Study on the Bioactive Phospholipids of Komatsuna (*Brassica rapa* var. *perviridis*) Juice Processed by Micro Wet Milling

July 2017

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A Dissertation Submitted to

the Graduate School of Life and Environmental Sciences,

the University of Tsukuba

in Partial Fulfillment of the Requirements

for the Degree of Doctor of Philosophy in Biotechnology

(Doctoral Program in Bioindustrial Sciences)

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CONTENTS

ABBREVIATIONS

PLs	Phospholipids
PC	Phosphatidylcholine
PLD	Phospholipase D
PA	Phosphatidic acid
PLA_2	Phospholipase A_2
LPA	Lysophosphatidic acid
GI	Gastrointestinal
MWM	Micro wet milling

CHAPTER 1
INTRODUCTION

1.1 Background

Japanese mustard spinach (*Brassica rapa* var. *perviridis*) is also known as Komatsuna in Japanese. It is a leaf vegetable, traditional and popular in the Japanese diet, and normally eaten as a kind of salad (boiled). In recent years, the Komatsuna amounts of production and consumption both increased (Fig. 1.1), and the proportion of Komatsuna consumption to production in Japan increased to approximately 86.8%, due to people pay more attention to the beneficial diet for healthy because of some studied health benefits. Thus, it is expected to improve the functional value of Komatsuna. Like, Aiso *et al.* (2014) had produced a juice mixture of fresh fruit and Komatsuna is highly effective in reducing serum cholesterol, compared to the commercial vegetable juice. In this study, the objective bioactive component was named phospholipids. Previous studies showed that, the amount of bioactive phospholipids in fresh Komatsuna has been detected to be approximately 0.89 μmol/g (wet weight), especially the content of phosphatidic acid (PA) in fresh Komatsuna has been estimated to be around 43.6% of the total phospholipids, which is high in the leafy vegetables belonging to *brassica* genus (Tanaka et al., 2012). An interesting phenomenon was observed that abundant PA was produced in the masticated and milled foodstuff (Tanaka et al., 2012), and the PA content of raw cabbage was increased more after mastication that compared to the boiled cabbage (Tanaka et al., 2009). Moreover, oral administration of PA showed an opportunity to prevent gastric

ulcer in mice, due to the formation of lysophosphatidic acid (LPA) from PA in the gastrointestinal (GI) tract by gastric phospholipase A_2 (Tanaka et al., 2013), due to LPA plays the important role in the integrity of GI tract epithelium. Therefore, a PA-rich food using fresh Komatsuna is appreciated to manufacture through the milling processing, for the preventive or therapeutic purposes of gastric ulcers, the improvement of Komatsuna functional value, and the facilitation of Komatsuna consumption.

1.2 Phospholipids

Phospholipids (PLs), the most important class of polar lipids in foods, are ubiquitous nutritional compounds because they are the major components of animal and plant cell membranes (Restuccia et al, 2012). Phosphatidic acid (PA) is a diacyl-glycerophospholipid that serves as a precursor for the biosynthesis of complex functional lipids and acts as a cellular signaling molecule (Ohlrogge and Browse, 1995; Testerink and Munnik, 2005), it has a critical role in cell membrane structure and function. For those reasons it has a possible application in the treatment of some health disorders in humans, can be used as a natural and nontoxic emulsifier and the component of drug carriers in pharmaceuticals and cosmetics as well as a component for synthesis of some new phospholipids (Beata et al., 2015). PA is produced in plant cells mainly via the hydrolysis of PLs, in particular phosphatidylcholine (PC) by phospholipase D (PLD); PA is also a precursor of lysophosphatidic acid (LPA), a simple PLs, which is generated by phospholipase A_2 (PLA_2) in gastrointestinal (GI)

tract. The transformation pathway of PC, PA and LPA, which mainly occurs during homogenization and digestion of plant tissues in the GI tract, is shown in the Fig 1.2.

LPA is a simple PLs that induces diverse biological responses such as brain development, tumor cell invasion, and wound healing in numerous types of cells through activating G protein-coupled LPA receptors (Moolenaar et al., 2004). However, the availability of endogenous LPA in animal cells or body fluids is very low. If exogenous LPA derived from plants could be applied to animal systems, it will facilitate various related biological effects of the LPA receptor (Lee et al., 2016). Recent findings revealed that the supplementation of LPA shows a protective effect on the gastrointestinal (GI) mucosa (Tokumura, 2011). Tanaka *et al*. reported that the application of LPA promotes the motility and proliferation of gastric epithelial cells and fibroblasts (HGC-27 cells and Swiss 3T3 cells), and the ingestion of LPA may influence the healing process of gastric ulceration (Tanaka et al., 2009). Adachi *et al.* also showed that oral administration of LPA is effective in avoiding stress-induced stomach ulcer in rats (Adachi et al., 2011). LPA derived from plants is equally bioactive compared to LPA in animals, and has been quantified in various parts of the plant such as leaf, seeds and root (Liu et al., 2007; Hwang et al., 2012).

Some LPA are produced from PA by PLA_2 during the digestion of foodstuff (Figure 2). LPA2, a type of LPA receptor, is presented in gastric surface lining cells, which locates just below the mucus gel layer (Tanaka et al., 2013). PLA_2 activity is highest in the lavage fluid of stomach, and hydrolyzes PA at the highest rate, due to the bicarbonate secretion by the epithelium, and because the mucosal gel layer is kept

at neutral pH. LPA can be produced from PA if the enzyme PLA_2 and the ingested PA interact in the gel layer (Tanaka et al., 2013). Furthermore, PLA_2 hydrolyzes PA to LPA more effectively than hydrolysis of PC, because gastric PLA_2 in stomach lavage fluid demands sodium deoxycholate for the hydrolysis of PC, while the enzyme does not require it when the substrate is PA. The large part of LPA provided to the GI tract upon the ingestion of foodstuff may be attributable to the PA hydrolysis (Uthe and Magee, 1971; Tanaka et al., 2013). In view of the effect of PA demonstrated here, PA-rich foodstuffs, such as cabbage leaves, radish root, and Komatsuna, are believed to be effective on gastric ulcer or diarrhea (Tanaka et al., 2012). A previous study has shown that the ingestion of fresh cabbage juice reduces the period required for the healing of stomach ulcers (Cheney, 1949). Accordingly, the exogenous plant-derived PA is worth utilizing for restoration or prevention of GI disorders.

PA-rich foodstuffs, such as cabbage leaves, radish root, and Japanese mustard spinach leaves (Komatsuna), are thought to be good sources of LPA, which is effective against gastric ulcer or diarrhea (Li et al., 2005). PA is the major structural component of cell membranes but does not accumulate at high levels in the membrane under normal conditions (Tanaka et al., 2012). However, it is rapidly and transiently generated in response to biotic and abiotic stresses in plants, such as osmotic, salinity, water deficit and temperature stresses (Hou et al., 2016). Recent studies reported that a substantial amount of PA is produced from many plant foodstuffs, especially *Brassica* vegetables, by repeated grinding, and some herbs release high amounts of PA when they are mashed. The masticated cabbage leaves contained similar PA levels

to those contained in mechanically homogenized cabbage leaves (Tanaka et al., 2009, 2012). These observations suggest that PLD is activated during grinding and mastication to form PA from endogenous PLs (Hanahan and Chaikoff, 1948). Thus, the development of PA-rich foodstuffs of Komatsuna is expected to involve the use of processing techniques such as grinding or milling. Accordingly, the formation of PA enrichment in Komatsuna during the milling is uncovered.

1.3 Micro Wet Milling System

There is wide utilization of wet milling in food processing and the pharmaceutical industry. Wet milling facilitates improvement of the physiochemical characteristics of materials, e.g., particle size, nutritional yield, water holding capacity, emulsion, foamability, and nutrition bioavailability (Aluko et al., 2009; Kethireddipalli et al., 2002; Maphosa and Jideani, 2016; Müller and Polke, 1999; Wall and Paulis, 1978). Most of the changes in properties arise from the reduction of particle size, which is a basic physical property of food products. Commercial yellow pea seed was processed to flour with a smaller particle size through wet milling, and the food emulsion subsequently showed high stability (Aluko et al., 2009). Grain was milled into smaller particles, which facilitated starch digestion (Al-Rabadi et al., 2009). Furthermore, the mean particle size was one of the dependent factors that influenced the bioavailability of β-carotene (Müller and Polke, 1999).

Since wet milling can achieve an efficient decrease in particle size (Kwade, 1999), a new wet milling technique named Micro Wet Milling (MWM) system is

adopted in this research. MWM is a modified electric stone mill system, as shown in the Fig. 1.3. In the MWM system, the pump feeds materials to the stone mill with an adjustable feeding rate, and the lower millstone with grooves can cleave materials to small particles efficiently at a certain milling rotation. In the present study, Koyama and Kitamura (2014) applied MWM to develop a new rice slurry with a fine rice particle smaller than 20 μm. Nakamura *et al.* also reported a cheese-type food using rice milk that milled by MWM system (Nakamura et al., 2016). Islam *et al.* produced the concentrated orange juice with smaller particle size and higher values of nutrition and antioxidant activity by using MWM (Islam et al., 2017). In this study, the fresh Komatsuna is explored to processed into Komatsuna juice with smaller particles and higher PA content using the MWM system.

The millstones have three types with different external contact surface areas of Mill 38 cm^2, Mill 111 cm^2 and Mill 350 cm^2. The same radius of millstone is 12 cm. The smaller contact surface mills materials with particle sizes of millimeters, while the larger contact surface mills particles of micrometer sizes (Koyama and Kitamura, 2014). Since the milling parameters can influence the reduction in particle size, such as the milling rotational speed, milling time, the volume fraction of the milling particles relative to the milling zone and flow rate (Cerdeira et al., 2011; Kwade, 1999), the importance of the milling parameters of MWM for the physiochemical properties of Komatsuna juice are investigated, in order to provide a reference for MWM operation to produce products with beneficial properties.

1.4 Research objectives

The experiments designed for this study aims to process the Komatsuna juice with rich PA by the application of micro wet milling. Fig. 1.4 shows the flow chart that explains the structure of this thesis, including 5 chapters.

In chapter 2, through the measurements of particle size and PA contents of Komatsuna under different processing conditions, the effects of steaming and milling on the PA contents of Komatsuna with inactivated and activated PLD are investigated, and the mechanism responsible for abundant PA formation in Komatsuna during grinding is discussed.

In chapter 3, based on the results of chapter 2, the influences of the MWM milling parameters (milling time, milling rotational speed, material feed rate and gap size) on the particle size reduction and the PA content enrichment of Komatsuna juice are studied. Designed to provide a reference for MWM operation to produce the products with appreciative properties.

In chapter 4, the MWM conditions for processing Komatsuna juice with small particle sizes and high PA contents are optimized. Meanwhile, The interactive effects and the importance of milling parameters on reducing particle size and increasing PA content are analyzed.

In chapter 5, the conclusion and expectation are summarized.

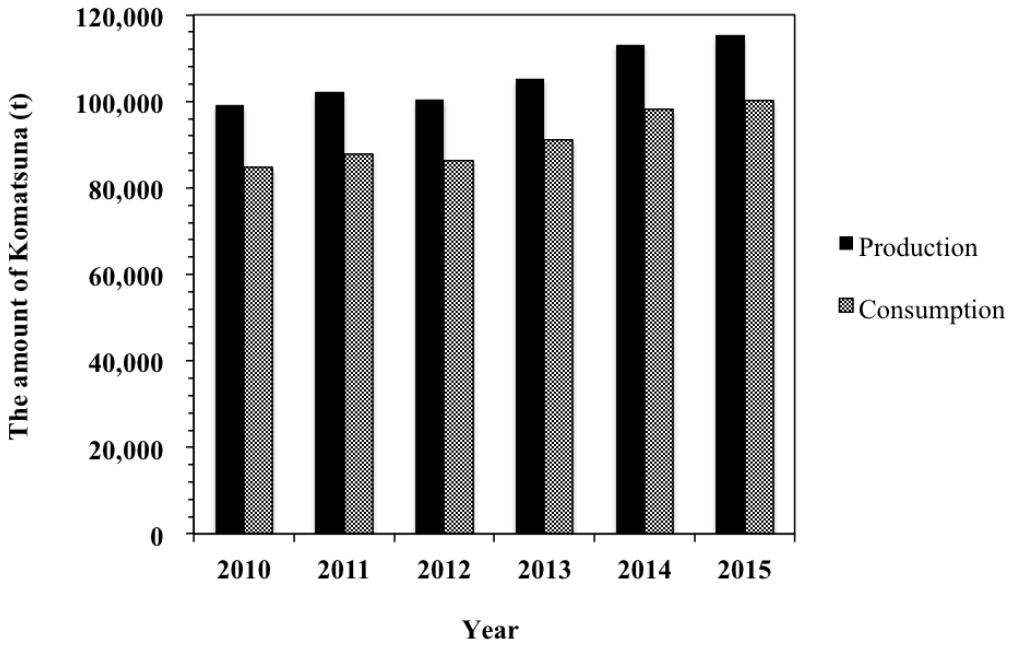

Fig. 1.1 The amounts of Komatsuna production and consumption from the year 2010 to 2015.

Source: Ministry of Agriculture and Forestry and Fisheries, Japan, 2016.

(http://www.maff.go.jp/j/tokei/kouhyou/sakumotu/sakkyou_yasai/index.html)

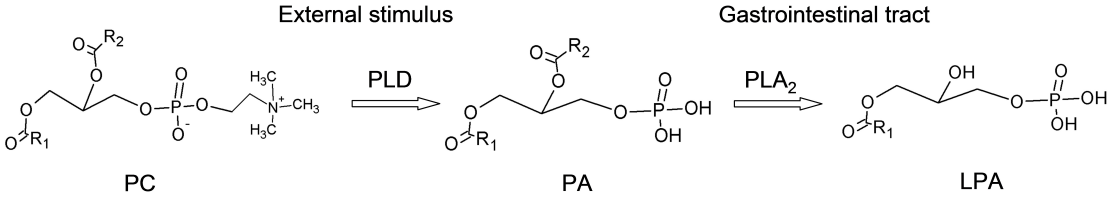

Fig. 1.2 The transformation pathway of PC, PA and LPA, which mainly occur during the homogenization and digestion of plants tissue in GI tract. Abbreviations used: PC, phosphatidylcholine; PLD, phospholipase D; PA, phosphatidic acid; PLA_2, phospholipase A_2; LPA, lysophosphatidic acid.

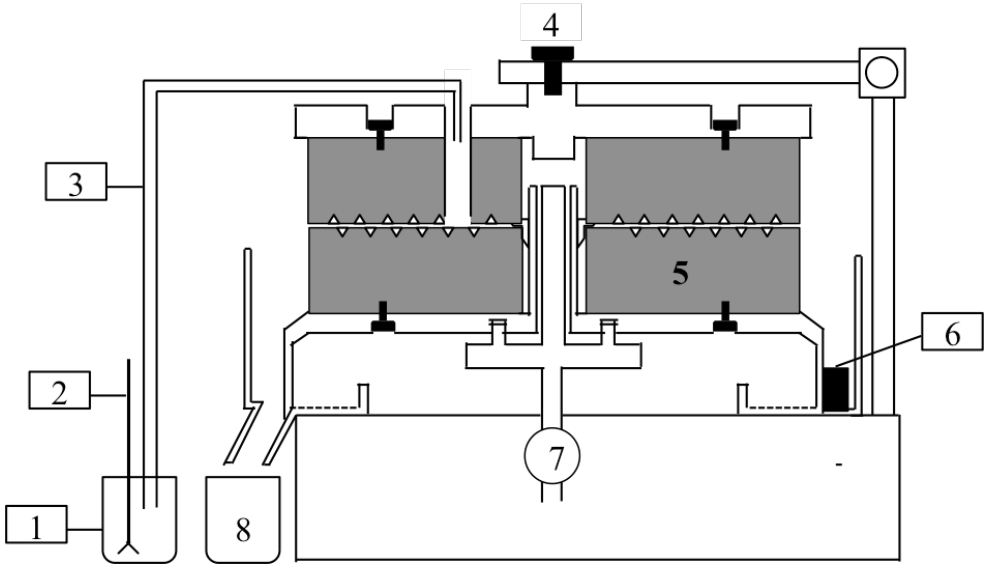

Fig. 1.3 Micro wet milling (MWM) system. (1) Raw material; (2) Stirrer; (3) Tubing pump; (4) Gap controller; (5) Lower millstone; (6) Rubber spatula; (7) Electric motor; (8) Sample receiver.

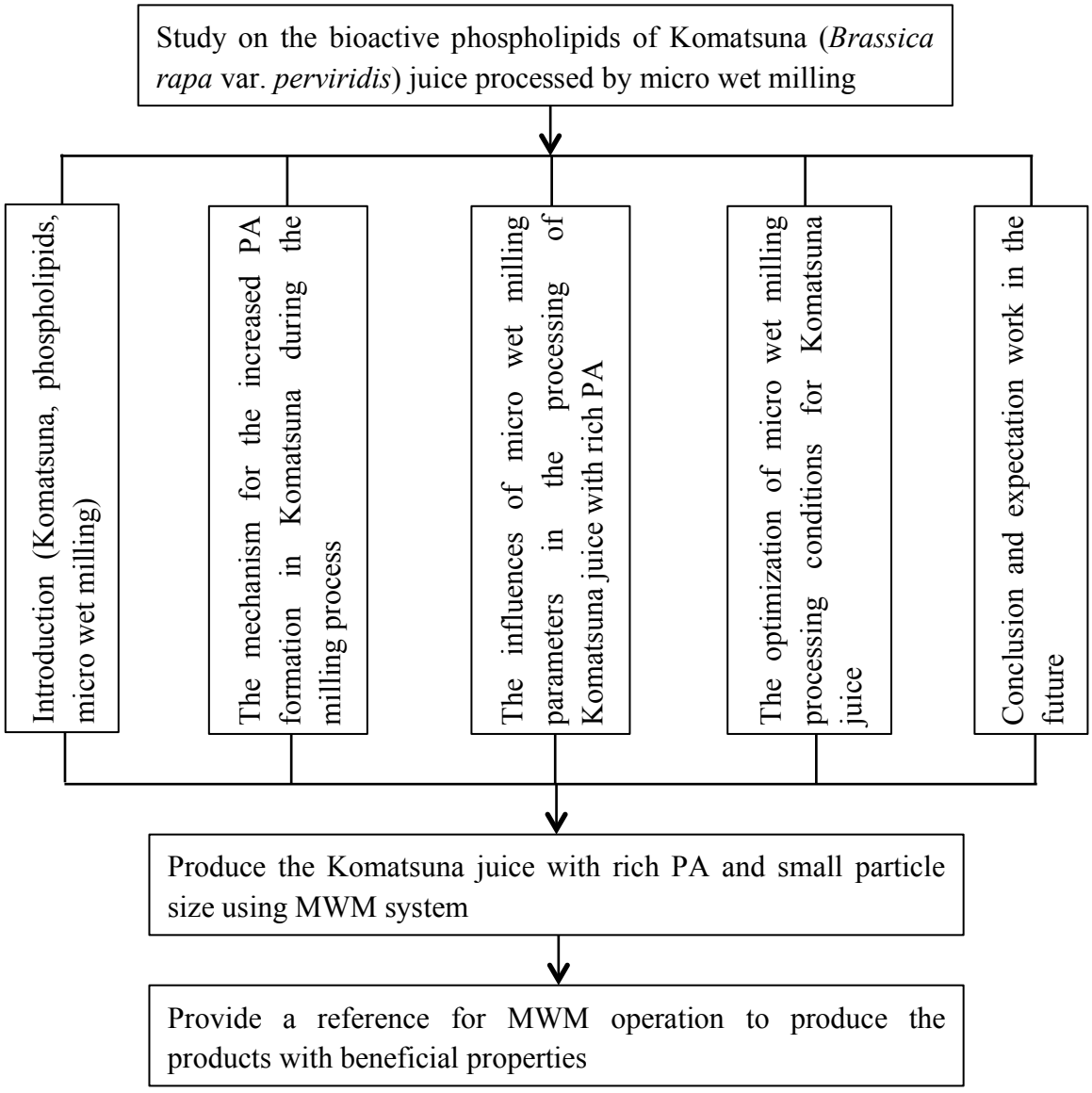

Fig. 1.4 The flow chart of the explanation and design conception for this thesis structures, including 5 chapters.

CHAPTER 2

FORMATION OF PHOSPHATIDIC ACID IN KOMATSUNA (*BRASSICA RAPA* VAR. *PERVIRIDIS*) DURING THE MILLING PROCESS

2.1 Introduction

Phosphatidic acid (PA) is a diacyl-glycerophospholipid that serves as a precursor for the biosynthesis of complex functional lipids and acts as a cellular signaling molecule (Ohlrogge and Browse, 1995; Testerink and Munnik, 2005), it has a critical role in cell membrane structure and function. PA was once reported as a component of cabbage leaf cytoplasm and is an acid, negatively charged phospholipid, and the structural component of many cell membranes (Beata et al. 2015). PA is hydrolyzed from phosphatidylcholine (PC) by phospholipase D (PLD), and is also a precursor of lysophosphatidic acid (LPA), a simple PL, which is generated by phospholipase A_2 (PLA_2). Recent findings revealed that oral administration of PA showed an opportunity to prevent gastric ulcer in mice, due to the formation of lysophosphatidic acid (LPA) from PA in the gastrointestinal (GI) tract by gastric phospholipase A_2 (Tanaka et al., 2013), due to LPA plays the important role in the integrity of GI tract epithelium. However, PA does not accumulate at high levels in the membrane under normal conditions, but abundantly emerge in the masticated and milled foodstuff (Tanaka et al., 2012), and the PA content of raw material was increased more after mastication that compared to the boiled foodstuff (Tanaka et al., 2009).

Therefore, we proposed that destroy the structure of cell membranes by efficient

grinding and milling, the more native PA will be released. In order to prove this, it is necessary to inactivate PLD activity by steaming (heating), to exclude the influence of PLD on PA increase.

Based on the above description, in this section, the PLD activities of Komatsuna with different steaming time were measured firstly, to find the thermostable endpoint of steaming time for PLD inactivation, to exclude the influence of PLD on PA content. Then, through measurements of particle size and PA contents of Komatsuna under different processing conditions, the effects of steaming and grinding on the PA contents of Komatsuna with inactivated and activated PLD were investigated, the relationship between particle size and PA content of Komatsuna was explored, to reveal the mechanism responsible for abundant PA formation in Komatsuna during milling.

2.2 Materials and Methods

2.2.1 Materials

Japanese mustard spinach (*B. rapa*; named Komatsuna in Japan) was purchased from a local market. Standard PA of 98% purity was purchased from Sigma-Aldrich (St. Louis, MO, USA). Chemicals, including chloroform, methanol, HCl, and 28% ammonia solution, were from Wako (Osaka, Japan). TLC Silica gel 60 was from Merck (Darmstadt, Germany).

2.2.2 Steaming of Komatsuna

To investigate the effects of steaming on the particle size and PA content of Komatsuna, preparations were conducted using whole Komatsuna. After washing with tap water, Komatsuna (100 g of wet weight) was steamed (99.7 ~ 100.4 ^{o}C) for different durations ranging from 1 to 30 min, and quickly cooled to 4 ^{o}C. The steamed Komatsuna was mixed with water (100 g, 4 ^{o}C) and ground using a blender (SBC-1000J; Cuisinart, Tokyo, Japan) for 2 min at room temperature. The obtained Komatsuna juice samples were analyzed for the following properties.

2.2.3 Grinding of Komatsuna

To investigate the effects of grinding on the particle size and PA content of Komatsuna, preparations were conducted using whole Komatsuna. After washing with tap water, Komatsuna (100 g of wet weight) was steamed (99.7 ~ 100.4 ^{o}C) for 5 min, and quickly cooled to 4 ^{o}C. The steamed Komatsuna was mixed with water (100 g, 4 ^{o}C) and ground using a blender (SBC-1000J; Cuisinart, Tokyo, Japan) for 2 to 25 min at room temperature. The obtained Komatsuna juice samples were analyzed for the following properties.

2.2.4 PLD assay of Komatsuna

PLD activity was detected *in vitro* using the Amplex Red assay kit (Molecular Probes, Eugene, OR, USA). For the PLD assay, Komatsuna was steamed for 1 to 5 min and ground for 2 min as above. The mixture was centrifuged at 1300 × *g* for 5 min at 4 ^{o}C, and the supernatant was incubated with 250 μM PC, 1 unit/mL horseradish peroxidase, 0.1 unit/mL choline oxidase, and 50 μM Amplex Red reagent

in 200 μL of a reaction buffer consisting of 5 mM $CaCl_2$ and 50 mM Tris-HCl (pH 8.0). PLD activity was measured after 2 h and 10 h incubation using a Benchmark Plus microplate reader (Bio-rad, Hercules, CA, USA) at 530 nm excitation and 590 nm emission (Fig. 2.1) (Kato et al., 2005; Vogt et al., 2002). Non-steamed raw Komatsuna was used as the control.

2.2.5 Particle size and particle size distribution

The particle size (D50) and particle size distribution were determined using a laser diffraction particle size analyzer (SALD-2200; Shimadzu Corporation, Japan) in wet measurement mode (Fig. 2.2). The D50 value was chosen as the particle size, termed the median diameter, and is defined as the average particle size by mass. The particle size distribution profile was described by the particle size and the frequency.

2.2.6 Measurement of PA content

Lipids were extracted from the Komatsuna juice according to the Bligh and Dyer method with minor modifications (Bligh and Dyer, 1959; Tanaka et al., 2009). Komatsuna juice (8 mL, containing approximately 97% moisture) was added to a mixture consisting of 10 mL chloroform, 20 mL methanol and 0.24 mL water, in order to form a chloroform/methanol/water phase (10:20:8, V/V/V). The mixed homogenate was vortexed (Vortex Mixer, VM-96B; Jeio Tech, Daejeon, Korea) for 20 s and centrifuged at 1300 × *g* for 10 min, and the supernatant portion was then collected. The supernatant was diluted with 15 mL of chloroform and water (1:1, V/V) to produce a biphasic system for phase-separation. After adding 0.05 mL of 5N HCl

for acidification, the mixed homogenate was vortexed for 20 s and centrifuged at 1300 × *g* for 10 min. The lipid extract was collected from the chloroform layer. PA was isolated from the lipid extract by TLC with a developing agent consisting of chloroform/methanol/28% ammonia (60:35:8, V/V/V). Then, two-phase separation was conducted to extract PA from the scraped silica gel. The recovered PA was first dissolved in 5.7 mL of a mixed solvent consisting of chloroform/methanol/water (10:20:8, V/V/V), then 3.0 mL of chloroform and water (1:1, V/V) were added for phase separation, and finally, 0.02 ml of 5N HCl was added. The mixture was vortexed, centrifuged, and the purified PA was collected from the lower phase. The amount of PA was spectrophotometrically quantified using a colorimetric method based on phosphomolybdenum-malachite green formation (Chalvardjian and Rudnicki, 1970).

2.2.7 Characteristics of micro wet milling of Komatsuna

Because the particle size of Komatsuna does not decrease obviously under long grinding times, the MWM system was used to produce smaller particles. For detailed observation of the mechanisms of PA production, experiments were conducted using active and inactive PLD, and the particle size and PA content of Komatsuna between the two groups were comparisons. The two samples were subjected to different processing sequences. The first sample was processed by steaming for 5 min, mixed with water (4 ^{o}C) at a ratio of 1:1 (w/w), and ground by a blender for 2 min, then milled by MWM at room temperature. The other sample was mixed with water,

ground for 2 min and milled by MWM, and steamed for 5 min after the grinding process. The MWM parameters were as follows: feeding rate, 10 mL/min; milling rotation, 40 rpm; contact surface area of the mill stone, 350 cm^2; groove width, 4.5 mm; gap size, 60μm. The measurements of particle size distribution and PA content were conducted as described above.

2.2.8 Statistical analyses

Student's *t*-test was used for comparisons between two groups, and one-way ANOVA was conducted using SPSS software (version 22.0; IBM, Armonk, NY, USA). The p value of < 0.05 was considered to indicate statistical significance.

2.3 Results and Discussion

2.3.1 Effect of steaming time on PLD activity

This study aimed to elucidate the relationship between the solid particle size of Komatsuna juice and the amount of PA available to the body when consumed. However, the amount of PA is also related to PLD activity, as PA is mainly converted from PC by the enzyme PLD. When PLD is inactivated, the influence of PLD on PA increase or content can be excluded. Therefore, in order to understand the inactivation conditions of PLD, the PLD activity of Komatsuna at different steaming times was investigated.

The production of PA is dependent on the different PLD isoforms and their catalytic properties (Ruelland et al., 2014). Previous studies showed different

thermostable properties of PLD; for example, approximately 30% of PLD activity was retained following exposure to a temperature of 100 ^{o}C for 15 min (Hanahan and Chaikoff, 1948), whereas Tanaka *et al.* (2009) adopted 3 min of heating time to inactivate PLD activity. Therefore, it is necessary to confirm the exact steaming time of Komatsuna for PLD inhibition. In this study, the steaming time ranged from 1 to 5 min, and PLD activity was measured after incubation for 2 h and 10 h at 37 ^{o}C in darkness. The effect of steaming time on PLD activity is shown in Fig. 2.3. Although the standard incubation time for the detection of PLD activity was 2 h, the results showed that the reaction of PC to PA by PLD was not completely finished at 2 h. Therefore, PLD activity was also detected after 10 h incubation, which is the time that the enzyme reaction remains stable. The PLD activity result of 10 h incubation was used to confirm the results observed at 2 h. The initial PLD activity of non-steamed raw Komatsuna was defined as 100%, and relative PLD activity of Komatsuna at different steaming times was determined. The effect of steaming on PLD activity at 2 h incubation was similar to that at 10 h. About 60% of PLD activity was inhibited following steaming for 1 min, whereas PLD was totally inactivated following steaming for 4 min.

The chemical reaction of PLD, which cleaves PC to choline, and PA continued after 2 h, because the excess PC stimulated PLD. The reaction was stable when incubated for 10 h, and this result was similar to that reported by Kato *et al.* (2005). Those researchers measured PLD activity with different incubation times and showed that PLD activity changed sharply in the first 3 h, reached a plateau at 6 h and lasted

up to 24 h. In this study, from the combined results of PLD activity with 2 h and 10 h incubation, it was clearly observed that PLD was inactivated after steaming of Komatsuna for 4 min. The result of PLD inactivation differed with Tanaka (2009) and Hanahan (1948), who boiled and steamed cabbage for 3 min and 15 min respectively. In plants, PLDs comprise a large and diverse family of enzymes (Bargmann et al., 2009). The differences in PLD primary structure and isoforms contribute to the different enzymatic properties (Ruelland et al., 2014). The high amount of vegetable fiber in Komatsuna may also be the reason for the different results (Aiso et al., 2014).

2.3.2 Effect of steaming time on particle size and PA content of Komatsuna

Steaming or heating not only inactivates PLD, but also influences the milling characteristics and resulting particle size of Komatsuna juice. Fig. 2.4 shows the relationship between steaming time and particle size of Komatsuna juice. The particle size decreased with increasing steaming time. Steaming of Komatsuna resulted in tissue that was more degradable, making the tissues more amenable to breaking down to smaller fragments upon grinding.

In order to confirm the inactivation of PLD, PA content was also determined at different steaming times. The result of PA content obtained at different steaming times is also shown in Fig. 2.4. The PA content of steamed Komatsuna was much lower compared to non-steamed Komatsuna. When PLD was inactivated, the conversion of PC to PA by PLD was prevented, and the PA content was the lowest after Komatsuna was steamed for 4-5 min. However, after 5 min of steaming, PA

content increased. Although PLD was inactivated, the particle size of Komatsuna decreased slightly with longer steaming times, allowing more PA to be released.

It is assumed that when biomembranes are broken down to smaller particles, the structural membrane PA is released. In order to investigate this assumption, Komatsuna was processed using various grinding times in the next experiment.

2.3.3 Effect of grinding time on particle size and PA production of Komatsuna

The particle size and PA content of steamed Komatsuna at different grinding times were quantified (Figs. 2.5 and 2.6). As shown in Fig. 2.5, particle size generally decreased with increasing grinding time, and the relationship can be expressed with a logarithmic function. The equation is as follows:

$$Y = -15.167\ Ln\ (X) + 147.77,\ R^2 = 0.9853 \quad \ldots\ldots \text{Eq. (1)}$$

where Y is the particle size (μm) and X is the grinding time (min).

Fig. 2.5 also shows that PA content increased gradually with grinding time. With longer grinding times, the fluid has sufficient time to break all bonds of the inner structure within the flocs (Blaser, 2000), and the fragments are degraded to smaller particles. Since PA is a structural component of many cell membranes, PA is released upon membrane disruption. In other words, with increasing grinding time, the particle size of Komatsuna decreased and the PA content increased. Moreover, the relationship between PA content and particle size was observed (Fig. 2.6). With PLD inhibition, the particle size and PA content showed a linear relationship; in other words, the smaller the particle size, the larger the PA content. The linear equation is

as follows:

$y = -0.1897x + 34.186$, $R^2 = 0.9755$ …… Eq. (2)

where y is the PA content (μg/mL) and x is the particle size (μm).

In the food sciences, a food particle is deemed fit for consumption when it is smaller than 20 μm (Inoue, 2011). Using Eqs. 1 and 2, it was calculated that to obtain a particle size of 20 μm, a grinding time of 4556 min would be required, with a predicted PA content of 29 μg/mL. Not only is this PA content considered to be low, the grinding time is too long for industrial production. Therefore, a new technique, namely micro wet milling (MWM) was applied to produce smaller sized particles in a short time.

2.3.4 Characteristics of micro wet milling of Komatsuna

MWM involves electric stone milling of wet material to minimize the particle size. With MWM processing, the PA content improved obviously and the particle size decreased to approximately 20 μm (Figs. 2.7 and 2.8). The particle size distributions of Komatsuna following ordinary blender grinding and MWM are shown in Fig. 2.7. The different processing sequences (steaming of Komatsuna before or after milling) had a minimal effect on particle size; in another words, compared to the differences between grinding and milling, the effect of steaming on particle size was small. Most particles of Komatsuna processed by ordinary blender grinding were 140 μm~170 μm, whereas the majority of particles following MWM were 20 μm. These results indicate that the MWM system is more efficient than ordinary blender grinding for producing

smaller particles.

The PA contents of Komatsuna with ordinary blender grinding and MWM system before and after steaming were quantified respectively (Fig. 2.8). The PA content of Komatsuna milled before steaming was much higher than that after steaming. The low PA content of Komatsuna steamed before milling suggests that steaming inhibited the PLD activity. High PA content was obtained when Komatsuna was milled before heating, which can be explained by the conversion of PC to PA by PLD, which was activated during grinding and milling. Similar results have also been observed in cabbage. Tanaka *et al.* (2009) reported that the PL profile of cabbage leaves boiled after grinding was characterized by high PA and low PC contents. PLs contain PA, PC and other phospholipids, which are major components of cell membranes (Hou et al., 2016). When structural membranes are disrupted, PA is released, and a similar situation is also expected with PC. PC serves as a substrate for the generation of PA by PLD, and the released endogenous PC stimulated PLD activity during milling. This suggestion is in agreement with the observations reported by Hanahan and Chaikoff (1948), in which repeated grinding of raw cabbage leaves activated PLD to form PA from endogenous PLs. Nguyen *et al.* (2014) also observed that the concentrations of PC and PA are complementary, and inversely proportional to each other (Nguyen et al., 2014). In future work, the amount of PC in Komatsuna subjected to different processes should be determined, which could clarify the conversion of PC to PA, and the mechanism responsible for PA increases during grinding would be explained more directly.

The PA contents of Komatsuna processed by MWM were much higher compared to ordinary blender grinding, and the particles following MWM were smaller compared to ordinary grinding. This result was obtained for samples that were steamed both before and after milling. Therefore, the MWM system was shown to be more effective than ordinary blender grinding in reducing particle size and increasing PA content, and could be developed as an efficient processing technology to produce Komatsuna juice with high PA content.

The bioavailability of a component is a fundamental issue in nutrition as opposed to the supply of a sufficient amount in the diet, which can be influenced by exogenous factors such as the properties of the bioactive ingredients, and endogenous host factors such as genetic conditions and physiological status (Skibniewska et al., 2012). Rabadi *et al.* (2009) examined the influence of milled grain particle size on starch digestion, and observed a decrease in the digestion rate with increasing particle size (Al-Rabadi et al., 2009). The digestion and absorption of phospholipids in the GI tract are also determined by their physicochemical characteristics (Thomson et al., 1993). Moreover, lipids are generally absorbed via diffusion in a monomolecular state rather than the bulk transfer of lipid molecules from mixed micelles to the brush border membrane of the enterocyte (Simmonds, 1972; Westergaard and Dietschy, 1976). Therefore, the amount of dissociated PA that is released from the cell membrane or micelle, rather than the total PA including that inside the intact plant cell, is directly connected to the LPA content that can be produced and utilized for the restoration or prevention of GI disorders. This study shows that by decreasing the particle size of

Komatsuna by effective milling, the amount of PA available to the body increases.

2.4 Conclusion

For the manufacture of Komatsuna PA-rich food, Komatsuna was processed using different steaming and milling conditions, and the particle size and PA content of Komatsuna juice were quantified. The study's main findings are as follows: (i) with longer grinding times, Komatsuna tissues were cleaved into smaller particles; (ii) sufficient amounts of PA were released from Komatsuna; and (iii) the smaller the particle size, the higher the PA content; and (iv) the MWM system more effectively manufactured PA-rich Komatsuna juice. Taken together, these findings suggest that the high PA content was primarily due to the release of native PA from the Komatsuna biomembrane by particle disruption, and secondarily, to the release of endogenous PC during the grinding process, which resulted in the conversion to PA by PLD. Foodstuff-derived PA is an efficient exogenous source of LPA for the human body, which has potential use as a dietary treatment for the restoration or prevention of GI disorders. Therefore, the application of milling for increasing PA content is worthy of further investigation.

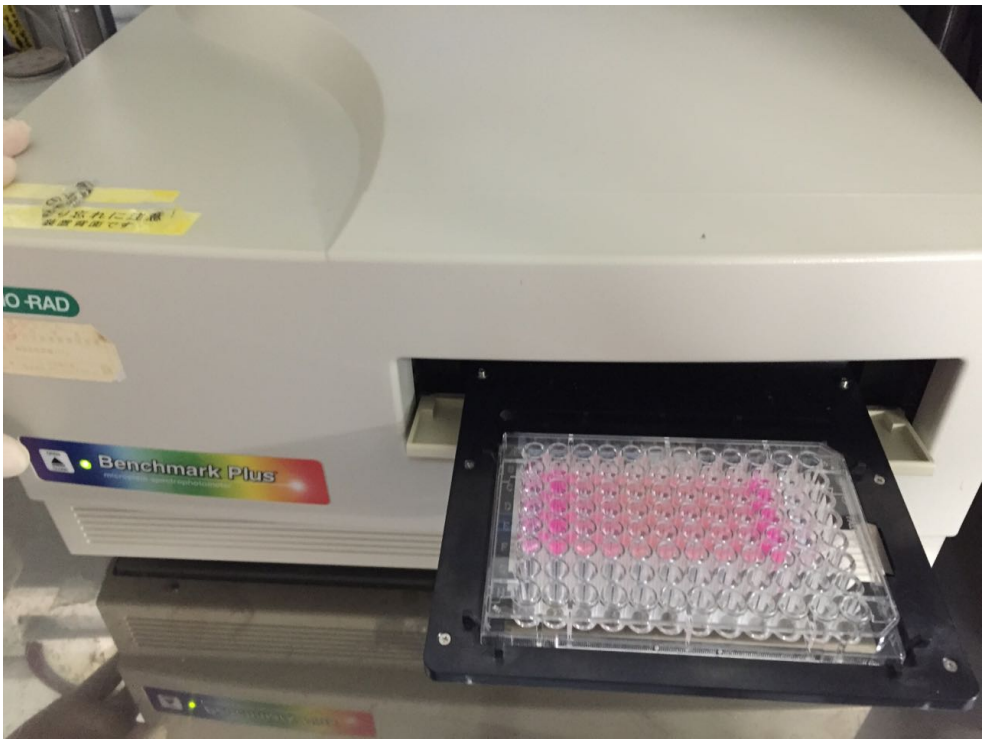

Fig. 2.1 The detection of PLD activity using the Amplex Red assay kit (Molecular Probes, Eugene, USA) by Benchmark Plus microplate reader (Bio-rad, Hercules, CA, USA).

Fig. 2.2 SALD-2200 Laser diffraction particle size analyzer (Shimadzu Corporation, Japan)

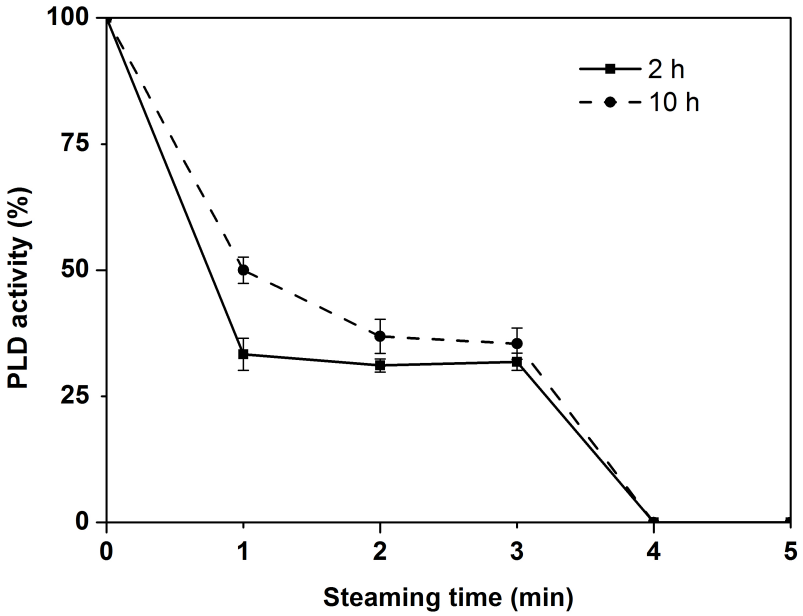

Fig. 2.3 Phospholipase D (PLD) activity was as a function of steaming time.

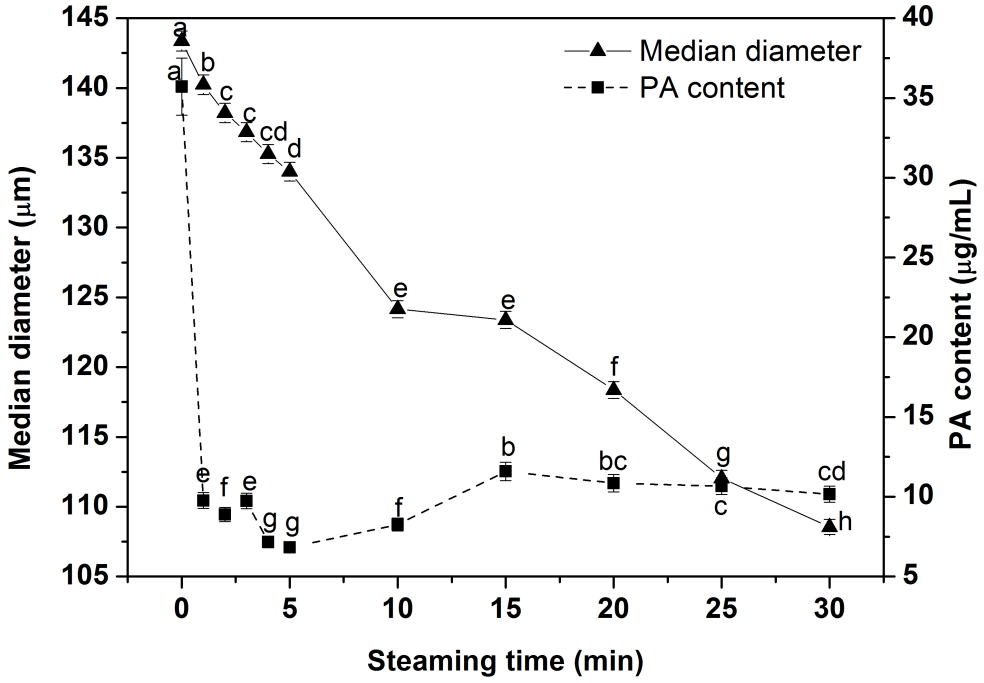

Fig. 2.4 The particle sizes and phosphatidic acid (PA) contents of Komatsuna juice with different steaming time. Different lowercase letters reflect significant differences ($p < 0.05$) in particle size and PA content, respectively.

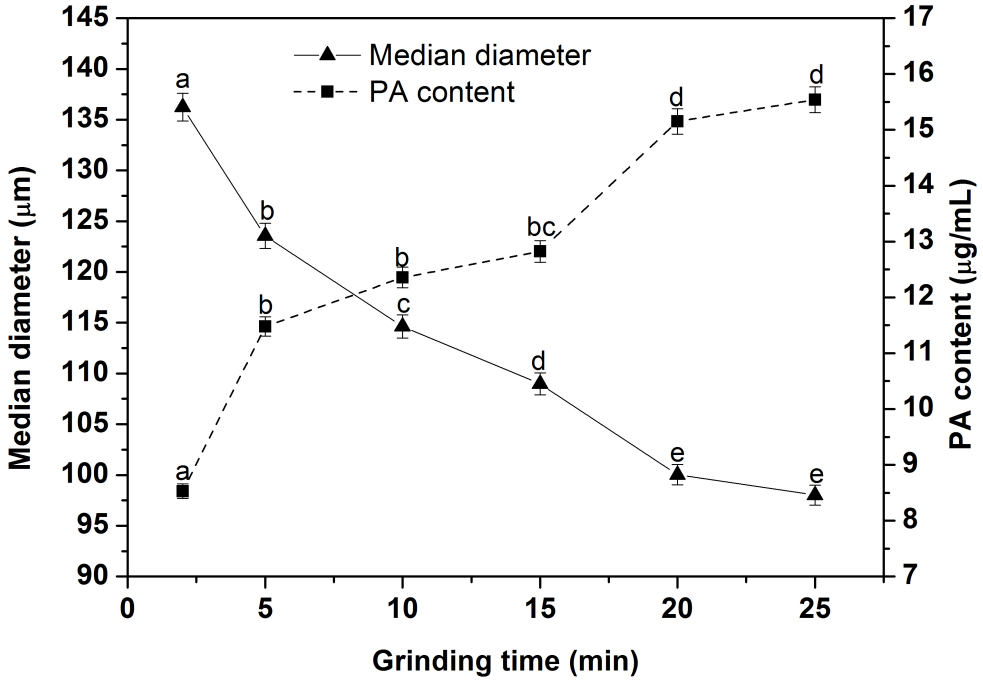

Fig. 2.5 The particle sizes and phosphatidic acid (PA) contents of steamed Komatsuna juice with different grinding time. Different lowercase letters reflect significant differences ($p < 0.05$) in particle size and PA content, respectively.

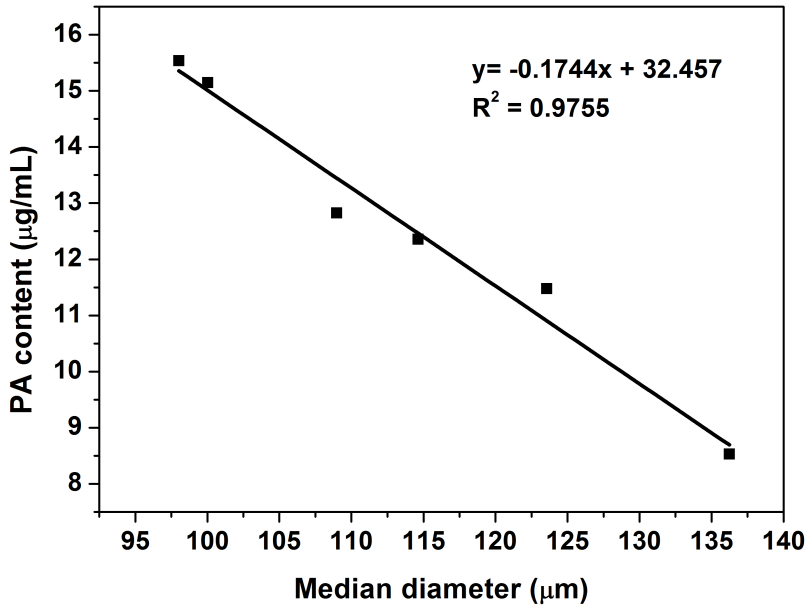

Fig. 2.6 The relationship of phosphatidic acid (PA) content and particle size (PLD inactive).

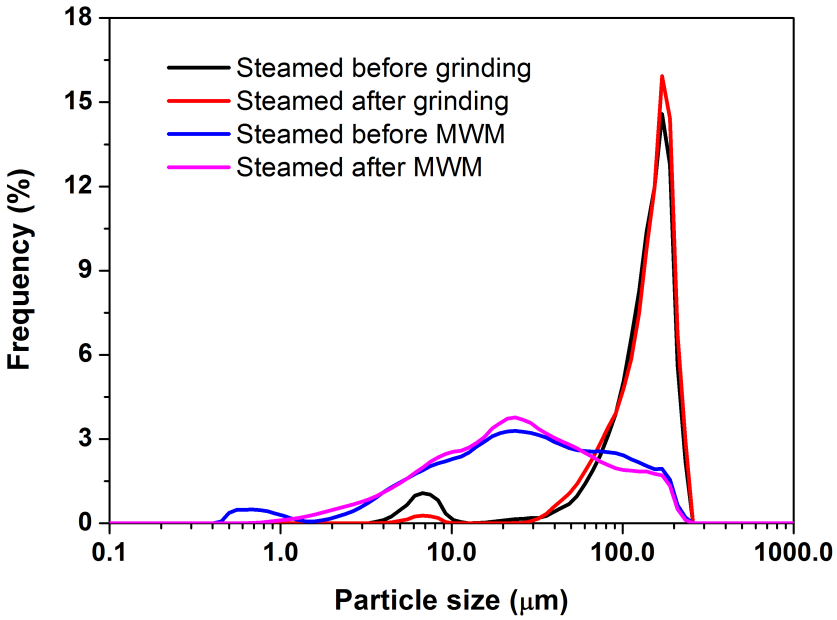

Fig. 2.7 Particle size distributions of Komatsuna juice steamed before or after blender grinding/Micro Wet Milling (MWM).

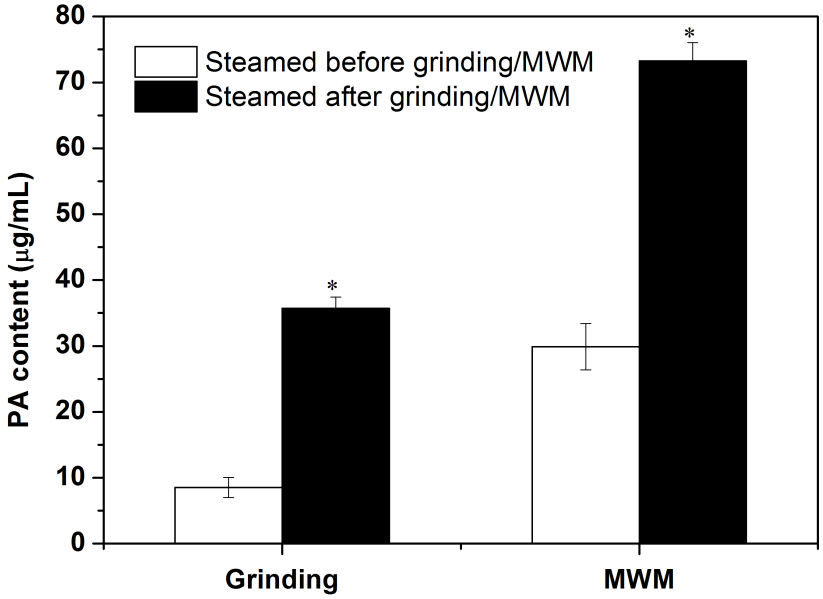

Fig. 2.8 The phosphatidic acid (PA) contents of Komatsuna steamed before or after grinding/Micro Wet Milling (MWM). *, $p < 0.05$ (student's *t*-test).

CHAPTER 3

THE INFLUENCE OF MICRO WET MILLING PARAMETERS IN THE PROCESSING OF KOMATSUNA (*BRASSICA RAPA* VAR. *PERVIRIDIS*) JUICE WITH RICH PHOSPHATIDIC ACID

3.1 Introduction

According to the previous studies, abundant PA was produced in the masticated and milled foodstuff (Tanaka et al., 2012), and the PA content of raw cabbage was increased more after mastication that compared to the boiled cabbage (Tanaka et al., 2009). Based on the results of chapter 2 suggested a similar phenomenon that PA in fresh Komatsuna emerged sufficiently after effective milling, due primarily to the release of native PA of Komatsuna from the biomembrane by particle splitting, and secondarily to the release of endogenous PC in the process of grinding, which stimulate the hydrolysis of PC to PA by PLD. And the smaller the particle size of Komatsuna juice, the higher the PA content. Thus, there is a possibility to manufacture a healthy diet of PA-rich Komatsuna juice with milling processing, for the preventive or therapeutic purposes of gastric ulcers.

Most of the changes in properties of materials are respect to the reduction of particle size that is the basic physical property of food products. The wet milling can effectively decrease the particle size of material tissue (Kwade, 1999), a new wet milling technique named Micro Wet Milling (MWM) system was adopted in this study. MWM is a modified electric stone mill system, as shown in the Fig. 1.3 (chapter 1). Reductions in particle size are influenced by milling parameters such as

the milling rotational speed, milling time, the volume fraction of the milling particles relative to the milling zone and flow rate, during ball milling in a wall chamber (Cerdeira et al., 2011; Kwade, 1999). The MWM system is a continuous process that is similar to the colloid mill, the milled juice is collected simultaneously while the material is fed into the stone mill, and the particle size also depends on the gap between the upper and lower millstones (Vishwanathan et al., 2011).

Therefore, the aim of this work was to study the importance of the milling parameters of MWM for particle size reduction and PA content enrichment in Komatsuna juice. Komatsuna juice with smaller particle size and higher PA content was produced under the optimum MWM conditions, and the resulting product could support a beneficial diet for the prevention or restoration of GI disorders. The investigation results of milling time, milling rotational speed, material feeding rate and gap size, can provide a reference for MWM operation to produce the products with appreciative properties.

3.2 Materials and Methods

3.2.1 Materials

Japanese mustard spinach (*B. rapa*; named Komatsuna in Japan) was purchased from a local market. Standard PA with a purity grade of 98% was purchased from Sigma-Aldrich (St. Louis, USA). Chemicals: chloroform, methanol, hydrochloric acid, 28% ammonia solution were from Wako (Osaka, Japan). TLC Silica gel 60 was from Merck (Darmstadt, Germany).

3.2.2 Preparation of Komatsuna juice with MWM

In order to investigate the influences of MWM operational parameters on the particle size and PA content of Komatsuna, the preparation was conducted on the whole Komatsuna. After washing with tap water, Komatsuna was mixed with water (4 ^{o}C) at a ratio of 1:1 (w/w), blended by a mixer (SBC-1000J, Cuisinart, Japan) for 5 min at room temperature. The Komatsuna mixture (particle size of approximately 120 μm) was fed to the stone mill of the MWM with a pump with adjustable feeding rate, and milled with a certain rotational speed by controlling the lower millstone. The feed rate of the pump, milling rotation speed and milling time are explained below. The processed juice was collected simultaneously by rubber spatula during the successive milling. Finally, the collected Komatsuna juice was boiled for 5 min to inhibit enzyme activity and then analyzed.

3.2.3 Milling conditions for MWM

In this study, a milling stone with external contact surface area of 350 cm^2, a radius of 12 cm and a groove width of 4.5 mm was used, as shown in the Figs. 3.1 and 3.2. The minimum inner distance between the upper and lower millstones is 60 μm (groove depth), and this distance was set as the initial gap size. The milling parameters were investigated as below description.

3.2.3.1 Effect of milling time on the properties of Komatsuna juice

Komatsuna was milled by MWM with different milling time (0, 10, 20, 30, 40, 50, 60 min), milling rotation 40 rpm, feeding rate 10 mL/min and gap size 60 μm. The

particle size, yield and PA content of Komatsuna juice with different milling time were measured.

3.2.3.2 Effect of milling rotational speed on the properties of Komatsuna juice

Komatsuna was milled by MWM with different milling rotational speed (10, 20, 30, 40, 50 rpm), milling time 50 min, feeding rate 10 mL/min and gap size 60 μm. The particle size, yield and PA content of Komatsuna juice with different mill rotation were measured.

3.2.3.3 Effect of material feed rate on the properties of Komatsuna juice

Komatsuna was milled by MWM with different material feed rate (10, 20, 30, 40 mL/min), milling time 50 min, milling rotation 40 rpm and gap size 60 μm. The particle size, yield and PA content of Komatsuna juice with different material feed rate were measured.

3.2.3.4 Effect of gap size on the properties of Komatsuna juice

Komatsuna was milled by MWM with different gap size (60, 200, 300, 400 μm), milling time 50 min, milling rotation 40 rpm and feed rate 10 mL/min. The particle size, yield and PA content of Komatsuna juice with different gap size were measured and analyzed.

3.2.4 Measurement of particle size

The particle sizes were determined using a laser diffraction particle size analyzer (SALD-2200, Shimadzu Corporation, Japan) in the wet measurement mode. The D50

value of micrometers was chosen as particle size, termed the median diameter, and is defined as the average particle size by mass. Three independent determinations were conducted, and the results were presented as an average ± standard deviation.

3.2.5 Quantification of phosphatidic acid

Lipids were extracted from the Komatsuna juice by the Bligh and Dyer method with minor modifications (Bligh and Dyer, 1959; Tanaka et al., 2009). Komatsuna juice (8 mL, containing approximately 97% of moisture) was added into a mixture consisting of 10 mL chloroform, 20 mL methanol and 0.24 mL water, in order to form a phase of chloroform/methanol/water (10:20:8, V/V/V). The mixture homogenate was vortexed (Vortex Mixer, VM-96B, Jeio Tech, Korea) for 20 s and centrifuged at 1300 × *g* for 10 min, and the supernatant portion was collected. The supernatant was diluted with 15.0 mL of chloroform and water (1:1, V/V) to produce a biphasic system for the phase-separation. After adding 0.05 mL of 5N HCl for acidification, the mixed homogenate was vortexed for 20 s and centrifuged at 1300 × *g* for 10 min. The lipid extract was collected from the chloroform layer. PA was isolated from lipids extract by TLC with the developing agent consisting of chloroform/methanol/28% ammonia (60:35:8, V/V/V). Then, the two-phase separation was conducted to extract PA from the scraped silica gel. The recovered PA was first dissolved in 5.7 mL of a mixed solvent consisting of chloroform/methanol/water (10:20:8, V/V/V), 3.0 mL of chloroform and water (1:1, V/V) were added for phase separation, and finally, 0.02 ml of 5N HCl was added. The mixture was vortexed, centrifuged, and the purified PA

was collected from the lower phase. The amount of PA was quantified by a colorimetrical method based on the phosphomolybudenum-malachite green formation with a spectrophotometer (Chalvardjian and Rudnicki, 1970). Three independent measurements were repeated.

3.2.6 Statistical analyses

Student's *t*-test was performed between two groups for comparison; one-way ANOVA was conducted by SPSS software (version 22.0; IBM, Armonk, NY, USA). The p value of < 0.05 was considered as statistically significant.

3.3 Results and Discussion

3.3.1 Effect of milling time on the particle size and PA content of Komatsuna juice

In order to observe the influences of MWM adjustable parameters on the physiochemical properties of Komatsuna juice, a suitable milling time that assures stable MWM milling status needs to be found. The particle size, yield and PA content of Komatsuna juice with different milling time were measured under the process conditions of milling rotation 40 rpm, feeding rate 10 mL/min and gap size 60 μm.

Figs. 3.3a and 3.3b show that the particle size (D50) clearly decreased in the first 10 min of milling time, then decreased slowly during the milling time of 10-50 min, but became constant after 50 min of milling time. The yield of Komatsuna juice increased in linearly tendency with the equal extension of milling time when the

milling time exceeded 20 min. The PA content of Komatsuna juice increased with longer milling time, whereas the PA contents were constant between the milling times of 50 min and 60 min.

Compared to the properties of the initial material of Komatsuna juice ground by a mixer (0 min of milling time), the properties of Komatsuna juice milled for 10 min were much different. The decrease in particle size was almost three fold, and PA content increased approximately two fold. This proved the effect of MWM on reducing particle size and improving PA content. The MWM system is a successive processing system, and the milled juice is collected simultaneously while the material is fed into the millstones. In the first 10 min of milling time, material was mainly filling into the grooves of the millstones and being milled at the same time, therefore the yield of milled juice was low, the particles of Komatsuna juice were not milled sufficiently, and the PA was not released and produced absolutely. The particle size and PA content of Komatsuna juice changed gradually with longer milling times, then showed no differences between 50 min and 60 min of milling time. Similarly, the yield of processed Komatsuna juice was stable after 50 min, which means the successive milling process was in a stable situation. Therefore, 50 min of milling time was adopted for the following experiments.

3.3.2 Effect of milling rotational speed on the particle size and PA content of Komatsuna juice

The particle size, yield and PA content of Komatsuna juice with different milling

rotational speeds were measured under the process conditions of milling time 50 min, feeding rate 10 mL/min and gap size 60 μm, to investigate the effect of rotational speed on the properties of Komatsuna juice.

As shown in the Figs. 3.3c and 3.3d, the particle size (D50) of Komatsuna juice reduced with the higher milling rotational speeds, but increased when the rotational speed exceeded 40 rpm. The yield of Komatsuna juice was higher when the rotational speed was higher, but yields did not increase linearly with the increase of rotational speed. The PA contents of Komatsuna juice with the milling rotation between 20 rpm and 40 rpm were higher than that processed with milling rotational speeds of 10 rpm and 50 rpm.

The reduction of particle size and increase of yield to a certain extent with the higher milling rotational speed could be attributed to the higher milling forces, that are predominantly compression and less of shear in stone mills (Sharma et al., 2008). However, when the milling rotation exceeded 40 rpm, the particle size was not reduced continuously, and the yield of juice increased obviously, which shows that the materials were pushed out of millstones by the higher milling rotation, and the particles were not milled sufficiently before they were pushed out. This could be because the increasing rotational speed leads to the increase of centrifugal force and helix angle. This result is different with that of Koyama *et al.* (2014), where the particle size of rice decreased with the higher milling rotation by strong shear stress. The particle size reduction also depends on the properties of materials, and some excipients and stabilizers are appreciated to improve the reduction of particle size by

stabilizing the physical property of milling particles (Cerdeira et al., 2011). In addition, the result of PA content was agreement with the phenomenon reported previously in the chapter 2, where the particle size was smaller, and the PA content was higher. In this study, with the different milling rotational speeds, the PA contents were changing within a limited range.

3.3.3 Effect of material feeding rate on the particle size and PA content of Komatsuna juice

Since the volume fraction of the milling particles relative to the milling zone influences the reduction of particle size, the material feeding rate, one of the parameters that affect the volume fraction was investigated. Thus, the particle size, yield and PA content of Komatsuna juice with different feeding rate were detected under the process conditions of milling time 50 min, milling rotation 40 rpm and gap size 60 μm.

Figs. 3.3e and 3.3f show that the particle sizes and yields of Komatsuna juice increased with the higher feeding rate, whereas the PA contents decreased. The results of the feeding rate between 8 mL/min and 10 mL/min showed little difference compared to those of the other higher feeding rates.

At the beginning of the MWM process, the milling zone is filled gradually by feeding the materials, the volume fraction of milling particles in the milling zone increases, and the friction and collisions between the particles-millstone and particles-particles become stronger to accomplish the breaking up of particles.

However, the higher feeding rate also influences the flow of Komatsuna juice in the milling system that affects the effective exposure of particles to the milling zone (Sharma et al., 2008), which means the particles will flow out before they are fully milled. MWM is a continuous milling system that is similar to the colloid mill (Vishwanathan et al., 2011), but not the ball milling in the wall chamber (Cerdeira et al., 2011).

3.3.4 Effect of gap size on the particle size and PA content of Komatsuna juice

In this experiment, the influences of gap size on the particle size, yield and PA content of Komatsuna were observed, with the process conditions of milling time 50 min, material feeding rate 10 mL/min and milling rotation 40 rpm.

Figs. 3.3g and 3.3h show that the particle size and yield of Komatsuna juice increased gradually and PA content decreased with the larger gap size. The gap size is also a parameter that influences the volume fraction of milling particles in the milling zone, the gap size was larger, and the volume fraction was lower, the friction and collisions between milling particles were lower. The predominant milling force of stone grinders is the compressive force (Vishwanathan et al., 2011), and when the volume fraction of particles in milling zone was small, the effective milling force was low, the reduction of particle size was low. The particles could flow out of the milling zone by the centrifugal force of rotation, compressive and flow force, and the particles were not milled sufficiently. Vishwanathan *et al.* (2011) also suggested that the particle size of the ground material in the colloid mill depends on the gap between the

grinding stones, and that the volume fraction of grinding particles in grinding zone increased by the increased volumetric size of soaked beans. Therefore, the interaction of high feeding rate and small gap size was presumed to achieve the large reduction of particle size.

3.4 Conclusion

The influence of milling parameters of a new wet milling technique named micro wet milling system, for the particle size reduction and the PA content enrichment of Komatsuna juice was studied. The evaluation of milling characteristics of Komatsuna juice with different MWM conditions suggested that the milling time, milling rotational speeds, gap size, material feed rate had different effects on the reduction of particle size of Komatsuna juice, and the interactions among the parameters were presumed to achieve the large reduction of particle size. Moreover, the reduction of particle size had a profound benefit on the increase of PA content in the milled Komatsuna juice. This will improve the bioaccessibility of PA that converts to LPA in the GI tract. Compared to the Komatsuna juice ground by an ordinary mixer, the Komatsuna juice processed by MWM with the smaller particle size and higher PA content. In the future work, the interactive effects of MWM parameters are detailed investigated, suitable combination conditions of MWM are optimized.

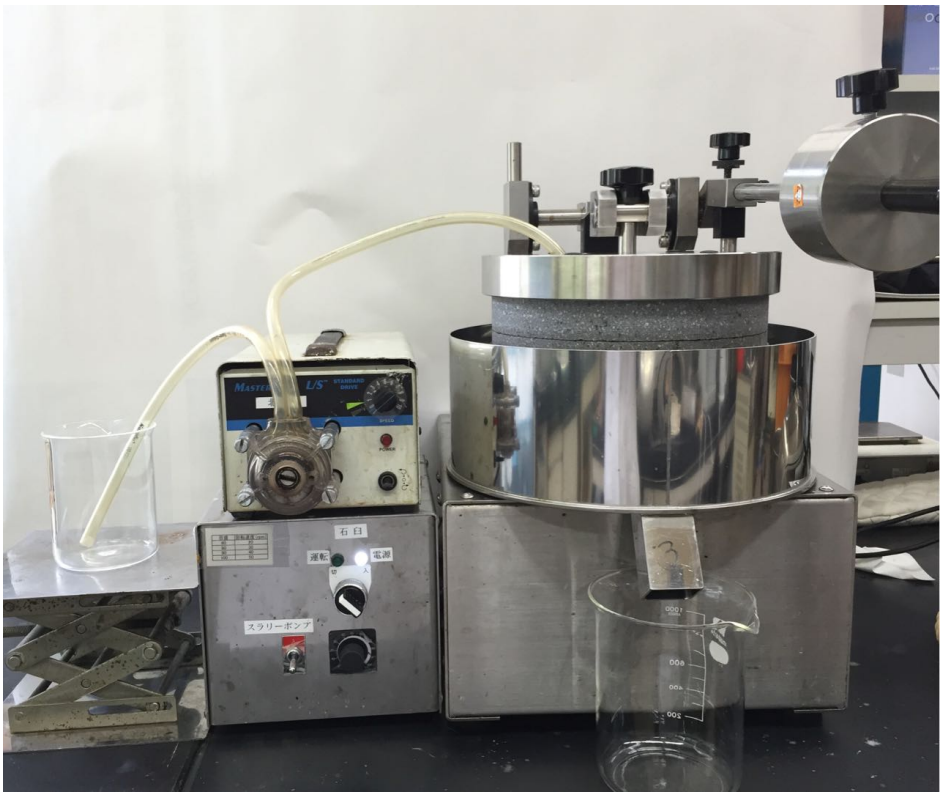

Fig. 3.1 The actual operational MWM system. In the MWM system, the pump feeds materials to the stone mill with an adjustable feeding rate, and the lower millstone with grooves can cleave materials to small particles efficiently at a certain milling rotation.

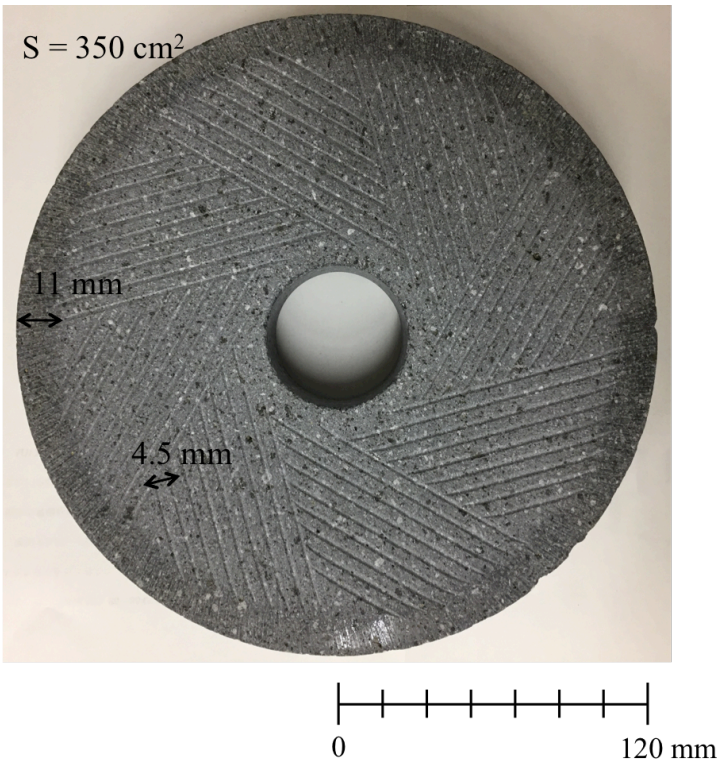

Fig. 3.2 The lower millstone of the Mill with contact surface area of 350 cm^2, millstone radius of 12 cm, edge distance of 11 mm, and groove width of 4.5 mm. The upper millstone is the same with the lower millstone.

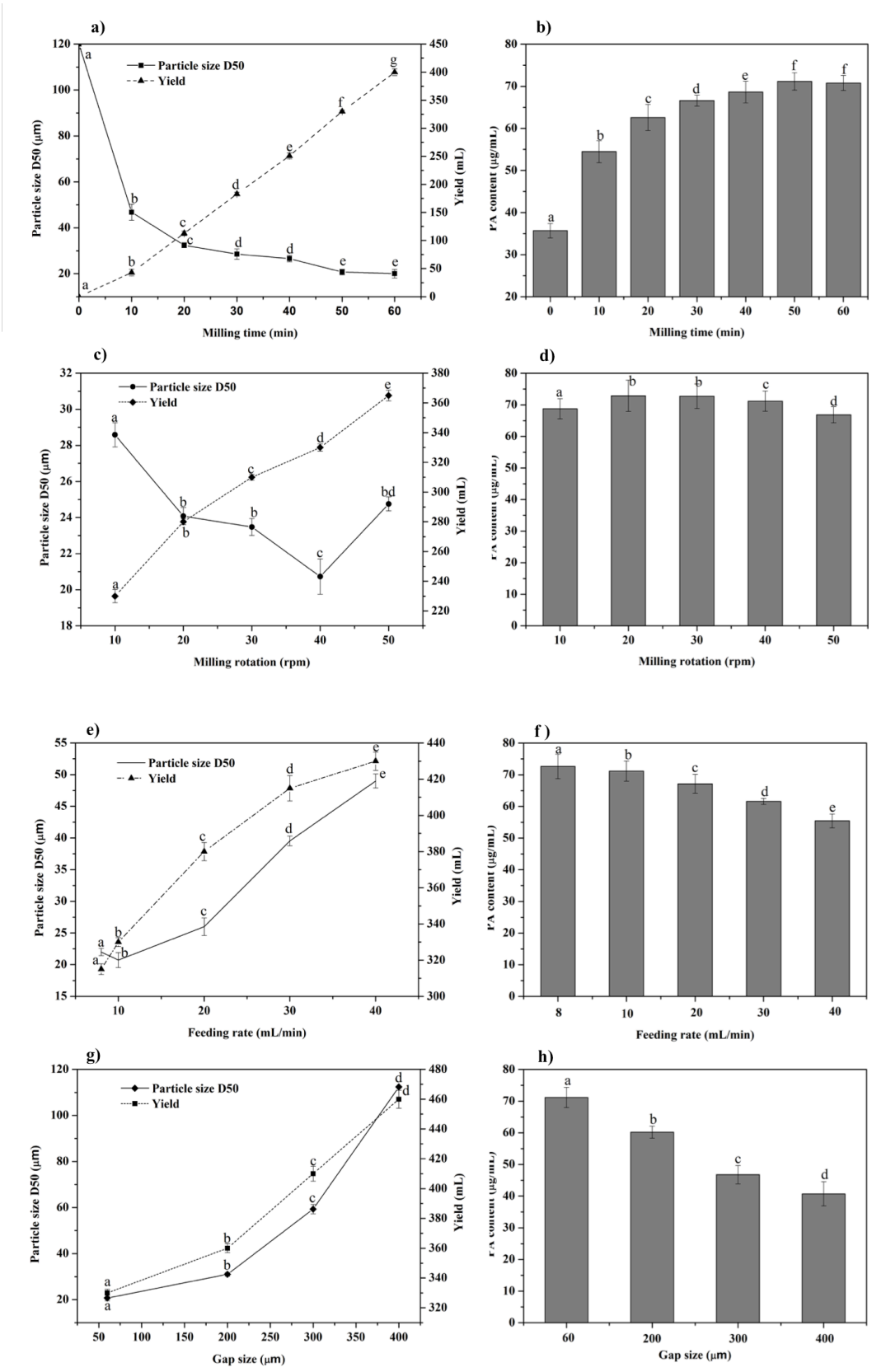

Fig. 3.3 The results of particle size, yield and PA content of Komatsuna juice with different MWM parameters. Significant differences ($p < 0.05$) were analyzed.

CHAPTER 4

THE OPTIMIZATION OF MWM PROCESSING CONDITIONS FOR KOMATSUNA (*BRASSICA RAPA* VAR. *PERVIRIDIS*) JUICE

4.1 Introduction

Based on the results of chapter 3, the particle sizes and PA contents of different milling rotational speeds were changing in a limit range, whereas the particle sizes and PA contents of different feeding rates and gap sizes were changing obviously. The milling parameters show different importance on reducing particle size and increasing PA content. Islam *et al.* (2017) reported that the higher rotational speed and higher gap produced the orange juice with larger particles, and Koyama (2014) also suggested that reducing the gap size and increasing the milling rotational speed benefit for the reduction of particle size. Therefore, the phenomenon that interaction effect of milling parameters on particle breakage and PA enrichment were observed. In this study, the interaction effects of different milling parameters on the particle size and PA content of Komatsuna juice were investigated, the sequence of primary parameters was analyzed, and the optimum process conditions were obtained.

4.2 Materials and Methods

4.2.1 Materials

Japanese mustard spinach (*B. rapa*; named Komatsuna in Japan) was purchased from a local market. Standard PA with a purity grade of 98% was purchased from

Sigma-Aldrich (St. Louis, USA). Chemicals: chloroform, methanol, hydrochloric acid, 28% ammonia solution were from Wako (Osaka, Japan). TLC Silica gel 60 was from Merck (Darmstadt, Germany).

4.2.2 Preparation of Komatsuna juice with MWM

Komatsuna was washing with tap water, mixed with water (4 ^{o}C) at a ratio of 1:1 (w/w), and blended by a mixer (SBC-1000J, Cuisinart, Japan) for 5 min at room temperature. The Komatsuna mixture (particle size of approximately 120 μm) was fed to the stone mill of the MWM with a pump with adjustable feeding rate, and milled with a certain rotational speed by controlling the lower millstone. The feed rate of the pump, milling rotation speed and milling time are explained below. The processed juice was collected simultaneously by rubber spatula during the successive milling. Finally, the collected Komatsuna juice was boiled for 5 min to inhibit enzyme activity and then analyzed.

4.2.3 The optimization of MWM conditions for Komatsuna juice

According to the results of the investigation of milling parameters, the milling parameters show different importance on reducing particle size and increasing PA content, and interactive effects of milling parameters on particle breakage and PA enrichment were also observed. Thus, the sequence of primary parameters and the interaction of milling parameters were investigated by an orthogonal array L_8 (2^7) to find optimum MWM processing conditions for Komatsuna juice with the properties of fine sensory acceptable particle size and high PA content.

The three variables, mill rotational speed, gap size, and feeding rate are reported to play a dominant role on the particle size and PA content. The particle size and PA content were set as the response value, respectively. The range and levels of the variables are listed in Table 4.1, and the design matrix for the experiment is shown in Table 4.2 and Table 4.4, followed by the results of range analysis and ANOVA analysis.

4.2.4 Measurement of particle size

The particle sizes were determined using a laser diffraction particle size analyzer (SALD-2200, Shimadzu Corporation, Japan) in the wet measurement mode. The D50 value of micrometers was chosen as particle size, termed the median diameter, and is defined as the average particle size by mass. Three independent determinations were conducted, and the results were presented as an average ± standard deviation.

4.2.5 Quantification of phosphatidic acid

Lipids were extracted from the Komatsuna juice by the Bligh and Dyer method with minor modifications (Bligh and Dyer, 1959; Tanaka et al., 2009). PA was isolated from lipids extract by TLC with the developing agent. The amount of PA was quantified by a colorimetrical method based on the phosphomolybudenum-malachite green formation with a spectrophotometer (Chalvardjian and Rudnicki, 1970). Reference the description of method 3.2.5 in chapter 3. Three independent measurements were repeated.

4.2.6 Statistical analyses

Student's *t*-test was performed between two groups for comparison; one-way ANOVA was conducted by SPSS software (version 22.0; IBM, Armonk, NY, USA). A p value of < 0.05 was considered as statistically significant.

4.3 Results and Discussion

4.3.1 The optimization of MWM milling parameters for the particle size Komatsuna juice

The orthogonal test is a simple method that analysis the influence of single variables and interaction of variables on the response value by range analysis and ANOVA analysis, suggesting the optimum combination of tested parameters in the several treatments. In this study, for optimizing the MWM processing conditions of Komatsuna juice, the orthogonal array L_8 (2^7) was designed, and 8 treatments were implemented with the different combinations of three factors in two levels (Table 4.1). The results of response values (particle size and PA content) and ANOVA analysis are shown in the Tables 4.2-4.5.

Table 4.2 shows the effects of milling rotational speed, gap size and material feeding rate on the particle size, and the result of range analysis expressed by the calculation values of $\overline{K}$ and R. $\overline{K}$ is the average value of all the particle sizes of one variable with one level, and R is an absolute value, the difference between $\overline{K_1}$ and $\overline{K_2}$. A high R value shows that the influence of the corresponding variable is more important. Therefore, the result of range analysis shows the primary and secondary sequence of different parameters effect on the reduction of particle size is in the order

of X_2, X_2X_3, X_3, X_1, X_1X_3, X_1X_2 and $X_1X_2X_3$. The interactive effect between gap size and feeding rate showed an importance effect on the reduction of particle size, which is similar to the results described in chapter 3. By decreasing the gap size and increasing the material feeding rate, the volume fraction of milling particles in the milling zone increases, the friction, collisions and the effective milling force of compression are also increased, and the particle size decreases. In order to reduce particle size effectively, the suitable variables combination in the tested range is $X_{1\text{-}1}X_{2\text{-}1}X_{3\text{-}1}$ or $X_{1\text{-}2}X_{2\text{-}1}X_{3\text{-}2}$.

To further investigate which factor significantly affects the reduction of particle size, ANOVA was carried out and the results are showed in Table 4.3. Statistical analyses indicated that gap size, the interaction of gap size and feeding rate, and feeding rate were the principal factors which have the most significant effects on the particle size ($p < 0.01$), whereas the milling rotational speed shows no significant effect on the particle size ($p > 0.05$). These results are in good agreement with the range analysis. Based on above analysis, the parameters of gap size and feeding rate show interactive effect on the particle size, the optimal MWM processing conditions of Komatsuna juice with smaller particles can be set as the milling rotational speed 20 rpm, gap size 60 μm and feeding rate 10 mL/min or the milling rotational speed 40 rpm, gap size 60 μm and feeding rate 20 mL/min, and particle size was verified at 21.803 $\pm$ 0.2 μm and 22.084 $\pm$ 0.4 μm according to the conditions, respectively.

4.3.2 The optimization of MWM milling parameters for the PA content of

Komatsuna juice

The orthogonal array L_8 (2^7) and ANOVA analysis were also conducted to investigate the effect of milling parameters on the response value of PA content. Similar to the above analysis, the result of range analysis (Table 4.4) shows that the importance of principal variables is in the order of X_2, X_1X_3, $X_1X_2X_3$, X_3, X_1, X_1X_2, X_2X_3, and that the interactions between milling rotation, gap size and feeding rate have a large effect on the PA content. ANOVA analysis (Table 4.5) shows the primary parameter of gap size has a significant effect on the PA content ($p < 0.05$), whereas the interactions among the factors show no significant effect on the PA content ($p > 0.05$). The suitable combination of variables for high PA content is $X_{1\text{-}1}X_{2\text{-}1}X_{3\text{-}1}$ or $X_{1\text{-}2}X_{2\text{-}1}X_{3\text{-}2}$, which is agreement with the analyzed result of particle size. These results verified the previous study reported in chapter 2, that the increase of PA content depends on the reduction of particle size, which can be explained by the high proportion of native Komatsuna PA released from the biomembrane by particle splitting, and the release of endogenous phosphatidylcholine during grinding that is converted to more PA by phospholipase D. Moreover, the PA contents of optimum conditions were verified to be 69.878 $\pm$ 2.6 μg/mL and 70.089 $\pm$ 3.4 μg/mL. Therefore, the MWM system is more effective in reducing the particle size and increasing PA content of Komatsuna juice to approximately 21 μm and 70 μg/mL, which is a six fold decrease of particle size and two fold increase of PA content compared to the ordinary grinding by a mixer (Figs. 3.3a and 3.3b, 0 min of milling time).

4.4 Conclusion

In this study, the importance and sequence of MWM parameters were investigated. The results revealed that the milling rotational speeds, gap size, material feeding rate and the interactions among the parameters had different significant effects on the reduction of particle size of Komatsuna juice. The optimum combination of MWM conditions were milling rotational speed 20 rpm, gap size 60 μm, material feeding rate 10 mL/min or milling rotational speed 40 rpm, gap size 60 μm, material feeding rate 20 mL/min. Correspondingly, Komatsuna juice was processed with the obtained optimum MWM conditions, and the particle size of Komatsuna juice was approximately 21 μm which is suitable for consumption (Inoue, 2011), the PA content of Komatsuna juice was approximately 70 μg/mL which is twice of that of the Komatsuna juice ground by an ordinary mixer. This will improve the bioaccessibility of PA that converts to LPA in the GI tract. Therefore, the MWM system is a successful processing method that is able to the produce Komatsuna juice with smaller particle size and rich PA content effectively. In the future work, MWM system is expected to be a potential facility of continuous industrial scale operation for producing the PA-rich food of Komatsuna juice, which can be a beneficial diet for the restoration or prevention of certain kinds of GI disorders.

Table 4.1 The Level and code of variables in the orthogonal array L_8 (2^7) for the MWM processing of Komatsuna juice

Codes levels	Variables		
	Milling rotational speed	Gap size	Material Feeding rate
	X_1 (rpm)	X_2 (μm)	X_3 (mL/min)
1	20	60	10
2	40	200	20

Table 4.2 The orthogonal array design and the analysis for the response value of particle size

Treatment	Independent variables							Respond value
	X_1	X_2	X_1X_2	X_3	X_1X_3	X_2X_3	$X_1X_2X_3$	Median diameter (μm)
1	1 (20)	1 (60)	1	1 (10)	1	1	1	21.271
2	1	1	1	2 (20)	2	2	2	22.997
3	1	2 (200)	2	1	1	2	2	37.704
4	1	2	2	2	2	1	1	90.641
5	2 (40)	1	2	1	2	1	2	24.865
6	2	1	2	2	1	2	1	22.614
7	2	2	1	1	2	2	1	42.369
8	2	2	1	2	1	1	2	93.444
$\overline{K_1}$	43.153	22.937	45.020	31.552	43.758	57.555	44.224	
$\overline{K_2}$	45.823	66.040	43.956	57.424	45.218	31.421	44.753	
R	2.670	43.103	1.064	25.872	1.460	26.134	0.529	

Table 4.3 Analysis of variance (ANOVA) showing the significance of independent variables on particle size

Source	SS	df	MS	F	Sig.
Corrected model	6434.642[a]	4	1608.661	681.044	0.000**
X_2	3715.694	1	3715.694	1573.080	0.000**
X_2X_3	1365.998	1	1365.998	578.310	0.000**
X_3	1338.695	1	1338.695	566.751	0.000**
X_1	14.255	1	14.255	6.035	0.091^{NS}
Error	7.086	3	2.362		

a. R^2=0.999

b. **: $p < 0.01$; *: $p < 0.05$; ^{NS}: non-significant.

Table 4.4 The orthogonal array design and the analysis for the response value of PA content

Treatment	Independent variables							Respond value
	X_1	X_2	X_1X_2	X_3	X_1X_3	X_2X_3	$X_1X_2X_3$	PA content (μg/mL)
1	1 (20)	1 (60)	1	1 (10)	1	1	1	66.403
2	1	1	1	2 (20)	2	2	2	61.282
3	1	2 (200)	2	1	1	2	2	47.197
4	1	2	2	2	2	1	1	50.859
5	2 (40)	1	2	1	2	1	2	55.449
6	2	1	2	2	1	2	1	65.194
7	2	2	1	1	2	2	1	46.550
8	2	2	1	2	1	1	2	50.701
$\overline{K_1}$	56.435	62.082	56.234	53.900	57.374	55.853	57.252	
$\overline{K_2}$	54.474	48.827	54.675	57.009	53.535	55.056	53.657	
R	1.962	13.255	1.559	3.109	3.839	0.797	3.594	

Table 4.5 Analysis of variance (ANOVA) showing the significance of independent variables on PA content

Source	SS	df	MS	F	Sig.
Corrected model	426.047[a]	4	106.512	23.103	0.014*
X_2	351.403	1	351.403	76.223	0.003**
X_1X_3	29.472	1	29.472	6.393	0.086^{NS}
$X_1X_2X_3$	25.837	1	25.837	5.604	0.099^{NS}
X_3	19.335	1	19.335	4.194	0.133^{NS}
Error	13.831	3	4.610		

a. R^2=0.969

b. **: $p < 0.01$; *: $p < 0.05$; ^{NS}: non-significant.

CHAPTER 5

CONCLUSIONS

In this thesis, the Komatsuna juice with rich PA was produced using the application of micro wet milling system.

In chapter 1, the background of this research was introduced. The material adopted in this research is Komatsuna, an important source of foodstuff-derived phosphatidic acid (PA). The phospholipids, lysophosphatidic acid (LPA) has a role in protecting the gastrointestinal tract, and is formed from the digestion of exogenous foodstuff-derived PA in the human digestive tract. Furthermore, the new wet milling technique named micro wet milling (MWM) system was illustrated in detail.

In chapter 2, the effects of steaming and milling on the PA contents of Komatsuna with inactivated and activated PLD were observed, and the mechanism responsible for abundant PA formation in Komatsuna during grinding was demonstrated. Acccording to the results of particle sizes and PA contents of Komatsuna juice under different processing conditions, the main findings are as follows: (i) with longer grinding times, Komatsuna tissues were cleaved into smaller particles; (ii) sufficient amounts of PA were released from Komatsuna; and (iii) the smaller the particle size, the higher the PA content; and (iv) the MWM system more effectively manufactured PA-rich Komatsuna juice. Taken together, these findings suggest that the high PA content was primarily due to the release of native PA from the Komatsuna biomembrane by particle disruption, and secondarily, to the release of

endogenous PC during the grinding process, which resulted in the conversion to PA by PLD.

In chapter 3, the influences of the MWM milling parameters (milling time, milling rotational speed, material feed rate and gap size) on the particle size reduction and the PA content enrichment of Komatsuna juice are studied. The evaluation of milling characteristics of Komatsuna juice with different MWM conditions suggested that the milling time, milling rotational speeds, gap size, material feed rate had different effects on the reduction of particle size of Komatsuna juice, and the interactions among the parameters were presumed to achieve the large reduction of particle size. Moreover, the reduction of particle size had a profound benefit on the increase of PA content in the milled Komatsuna juice. Compared to the Komatsuna juice ground by an ordinary mixer, the Komatsuna juice processed by MWM with the smaller particle size and higher PA content.

In chapter 4, the importance and sequence of MWM parameters were analyzed, and the MWM conditions for processing Komatsuna juice with small particle sizes and high PA contents are optimized. The results revealed that the milling rotational speeds, gap size, material feeding rate and the interactions among the parameters had different significant effects on the reduction of particle size of Komatsuna juice. The optimum combination of MWM conditions were milling rotational speed 20 rpm, gap size 60 μm, material feeding rate 10 mL/min or milling rotational speed 40 rpm, gap size 60 μm, material feeding rate 20 mL/min. Correspondingly, Komatsuna juice was processed with the obtained optimum MWM conditions, and the particle size of

Komatsuna juice was approximately 21 μm which is suitable for consumption, the PA content of Komatsuna juice was approximately 70 μg/mL which is twice of that of the Komatsuna juice ground by an ordinary mixer. This will improve the bioaccessibility of PA that converts to LPA in the GI tract. Therefore, the MWM system is a successful processing method that is able to the produce Komatsuna juice with smaller particle size and rich PA content effectively. In the future work, MWM system is expected to be a potential facility of continuous industrial scale operation for producing the PA-rich food of Komatsuna juice, which can be a beneficial diet for the restoration or prevention of certain kinds of GI disorders.

Therefore, based on the revealed mechanism that responsible for the formation of increased PA in Komatsuna during milling process, the Komatsuna juice was manufactured to the beneficial properties of small particle size and rich PA content with the utilization of optimal process conditions of MWM, and the resulting product can support a beneficial diet for the prevention or restoration of GI disorders. Moreover, the optimal parameter conditions of MWM are different according to the different characteristic materials. Previously, Koyama (2014) had discussed the application of MWM system on processing a non-Newtonian fluid of rice slurry, whereas the Komatsuna juice is a Newtonian fluid, which results in different milling conditions of MWM system in this study, compared to rice slurry. Results of the influences of milling time, milling rotational speed, material feeding rate and gap size on Komatsuna processing that described here, can provide a reference for MWM operation to produce the products with beneficial properties.

The final purposes of improving PA content and reducing particle size with the utilization of MWM system are to enhance the bioaccessibility of PA and bioavailability of LPA, in the human digestion tract. In the future work, the bioaccessibility of PA in milled Komatsuna juice can be evaluated by in vitro simulated gastrointestinal digestion, the bioavailability of LPA is suggested to investigate by in vivo test.

SUMMARY

Komatsuna (*Brassica rapa* var. *perviridis*) is a leaf vegetable, traditional and popular in the Japanese diet, and normally eaten as a kind of salad (boiled). The content of phosphatidic acid (PA) in fresh Komatsuna has been estimated to be around 43.6% of the total phospholipids, which shows Komatsuna is an important source of foodstuff-derived PA. Oral administration of PA showed an opportunity to prevent gastric ulcer in mice, due to the formation of lysophosphatidic acid (LPA) from PA in the gastrointestinal (GI) tract by gastric phospholipase A_2. LPA plays the important role in the integrity of GI tract epithelium. Moreover, PA does not accumulate at high levels in Komatsuna, but is abundantly released upon grinding of Komatsuna. Therefore, in this research, the new wet milling technique named Micro Wet Milling (MWM) system was adopted to process Komatsuna juice with small particles and rich PA content, which has potential use as a dietary treatment for the restoration or prevention of GI disorders.

In chapter 1, the background of this research was mentioned initially and the new wet milling technique of MWM system was illustrated in detail. At last, the research objective was proposed.

In chapter 2, to explore the mechanism responsible for abundant PA formation in Komatsuna during milling, the particle sizes and PA contents of Komatsuna juice under different processing conditions were measured. The results suggested that (i) with longer grinding times, Komatsuna tissues were cleaved into smaller particles; (ii)

sufficient amounts of PA were released from Komatsuna; and (iii) the smaller the particle size, the higher the PA content; and (iv) the MWM system more effectively manufactured PA-rich Komatsuna juice. Taken together, these findings suggest that the high PA content was primarily due to the release of native PA from the Komatsuna biomembrane by particle disruption, and secondarily, to the release of endogenous PC during the grinding process, which resulted in the conversion to PA by PLD.

In chapter 3, to investigate the influences of the MWM milling parameters (milling time, milling rotational speed, material feed rate and gap size) on the particle size reduction and the PA content enrichment of Komatsuna juice, the milling characteristics of Komatsuna juice with different MWM conditions were evaluated. The milling time, milling rotational speeds, gap size, material feed rate had different effects on the reduction of particle size of Komatsuna juice, and the interactions among the parameters were presumed to achieve the large reduction of particle size. Moreover, the reduction of particle size had a profound benefit on the increase of PA content in the milled Komatsuna juice. Compared to the ordinary mixer, the Komatsuna juice processed by MWM with the smaller particle size and higher PA content.

In chapter 4, in order to obtain the optimum MWM conditions for processing Komatsuna juice with small particle sizes and high PA contents, the importance and sequence of MWM parameters were analyzed by an orthogonal array L_8 (2^7). The results showed that the milling rotational speed, gap size, material feeding rate and the

interactions among the parameters had different significant effects on the reduction of particle size of Komatsuna juice. The optimum combination of MWM conditions were milling rotational speed 20 rpm, gap size 60 μm, material feeding rate 10 mL/min or milling rotational speed 40 rpm, gap size 60 μm, material feeding rate 20 mL/min. Correspondingly, Komatsuna juice was processed with the obtained optimum MWM conditions, and the particle size of Komatsuna juice was approximately 21 μm which is suitable for consumption, the PA content of Komatsuna juice was approximately 70 μg/mL which is twice of that of the Komatsuna juice ground by an ordinary mixer. This will improve the bioaccessibility of PA that converts to LPA in the GI tract. Therefore, the MWM system is a successful processing method that is able to the produce Komatsuna juice with smaller particle size and rich PA content effectively.

Taken together, these findings suggest that using the application of optimal process conditions of MWM system, the Komatsuna juice was produced with the properties of small particle size and rich PA content, which can be a beneficial diet for the restoration or prevention of certain kinds of GI disorders. MWM system is expected to be a potential facility of continuous industrial scale operation for producing the products with appreciative properties.

ACKNOWLEDGEMENT

Firstly, I would like to express my deep gratitude to my supervisor, Professor Dr. Yutaka Kitamura, Life and Environmental Science at University of Tsukuba, for his generous guidance and precious advice throughout my research work. I am also thankful to him for providing me all the facilities regarding this research work.

I am grateful to the members of my supervisory committee, Prof. Zhang, Prof. Yang and Prof. Miyazaki in Graduate School of Life and Environmental Science at University of Tsukuba, for their worthy suggestions to improve this manuscript.

I owe my thanks and appreciation to Dr. Mito Kokawa, Assistant professor, Life and Environmental Science at University of Tsukuba, for her detailed guidance and support, and many valuable suggestions for my research work.

I also would like to thank the China Scholarship Council (CSC). I was enjoyed a comfortable study life in the three years due to the support of China government.

Thanks to all the members of our laboratory, the laboratory of Food and Biomass Engineering, Life and Environmental Science. Thanks for their help in my study life.

In the end, I would express my sincere gratitude to my parents and friends. Thank you so much for their constant support, care and encourage.

PUBLICATIONS

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