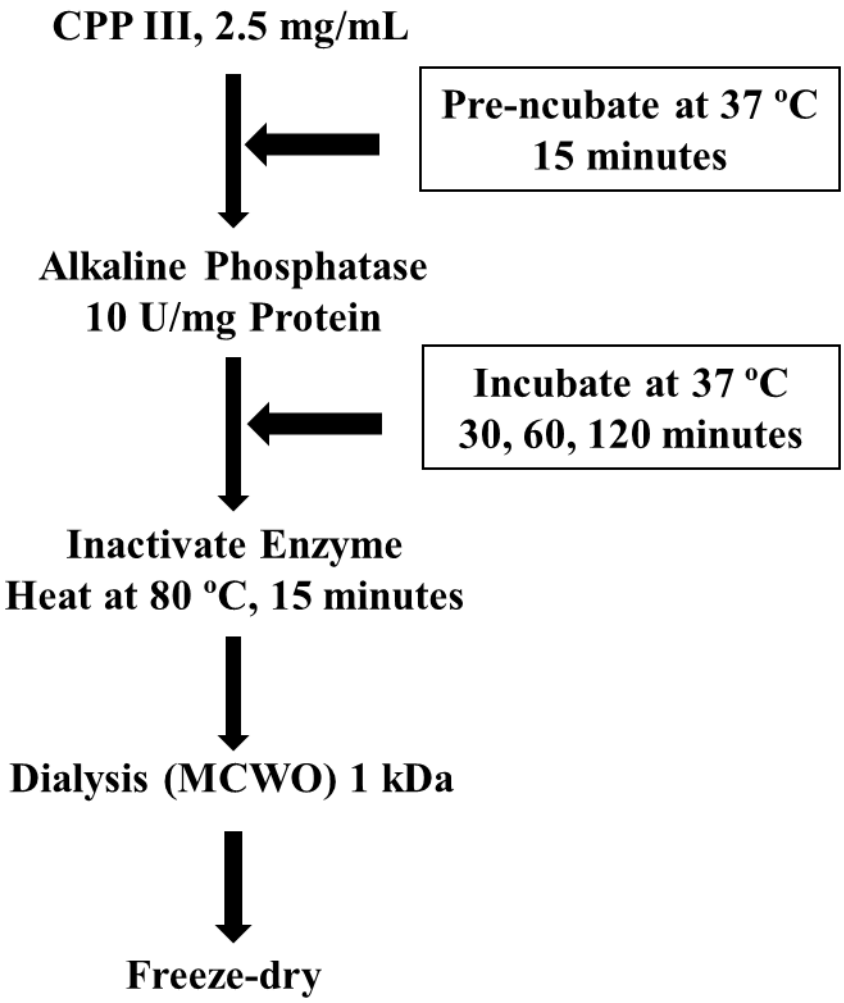


Figure10B. Dephosphorylation of CPP III by bovine alkaline phosphatase at different incubation times.

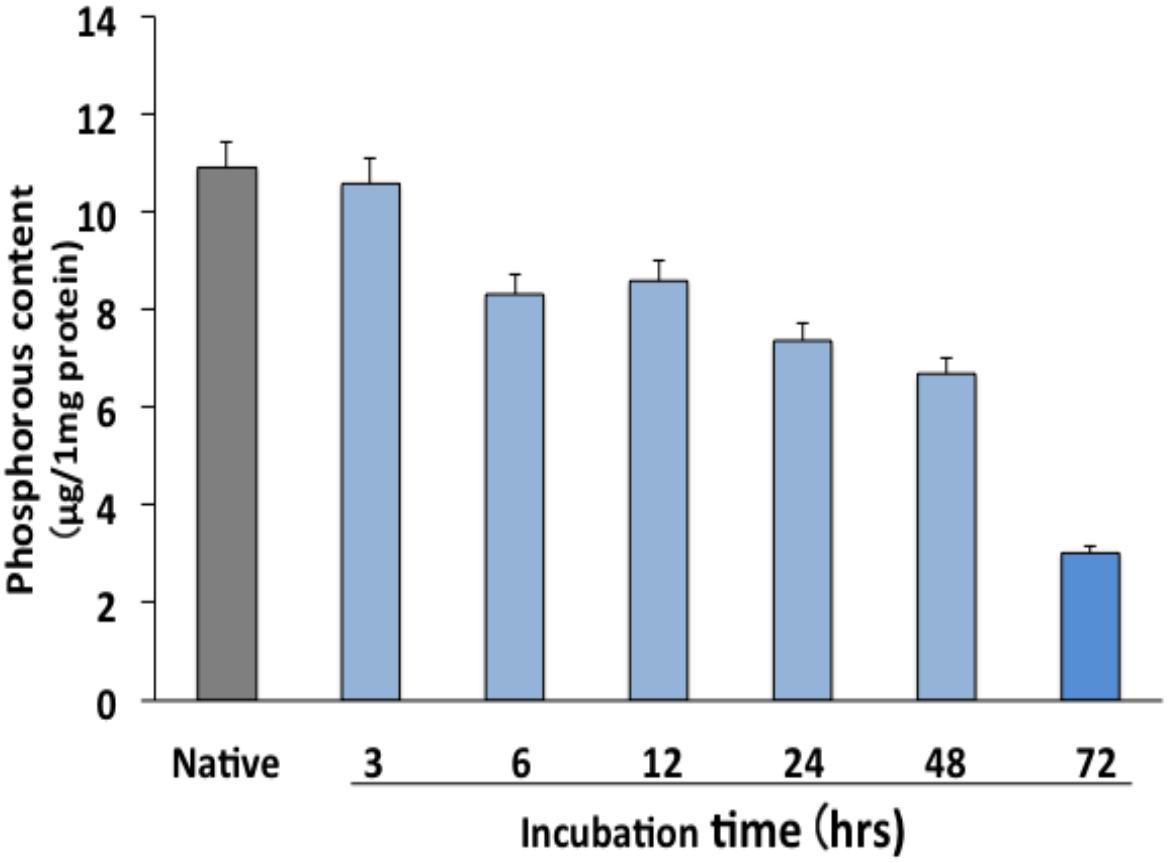


Figure 10C. Effect of incubation time on the dephosphorylation rate of CPP III by sodium hydroxide.

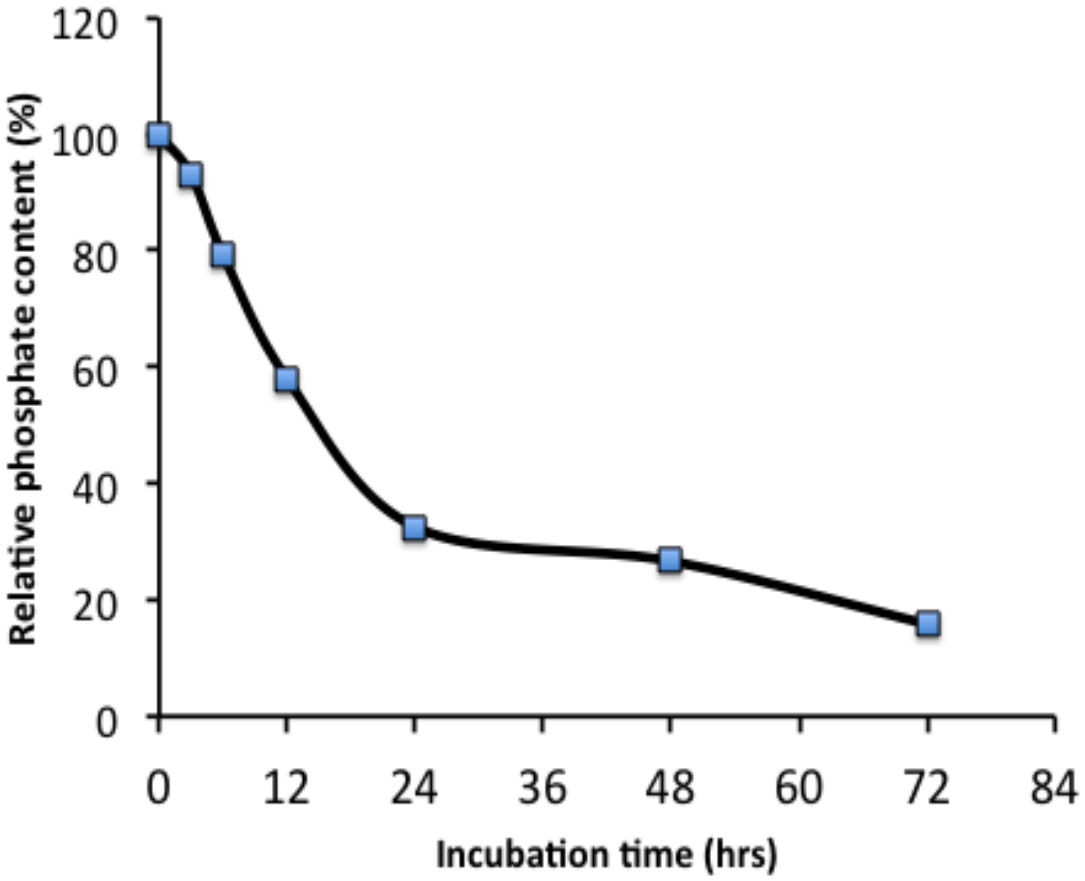


Figure 10D. Relationship between incubation time and dephosphorylation rate of CPP III by 0.4 M sodium hydroxide.

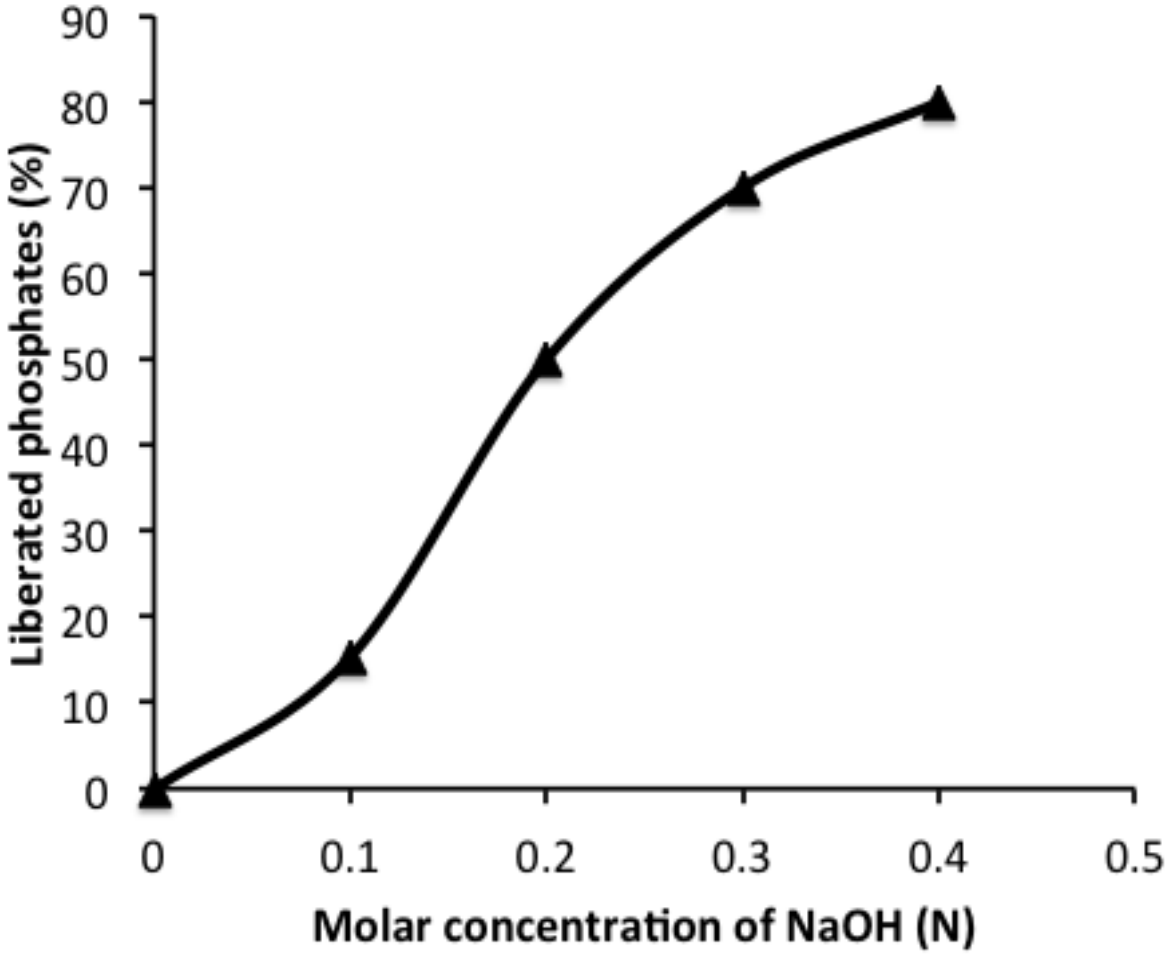


Figure 10E. Correlation between different alkali (NaOH) concentration and the dephosphorylation rate of CPP III.

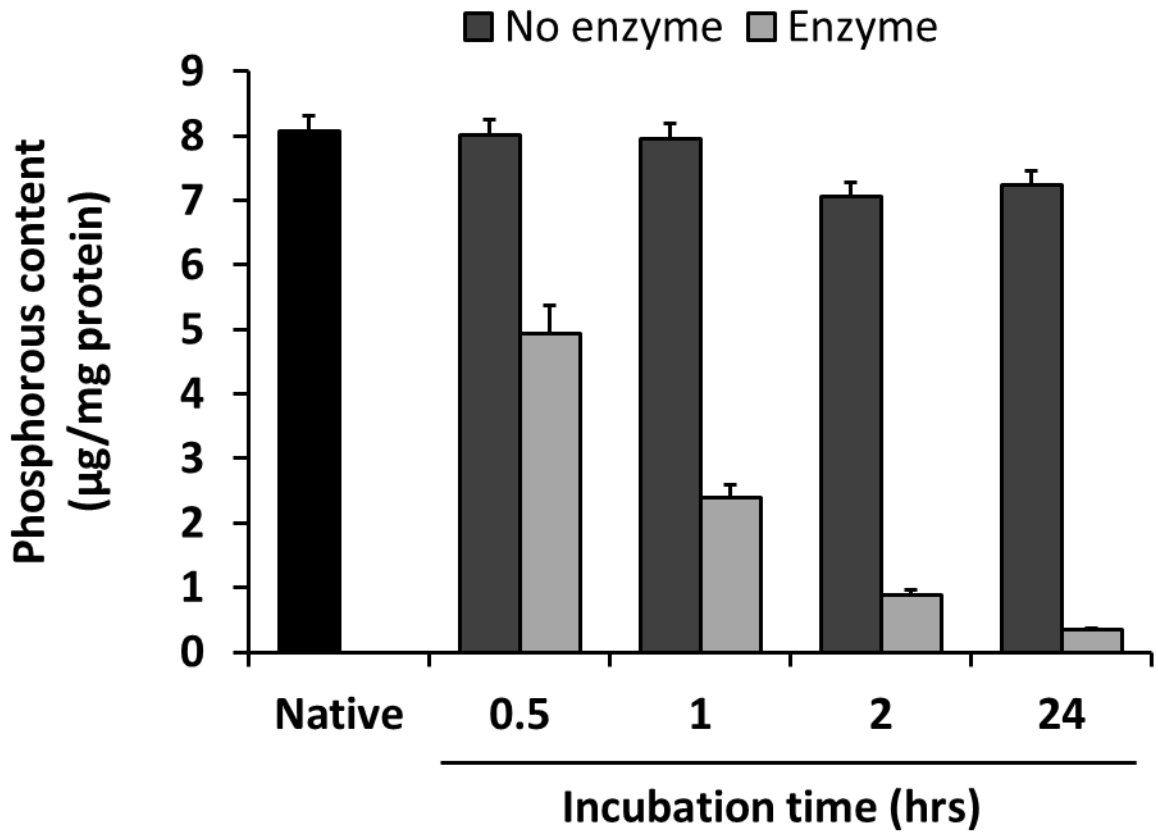


Figure 10F. Changes in phosphates content by enzymatic dephosphorylation of CPP III by bovine alkaline phosphatase.

4.3 Protective effect of CPP III on CRFK cells

The effect of pre-treating the host cells with differently phosphorylated CPP III on cell viability against virus infection was determined using MTT colorimetric assay. 10 μg/mL and 100 μg/mL of differently phosphorylated CPP III (N-CPP III, P-CPP III, and D-CPP III) were used to pretreat the cells for 48 hours before virus infection. Cells pre-treated with 100 and 10 μg/mL concentrations of P-CPP exhibited significantly higher percentage (92.7% and 82.6%) respectively of cell viability as compared to host cells pre-treated with N-CPP (65.8% and 53.9%) and D-CPP (56.5% and 50.4%) respectively **(Figure 11A)**. However, 100 μg/mL sample concentration showed higher viability across all treatment groups than 10 μg/mL sample concentration. Thus, the 100 μg/mL of sample concentration was used as a protective and non-cytotoxic concentration on host cells. This clearly hinted that the sequence containing SerP-X-SerP rich in phosphoserine (SerP) as observed by Kawahara *et al* [9] indeed has some bioactivities. It has been reported by Otani *et al* [107] that this tripeptide sequence SerP-X-SerP within CPP III is essential for IgA enhancing effect of CPP III. This enhancement of IgA evolved the IL-6 that is known to promote the growth of B-cells. Kreutz *et al* stated that the interaction of FCV with host cells was mediated by the specific receptor on the cells [108]. This suggests that P-CPP III might have interfered with the FCV infection at an attachment stage since it was added

to the cell before infection. McCann *et al* reported a similar finding of Lactoferrin, where it was active against FCV when it was pre-treated and or co-treated together with the viral inoculation [109]. It is thought that the charge on the anti-viral protein plays an indispensable role in their interaction with cell receptors [49]. Many researchers have proposed that negatively charged proteins have a stronger attraction to the viral target cell receptors and or to the viral envelope proteins [110–112]. Hata *et al* also suggested that the stimulation and immunoglobulin production in mouse spleen cell cultures by CPPs is attributed to *o*-phospho-*l*-serine residue [113]. $TCID_{50}$ assay also shown the reduction of viral titer by P-CPP III compared to N-CPP III (**Figure 11B**).

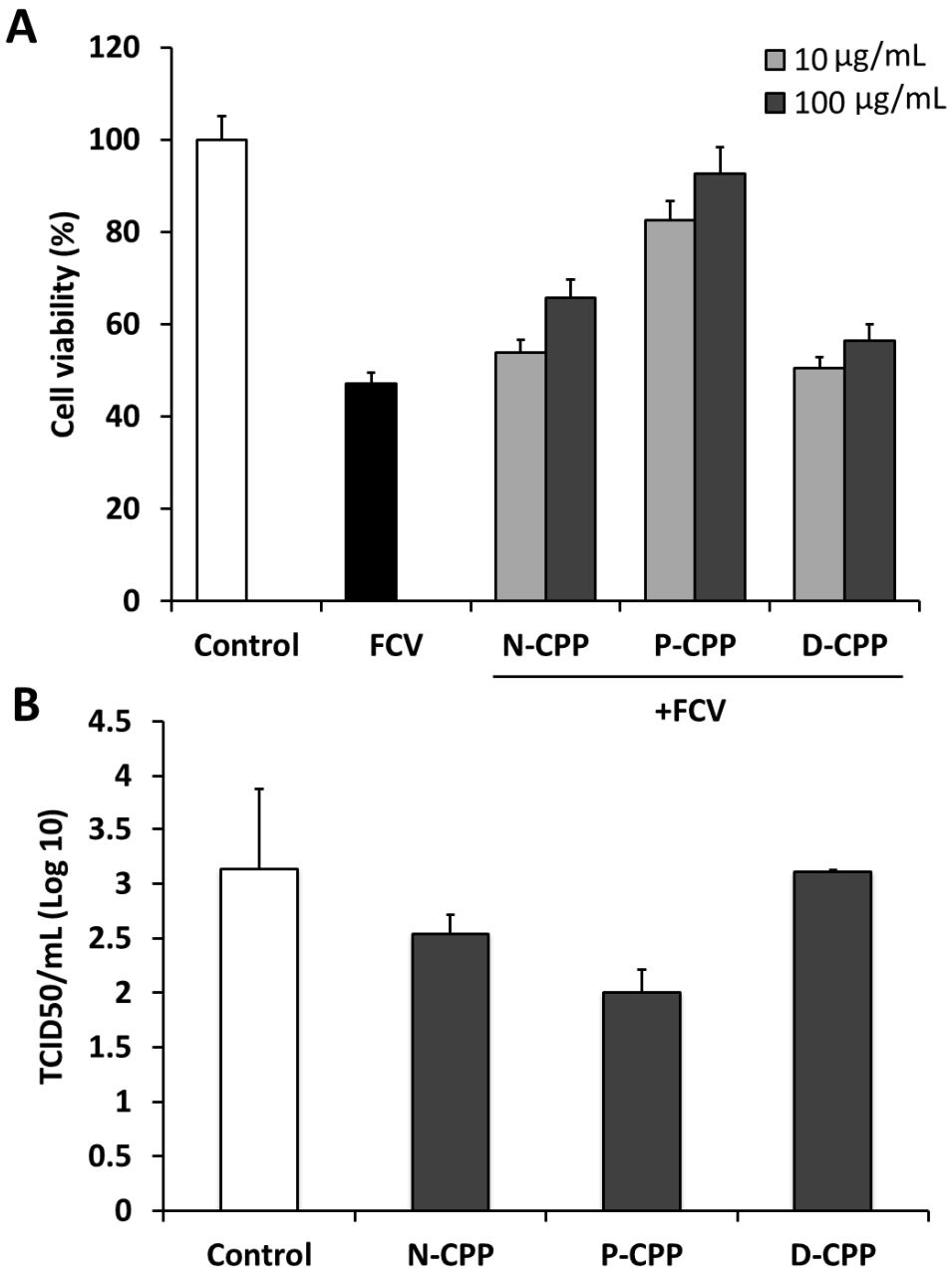


Figure 11. Effect of the pretreatment with N-CPP III, P-CPP III and D-CPP III on FCV infection. Virus infectivity was measured with **(A)** MTT assay and **(B)** TCID50 assay.

4.4 Induction of type I interferons on CRFK cells pretreated with differently phosphorylated CPP III

The relative expression of IFN-α and IFN-β mRNA in CRFK cells pre-treated with differently phosphorylated CPP III followed by FCV infection are shown in **Figures 12A and 12B** respectively. Both antiviral cytokines were more expressed in the sample pre-treated cells combined with virus infection as compared to cells infected by virus alone. The negative control showed an insignificant gene expression. Type I interferons are produced by cells in direct response to virus infection [114] or stimulus provided by viral nucleic acid [86]. Interferons are cytokines that have antiviral, antiproliferative and immunomodulatory effects [115]. The interferon system has been described by Randall and Goodbourn as an extremely powerful antiviral response that is capable of controlling most, if not all, virus infection in the absence of adaptive immunity [116].

Among the type 1 IFN, it is reported that IFN -α and IFN-β exhibits some form of antiviral activities [116]. They have been shown, more specifically IFN-α to counter different types of viruses including hepatitis A, B, and C by reducing hepatitis virus antigen expression and its replication [117]. Lenschow *et al.* observed that IFN -α/β plays a critical role in the control of viral infections and they exert their antiviral effect through inducing the transcription of many interferon-stimulated genes (ISGs) [118]. IFN-α/β

uses the janus kinase signal transducer and activator of transcription (JAK-STAT) signal transduction pathway. It has been stated that the signaling proteins transduce intracellular signals from IFN receptors to the nucleus and activate transcription of ISGs [119]. The interferon genes encode anti-viral proteins that inhibit viral replication and modulate immune function [120]. They are reported to be capable of targeting almost any step in a virus life cycle [121].

They are believed to possess some synergistic effects. The effect of anti-viral drugs has been increased by their cooperative use with type I IFNs. It is reported that they have also been used in synergic regiments where administration of IFN-α2 or -β2 and anti-viral drugs (e.g., ribavirin and fadaprevir) could effectively reduce viral loads of certain hepatitis C virus and is currently the best treatment for hepatitis C virus (HCV) infected patients [122,123]. Animal cells respond and counter viral or pathogen infection through the production of type 1 IFN [124]. Apart from the treatment of pathogen infections they have also been used for treatment and regression of various cancers including leukemia, prostate cancer [125,126]. The up-regulated antiviral cytokine gene expression induced by P-CPP III is expected to contribute to the reduction of norovirus infections.

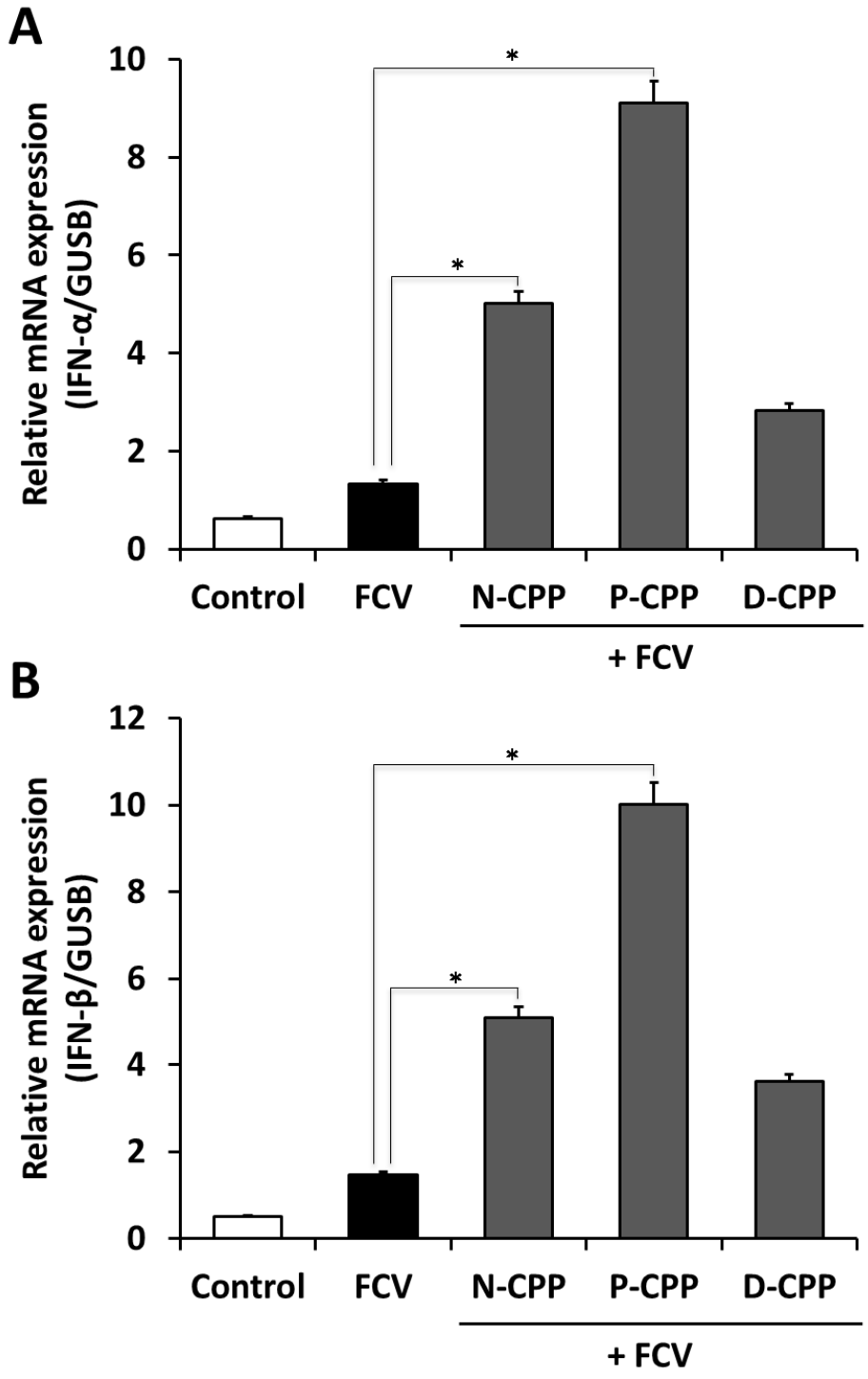


Figure 12. Relative mRNA expression of **(A)** IFN-α and **(B)** IFN-β on cells pretreated for 48 hrs followed by 24 hrs FCV infection. *$p<0.05$.

4.5. Immunostimulatory activity of CPP III

Relative gene expression at mRNA level of type I IFN (IFN-α and IFN-β) in host cells pre-treated with differently phosphorylated CPP III without virus infection is shown in **Figure 13A and 13B** respectively. It was interestingly noted that the mRNA expression of type I IFN was also up-regulated where cells were only pre-treated with the sample without virus infection. Though the gene expression was lower than the sample pre-treatment and virus combination groups in previous **Figures 12A and 12B**, it gives a hint that CPP III may be embodied with type I IFN- inducing factors.

Bioactive compounds from milk are considered an active subject of research for the development of new and potent nutraceutical products. Currently, health and food industries are placing an increased emphasis on the exploitation of food proteins as ingredients for specific functional properties to different formulated foods and food-based supplements. Modification of proteins has been used considerably to enrich the functional properties of food proteins offering them the potential to be used in different applications of food and pharmaceutical industries. It comes in different methods including succinylation, deamidation, glycosylation, phosphorylation, dephosphorylation, and many other methods. In phosphorylation, Serine, Threonine, tyrosine, Arginine, and Lysine are reported to be the primary amino acids susceptible to phosphorylation. **Figure**

14 showed some of the major constituents of CPP III. Modified proteins have enhanced functionalities as compared to their native counterparts. This work will not only generate data on the nutraceutical and health-promoting properties of CPP III but will also encourage its maximum utilization.

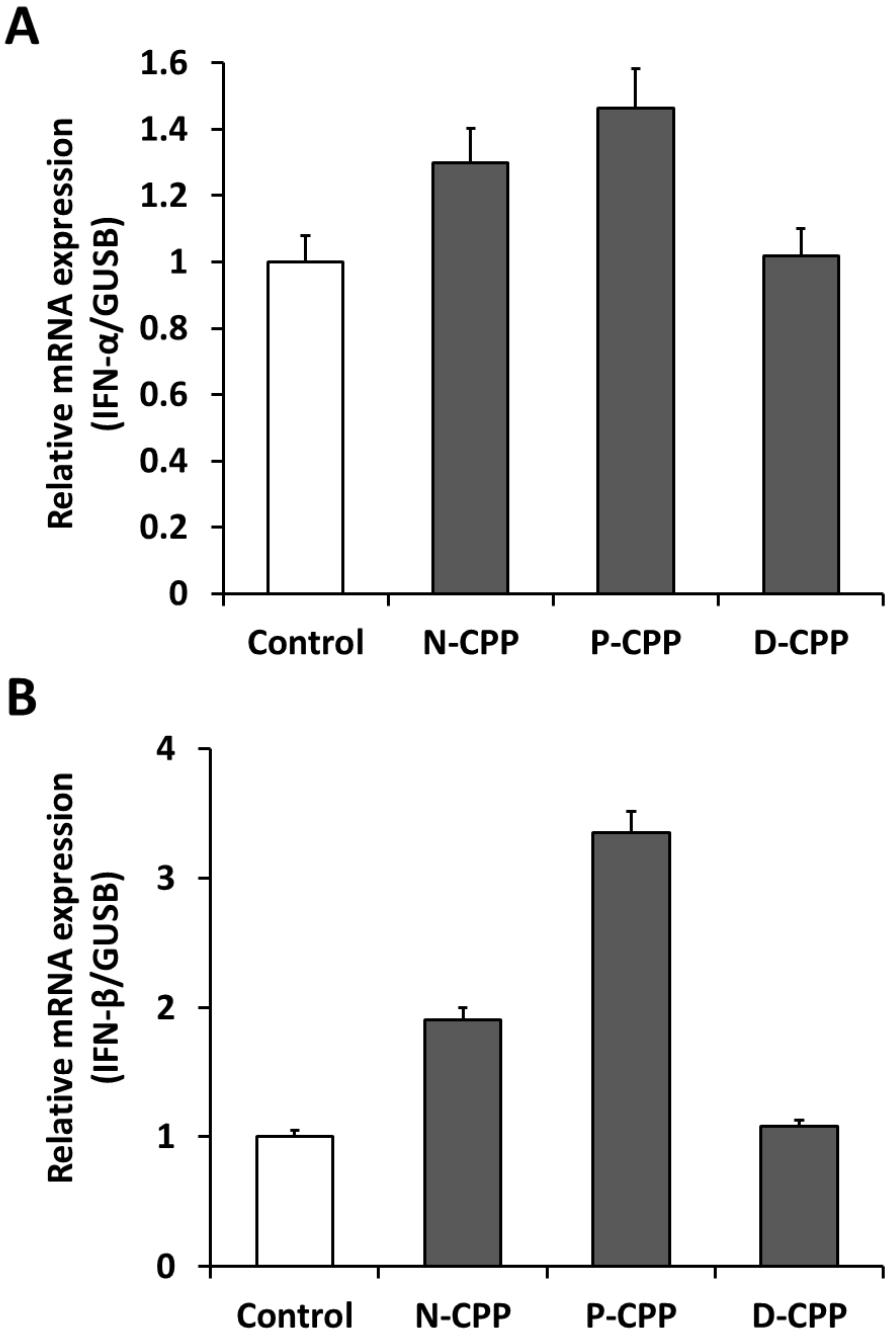


Figure 13. Relative mRNA expression of **(A)** IFN-α and **(B)** IFN-β on pretreated cells without FCV infection.

Parent protein	Molecular mass (Da)		Start		End	Peptide sequence	Peptide modifications
	Expected	Calculated					
α_{s1}-CN	1222.5	1222.5	110	-	119	(L)EIVPNSAEER(L)	1P
	1517.8	1517.6	68	-	79	(S)SEEIVPNSVEQK(H)	2P
	1525.7	1525.5	41	-	53	(L)SKDIGSESTEDQA(M)	2P
	1586.6	1586.5	43	-	55	(K)DIGSESTEDQAME(D)	2P; Oxidation (Met)
	1672.7	1672.6	41	-	54	(L)SKDIGSESTEDQAM(E)	2P; Oxidation (Met)
	1785.8	1785.6	41	-	55	(L)SKDIGSESTEDQAME(D)	2P
	1801.7	1801.6	41	-	55	(L)SKDIGSESTEDQAME(D)	2P; Oxidation (Met)
	1963.9	1963.8	39	-	55	(N)ELSKDIGSESTEDQAME(D)	1P; Oxidation (Met)
	1989.9	1989.7	37	-	52	(K)VNELSKDIGSESTEDQ(A)	3P
	2060.7	2060.7	37	-	53	(K)VNELSKDIGSESTEDQA(M)	3P
α_{s2}-CN	900.4	900.3	58	-	65	(S)SEESAEVA(T)	P
	937.4	937.3	141	-	147	(D)MESTEVF(T)	1P; Oxidation (Met)
	1067.4	1067.3	57	-	65	(S)SSEESAEVA(T)	2P
	1089.5	1089.4	138	-	146	(K)TVDMESTEV(F)	1P
	1105.4	1105.4	138	-	146	(K)TVDMESTEV(F)	1P; Oxidation (Met)
	1252.6	1252.5	138	-	147	(K)TVDMESTEVF(T)	1P; Oxidation (Met)
	1410.6	1410.5	126	-	136	(R)EQLSTSEENSK(K)	2P
	1538.7	1538.6	126	-	137	(R)EQLSTSEENSKK(T)	2P
	1623.7	1623.6	1	-	13	KNTMEHVSSSEES(I)	2P
	1639.7	1639.5	1	-	13	KNTMEHVSSSEES(I)	2P; Oxidation (Met)
	1680.8	1680.6	124	-	136	(L)NREQLSTSEENSK(K)	2P
	1719.7	1719.5	1	-	13	KNTMEHVSSSEES(I)	3P; Oxidation (Met)
	1808.9	1808.7	124	-	137	(L)NREQLSTSEENSKK(T)	2P
β-CN	639.3	639.3	12	-	16	(E)IVESL(S)	1P
	900.5	900.4	19	-	25	(S)SEESITR(I)	1P
	1067.4	1067.4	18	-	25	(S)SSEESITR(I)	2P
	1354.6	1354.5	15	-	25	(E)SLSSSEESITR(I)	2P
	1434.6	1434.5	15	-	25	(E)SLSSSEESITR(I)	3P
	1447.7	1447.5	33	-	43	(K)FQSEEQQQTED(E)	1P
	1576.7	1576.6	33	-	44	(K)FQSEEQQQTEDE(L)	1P
	1689.8	1689.6	33	-	45	(K)FQSEEQQQTEDEL(Q)	1P
	1775.8	1775.7	12	-	25	(E)IVESLSSSEESITR(I)	3P
	1855.6	1855.6	12	-	25	(E)IVESLSSSEESITR(I)	4P
κ-CN	968.4	968.4	145	-	152	(A)TLEDSPEV(I)	1P
	1734.7	1734.7	147	-	161	(L)EDSPEVIESPPEINT(V)	1P
Lactophorin	1226.5	1226.51	34	-	43	(L)SKEPSISRED(L)	1P
	1306.6	1306.5	34	-	43	(L)SKEPSISRED(L)	2P
	1419.6	1419.56	34	-	44	(L)SKEPSISREDL(I)	2P
	1499.7	1499.5	34	-	44	(L)SKEPSISREDL(I)	3P

Figure 14. Major constituent of CPP III. The red letters depict phosphorylated serine residues and possible phosphorylation sites in non-phosphorylated serine, threonine and tyrosine amino acids. Adapted from [127].

CHAPTER III

Improvement of the anti-allergic effect of casein phosphopeptide (CPP III) in vivo by phosphorylation

1. Abstract

Bovine milk protein-derived peptides are considered potential modulators of various immunological responses in our bodies. Among them, a proteolytic digest of milk casein named CPP III has exhibited diverse biological activities related to the phosphate groups attached to its amino acid sequence. We hypothesized that modification of this peptide by phosphorylation will enhance its bioactivity towards altering allergy associated cytokine profile and antigen-specific immune response. This present study aimed to assess whether oral intake of P-CPP III can inhibit OVA-induced IgE mediated allergic reactions through enhancing a dominant Th1 immune response. Female BALB/c mice were sensitized twice intraperitoneally with OVA at an interval of 14 days then orally fed P-CPP III for 6 weeks. After feeding duration, the mice were orally challenged with 50 mg of OVA. Total and specific IgE and IgA, specific IgG1 and IgG2a in serum and IL-4 in OVA primed spleen (SP) cell supernatants were measured by ELISA. P- CPP III suppressed Total and specific IgE secretion in serum. Mice fed P-CPP III exhibited low levels of OVA-specific IgG1 and increased OVA-specific IgG2a. Further, P-CPP III suppressed IL-4 production in SP cells. The results suggest that P-CPP III treatment can inhibit allergen-specific IgE modulated allergic reactions in murine food allergy model through shifting from Th2 to Th1 immune response.

2. Introduction

Even though food is very essential for life, it is considered to be the major determinant of most chronic diseases such as allergies. Severe IgE food-induced allergic reactions are responsible for a variety of symptoms that involve the skin, gastrointestinal tract, and respiratory tract [128]. Food allergy affects between 5% and 7.5% of children and between 1% and 2% of adults [129]. The prevalence rates of allergic diseases have recently plummeted in rapidly developing societies like Japan becoming a major health problem. The number of people suffering from allergies to food and other allergens in developing countries also continue to rise considerably to match those of the developed world. The expansion in population with allergic disorders brings about inconvenient symptoms such as dermatitis, Fever, sneezing, running nose and itching eyes which reduces the quality of life and exhaust the patients. This dramatically increased burden of allergic diseases will result in substantial financial costs incurred by affected individuals. It will also impact the world's economies through; an escalation in economic costs related to health care and loss of productivity at workplaces. Antigen-specific IgE antibodies and mast cells reactions are the main suspects in facilitating most immediate hypersensitivity reactions or type I allergic reactions. We cannot deny that therapies for allergic diseases have improved over the years as far as battling the inflammatory

processes and providing symptomatic relief, but those therapies remained non-curative. Therefore, Food dietary compounds that can safely and effectively suppress or inhibit the formation of antigen-specific IgE, prevent severe IgE-mediated, food-induced anaphylactic reactions or promote oral immune tolerance will be an important discovery in the fight against food allergic diseases [130].

Bovine milk protein is a major source of valuable bioactive peptides encrypted within its amino acid sequences and released upon enzymatic hydrolysis during gastrointestinal transit or upstream during food processing [131]. The enzymatic or gastrointestinal digestion of milk protein results in the production of functionally and physiologically active peptides. Meisel stated that bioactivities of peptides encrypted within milk protein are latent until released and activated [132,133]. This was also alluded to by Park and Nam who emphasized that most of the bioactivities of milk proteins are absent or incomplete in the original native protein, but full activities are manifested upon proteolytic digestion to release and activate bioactive peptides from the original protein [134]. A wide variety of biological properties from this milk peptide has been reported. This includes anti-viral effect [135], immunomodulatory/immunostimulatory [7], anti-microbial [136], Angiotensin – I – Converting Enzyme (ACE) Inhibitory [137], Allergic symptoms reduction [138], Opioid activities, and also enhance calcium absorption

[127].

CPPs are phosphorylated peptides which result from the proteolysis of calcium-sensitive caseins and they possess a phosphoserine (SerP) rich region [7]. These peptides have multiple bioactive functionalities. Various researchers have widely reported on the bioactivities of CPP especially the commercial CPP III, which consist mainly of β-casein (1-28) and α-s2 casein (1-32). Previously other researchers reported how CPP III enhances intestinal IgA through promoting the production of IL-6 in mice [17] [139], and also its immune-enhancing activities by stimulating proliferation by mouse SP cells [140]. However, they did not fully explore and elucidate its mechanism of action relating it to its possession of phosphate groups.

In this research study, we scrutinized the effects of chemically modified CPP III through phosphorylation on serum IgE production from OVA-sensitized BALB/c mouse. Additionally, we observed the effect of orally fed CPP III on the OVA-induced allergic response through immunoglobulin production, cytokine expression, and histamine level measurement. We further determined its preventative effect against mast cell degranulation.

3. Materials and Methods

3.1 Materials

CPP III consisting of 90% of casein phosphopeptides, such as bovine α-s2 casein (1-32) and β-casein (1-28), was provided courtesy of Meiji Co., Ltd. (Tokyo, Japan). Antibodies and reagents used for flow cytometry analyses were purchased from BD Biosciences Co., Ltd. (San Jose, CA, USA). All other reagents were of analytical grade.

3.2 Preparation of phosphorylated CPP III and dephosphorylated CPP III

CPP III was phosphorylated according to the method of Li. *et.al* which we also utilized in our previous study [135]. Briefly, CPP III was dissolved at a concentration of 1mg/mL in 0.1M sodium pyrophosphate buffer at pH 4.0, and then lyophilized. The lyophilized sample was dry heated at 85 °C for 5 days. The dry-heated sample was dissolved in deionized water and dialyzed to remove free phosphate for 2 days using spectra/Por regenerated cellulose of 1 kDa MWCO. It was then lyophilized to obtain phosphorylated CPP III. On the other hand, CPP III was dephosphorylated by incubating it for 2 days with bovine alkaline phosphatase at 37°C as reported by [88]. Hereafter, native CPP III, phosphorylated CPP III and dephosphorylated CPP III are referred to as N-CPP, P-CPP, and D-CPP, respectively.

3.3 Animals

Female BALB/c mice were purchased from Charles River (Tokyo, Japan) at five weeks of age. All the mice were housed in a group of six per cage and the animal room was maintained at a controlled temperature of (20-24°C), humidity (40-70%), with an alternating 12 hr/12 hr light-dark cycle (lights on at 8:00 am and off at 8:00 pm). All animal experiments were performed following the animal experiment protocol approved by the Institutional Animal Care and use committee.

3.4 OVA-sensitized allergic model mouse

Model mice sensitized with OVA were prepared according to previously described protocols [141,142]. The mice were divided into five groups (six mice per group). For the OVA sham-treated control, N-CPP, P-CPP, and D-CPP groups, naïve mice were immunized with 50 μg OVA dissolved in 100 μL of phosphate-buffered saline (PBS) and alum adjuvant by intraperitoneal injection. Mice were then given a booster dose by intraperitoneal injection with 50 μg OVA and alum adjuvant at 14 days after the initial immunization. On day 21, after confirming the elevated serum levels of specific IgE antibodies via enzyme-linked immunosorbent assay (ELISA), the mice were provided *ad libitum* access to homemade feed containing 50 mg N-CPP, P-CPP, or D-CPP per 100 g

MF pellet for 6 weeks. Mice were then orally challenged with 50 mg of OVA on day 72. The body temperature of the mice was measured from the rectum at 0, 10, 20, 30, 40, 50, and 60 minutes using a Weighing environment logger (AD1687, A&D Company, Limited, Tokyo, Japan). Mice in the naïve control group were provided a commercial pellet diet on the same schedule. All mice were sacrificed by asphyxiation with CO_2, and their sera and small intestines were harvested. For cytokine analyses, SP cells harvested from mice in the naïve, OVA sham-treated control, N-CPP, P-CPP, and D-CPP groups were incubated with 50 μg/mL (final concentration) OVA at 37 °C in a humidified atmosphere with 5% CO_2 for 72 h. Cells in the naïve control group were incubated with PBS rather than with allergens.

3.5 ELISA quantification of cytokine levels in splenocytes

Levels of cytokines secreted in murine splenocyte and payer's patch cell culture supernatants including IL-4, and Interferon- γ were measured using sandwich ELISA. Specific antibody sets (Pharmingen, San Diego, CA, USA) were used to assay IL-4, and Interferon- γ. High binding 96 microwell plates (Greiner bio-one, Germany) were coated with 100μL of rat anti-mouse IL-4, IL-6 and IFN-γ (BD Biosciences) in 1 M bicarbonate buffer pH 6. The coated plates were incubated overnight at 4°C. Plates were washed three

times with 200 µL of PBST and blocked with 200µL of 3% Skim milk in PBS at 37°C for 90 minutes. The plates were further washed three times with PBST and 100 µL of standard cytokines.

3.6 Temperature measurement post oral challenge

After oral challenge with OVA the body temperature of the mice was measured from the rectum at 0, 10, 20,30,40,50 and 60 minutes using Weighing environment logger (AD1687, A&D Company, Limited, Japan).

3.7 Allergic score

Mouse allergic symptoms were observed and scored from 40 to 90 minutes post oral challenge dose according to the previous description by Li *et al.* [143]. Anaphylactic symptoms were scored as follows: 0, no symptoms; 1, scratching and rubbing around the nose and head; 2, puffiness around the eyes and mouth, diarrhea, pilar erecti, reduced activity, and or decreased activity with increased respiratory rate; 3, wheezing, labored respiration, and cyanosis around the mouth and the tail; 4, no activity after prodding or tremor and convulsion; and 5, death.

3.8 ELISA analysis for OVA-specific IgE, IgA, total IgE, IgA, and OVA-specific IgG1, IgG_{2a}

Blood samples attained were kept at room temperature for one hour. They were then centrifuged at 1000 x g, 4°C for 15 minutes to collect the serum. Levels of Total IgE and IgA, OVA-specific IgE and IgA, IgG_1 and IgG2a in the blood serum were measured via the sandwich ELISA. HRP-labeled antibodies and hydrogen peroxide with o-phenylenediamine were used as the substrate. Anti-mouse IgE and IgA, and HRP-Conjugated anti-mouse IgE and IgA antibodies used in this experiment were purchased from Pierce (Rockford, IL, USA). OVA-specific IgE, IgA, IgG1, and IgG2a were detected following the same protocol as described for total IgE and IgA, except in OVA-specific IgE and IgA the coating antigen was substituted with OVA (1mg/mL).

3.9 Flow cytometric analysis for Treg and Tfh cell populations

We employed flow cytometry in assessing changes in cells population which included the number of Tregs and T follicular helper (Tfh) cells. OVA-sensitized mice were orally fed differently phosphorylated CPP III for 6 weeks. They were then sacrificed and PP and SP cells were collected. Tfh cells were stained with FITC-labelled anti-CD4, PE-labelled anti-CXCR5, and APC Anti-mouse CD279 (PD-1) antibodies (1:1000) (BD

Biosciences) for detection. The resultant cell samples were examined with a FACSCalibur flow cytometer with cell quest software (BD Biosciences).

3.10 Statistical analysis

Data were expressed as means ± SD. The data were also subjected to analysis of variance (ANOVA) and Tukey's multiple comparison tests. The significance level of $P<0.05$ was considered significantly different

4. Results

4.1 Phosphorylation and dephosphorylation of CPP III

P-CPP was prepared by dry-heating in the presence of a pyrophosphate buffer. After dry-heating at 85 °C for 5 days, the phosphorous content of P-CPP III was 18.82 μg/mg of protein which was significantly higher than that of N-CPP which had approximately 9.8 μg/mg of protein and D-CPP which had levels below detection. In contrast, dephosphorylation was conducted through incubating CPP III with bovine alkaline phosphatase at 37 °C for 3 days to remove phosphate groups. D-CPP III had significantly below detectable phosphorus content as compared to N-CPP III.

4.2 Effects of oral administration of P-CPP against OVA-induced IgE and allergic reactions

Ingestion and absorption of OVA are capable of inducing severe systemic anaphylactic reactions in the OVA allergy model mouse. The effect of orally administered P-CPP on food-allergic reactions triggered by exposure to allergen was studied. Temperature changes were recorded immediately after oral challenge with OVA and significant body temperature decreases of −1.47±0.94°C and −1.67±1.71 °C were observed at 10 and 20 minutes in the sham-treated control and D-CPP groups, respectively; the naïve, N-CPP, and P–CPP groups showed body temperature changes of 0.93±1.04, −0.37±0.47, and −0.43±0.43°C, respectively (**Figure 15B**). Systemic anaphylactic symptoms were recorded in the sham-treated control and D-CPP groups after 40 min, which showed average scores of 1.67±0.56 and 2.0±0.37, respectively. In contrast, the average scores were significantly lowered in the N-CPP and P-CPP groups to 0.33±0.21 and 0.66±0.21, respectively compared to the sham and D-CPP group ($P < 0.05$) (**Figure 15C**). No symptoms were observed for the naïve group.

We investigated whether the development of antigen-specific immune response could be regulated by oral treatment with P-CPP. All mice except the naïve were sensitized twice by intraperitoneal immunization with 50 μg of OVA at an interval of 2

weeks before oral challenge with 50 mg of food allergy model OVA and only PBS for the naïve group. Mice were then provided an ad-lib oral access to feed containing 0.05% N-CPP, P-CPP, D-CPP, and commercial feed for the naïve group and sham-treated group **(Figure 15A), (Table 1V).**

Table IV Treatment groups and diets. The naïve group was not sensitized, not orally challenged and fed a commercial mice diet; the Sham-treated group was sensitized with OVA, orally challenged with OVA and not treated only fed a commercial diet. N – CPP III, P -CPP III and D – CPP III were all sensitized and orally challenged with OVA and then fed homemade diet containing 0.05% of N-CPP, P-CPP, and D-CPP III respectively.

Group	Sensitization	Oral challenge	Treatment
Naïve	**PBS**	**PBS**	**Normal diet**
Sham	**OVA + Al(OH)3**	**OVA**	**Normal diet**
N – CPP III	**OVA + Al(OH)3**	**OVA**	**0.05% N – CPP III**
P – CPP III	**OVA + Al(OH)3**	**OVA**	**0.05% P – CPP III**
D – CPP III	**OVA + Al(OH)3**	**OVA**	**0.05% D – CPP III**

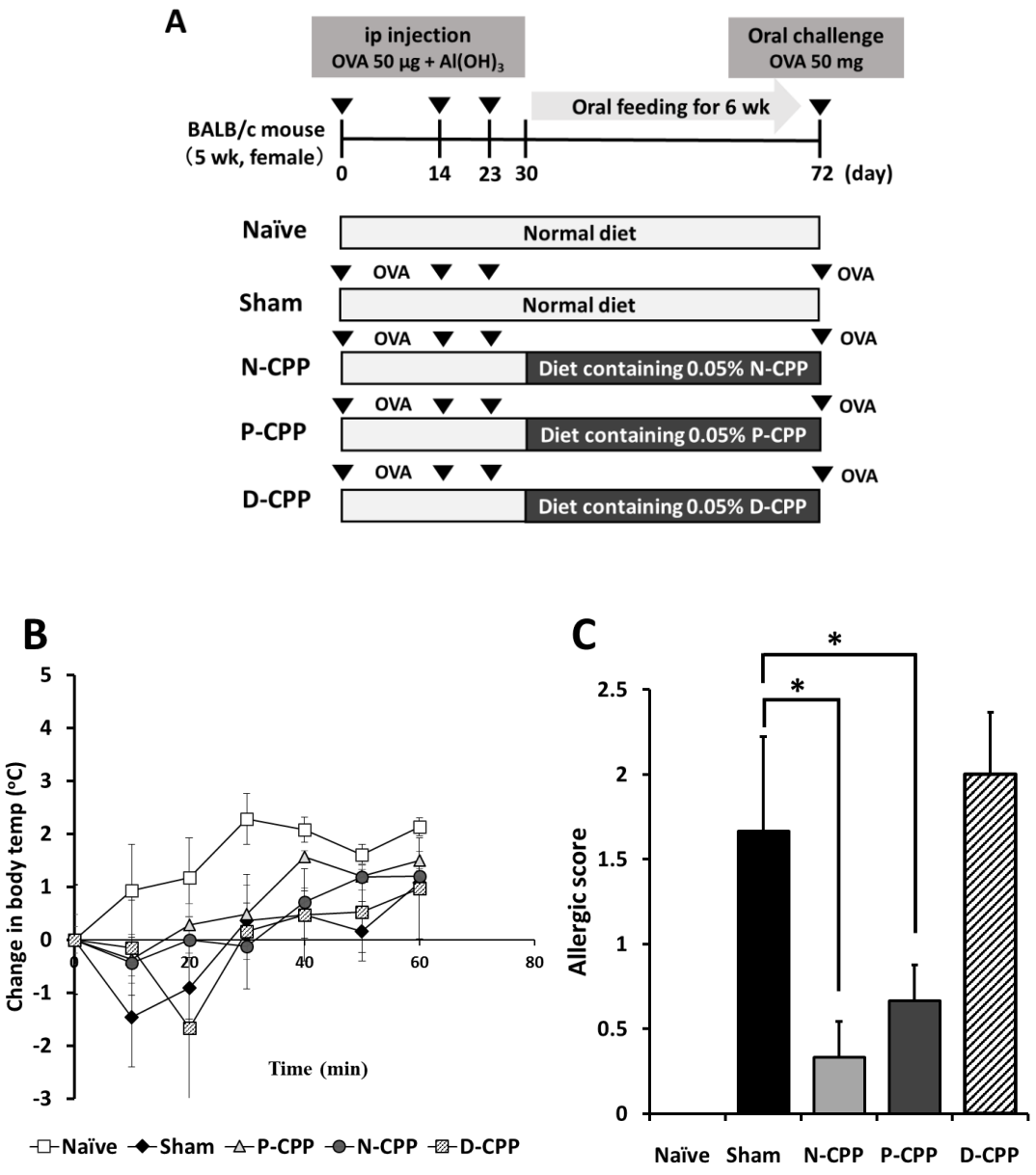


Figure 15. Experimental layout for the in vivo experiment and systemic anaphylactic reactions post oral challenge with OVA. **(A)** BALB/c mice (n=6) were sensitized with 50 μg of OVA plus Aluminum hydroxide $Al(OH)_3$ as an adjuvant. **(B)** Body temperatures changes were recorded at 0, 10, 20, 30, 40, 50, and 60 minutes, **(C)** Allergic scores and anaphylactic symptoms were evaluated and scored from 40-90 minutes after oral challenge, Bars represent mean ± SE of individual mice in the group.

4.3 Effects of oral feeding of CPP III on immune response of OVA sensitized mice

Mice orally treated with P-CPP significantly suppressed the production of the total IgE antibody in blood serum as compared to the sham-treated and D-CPP group **(Figure 16A)**. A similar decrease was also noticed in the levels of OVA-specific IgE in serum **(Figure 16B)**. The mice in the P-CPP treated group exhibited higher levels of Total IgA compared to sham-treated, N-CPP and D-CPP treated groups. In contrast, specific IgA levels did not significantly differ among the groups (**Figure 16C and 16D**) respectively.

The OVA-specific IgG1 (Th2 associated) levels in P-CPP treated group was significantly lower ($p < 0.05$) than the sham-treated group while on the other hand the OVA-specific IgG2a (Th1 associated) was remarkably increased ($p < 0.05$) in P-CPP group as compared to the sham-treated group **(Figure 16E and 16F)** respectively.

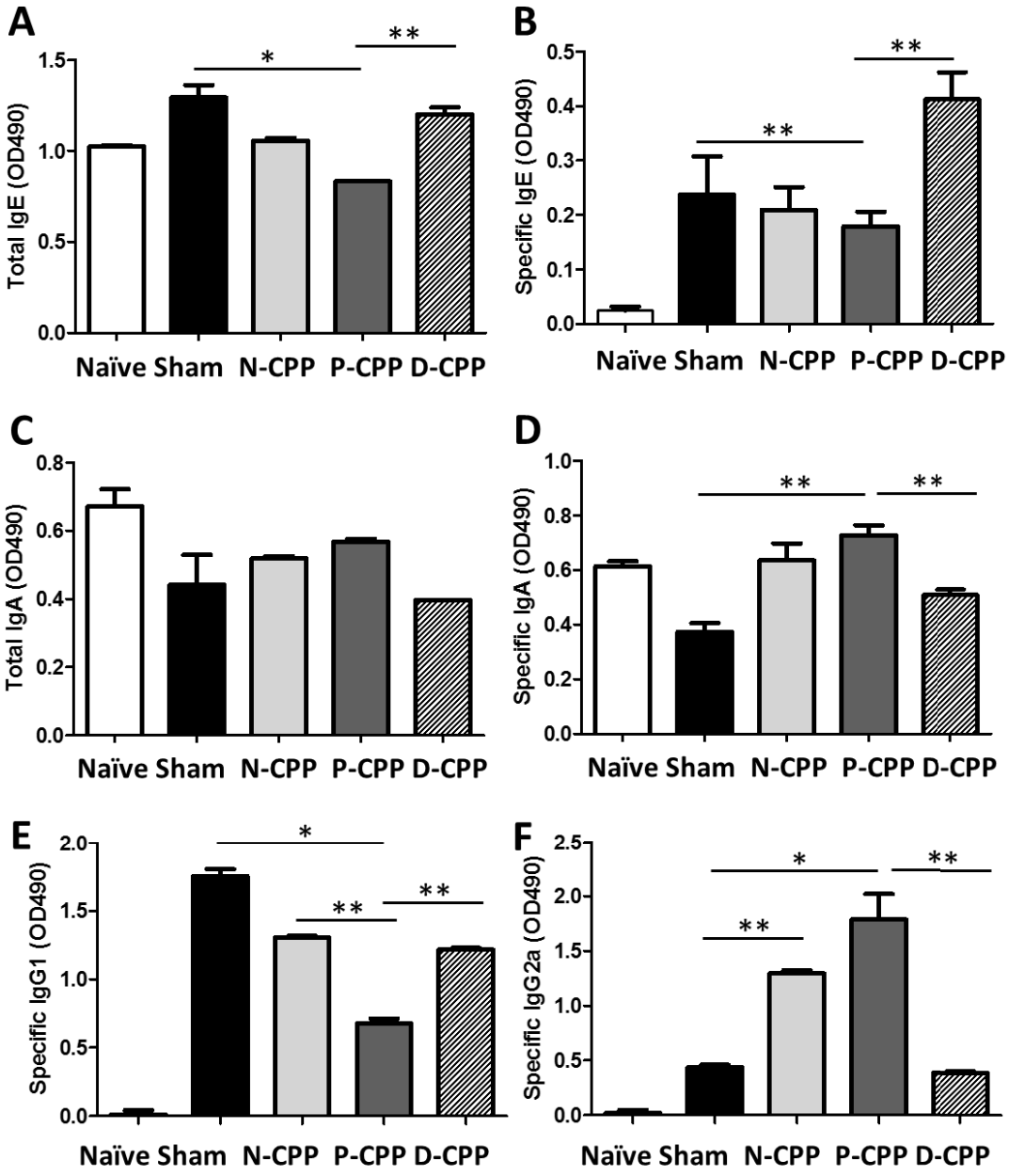


Figure 16. Effects of oral feeding differently phosphorylated CPP III on the immune response of OVA-sensitized mice. As depicted by serum levels of OVA-specific or total IgE and IgA in OVA-immunized mouse. **(A)** Serum levels of total IgE, (**B)** specific IgE, **(C)** total IgA **(D)** specific IgA. The subclasses including **(E)** Specific IgG1 and **(F)** specific IgG2a were detected by Sandwich ELISA. Bars represent mean ± SE of individual mice in the group.

4.4 Effects of orally fed phosphorylated CPP III on OVA-induced cytokine production in vitro

To establish the regulatory effects of P-CPP treatment upon Th1/ Th2 cell responses, we measured the production of IFN-γ (Th1- associated cytokine) and IL- 4 (Th2-associated cytokine) in SP culture cells. We also measured Transcription factor GATA-3 and IL-4 mRNA expression. The oral administration of P-CPP III to the mice significantly enhanced the secretion of IFN- γ , while it inhibited the production of IL – 4 cytokines **(Figures 17A and 17B)**, respectively. It also downregulated transcription factor GATA-3 and IL-4 gene induction **(Figure 17C)**.

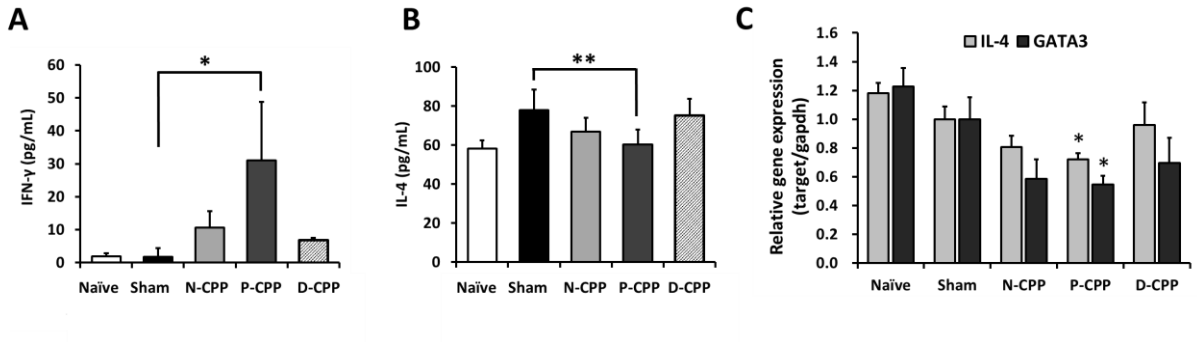


Figure 17. Effects of oral feeding differently phosphorylated CPP III on cytokine production of OVA-sensitized mice. OVA-sensitized mice were fed different phosphorylated CPP III for 6 weeks and spleen and peyer's patch cells were harvested and incubated with at 37℃ in a humidified atmosphere with 5% CO_2 for 72 hrs to determine cytokine levels. **(A)** IFN-γ and **(B)** IL-4, **(C)** GATA-3 and IL-4 mRNA expression.

4.5 Effects of orally fed phosphorylated CPP III on differentiation and population changes of Treg and Tfh

We evaluated the effect of orally administered P – CPP on the differentiation level of Tregs and Tfh cell population changes from SP and payer's patches (PP) of murine allergy model. This was based on the flow cytometric analysis after feeding P-CPP for 6 weeks. In this paper, we counted $CD25^{+}$ $Foxp3^{+}$ T cells as Tregs. The results showed that the population of Tregs in cultured splenocytes of P – CPP treated group was higher (16.63±0.83%) compared to that of sham-treated group (12.9±0.29%) (**Figure 18A**) and the resulting representative flow cytometric images depicting percentage of $CD25^{+}$ $Foxp3^{+}$ cells for each treatment group as determined by fluorescence-activated cell sorting (FACS) is shown in **Figure 18B**. The Tregs were comparatively lower in PP cultured cells compared to the SP cultured cells. The percentage of cells in P-CPP treated group 9.7±0.64 % was higher than that of the sham-treated group 7.1±0.5 % but lower than that of N-CPP and D-CPP at 10.35±0.23 and 10.2±0.38 %, respectively (**Figure 18C**). The resultant cytometric image is shown in **Figure 18D**.

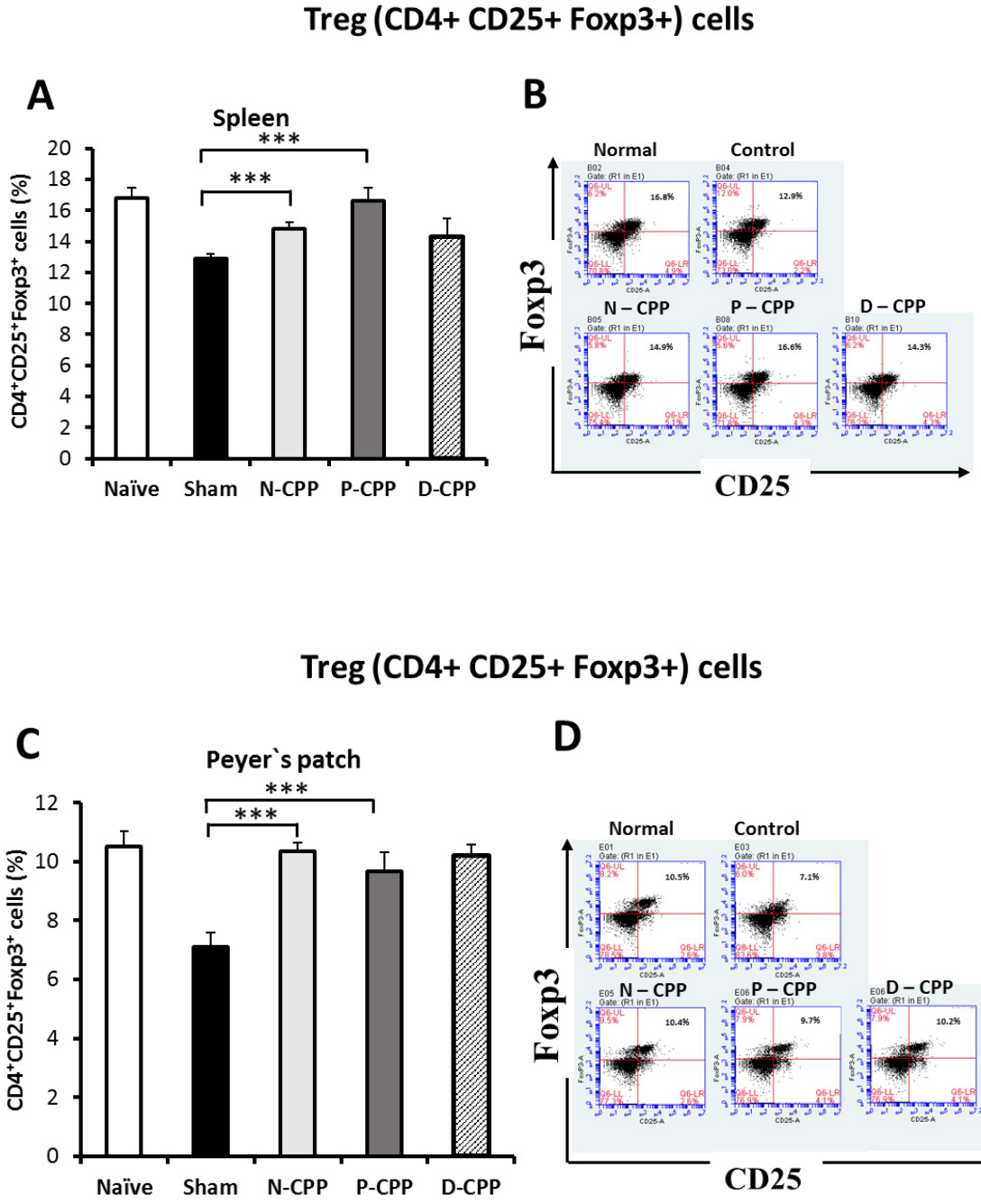


Figure 18. Effects of oral feeding differently phosphorylated CPP III on Treg cells population. Percentages of Treg cell populations in cultured **(A)** spleen and **(C)** peyer's patch cells of OVA-sensitized mice. Representative flow cytometric image by FACS for treatment groups **(B)** SP and **(D)** PP.

We also determined the proportion of Tfh cell population changes from SP and PP after the oral administration of P-CPP III. The population of Tfh cells in splenocytes of mice orally fed P-CPP was higher (3.02%) compare to that of sham-treated mice group (1.8%) (**Figures 19A**). The same trend was observed in PP (3.56%) Tfh population for P-CPP against (2.35%) for sham-treated mice (**Figures 19C**). The resulting flow cytometric representative image for SP and PP Tfh cell population across the groups is depicted in **Figures 19B and 19D,** respectively.

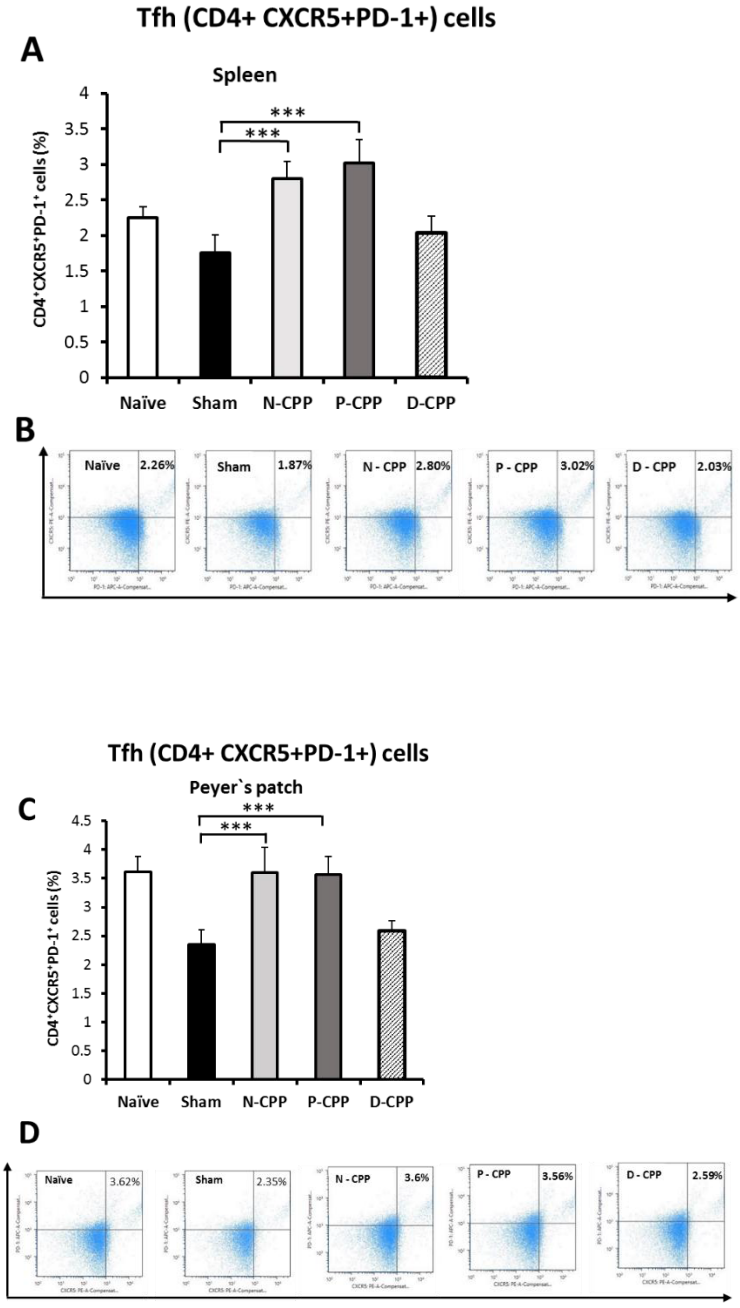


Figure 19. Effects of oral feeding differently phosphorylated CPP III on T follicular helper (Tfh) cell populations. Changes in Tfh cell populations in cultured **(A)** SP and **(C)** PP cells of OVA-sensitized mice. Representative flow cytometric image by FACS for treatment groups **(B)** SP and **(D)** PP. $p<0.001$.

5. Discussion

Allergic diseases are brought about by the failure to develop and sustain oral immune tolerance to well-known and harmless allergens including aero-allergens and foods [144]. Overall, food-based allergies are characterized as IgE-mediated hypersensitivity reactions. This is whereby naive $CD4^+$ T cells are induced to differentiate into helper Th2 cells that sequentially promote the production of IgE via the production of cytokines like IL-4 and IL-5. Effective tolerogenic immune response towards allergens is a key factor in preventing the pathogenesis of allergic diseases, In this regard, numerous research works [145–147] have recommended Treg cell-mediated immune suppression as a likely ameliorative remedy.

In the present study, I established that oral intake of highly phosphorylated casein phosphopeptide could attenuate type-I allergic response in OVA model mice through the induction of Tfh and Treg which are inducers of IL-6 and promoter of oral tolerance respectively. Bioactivities of casein hydrolysates have been widely studied. In a previous report, Bamdad *et al*. showed that casein hydrolysates exhibited anti-inflammatory and antioxidant properties by significantly reducing nitric oxide and suppressing the synthesis of pro-inflammatory cytokines (TNF-α and IL-1β) in lipopolysaccharide-stimulated RAW 264.7 macrophage cells [148].

In this study after oral challenge with OVA, a marked decrease in body temperature or hypothermia was observed in the Sham and D-CPP treated group, while it was practically unchanged in the naïve, N-CPP and P-CPP group. Mice orally provided P-CPP also exhibited a lower allergic score as compared to the sham-treated and D-CPP group. This similar IgE systemic anaphylaxis reaction was reported by Makabe-Kobayashi *et al*. who linked it to the activities of mast cell-derived histamine using Histidine decarboxylase gene knockout (HDC -/-) mice, which lacks histamine [149].

IgE antibodies are the mediators of most food allergic reactions and overall, food-based allergies are characterized as IgE-mediated hypersensitivity reactions [150]. Burton and Oettgen emphasized that IgE binds to two main receptors, the high affinity FcεRI and FcεRII. And this interaction of IgE with its receptors expressed on mast cells is considered to have a significant role in maintaining a sensitized state in food allergic patients by focusing on stimulating memory T and B cell responses, it is also considered to amplify the Th2 and IgE response [144] [151]. As such discoveries of safer and effective methods to inhibit allergen specific-IgE production are vital in controlling type-I allergic reactions. After oral feeding of P-CPP for 6 weeks, we noticed a significant decrease in levels of total IgE and OVA-specific IgE in serum, in contrast, there was an increase in total IgA and OVA-specific IgA. IgA is reported to mediate both pro and anti-inflammatory effects

in innate immune cells [152]. The protective effect of IgA antibodies against infectious antigens in the gut is well documented but little is elucidated on the role of IgA in food allergy. Previously, some researchers showed that oral ingestion of different preparations of commercial casein phosphopeptides, including CPP-I and CPP-III, by mice, enhanced intestinal and milk IgA and IL-6 expression [7,17,139]. Recently Kiewiet *et al*. in their review emphasized that the administration of different protein hydrolysate like common carp egg hydrolysate in vivo can increase the secretion level of IgA and IgA^+ cells. They point out that IgA functions in the clearance of toxins when it is released in the gut and it is easily measured in feces [75]. This suggests that the peptide might not enhance only IgA, but also various immunoglobulins in the immune system including the T cell family. In our previous study, we found that P-CPP enhanced antiviral activities against feline calicivirus infection by inducing anti-viral cytokines such as IFN-α and IFN-β. Phosphorylated CPP-III exhibited stronger anti-viral activities than its native and dephosphorylated forms. We next demonstrated that the stronger anti-viral activities of P-CPP were dependent on the negative electrostatic charge of the phosphate groups attached to the amino acid chain [135]. Overview of recent researches [75] has indicated that dietary ingredients especially immunomodulatory protein hydrolysates exhibit the capacity to attenuate food allergic reactions. Included among this protein hydrolysate are

milk casein hydrolysates, egg yolk digests, yellow pea, shark protein, and soybean hydrolysates. They exhibit their immunomodulatory properties through the upregulation of pro-inflammatory cytokines (IL-10, TNF-α, IL-6, and IFN-γ), an increase of IgA^+ cells, elevation of secretory IgA in the gut, increase in Treg in the SP and reduction of IgE production [153–155]. We also observed that the secretion of Th2-driven IgG1 was markedly inhibited by oral administration of P-CPP, whereas secretion of Th1-driven IgG2a was significantly increased. This hypoallergic properties of some milk protein hydrolysates including caseins and whey are in agreement with studies by Kiewiet *et al*. and Pan *et al.* who reported on the immunomodulatory and hypoallergenic properties of milk protein hydrolysates through enhancing regulatory T and B cell frequencies [154,156].

We then determined the cytokine levels in culture supernatants of P-CPP administered mice splenocytes. We observed that the oral ingestion of P-CPP by mice profoundly increased the splenocytes production of Th1 related IFN-γ and significantly suppressed the secretion of Th2 related IL-4 cytokine. Naturally, IgE synthesis is considered to be due to the development and activation of Th2 cells and B cells. Naive $CD4^+$ T cells are induced to differentiate into helper Th2 cells that sequentially promote the production of IgE via the predominant production of cytokines such as IL-4. The IL-

4 plays an important role in inducing class switching of the IgE isotype and its production [157]. Excessive secretion of IL-4 by Th2 is associated with increased IgE levels and subsequent allergic reactions. In contrast, based on the production patterns of cytokines by helper T cells, a Th1 type immune response occurred that mainly involved secretion of cytokines such as IFN-γ, which inhibit IgE and IgG1 secretion and enhance IgG2a secretion [158]. Th1 cytokines are possibly the ones that inhibited the secretion of IgG1 and enhanced that of IgG2a above. Our results suggest that the administration of P-CPP skewed the balance from Th2 towards Th1 dominance. The development and activation of Th2 cells and B cells are thought to increase IgE synthesis and IL-4 and IL-5 cytokine production [130]. This shift also confirms that P-CPP has a modulating ability on Th1/Th2 balance to down-regulate Th2 response.

Numerous studies [145–147] have recommended Treg cell-mediated immune suppression as an ameliorative remedy. In this study, the ability of P-CPP to induce Treg differentiation was established in an OVA mouse model and egg allergy model. Treg is considered a specialized subpopulation of T cells that is capable of regulating both Th1 and Th2 immune response to allergens. Their suggested functions according to previous literature include the prevention of autoimmune diseases by maintaining oral tolerance, suppression of allergy, asthma and pathogen-induced immunopathology [159]. The

differentiation level of Treg was assessed based on the flow-cytometric analysis in the P-CPP feeding regime. P-CPP ingestion for 6 weeks displayed a very lethal immunosuppressive effect by significantly increasing the Treg cell population. Treg is said to suppress a variety of physiological and pathological immune responses. It is proposed that they achieve this through suppressing other lymphocytes at the molecular level *in vivo* and *in vitro*. And it is proposed there may be a key suppressive mechanism shared by each foxP3 + *in vivo* and *in vitro* in mice and humans [160].

We also observed the induction and differentiation of precursor cells into Tfh cells, leading to an increase in their numbers in the SP and PP cells. These cells are specialized providers of T cell help to B cells to support their activation, expansion, differentiation, and formation of the germinal centers (GC) [161]. In our study, the feeding of P-CPP to mice significantly increased the population of Tfh both in supernatants of cultured SP and PP cells. Tfh cells were reported to promote the secretion and production of IgA by enhancing the differentiation of IgA^+ B cells [162]. This increase in the Tfh cell population may have been responsible for the suppression of Th2-induced allergic reactions, including IgE production, following sensitization with OVA. Previous studies showed that Tfh could suppress Th2 type immune response with minimal negative effects on the Th1 response. Achieving a balance and modulation between the Th1/Th2 immune

response is regarded as the best immunotherapy strategy for allergic diseases [163,164]. While Tfh cells have been notably known to produce IL-21 which inhibits class switching to IgE, there are other existing reports of Tfh cells producing other cytokines, among them IL-4 which may determine the antibody produced [165,166]. Recent studies, which are consistent with these findings observed the development of IL-4 producing Tfh cells and how they might be playing a critical role in IgE production in peanut allergy [167], [168], and also the discovered type 2 subset (Tfh2) within the Tfh which are considered as the major player that secretes IL-4 and promotes the isotype switching to IgE after allergen exposure and during intestinal helminth infection [169]. Despite all these recent findings, the functions of Tfh remains less clear and even though it's indicative from most researches that their secretion of IL-4 is necessary for IgE production it does not definitely rule out a contribution of Th2 cell-derived IL-4 in this particular immune response. As such further investigation is required to understand the development and/ or functions of Tfh and its relationship with effector Th1 and Th2.

Peptides liberated from milk by enzymes were demonstrated to possess immunomodulatory properties [170] and should be considered as potential modulators of various regulatory processes in the body. Casein phosphopeptides are released from casein through proteolytic digestion in the small intestine. They were reported to resist

further digestion by intestinal proteinases or bacterial proteinases in the digestive tract and accumulate in the most distal part of the small intestine [10,171]. Review by Scherer *et al.* pointed out that allergen-specific immunotherapy also includes immunotherapy with modified proteins that are designed to be hypoallergenic to reduce the risk of immune reactions towards food proteins [172]. Ueno et al developed an edible hypoallergenic casein hydrolysate which reduced the antigenicity for casein-specific antibodies while retaining the immunogenic epitopes. They achieved this through digesting casein at alkaline pH [173]. Recently Kim *et al.* using intact casein as an allergen demonstrated that mesenteric lymph node (MLN) IL-10 producing $CD5^+$ B cell suppressed casein induced allergic responses in mice [174]. It was deduced from these findings by Kiewiet *et al.* that casein might have been digested in the intestine of mice, after which the newly formed peptides derived from casein increased the Bregs which induced oral tolerance [75]. These findings suggest the high possibility of casein and its hydrolysates including P-CPP`s hypoallergenic properties against cow`s milk allergy.

In the past, several studies have assessed the modification of allergenic proteins to be used in immunotherapeutic treatments. Of these modifications, Maillard-type glycosylation demonstrated some efficacy in modifying surfaces of target proteins [175]. In this method attachment of polysaccharides such as glucomannan and xyloglucan

through Maillard reaction exhibited some masking of IgE epitopes of allergenic proteins resulting in reduced IgE binding capacity in sera of allergic patients. This conjugate also was shown to shift the Th1/Th2 balance in spleen towards a Th1 dominated immune response [176]. Polysaccharide moieties by nature have long side chains, as such this might hamper their ability to reach and mask epitopes located at the interior of proteins. The addition of phosphate groups to a protein might not only function as a more effective method to mask epitope site of allergenic proteins but also as a strong enhancer of immunomodulation as evidenced by its previous promotion of type-I IFN secretion [135].

A recent study showed that phosphorylated buckwheat hypoallergenic protein P-Fag e 2 more strongly suppressed some Th2-induced allergic responses in a murine model of buckwheat allergy compared to its native form [177]. This suppressive capability occurred because of increased secretion of total and specific IgA and the induction of Tfh cells regulated by dendritic cell-derived IL-6. Thus, the increase in IgA and induction of Tfh and Treg suppressed OVA-specific and total IgE by P-CPP possibly through a similar mechanism as used by P-Fag e 2. In this study, we have not evaluated the immunomodulatory activities of P-CPP against cow`s milk allergy and its hypoallergenecity. Further study will be needed to elucidate that and specific pathways involved in shifting the immune response.

Chapter IV

Conclusions

In this study, it was demonstrated that enhanced phosphorylated CPP III shows anti-viral activity against FCV infection, and this activity is dependent on the phosphate groups. These findings suggest that CPP III holds potential as an accessible and cheaper alternative to the food-based nutraceutical ingredients and supplements used to boost the immune system against viral infections. This information will contribute to the development of safe and effective anti-viral agents using natural dietary compounds in conjunction with modification techniques to enhance their functionality. Further investigation of the potential anti-viral and immunostimulatory benefits of Casein`s bioactive peptides and other functional compounds are deemed necessary. Besides, investigations aiming to clarify the underlying mechanisms of this activity are necessary. Allergy is a worldwide social problem, and current therapeutic strategies cannot halt the explosion of allergic patients. Moreover, current anti-allergic therapies still have problems such as varied efficacy among individual patient and high medical expenses. Those situations strongly suggest a necessity for the development of safer and more effective strategies for the intervention of allergic disorders. In the present study, I aimed to explore novel allergy preventive and therapeutic molecules upon targeting IgE-mediated mast cell response.

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Acknowledgments

First, I would like to thank God above for the precious and wonderful gift of life and vigor to complete my studies. I wish also to express my sincere gratitude to the Government of Japan through the Ministry of Education, Culture, Sports, Science and Technology (MEXT) for the financial assistance on my entire studies, research needs and general life in Japan. I would not forget to thank the Government of Botswana, Ministry of Agriculture, and Department of Agricultural Research (DAR) for having seen it fit to release me from my duties to upgrade my skills to benefit the great Republic of Botswana.

My most sincere appreciation goes to my supervisors Professors Soichiro Nakamura, and Shigeru Katayama first for taking me in as their second Ph.D. student from Africa and for their enduring support, guidance, and motivation that made my research possible. I would also extend thanks to my fellow Food chemistry lab members for creating a conducive and enjoyable working time in the lab. The language was a challenge but they tried to meet halfway through Japanese and English.

I would particularly mention my colleague and junior Mr. Yuta Suzuki who we collaborated in my second study. His hardworking and persistent spirit motivated me in never giving up attaining my goal. I wish him success in all his desires in life.

Particularly I would also like to thank my fellow international students in the Food

Chemistry Laboratory; Mr Henry.M. Corpuz (Philippines), Ahmad M. Al Athmneh (Jordan), and Abdel Fawaz Bagodudo (Benin) who together from different corners of the globe we faced and conquered the challenges of unique language and culture of Nihon.

Lastly, I would like to thank my family more especially my wife Kempho Lebetwa and my two daughters Poneso Refilwe Lebetwa and Esaleone Mia Lebetwa for the unshaking moral support through prayers and emotional encouragement. To all, I say God abundantly bless you.

Ntshepisa Lebetwa