Physico-Chemical and Functional Properties of Leaf Protein Concentrates Isolated Using Different Techniques

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Pakawan Meesiri

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Abstract

Leaf proteins are a potential sustainable protein source for human food as they contain RuBisCo, the most abundant protein in the world (Ellis, 1979). However, these proteins, particularly grasses, are still underutilized due to their undesirable characteristics, such as dark green colour (Di Stefano et al., 2018) and grassy flavour, limiting food applications (Hatanaka, 1996). This thesis studies the effect of different isolation methods on leaf protein concentrates (LPCs) and determines the physico-chemical characteristics of the selected LPCs.

In the first part of the study, leaf proteins were extracted through different extraction methods using perennial ryegrass. The result shows that alkali-acid precipitation and alkali-acid precipitation combined with activated carbon (AC) treatment could not remove the green colour and grassy flavour of LPCs. The highest protein content (53.94 % dry basis) and yield (9.64 % of dried grass) of LPC were obtained through alkali-acid precipitation without $CaCl_2$ treatment. The green colour and grassy flavour were improved using the heat coagulation method without $CaCl_2$ and NaOH. The protein content was 48.16 % (dry basis); however, a low extraction yield was obtained (1.12 % of dried grass). Although ultrafiltration and chromatography techniques could isolate the brown and colourless LPC, a low yield and protein content were observed. Combining $CaCl_2$ or AC with the extraction process enhanced the removal of green colour and grassy flavour, but the protein content and extraction yield decreased. Alkali assisted the protein extractability when used with acid precipitation. In contrast, the adverse effects of alkali combined with heat coagulation were obtained.

Two leaf protein concentrates (LPCs) were selected to be analysed in the second part: LPC extracted through alkali-acid precipitation without $CaCl_2$ (AAP-0), and LPC obtained through heat coagulation without $CaCl_2$ and NaOH (HC-0). AAP-0 had protein solubility higher than HC-0 at pH below 2 and above 5. The protein solubility of AAP-0 was significantly improved (p <0.05) after the treatment of 80 °C for 10 minutes combined with pH shift, while the protein solubility of HC-0 was rarely affected using the same treatment. The denaturation temperature and enthalpy of HC-0 were lower than AAP-0. SDS-PAGE results show that the large and small subunits of RuBisCo protein were detected in HC-0, while the large subunit was not detected in AAP-0 due to less protein purity recovered through alkali-acid precipitation. During digestion, AAP-0 had higher solubility than HC-0, whereas its free amino N was lower than HC-0. SDS-PAGE show that AAP-0 had lower digestibility than HC-0 as 10 kDa proteins still appeared in AAP-0 at 180 minutes, while all protein bands were not observed in HC-0.

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Abbreviations

AAP-0 = Leaf protein concentrate: Through alkali-acid precipitation with 0 mM $CaCl_2$

AC = Activated carbon

ANZFA = Australia New Zealand Food Authority

BW = Body weight

$CaCl_2$ = Calcium chloride

CPA = Cyclopiazonic acid

CT = Condensed tannins

DM = Dry matter

DSC = Differential scanning calorimetry

EAA = Essential amino acids

EC = Emulsion capacity

ES = Emulsion stability

FAO = Food and Agricultural Organization of The United Nations

FSANZ = Food Standards Australia New Zealand

GPC = Grass protein concentrates

HC-0 = Leaf protein concentrate: Through heating at 50 °C, 15 minutes with 0 mM $CaCl_2$, followed by heat coagulation.

HCl = Hydrochloric acid

IEX = Ion exchange chromatography

LOX = Lipoxygenase

LPC = Leaf protein concentrate

LPI = Lentil protein isolate

MW = Molecular weight

MWCO = Molecular weight cut-off

N = Nitrogen

NaCl = Sodium chloride

NaOH = Sodium hydroxide

ND = Not detected

NOAEL = No observed adverse effect level

PAs = Pyrrolizidine alkaloids

pI = Isoelectric point

PPO = Polyphenol oxidase

RuBisCo = Ribulose-1,5-diphosphate carboxylase oxygenase

SDS-PAGE = Tricine-Sodium Dodecylsulfate Polyacrylamide Gel Electrophoresis

SEC = Size exclusion chromatography

SGF = Simulated gastric fluid

SIF = Simulated intestinal fluid

SSF = Simulated salivary fluid

T_d = Denaturation temperature

UF = Ultrafiltration

UNU = United Nations University

VOCs = Volatile organic compounds

WHO = World Health Organisation

CHAPTER 1

Introduction

The demand for protein consumption and low-cost protein sources is on the rise due to the world population growth (estimated to be 10 billion by 2050) (United Nations, 2019) and a reduction goal of child undernutrition (Di Stefano et al., 2018). Vegetarian and vegan dietary trends (Best, 2015) and the rise in people's consciousness of food diversity (Bohrer, 2017) have increased the demand for plant-based proteins. As a result of the rising protein need, sustainable protein sources are required. Although meat and dairy are common protein sources due to their high nutritional value, plant proteins are considered more sustainable than animal proteins (Sutton et al., 2018).

Legumes, cereals, nuts, and seeds are common plant proteins (González-Pérez & Arellano, 2009). However, novel plant protein sources should be explored to contribute to sustainable food goals (United Nations, 2020). Plant leaves are an interesting protein source as their protein content is comparable to other proteins, such as cow milk (Barbeau & Kinsella, 1988). The main soluble protein in plant leaves is RuBisCo which accounts for 50 % of total soluble leaf proteins and is recognised as the most abundant protein in the world (Ellis, 1979). Sugar beet leaves, spinach, and alfalfa are also common sources in this area of research. However, pasture leaves, the most widespread plant used for animal feed, have not been studied. Perennial ryegrass is the principal forage for ruminants in New Zealand and several countries. It is abundant worldwide and easy to cultivate (Charlton & Stewart, 1999); thus, it is potentially a novel protein source.

Although the abundance and sustainability of plant leaves are acknowledged, using leaf proteins is still unavailable for humans. This is due to the laborious isolation process and unacceptable sensory attributes, such as dark green colour caused by chlorophyll (Di Stefano et al., 2018) and grassy flavour generated by lipid oxidation (lipoxygenase and unsaturated fatty acids) (Hatanaka, 1996). Many techniques: chemical solvents, mechanical extraction, enzymatic assistance, and a combination of those techniques, have been studied. However, the final product was green in colour (Kaur et al., 2021). Consequently, using leaf protein concentrate (LPC) as a food ingredient is challenging (Di Stefano et al., 2018). Hence, a more effective extraction is needed to remove chlorophyll and grassy flavour.

This project was divided into two sections based on the objectives:

Objective (1): To evaluate the different extraction processes to isolate the colourless and odourless LPC

Perennial ryegrass (cultivar-*One50 Ar1*) was selected for this study as it delivers excellent persistence from summer through winter. *One50 Ar1* has a high protein content (18 % protein dry basis) and provides a higher total dry matter (DM) yield by producing 3 % more than standard ryegrass across all seasons (Agriseeds, 2013; Kerr et al., 2012). Different extraction techniques (alkali-acid precipitation, heat treatment, divalent cations treatment, activated carbon, ultrafiltration, and chromatography) were studied to isolate the colourless and odourless LPC. Protein content and the final yield of LPC using the above methods were determined. The colour and flavour of the final products were also evaluated. Furthermore, the possibility of using ethanol for chlorophyll removal from grass leaves and final LPC was explored.

Objective (2): To determine the physico-chemical and functional characteristics of the selected LPC.

The selected LPCs were analysed for thermal denaturation, protein profile, protein solubility, and protein digestibility. Differential scanning calorimetry (DSC), Tricine-Sodium Dodecylsulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE), Kjeldahl, and *in vitro* oral-gastro-small intestinal digestibility analyses were conducted to determine the above characteristics.

CHAPTER 2

Literature review

2.1. Introduction

Protein plays a vital role in growth, cell repair, muscle regeneration, and immunity development (Sá et al., 2020), making it an essential nutrient. It is also utilised as a food additive in a wide range of food products since its functional properties (e.g., emulsifying, gelation, foaming, and solubility) contribute to the sensory attributes and stability of food products (Day, 2013).

A double demand for proteins has been estimated since the world population is going to be over 9 billion by 2050 (United Nations, 2019). A growing trend of vegetarianism, veganism and a healthy lifestyle has also been observed (Best, 2015), resulting in the rising demand for plant-based proteins. Another global issue is undernutrition which causes death in children globally and health issues for 795 million people in low-income countries (Di Stefano et al., 2018). This relates to the high cost of animal agriculture and meat production (Müller & Krawinkel, 2005). Thus, all the challenges mentioned above result in higher demand for protein consumption and affordable proteins.

Meat and dairy have been used as the primary protein sources for humans since they are good sources of essential amino acids (EAA), vitamin B, and many minerals (Bohrer, 2017). However, the environmental impacts from livestock (such as greenhouse gas, nitrogen footprint, and wastewater) make meat products less sustainable proteins and less profitable due to carbon emission tax. In contrast, plant proteins are more sustainable than meat proteins since plant farming causes fewer environmental impacts (Sutton et al., 2018). The agricultural cost of plant proteins is also lower than livestock farming (Müller & Krawinkel, 2005). Hence, plant proteins should be further studied as alternative proteins to supply future demand and relieve malnutrition issues.

2.2 Plant proteins

Many studies have reported that plant proteins contain lower protein density and EAA than animal products (Bohrer, 2017; Di Stefano et al., 2018 Sá et al., 2020). Thus, consuming a higher quantity of various plant proteins is suggested to compensate for the proteins and EAA (Day, 2013). Nevertheless, some plant protein isolates (pea, soy, potato, chickpea, maize, and brown rice) have sufficient EAA as the requirement of 0.66 g/kg body weight/day for adults, as stated by the Joint World Health Organization/ Food and Agricultural Organization of The United Nations/ United Nations University (WHO/FAO/UNU) (Gorissen et al., 2018). A healthy vegetarian diet containing beans, peas, soy products, nuts, and cereals, is a well-planned diet that can replace meat, seafood, and poultry in terms of nutrition (U.S. Department of Health and Human Services, 2016).

2.2.1 Legumes

Legumes have been consumed as a universal food for over 20,000 years as they are excellent sources of proteins, carbohydrates, fibre, vitamins, minerals, and energy. (González-Pérez & Arellano, 2009). Soybean, pea, chickpea, and lupin are commercial legumes consumed worldwide (Sá et al., 2020). Soybean is a major crop among all legumes and contains approximately 40 % protein and 32 to 50 % protein among all cultivars (González-Pérez & Arellano, 2009; Sá et al., 2020). Peas, lupin, and chickpeas are attractive plant-based proteins in Europe due to their low allergen and non-GMO status (Amarakoon, 2012; Mattila et al., 2018). Gorissen et al. (2018) reported that soy and pea protein isolates contained sufficient EAA according to the WHO/FAO/UNU (2007) reference pattern. Most legumes contain a high amount of lysine (Lys); however, the limiting amino acids are sulfur-containing amino acids (methionine (Met) and cysteine (Cys)) and tryptophan (Trp) (Sá et al., 2020).

2.2.2 Cereals

Cereals or grains are edible plant seeds accounting for 50% of total plant-based protein consumption worldwide. The most commonly consumed cereals are rice, wheat, barley and maize. The protein content ranges from 7 to 13 % (7, 10, 11, and 11 % for rice, maize, wheat, and barley, respectively) (González-Pérez & Arellano, 2009). The EAA of maize protein isolate is sufficient to meet the WHO/FAO/UNU amino acid requirements, whereas wheat protein isolate provides insufficient EAA (Gorissen et al., 2018). Similarly, rice and barley are

good nutrient sources (carbohydrates, vitamins, and minerals), but Lys is their limiting amino acid (Sá et al., 2020).

2.2.3 Nuts

Nuts are excellent sources of fatty acids and proteins (18.22 g – 25.80 g/100g serving). Some species of almonds (e.g., Baru almonds) exhibit complete EAA profiles (Sousa et al., 2011). Consequently, the use of almonds to substitute milk in dairy beverages has increased (Qamar et al., 2020). Almond milk, with a 1:3 nut and water ratio, contained 1.7 % protein (Alozie Yetunde and Udofia (2015), as cited in Qamar et al., 2020). Similarly, Shakerardekani et al. (2013) reported that 4 % of proteins were observed in pistachio milk. Thus, the protein content in nut milk would be comparable to cow milk (3.5 % on average) (Barbeau & Kinsella, 1988). However, consuming nuts provides higher energy than other food proteins. For instance, nuts provide 553-579 kcal per 100 g, while soy-based products and raw meat provide 61-78 kcal and 166 – 310 kcal per 100 g, respectively (Bohrer, 2017).

2.2.4 Seeds

A growing trend in seed consumption has been reported due to their high nutritional value. Flaxseeds, chia, watermelon seeds, and paprika seeds have been used in human diets (El-Adawy & Taha, 2001; Giacomino et al., 2013; Lakshmi & Kaul, 2011). Flaxseeds are a rich source of protein, phenolic compounds, and fibre; however, Lys is the limiting amino acid (Giacomino et al., 2013). Lys deficiency is also observed in chia (Olivos-Lugo et al., 2010). Paprika seeds are rich in Lys, Met, and Cys; however, isoleucine (Ile) and sulfur amino acids are insufficient compared to the WHO/FAO/UNU reference pattern (El-Adawy & Taha, 2001). Watermelon seeds are an excellent source of leucine (Leu) and arginine (Arg); nevertheless, their EAA content is below the WHO/FAO/UNU recommendation (Lakshmi & Kaul, 2011).

In conclusion, plant proteins are used as alternative proteins to substitute meat and dairy products. Consuming a single plant protein provides lower proteins and EAA than animal proteins; however, obtaining EAA from plant proteins per WHO/FAO/UNU recommendations may be achieved by consuming various plant protein sources or higher quantities. Some plant proteins (e.g., soy protein isolate, maize protein isolate, and pea protein isolate) also have a complete EAA profile. The protein content and EAA profiles of plant proteins are shown in Table 2.1.

Table 2.1: Proteins and essential amino acid profiles of various food proteins.

Protein sources	Soybean	Pea	Chickpea	Lupin	Barley	Maize	Rice
Proteins (g/100g)	44.53	n/a	24.7 ± 1.7	30.5 ± 1.6	10.0	10.6	9.57 ± 0.11
Essential amino acids (g/100g protein)							
Isoleucine (Ile)	4.45	4.23 ± 0.09	6.0 ± 0.1	3.94 ± 0.01	3.5	3.9	3.25 ± 0.11
Leucine (Leu)	7.82	7.11 ± 0.16	10.0 ± 0.1	6.85 ± 0.04	7.7	11.6	6.51 ± 0.32
Lysine (Lys)	5.91	6.93 ± 0.20	8.5 ± 0.1	4.73 ± 0.05	3.9	3.3	2.48 ± 0.25
Valine (Val)	4.58	4.72 ± 0.07	5.7 ± 0.1	3.95 ± 0.05	5.4	4.9	3.52 ± 0.13
Histidine (His)	2.58	2.22 ± 0.12	3.3 ± 0.1	2.56 ± 0.01	2.4	3.3	1.73 ± 0.09
Threonine (Thr)	3.61	3.45 ± 0.03	4.7 ± 0.1	3.49 ± 0.04	3.9	3.9	1.56 ± 0.04
Tryptophan (Trp)	0.33	n/a	n/a	n/a	n/a	1.0	n/a
Phenylalanine (Phe)	n/a	4.87 ± 0.27	7.9 ± 0.1	3.86 ± 0.03	5.7	4.9	4.13 ± 0.17
Methionine (Met)	n/a	5.00 ± 0.06	2.1 ± 0.1	0.80 ± 0.01	2.1	2.0	1.74 ± 0.03
Source	Vasconcelos et al. (2006)	Amarakoon (2012)	Sánchez-Vioque et al. (1999)	Mattila et al. (2018)	Ejeta et al. (1987)	Ejeta et al. (1987)	Liu et al. (2017)

n/a data was not reported in the respective study.

Table 2.1 (Continued)

Protein sources	Flaxseeds	Chia	Watermelon seeds	Peanut	Baru almond	Cashew nut
Proteins (g/100g)	26.35 ± 0.05	24.6 ± 2.5	35.7	29.59 ± 0.05	29.92 ± 0.37	22.67 ± 0.20
Essential amino acids (g/100g protein)						
Isoleucine (Ile)	3.98 ± 0.06	3.66 ± 0.28	2.8	3.27	3.25	4.19
Leucine (Leu)	6.08 ± 0.13	5.55 ± 0.77	7.7	6.71	7.44	7.31
Lysine (Lys)	3.66 ± 0.09	3.70 ± 0.43	3.14	4.38	6.64	4.46
Valine (Val)	4.80 ± 0.02	4.80 ± 0.23	3.98	3.55	5.56	5.38
Histidine (His)	2.5 ± 0.05	2.24 ± 0.11	3.21	2.68	2.34	2.33
Threonine (Thr)	4.35 ± 0.04	2.91 ± 0.20	3.09	3.18	5.53	3.60
Tryptophan (Trp)	1.55 ± 0.02	n/a	1.15	0.71	1.12	2.65
Phenylalanine (Phe)	4.88 ± 0.09	4.01 ± 0.37	5.76	n/a	n/a	n/a
Methionine (Met)	1.52 ± 0.01	2.50 ± 0.25	1.71	n/a	n/a	n/a
Source	Giacomino et al. (2013)	Olivos-Lugo et al. (2010)	El-Adawyand and Taha (2001)	Sousa et al. (2011)	Sousa et al. (2011)	Sousa et al. (2011)

n/a data was not reported in the respective study.

2.2.5 Emerging proteins

Green biomass, such as plant leaves and algae, is an emerging alternative protein source. It has been studied due to its abundant availability and high protein yield per cultivated area (Tenorio et al., 2018). Many types of plant leaves, such as sugar beet leaves, spinach (Martin et al., 2014, 2019), and alfalfa (Lamsal et al., 2007), have been studied to extract the leaf proteins. Protein content in plant leaves is similar to cow milk (3.5% protein), ranging from 1.2 to 8.2% protein (3.5% on average) wet weight basis. However, the extraction process of leaf proteins is more laborious than other food proteins, and half of the total leaf proteins are insoluble (Barbeau & Kinsella, 1988). In addition, the difficulty of green colour removal makes the final isolated proteins difficult to apply to food (Di Stefano et al., 2018). For these reasons, plant leaves have been neglected as human protein sources.

Several limitations of leaf protein extraction still exist, one of which is low extraction yield. The extraction yield of common plant proteins is around 50 to 60 %, with a high protein content: 70 % in protein concentrates and 90 % in protein isolates, respectively. In comparison, the extraction yield of green biomass is usually below 10 %, with 60 to 80 % protein purity (Dale et al., 2009). Moreover, due to differences in the tissue structure, some current processes used for seeds, legumes, and cereals cannot be applied to plant leaves and other green biomass. For instance, dry fractionation is suitable for pulse and seeds (Tenorio et al., 2018). Transportation and storage management for green biomass is costly due to its high moisture content (Kammes et al., 2011).

Although current plant proteins can alleviate protein scarcity, researching novel protein sources, such as plant leaves, is imperative to support future demand. However, pasture leaves (such as grass and white clover) have not been widely explored. Thus, the extraction process of leaf proteins, focusing on pasture leaves, should be studied and optimised to improve the quality of products and other limitations.

2.2.5.1 White clover

White clover (*Trifolium repens L.*) is a common pasture for feeding cattle worldwide. It is usually sown with ryegrass, lucerne, chicory, and plantain to increase total nitrogen in forage. White clover has protein content ranging from 17 to 33 % dry basis (around 23 % on average) (FAO, 2011). It has high protein, and its amino acid balance is comparable to soy, as shown in Table 2.2; nevertheless, Met and Cys are the limiting amino acids. A good digestibility of white

clover leaves has been reported in monogastric young animals (e.g., rats) (Stødkilde et al., 2018). However, low digestibility has been found in flowers, which make up a considerable part of leave (up to 50 %) in summer (Søegaard, 1993). Considering white clover leaves as the raw material of protein extraction, they may be inconstant across all seasons.

Table 2.2: Protein content and essential amino acid profiles of white clover and soybean.

Plant source	White clover		Soymeal
	plant	leaves	
Crude protein (% dry basis)	32.56	39.86	n/a
Essential amino acids (g/16g N)			
Isoleucine (Ile)	4.09	4.18	5.1
Leucine (Leu)	7.03	7.03	7.7
Lysine (Lys)	5.23	5.29	6.9
Valine (Val)	5.06	5.21	5.4
Histidine (His)	2.06	2.06	n/a
Threonine (Thr)	4.19	4.33	4.3
Tryptophan (Trp)	1.66	5.72	1.3
Phenylalanine (Phe)	4.68	5.89	9.4[1]
Methionine (Met)	1.36	1.40	2.4[2]
Source	Stødkilde et al. (2018)	Stødkilde et al. (2018)	Barbeau and Kinsella (1988)

[1] Phe+Tyr. [2]Met+Cys.

n/a data was not reported in the respective study.

2.2.5.2 Grass

Grass has been used for animal feed worldwide and is one of the popular forages in New Zealand. Perennial ryegrass (*Lolium perenne* L.) is the most widespread grazing pasture due to the simplicity of planting (Charlton & Stewart, 1999). It contains approximately 17 to 21 % protein (Jensen, 2003) depending on plant cultivar, irrigation level, temperature, humidity, nutrient supply, and season (Barbeau & Kinsella, 1988). Although it is fast-growing, some cultivars have poor quality in drought conditions (Charlton & Stewart., 1999).

One50 cultivar is the largest-selling perennial ryegrass in New Zealand due to its well-proven persistence and smooth growth from summer through winter (Agricom research, 2020). The

persistence is evaluated by the percentage of ground cover and average plant pulling score. These indicators dedicate the plant loss through animal grazing and pulling, leading to pasture density loss. A study from nine trials in the North Island (New Zealand) from 2003 to 2011 reported that *One50 Ar 1 (One 50* cultivar with endophyte *Ar1)* had a moderate ground cover percentage (66 %) among all cultivars (54 to 71 %). The average plant pulling score was 7.7 (the top five cultivars among all studied samples). Also, the highest rust score was reported, indicating excellent crown rust resistance (a pathogen reducing the photosynthetic area) (Kerr et al., 2012). Moreover, *One50 Ar1* provides a 3 % DM higher than the standard ryegrass (Agriseeds, 2013; Kerr et al., 2012). It also has high protein content (17.8 % dry basis) (Agriseeds, 2013).

Perennial ryegrass is the most widely used pasture in New Zealand, and its persistence in drought conditions is better than white clover (Søegaard, 1993). *One50 Ar1*, a perennial ryegrass cultivar, has good persistency, smooth seasonal growth, high proteins, and high DM, making this cultivar an attractive candidate as an alternative protein source. Thus, *One50 Ar1* was selected for the present study.

2.3 Nutritional compositions of plant leaves

The major components in plant leaves are carbohydrates, fat, protein, and fibre. Other compounds, such as antioxidants, bioactive compounds, vitamins, and minerals, are minor components (González-Pérez & Arellano, 2009). The protein is mainly discussed in the present study as it is relevant to the thesis. Therefore, two main areas are categorised: protein and non-protein sections.

2.3.1 Proteins in plant leaves

Plant leaves have various proteins, such as membrane proteins, chlorophyll-protein complexes, structural proteins, and enzymes. They are categorised into soluble cytoplasmic and insoluble chloroplastic proteins or white and green proteins, respectively (Barbeau & Kinsella, 1988).

2.3.1.1 Non-soluble proteins

Non-soluble proteins account for 50% of total proteins in plant leaves, mainly lipoproteins (membrane proteins) (Barbeau & Kinsella, 1988) and chlorophyll-protein complexes (Liu et al., 2004). Membrane proteins are usually lost in the step of discarding leaf pulp due to their low solubility (Lamsal et al., 2007), heterogeneity of proteins, and the interaction between

proteins and other components (e.g., protein-lipid membrane and protein-enzyme cofactors) (Wang et al., 2008).

The chlorophyll-protein complexes are located in the thylakoid membrane of chloroplast (Liu et al., 2004). These complexes are crucial for plants' photosynthesis, changing solar energy to chemical energy. One of the strong bonds in these complexes is the hydrogen bond, which strengthens the linkage between proteins and pigments. In photosystem I, a hydrogen bond binds glutamic acid (Glu) with chlorophyll (Qin et al., 2015). Similarly, hydrogen bonds bound the polypeptide's side chains in a light-harvesting complex (LHCI) with the C7-formyl group of chlorophyll. This interaction makes separating proteins from the chlorophyll difficult, resulting in many proteins being lost while removing the chlorophyll from leaf extracts (McLuskey et al., 2001).

2.3.1.2 Soluble proteins (RuBisCo)

Soluble proteins in plant leaves are classified into Fractions I and II based on the molecular weight (MW). Fraction I is a homogeneous protein with an MW of approximately 500 kDa, whereas fraction II proteins are heterogeneous groups with MW between 10 to 200 kDa (Horwarth et al., 1973). A major soluble protein in plant leaves is Ribulose-1,5-diphosphate carboxylase oxygenase (RuBisCo) (Kobbi et al., 2017). RuBisCo is a metabolic enzyme in the Calvin cycle, which catalyses the carbon fixation in the photosynthetic system to convert carbon dioxide to organic carbon compounds (Barbeau & Kinsella, 1988; De Jong et al., 2014; Kobbi et al., 2017). It also catalyses the first process of photorespiration on the condition that the affinity between enzyme and oxygen molecule is higher than carbon dioxide (Barbeau & Kinsella, 1988).

RuBisCo is densely packed, around 300 mg/ml in the stroma part (the mobile phase in the chloroplast). It is presented abundantly in plant leaves (15-30% of total nitrogen or up to 25% of total leaf proteins) since the first stage of photosynthesis reaction is slow; thus, plants create a large amount of RuBisCo to overcome this inefficiency (De Jong & Nieuwland, 2011; Ellis, 1979). RuBisco has been considered the most abundant protein in the world (Ellis, 1979). It has eight small (12-18 kDa) and eight large (50-55 kDa) protein subunits with a molecular weight of 550 – 560 kDa (De Jong & Nieuwland, 2011; Udenigwe et al., 2017). It is a globular, mildly acidic, negatively charged protein at neutral pH. Its isoelectric point is around pH 4.5 (De Jong et al., 2014).

2.3.1.2.1 Nutritional value

As RuBisCo is the main soluble protein in plant leaves, it is used to determine the nutritional value of the leaf proteins. Its EAA has been reported to meet the FAO/WHO nutritional guidelines, and it is very rich in Leu, Phe, Trp, and Try compared with other food proteins, as shown in Table 2.3 (Barbeau & Kinsella, 1988). Similarly, a study stated that leaf proteins could compensate for the low Lys food, such as cereal-based food; however, Met is a limiting amino acid (Takeiti et al., 2009)

Table 2.3: Essential amino acids in RuBisCo compared with other common protein sources and FAO/WHO reference.

Amino acid	FAO/ WHO	Whole egg	Casein	Soybean	RuBisCo	Chemical score
			(g/16 g N)			
Lysine	5.5	6.4	8.0	6.9	6.5	>100
Tryptophan	1.0	1.2	1.3	1.3	2.7	>100
Threonine	4.0	5.0	4.3	4.3	6.5	>100
½ Cystine and methionine	3.5	5.5	3.5	2.4	3.4	98
Valine	5.0	7.4	7.4	5.4	6.7	>100
Isoleucine	4.0	6.6	6.6	5.1	4.9	>100
Leucine	7.0	8.8	10.0	7.7	9.4	>100
Tyrosine and phenylalanine	6.0	10.1	11.2	9.4	12.8	>100

(Barbeau & Kinsella, 1988)

2.3.1.2.2 Digestibility property

Protein digestibility is another factor in determining the nutritional value, demonstrating the ability of proteins to be broken down and the amino acid absorption in the gastrointestinal tract. Non-soluble proteins (green proteins) in leaves constitute chlorophyll-protein complexes (presented in the thylakoid membrane) and membrane proteins. This protein group is usually bound to insoluble compounds, making it less soluble. As a result, it is usually removed through the discarding process (e.g., filtering and centrifuging) along with non-proteinous components, such as insoluble fibre (Ramírez-Rodrigues et al., 2022). Eggum (1995) reported that dietary

fibre impairs protein digestion in the gastrointestinal tract, resulting in low protein digestibility of the non-soluble protein fraction.

Many studies have reported that a purified RuBisCo has good digestibility (De Jong & Nieuwland, 2011; Ofori-Anti et al., 2008). For this reason, the nutritional value of the extracted proteins is determined based on RuBisCo (soluble proteins). The protein digestibility of purified RuBisCo was compared with six proteins (β-lactoglobulin, bovine serum albumin, ovalbumin, jack bean concanavalin, and egg lysozyme). The result presented that the large subunit of RuBisCo fully disappeared after 30 seconds of reacting with pepsin in pH 1.2 and 2. This result indicated that a purified RuBisCo has good digestibility (Ofori-Anti et al.,2008).

However, protease inhibitors, phenolic compounds, and toxins generally decrease protein digestibility as these compounds can reduce protein availability and digestive enzyme activities. Consequently, the nutritional value of isolated proteins is reduced (Day, 2013; De Jong & Nieuwland, 2011). For instance, leaf proteins bound to chlorogenic acid had low protein digestibility due to the decrease in trypsin rate (Lahiry et al., 1977). It would be said that several parameters impact the digestibility of leaf proteins. A good digestibility of leaf proteins could obtain by preventing contamination of these impurities mentioned above and retaining the purity of RuBisCo.

2.3.1.2.3 Allergenicity

Food allergy is a vital issue impacting people's health, especially those allergic to proteins, such as dairy, fish, shellfish, eggs, soybeans, wheat, and nuts (González-Pérez & Arellano, 2009; Gupta et al., 2017). For instance, a survey conducted between 2009 to 2010 presented that 8% of children in the United States had suffered from food allergies that might be life-threatening (Gupta et al., 2011). No medical treatment can directly cure food allergies. Thus, avoiding the ingestion of food allergens is suggested. Moreover, food allergens also impact manufacturing costs since manufacturers must apply food allergen management to produce safe food (Gupta et al., 2017). Thus, a novel protein source should be from non-allergen or low-allergen raw materials.

Criteria for determining the potential allergen have not been clarified. However, food containing indigestible or low-digestible proteins, which cause food allergies, is suggested to determine food allergens (De Jong & Nieuwland, 2011). β-conglycinin, a primary protein in soybean, requires 60 minutes to be digested; hence high allergic reactions in humans have been observed. In contrast, RuBisCo was digested in 30 seconds (Ofori-Anti et al., 2008). This

indicates that RuBisCo has good digestibility and low allergenicity than other food proteins. However, four people reported an incident in 2008 of food allergy after consuming vegetable leaves (Genkov & Spreitzer, 2009). It was assumed that RuBisCo in vegetable leaves still does not undergo modification, as happens during purification. Removing polyphenols and carbohydrates during extraction might reduce the reactions with RuBisCo, which may trigger allergenicity (De Jong & Nieuwland, 2011). Up to now, no new cases have been reported. Thus, plant leaves could be a potential protein source due to their low allergen.

2.3.1.2.4 Functional properties

Protein functionalities significantly affect the food characteristics, contributing to the final products' appearance, texture, mouthfeel, and shelf life (De Jong et al., 2014). Green proteins are usually bound to non-proteinous compounds, making them less soluble (Kaur et al., 2021; Ramírez-Rodrigues et al., 2022). Consequently, they are discarded during extraction; hence, soluble proteins are assumed to be recovered into the isolated protein. RuBisCo, a main soluble protein, has been reported to provide good solubility, gelation, foaming and emulsion; however, its functionalities are influenced by purification methods and drying conditions (De Jong et al., 2014; Kobbi, 2017). A review of RuBisCo's functional properties and the relevant factors is provided in the following sections.

Solubility

Protein solubility is defined as the dissolved proteins in a solution or supernatant after centrifugation. In other words, it is defined as the total proteins in the liquid phase compared with the total proteins in protein powder (Pelegrine & Gasparetto, 2005). This functionality is a significant property of proteins since it relates to food processing and other functionalities (e.g., emulsion, gelation, and foaming) (De Jong & Nieuwland, 2011).

RuBisCo provides good solubility comparable with other proteins; for instance, tobacco RuBisCo provides better protein solubility than soy protein isolate (Sheen & Sheen, 1985). Many studies have reported that RuBisCo has a good solubility at pH 6 to 9, which is the normal pH of many foods (Prevot-D'Alvise et al., 2004; Van De Velde et al., 2011a). Similar results for alfalfa leaf proteins have been reported. Its protein solubility increased from 10 % to 90 % at pH 7 to 9, while the lowest solubility was observed at pH 3.5 to 5 (Prevot-D'Alvise et al., 2004). Similarly, De Jong et al. (2014) stated that the isoelectric point (pI) of RuBisCo is around 4.5.

pH is a vital factor relating to protein solubility, the same as RuBisCo since it impacts the charges and electrostatic forces of protein molecules (De Jong & Nieuwland, 2011). The lowest solubility of protein molecules is observed at the pH closest to their pI (Di Stefano et al., 2018; Martin et al., 2019; Van De Velde et al., 2011a). This is due to the lowest electrostatic forces at this point, where the charge of amino acids reaches net zero. As a result, increased protein-protein interactions lead to proteins aggregating and having lower solubility (Damodaran et al., 2007). An increase in protein solubility is observed on either side of the protein's pI (acid and alkali pH) since the electrostatic forces of protein molecules are increased (Kobbi, 2017).

Furthermore, the solubility of proteins is impacted by other factors, such as temperature, salt concentration (Kaur et al., 2021), plant species, and isolation methods (Sheen, 1991). These factors also would affect the solubility of leaf proteins. Kaur et al. (2021) reported that the solubility of grass protein concentrate (GPC) extracted by enzyme treatment and acid precipitation increased when the temperature of the GPC solution was increased to 60 and 80 °C, respectively. Their study also showed that a decreased protein solubility of GPC was detected with increasing salt concentration. The effect of plant species on protein solubility has been studied by De Jong & Nieuwland (2011). They found that RuBisCo of tobacco leaves treated with magnesium ions was insoluble at pH below 7.5, whereas the solubility of spinach leaf proteins was much less affected.

Foaming properties

Foam is gas suspended in a continuous phase (liquid or solid materials), stabilised by proteins. The foam formation is induced by proteins decreasing the interfacial tension and rapidly absorbing at the freshly formed air/water interface. Subsequently, the viscosity in the continuous phase changes and the interfacial films form the foam structure. Hence, proteins play a vital role in various food foams, contributing to the product's texture, volume, and stability (Foegeding & Davis, 2011).

The foaming properties of RuBisCo are comparable to other proteins, such as soybean, whey, and egg white (Barbeau & Kinsella, 1988; Van De Velde et al., 2011a). Van De Velde et al. (2011a) reported that the foaming capacity of RuBisCo isolate was higher than soy and whey protein isolates. Better foam stability also was observed in the RuBisCo isolate. However, pH and ionic strength are vital factors of foam stability. Foam is the most stable in conditions close to pI, high ionic strength, and low temperature due to a minimal repulsive force of proteins (Barbeau & Kinsella, 1988).

Emulsifying properties

Emulsification is a surface-active property of food proteins. It refers to the ability of proteins to form and stabilize emulsions in various food products (e.g., mayonnaise, butter, sausage, and cake) (Barbeau & Kinsella, 1988). Emulsifying properties of RuBisCo have been studied in many isolated leaf proteins. Barbeau & Kinsella (1998) reported that spinach RuBisCo provided a lower emulsifying activity index than soy proteins and bovine serum albumin but higher than ovalbumin. Lamsal et al. (2007) observed that the emulsion capacity (EC) of RuBisCo was similar to the egg white proteins. Similarly, Martin et al. (2019) reported that emulsifying properties of sugar beet leaf proteins were better than soy protein isolate.

Emulsifying properties are affected by pH, protein concentration, and purification methods (Lamsal et al., 2007; Martin et al., 2019). Proteins rapidly absorb the oil droplets at pH close to their pI, resulting in good EC and emulsion stability (ES) (Phillips et al., cited in Martin et al., 2019). A study reported that the EC of RuBisCo was better at pH 4 than 7 since this condition is close to RuBioCo's pI (Lamsal et al., 2007). Protein concentration also influences the ES of the emulsion food system. A decrease in ES was observed in the sample containing low proteins (0.2 % RuBisCo protein isolate; [RPI]) due to the aggregation of oil droplets stabilised by insufficient protein quantities. In contrast, a high protein sample (1 % RPI) provided a smaller oil droplet size and better ES (Martin et al., 2019). Moreover, different extraction methods provide different emulsifying properties. Lamsal et al. (2007) reported that the alfalfa leaf proteins extracted through ultrafiltration provided the ES almost twice better than the acid-precipitated sample.

Gelling properties

Gelation in food systems is influenced by proteins, which contribute to the food texture (e.g., elasticity and brittleness), and change in food characteristics (e.g., fat/moisture release) (Foegeding & Davis, 2011). The gelling structure develops when the proteins denature; subsequently, the intermolecular interactions of protein-protein or protein-other compounds form, contributing to the gelling networks (De Jong & Nieuwland, 2011).

Gelling properties of RuBisCo have been studied in various leaf proteins: alfalfa, sugar beet leaves, tobacco, and spinach. RuBisCo provides better gelling properties than other food proteins, such as soy and egg white proteins. In addition, it needs a significantly lower protein concentration to develop gelation compared with other proteins (Barbeau & Kinsella, 1988; Martin et al., 2014, 2019). Barbeau & Kinsella (1988) reported that spinach proteins could

form a gel using only 2.5 %, and a similar result was observed by Martin et al. (2019). In comparison with soy protein isolate (SPI) and whey protein isolate (WPI), both proteins required higher concentrations (approximately 10 %) to provide gel strength as same as RuBisCo protein isolate (RPI) (Van De Velde et al., 2011a).

Many factors, such as temperature, protein concentration, and ionic strength, affect the gelling properties (De Jong & Nieuwland, 2011). Protein denaturation temperature (T_d) is vital because it is relevant to protein network formation (Martin et al., 2014). The study showed that RPI gel formed at a temperature lower than WPI since the T_d of RPI (65 °C) was lower than WPI (72 °C). Adding calcium salt can reduce gelation time since high ionic strength reduces the T_d. Moreover, higher protein concentration leads to higher gel strength (Van De Velde et al., 2011a).

It can be seen that leaf proteins, mainly RuBisCo, provide good nutritional value, digestibility, and functional properties comparable to conventional proteins. Leaf proteins also provide a low risk of food allergy. Thus, plant leaves can be an alternative protein source for food applications. However, many factors (e.g., purification, pH, ionic strength, and temperature) relate to the functional properties of the isolated proteins (Martin et al., 2019).

2.3.2 Non-proteins in plant leaves

Apart from proteins, polysaccharides, cellulose, and lignin are the other main components in plant leaves. Water-soluble carbohydrates are ruminants' primary carbohydrate and energy source, which correlates to protein utilisation in animals' rumen (Smith et al., 1999). Cellulose, hemicellulose, and lignin are insoluble dietary fibres that absorb fluid and waste in the intestine. They enhance intestine movement and prevent human gastrointestinal blockage, constipation, haemorrhoids, and colon cancer. Consuming insoluble dietary fibres also decreases the risk of obesity and diabetes (Ötles & Ozgoz, 2014). Carotenoids, calcium, iron, magnesium, vitamin C, folic acid, and riboflavin are the minor components in plant leaves (González-Pérez & Arellano, 2009). The chemical compositions of perennial ryegrass and white clover are presented in Table 2.4.

Table 2.4: Chemical compositions of perennial ryegrass and white clover (% dry basis).

Compositions	Perennial Ryegrass	White Clover
Dry matter	19.4[1]	19.07[2]
Water-soluble carbohydrates[3]	12.62	7.94
Hemicellulose[3]	12.63	9.70
Cellulose[3]	25.43	20.93
Lignin[3]	6.82	8.23
Nitrogen[3]	3.28	4.14
Crude protein	19.0[4]	23.0[5]
Ash[3]	12.72	10.32
- Calcium	0.65[4]	1.01[5]
- Phosphorus	0.40[4]	0.33[5]
- Copper	n/a	1.20[5]

[1] Minneé et al. (2019). [2] Søegaard et al. (1993). [3] Rattray and Joyce (1974). [4] Hannaway et al. (1999). [5] FAO (2011).

2.4 Antinutritional components and toxins in plant leaves

Plant leaves naturally comprise antinutritional compounds, such as protease inhibitors, polyphenol-protein complexes, and natural toxins. These compounds reduce protein digestibility and may cause adverse health effects (Day, 2013; De Jong & Nieuwland, 2011; Tadele, 2015). The effect of these compounds on protein properties and human health, including inactivation methods, are discussed in the following subsection.

2.4.1 Protease inhibitors

Protease inhibitors (e.g., trypsin inhibitors and chymotrypsin inhibitors) are generally found in the plant kingdom and usually interrupt digestive enzyme activity in the gastrointestinal tract (Day, 2013; Thakur et al., 2019). Hazlewood et al. (as cited in Barbeau & Kinsella, 1988) found that trypsin inhibitors from alfalfa leaves could reduce the activity of 30, 60, and 90 % of pepsin pronase E, and trypsin, respectively. In addition, trypsin inhibitors can form the irreversible enzyme-trypsin complex, causing lower protein digestibility and growth rate reduction (Thakur et al., 2019).

However, some food processing can inactivate the activity of protease inhibitors. Boiling and autoclaving are effective methods to inhibit the activity of enzyme inhibitors; thus, cooking raw plant food before consumption is recommended (Thakur et al., 2019). Furthermore, Smit et al. (2006) reported that the protease inhibitors found in perennial ryegrass could be inactivated by freeze-drying. Hence, enzyme inhibitors would not be a significant issue for leaf proteins as food ingredients since they are inhibited during the food process.

2.4.2 Polyphenol-protein complexes

In general, polyphenols provide various benefits for human health since they act as antimicrobial substances and antioxidants. Polyphenols also are used as preservatives in food products (Winters et al., 2003). Nevertheless, polyphenols in food proteins are undesirable since they can bind with proteins, reducing protein digestibility and absorption (De Jong & Nieuwland, 2011; De Jong et al., 2014).

Polyphenols are found abundantly in plant materials, especially plant leaves, and react instantly with polyphenol oxidase (PPO) once the plant cells are disrupted (Jang & Moon, 2011). Subsequently, quinone products, the highly reactive substances, are generated. These compounds can form covalent and non-covalent bonds with the nucleophilic site of amino acids or proteins known as “cross-linked protein polymers” (Winters et al., 2003, p. 121). Non-covalent polyphenol-protein complexes can retard the protease enzyme, whereas covalent bonding complexes change amino acids to an unhydrolyzed form. Thus, bound proteins are less digestible, causing their nutrition loss (Winters et al., 2003). Many polyphenols are detected in plant materials; however, the primary phenolic compound found in forage is tannin (Tadele, 2015).

Tannin is a water-soluble and heat-stable polyphenol in plant vacuole cells (Patel et al., 2013; Tadele, 2015). It can bind with proteins and amino acids through hydrogen bonds and hydrophobic interactions, making proteins unabsorbable and less digestible. It reduces the activity of various enzymes (e.g., trypsin, amylase, and lipase) and interferes with iron absorption (Natesh et al., 2017).

Tannin is categorised into hydrolysable and condensed tannin (CT). This review mainly discusses CT since its effects on protein digestibility (Patel et al., 2013) and hydrolysis resistance (Natesh et al., 2017) are higher than hydrolysable tannin. The effect of CT on ruminant health has been studied; worse protein adsorption and swallowing problems were

observed in ruminants consuming 2 to 4 % CT (Escaray et al., 2012). Similarly, laying hens obtaining 0.5 to 2 % CT had a reduction in egg production and growth rate (Chung et al., 1998). However, a small amount of CT is detected in perennial ryegrass (0.02 – 0.25 %) (Jackson et al., 1996) and white clover (Burggraaf et al., 2003). Perennial ryegrass and white clover contain CT almost ten times lower than that impact on animal health.

2.4.3 Natural toxic compounds

All ruminants obtain nutrients and energy from grasses, fodders, and legumes (e.g., clovers). Nevertheless, animals sometimes get adverse illnesses due to the high level of natural toxins in forage. Alkaloids, mycotoxins, and nitrates are occasionally found in grasses (Reed et al., 2011; Stone, 1994). In comparison, saponin and cyanogenic glycosides are detected in legumes, such as clovers (Naydenova et al., 2018; Sakamoto et al., 1992). These toxins interrupt nutrient adsorption and reduce enzyme activity, affecting animal health (Samitiya et al., 2020; Tadele, 2015). Understanding these toxins' health effects, limitation levels, and inactivation methods is critical for producing safe food.

2.4.3.1 Alkaloids

Alkaloids are organic nitrogen-containing bases in vascular plant cells, directly impacting the neurological system by inhibiting electrochemical neurotransmission in humans and animals (Tadele, 2015). Paralysis, rapid heartbeat, and fatal cases are found in animals that consume alkaloid-contaminated feeds (Fekadu Gemede & Retta, 2014). This toxin group is detected in perennial ryegrass (Stone, 1994) and white clover in small amounts (approximately 9 µg/kg) (Burkin et al., 2017).

Pyrrolizidine alkaloids (PAs) have been important natural toxins in recent years due to the increased occurrence of PAs in food matrices (Casado et al., 2022). Consuming food containing PAs leads to liver and kidney damage (Australia New Zealand Food Authority (ANZFA), 2011). The European Commission has regulated the maximum levels of PAs contaminated in various food products (e.g., herbal infusion, tea, food supplement, and pollen products) at 75 – 1000 mg/kg. However, the maximum contamination level of liquid products for infants and young children is allowed only 1 mg/kg (The European Commission, 2020).

Food process, such as boiling and soaking, has been reported to decrease the concentration of alkaloids. A debittering process (soaking and washing with water) could reduce lupin's alkaloid

content from 1-2 % to 0.05% (ANZFA, 2011). Also, alkaloids could be removed by boiling since they are water-soluble. Thus, boiling and soaking accelerate the removal of these compounds from seeds (Mohammed et al., 2017). It could be seen that alkaloids in perennial ryegrass and white clover are lower than the maximum level regulated by The European Commission. Also, these compounds are eliminated during food processing.

2.4.3.2 Mycotoxins

Mycotoxins are secondary metabolites and act as insecticides, increasing pasture persistence. Nevertheless, these toxins cause health issues for grazing animals (Meat & Livestock Australia, 2020). Lolitrem B (Reed et al., 2011) and cyclopiazonic acid (CPA) (Burkin et al., 2017) are the most critical mycotoxins in perennial ryegrass and white clover, respectively.

Lolitrem B

Lolitrem B is a mycotoxin produced by *Neothyphodium lolii*, a fungus that infects perennial ryegrass. The amount of lolitrem B in perennial ryegrass relies on the season and the plant's part (Reed et al., 2011). The highest concentration of lolitrem B is usually detected in March or between summer and autumn (Meat & Livestock Australia, 2020; Reed et al., 2011). A study in France presented that lolitrem B in leaf base and the inflorescence is found at 0.01 to 3 mg/kg, while leaves contain lower than these levels (Repussard et al., 2014). Ingestion at high concentrations leads to a neurological disorder and abnormal clinical symptoms (e.g., staggering and tremors), including slow weight gaining (Shimada et al., 2013).

The effect of lolitrem B on human health has been evaluated since people may consume meat from cattle fed by infected grass (Shimada et al., 2013). The study also demonstrated that lolitrem B at 12 µg/kg/body weight (BW) per day did not harm Japanese black cattle. This concentration is designated as no observed adverse effect level (NOAEL). Moreover, the study showed that humans might obtain this toxin at only 109 ng/kg/BW per day when consuming contaminated beef. This level is much lower than NOAEL; thus, Shimada and his teams concluded that ingesting lolitrem B from contaminated beef would not impact human health.

Furthermore, Lolitrem B is rarely observed in human food as common mycotoxins are ochratoxin A, aflatoxins, patulin, zearalenone, and fumonisins (WHO, 2018). However, minimising the risk of mycotoxins is discussed in this study. The ideal prevention is controlling a properly dried environment during cultivation to prevent mould growth, as mycotoxins are products of mould infection (WHO, 2018). Mycotoxins are difficult to eradicate once they

contaminate the food as they can resist various processes. Many methods have been studied to reduce mycotoxins concentration. A chemical agent, such as acid, quaternary ammonium, peroxide, sorbate, sodium hypochlorite, and propionate, can decontaminate mycotoxins before storage. Physical treatment (e.g., non-ionising irradiation, UV light, electron-beam irradiation, ozone, microwave, ultrasonication, and high hydrostatic pressure) also is an effective decontamination method (Cattini, 2019).

Cyclopiazonic acid

Cyclopiazonic acid (CPA) is the greatest mycotoxin detected in white clover (0.175 – 0.350 µg/g) (Burkin et al., 2017). CPA is a mycotoxin produced by *Aspergillus* and *Penicillium*. It is not an acute toxin because of its low toxicity; however, some animals are sensitive to CPA (e.g., rodents, chickens, and pigs). CPA is naturally found in various food (legumes, milk, cheese, and meat), and the acceptable daily intake is 10 µg/kg/day or 700 µg/day (Burdock & Flamm, 2000). It can be seen that the amount of contaminated CPA in white clover is much lower than the acceptable daily intake.

2.4.3.3 Nitrates

Some forages, such as sudan grass and millet, can accumulate nitrate to toxic levels. A perennial ryegrass/clover mixture also has been reported as a source of the outbreak in cattle (Stone, 1994). A high level of nitrates in plants causes life-threatening in cattle since nitrates are converted to nitrite and cannot be converted to protein completely. The remaining nitrite is accumulated and absorbed through the bloodstream, converting haemoglobin (oxygen carrier) to methaemoglobin (non-oxygen carrier). Consequently, a low oxygen level leads to fatal death in grazing animals (Tadele, 2015).

Furthermore, nitrate toxicity in humans has been reported: consuming more than 45 ppm of nitrate-rich food leads to methemoglobinemia, causing a lack of oxygen. Nevertheless, it rarely occurs in adults since nitrates are converted to nitrite by nitrate-reducing bacteria in the lower intestine, where nitrite is not absorbed into the bloodstream. The human body also excretes nitrates through urine, sweat, and faeces within 24 hours. However, nitrates are harmful to infants since the conditions in the upper gastrointestinal tract are optimal for nitrate-reducing bacteria, and this area is excellent for nitrite adsorption (Majumdar, 2003). It can be seen that obtaining nitrates from leaves would not be harmful to humans; however, it should be considered carefully for infant food products.

2.4.3.4 Saponins

Saponins are natural secondary metabolites in many plant species, especially legumes (Samitiya et al., 2020). White clover (large-leaf species) also contains several saponins. Nevertheless, the contamination level in clover leaves is very low, and the whole plant contains 1.5 to 75 mg/kg (Sakamoto et al., 1992). Saponins can impair vitamin E and lipid adsorption since they bind the sterols; consequently, their structure is similar to fat-soluble vitamins (Samitiya et al., 2020). Saponins also inhibit various enzyme activities, such as glucosidase, amylase, lipase, chymotrypsin, and trypsin, leading to indigestion problems (Lee et al., 2015).

A study reported that consuming more than 150 mg/kg BW caused fatal cases in sheep; however, saponins content in white clover is much lower than the fatal dosage mentioned above. Samitiya et al. (2020) stated that consuming a low amount of saponin food would not impact human health. It also enhances people's health, such as anticancer, decreased risk of heart disease, and reduced cholesterol. A high concentration of saponins also provides a bitter taste, limiting food intake (Thakur et al., 2019). Moreover, a reduction in the content of saponins in legumes was observed after the cooking process (Samitaya et al., 2020). Hill (2003) reported that saponin removal in quinoa grains was achieved by soaking and washing.

2.4.3.5 Cyanogenic glycosides

Cyanogenic glycosides (cyanogen) are toxic substances found in white clover at 19.4, 28.4, and 19.2 mg/100g dried leaves of small, middle, and large-leaf species, respectively (Naydenova et al., 2018). Cyanogen is hydrolysed and converted to hydrogen cyanide, causing human health problems, such as blood pressure drop, dizziness, vomiting, rapid pulse, and respiration disorder (Thakur et al., 2019). However, health effects occur when a high dosage is ingested. Furthermore, cyanide detoxification is a normal process in the human body by changing cyanide to thiocyanate and excreting it through urine. Indeed, people currently consume some plants containing cyanogen, such as cassava, almonds, sorghum, stone fruits, and bamboo shoot (WHO, 2014).

Various methods, such as soaking, fermentation, and cooking process, can reduce the concentration of hydrogen cyanide before consumption. Cyanogen is changed to cyanide during soaking and fermentation, and it is dissipated out of food by air or water. Also, it is a heat-labile substance; thus, it is degraded at high temperatures (Thakur et al., 2019). Ngudi et al. (2003) found that the amount of cyanogen in cassava leaves was reduced by up to 99 %

after boiling. Similarly, Ferreira et al. (1995) reported that cyanogen in the bamboo shoots was reduced by 97 % after the cooking process (98 - 102 ℃ for 148-180 minutes).

According to Food Standards Australia New Zealand [FSANZ] (2015), there is no regulation of antinutrients and natural toxins for leaf proteins. However, heating, soaking, washing, and freeze-drying can inactivate protease inhibitors and decrease the content of antinutrients. The concentration of polyphenols and some toxins (e.g., alkaloids) is lower than the dosage that causes health problems. The human body has the natural process of excreting toxins out of the body (e.g., nitrates and cyanogen). In addition, chemical use and physical treatments can also reduce the risk of toxin contamination. Thus, using pasture leaves as a human protein source should be further studied since plant leaves are potentially safe raw materials for food applications.

2.5 Isolation process of leaf proteins

The nutritional value and functionality of RuBisCo make plant leaves an interesting protein source. As a result, the extraction process of leaf proteins has been studied. However, leaf proteins are still unutilised since removing chlorophyll and off-flavour requires laborious processes (Di Stefano et al., 2018). The extraction process of leaf proteins comprises harvesting, cell disruption, protein fractionation, and purification and concentration (Tenorio et al., 2018), as shown in Figure 2.1.

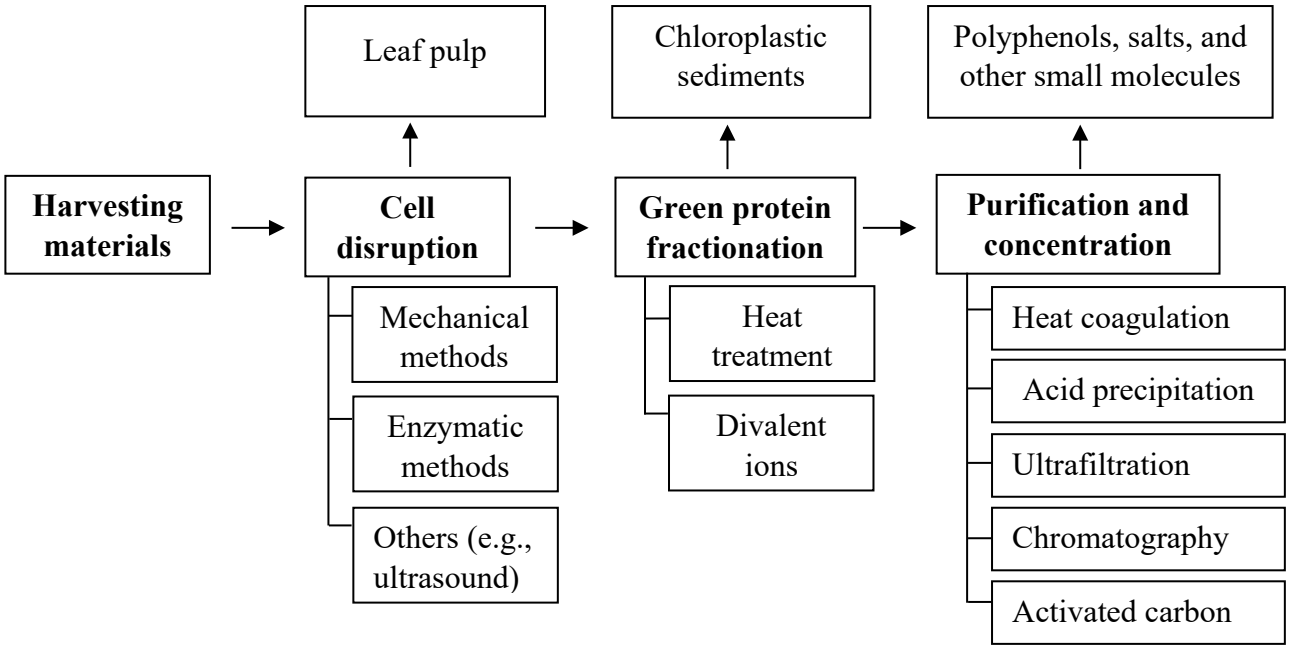


Figure 2.1: Extraction process of leaf protein concentrate.

2.5.1 Harvesting plant materials

Harvesting is the process of gathering plant materials from the field. This process is vital to maintain intact plant cells and retain the original quality since the damaged cells initiate undesirable reactions. For instance, the oxidation reaction of PPO leads to low protein digestibility (De Jong et al., 2014), and lipid oxidation generates a grassy flavour (Hatanaka, 1996). Moreover, the storage and handling procedure relates to the plant's quality. Avoiding light and air exposure is suggested to minimise the effect of the oxidation reactions mentioned above (Baysal & Demirdöven, 2007). In addition, a storage temperature should be less than 15 °C to decrease enzyme activity; consequently, the original quality of plant cells is retained (De Jong et al., 2014).

2.5.2 Plant cell disruption

Proteins in plant leaves are located inside plant cells. Particularly RuBisCo, the main soluble protein in plant leaves, is enclosed inside the chloroplast (Barbeau & Kinsella, 1988). Thus, a disruption process is essential to separate the proteins from plant tissue. At the same time, other compounds (e.g., pigments, antinutrients, and enzymes) also are released during this process, which reduces the purity of isolated proteins (Di Stefano et al., 2018). Moreover, stems or other parts increase the impurities into isolated proteins, which may require a higher amount of food additive or more process steps. Thus, removing non-leaf parts before the extraction process is recommended (De Jong et al., 2014).

Disrupting plant tissues is achieved by mechanical instruments, such as a blender, high-pressure homogeniser, screw-pressing homogeniser, and grinder. Kitchen homogenisers would disrupt low-fibre plants (e.g., sugar beet leaves). High-pressure homogenisers, screw-pressing homogenisers, and grinders are appropriate for high-fibre plants (De Jong et al., 2014). A screw-pressing homogenizer has been used in several studies (De Jong et al., 2014; Martin et al., 2014, 2019) for disrupting plant cells since it is an efficient machine for leaf juice extraction without adding water. It also separates fibres from the soluble fraction in one step (De jong et al., 2014; Di Stefano et al., 2018). Barbeau & Kinsella (1998) reported that leaf juice extracted by this machine without water had good recovery of dry matter and proteins (up to 10 % DM and 2 to 3 % protein (wet basis)).

Nonetheless, a high amount of foam is observed, which could induce covalent bonding between polyphenols and proteins, reducing protein digestibility. Furthermore, non-mechanical

methods have been studied for disrupting plant materials, for instance, enzymatic lysis, freeze-thaw cycles, and ultrasound treatment. Nevertheless, these techniques are still unutilised due to the difficulty of scaling up (De Jong et al., 2014).

2.5.3 Green proteins fractionation

Green protein fractionation is a process to separate insoluble from soluble fractions, which comprises three steps: discarding leaf pulp, aggregating chloroplastic curd (chlorophyll), and centrifuging or filtering. Discarding leaf pulp is done after cell disruption by filtering. It is the first step to separating the insoluble fraction from the soluble (leaf juice). Subsequently, chlorophyll is removed from the obtained green juice by aggregating chloroplast cells and centrifuging to separate the precipitated chloroplastic curd from the solution. However, some residual chlorophyll may not be eliminated. Therefore, microfiltration or activated charcoal is used to remove the remaining chlorophyll (De Jong et al., 2014). As the aggregation of the chloroplastic curd is the most challenging, the following subsection focus on this step.

Chlorophyll has various advantages, such as non-toxic, antioxidant, anti-mutagen, wound healing, and anti-inflammatory prevention. It is also used as a colourant in food and cosmetic products (Fu et al., 2017). However, the dark green colour is the main obstacle to consumer acceptance and food applications (e.g., beverage products) (Van De Velde et al., 2011a). Thus, chloroplastic curd fractionation is necessary to remove chlorophyll.

Chlorophyll is the primary pigment of plants responsible for green colour and a significant component of photosynthesis. The structure of chlorophyll comprises a porphyrin ring (head) and phytol chain (tail) with the Mg-atom at the centre. It is located in the chloroplast between proteins and lipid layers, where the proteins link the porphyrin part, and the lipids link the phytol tail (Attokaran, 2011).

The amount of chlorophyll depends on plant species (Kholmanskiy & Zaytseva, 2020), nutrient supply, and environmental stress, such as drought, salinity, and temperature (Attokaran, 2011). Kholmanskiy & Zaytseva (2020) reported that chlorophyll content in grass species and red clover grown under sunlight was 0.85 - 0.95 and 0.9 mg/g, respectively. Zhang et al. (2020) found that perennial ryegrass exposed to light for 14 hr had chlorophyll of 1.67 to 1.95 mg/g. Although leafy species have less chlorophyll, about 1.6 times, than fruit plants (Kholmanskiy & Zaytseva, 2020), separating the chlorophyll and retaining the proteins simultaneously is

challenging. As proteins are naturally bound with the photosynthetic system on the thylakoid membrane (Liu et al., 2004), discarding chlorophyll also causes protein loss.

2.5.3.1 Chlorophyll fractionation by heat treatment

Heat treatment is a method to eliminate chlorophyll from leaf juice. Since some parts of photosynthesis link with the proteins, protein denaturation leads to irreversible chloroplastic curd formation (Östbring et al., 2014). Udenigwe et al. (2017) stated that heating leaf juice at 40 – 60 ℃ could remove abundant green pigments, non-soluble proteins, and cell debris. Similarly, De Jong et al. (2014) reported that plant juice is preferably heated at 40 – 60 ℃ for 15 - 30 minutes to precipitate chloroplast. This temperature range has been suggested for aggregating chlorophyll to avoid RuBisCo denaturation, as its T_d is around 61.85 - 72.85 ℃ (Béghin et al., 1993). The heated juice must be cooled down to less than 15 ℃ in a short time to stop the heating effect. The efficiency of heat-induced chloroplast precipitation reduces when the storage time increases (De Jong et al., 2014).

2.5.3.2 Chlorophyll fractionation divalent ions

Thylakoid membranes and organelles inside chloroplast have anionic binding sites; thus, they can bind with divalent cations (Camm & Green, 1982); consequently, these negative charges are neutralised. This results in thylakoid membrane stacking and chloroplast cell flocculation (Chow et al., cited in Camm & Green, 1982). The interaction of thylakoid membranes and cations has been studied since 1982. Chloroplast initially aggregated after adding 5 mM of Mg^{2+}, Ca^{2+}, and Mn^{2+} in a thylakoid solution, leading to the aggregated green layer (Camm & Green, 1982). Similarly, De Jong et al. (2014) demonstrated that using 100 – 300 mM divalent salts (based on plant materials weight) resulted in thylakoid membrane flocculation. Increasing the concentration of cations enhanced the degree of thylakoid stacking; however, it decreased protein extractability (Camm & Green, 1982). Moreover, the study also reported that divalent cations, such as Ca^{2+}, Mg^{2+}, and Fe^{2+}, enhanced the effectiveness of heat-induced chloroplast aggregation.

Removing chlorophyll by heat treatment may lead to partial RuBisCo denaturation since the recommended temperatures (40 – 60 ℃) are close to its T_d. However, the thermal treatment reduces antinutritional compounds in plant leaves (Samitiya et al., 2020). Using divalent cations to remove chlorophyll is an interesting approach due to no involvement of heat. In addition, combining both techniques should be further studied to evaluate the effectiveness of green colour removal and the effects on the protein content of extracted proteins.

2.5.4 Protein purification

Purification aims to eliminate small molecules and impurities, such as pigments, polyphenols, salts, and polysaccharides, from the initial source to purify the target proteins and enrich protein content by removing the moisture content (Xie, 2017). Various techniques used for protein purification are discussed in the following subsection

2.5.4.1 Heat coagulation

Heat coagulation is defined as the thermal precipitation method. Thermal treatment results in protein denaturation; consequently, the hydrophobic sites of proteins are exposed, and the proteins are aggregated (De Jong et al., 201). A study reported that protein precipitation from chlorophyll-free alfalfa juice could be achieved by heating at 80 ℃, followed by centrifugation to obtain the protein precipitates. The obtained proteins were light-tan and bland; however, they had irreversible structures, and their solubility was low (Edward et al., 1975). In contrast, a good result in emulsion and foaming properties were observed in heat-coagulated proteins. The isolated duckweed proteins by heat coagulation showed 93.33 %, 60.12 %, and 50.67 % of foam capacity, foam stability, and emulsion stability. Nevertheless, only 34.41 % of crude protein was recovered (Kalburgi, 2019). It could be seen that heat coagulation is a straightforward and low-cost method.

2.5.4.2 Acid precipitation

Acid precipitation has been used extensively for protein recovery as it is a simple and low-cost process. The crucial point of this technique is reducing pH to the isoelectric point of target proteins, resulting in protein aggregation. At the isoelectric point, the net charge of proteins is near zero (no net charge), which leads to protein molecules having the lowest electrostatic forces. Subsequently, the water-protein interaction reduces, and the protein-protein interaction increases; consequently, proteins are aggregated and lose the ability to solubilise (Pelegrine & Gasparetto, 2005). Nevertheless, acid-precipitated proteins retain their solubility property more than those obtained through heat coagulation (Knorr, 1982).

Improving the protein solubility has been conducted by pH shifting. Proteins are subjected to an extremely low or high pH before adjusting to the neutral condition, resulting in partially unfolded and refolded proteins. This structural change leads to molten globule conformation and specific surface properties formation, enhancing the proteins' solubility (Jiang et al., 2014). An increase in grass protein concentrates (GPC) solubility by using the pH shifting technique

was observed. The study reported that GPC treated with strong alkali (pH 12 at 20 ℃) before shifting to pH 7 had protein solubility 5.3 times higher than untreated GPC (Kaur et al., 2021). It can be seen that the pH-shifting process would be a method to improve protein solubility. However, the structure of proteins is not reversed entirely since the structural conformation appears in the tertiary structure (Jiang et al., 2009).

2.5.4.3 Ultrafiltration

Ultrafiltration is a process that allows products to pass through the three-dimensional structure of the membrane to separate the contaminants from the target compounds. It is defined as a separation technique based on the size of target molecules against the pore size of filters. Its purification efficiency involves filtration mode, membrane type, hydrophobicity, and electrostatic interaction. A study showed that low protein purity was obtained due to the membrane blockage, leading to the impurities retained on the membrane. It usually occurs with static filtration mode, where the separation is operated only by pressure force. Tangential flow filtration is another design with swiping flow over the membrane; consequently, membrane fouling decreases (Walter et al., 2011). The schematic flow of both filtration modes is presented in Figure 2.2.

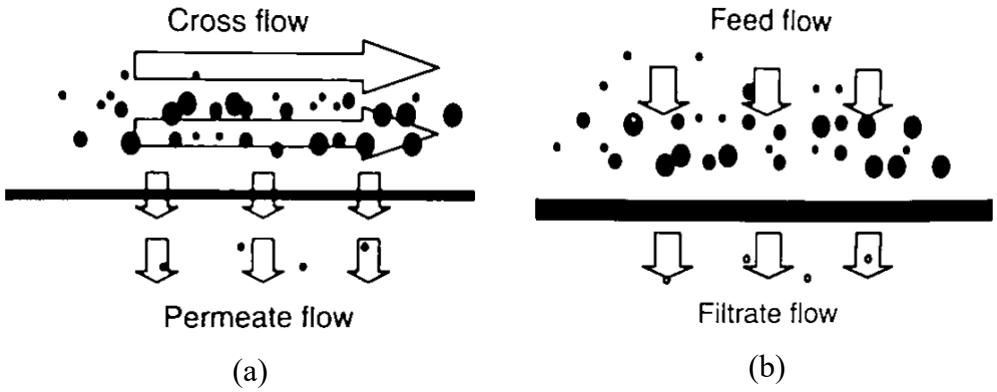


Figure 2.2: Schematic flow of tangential filtration (a) and static filtration (b). (Walter et al., 2011).

Unspecific protein adsorption can occur with some materials since the chemical surface treatment can enhance the hydrophobicity and alter the membrane's electrical charge. This increases protein-membrane interaction and protein binding capacity. Synthetic membranes, such as polysulfone, polyethersulfone, and polyvinylidene difluoride, are widely used due to their excellent chemical compatibility and pH stability. However, a high membrane fouling is observed due to its hydrophobic characteristic. Cellulose membranes, such as regenerated

cellulose and cellulose acetate, contain hydrophilic molecular structures; consequently, protein binding capacity is lower than synthetic polymers (Walter et al., 2011). Normal membranes used in protein research are shown in Table 2.5.

Table 2.5: Commercial membrane polymers for protein research using in ultrafiltration.

Cellulose membranes	Synthetic membranes	Inorganic membranes
Cellulose acetate	Polysulfone	Ceramic
Regenerated cellulose	Polyethersulfone	Stainless steel
Cellulose nitrate	Polyvinylidene difluoride	
Hydrated cellulose	Polypropylene	
	Polyamide	

(Adapted from Walter et al., 2011)

Ultrafiltration has been studied for protein purification and concentration since extreme conditions, such as heat and acid, are irrelevant; consequently, native proteins' properties remain (De Jong et al., 2014). The purification and concentration of some leaf proteins have been studied by ultrafiltration. Koschuh et al. (2004) reported that 59% of crude proteins could be recovered from ryegrass juice using ultrafiltration at a molecular weight cut-off (MWCO) of 1 kDa. Moreover, the study also reported that this membrane could not retain the silage ryegrass proteins since plants naturally contain proteases, which could hydrolyse the proteins in silage juice into small oligopeptides or amino acids. Hence, proteases would be a noticeable factor leading to low protein recovery.

2.5.4.4 Chromatography technique

Chromatography has been used in various protein research, such as therapeutic use, biophysical analysis, enzyme study, proteomics research, drug discovery, and food analysis. It is a physical method to separate the target compounds in the mixture based on the retardation mechanism between the target molecules in the mobile and stationary phases (Labrou, 2018). Chromatography for protein purification is done using techniques based on the target proteins' characteristics, such as affinity, hydrophobicity, net charge, and protein size (GE Healthcare, 2016). The present study focused on the separation techniques based on net charge and protein size due to their uncomplicated operation and extensive protein purification use. Therefore, ion exchange and size exclusion chromatography are discussed.

Ion-exchange chromatography

Ion-exchange chromatography (IEX) is frequently used to purify proteins, peptides, and charged molecules since it offers a good result with a high sample loading capacity (micrograms to kilograms of samples) (GE Healthcare, 2012). Its fundamental principle involves the interaction between the charge of proteins and the stationary phase. The process of ion-exchange chromatography is illustrated in Figure 2.3.

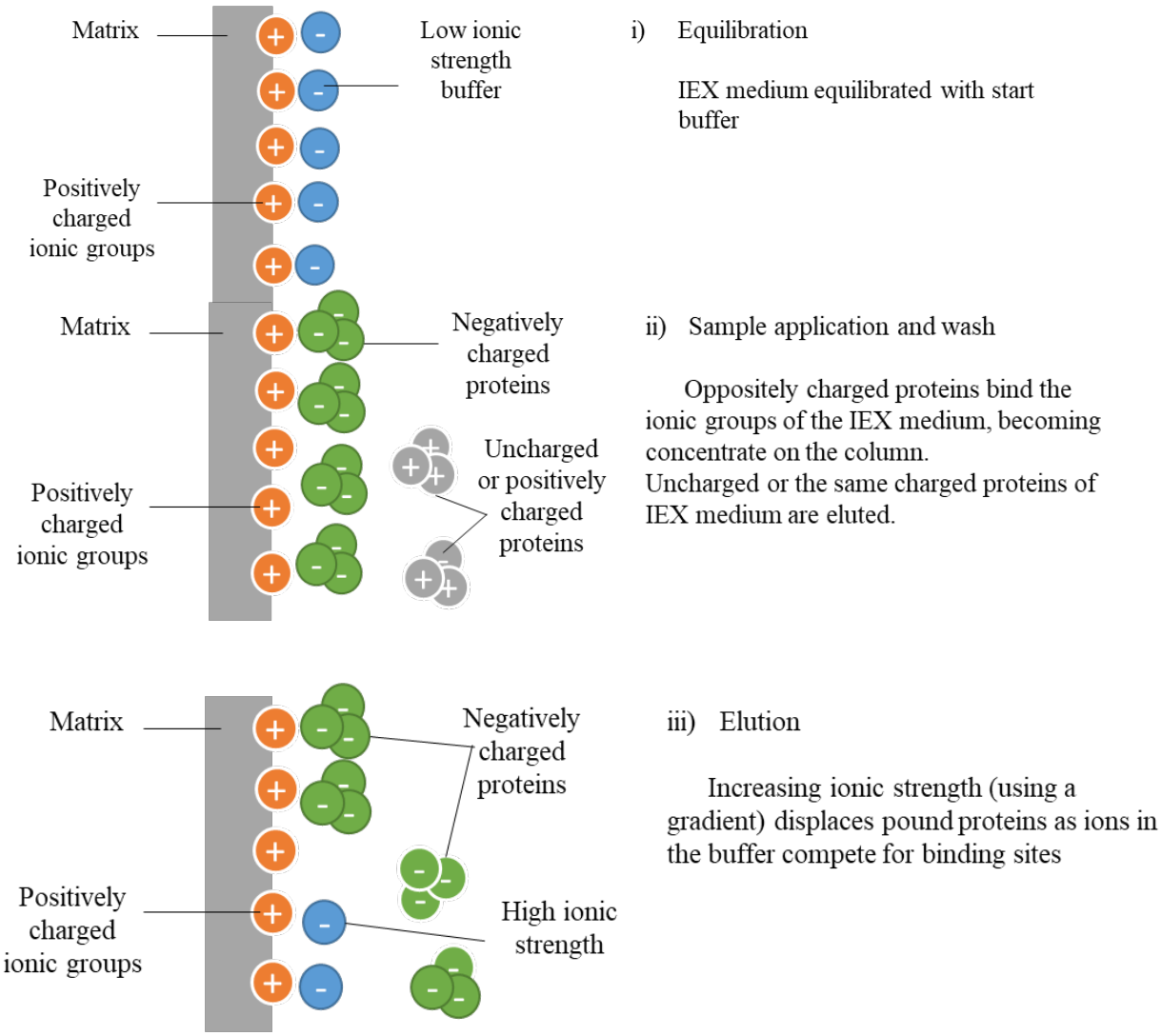


Figure 2.3: Ion-Exchange chromatography process.

Positively charged proteins bind to the negatively charged stationary phase (cation exchanger). In other words, negatively charged proteins bind to the positively charged stationary phase (anion exchanger) (Labrou, 2018). Hence, the impurities, oppositely charged molecules from proteins and non-charge molecules, are firstly washed from the column. Subsequently, the

adsorbed proteins are eluted using 0.1 – 1.0 M NaCl. The difference in protein's pI and pH buffer enhances the strength of interactions, which requires high ionic strength to elute the target proteins. Alternatively, altering the pH to the protein's pI also elutes the adsorbed proteins since their net charge is close to zero; consequently, proteins are eluted (GE Healthcare, 2012).

As the principle of IEX is based on the net surface charge, pH is a significant factor. Proteins comprise several amino acids containing the acidic carboxyl group (-COOH) and basic amino group (-NH2); thus, protein charges change as a function of pH. Proteins have positive charges if the pH is below their pI. In contrast, the proteins have negative charges if the pH of the environment is higher than their pI (GE Healthcare, 2012). Moreover, the buffer and stationary phase used for protein purification should be used in conditions where the most stable target proteins are. RuBisCo is stable around pH 7 to 8, with a negative net charge (De Jong et al., 2014). Thus, an anion exchanger, such as Q-sepharose, was selected for the present study.

IEX efficiency is also affected by other factors, such as salts, column packing, and enzymes. High salt concentration induces the hydrophobic interactions between proteins and stationery matrics, causing nonspecific protein adsorption. Moreover, some proteins are precipitated at a high salt concentration, resulting in lower protein yield. Improper column packing, such as too tight, loose and uneven, affects the separation efficiency due to loss of resolution. Protease in protein extracts is another factor that leads to protein degradation; consequently, protein recovery is low. Thus, enzyme inhibitors or chelators (e.g., EDTA) are recommended to add to juice extracts (GE Healthcare, 2012).

Although IEX is an efficient method for separating substances, it requires a long operation time which could take several hours or up to a day. The process duration relates to the mobile phase's flow rate influenced by temperature, bead type, and separation efficiency (Dudás, 2011). The temperature significantly affects the kinetic adsorption-desorption reaction of the mobile and stationary phases. Moreover, increasing temperature enhances the diffusion rate, making molecules move faster (Wirth, 2011). Bead size also impacts the speed of the solution moving through stationary matric. Coarse resin beads provide a larger inter-space among the beads, decreasing the capillary effect. Thus, coarser beads have faster fluid flow than fine-grain resins. However, faster fluid flow results in insufficient separation (Dudás, 2011).

Size exclusion chromatography

Size exclusion chromatography (SEC) or gel filtration distinguishes the components based on molecular size. Large molecules (e.g., proteins) are eluted out of the column with the void volume, while the small molecules (e.g., salts) penetrate the bead's pores and are retained in the column longer. SEC has been used in various applications, such as fractionation (monomer separation from multimers), desalting (group separation), and molecular size analysis (Harvard apparatus, 2012).

SEC has been used for desalting after IEX due to an uncomplicated technique. Nevertheless, sample loading is still a limitation for SEC (Jason & Jönsson, 2011). Loading volume is usually only 0.5 to 2% of the total column volume to obtain the maximum resolution; however, up to 5% is acceptable for group separation. Hence, concentrating the large sample volume is suggested before loading to SEC to increase the separation efficiency and reduce the operation time (Harvard apparatus, 2012).

Although SEC is a straightforward method, the efficiency of SEC is affected by pH, ionic strength, solvents, SEC support, column dimension, and packing efficiency (Jason & Jönsson, 2011). SEC is irrelevant to any binding mechanism between proteins and the stationary phase, which is different from other chromatography. However, unexpected protein adsorption may occur (Labrou, 2018). Low ionic strength solvent, 25 – 150 mM NaCl, is suggested to avoid since it induces weak electrostatic interactions between proteins and stationery matrics. Moreover, avoiding high salt concentration and pH at protein's pI is suggested to prevent protein precipitation (Harvard apparatus, 2012).

2.5.4.5 Impurity removal by activated carbon

Activated carbon (AC), an inexpensive absorbent, has been used in various industries to remove impurities, such as water purification, beverage manufacturing, and oral poisoning treatment. It is also used in the protein separation process to remove small molecules from the protein extracts (Stone & Kozlov, 2014). It can remove some residue chlorophyll that is not eliminated in the fractionation step (Di Stefano et al., 2018). Therefore, AC is a feasible material to remove phenolic compounds and other impurities in leaf protein extracts.

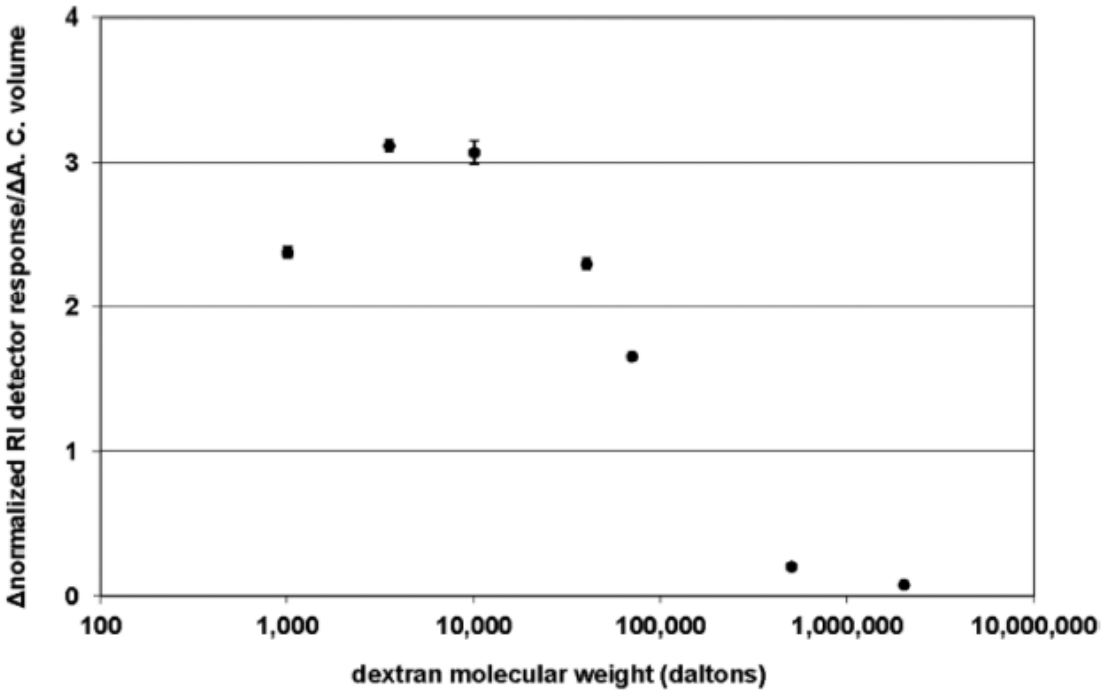


Figure 2.4: The Δnormalized RI detector response/Δactivated carbon volume represents the binding capacity of activated carbon as a function of dextran molecular weight (Stone & Kozlov, 2014).

The adsorption capacity of AC is due to its porous structures and the interactions of AC and molecules. Large proteins possibly have a strong binding with AC; however, they cannot penetrate through the micropore (less than 2 nm) and mesopore (2-50 nm) surface of AC (Marsh & Rodríguez-Reinoso, 2006). Thus, the adsorption capacity between AC and large proteins is limited. As shown in Figure 2.4, Stone and Kozlov (2014) reported that the adsorption capacity increased when the molecular weight of dextran was small (1 – 3 kDa). However, a decrease in adsorption capacity was observed in molecules with MW larger than 10,000 Da. As RuBisCo is a large protein with an MW of 550-560 kDa, using AC to remove impurities and flavour along with retaining proteins should be further studied.

pH also affects the AC-protein binding capacity. A function of pH has been studied to determine the effect of protein's charge on AC-protein binding capacity. Proteins are less adsorbed at the pH that is far from their pI. In contrast, proteins are much absorbed when the pH is near their pI. (Stone & Kozlov, 2014). Proteins have minimum overall charges at pI, leading to a reduction of electrostatic repulsion. Consequently, non-covalent bonds are formed between proteins and AC (Vinu et al., 2004). However, Stone & Kozlov (2014) reported that an increase in binding capacity at near pI occurred with low molecular weight proteins, such

as cytochrome C (13400 Da), α-lactalbumin (14,175 Da), lysozyme (14,307 Da), and bovine serum albumin (BSA) (66,430 Da). In contrast, MAb I (monoclonal antibody), a large protein with 145 kDa, was bound with AC less than small proteins, as shown in Figure 2.5.

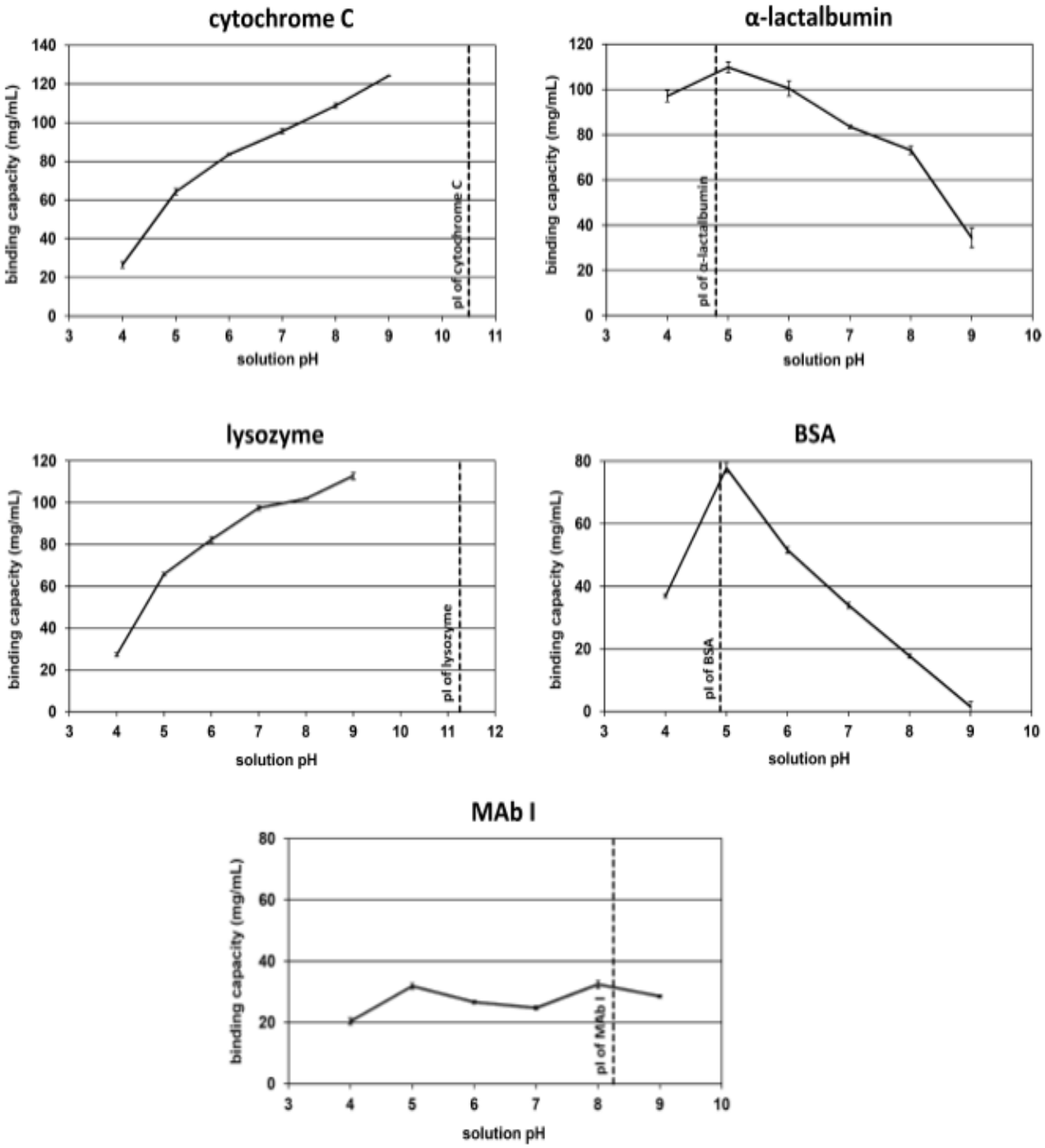


Figure 2.5: The effect of pH solution on the binding capacity of activated carbon with different proteins. (Stone & Kozlov, 2014)

AC-protein binding capacity is also influenced by salt concentration since it relates to the electrostatic repulsion of proteins. AC binding capacity at the pH far from the protein's pI (high electrostatic repulsion) increases when salt concentration increases. However, the binding capacity at the pH near the protein's pI (low electrostatic repulsion) is slightly impacted despite a high salt concentration (Stone & Kozlov, 2014). Thus, AC binding capacity at the pH far from the protein's pI is significantly affected by salt concentration.

Using AC to remove antinutrients and improve off-flavour from protein extracts obtained from napiergrass was studied by Xie, 2017. Applying activated carbon to protein extracts decreased 90 % of tannin and removed off-flavour from protein concentrate. However, the protein yield was reduced in the protein concentrate extracted through alkali-acid precipitation along with AC treatment. This might be due to improper conditions used in the AC column method, leading to unspecific AC-protein absorption (Xie, 2017).

2.5.5 Other methods to enhance the protein purity

Grassy flavour and dark green colour are the characteristics of plant leaves. The colour mainly relates to chlorophyll pigments (Di Stefano et al., 2018), and the grassy flavour is produced because of lipid oxidation after cutting (Hatanaka, 1996). Both attributes significantly limit the incorporation into human food. Removing chlorophyll and off-flavour compounds from plant leaves at the initial step and final products is a feasible method for enhancing the final products' quality.

2.5.5.1 Organic Solvents

Chlorophyll is soluble in organic solvents, such as methanol, ethanol, acetone, and chloroform (Tsuji et al., 1985). Removing chlorophyll from plant leaves and LPC by organic solvents has been studied. Fu et al. (2017) reported that chlorophyll of bamboo leaves was extracted successfully by soaking them in acetone. The chlorophyll extractability increased when the temperature rose from 35 to 45 ℃ but reduced at 55 ℃. A similar result was reported; the chlorophyll pigments of alfalfa and white clover protein concentrates were extracted effectively by methanol, ethanol, and chloroform; however, only ethanol is considered the food-grade solvent. The nitrogen content of treated samples increased while lipid content decreased, as shown in Table 2.6. The grassy flavour of treated samples also was removed. Nevertheless, the colour of LPC after treatment changed from green to brown without explicit ness improvement

(Tsuji et al., 1985). It indicates that using organic solvents can remove chlorophyll and other water-insoluble compounds (e.g., lipids) and improve flavour, enriching the protein content.

Table 2.6: Protein and lipid contents of green leaves and leaf protein concentrate (LPC) of white clover and alfalfa after ethanol extraction.

Samples	Protein (%)		Lipid (%)	
	Leaves	*LPC*	*Leaves*	*LPC*
White clover	55.1	70.8	10.0	1.7
Alfalfa	48.4	72.2	9.7	2.1

(Adapted from Tsuji et al., 1985)

2.6 Factors affecting the quality of extracted proteins

Since plant leaves contain many natural compounds which can react once the plant cells are damaged, causing undesirable reactions. Moreover, the properties of proteins rely on many factors, such as pH and chemicals. Therefore, many factors during the extraction process affect the quality of isolated proteins.

2.6.1 Lipid oxidation

Lipid oxidation is an undesirable reaction resulting in a grassy flavour (Hatanaka, 1996). This reaction occurs after disrupting plant cells, leading to the interaction between unsaturated fatty acids (such as α-linolenic and linoleic) in plant leaves and lipoxygenase (LOX) in the chloroplast membrane. Consequently, hydroperoxide of fatty acids is generated and degraded by hydroperoxide lyase. Subsequently, volatile organic compounds (VOCs) are developed, and a grassy flavour (smell of freshly cut grass) is released (Baysal & Demirdöven, 2007; Hatanaka, 1996). Eight VOCs responsible for grassy flavour are C6-aldehyde, C6-alcohol, leaf aldehyde, leaf alcohol, (2E)-hexenal, and (3Z)-hexanol (Hatanaka, 1996).

Heat treatment has been used widely to inhibit LOX activity as it is a cost-effective and uncomplicated process. The heating process is an effective enzymatic inactivation method; however, protein functionalities are affected due to protein denaturation. Additionally, the cooked flavour is developed, causing undesirable flavour in the final products (Chang et al., 2019). Chang et al. (2019) also used acetone, ethanol, and isopropanol to remove grassy flavour since they are common organic solvents used to remove off-flavour from legumes, soybean, and lupin. Nevertheless, the effectiveness depends on the type and concentration of solvents.

They reported that lentil protein isolate (LPI) treated with acetone had higher VOCs, resulting in an unfavourable flavour. In contrast, 75% ethanol and 70% isopropanol could remove off-flavour from LPI, but the protein solubility reduced from 60 to 40% compared with the untreated LPI.

2.6.2 pH

pH is a vital parameter for protein extraction since it relates to protein properties (e.g., surface charge) and PPO activity. The optimum pH of extracted juice is 6.5 to 7.5 due to high RuBisCo solubility. RuBisCo can link to the Mg^{2+} at the surface of the thylakoid membrane maximally at pH 8, causing protein loss. Nevertheless, this bonding can be cleaved by shaking with a vortex mixer or adding a chelating agent (e.g., EDTA). pH under 6 should be avoided as it causes co-precipitation of proteins and chloroplast membranes (De Jong et al., 2014). A similar result was observed by Camm and Green (1982). The extractability of leaf proteins decreased significantly at pH 4.5 since it was near the RuBisCo pI.

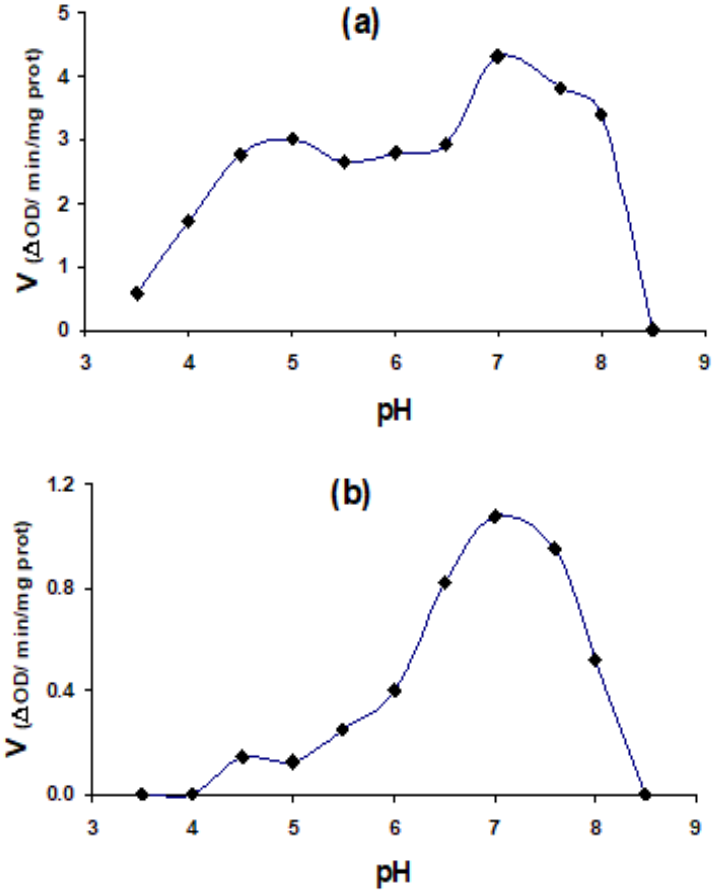


Figure 2.6: PPO activities in the pH range 3 to 9 against methyl catechol in red clover (a) and perennial ryegrass (b) (Winters et al., 2003).

pH is a crucial factor related to PPO activity, which causes polyphenol-protein interactions and reduces the nutritional value of target proteins. Winter et al. (2003) stated that high activity of PPO in both perennial ryegrass and red clover was observed at neutral pH. As Figure 2.6, the highest PPO activity reacting to the substrate (methyl catechol) was observed at pH 7 to 8 in red clover and 6.5 to 8 in perennial ryegrass. A similar result was demonstrated by De Jong et al. (2014); high pH enhanced the linkage between polyphenols and proteins, which led to lower protein digestibility (De Jong et al., 2014). Furthermore, this enzyme is the primary cause of browning reactions in vegetables and fruits, leading to economic loss and product development limitations (Jang & Moon, 2011).

Thermal treatment (e.g., blanching) is the most effective method to inactivate enzymes; however, it impacts end-product quality (Thakur et al., 2019). Thus, reducing agents are widely used for enzyme inactivation (Jang & Moon, 2011). Sodium metabisulfite is an effective reducing agent used in vegetable and fruit products. It is suggested to spray the solution on the freshly harvested leaves before storage. Adding reducing agents during the cell disruption process is another method. However, a high amount of reducing agents decreases the extraction yield and protein purity due to salt contamination. Thus, the suitable concentration ranges from 0.2 to 1.5% of fresh plant materials (De Jong et al., 2014). Alternatively, ascorbic acid is also used to inactivate PPO. However, Rojas-Graü et al. (2006) reported that the effectiveness of ascorbic acid is temporary since it is easily oxidised. Thus, controlling pH during protein isolation is significant since it is vital in protein extraction yield and controlling the oxidation reaction of PPO.

2.6.3 Alkaline solution

Using an alkaline solution is a common practice to assist protein extraction. Alkali enhances disruption of the leaf tissue (epidermis and lamella layer), resulting in more mesophyll proteins being extracted (Zhang et al., 2015). Moreover, alkali pH increases the solubility of proteins; therefore, a higher protein extraction yield is obtained (Di Stefano et al., 2018). Nevertheless, strong alkali (pH>11) causes protein hydrolysis, impacting protein functionalities and losing proteins. For instance, hydrolysed proteins provide a good emulsifier, while non-hydrolysed and partially hydrolysed proteins contribute to gelling properties (Tenorio et al., 2018). Therefore, mild alkali has been suggested for protein isolation, such as 0.1 M sodium hydroxide (NaOH) and 0.1 M ammonium hydroxide (NH_4OH) (De Jong et al., 2014).

2.6.4 Chemical use (food additives)

Leaf membrane proteins are usually abandoned during the discarding leaf pulp process (De Jong et al., 2014; Lamsal et al., 2007); thus, a high extraction yield would be obtained by extracting more insoluble proteins. However, the extraction of leaf membrane proteins is more complicated than a soluble fraction due to their heterogeneity (Tamayo Tenorio et al., 2017). Tween 80 (polysorbate 80), a food-grade surfactant, is a chemical that assists in extracting membrane proteins. It is allowed to use in food products at 1 – 10 % (w/w) (European Commission as cited in Tamayo Tenorio et al., 2017) or at the GMP level (FSANZ, 2016). However, its selectivity is not only for proteins; other compounds are co-solubilised with proteins. Consequently, the protein purity decreases, resulting in more processes required to remove the impurities (Tamayo Tenorio et al., 2017).

2.6.5 Process time and temperature

Time and temperature are significant factors for protein isolation since they relate to enzymatic reactions and protein degradation, affecting the quality of final products. Winter et al. (2003) presented that a long process time increased PPO activity, causing more polyphenol-protein complexes. As shown in Figure 2.7, a higher molecular weight protein was observed in perennial ryegrass and red clover after 24 hr of incubation. It demonstrated that proteins were bound with polyphenols; consequently, larger proteins were observed. Although the advantage of the large proteins is retarding proteolysis and increasing protein stability, the protein digestibility decreases (Winter et al., 2003).

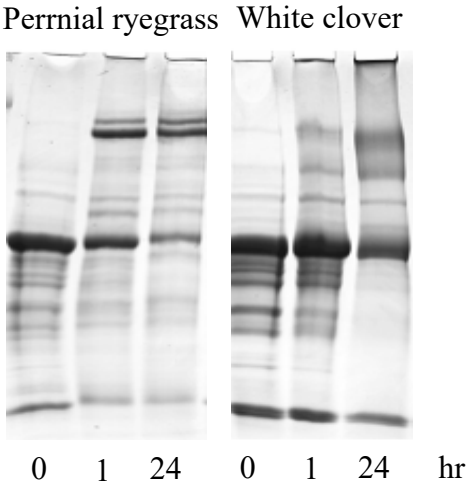


Figure 2.7: Protein size distribution of protein extracts from perennial ryegrass and red clover during 24 hours of incubation, as analysed by SDS-PAGE (adapted from Winter et al., 2003).

Time and temperature are relevant factors in recovery protein yield. Koschuh et al. (2004) studied the content of RuBisCo during 24-hour storage at different temperatures. A high amount of RuBisCo was observed at low temperatures, while the lowest was observed at 30 ℃, as shown in Table 2.7. Furthermore, they also studied the degradation rate of RuBisCo stored at 20 ℃. The result showed that RuBisCo decreased from 100 to 2 % after 24 hr due to the protease in plant materials hydrolysing proteins to small peptides. This may cause protein loss during extraction (De Jong et al., 2014). The above studies indicate that long processing times and high temperatures accelerate protein degradation. Hence, leaf protein isolation should be done at low temperatures and short cycles to avoid protein loss.

Table 2.7: Degradation rate of RuBisCo at different temperatures.

Temperature (℃)	% RuBisCo	
	Fresh green juice	24-hr green juice
0	100	80
4	100	65
20	100	1
30	100	1

(Adapted from Koschuh et al., 2004)

2.7 Conclusions

Plant proteins are more sustainable than animal proteins, as the environmental impacts affected by plant farming are less than animal agriculture. Several plant proteins, such as soybean, pea, chickpea, wheat, and seeds, have been used globally for the human diet. However, novel plant proteins should be studied due to the high protein demand in 2050. Plant leaves contain a valuable protein called RuBisCo, providing good nutritional value and functionalities. Therefore, leaf proteins are projected as a good protein source. Nevertheless, leaf proteins, especially pasture leaves, have not been studied extensively. Although *One50 AR1*, a perennial ryegrass cultivar, was selected for the present study, the isolation process could apply to other plant leaves (e.g., white clover) with some modifications.

The protein isolation process consists of four steps: harvesting, cell disruption, protein fractionation, and protein purification and concentration. Several factors, such as pH, temperature, process time, alkaline, and chemicals, should be set at proper conditions to ensure that RuBisCo is stable. The isolation conditions for RuBisCo should avoid extreme pH, high

temperature, long process time, and high ionic strength. Consequently, high recovery protein yield, good nutritional value, and good protein functionalities are obtained.

Different protein isolation methods also affect protein functionalities, nutritional value, and recovery yield. In this study, various techniques: alkali-acid precipitation, heat coagulation, ultrafiltration, chromatography, and activated charcoal treatment, have been studied. In addition, different conditions of the relevant factors, such as salt concentration, temperature, buffer ratio, cell disruption degrees, and a combination of those factors, have been varied. Kjeldahl, SDS-PAGE, DSC, and *in-vitro* gastro-small intestinal digestion have been used to determine crude protein, protein purity, and protein digestibility of leaf protein concentrate.

As there is no regulation for LPC, this study referred to an international general standard for vegetable protein products (VPP). The final products from vegetables that are removed non-protein constitutes should contain 40 % protein (dry weight basis) (Joint WHO/FAO, 2019).

CHAPTER 3

The Effect of Different Extraction Methods on Physico-Chemical Properties of Leaf Proteins

3.1 Introduction

Leaf proteins are projected as new alternative proteins for humans since they contain a valuable protein called RuBisCo. It accounts for 50 % of total soluble proteins in leaves and has a good nutritional value and functionalities (De Jong & Nieuwland, 2011). Scientists have studied leaf proteins from many plant sources, such as spinach, sugar beet leaves (Martin et al., 2014, 2019), and tobacco; however, pasture leaves have not been studied extensively. *One50 AR1* grass, a perennial ryegrass cultivar, is the largest-selling grass cultivar in New Zealand (Agricom research, 2020). It has dry matter and protein content higher than standard ryegrass across all seasons (Agriseeds, 2013; Kerr et al., 2012); thus, it was selected as the raw material for this study.

Different protein isolation methods affect the physico-chemical properties of isolated proteins, such as recovery yield, protein content, functionalities, nutritional value, appearance and flavour (Kaur et al., 2021, De Jong et al., 2014; Kobbi, 2017). Alkali-acid precipitation and heat coagulation were conducted in this study as both are uncomplicated and cost-effective extraction processes. However, these methods result in a loss of protein functionalities due to harsh conditions used during extraction (Knorr, 1982; Pelegrine & Gasparetto, 2005). Therefore, the effect of activated carbon, ultrafiltration, and chromatography on leaf protein isolation was also studied as they involve mild conditions. Alkali-assisted extraction was also studied as it increases the efficiency of protein extractability.

However, leaf proteins are still underutilized due to their dark green colour and grassy flavour associated with chlorophyll; consequently, their food applications are limited. Heating and divalent ions treatment have been studied for chloroplast aggregation, resulting in the potential removal of green colour and grassy flavour. Therefore, different concentrations of divalent ions and thermal treatment conditions were tested to remove chlorophyll from the isolated proteins.

Solvent extraction has been reported to remove green colour from green biomass, such as algae and plants. Chloroform, methanol, acetone, and propanol, can extract chlorophyll and remove

lipid-soluble components, increasing the protein content. However, only ethanol extraction is considered food grade (Tsuji et al., 1985); thus, removing green colour and improving grassy flavour by ethanol extraction was conducted in the present study. This chapter presents the protein content, recovery yield, colour, and flavour of the isolated leaf proteins extracted through each method mentioned above.

3.2 Materials and methods

3.2.1 Materials

Perennial ryegrass (cultivar-*One50 Ar1*), native grass in New Zealand, was used for the protein extraction experiments. The grass was harvested and stored immediately at -20 ℃ until further use. The protein content of grass was determined using the Kjeldahl method, AOAC 978.04 (Horwitz, 1978) and was found to be 18.50 % (dry basis), using a nitrogen conversion factor of 5.83 (Jiang et al., 2014). All chemicals used for protein extraction were food and USP-grade. Other chemicals were analysis grade.

3.2.2 Protein extraction

3.2.2.1 Alkali-acid precipitation

Leaf protein concentrate (LPC) was extracted using a method of Kaur et al., 2021, with slight modifications (Figure 3.1). Frozen grass was mixed with water and 0.1 M NaOH at the ratio of 1:1.7:0.7. The mixture was ground by a wet disintegrator (C200, JEFFRESS Engineering Pty Ltd., Dry Creek, Australia) at 6000 rpm for 10 minutes. The obtained slurry mixture was filtered through a nylon cloth (80 mesh), and the grass juice was collected and kept in the ice bath until further use. Grass pulp was ground again using the same conditions to extract any remaining proteins. The final grass juice was mixed with calcium chloride ($CaCl_2$) at 0, 100, 200, or 300 mM based on frozen grass weight. Subsequently, the grass juice was centrifuged at 17,200 *x g* for 20 minutes at 4 ℃ (Sorvall RC6+, Thermo Fisher Scientific Inc., Langenselbold, Germany). The pH of the recovered solution was reduced to 3.5 by using 0.1 M HCl, followed by centrifuging under the same conditions described above. The precipitated proteins were freeze-dried (FD18LT Freeze Drier, Cuddon, Blenheim, New Zealand). Dried LPC was ground in a coffee grinder (BCG200, Breville®, Sydney, Australia) and stored at -20 ℃ until further analysis.

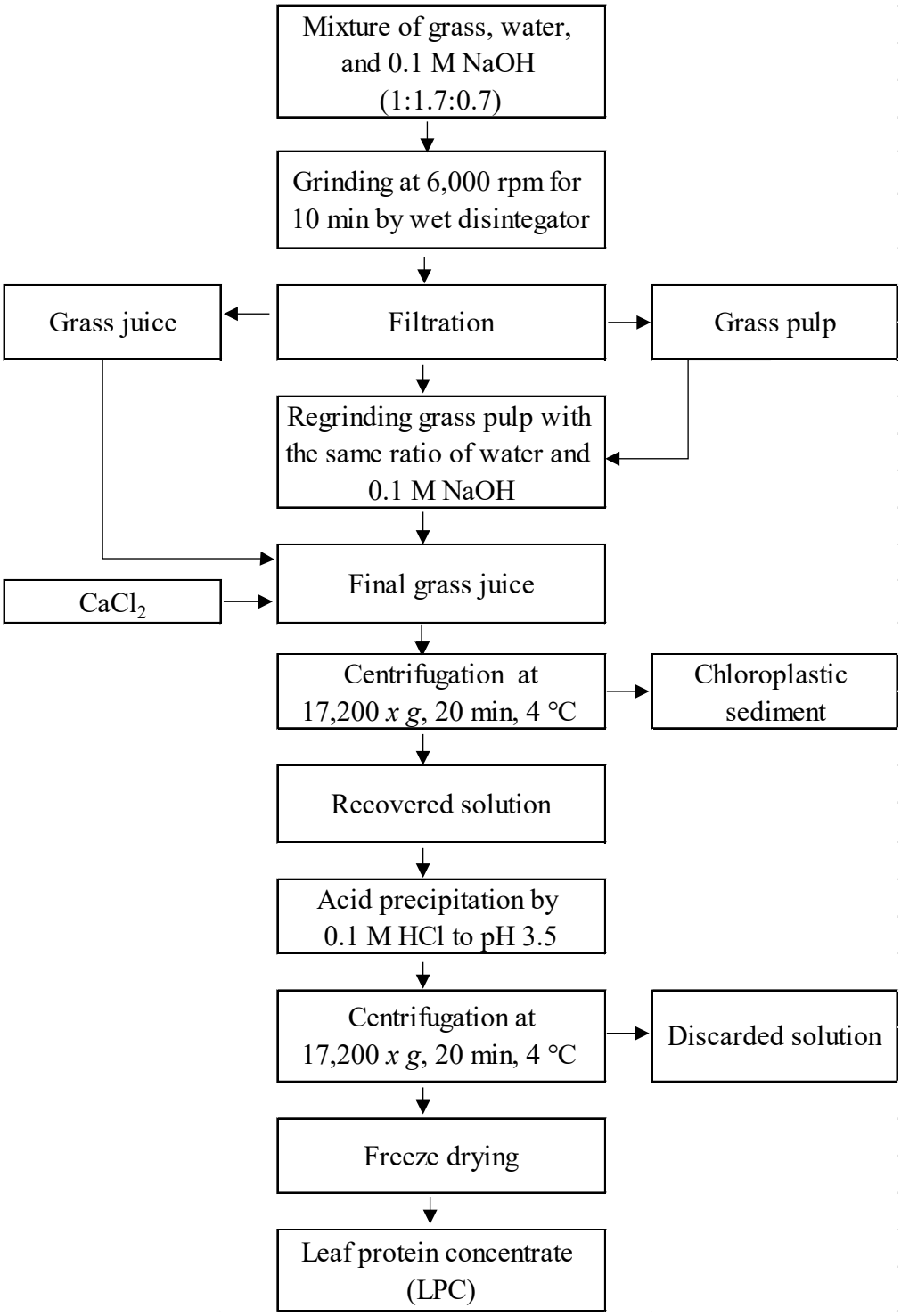


Figure 3.1: Extraction process of leaf protein concentrate: Alkali-acid precipitation.

3.2.2.2 Protein extraction combined with activated carbon

Leaf protein concentrate (LPC) was extracted using a method of Van De Velde et al. (2011b) with slight modifications (Figure 3.2). Frozen grass was mixed with water and 0.2 M Tris/HCl (pH 6) containing 2 % sodium metabisulfite at the ratio of 1:1.6:0.8. The mixture was ground by a wet disintegrator (C200, JEFFRESS Engineering Pty Ltd., Dry Creek, Australia) at 6000 rpm for 10 minutes. The obtained slurry mixture was filtered through a nylon cloth (80 mesh), and the grass juice was stored in the ice bath until further step. The final grass juice was heated at 60 ℃ for 5 minutes while stirring to distribute the heat uniformly and cooled down in the ice bath to below 10 ℃ immediately. The heated and cooled grass juice was centrifuged at 14,000 *x g* for 20 minutes at 5 ℃ (Sorvall RC6+, Thermo Fisher Scientific Inc., Langenselbold, Germany). The clear brown recovered solution was obtained, followed by dialysis overnight at 4 ℃ using a dialysis tube (MWCO 14 kDa). Activated carbon (AC) (powder form; particle size ≤ 200 µm) was added into the solution at different concentrations: 1.9 %, 3.8 %, and 5.7 % (w/w). The mixture was stirred for 5 minutes before centrifuging under the same conditions and filtered through 0.45 and 0.22 µm PVDF filters. The final solution was then freeze-dried (FD18LT Freeze Drier, Cuddon, Blenheim, New Zealand), followed by grinding in a coffee grinder (BCG200, Breville®, Sydney, Australia) and storage at -20 ℃ until further analysis.

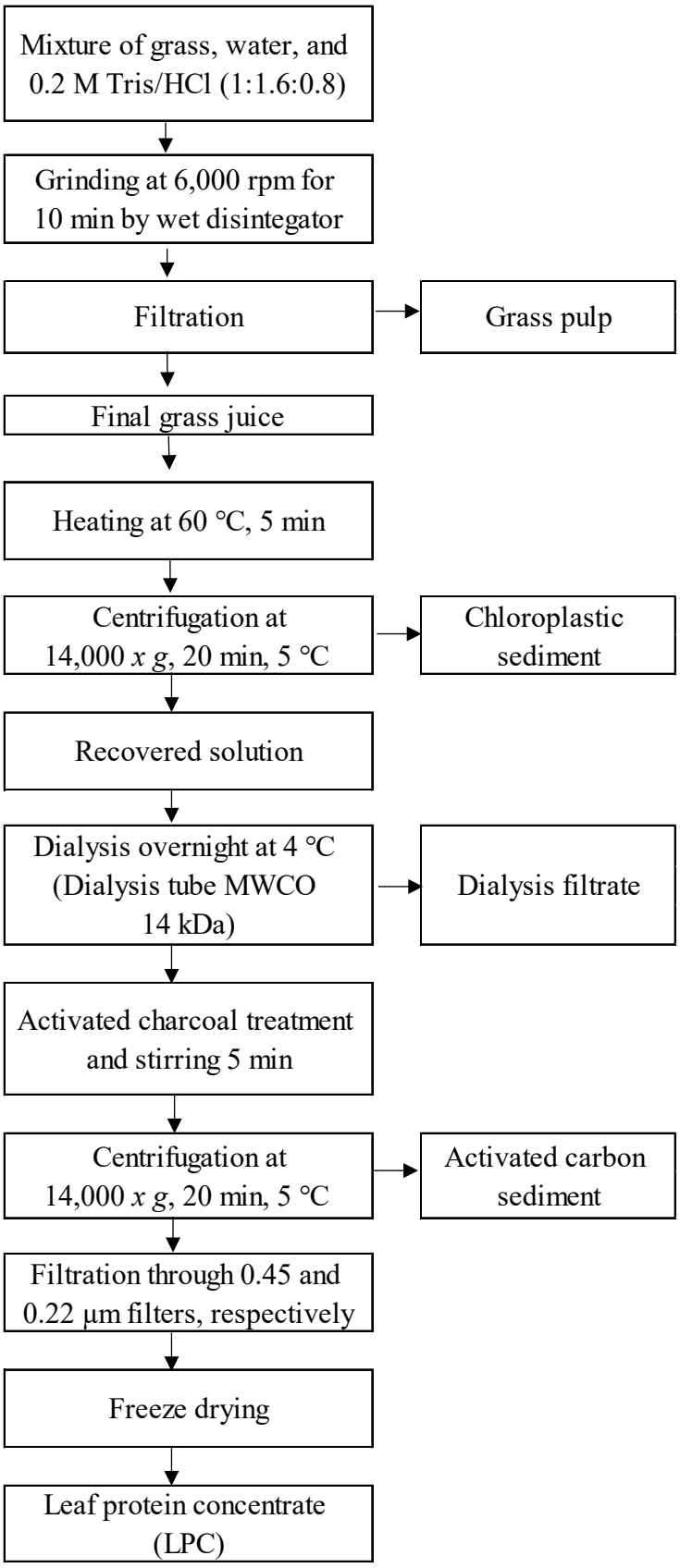


Figure 3.2: Extraction process of leaf protein concentrate: Protein extraction combined with activated carbon (powder form) treatment.

3.2.2.3 Alkali-acid precipitation combined with activated carbon

Leaf protein concentrate (LPC) was extracted using the method of Kaur et al. (2021) and Van De Velde et al. (2011b) with slight modifications (Figure 3.3). Frozen grass was mixed with water and 0.1 M NaOH at two ratios (1:2.4:0 and 1:1.7:0.7), and then the mixture was ground by a wet disintegrator (C200, JEFFRESS Engineering Pty Ltd., Dry Creek, Australia) at 6000 rpm for 10 minutes. The obtained slurry mixture was filtered through a nylon cloth (80 mesh), and the grass juice was stored in the ice bath until further use. Grass pulp was ground twice using the same conditions to extract any remaining proteins. The final grass juice was centrifuged at 14,000 *x g* for 20 minutes at 5 ℃ (Sorvall RC6+, Thermo Fisher Scientific Inc, Langenselbold, Germany). Subsequently, the obtained recovered solution was mixed with activated carbon (AC) (powder form; particle size ≤ 200 µm) at 1.9 % (w/w) for 5 minutes. The mixture was then centrifuged under the same conditions and filtered through filter paper (LabServ qualitative filter paper; code: LBS0001.150; size: 150 mm). The pH of the solution was reduced to 3.5 using 0.1 M HCl, followed by centrifuging under the same conditions described above. The precipitated proteins were solubilised with water at the ratio of 1:20 before adjusting pH back to 7 using 0.1 M NaOH and stirring for 20 minutes at 20 ℃. The final solution was freeze-dried (FD18LT Freeze Drier, Cuddon, Blenheim, New Zealand), ground in a coffee grinder (BCG200, Breville®, Sydney, Australia) and stored at -20 ℃ until further analysis.

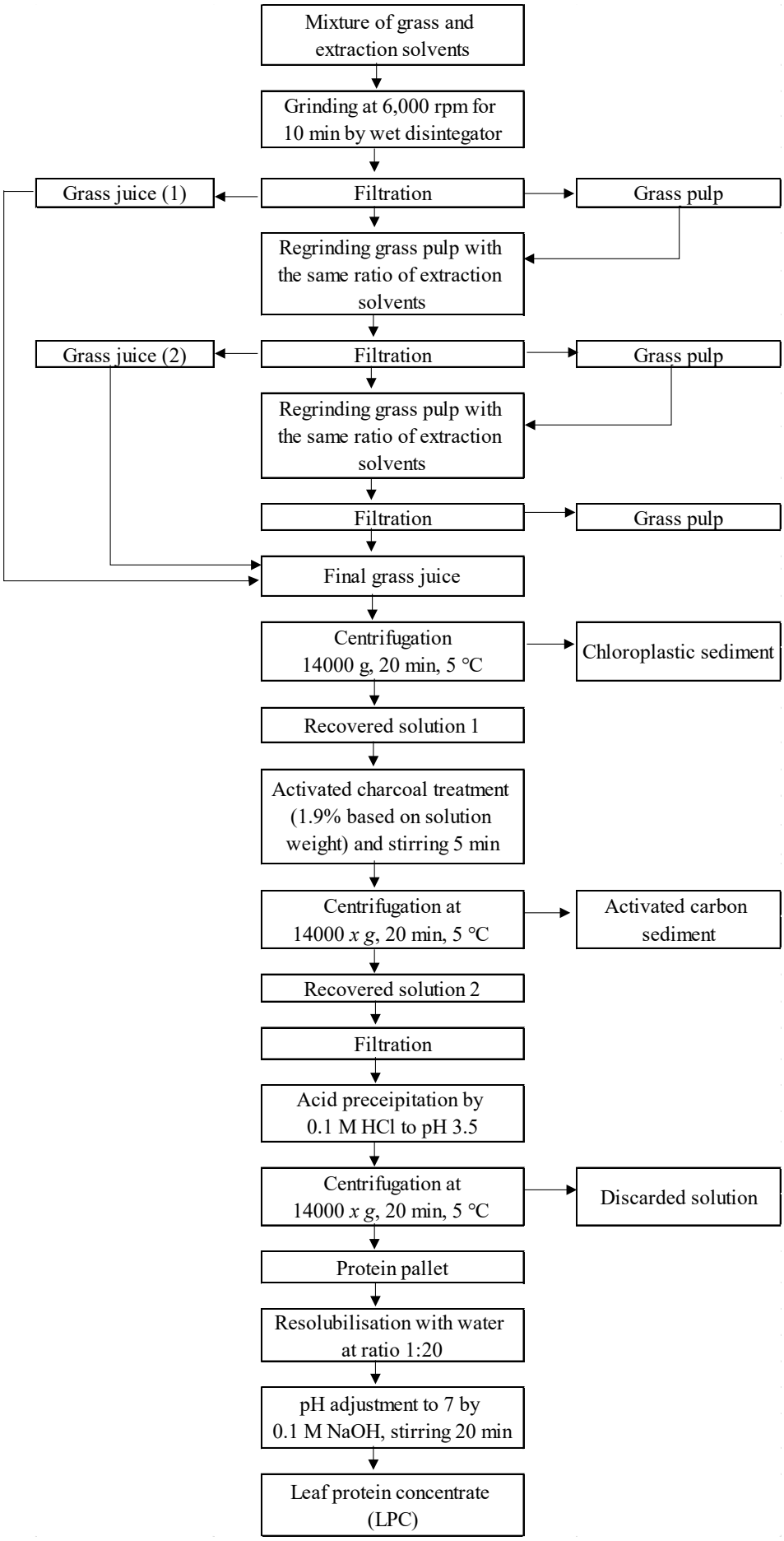


Figure 3.3: Extraction process of leaf protein concentrate: Alkali-acid precipitation combined with activated carbon (powder form).

3.2.2.4 Heat coagulation (effect of $CaCl_2$ treatment along with heating)

Slight modifications were made to the method of Damborg et al. (2020) to extract leaf protein concentrate (LPC), as shown in Figure 3.4. Frozen grass was chopped into around 1 cm pieces and sprayed with water containing 1 % sodium metabisulfite at a ratio of 1:0.2. Subsequently, the chopped grass was pressed twice using a single screw presser (Titanium Major KMM020, Kenwood, United Kingdom). The final grass juice was filtered through a nylon cloth (80 mesh) and stored in the ice bath. Calcium disodium EDTA (0.76 mM based on grass juice weight) and $CaCl_2$ at different concentrations (Table 3.1) were added to the grass juice, and its pH was adjusted to 7 using 0.1 M NaOH. The juice was heated under different conditions, as shown in Table 3.1. Nitrogen was flushed in the headspace while stirring to avoid heat accumulation. The heated juice was cooled down in the ice bath to lower the temperature below 10 °C.

Table 3.1: $CaCl_2$ concentrations and heating conditions.

Heating conditions	Concentration of $CaCl_2$ (mM based on grass juice weight)
50 °C, 15 minutes	0
	100
	150
58 °C, 2 minutes	0
	100
	150

The grass juice was centrifuged at 5,000 *x g* for 10 minutes at 4 °C (Sorvall RC6+, Thermo Fisher Scientific Inc., Langenselbold, Germany). The recovered solution was heated at 80 °C for 30 seconds and immediately cooled down in an ice bath to lower the temperature below 10 °C. The cooled juice was centrifuged at 17,200 *x g* for 20 minutes at 4 °C to collect precipitated proteins, followed by freeze-drying (FD18LT Freeze Drier, Cuddon, Blenheim, New Zealand). Dried LPC was ground by a coffee grinder (BCG200, Breville®, Sydney, Australia) and stored at -20 °C until further use.

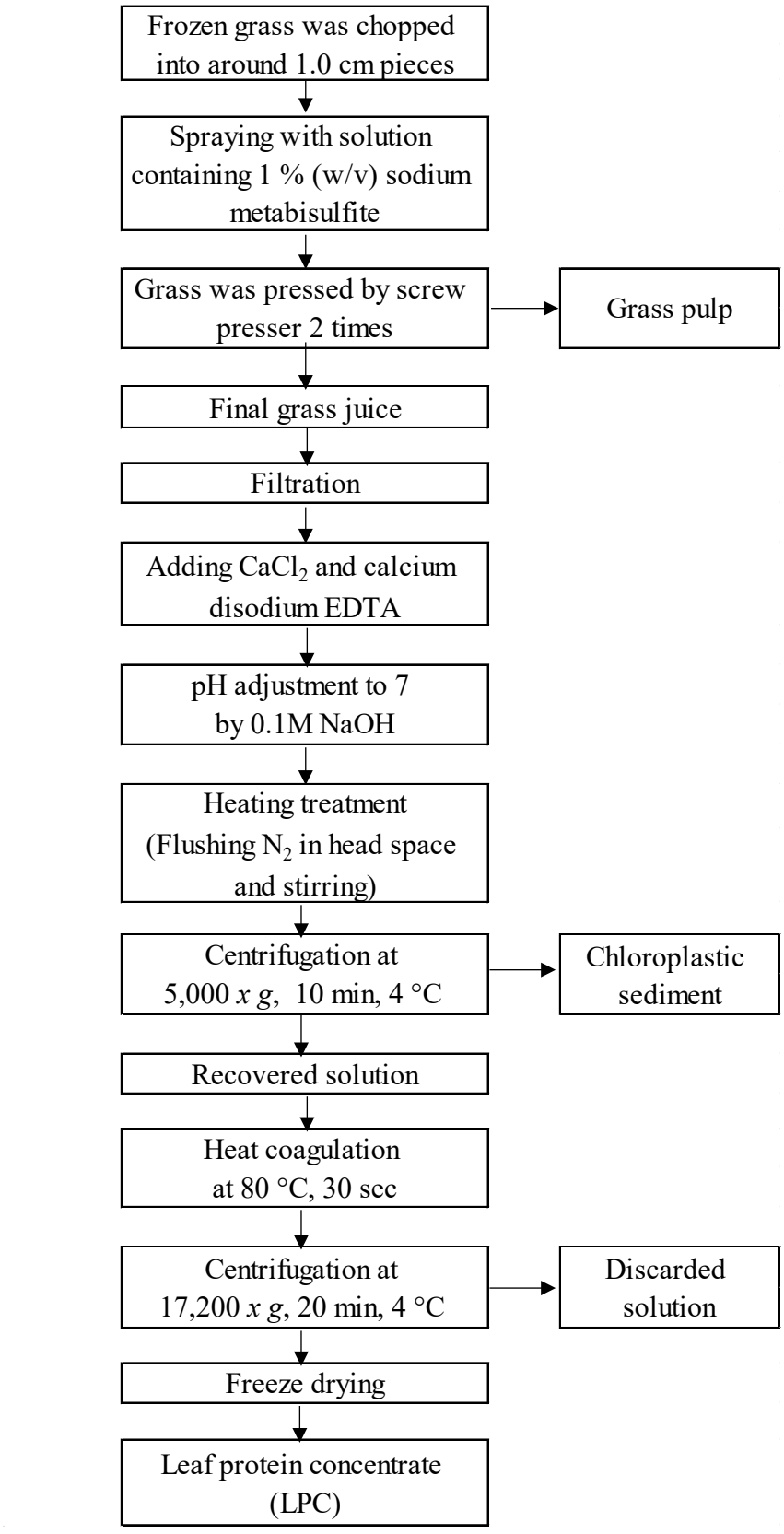


Figure 3.4: Extraction process of leaf protein concentrate: Heat coagulation with different $CaCl_2$ treatments along with heating.

3.2.2.5 Heat coagulation (effect of alkali solution)

Slight modifications were made to the method of Damborg et al. (2020) to extract leaf protein concentrate (LPC), as shown in Figure 3.5. Extraction solutions were prepared by mixing water and 0.1 M NaOH, as given in Table 3.2. All solutions contained 1 % sodium metabisulfite.

Table 3.2: Mixture ratio of extraction solutions.

Solutions	The final concentration of NaOH (M)	Mixture ratio	
		Water	0.1 M NaOH
1	0.00	1.0	0.0
2	0.03	0.7	0.3
3	0.10	0.0	1.0

Frozen grass was chopped into around 1 cm pieces and sprayed with solutions at the ratio of 1:0.5. Subsequently, the chopped grass was pressed twice using a single screw presser (Titanium Major KMM020, Kenwood, United Kingdom). The final grass juice was filtered through a nylon cloth (80 mesh) and stored in the ice bath. Calcium disodium EDTA (0.76 mM based on grass juice weight) was added to the grass juice, and its pH was adjusted to 7 using 0.1 M NaOH. The obtained grass juice was heated at 50 °C for 15 minutes. Nitrogen was flushed in the headspace while stirring to avoid heat accumulation. The heated juice was cooled down in an ice bath to lower the temperature below 10 °C. The cooled grass juice was centrifuged at 5,000 *x g* for 10 minutes at 4 °C (Sorvall RC6+, Thermo Fisher Scientific Inc., Langenselbold, Germany). The recovered solution was heated at 80 °C for 30 seconds and immediately cooled down in the ice bath to lower the temperature below 10 °C. The cooled solution was centrifuged at 17,200 *x g* for 20 minutes at 4 °C using the same centrifuge machine. The precipitated proteins were freeze-dried (FD18LT Freeze Drier, Cuddon, Blenheim, New Zealand), ground in a coffee grinder (BCG200, Breville®, Sydney, Australia) and stored at -20 °C until further use.

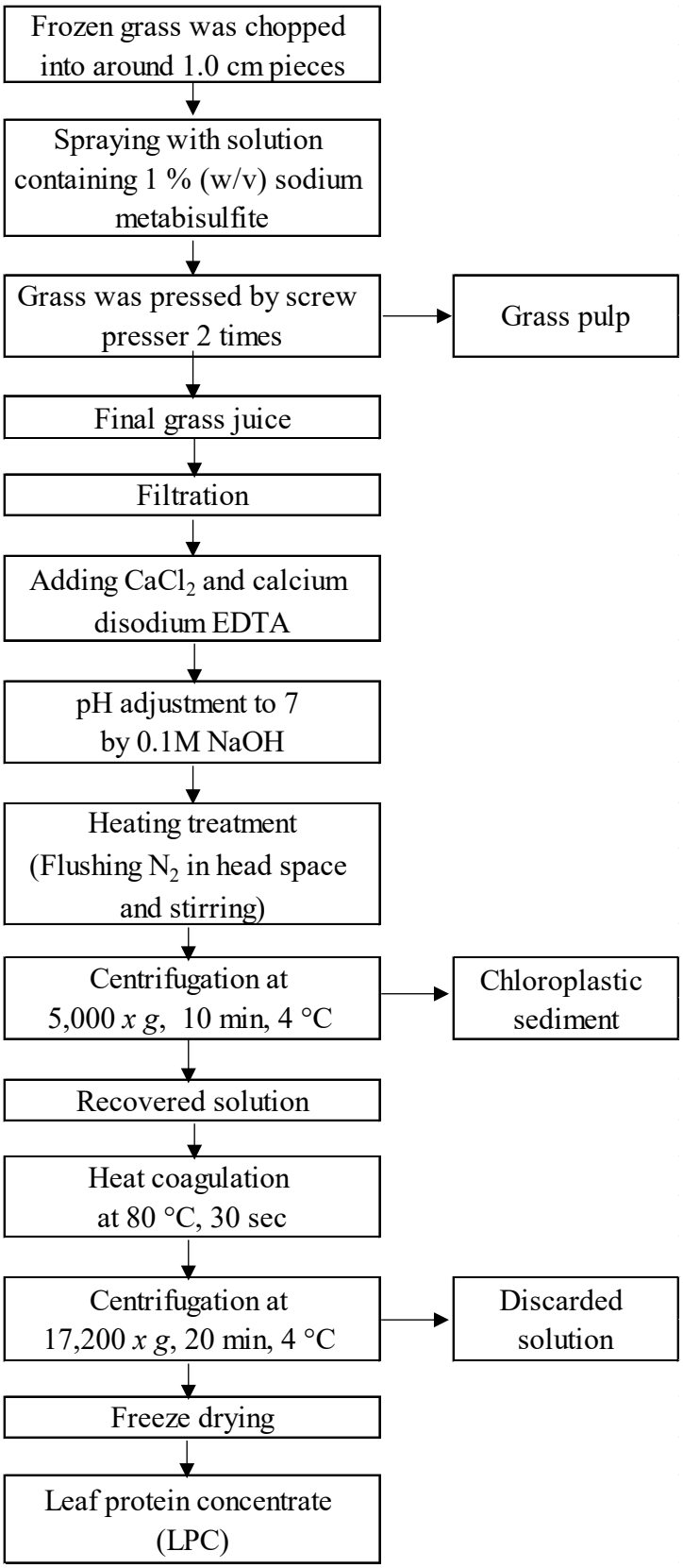


Figure 3.5: Extraction process of leaf protein concentrate: Heat coagulation with the different alkali concentrations of extraction solutions.

3.2.2.6 Ultrafiltration

Slight modifications were made to the method of Martin et al. (2019) to extract leaf protein concentrate (LPC), as shown in Figure 3.6. Frozen grass was chopped into around 1 cm pieces and sprayed with water containing 1 % sodium metabisulfite at a ratio of 1:0.5. Subsequently, the chopped grass was pressed twice using a single screw presser (Titanium Major KMM020, Kenwood, United Kingdom). The grass juice was filtered through a nylon cloth (80 mesh) and stored in the ice bath. Calcium disodium EDTA (0.76 mM based on grass juice weight) was added to the grass juice, and its pH was adjusted to 7 using 0.1 M NaOH. The final grass juice was heated at 50 ℃ for 15 minutes. Nitrogen was flushed in the headspace while stirring to avoid heat accumulation. The heated juice was cooled down in an ice bath to lower the temperature below 10 ℃. The cooled juice was centrifuged at 5,000 *x g* for 10 minutes at 4 ℃ (Sorvall RC6+, Thermo Fisher Scientific Inc., Langenselbold, Germany). The recovered solution was filtered through a 0.45 µm PVDF filter before concentrating by Amicon stirred cell ultrafiltration (regenerated cellulose at MWCO 100 kDa, Merck Millipore, Massachusettes, United States of America). After the final volume was decreased to 1/10 of the original volume, water was added to the retentate until reaching half of the original volume. Subsequently, the mixture was dialysed to remove sodium metabisulfite and other salts (using the same ultrafiltration) until the volume was 1/10 of the original volume. The final retentate was freeze-dried (FreeZone^{12Plus}, Labconco, Kansas City, Missouri, United States of America), ground in a coffee grinder (BCG200, Breville®, Sydney, Australia) and stored at -20 ℃ until further analysis.

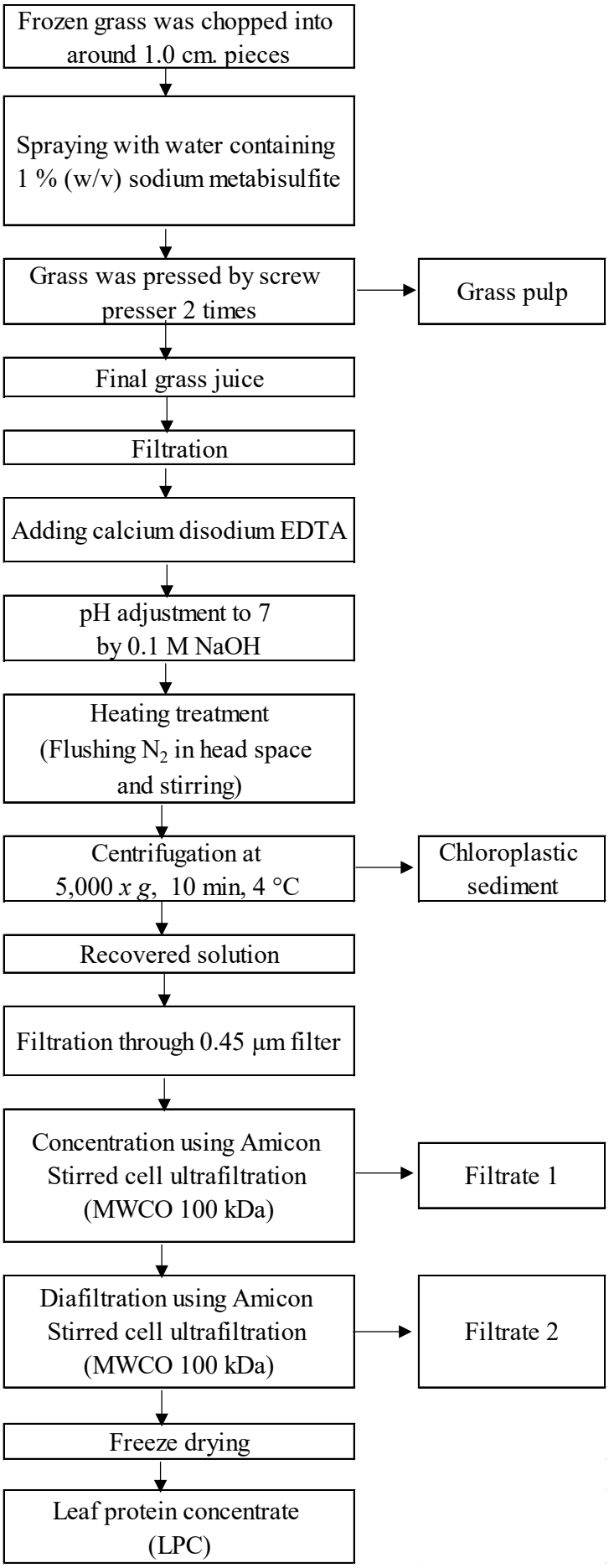


Figure 3.6: Extraction process of leaf protein concentrate: Through the use of ultrafiltration.

3.2.2.7 Chromatography technique

Slight modifications were made to the method of Martin et al. (2014) to extract leaf protein concentrate (LPC), as shown in Figure 3.7. 500 g of frozen grass was chopped into around 1 cm pieces and sprayed with 250 g of water containing 1 % sodium metabisulfite. Subsequently, the chopped grass was pressed twice using a single screw presser (Titanium Major KMM020, Kenwood, United Kingdom). The final grass juice was filtered through a nylon cloth (80 mesh) and stored in the ice bath. Calcium disodium EDTA (0.76 mM based on grass juice weight) was added to the grass juice, and its pH was adjusted to 7 using 0.1 M NaOH before heating at 50 ℃ for 15 minutes. Nitrogen was flushed in the headspace while stirring to avoid heat accumulation. The heated juice was cooled down in an ice bath to lower the temperature below 10 ℃. Before collecting the recovered solution, the grass juice was centrifuged at 5,000 *x g* for 10 minutes at 4 ℃ (Sorvall RC6+, Thermo Fisher Scientific Inc., Langenselbold, Germany). The pH of the recovered solution was adjusted to 8 by adding 2.23 g/L of Tris base before filtering through a 0.45 µm PVDF filter. The filtered juice was diluted with Milli Q water to decrease the conductivity until below 6.5 mS/cm.

The solution was mixed with 150 mL of Q-sepharose st-flow resin for 1 h in a cold room (resin was equilibrated with 20 mM Tris/HCl (pH 8) before loading the sample). The mixture was loaded into a column (diameter = 50 mm, height = 30 cm); subsequently, 500 mL of 0.1 – 1.0 M NaCl were loaded into a column to elute the target protein. The filtrate from the elution process was analysed for the protein's molecular weight (MW) using SDS-PAGE. Only the filtrate from 0.3 M NaCl elution, which contained the highest content of RuBisCo, was concentrated by centrifuging at 3,500 *x g* for 25 minutes at 20 ℃ using a centrifugal filter (Centricon® Plus-70, regenerated cellulose 100 kDa, Merck Millipore, Carrigtwohill, Ireland). The retentate was desalted into 50 mM sodium phosphate buffer (pH 7) using a Sephadex G75 column (70 ml, XK20/20, GE Healthcare, Uppsala, Sweden). The desalted fractions were analysed by SDS-PAGE to determine the extracted protein molecular weight. The fractions containing small and large subunits of RuBisCo were freeze-dried (FD18LT Freeze Drier, Cuddon, Blenheim, New Zealand) and stored at -20 ℃ until further use.

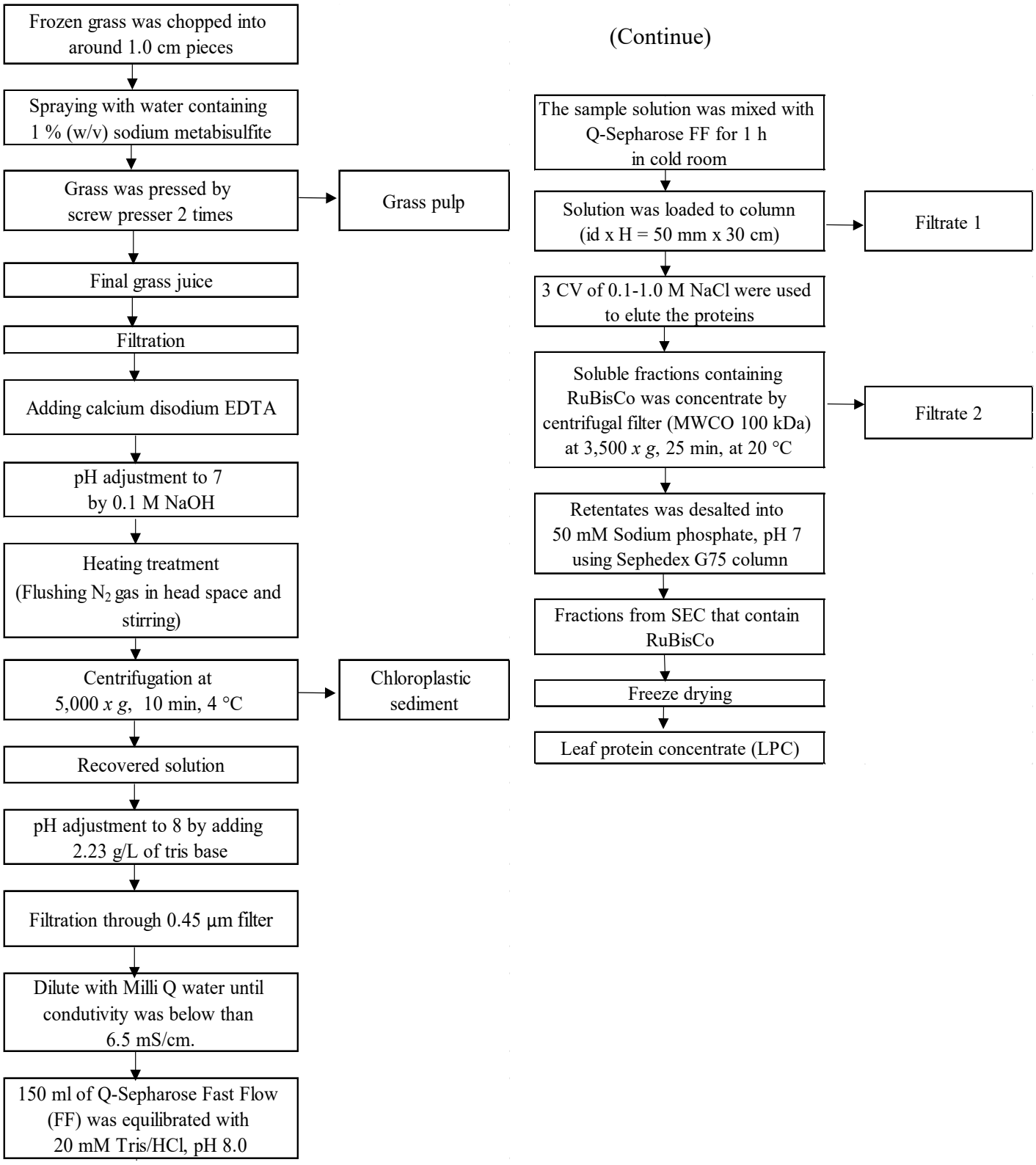


Figure 3.7: Extraction process of leaf protein concentrate: Through chromatography technique.

3.3 Removal of green colour and grassy flavour by ethanol extraction

3.3.1 Materials

Perennial ryegrass (cultivar-*One50 Ar1*), native grass in New Zealand, was freeze-dried and ground by Nutri Ninja™ Slim Blender (QB3001, Ninja, Brand Developers Ltd., New Zealand). Leaf protein concentrate (LPC) was obtained from the protein extraction method as stated in section 3.2.2.3 using water and 0.1 M NaOH at the ratio of 1.7:0.7. Both samples were stored at -20 °C until further use. The protein content of freeze-dried grass leaves and LPC was determined using the Kjeldahl method, AOAC 978.04 (Horwitz, 1978) and was found to be 18.50 % and 50.03 % (dry basis), respectively, using a conversion factor of 5.83 (Jiang et al., 2014).

3.3.2 Ethanol treatment of freeze-dried grass leaves (ground form)

Slight modifications were made to Tsuji et al. (1985) method to remove the green colour and grassy flavour from the grass leaves. In short, the freeze-dried and ground grass leaves were mixed with 90 % ethanol with a ratio of 1:20 (w/w), followed by stirring at 45 ℃ in the water bath under different conditions (Table 3.3). The heated mixture was cooled down in the ice bath until the temperature was 20 ℃. The cooled sample was centrifuged at 10,000 *x g* for 10 minutes at 20 ℃ (Sorvall RC6+, Thermo Fisher Scientific Inc., Langenselbold, Germany). The precipitates were freeze-dried (FD18LT Freeze Drier, Cuddon, Blenheim, New Zealand) and stored at -20 ℃.

Table 3.3: Removal of green colour and grassy flavour from grass leaves by ethanol extraction at different conditions.

Experiment	Time (minutes)	Repetition
1	60	1
2	20	3

3.3.3 Ethanol treatment of leaf protein concentrate

Slight modifications were made to Tsuji et al. (1985) method to remove the green colour and grassy flavour from the leaf protein concentrate (LPC). In short, LPC was mixed with 90 % ethanol with a ratio of 1:20 (w/w), followed by stirring at 45 ℃ for 60 minutes in a water bath. The heated mixture was cooled down in the ice bath until the temperature was 20 ℃ before

centrifuging at 10,000 *x g* for 10 minutes at 20 °C (Sorvall RC6+, Thermo Fisher Scientific Inc., Langenselbold, Germany). The precipitates were mixed with 90 % ethanol at the same ratio, stirred at room temperature for 20 minutes, and centrifuged under the conditions described above. This process was repeated twice before collecting the final precipitates to freeze dry (FD18LT Freeze Drier, Cuddon, Blenheim, New Zealand) and storing at -20 °C until further analysis.

3.4 Physico-chemical analysis

Leaf protein concentrates (LPCs) using the different extraction methods, grass leaves and LPC treated by ethanol extraction were analysed for their physico-chemical properties.

3.4.1 Moisture analysis

The moisture content of the liquid samples obtained from the extraction process was determined using the hot air oven method (AOAC.930.15925.10). In short, 2 g of samples were dried in an air oven at 108 °C for 4 h (Cat. No. 240, Contherm Scientific Ltd., New Zealand). The moisture dishes were cooled down, and their weight was recorded. The moisture content was calculated from the weight difference between the samples before and after drying.

3.4.2 Ash

Ash content was determined using a chamber furnace (Carbolite™, Thermos Fisher Scientific, China) (Smith et al., 2011). In short, 2 g of samples was placed into the crucible and dried in the furnace at 550 °C for 5 h. The crucibles were cooled in a desiccator and weighed. The percentage of ash was calculated according to the following formula:

$$\% \text{ Ash} = \frac{W2 - W1\,(g)}{W3\,(g)} \times 100 \qquad (1)$$

where *W1* is the weight of the crucible and sample after drying (g), *W2* is the weight of the crucible and sample before drying (g), and *W3* is the weight of the initial sample (g).

3.4.3 Protein content

The protein content of samples was determined using the Kjeldahl method, AOAC 978.04 (Horwitz, 1978). The nitrogen to a protein conversion factor of 5.83 was used (Jiang et al., 2014). The procedure involved the conversion of the total organic nitrogen to ammonium sulfate through digestion with concentrated sulfuric acid. Ammonia was distilled to boric acid

under alkali conditions, and the borate anions were titrated with standardized 0.1 M HCl. The nitrogen content representing the amount of crude protein in samples was calculated (Jiang et al., 2014). The crude protein content was calculated via the equations below:

$$\% \text{ N} = \frac{(A-B)\times C \times 14 \times 100}{D \times 1000} \text{.....(2)}$$

$$\% \text{ protein} = \% \text{ N} \times 5.83 \text{.....(3)}$$

where *A* is the volume of HCl (0.1 M) in the sample (mL), *B* is the volume of HCl (0.1 M) in the blank (mL), *C* is the molarity of HCl (0.1 M) and *D* is the weight of the initial sample (g).

3.4.4 Colour and flavour

Leaf protein concentrates (LPCs) extracted through alkali-acid precipitation without $CaCl_2$ treatment are determined as a control sample: characterized by dark green colour and strong grassy flavour. The colour and grassy flavour of LPCs extracted through different methods were compared visually and by smelling the samples. The observations are reported in the result and discussion section.

3.4.5 Tricine-Sodium Dodecylsulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) of filtrates from chromatography technique

The filtrates were concentrated using ultra 2 mL centrifugal filters (Amicon® ultrafilters, Darmstadt, Germany) by centrifuging at 2500 *x g* for 5 minutes at 5 ℃ or until the final volume was 1/10 of the original volume. The solutions were diluted (1:1) with sample buffer (containing 5% β-mercaptoethanol) and heated at 95 ℃ for 5 minutes. Standard marker (10 µL) and samples (25 µL) were loaded into 16.5 % gradient Tricine gels (Criterion™ Precast gel, Bio-Rad Laboratories Pty. Ltd., United States of America). The molecular weight of standard markers was 10, 15, 20, 25, 37, 50, 75, 100, 150, and 250 kDa (Precision Plus Protein™ Dual Xtra Prestained Protein Standards). The gels were run with a running buffer at 125 V voltage until the samples reached the bottom of the gels. A fixing solution (40 % methanol, 10 % acetic acid) was used to treat gels for 30 minutes, then stained with a staining solution (Bio-Safe™ Coomassie G-250) for 1 h. After this, the gels were de-stained in Milli-Q water overnight and scanned using a gel scanning densitometer (Molecular Imager Gel Doc XR, Bio-Rad Laboratories Pty. Ltd., New Zealand). The images were analysed using Image Lab™ (Bio-Rad Laboratories Pty. Ltd., New Zealand).

3.4.6 Statistical analysis

All the data reported in this study were performed at least in triplicate, except the extraction yield (one replicate). The data are presented as mean ± standard deviation. All data were analysed with Minitab statistical software version 19 (Minitab Inc., State College, PA). One-way ANOVA and Tukey's test at the 95 % confidence level identified significant differences ($p < 0.05$) for more than two sample groups. A two-sample t-test at a 95 % confidence level was used to identify the significant difference between the two sample groups.

3.5 Results and discussion

3.5.1 Physico-chemical properties of the isolated leaf proteins

Protein content, protein yield, colour, and flavour of extracted proteins are significant factors determining consumer acceptability and protein commercialisation. This study aimed to remove the green colour and grassy flavour from the leaf protein concentrate (LPC). Hence, the colour and flavour of LPC extracted through each method were virtually evaluated using LPC extracted through alkali-acid precipitation without $CaCl_2$ as a control sample (dark green and strong grassy flavour).

Protein content determination is crucial for food proteins to determine their purity. This project aimed to investigate the effect of extraction methods on protein extractability and protein loss during the extraction process. Thus, the protein content of LPC from each method was analysed and reported as a percentage (dry basis). Also, the selected fractions obtained during the extraction process were analysed as the amount of recovered protein and reported in a unit of g. The extraction yield of LPC was determined to study the effect of different extraction methods on the yield of isolated proteins. The results were reported as a percentage (%) based on the dried-weight grass.

Leaf protein concentrate (LPC) used for further studies (in Chapter 4) was selected depending on high protein content, good extraction yield, and the most significant colour and flavour improvement compared among all LPCs.

3.5.1.1 Alkali-acid precipitation with or without the addition of $CaCl_2$

Acid precipitation has been used extensively in commercial products (e.g., cheese) to concentrate proteins. Alkali pH increases protein extractability, resulting in more recovered proteins in the final product. Nevertheless, leaf protein concentrate (LPC) obtained through

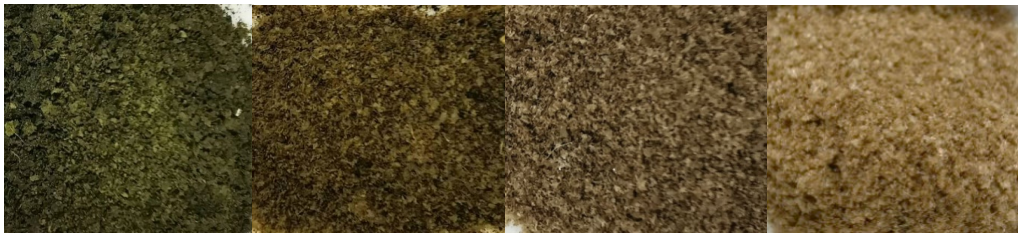
alkali-acid precipitation had dark green colour and grassy flavour (Kaur et al., 2021). $CaCl_2$ has been studied to agglomerate chloroplast cells since its positive charges (Ca^{2+}) can neutralize negative charges on the thylakoid membrane; consequently, the chloroplast cells aggregate (Camm & Green, 1982). Thus, adding $CaCl_2$ in this experiment aimed to remove the green colour and improve the grassy flavour. The effect on protein content and extraction yield also was determined.

Green colour and grassy flavour

The dark green colour of leaf protein concentrate (LPC) was partially removed when 100 mM $CaCl_2$ was used. The green colour was eliminated and changed to brown and medium brown when the concentration of $CaCl_2$ was 200 and 300 mM, respectively (Table 3.4). These results were due to the chloroplast flocculation phenomena. As divalent ions (Ca^{2+}) neutralise the negatively charged thylakoid membrane, chloroplast aggregation occurs, and the chloroplast cells flocculate (Camm & Green, 1982). Thus, an appropriate amount of divalent ions can effectively remove the green colour.

The grassy flavour was reduced as the green colour was eliminated (Table 3.4). Leaf protein concentrate (LPC) treated with 100 mM $CaCl_2$ still had a grassy flavour as strong as without $CaCl_2$ treatment. However, a light grassy flavour was observed in LPCs treated with 200 and 300 mM $CaCl_2$. A study by Lelyveld & Smith (1989) can support the present observation. They reported that the grassy taste of black tea leaves was related to chlorophyll. Hence, the more chlorophyll removal, the less grassy flavour in the final LPC.

Table 3.4: Colour and flavour of leaf protein concentrate: Through alkali-acid precipitation with different concentrations of $CaCl_2$.

$CaCl_2$ (mM)	**0**	**100**	**200**	**300**
Colour				
	Dark green	Brownish green	Brown	Medium brown
Grassy flavour	Strong	Strong	Light	Light

Protein content

Figure 3.8 shows the protein content of LPCs extracted through alkali-acid precipitation with different $CaCl_2$ concentrations. A significant decrease ($p < 0.05$) in protein content was detected when $CaCl_2$ concentration was increased. The protein content of LPCs treated with 0, 100, 200, and 300 mM $CaCl_2$ was 53.94 %, 51.14 %, 48.70 %, and 44.76 % (dry basis), respectively. The salting-out effect might decrease the protein content of LPCs since ions of salts are dissociated in the solution, reducing the electrostatic forces between the charge of protein molecules (Mu et al., 2008). Consequently, protein aggregation occurs and protein loss along with the chloroplastic sediments. Therefore, the amount of protein in the recovered solutions (after the first centrifugation) significantly decreased ($p < 0.05$) when $CaCl_2$ concentration was increased, as shown in Table 3.5.

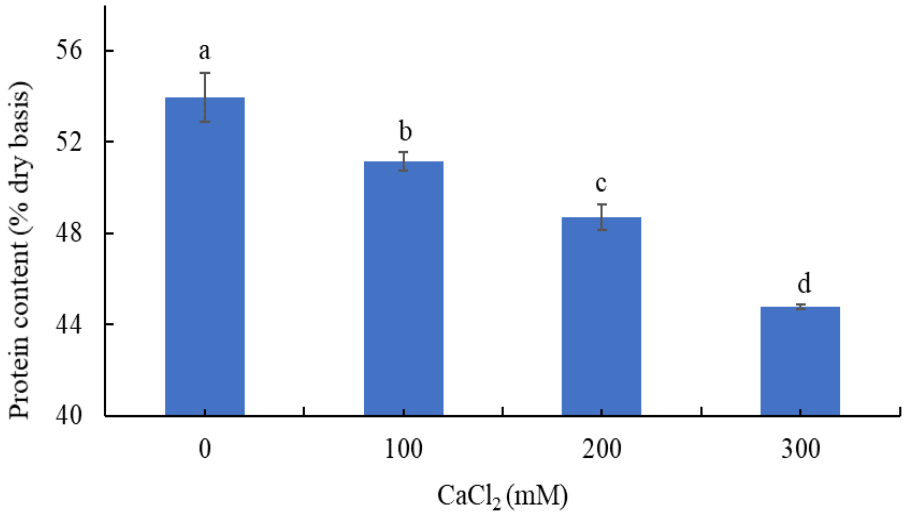


Figure 3.8: Protein content (% dry basis) of leaf protein concentrate: Through alkali-acid precipitation with different concentrations of $CaCl_2$.

Means ($n = 3$) that do not share a letter are significantly different ($p < 0.05$) (Tukey's HSD multiple comparisons and 95 % confidence). HSD (Honest Significance Test).

The above result could be supported by the protein content of different fractions obtained during the extraction process. Table 3.5 shows that the amount of protein recovered in the final grass juice was 6.21 g on average (6.15 to 6.23 g between 4 conditions). The similar protein results in the grass juice might be due to using the same conditions and materials in the disruption process. However, the proteins in the recovered solution were reduced from 3.74 to 2.44 g. $CaCl_2$ concentration increased from 0 to 300 mM. This result was due to the salting-out effect, reducing protein solubility in high-salt solutions (Mu et al., 2008). The same finding

was investigated in the grass protein concentrate (GPC) solution reported by Kaur et al. (2021). The solubility of GPC solutions decreased significantly ($p < 0.05$) from 27 % to 13 % in the solutions containing 0 to 1 M sodium chloride. It could be seen that using high salt decreases protein solubility in the solution, resulting in less available proteins being concentrated by acid precipitation. Thus, a high concentration of $CaCl_2$ leads to lower protein recovery in LPC.

Table 3.5: The amount of protein in the recovered fractions obtained from the extraction process: Through alkali-acid precipitation with different concentrations of $CaCl_2$.

Sample	$CaCl_2$ (mM)	Recovered proteins (g)
Final grass juice	-	6.21
Recovered solution	0	3.74[a]
	100	2.73[b]
	200	2.64[b]
	300	2.44[c]
LPC	0	2.59[a]
	100	0.70[b]
	200	0.65[b]
	300	0.36[c]

Note: The starting grass was 250 g (wet weight), and the initial protein content was 7.84 g (dry weight).
Different letters in the same column of each sample represent a significant difference ($p < 0.05$). Results are expressed as means ($n = 3$) with Tukey's HSD multiple comparisons and 95 % confidence. HSD (Honest Significance Test).

Moreover, proteins were also detected in the discarded fractions: grass pulp (18.03 to 20.45 % dry basis) and chloroplastic sediments (30.80 to 38.82 % dry basis). The proteins in these samples are non-soluble proteins in plant leaves, which account for up to 50 % of total leaf proteins (Barbeau & Kinsella, 1988). They mainly are lipoproteins (membrane proteins) (Barbeau & Kinsella, 1988) and chlorophyll-protein complexes (Liu et al., 2004). In addition, the interactions between proteins and other components (e.g., protein-lipid membrane and protein-enzyme cofactors) (Wang et al., 2008) make protein less soluble. Consequently, non-soluble proteins are usually lost through the waste fractions.

Adding $CaCl_2$ leads to chloroplast cell agglomeration; therefore, chlorophyll-protein complexes associated with chloroplast might also be precipitated (Camm & Green, 1982). Therefore, a high protein loss along with chloroplastic sediments could occur. Nevertheless,

protein content in chloroplastic sediments was decreased when $CaCl_2$ concentration was increased. This result contradicts the theory of the salting-out effect that leads to high protein precipitation. Thus, the percentage of ash in chloroplastic sediments was determined. The ash content of chloroplastic sediments significantly increased ($p < 0.05$) with increasing $CaCl_2$ concentration. The results were expressed as mean ± standard deviation in the unit of % (dry basis) as follows: 13.92 ± 0.03^{d}, 20.11 ± 0.03^{c}, 23.52 ± 0.28^{b}, and 25.89 ± 0.69^{a} at 0, 100, 200, and 300 mM $CaCl_2$. It indicates that decreasing protein content in chloroplastic sediments might be due to salt contamination.

Extraction yield

The extraction yield of LPCs extracted through alkali-acid precipitation with different $CaCl_2$ concentrations is shown in Figure 3.9. The yield of LPCs treated with 0, 100, 200, and 300 mM $CaCl_2$ were 9.61 %, 3.2 %, 3.14 %, and 1.96 % (based on dried grass), respectively. A notably decreasing yield was observed when the concentration of $CaCl_2$ was increased. This might involve lower protein solubility due to the salting-out effect (Mu et al., 2008). In addition, a higher concentration of divalent ions (Ca^{2+}) leads to a higher stacking degree of thylakoid membranes, reducing green protein extractability (Camm & Green, 1982). Their study presented that the maximum and minimum protein extractability were detected in the solutions without and with Mg^{2+}, respectively. Thus, increasing divalent ions would reduce protein extractability, leading to a lower yield of LPC.

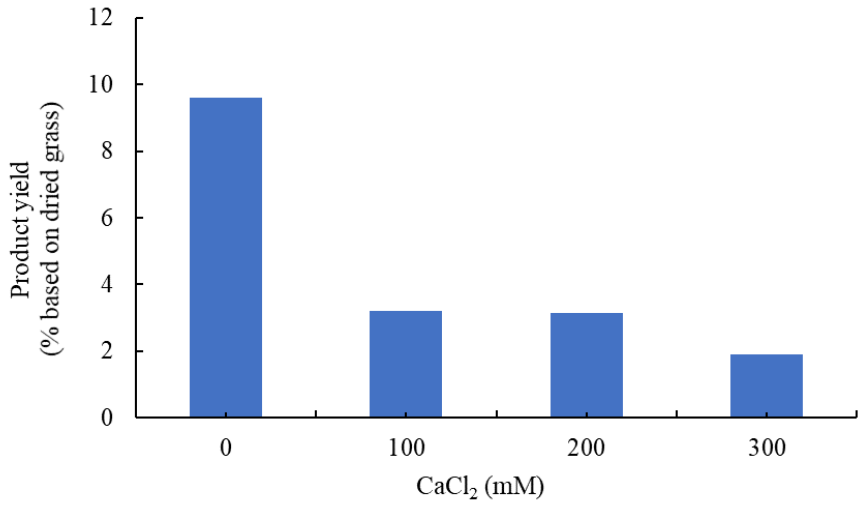


Figure 3.9: Extraction yield of leaf protein concentrate: Through alkali-acid precipitation with different concentrations of $CaCl_2$.

It could be seen that the addition of $CaCl_2$ ranging from 100 to 300 mM could partially remove the green colour and grassy flavour. Protein content and extraction yield were decreased when the concentration of $CaCl_2$ was increased. In conclusion, the green colour and grassy flavour were improved by increasing the concentration of $CaCl_2$. The high amount of $CaCl_2$ reduced the protein content and extraction yield of LPC.

3.5.1.2 Protein extraction combined with activated carbon

Thermal treatment was applied in this experiment to enhance chlorophyll fractionation. As some parts of photosynthesis link with proteins, protein denaturation would cause chlorophyll-protein complexes aggregation. This leads to irreversible chloroplastic sediments (green protein sediments) (Östbring et al., 2014). Removing chloroplastic sediments can improve the appearance and flavour of the isolated LPC.

Activated carbon (AC) is an absorbent used to adsorb the impurities from the solutions due to its porous structures. AC has been used to remove impurities in various processes, such as water purification, beverage production, and protein purification (Stone & Kozlov, 2014). Residue chlorophyll that cannot be eliminated in the fractionation step could be removed by activated carbon (Di Stefano et al., 2018). Therefore, adding AC in the extraction process expects to improve LPC's green colour and grassy flavour by removing chlorophyll and other impurities. The combination of thermal and activated carbon treatment was studied to extract colourless and odourless LPC. The Colour, flavour, protein content and extraction yield of LPC are discussed below.

Green colour and grassy flavour

Table 3.6 shows that the colour of all LPCs was light brown and yellow, indicating that chlorophyll was remarkably removed. This might result from the thermal treatment used for chloroplast fractionation since the T_d of green protein fraction (chloroplastic and membrane proteins) ranges from 50 to 65 ℃ (Nynäs et al., 2021). Thermal treatment in this study (60 ℃ for 5 minutes) might promote green protein coagulation; consequently, chloroplast and its related proteins were flocculated. Similarly, Fiorentini and Galoppini (1983) observed that the green protein fraction was destabilized by heating around 60 ℃ and removed by centrifuging; consequently, a clear brown juice containing soluble proteins remained.

The pI of leaf proteins, such as sugar beet leaves proteins (Martin et al., 2019) and grass protein concentrate (Kaur et al., 2021), is around pH 3.5 to 5. RuBisCo co-precipitation has been

observed at a pH lower than 6 (De Jong et al., 2014). Thus, chlorophyll removal also relates to the pH of solutions. The pH of the extraction buffer used in this experiment was 6, close to the isoelectric point (pI) of leaf proteins (where the lowest protein solubility occurs). Hence, the current pH of the extraction buffer also promoted green protein precipitation.

Since chlorophyll was removed, the grassy flavour was not detected in all LPCs extracted through this process. Lelyveld & Smith (1989) reported that grassy flavour was associated with chlorophyll; hence, flavour improvement was related to chlorophyll removal. Furthermore, Xie (2017) reported that an improvement of off-flavour in grass protein extracts was observed after AC treatment. Therefore, the grassy flavour was removed due to chlorophyll removal and AC treatment.

Table 3.6: Colour and flavour of leaf protein concentrate: Through protein extraction combined with activated carbon.

Activated carbon (%)	**1.9**	**3.8**	**5.7**
Colour			
	Light brown	Light yellow	Light yellow
Grassy flavour	Not detected	Not detected	Not detected

Protein content

Figure 3.10 shows the protein content of LPCs treated with AC at different concentrations. A significant decrease ($p < 0.05$) in the protein content of LPCs was detected: 16.50 %, 14.65 %, and 13.30 % (dry basis) when the amount of AC was increased. Table 3.7 shows no significant difference in the amount of recovered protein between the final grass juice and the recovered solution due to using the same raw materials and conditions. However, proteins were recovered slightly higher than in the previous method (alkali-acid precipitation with and without $CaCl_2$). This might be due to a higher quantity of starting material (300 g grass) than the previous method used (250 g grass).

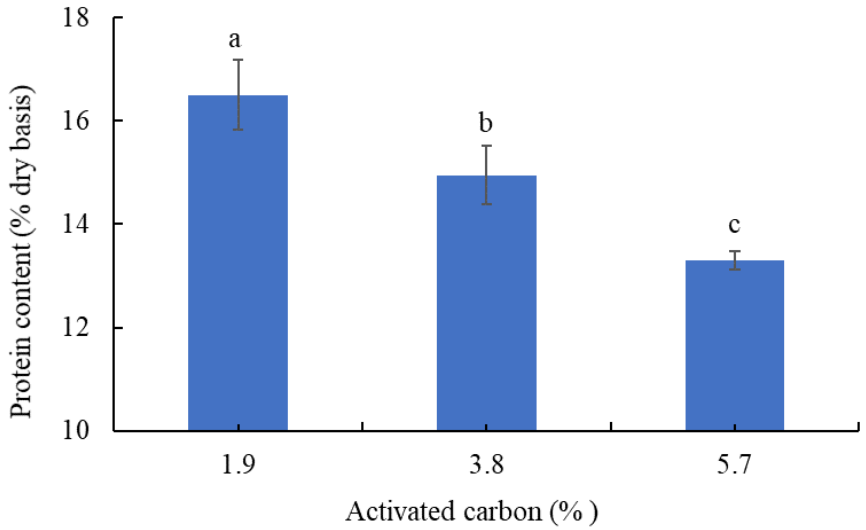


Figure 3.10: Protein content (% dry basis) of leaf protein concentrate: Through protein extraction combined with activated carbon.

Means (n = 3) that do not share a letter are significantly different ($p < 0.05$) (Tukey's HSD multiple comparisons and 95 % confidence). HSD (Honest Significance Test).

Table 3.7: The amount of protein in the recovered fractions obtained from the extraction process combined with activated carbon.

Sample	**AC (%)**	**Recovered proteins (g)**
Final grass juice	-	6.58
Recovered solution	-	3.81
LPC	1.9	1.07^{a}
	3.8	0.91^{b}
	5.7	0.40^{c}

Note: The starting grass was 300 g (wet weight), and the initial protein content was 9.41 g (dry weight). Different letters in the same column of each sample represent a significant difference ($p < 0.05$). Results are expressed as means (n = 3) with Tukey's HSD multiple comparisons and 95 % confidence. HSD (Honest Significance Test).

Furthermore, the protein content of all LPCs from this experiment was lower than the starting raw material (18.50 % proteins (dry basis)). Proteins were detected in the discarded fractions, such as grass pulp (18.91 to 19.78 % dry basis) and chloroplastic sediments (37.33 to 38.27 %

dry basis). These results indicated that non-soluble proteins were lost through the waste stream during extraction (Barbeau & Kinsella, 1988; Lui et al., 2004). Also, an inappropriate pH, long processing time, and unexpected binding of AC could lead to more protein loss. The pH of the extraction buffer was 6, as mentioned in the method section 3.2.2.2. This condition might induce more protein precipitation, including, RuBisCo co-precipitation. Since the pH is near RuBisCo's pI (pH 4.5) (De Jong et al., 2014). Van De Velde et al. (2011b) stated that a suitable pH for leaf protein extraction should be 6.5 to 8.5.

Proteins were detected in dialysis filtrate with protein content ranging from 11.70 to 12.08 % (dry basis). This result indicated that some proteins were lost during the dialysis step. This might be due to the long processing time (overnight), leading to protein hydrolysis induced by proteases in plant leaves (De Jong et al., 2014). A similar result studied by Koschuh et al. (2004) was reported; a reduction of RuBisCo content was investigated from 100 % to 40 % after storage at 4 °C for 24 h. Hence, proteins could be hydrolyzed during dialysis to small proteins than 14 kDa (MWCO used in the study). Consequently, the hydrolyzed proteins could pass through the dialysis membrane.

Some proteins were detected in AC sediments ranging from 2.31 % to 5.27 % (dry basis). It indicated that AC might adsorb proteins, resulting in protein loss through AC sediments. This could be supported by a study by Xie (2017). Grass proteins treated with AC had lower proteins than the untreated sample. Although RuBisCo is a large protein with a MW of 550 kDa, it is degraded to large and small subunits during long extraction time by the endogenous proteases present in plant materials (Koschuh et al., 2004). Protein degradation might cause an unexpected binding between AC and small proteins (Stone & Kozlov, 2014). Hence the high amount of AC could lead to more proteins being bound; consequently, lower proteins remained in the final LPC.

Extraction yield

Figure 3.11 presents the LPCs' yield obtained from protein extraction combined with activated carbon treatment. The extraction yield was reduced from 10.77 % to 5.07 % when AC concentration was increased from 1.9 % to 5.7 % (w/w). This indicated that product yield was associated with the AC quantity. Since AC can adsorb small impurities (i.e., tannin and phenolic compounds) (Xie, 2017) and small proteins (Stone & Kozlov, 2014), using a high quantity potentially leads to a higher binding capacity between AC and the hydrolyzed proteins, as mentioned above. Consequently, lower proteins (Stone & Kozlov, 2014) and other soluble

compounds (i.e., minerals and vitamins) (Bardar et al., 2011) would be recovered in the final products, reducing extraction yield.

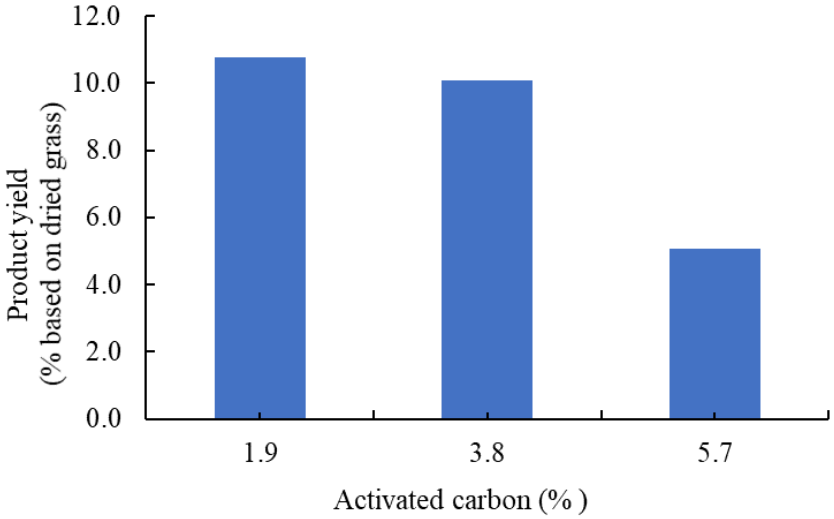


Figure 3.11: Extraction yield of leaf protein concentrate: Through protein extraction combined with activated carbon.

In conclusion, combining thermal and activated carbon treatment could enhance the efficiency of chlorophyll removal. Thus, the green colour and grassy flavour were not observed in the isolated LPCs. However, the protein content and extraction yield of the LPCs were reduced due to the inappropriate buffer pH and long operation time. In addition, protein content and extraction yield of LPCs were decreased when the amount of AC was increased. This was due to the unexpected binding of AC with small proteins hydrolysed along the process.

3.5.1.3 Alkali-acid precipitation combined with activated carbon

Alkali-acid precipitation method without $CaCl_2$ treatment, as mentioned in section 3.5.1.1, could isolate the leaf protein concentrate (LPC) with high protein content (53.94 % protein dry basis). However, the green colour and grassy flavour remained. According to the previous method (section 3.5.1.2), activated carbon (AC) could improve the green colour and grassy flavour. Therefore, combining alkali-acid precipitation and AC treatment was studied to extract high protein content, odourless and colourless LPC. Also, the non-alkali condition was conducted to compare the results with the alkali-assisted extraction process.

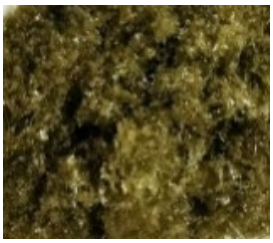
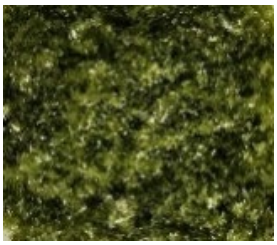
Green colour and grassy flavour

The green colour was observed in LPCs extracted through both non-alkali and alkali-assisted extraction processes, as presented in Table 3.8. The remaining green colour in both LPCs might be due to the thermal process not being used in this protocol, which might not induce chloroplast coagulation (De Jong et al., 2014). Consequently, chlorophyll and cell membrane debris were co-precipitated by acid precipitation and presented in LPC.

The condition using 0.1 M NaOH mixed with water as an extraction solvent resulted in LPC having a darker green colour. This was because of the chlorophyll saponification reaction. Chlorophyll is saponified in the presence of NaOH; consequently, chlorophyllin and phyton are produced. The solubility property of chlorophyll is changed from water-insoluble to water-soluble (Li et al., 2016), increasing chlorophyll solubility.

A decrease in grassy flavour was observed in both LPCs compared to the LPC extracted through alkali-acid precipitation without AC treatment (section 3.5.1.1). The less grassy flavour was due to the porous structures of AC, adsorbing off-flavour and impurities from the protein solution. Similarly, Xie (2017) reported that the flavour of grass protein extracts was improved after activated carbon treatment.

Table 3.8: Colour and flavour of leaf protein concentrate: Through alkali-acid precipitation with activated carbon varied water and 0.1 M NaOH ratio.

Water:0.1M NaOH	**2.4:0**	**1.7:0.7**
Colour		
	Brownish green	Dark green
Grassy flavour	Light	Light

Protein content

Figure 3.12 shows the protein content of LPC extracted through alkali-acid precipitation combined with activated carbon treatment. Leaf protein concentrate obtained from the non-alkali process (water:0.1 M NaOH at 2.4:0) had 47.51 % protein (dry basis). Whereas LPC extracted through the process using water and 0.1 M NaOH at the ratio of 1.7:0.7 had protein

content significantly higher ($p < 0.05$): 50.03 % (dry basis). Alkali aids protein extraction, enhancing cell disruption at the epidermis and lamella layer of leaf tissue (Zhang et al., 2015). Consequently, more mesophyll proteins are extracted.

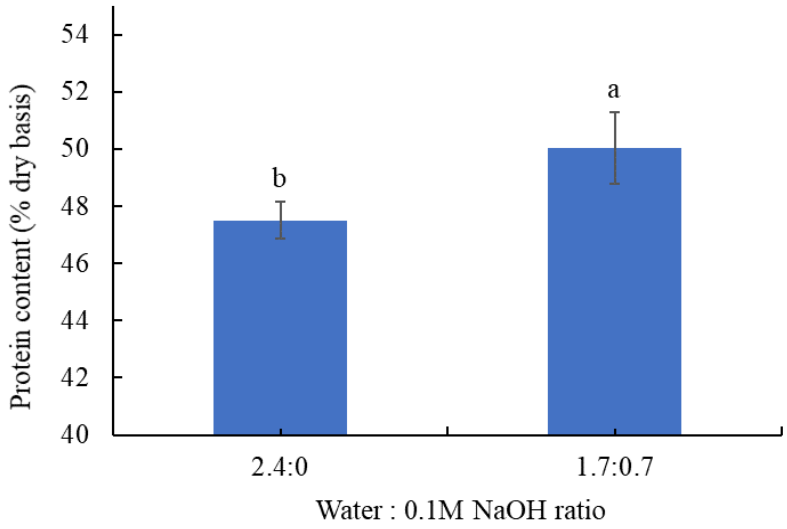


Figure 3.12: Protein content (% dry basis) of leaf protein concentrate: Through alkali-acid precipitation with activated carbon and varied water and 0.1 M NaOH ratios.

Means ($n = 3$) that do not share a letter are significantly different ($p < 0.05$) (Two-sample t-test comparison and 95 % confidence).

Table 3.9: The amount of protein in the recovered fractions obtained from the extraction process: Alkali-acid precipitation combined with activated carbon at different ratios of water and 0.1 M NaOH.

Sample	**Ratio of water and 0.1 M NaOH**	**Recovered proteins (g)**
Final Grass juice	2.4:0	5.97^{b}
	1.7:0.7	8.00^{a}
Recovered solution 1	2.4:0	2.99^{b}
	1.7:0.7	4.55^{a}
Recovered solution 2	2.4:0	1.54^{b}
	1.7:0.7	2.93^{a}
LPC	2.4:0	0.76^{b}
	1.7:0.7	1.95^{a}

Note: The starting grass was 250 g (wet weight), and the initial protein content was 7.84 g (dry weight). Different letters in the same column of each sample represent a significant difference ($p < 0.05$). Results are expressed as means ($n = 3$) with Two-sample t-test and 95 % confidence.

This reason is in accordance with the result of protein content in the final grass juice, the recovered solution 1, and the recovered solution 2, as shown in Table 3.9. These solutions extracted through the alkali-assisted process had significantly higher proteins (p <0.05) than those using only water. In addition, the increased protein content was due to high protein solubility at higher pH, leading to more proteins being extracted (De Jong et al., 2914). Similarly, Kaur et al. (2021) reported that an increase in grass protein concentrate solubility was detected when the pH of the protein solution was increased from 5 to 9. Furthermore, the protein content in waste fractions (grass pulp, chloroplastic sediments, and AC sediments) was significantly decreased when 0.1 M NaOH was used. Hence, alkali-assisted extraction could increase protein solubility and extractability, reducing protein loss along with the discarded fractions. Consequently, more proteins were recovered in the final LPC.

It could be seen that the protein content of LPC extracted through alkali-assisted extraction in this experiment was higher than the LPC obtained from the previous method (section 3.5.1.2). This result could be explained through the recovered proteins in the final grass juice, as shown in Tables 3.7 and 3.9. The amount of protein in grass juice obtained from the extraction process using water and 0.1 M NaOH at the ratio of 1.7:0.7 was 8.00 g, while the extraction process, as stated in section 3.5.1.2, could recover protein in grass juice at 6.58 g. This result supports that using alkali pH enhances the amount of extracted proteins, leading to high proteins being recovered into the final product.

Activated carbon (AC) was used in this protocol to improve LPC's colour and flavour. The result shows that the green colour remained in the LPCs; however, the grassy flavour was reduced, as shown in Table 3.8. Lower protein content was observed in LPC treated with AC. This result is similar to the result mentioned in section 3.5.1.2 that the protein content of LPC was decreased when AC concentration was increased. It could be said that the grassy flavour could be improved by AC treatment; however, it could not remove the green colour when the alkali solution was used. Also, the addition of AC reduced protein content.

Extraction yield

An increasing yield of LPC was obtained when using 0.1 M NaOH as a co-extraction solvent (Figure 3.13). The extraction yield of LPC using the alkali-assisted extraction was approximately 2.5 times higher than the non-alkali process. This result indicated a higher recovery yield related to the alkali solution used in the extraction process since protein solubility increases when the pH of the solution is alkali (Kobbi, 2017). Increased protein

solubility is due to high electrostatic repulsion among protein molecules, less protein-protein interactions, and more protein-water interactions under alkali pH conditions (Ferreira Machado et al., 2017). Hence, proteins in plant materials are highly solubilized and extracted into extracted leaf juice.

Moreover, the increasing yield might result from non-proteinaceous components (Kaur et al., 2021), such as chlorophyll. Since the darker green colour was observed in the LPC extracted through the alkali-assisted extraction process shown in Table 3.8. Alkali enhances chlorophyll saponification, producing chlorophyllin and phyton (Ferruzzi & Blakeslee, 2007). These compounds change the solubility property of chlorophyll from water-insoluble to water-soluble (Li et al., 2016). Consequently, more green pigments were solubilized and recovered in LPC. Thus, the alkali-assisted extraction process would yield more LPC than non-alkali extraction. However, using AC with alkali-acid precipitation as the current experiment resulted in a lower extraction yield than only the alkali-acid precipitation method (section 3.5.1.1 without $CaCl_2$). This was because AC can adsorb small impurities (i.e., tannin and phenolic compounds) and small proteins (Xie, 2017; Stone & Kozlov, 2014).

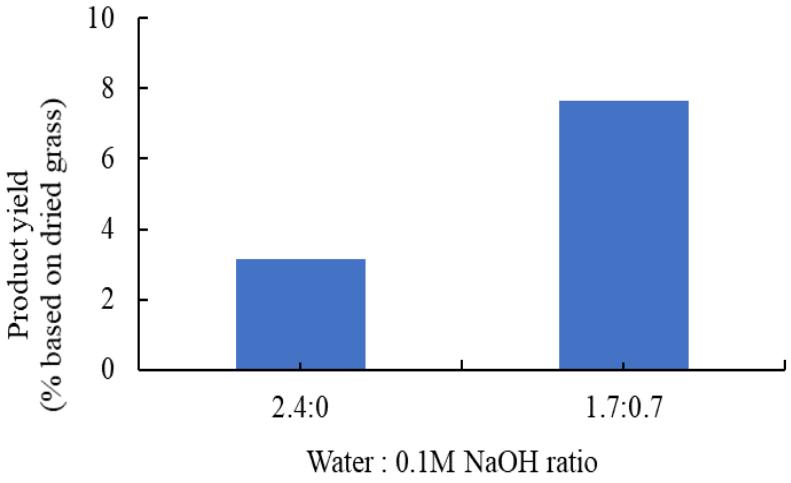


Figure 3.13: Extraction yield of leaf protein concentrate: Through alkali-acid precipitation with activated carbon at different water and 0.1 M NaOH ratio.

In conclusion, combining an alkali solution with the extraction solvent aids protein extractability, increasing protein content and product yield. However, a darker green colour was obtained since the saponification reaction changed the solubility of chlorophyll. The grassy flavour was improved noticeably due to AC treatment. However, using only AC treatment in the presence of an alkali solution could not improve the green colour of LPC.

3.5.1.4 Heat coagulation (effect of $CaCl_2$ treatment along with heating)

Thermal treatment has been reported to enhance chlorophyll fractionation. The heating process denatures the proteins bound with photosynthesis; consequently, proteins are aggregated. This leads to chloroplast cell aggregation, causing irreversible chloroplastic protein sediments (Östbring et al., 2014). Section 3.5.1.2 indicates that thermal treatment was a factor promoting chlorophyll aggregation. The addition of $CaCl_2$ in the extraction process, as mentioned in section 3.5.1.1, has proven that it could partially remove chloroplastic proteins, reducing the isolated LPC's green colour and grassy flavour. In addition, it could enhance the efficiency of thermal coagulation (De Jong et al., 2014). Thus, combining two parameters (thermal and $CaCl_2$ treatment) has been studied to remove the green colour and grassy flavour of LPC. The effect on protein content, extraction yield, and improvement of colour and flavour were determined.

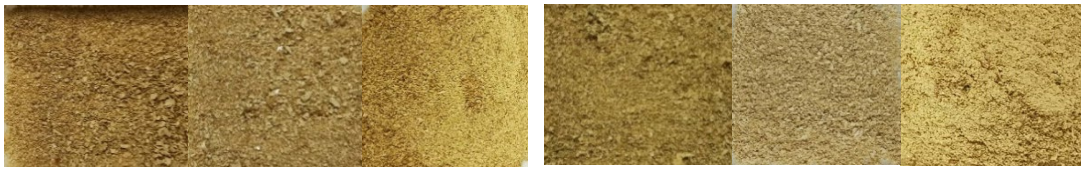
Green colour and grassy flavour

Table 3.10 shows that the green colour of all LPCs was removed entirely. This was due to the extraction process's thermal and $CaCl_2$ treatment effect. The heating conditions used in this experiment (50 ℃, 15 minutes and 58 ℃, 2 minutes) could induce green protein coagulation (chloroplastic protein aggregation). As a result, the brown and clear recovered solutions containing white protein fraction (Fraction-1 protein) were obtained (De Jong et al., 2014; Udenigwe et al., 2017; Nynäs et al., 2021).

The brighter colour of LPC was obtained as an increasing concentration of $CaCl_2$ (Table 3.10.). Similarly, the green colour of LPC extracted through alkali-acid precipitation decreased when using high $CaCl_2$ concentration, as reported in section 3.5.1.1. This is related to anion binding sites of the thylakoid membrane, and chloroplast can be neutralised by cations, such as Mg^{2+}, inducing chloroplast cell agglomeration and precipitation (Camm & Green (1982). Also, divalent ions enhance the effectiveness of heat-induced chloroplast aggregation since cations decrease protein denaturation temperature (T_d) (Farkas and Mohácsi-Farkas, 1996). As a result, more chlorophyll-protein complex precipitation would be obtained.

The less grassy flavour was observed in all LPCs, as presented in Table 3.10, since abundant chlorophyll was removed (Lelyveld & Smith, 1989) with increasing $CaCl_2$. This result was in accordance with the result obtained through alkali-acid precipitation (section 3.5.1.1). Moreover, lipoxygenase activity was inhibited due to the thermal effect (Chang et al., 2019), resulting in a reduced grassy flavour.

Table 3.10: Colour and flavour of leaf protein concentrate: Through heat coagulation with different $CaCl_2$ concentrations and heating conditions.

Heating condition	**50 ℃, 15 minutes**			**58 ℃, 2 minutes**		
$CaCl_2$ (mM)	**0**	**100**	**150**	**0**	**100**	**150**
Colour						
	Light brownish yellow	Light brown	Yellow	Light greenish yellow	Cream	Light yellow
Grassy flavour	Light	Light	Not detected	Light	Not detected	Not detected

Protein content

Figure 3.14 shows the protein content of LPC extracted through heat coagulation with different $CaCl_2$ concentrations and heating conditions. At the same $CaCl_2$ concentration, the protein content of LPC treated at 58°C for 2 minutes was significantly lower (p <0.05) than LPC treated at 50 °C for 15 minutes. A similar finding was reported by Nynäs et al. (2021); protein bands of green proteins analysed by SDS-PAGE started to disappear around 50 to 55 °C and fully disappeared around 60 to 65 °C. This finding indicates that more protein precipitation occurred at higher-temperature conditions. Protein precipitation is observed during thermal treatment due to protein denaturation. High temperature induces protein conformation changes, such as weakening electrostatic forces and hydrogen bonds. Consequently, the protein's secondary, tertiary or quaternary structure unfolds, and proteins subsequently aggregate (Farkas & Mohácsi-Farkas, 1996). Hence, heating at 58 °C, close to 60 °C, causes more protein denaturation. As a result, the denatured proteins are precipitated and lost through the waste stream (chloroplastic sediments).

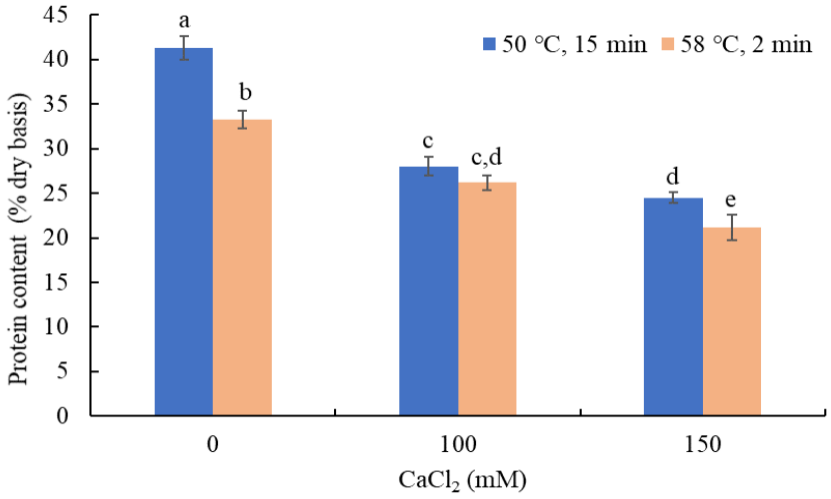


Figure 3.14: Protein content (% dry basis) of leaf protein concentrate: Through heat coagulation with different $CaCl_2$ concentrations and heating conditions.

Means ($n = 3$) that do not share a letter are significantly different ($p < 0.05$) (Tukey's HSD multiple comparisons and 95 % confidence). HSD (Honest Significance Test).

Table 3.11: The amount of protein in the recovered fractions obtained from the extraction process through heat coagulation with different $CaCl_2$ concentrations and heating conditions.

Sample	Heating condition	$CaCl_2$ (mM)	Recovered proteins (g)
Final grass juice	-	-	6.17
Recovered solution	50 °C, 15 minutes	0	3.50 [a]
		100	3.43 [a]
		150	3.15 [a]
	58 °C, 2 minutes	0	3.39 [a]
		100	3.18 [a]
		150	2.71 [b]
LPC	50 °C, 15 minutes	0	0.30 [a]
		100	0.16 [b]
		150	0.08 [d]
	58 °C, 2 minutes	0	0.10 [c]
		100	0.10 [c]
		150	0.06 [e]

Note: The starting grass was 500 g (wet weight), and the initial protein content was 15.68 g (dry weight). Different letters in the same column of each sample represent a significant difference ($p < 0.05$). Results are expressed as means ($n = 3$) with Tukey's HSD multiple comparisons and 95 % confidence.

The above discussion could be supported by the results observed in the recovered solution. Table 3.11 shows that all recovered solutions heated at 58 °C for 2 minutes had proteins lower than the recovered solutions heated at 50 °C for 15 minutes. This result confirms that high temperature leads to more protein denaturation; consequently, lower proteins are recovered in the recovered solution and LPC.

At the same heating condition, Figure 3.14 shows that the protein content of LPC was significantly decreased with the increasing concentration of $CaCl_2$ (p <0.05). Leaf protein concentrates (LPCs) heated at 50 °C for 15 minutes had 41.26 %, 28.02 %, and 24.49 % protein (dry basis) at $CaCl_2$ concentrations of 0, 100, and 150 mM, respectively. Similarly, LPCs heated at 58 °C for 2 minutes had 33.22 %, 26.21 %, and 21.16 % protein (dry basis) at $CaCl_2$ concentrations of 0, 100, and 150 mM, respectively. These results were similar to the LPCs extracted through alkali-acid precipitation with $CaCl_2$ (0 to 300 mM), as presented in Figure 3.8. A decrease in protein content at high salt concentrations was due to the salting-out effect (Mu et al., 2008), leading to less soluble proteins in the recovered solution, as shown in Table 3.11. As a result, lower proteins were recovered in the LPC.

Furthermore, high salt concentration can reduce protein denaturation temperature (T_d), causing more precipitated proteins (Farkas and Mohácsi-Farkas, 1996). Their study found that increasing ionic strength by adding 2 % curing salt to muscle proteins reduced T_d value. A similar effect was also observed in other meats treated with phosphate and chloride salts. The present study shows a similar observation (Table 3.11). At the same heating condition, a reduction of proteins was detected in the recovered solution as $CaCl_2$ concentration was increased. Therefore, high $CaCl_2$ concentration decreases protein solubility and T_d, leading to less protein being recovered in the recovered solution and LPC.

According to the salting-out effect, proteins are highly precipitated at a high salt concentration (Mu et al., 2008) and lost through chloroplastic sediments. However, a decrease in protein content was detected in chloroplastic sediments (waste fraction). Therefore, the percentage of ash was analysed to determine the amount of salt contamination. The results are expressed as mean ± standard deviation in the unit of % dry basis. Higher ash content was found in the chloroplastic sediments when increasing $CaCl_2$ concentration. At heating condition 50 °C for 15 minutes, ash content (% dry basis) in the chloroplastic sediments was 13.74 ± 0.38^{e}, 25.95 ± 0.54^{c}, and 32.55 ± 0.10^{a} at the $CaCl_2$ concentration of 0, 100, and 150 mM, respectively. The same trend was observed in chloroplastic sediments obtained from the process using 58 °C for

2 minutes. Ash content (% dry basis) was 13.92 ± 0.03^{e}, 23.49 ± 0.21^{d}, and 29.80 ± 0.05^{b} at the $CaCl_2$ concentration of 0, 100, and 150 mM, respectively. Hence, lower protein content in the chloroplastic sediments might be due to salt contamination as a high amount of $CaCl_2$ was added.

Extraction yield

Figure 3.15 shows the extraction yield of LPC extracted through heat coagulation with different $CaCl_2$ concentrations and heating conditions. At the same $CaCl_2$ concentration, the low-temperature method yielded LPC higher than the high-temperature method. This might relate to the thermal coagulation behaviour of green proteins. As discussed above, Nynäs et al. (2021) reported that SDS-PAGE analysis did not detect green protein bands when the protein extract was heated higher than 60 °C. In contrast, a higher intensity of protein bands was detected when the protein extract was heated around 50 to 55 °C. This reason dedicates that high temperature induces more green protein denaturation, resulting in green proteins (membrane proteins and chlorophyll-bound proteins) being eliminated through chloroplastic sediments. Consequently, the high-temperature method would give a lower yield of LPC.

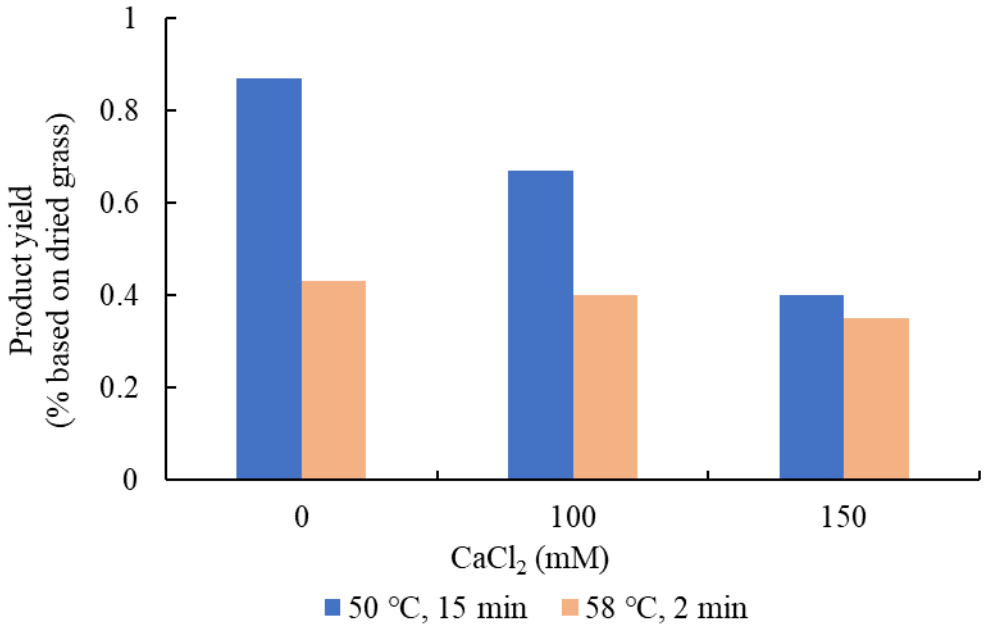


Figure 3.15: Extraction yield of leaf protein concentrate: Through heat coagulation with different $CaCl_2$ concentrations and heating conditions.

Figure 3.15 also shows a decreased yield of LPC when $CaCl_2$ concentration was increased. The extraction yield of LPCs treated at 50 °C for 15 minutes was reduced from 0.87 % to 0.4 % (based on dried grass) as a higher $CaCl_2$ concentration was used. The yield of LPCs treated at

58 °C for 2 minutes ranged between 0.35 % to 0.40 % (based on dried grass). This result might be due to a decrease in protein extractability at high salt concentrations. Thylakoid membrane agglomeration is promoted at high salt concentrations, decreasing chlorophyll-related protein extractability (Camm and Green, 1982). Consequently, the recovery yield would be reduced. A similar result was obtained through the alkali-acid precipitation method, as shown in Figure 3.9, confirming that yield of LPC decreased with increased $CaCl_2$ concentration.

In conclusion, heat coagulation with thermal and $CaCl_2$ treatment could remove LPC's green colour and grassy flavour. This was due to the thermal treatment and $CaCl_2$ enhancing the chlorophyll aggregation. The protein content and extraction yield of LPC depended on the amount of $CaCl_2$ and the temperature. The highest protein content (48.16 %) was detected in LPC treated at low temperatures (50 °C, 15 minutes) without $CaCl_2$ treatment. High temperature leads to more protein denaturation (Nynäs et al., 2021). Divalent ions reduce protein solubility, decrease the T_d of proteins, and increase thylakoid stacking. Therefore, high-temperature treatment (58 °C, 2 minutes) and high $CaCl_2$ concentration decreased protein content and extraction yield.

3.5.1.5 Heat coagulation (effect of alkali-solution)

A previous study has shown that heating at 50 °C for 15 minutes without $CaCl_2$ was the condition that could remove green colour, reduce grassy flavour, and recover the highest proteins. Therefore, heating at 50 °C for 15 minutes was used in this experiment. Heat coagulation at 80 °C for 30 seconds, the same condition used in the previous experiment, was used to concentrate white protein fraction (Fraction I). NaOH was applied in the current experiment to increase the protein content since alkali pH aids leaf tissue disruption (epidermis and lamella layer), resulting in more mesophyll proteins being extracted (Zhang et al., 2015). High pH also increases protein solubility; therefore, a higher extraction yield would be obtained (Di Stefano et al., 2018). The concentration of NaOH varied from 0 to 0.1 M.

Green colour and grassy flavour

Table 3.12 shows that using 0 and 0.03 M NaOH extraction methods could remove chlorophyll from LPC, whereas a green colour was observed in LPC using 0.1 M NaOH. Although green proteins can be removed by thermal treatment at 50 to 60 °C (Nynäs et al., 2021) or 40 to 60 °C (De Jong et al., 2014; Udenigwe et al., 2017), the green colour was observed in LPC using 0.1 M NaOH as an extraction solution. This might be because alkali promotes chlorophyll

saponification reaction, producing water-soluble chlorophyllin and phyton (Li et al., 2016). The mentioned compounds are potentially extracted, providing a green colour LPC.

The grassy flavour was rarely detected in the LPCs extracted using 0 and 0.03 M NaOH, while it was strong in LPC obtained from the process using 0.1 M NaOH. As grassy flavour is associated with the chlorophyll in plant leaves (Lelyveld & Smith, 1989); thus, grassy favour is detected in the LPC containing chlorophyll. In contrast, a reduction of grassy flavour would be observed when chlorophyll is removed. Furthermore, the high temperature (80 °C) used for protein concentration in this experiment also inhibits lipoxygenase (LOX) activity which catalyses lipid oxidation and subsequently produces volatile compounds that are responsible for grassy flavour (Chang et al., 2019).

Table 3.12: Colour and flavour of leaf protein concentrate: Through heat coagulation at different concentrations of NaOH.

NaOH (M)	**0**	**0.03**	**0.1**
Colour			
	Brownish yellow	Brownish yellow	Dark green
Grassy flavour	Light	Light	Strong

Protein content

Figure 3.16 shows the protein content of LPC extracted through heat coagulation with different concentrations of NaOH. Although the amount of recovered protein in the final grass juice and the recovered solution was increased when a higher concentration of NaOH was used, a decrease in the protein content of LPC was observed (Table 3.13). Figure 3.16 shows the protein content of LPC at 0, 0.03, and 0.1 M NaOH: 48.16 %, 42.94 %, and 38.81 % protein (dry basis), respectively. An alkali solution theoretically assists protein extraction since alkali pH aids plant tissue disruption and increases protein solubility. Consequently, more proteins are potentially excreted (Zhang et al., 2015). However, the results from this experiment are inconsistent with the theory mentioned above.

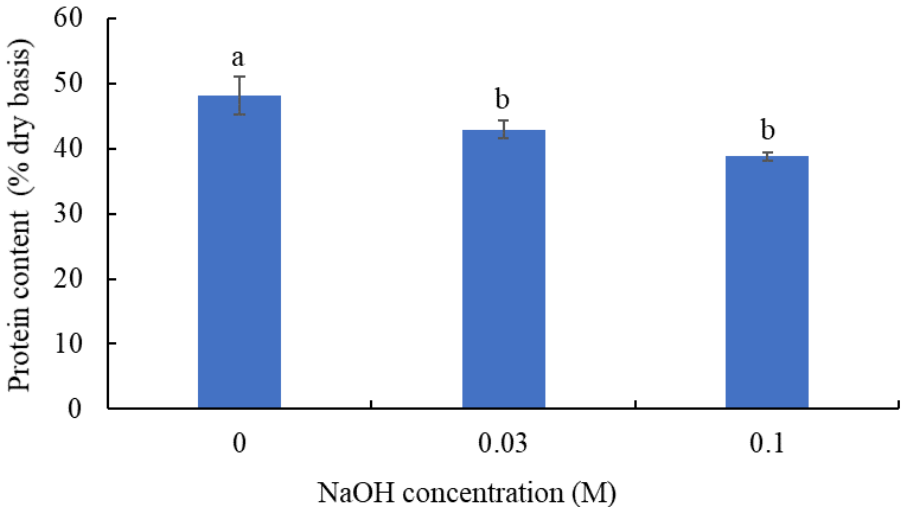


Figure 3.16: Protein content (% dry basis) of leaf protein concentrate: Through heat coagulation with different concentrations of NaOH.

Means ($n = 3$) that do not share a letter are significantly different ($p < 0.05$) (Tukey's HSD multiple comparisons and 95 % confidence). HSD (Honest Significance Test).

Table 3.13: The amount of protein in the recovered fractions obtained from the extraction process through heat coagulation with different concentrations of NaOH.

Sample	NaOH (M)	Recovered proteins (g)
Final grass juice	0.0	6.18^{b}
	0.03	6.24^{b}
	0.1	6.72^{a}
Recovered solution	0.0	2.90^{b}
	0.03	2.70^{b}
	0.1	3.74^{a}
LPC	0.0	0.46^{b}
	0.03	$0.50^{a,b}$
	0.1	0.52^{a}

Note: The starting grass was 500 g (wet weight), and the initial protein content was 15.68 g (dry weight). Different letters in the same column of each sample represent a significant difference ($p < 0.05$). Results are expressed as means ($n = 3$) with Tukey's HSD multiple comparisons and 95 % confidence. HSD (Honest Significance Test).

Lower protein content in LPCs using 0.03 and 0.1 M NaOH might involve the thermal coagulation behaviour of leaf proteins. Heat coagulation at 80 °C mainly recovers Fraction-I proteins (soluble protein fraction), resulting in green proteins not being recovered (Edward et al., 1975). This could be supported by the protein content in the discarded solution (after heat coagulation). The protein content in this solution was significantly increased (p <0.05) from 11.30 % to 16.02 % (dry basis) when NaOH concentration increased. It indicates that alkali pH enhances protein extraction; however, heat coagulation might not recover green proteins at this temperature. Consequently, protein recovery might be lower in the LPC extracted through heat coagulation with alkali conditions.

Moreover, the current results contradicted the findings obtained through alkali-acid precipitation with AC treatment (section 3.5.1.3). The current experiment shows a decrease in the protein content of LPC as increasing NaOH concentration. In contrast, the latter process shows a higher protein content in LPC that used NaOH as an extraction solution. This might relate to the thermal process used for chlorophyll fractionation and heat coagulation. The current experiment used a heating condition at 50 °C for 15 minutes to enhance the chlorophyll fractionation, removing the green protein fraction. In comparison, the heating process was not used in the latter process, resulting in more green proteins being remained and recovered in the final products. Also, heat coagulation recovers only Fraction-I protein (soluble proteins), whereas acid precipitation could recover soluble and insoluble proteins at their pI (Pelegrine & Gasparetto, 2005). Thus, the different concentration methods would lead to different recovered protein amounts.

The LPC extracted through the current experiment without NaOH had a protein content higher than LPC extracted through a similar method (heat coagulation without $CaCl_2$ and NaOH, as mentioned in section 3.5.1.4). This might be due to a higher ratio of water used in the current experiment. The current method's ratio of grass and water was 1:0.5, while the experiment in section 3.5.1.4 used 1:02. A higher extraction solution might result in more soluble proteins being extracted (De Jong et al., 2014).

Extraction yield

The extraction yield of LPC obtained from this experiment was increased from 1.12 % to 1.58 % (based on dried grass) as NaOH concentration was increased from 0 to 0.1 M (Figure 3.17). This was because alkali enhances protein extractability, resulting in high product yield. An increasing yield might result from recovering non-proteinous components, such as chlorophyll

(Kaur et al., 2021). The results presented in Table 3.12 also supported that the green colour was detected when a high concentration of alkali (0.1 M NaOH) was used due to chlorophyll saponification reaction (Li et al., 2016). In addition, a decrease in the protein content of LPC was observed (Figure 3.16). These results confirm that an increasing yield was due to non-proteinous compounds. Furthermore, the extraction yield of LPC extracted through a similar method (heat coagulation without $CaCl_2$ and NaOH), as mentioned in section 3.5.1.4, had slightly lower than LPC extracted through this experiment using 0 M NaOH. This was due to a lower amount of water used in the process mentioned in section 3.5.1.4. Therefore, that extraction yield also relates to the amount of extraction solution.

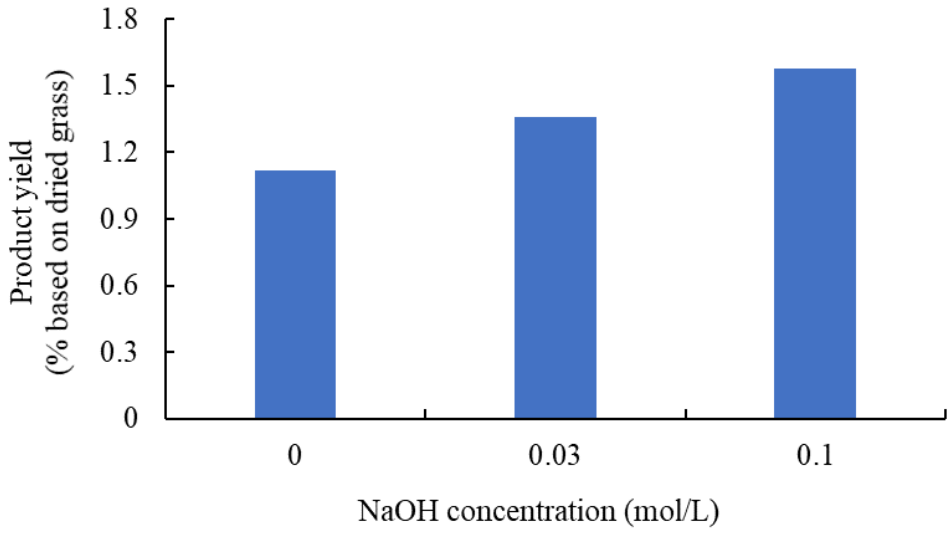


Figure 3.17: Extraction yield of leaf protein concentrate: Through heat coagulation with different concentrations of NaOH.

In conclusion, high pH enhances protein extraction, as the results presented in the final grass juice and the recovered solution. However, the protein content and yield of LPC extracted through heat coagulation decreased when high alkali was used. This might relate to the thermal process used for chlorophyll fractionation, which removes insoluble green proteins. Also, heat coagulation recovers only the Fraction-I protein (soluble proteins), resulting in other insoluble proteins not being recovered. Furthermore, green colour and grassy flavour were detected in LPC using 0.1 M NaOH due to chlorophyll saponification. In summary, using an alkali solution with heat coagulation could not increase the protein content and extraction yield of LPC and would increase the green colour and grassy flavour in LPC.

3.5.1.6 Ultrafiltration

Heat coagulation and acid precipitation are harsh and extreme methods that affect the isolated proteins' properties. In comparison, ultrafiltration (UF) is a mild condition used for protein purification and concentration; consequently, native proteins' properties would be retained (De Jong et al., 2014). Martin et al. (2019) have proven that RuBisCo protein isolate extracted through UF had high solubility (80%) at pH above 5.5. Furthermore, UF has been reported to remove the residual chlorophyll from protein solution (De Jong et al., 2014); hence, the colour and off-flavour of LPC are expected to be improved through UF.

Green colour and grassy flavour

As Figure 3.18, the green colour of leaf protein concentrate extracted through UF was removed; however, the brown colour was observed. A grassy flavour was not detected in LPC. The green colour was removed due to the thermal process heating at 50 ℃ for 15 minutes, enhancing the thermal coagulation of green proteins (Nynäs et al., 2021) and being eliminated through chloroplastic sediments. Also, UF can remove the remaining chlorophyll (DeJong et al., 2014), enhancing the removal of green pigments. In addition, ultrafiltration can remove polyphenols and polyphenol oxidase (PPO) (Walter et al., 2011), which induce an enzymatic browning reaction in plant extracts (Jang & Moon, 2011). However, a long process promotes this reaction, producing o-quinones and subsequently polymerising. An undesirable brown colour was observed (Espín et al., 1998).

The grassy flavour was not detected in the LPC obtained from this method. As grassy flavour is associated with the chlorophyll in plant leaves (Lelyveld & Smith, 1989); thus, grassy favour is detected in the presence of chlorophyll. In contrast, a reduction of grassy flavour is observed when chlorophyll is removed.

Figure 3.18: Colour and flavour of leaf protein concentrate: Through the use of ultrafiltration.

Protein content

The protein content of LPC extracted through ultrafiltration was 22.73 % (dry basis), which was lower than the heat coagulation method (as discussed in section 3.1.5.5). Similarly, the current LPC had a lower protein content than sugar beet leaves protein isolate (93 g kg^{-1} of crude protein) extracted through a similar method (Martin et al., 2019). However, protein recovery in the final grass juice and the recovered solution was similar to the results obtained through heat coagulation. This might be because the same materials and methods were used. Thus, low protein recovery from the present study might be due to the different plant sources (Nynäs et al., 2021), ultrafiltration mode (Walter et al., 2011), and protein degradation (De Jong et al., 2014; Koschuh et al., 2004).

Nynäs et al. (2021) found that using the same extraction method with nine crops has provided different qualities and quantities of resulting proteins. Only seven crops were successfully fractionated to obtain white proteins. Thus, they have confirmed that the origin of green leafy biomass significantly impacts the resulting proteins. However, comparing the results between different studies is rarely comparable in practice as the conditions of fractionation and calculation are generally different (Nynäs et al., 2021). The ultrafiltration mode used in the present study was static mode lacking swiping flow over the membrane, which might have resulted in membrane fouling, and other impurities could not pass through the filter (Walter et al., 2014). Hence, low protein recovery might be due to membrane blockage.

Also, low protein content in the final LPC might be due to protein degradation, leading to protein loss during the ultrafiltration process. This could be supported by Koschuh et al. (2004), RuBisCo was determined only 2 % out of 100 % after 24 h of storage time. They confirmed that a lower amount of RuBisCo was due to protein degradation. The ultrafiltration process in this study was operated for 6 h at 20 °C; hence, RuBisCo and other proteins might be degraded owing to the long processing time and high temperature. Therefore, the hydrolysed proteins smaller than the filter size (100 kDa) could pass through the filter. This can be confirmed by proteins detected in filtrates 1 and 2 with 10.06 % and 7.07 % (dry basis), indicating protein loss through waste fraction. Thus, fewer proteins were recovered in retentate, resulting in low protein content in LPC.

Table 3.14: The amount of protein in the recovered fractions obtained from the extraction process: Through the use of ultrafiltration.

Sample	Recovered proteins (g)
Final grass juice	6.71
Recovered solution	3.11
LPC	0.38

Note: The starting grass was 500 g (wet weight), and the initial protein content was 15.68 g (dry weight).

Extraction yield

The yield of LPC obtained from this process was 1.98 % based on dried grass or 0.34 % based on fresh grass. Similarly, Martin et al. (2019) reported that sugar beet leaf isolate extracted through ultrafiltration yielded 0.3 % based on fresh grass. A similar extraction yield might be because of the similar protein content of starting material: 18.42 % protein (dry basis) was detected in ryegrass used in this study, and 18.3 % protein (dry basis) was detected in sugar beet leaves used in their study.

In comparison, LPC extracted through the same conditions, except using heat coagulation to concentrate proteins (section 3.5.1.5 without NaOH), had an extraction yield of 1.12% (based on dried grass. A recovery yield by ultrafiltration was slightly higher than heat coagulation, possibly due to the recovery of non-proteinaceous components (Kaur et al., 2021). Since lower protein content was obtained (22.73%) compared to the LPCs extracted through heat coagulation (48.16%), as mentioned in section 3.5.1.5.

This study used ultrafiltration to purify leaf proteins due to its mild conditions; however, the protein content detected in the LPC was very low. This could be relevant to a static filtering mode used in this study, leading to membrane fouling and other impurities contamination. Long processing time and inappropriate temperature (room temperature) promote protein degradation, resulting in the hydrolysed proteins or amino acids that are smaller than the filter size being lost through the filtrate. Thus, non-proteinaceous components and protein loss lead to low protein content in the isolated LPC. LPC's colour was brown due to the browning reaction catalyzed by polyphenols oxidase and phenolic compounds (Jang & Moon, 2011).

3.5.1.7 Chromatography

Chromatography is a physical method to separate the target compounds in the mixture solution. It is a mild process irrelevant to heat or extreme conditions; therefore, it has been used in protein purification (Labrou, 2018). Consequently, native proteins' properties would be reminded (De Jong et al., 2014). Furthermore, chromatography is a highly selective separation method; thus, high purity of the isolated proteins would be obtained (GE Healthcare, 2012). The present study has chosen ion exchange and size exclusion chromatography due to their uncomplicated operation.

Green colour and grassy flavour

After the chromatography process, the white-cream colour and odourless LPC was obtained (Figure 3.19). After removing the green protein fraction by a thermal process, the unwanted residual compounds were eliminated by filtration and chromatography. De Jong et al. (2014) stated that the residual chlorophyll could be decontaminated through a 0.45 µm filter. Ion-exchange chromatography can remove colourants and small impurities (i.e., polyphenols) from protein extracts, as Labrou (2018) has stated. Although chromatography is a long process, which may induce an enzymatic reaction, brown colour was not observed in the LPC. Similarly, Martin et al. (2014) reported that polyphenol absorbance was not observed in the RuBisCo protein isolate extracted through a chromatography technique, indicating that polyphenols and browning pigments were decontaminated. Furthermore, they also reported that chromatography could successfully eliminate the off flavour of protein isolate; hence, the odourless LPC would be obtained.

Figure 3.19: Colour and flavour of leaf protein concentrate: Through the use of chromatography.

Protein content

The chromatography technique has been used for protein purification to obtain high purity of target protein (GE Healthcare, 2012). However, the obtained LPC from this experiment had only 14.24 % protein (dry basis), contradicting the theory mentioned above. As shown in Table 3.15, protein recovery in the final grass juice and the recovered solution was similar to the results obtained through heat coagulation and ultrafiltration. This might be due to using the same materials and methods. However, several factors could affect the efficiency of protein recovery, such as packing column, protease, and process conditions. Improper packing columns of ion-exchange chromatography (IEX), such as loose, tight, and short columns, might reduce the resolution efficiency. This could contaminate the target protein with impurities (i.e., salt, phenolic compounds, and other small molecules).

Table 3.15: The amount of protein in the recovered fractions obtained from the extraction process: Through the use of chromatography.

Sample	Recovered proteins (g)
Final grass juice	6.28
Recovered solution	3.05
LPC	0.03

Note: The starting grass was 500 g (wet weight), and the initial protein content was 15.68 g (dry weight).

During the chromatography process, some proteins were lost through waste fraction since 9.89 % protein (dry basis) was detected in filtrate 1 (the solution obtained after IEX). Uncharged proteins and the same charged proteins with column materials were first eluted at the same speed flow of sample buffer (GE Healthcare, 2012). SDS-PAGE was used to confirm protein loss during the IEX process. The results were presented in lanes 1 and 2 of Figure 3.20. Many protein bands were detected between the MW of 10 to 100 kDa, which could be green protein fraction, large and small subunits of RuBisCo (Nynäs et al., 2021), light-harvesting complex chlorophyll (Shu Min, 2017), and Fraction-II proteins (Horwarth et al., 1973). It confirms that some proteins were not recovered by IEX, resulting in protein loss.

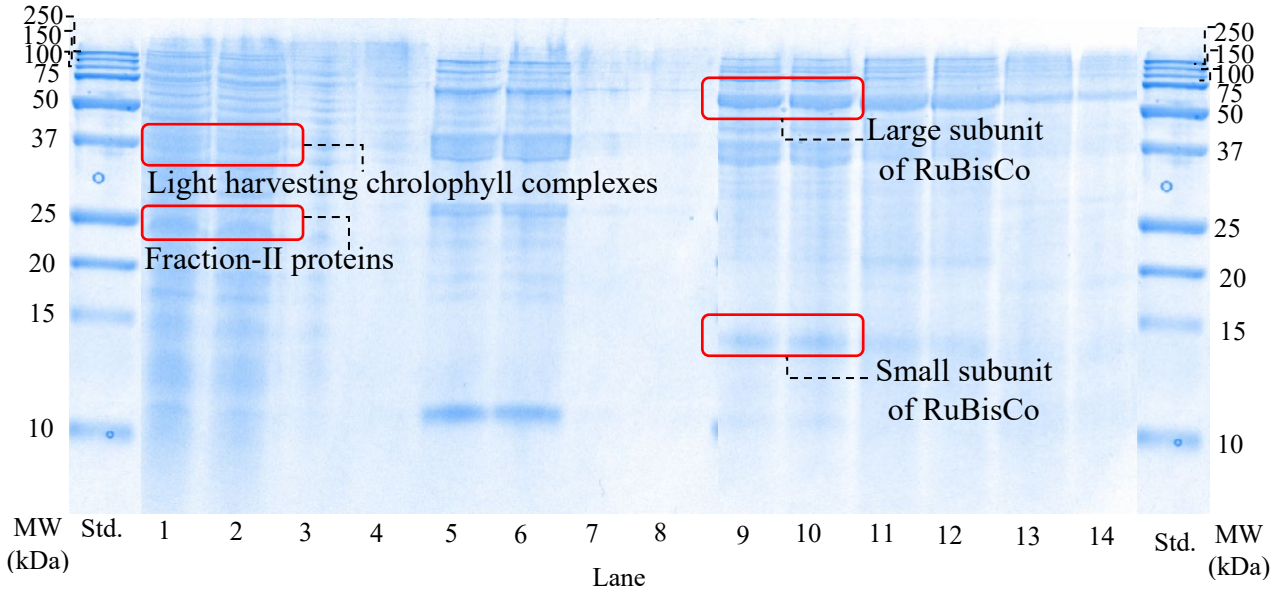


Figure 3.20: Tricine-SDS-PAGE electrophoretogram of filtrates obtained from ion-exchange chromatography. Filtrate 1 (lanes 1 to 2); Filtrate eluted by 0.025 M NaCl (lanes 3 to 4); Filtrate eluted by 0.1 M NaCl (lanes 5 to 6); Filtrate eluted by 0.2 M NaCl (lanes 7 to 8); Filtrate eluted by 0.3 M NaCl (lanes 9 to 10); Filtrate eluted by 0.5 M NaCl (lanes 11 to 12); Filtrate eluted by 1.0 M NaCl (lanes 13 to 14).

Using gradient buffer (0.25, 0.1, 0.3, 0.5, and 1.0 M NaCl) to elute the target protein from IEX and selecting only the fraction containing the highest RuBisCo content might cause low protein recovery. As shown in Figure 3.20 at lanes 9 and 10, the highest intensity bands of large and small RuBisCo subunits (55 kDa and 14 kDa, respectively) were observed in the fraction eluted by 0.3 M NaCl. Thus, this fraction was chosen to be purified further using size exclusion chromatography (SEC), while other fractions eluted by other NaCl concentrations were discarded. Proteins at MW of 10 to 100 kDa were observed in the discarded fractions, indicating protein loss along with these fractions. Thus, a single elution solvent (no gradient NaCl) should be used to avoid protein loss.

Furthermore, protein loss might be due to protein degradation during extraction. Target proteins could be degraded by protease in grass leaves to small peptides and amino acids; consequently, the hydrolysed proteins were lost through the waste fractions (De Jong et al., 2014). This could be supported by the observation in filtrate 2 (the fraction from concentrating process before loading to SEC). It had 8.92 % protein (dry basis), dedicating that some proteins

were hydrolysed during the extraction process. These hydrolysed proteins subsequently pass the filter and lose through the filtrate.

Extraction yield

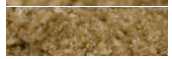
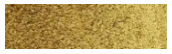
The extraction yield of LPC from the chromatography technique was 0.21 % based on dried grass, which was lower than other extraction methods used in this study. It might be due to a highly selective property of chromatography (GE Healthcare) and many steps used in this protocol, possibly leading to the loss of proteins and other components along the process. As mentioned above, there was protein loss through filtrates 1 and 2 with 9.89 % and 8.92 % protein (dry basis). In addition, the results from SDS-PAGE showed that many protein bands were detected in every fraction eluted by 0.1 – 1.0 M NaCl, as shown in Figure 3.20. It shows that proteins were also lost through the elution step. Moreover, small impurities (i.e., salts, phenolic compounds, uncharged proteins, and polysaccharides) were removed through microfiltration, centrifugal filtration, and chromatography.

In conclusion, this process successfully removed LPC's green colour and grassy flavour. However, it provided the lowest extraction yield (0.21 % of dried grass) and protein content (14.14 % protein dry basis) compared to other extraction methods. Many factors affect protein loss along the process, such as improper packing column, protease, and the number of processes. In addition, uncharged and the same charged proteins with the ion-exchange medium were lost through the filtrate; hence these proteins were not recovered in LPC. SDS-PAGE shows that a single elution should be used to elute the bound proteins since many proteins were detected in the filtrates eluted by gradient elution (0.1 to 1.0 M NaCl).

3.5.1.8 Summary

The different extraction methods were studied to isolate leaf protein concentrate with the objective of colour and flavour removal. Physico-chemical properties of the resulting proteins were determined to study the effect of isolation methods, including protein content (% dry basis), extraction yield (% of dried grass weight), and colour and flavour improvement.

Table 3.16: Physico-chemical properties of leaf protein concentrates using different extraction methods.

No.	Extraction method	Varied conditions				Protein content (% dry basis)	Extraction yield (% of dried grass)	Colour	Grassy flavour
		Thermal treatment	$CaCl_2$ (mM)	Alkali-assisted extraction	Activated carbon (% w/w)				
1	Alkali-acid precipitation with $CaCl_2$ treatment	-	0	Grass: water: 0.1M NaOH (1:1.7:0.7)	-	53.94	9.61		Strong
			100			51.14	3.20		Strong
			200			48.70	3.14		Light
			300			44.76	1.96		Light
2	Protein extraction with AC treatment	60 °C, 5 minutes	-	-	1.9	16.50	10.77		ND
					3.8	14.65	10.10		ND
					5.4	13.30	5.07		ND
3	Alkali-acid precipitation with AC treatment	60 °C, 5 minutes	-	-	1.9	47.51	3.14		Light
				Grass: water: 0.1M NaOH (1:1.7:0.7)		50.03	7.65		Light
4	Heat coagulation with $CaCl_2$ and thermal treatment	50 °C, 15 minutes	0	-	-	41.26	0.87		Light
			100			28.02	0.67		Light
			150			24.29	0.40		ND

No.	Extraction method	Varied conditions				Protein content (% dry basis)	Extraction yield (% of dried grass)	Colour	Flavour
		Thermal treatment	$CaCl_2$ (mM)	Alkali-assisted extraction	Activated carbon (% w/w)				
4	Heat coagulation with $CaCl_2$ and thermal treatment	58 °C, 2 minutes	0	-	-	33.22	0.40		Light
			100			26.21	0.40		ND
			150			21.10	0.35		ND
5	Heat coagulation with different concentrations of NaOH	50 °C, 15 minutes	-	0.0 M	-	48.16	1.12		Light
				0.03 M		42.94	1.36		Light
				0.1 M		38.81	1.58		Strong
6	Ultrafiltration	50 °C, 15 minutes	-	-	-	22.73	1.98		ND
7	Chromatography	50 °C, 15 minutes	-	-	-	14.14	0.21		ND

ND is referred to as Not detected.

The result shows that leaf protein concentrate (LPC) obtained through different methods had different protein content, extraction yield, colour, and flavour, as shown in Table 3.16. The highest protein content (53.94 %) was detected in LPC extracted through the alkali-acid precipitation method without $CaCl_2$ treatment at an extraction yield of 9.61 % (based on dried grass). However, dark green colour and grassy flavour were observed. At the same time, colour and flavour were improved as the concentration of $CaCl_2$ was increased. However, protein content and extraction yield decreased (Table 3.16, experiment 1).

Experiment 3 in Table 3.16 shows a significant increase in protein content ($p < 0.05$) and higher extraction yield in LPC using alkali-assisted extraction compared to the non-alkali process. It would confirm that alkali enhances cell disruption and protein extractability. The green colour was observed in LPCs from both processes. This might be due to the thermal process used for chlorophyll fractionation not being used. Hence, green protein fraction and membrane protein remained (De Jong et a., 2014; Nynäs et al., 2021). However, a darker green colour was observed in the LPC using alkali-assisted extraction since a chlorophyll saponification reaction occurred. The less grassy flavour was detected in both LPCs due to activated carbon treatment. This could be confirmed by experiment 2, as shown in Table 3.16. Using activated carbon reduced grassy flavour in LPC; however, protein content and extraction yield of LPC were decreased.

Leaf protein concentrate extracted through heat coagulation had protein content and extraction yield lower than the alkali-acid precipitation method; however, the green colour and grassy flavour were noticeably improved. These results depend on the amount of $CaCl_2$, heating conditions, and alkali concentration. High temperature (58 °C) and high $CaCl_2$ concentration decreased protein content and extraction yield. High temperature leads to more protein denaturation (Nynäs et al., 2021). $CaCl_2$ promotes thylakoid stacking, resulting in fewer proteins being extracted. Although alkali-assisted extraction enhances protein extraction, heat coagulation mainly recovers Fraction-I protein. Thus, combining alkali-assisted extraction and heat coagulation would not increase the protein content in the final LPC. Furthermore, chlorophyll saponification induced by the presence of NaOH leads to the removal of the green colour not being successful.

The protein content and yield of LPC extracted through ultrafiltration were very low (Table 3.16, experiment 6) compared to other studies. This could be relevant to the improper filtering mode, leading to membrane fouling and contamination of other impurities. Long processing

time and inappropriate temperature (room temperature) promote protein hydrolysis and browning reaction, leading to small proteins being lost and brown LPC being obtained.

Leaf protein concentrate (LPC) extracted through chromatography had the lowest extraction yield and protein content compared to other extraction methods. This might be affected by improper packing columns, numerous processes, long processing times, and high temperatures, which cause protein loss during the extraction process. In addition, uncharged and the same charged proteins with the ion-exchange medium were not recovered. A single elution should be used to elute the bound proteins to reduce protein loss. However, chromatography successfully removed the final product's colour and grassy flavour.

3.5.2 Physico-chemical properties of grass leaves and leaf protein concentrate after ethanol extraction

Grassy flavour and dark green colour are the natural characteristics of plant leaves, which relate to chlorophyll pigments (Di Stefano et al., 2018). Both attributes significantly limit the incorporation into human food. Chlorophyll is a water-insoluble compound but soluble in an organic solvent. Chlorophyll removal was achieved using organic solvents, such as methanol, ethanol, acetone, and chloroform. However, only ethanol is considered a food-grade solvent (Tsuji et al., 1985). Therefore, removing chlorophyll and grassy flavour from the starting material (grass leaves) and the final product (leaf protein concentrate: LPC) by ethanol treatment was studied.

3.5.2.1 Colour and flavour improvement

This study used ethanol extraction to remove colour and grassy flavour from grass leaves and LPC. The same trend of colour change was observed in both samples; the initial green colour of grass leaves and LPC was removed and changed to light brown after ethanol extraction (Table 3.17). A similar result was reported; the green colour of alfalfa and white clover protein concentrates were successfully removed by ethanol extraction. This was due to the soluble property of chlorophyll pigments in organic solvents (Tsuji et al., 1985). Chlorophyll removal by organic solvent involves three main mechanisms: diffuse through plant cells, increase the permeability of chloroplast membrane, and cleavage of the linkage of chlorophyll-protein complexes (Hosikian et al., 2010). Consequently, chlorophyll is excreted from plant tissue, and dechlorophyllised products are obtained. However, the obtained colour of grass leaves and LPC were brown. The colour change might be due to polyphenol oxidation, leading to an enzymatic

browning reaction. Also, chlorophyll decomposition stimulated by light, air, and acid produces pheophobide: a derived product responsible for brown colour (Tsuji et al., 1985).

Table 3.17: Colour and flavour of grass leaves and leaf protein concentrate treated by ethanol extraction.

Sample	**Grass leaves**		
Ethanol extraction	**Untreated leaves**	**60 min, 1 time**	**20 min, 3 times**
Colour			
	Green	Light green	Medium brown
Grassy flavour	Strong	Light	Not detected
Sample	**LPC**		
Ethanol extraction	**Untreated LPC**	**60 min, 1 time and 20 min, 3 times**	
Colour			
	Dark green	Brown	
Grassy flavour	Light	Not detected	

Furthermore, Table 3.17 shows that 1-time extraction (60 minutes) could partially eliminate green pigments from grass leaves. In comparison, 3-time extraction (20 minutes at each time) could successfully remove the green colour since the number of steps employed in the process significantly affects the extraction efficiency (Cubas et al., 2008).

After soaking in the ethanol solvent, the grassy flavour of grass leaves and LPC was evaluated. A reduction of grassy flavour was observed in both treated samples. Similarly, Chang et al. (2019) reported that the off flavour of lentil isolate was improved after ethanol extraction since the volatile compounds, which provide green and beany flavour in lentils, significantly reduced (p <0.05). Furthermore, a less grassy flavour was observed in grass leaves treated with 1-time extraction, whereas no grassy flavour was observed in the 3-time extracted sample. This result

presents the same trend as colour removal (more extraction, less off flavour), confirming that the degree of extraction affects flavour improvement.

3.5.2.2 Protein content and extraction yield

Protein content (% dry basis) was determined in treated grass leaves and LPC to compare with the untreated samples. Table 3.18 shows an increase in the protein content of grass leaves after ethanol treatment: 18.52 %, 19.08 %, and 19.74 % (dry basis) of untreated, 1-time, and 3-time extracted samples, respectively. Also, the same trend was observed: LPC after ethanol treatment had protein higher than untreated LPC. Similarly, the protein content of white clover and alfalfa protein concentrate was increased from 55.1 % to 70.8 % and from 48.4 % to 72.2 % (dry basis), respectively, after 5-time ethanol extraction (Tsuji et al. 1985). Chang et al. (2019) also reported the same observation. The protein content of lentil protein isolate was increased from 75.34 % to 79.07 % (dry basis) after soaking in 75% ethanol.

An increase in protein content was due to a reduction of lipid and ash after organic solvent extraction, as stated by Tsuji et al. (1985) and Chang et al. (2019). After 5-time ethanol extraction, lipids in alfalfa and white clover protein concentrates remarkably decreased from 9.7 % to 2.1 % and 10 % to 1.7 %, respectively. At the same time, protein content was unaffected (Tsuji et al., 1985). Change et al. (2019) detected a 3.31 % decrease in ash content from lentil protein isolate after organic solvent extraction using 35 to 55 % concentration.

Table 3.18: Protein content and yield of grass leave and leaf protein concentrate treated by ethanol extraction.

Sample		**Ethanol extraction**	**Protein (% dry basis)**	**Yield (% based on untreated sample)**
Grass leave	1	Untreated	18.52 ± 0.02^{b}	100
	2	60 min, 1 time	$19.08 \pm 0.50^{a,b}$	53.21
	3	20 min, 3 times	19.74 ± 0.25^{a}	51.15
LPC	1	Untreated	50.03 ± 1.25^{b}	100
	2	60 min, 1 time and 20 min, 3 times	55.07 ± 0.97^{a}	53.13

Although the green colour and grassy flavour were successfully removed, the yield of the treated samples was reduced. The final yield of grass leaves treated by 1-time, and 3-time extraction was reduced by almost 50% of the untreated grass. A similar observation was presented in Table 3.18. The treated LPC yielded 53.13 % (approximately 50%) of the untreated LPC. The decreasing yield of all treated samples might relate to the loss of chlorophyll pigments. Also, as mentioned above, a remarkable decrease in lipids and ash could be another cause of yield loss. Since they are organic soluble components, they were removed through waste fraction (Tsuji et al., 1985; Chang et al., 2019).

In conclusion, the present study shows that ethanol extraction can remove chlorophyll and grassy flavour from freeze-dried grass leaves and leaf protein concentrate. Protein content was increased in all treated samples as the organic solvent removed chlorophyll, lipids, ash, and other organic soluble compounds. However, the final colour was brown due to an enzymatic browning reaction induced by polyphenol oxidase, including chlorophyll decomposition, producing brown pigments known as pheophobide. The final yield was decreased by almost 50 % of the starting materials, which was relevant to removing pigments, lipids, and ash.

3.6 Final Summary

The above findings show that the quality and amount of isolated leaf proteins are associated with extraction methods and environmental conditions. Divalent ions and heat can enhance green protein fractionation, enhancing the removal of green colour and grassy flavour. However, a high concentration of divalent ions and high temperature reduce protein content and extraction yield of the isolated LPC. Alkali enhances protein extractability, increasing extraction yield and protein content; however, low protein content was detected in LPC when using alkali with heat coagulation. Activated carbon, ultrafiltration, and chromatography, mild-condition extraction processes, can remove grassy flavour. Nevertheless, short process time and low temperature should be controlled along with these processes as protein degradation stimulated by high temperature and time causes protein loss. Although green colour and grassy flavour removal were successful by ethanol extraction, the product's yield was decreased by about 50 %.

The present study has selected two LPCs with and without green colour to compare physico-chemical and functional properties. Leaf protein concentrate extracted through alkali-acid precipitation without $CaCl_2$ was selected to be represented as the LPC with green colour. Also, it had the highest protein content (53.64 % cry basis) and high extraction yield (9.61 % of dried

grass). Leaf protein concentrate extracted through heat coagulation treated at 50 ℃ for 15 minutes without $CaCl_2$ and NaOH was selected to be represented as the LPC without green colour. It had the highest protein content (48.13 % dry basis) and high extraction yield (1.12 % of dried grass) compared to other dechlorophyllised LPCs. Therefore, these two LPCs were analysed for their thermal denaturation properties, protein profile, protein solubility, and protein digestibility. The results are discussed in the next chapter (Chapter 4).

CHAPTER 4

Physico-Chemical and Functional Properties of The Selected Leaf Protein Concentrates

4.1 Introduction

Leaf protein concentrate (LPC) extracted by two different methods (see Chapter 3) was selected for further investigation. Leaf protein concentrate extracted through alkali-acid precipitation was selected due to the highest protein content (53.94 % dry basis) and high recovery yield (9.61% of dried grass) compared to other isolated-green LPCs. This LPC was represented as LPC with green colour and was designated as AAP-0. The LPC extracted through heat coagulation was selected for another LPC due to its less green colour and grassy flavour with 48.16 % protein (dry basis). It was represented as LPC without green colour and was designated as HC-0. Although both alkali-acid precipitation and heat coagulation may affect protein functionality, this study selected both methods due to their uncomplicated and low-cost nature (Knorr, 1982; Pelegrine & Gasparetto, 2005). Additionally, all materials used in both methods are food grade; thus, there is a potential to utilise both methods in the food industry.

Many studies have reported that the protein extraction method affects the physico-chemical properties of isolated proteins, such as functionalities and nutritional value (Kaur et al., 2021, De Jong et al., 2014; Kobbi, 2017). Hence, the effect of the extraction methods mentioned above on the properties of the selected LPCs was studied. AAP-0 and HC-0 were determined for protein solubility, thermal denaturation profile, protein types, and protein digestibility by the Kjeldahl method, Differential scanning calorimetry (DSC), Tricine-Sodium Dodecylsulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE), and *in vitro* gastro-small intestinal digestion, respectively.

4.2 Materials and methods

4.2.1 Materials

Leaf protein concentrate (LPC) was obtained from two extraction methods, as mentioned in Chapter 3, section 3.2.2.1 & 3.2.2.4.2. Leaf protein concentrate extracted through alkali-acid precipitation with 0 mM $CaCl_2$ was designated AAP-0. Leaf protein concentrate extracted through heating at 50 °C, 15 minutes with 0 mM $CaCl_2$ and then heat coagulation was designated as HC-0. The protein content of AAP-0 and HC-0 was determined to be 53.94 % and 48.16 % (dry basis), respectively. Both LPCs were stored at -20 °C until further analysis. All chemicals used for analysis in this chapter were analytical grade.

4.2.2 Protein solubility

4.2.2.1 Effect of pH

The solubility of LPC was determined using the method of Kaur et al. (2021) with slight modifications. In short, LPC was first dissolved (1 %, w/v) in distilled water. The pH of the LPC solutions was adjusted from 2 to 9 using 1 M HCl or NaOH to determine the effect of pH on LPC's solubility. All solutions were mixed at 300 rpm for 1 h at 20 ℃ using a benchtop shaker (Orbital shaker, OHAUS, New Jersey, United States).

4.2.2.2 Effect of temperature

The effect of temperature was determined by following the method of Pelegrine and Gasparetto (2005) with slight modifications. In short, 1 % (w/v) of LPC solutions were prepared using distilled water. The pH of the solutions was adjusted to 7 using 1 M NaOH, followed by mixing at 300 rpm for 1 h at 20 ℃ using a benchtop shaker (Orbital shaker, OHAUS, New Jersey, United States). The treated solutions were left to stir at 300 rpm using a magnetic stirrer for 10 minutes at 80 °C. The beakers were covered with aluminium foil to prevent moisture loss.

4.2.2.3 Effect of temperature in combination with pH shift

A pH-shift method along with thermal treatment was applied to LPC by following the method of Kaur et al. (2021) with slight modifications. In short, LPC solutions (1%, w/v) were prepared in distilled water. The pH of the solutions was adjusted to 12 using 1 M NaOH, followed by mixing at 300 rpm for 1 h at 20 °C using a benchtop shaker (Orbital shaker, OHAUS, New Jersey, United States). The pH of all solutions was adjusted back to 7 using 6 M HCl. The

resulting solutions were left to stir at 300 rpm for 10 minutes at 80 °C using a magnetic stirrer. The beakers were covered with aluminium foil to prevent moisture loss during heating.

All samples were centrifuged at 2400 *x g* for 15 minutes, and the obtained supernatants were tested for soluble protein concentration (Ps) using the Kjeldahl method, AOAC 978.04 (Horwitz, 1978). The results were shown as the percentage of solubility, %S:

$$\%S = \frac{Ps}{Initial\ protein\ concentration}\ x\ 100 \ldots\ldots\ldots\ldots\ldots\ldots(4)$$

4.2.3 Differential scanning calorimetry

The thermal properties of the LPC were assessed using differential scanning calorimetry (DSC) analysis adapted from Ahmad et al. (2018), with slight modifications. Leaf protein concentrate (12 mg) was weighed into aluminium pans (TA Instruments, TZero 901684.901), followed by the addition of 18 mg of Milli-Q water to obtain 30 mg of 40 % (w/v) LPC dispersion. The pans were then hermetically sealed and left overnight at 23 ± 2 ℃. Scans were then conducted in the differential scanning calorimeter (Q2000, TA Instruments, USA) in triplicates, with heating from 20 to 100 ℃ at a rate of 5 ℃/minutes. The DSC was calibrated with indium and gallium, with empty aluminium pans used as references. The thermal properties were analysed using analytical software (TA Universal Analysis, TA Instruments, USA). Denaturation temperature was taken at the maximum of the endothermic peak, and enthalpy was calculated from the area of the transition peaks.

4.2.4 SDS-PAGE of leaf protein concentrate

Tricine SDS-PAGE under reducing conditions was performed to determine proteins' molecular weight (MW) in the LPC. Samples were dispersed in milli-Q water at a concentration of 4 mg/mL protein content and shaken at 20 rpm for 1 h. Following this, the solutions were diluted further to 2 mg/mL protein content with sample buffer (5 % β-mercaptoethanol) at a ratio of 1:1. The solutions were heated at 95 ℃ for 5 minutes. Molecular weight marker (Precision Plus Protein™ Dual Xtra Prestained Protein Standards) (10 µL) and samples (25 µL) were loaded into 16.5 % gradient Tricine gels (Criterion™ Precast gel, Bio-Rad Laboratories Pty. Ltd., United States of America). The gels were run with a running buffer at 125 V voltage until the samples reached the bottom of the gels. The gels were kept in a fixing solution (40 % methanol, 10 % acetic acid) for 30 minutes, then stained with a staining solution (Bio-Safe™ Coomassie G-250) for 1 h. After this, the gels were de-stained in Milli-Q water overnight and scanned using a gel scanning densitometer (Molecular Imager Gel Doc XR, Bio-Rad Laboratories Pty.

Ltd., New Zealand). The images were analysed using Image Lab™ (Bio-Rad Laboratories Pty. Ltd., New Zealand).

4.2.5 Determination of *in vitro* gastro-small intestinal digestibility

4.2.5.1 Preparation of leaf protein concentrate paste

Leaf protein concentrates were mixed with Milli-Q water, and their pH was adjusted to 12 using 1 M NaOH to obtain 20 % (w/v) of LPC paste. The mixture was stirred at 20 °C for 1 h before heating at 80 °C for 10 minutes under constant stirring at 300 rpm, using a magnetic stirrer. Moisture lost in the heating process was then determined, and the amount of water lost was topped up. The samples were then used to determine *in vitro* protein digestibility.

4.2.5.2 *In vitro* protein digestion

The number of salts used to mimic the digestion fluids in the oral, stomach and small intestinal digestion phases during *in vitro* digestion are shown in Table 4.1, obtained from Minekus et al. (2014). All digestions were conducted at 37 ± 1°C.

Table 4.1: Electrolytes concentration of simulated salivary fluid (SSF), simulated gastric fluid (SGF), and simulated intestinal fluid (SIF).

Composition	Stock concentration (M)	SSF pH 7 Volume (mL)	SGF pH 3 Volume (mL)	SIF pH 7 Volume (mL)
KCl	0.5	15.1	6.9	6.8
KH_2PO_4	0.5	3.7	0.9	0.8
$NaHCO_3$	1	6.8	12.5	42.5
NaCl	2	0.0	11.8	9.6
$MgCl_2(H_2O)_6$	0.15	0.5	0.4	1.1
$(NH_4)_2CO_3$	0.5	0.06	0.5	0.0
$CaCl_2\ (H_2O)_2$	0.3	-	-	-

(Adapted from Minekus et al., 2014).

Note: The volume in this table was calculated for the final volume of stock solutions (400 mL). The simulated fluids were 1.25X concentration.

SSF, Simulated Salivary Fluid; SGF, Simulated Gastric Fluid; SIF, Simulated Intestinal Fluid

The digestion protocol was obtained from Kaur et al. (2010) and Minekus et al. (2014), with slight modifications. In short, 8 g of prepared LPC mixture was added to each reactor. SSF (3.4 mL) was added, followed by the addition of 40 µL $CaCl_2$. The pH was maintained at 7 ± 0.1 using 1 M NaOH. α-amylase (10025, Sigma Aldrich, USA) was added to obtain a concentration of 75 U/mL activity in the mixture, and the solution was stirred for 2 minutes.

Following this, 24.12 mL of SGF was added, followed by 12 µL of $CaCl_2$. The solution was adjusted to pH 3 ± 0.1 using 6 M HCl. Porcine gastric pepsin (P7125, Sigma Aldrich, USA) was added to obtain a concentration of 2.5 U/mg protein. Samples were drawn at 0, 30 and 60 minutes, and 10 µL of Pepstatin A (AB141416, Abcam Plc, New Zealand) (0.5 mg/ml methanol) was added to every mL of digest collected.

For the small intestinal phase, 32.4 mL of SIF, 96 µL of $CaCl_2$, and 6 mL of bile (10 mM in the final mixture) were added to the gastric digest, with the pH adjusted to 7 ± 0.1 using 1 M NaOH. Porcine pancreatin (4xUSP, P1750, Sigma Aldrich, USA) was added in a 1:100 ratio of enzyme to substrate, and digests were collected after another 10, 60 and 120 minutes. Protease inhibitor cocktail solution (SigmaFast™, Sigma Aldrich, USA) (350 µl/mL digest) was added (4.5 mL for every mL of the digests collected). All digests were then placed in an ice bath to stop the further enzymatic activity and stored at -20 °C until further analysis.

4.2.5.3 Digest sample preparation

All samples obtained from the *in vitro* digestion process were centrifuged at 14,100 *x g* for 10 minutes using a high-speed centrifuge (MiniSpin® plus centrifuge, Eppendorf South Pacific Pty. Ltd., Australia). The supernatant was then filtered through a 0.45 µm PVDF filter.

4.2.5.4 Soluble protein content

The soluble protein content in the digests was determined using the Kjeldahl method, as per AOAC 978.04 (Horwitz, 1978).

4.2.5.5 Free amino nitrogen content

The digested samples were treated with ninhydrin using a colourimetric method to determine the ninhydrin reactive amino nitrogen content by following the method of Moore (1968) and Kaur et al. (2021). In short, a standard curve was prepared using stock solutions of glycine in 0.05 % glacial acetic acid. The stock solutions and digest samples were mixed with ninhydrin reagent (N7285, Sigma Aldrich Pty Ltd, USA) and heated for 10 minutes at 97 ± 2 °C. The

solutions were then cooled immediately, and ethanol (95 %, v/v) (2.5 mL) was added. The absorbance of the solutions was then read at 570 nm using a spectrophotometer.

4.2.5.6 SDS-PAGE of digested leaf protein concentrate

Protein breakdown during digestion was done using tricine SDS-PAGE as described by Kaur et al. (2016). The digests were not subjected to centrifugation and filtration (as stated in section 4.2.5.3). A homogenous sample was drawn from the digests and diluted to 2.5 mg/mL protein content with Milli-Q water at pH 3 and 7 for the gastric and small intestinal digests, respectively. Following this, the solutions were diluted further to 1.25 mg/mL protein content using Tricine buffer (containing 2 % β-mercaptoethanol) at a ratio of 1:1. The solutions were heated at 97 ± 2 °C for 5 minutes. The sample solutions (25 µL) and standard (10 µL) (Precision Plus Protein™ Dual Xtra Prestained Protein Standards) were loaded into 16.5 % gradient Tricine gels. The gels were run with a running buffer at 125 V voltage until the samples reached the bottom of the gels. The gels were kept in a fixing solution (40 % methanol, 10 % acetic acid) for 30 minutes, then stained with a staining solution (Bio-Safe™ Coomassie G-250) for 1 h. After this, the gels were de-stained in Milli-Q water overnight and scanned using a gel scanning densitometer (Molecular Imager Gel Doc XR, Bio-Rad Laboratories Pty. Ltd., New Zealand). The images were analysed using Image Lab™ (Bio-Rad Laboratories Pty. Ltd., New Zealand).

4.2.6 Statistical analysis

All the analyses reported in this study were performed at least in triplicate. The data were represented as mean ± standard deviation. All data were analysed with Minitab statistical software version 19 (Minitab Inc., State College, PA). One-way ANOVA and Tukey's test at the 95 % confidence level was used to identify the significant differences ($p < 0.05$) for more than two groups.

4.3 Results and discussion

4.3.1 Protein solubility

Protein solubility is a vital property of food proteins as proteins with high solubility have a high potential to obtain a uniform colloidal system, increasing the potential of food application in various food products (Zayas, 1997). Thus, the selected LPCs determined the ability to be utilised in food by determining the protein solubility under different pH conditions. Furthermore, the solubility improvement was conducted under high temperature and pH-shifting conditions. The total soluble nitrogen was analysed to indicate the ability of protein solubility.

4.3.1.1 Effect of pH

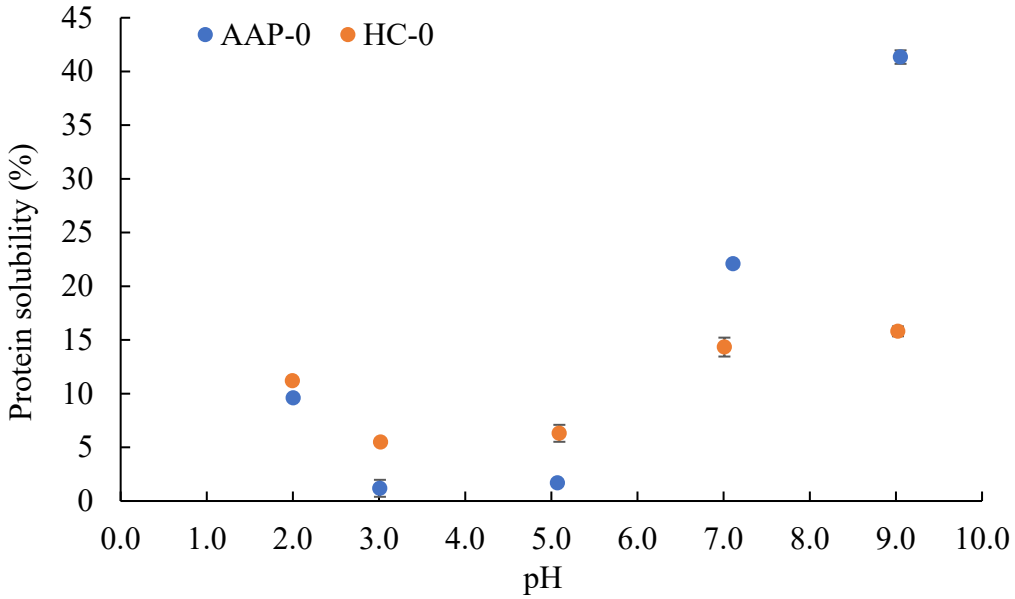


Figure 4.1: The effect of pH on protein solubility of leaf protein concentrates.

AAP-0, LPC extracted through alkali-acid precipitation with 0 mM $CaCl_2$.

HC-0, LPC extracted through heating at 50 °C, 15 minutes with 0 mM $CaCl_2$, followed by heat coagulation.

Figure 4.1 shows that the protein solubility of both LPCs was significantly affected (p <0.05) when the pH of the LPC solutions was varied, indicating that the pH of the environment highly impacts the protein solubility. The lowest protein solubility of LPC extracted through alkali-acid precipitation (AAP-0) was observed at the pH range of 3 to 5. The same result was reported by Kaur et al. (2021); the lowest protein solubility of grass protein concentrate (GPC) extracted through acid precipitation was found at the same pH range as the present study. Also, the

protein solubility of LPC extracted through heat coagulation (HC-0) was lowest in the pH range of 3 to 5. It has been concluded that proteins are least soluble at the pH nearest their isoelectric point (pI) (Pelegrine & Gasparetto, 2005; Nynas et al., 2021). In addition, no significant difference was observed between pH 3 and 5, as shown in Table 4.2, confirming that the isoelectric pH of both LPCs ranges between 3 to 5.

The lowest soluble proteins are due to the lowest electrostatic forces at this point, where the charge of amino acids reaches net zero, increasing protein-protein interactions. Consequently, proteins aggregate and have lower solubility (Damodaran et al., 2007). However, the isoelectric point of pure RuBisCo has been reported at pH 4 - 6, which is different from this study. This difference might result from the unpurified form of RuBisCo extracted in LPC. Other forms of proteins present in the plant leaves may be extracted, such as ATP synthase or protein kinase (Xeu et al., 2015). Nynas et al. (2021) also supported that these compounds would significantly impact the net charge of proteins, affecting their functionalities.

When the pH was increased from 7 to 9, the solubility of AAP-0 increased from 22.09 % to 41.34 %. In contrast, the solubility of HC-0 rarely changed. This was due to the extraction method affecting the protein solubility (Lamsal et al., 2007). Betschart (1974) and Farkas & Mohácsi-Farkas (1996) also supported that thermal coagulation and acid precipitation provided different results for RuBisCo's solubility. Furthermore, the protein solubility of HC-0 was lower than AAP-0, as shown in Figure 4.1. Similarly, mustard protein isolate concentrated by steam injection had 20 – 40 % protein solubility at pH ranging from 1 to 12, whereas protein isolate concentrated by isoelectric precipitation had a higher protein solubility (40 - 80%) at the same pH range (Sadeghi & Bhagya, 2009). The lower protein solubility of HC-0 might be due to a conformational change affected by thermal treatment. Protein denaturation occurs during heat coagulation, leading to exposure of hydrophobic sites of proteins (De Jong et al., 2014) and forming an irreversible structure (Edward et al., 1975). Therefore, a low protein solubility was observed in HC-0 as its structure was irreversible.

Nevertheless, both LPCs displayed a similar trend of having higher solubility on either side of their pI. This solubility curve was similar to the results obtained from other leaf sources (Betschart, 1974; Farkas & Mohácsi-Farkas, 1996). The solubility curve of leaf proteins involves a total negative or positive charge of proteins, which is acquired in acidic or alkali conditions. The charge on the protein surface increases the electrostatic repulsion between

molecules, resulting in decreased protein-protein interactions; thus, higher protein solubility is observed (Machado et al., 2007).

Table 4.2: The protein solubility of leaf protein concentrates against the pH.

pH	AAP-0	HC-0
2.0	9.60 ± 0.18^{c}	11.20 ± 0.04^{b}
3.0	1.18 ± 0.79^{d}	5.48 ± 0.05^{c}
5.0	1.68 ± 0.03^{d}	6.30 ± 0.99^{c}
7.0	22.09 ± 0.31^{b}	14.33 ± 0.88^{a}
9.0	41.34 ± 0.63^{a}	15.80 ± 0.46^{a}

Different letter in the same column represents a significant difference ($p < 0.05$). Results are expressed as means ($n = 3$) ± standard deviation with Tukey's HSD multiple comparisons and 95 % confidence. HSD (Honest Significance Test).

AAP-0, LPC extracted through alkali-acid precipitation with 0 mM $CaCl_2$

HC-0, LPC extracted through heating at 50 °C, 15 minutes with 0 mM $CaCl_2$, followed by heat coagulation.

4.3.1.2 Effect of temperature

The solubility of proteins is relevant to protein-water interactions, which are affected by the temperature of the protein solution (Kaur et al., 2021). Therefore, the effect of high temperature was studied to improve the solubility of both LPCs (AAP-0 and HC-0) at neutral pH.

At neutral pH, the protein solubility of AAP-0 was increased significantly (p <0.05) from 22.09 % to 57.69 % when it was subjected to 80 °C for 10 minutes (Figure 4.2). Kaur et al. (2021) reported a similar result: an increase in protein solubility of grass protein concentrate (GPC) extracted by alkali-acid precipitation was observed when the GPC was subjected to 40, 60, and 80 °C. The protein solubility increases with the increasing temperature due to the increased protein-water interactions. Also, thermal treatment leads to protein denaturation, weakening hydrogen bonds and electrostatic forces that hold proteins' structure (secondary, tertiary, and quaternary) (Pelegrine & Gasparetto, 2005). Hence, protein solubility increases by increasing the temperature of the medium that proteins are subjected to. However, the solubility of HC-0 after high-temperature treatment was rarely affected. This might be due to protein denaturation induced by heat coagulation, leading to irreversible structure (Edward et al., 1975).

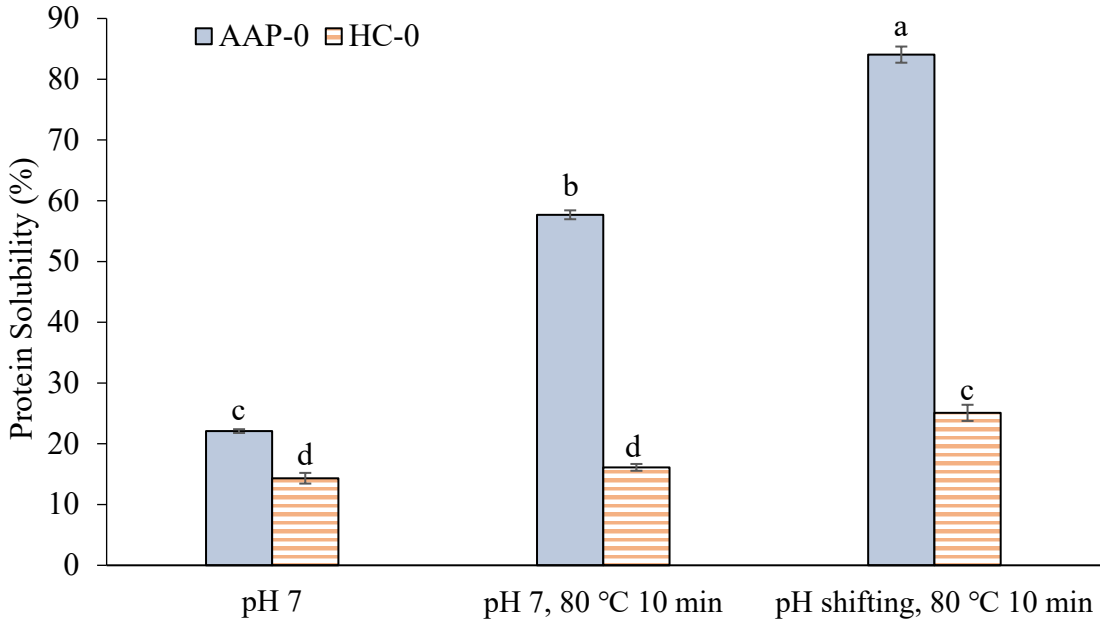


Figure 4.2: The effect of high temperature and high temperature in combination with pH shift on protein solubility of leaf protein concentrate.

Means (n =3) that do not share a letter are significantly different (p <0.05) (Tukey's HSD multiple comparisons and 95 % confidence). HSD (Honest Significance Test).

AAP-0, LPC extracted through alkali-acid precipitation with 0 mM $CaCl_2$.

HC-0, LPC extracted through heating at 50 °C, 15 minutes with 0 mM $CaCl_2$, followed by heat coagulation.

4.3.1.3 Effect of temperature in combination with pH shift

The process of pH-shifting involves subjecting the proteins to a highly acidic and alkali pH before neutral pH, allowing the proteins to unfold and refold partially. This structural change creates unique surface properties, enhancing the solubility of proteins. Also, the solubility of proteins increases at high temperatures. Thus, the effect of high temperature combined with pH-shifting was studied to improve the solubility of LPC.

The combination of temperature and pH shifting treatment significantly increased (p <0.05) the protein solubility of AAP-0 to 84.05 %, as shown in Figure 4.2. At neutral pH, the protein solubility of AAP-0 was increased approximately 4 times compared to the untreated sample and 1.4 times compared to the heated sample. The protein solubility of HC-0 after this treatment was also increased to 25.09 %, while only the high-treatment treatment rarely impacted its protein solubility. These results were similar to the result reported by Kaur et al. (2021). Their study showed that the solubility of GPC extracted by enzyme-assisted extraction was increased

5.3 times after subjecting to the pH-shifting process. An increase in protein solubility was due to thermal and pH effects. Thermal treatment leads to protein denaturation, weakening hydrogen bonds and electrostatic forces that hold proteins' structure (secondary, tertiary, and quaternary) (Pelegrine & Gasparetto, 2005). Hence, protein solubility increases. Jian et al. (2009) stated that proteins are partly unfolded in extreme pH conditions, resulting in the buried side chain being exposed to the polar surface. However, the unfolding structure involves primary and tertiary structures, which cannot be reversed entirely with refolding treatment at pH 7. This conformation, called "a molten globule" structure, induces the proteins to be more flexible. Consequently, the solubility improvement of the pH-shifting treated sample would be observed.

4.3.2. Thermal stability

The thermal process has been widely used in the food industry since it enhances food's digestibility, palatability, and shelf-life (Farkas & Mohácsi-Farkas, 1996). When proteins are heated, their structure undergoes conformational change and bond disruption, which is an endothermic process. Hydrogen bonds, van der Waals and electrostatic forces that hold the quaternary and tertiary protein's structure are weakened after thermal treatment, exposing the buried hydrophobic sites (Jiang et al., 2009). Consequently, protein molecules aggregate, which is an exothermic process. The differential scanning calorimeter (DSC) has been used to analyse thermal analysis in food research by measuring the difference between endo- and exothermic processes (Farkas & Mohácsi-Farkas, 1996). The present study determined protein denaturation profile and enthalpy using DSC analysis.

Table 4.3: Thermal denaturation temperature and enthalpy of leaf protein concentrate.

Samples	Thermal denaturation temperature (°C)	Enthalpy (J/g)
AAP-0	69.98 ± 0.14^{a}	5.11 ± 0.66^{a}
HC-0	65.26 ± 0.67^{b}	0.34 ± 0.59^{b}

Different letter in the same column represents a significant difference ($p < 0.05$). Results are expressed as means ($n = 3$) ± standard deviation with a Two-sample t-test and 95 % confidence.

AAP-0, LPC extracted through alkali-acid precipitation with 0mM $CaCl_2$.

HC-0, LPC extracted through heating at 50 °C, 15 minutes with 0 mM $CaCl_2$ and followed by heat coagulation

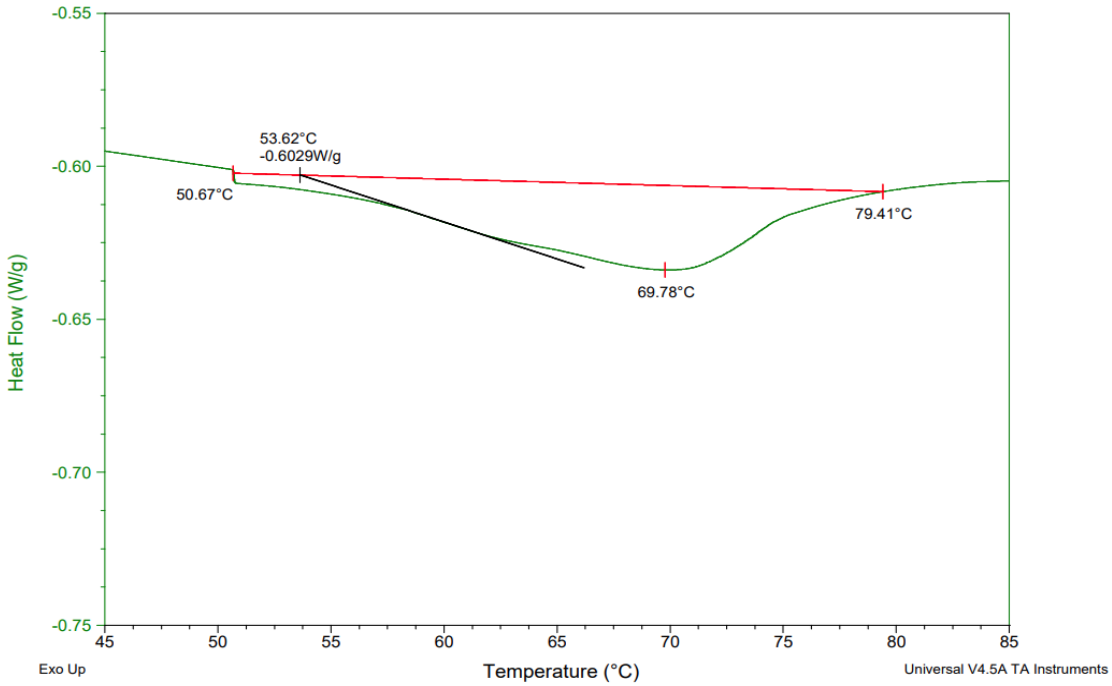


Figure 4.3: Thermal denaturation curve of leaf protein concentrate: Through alkali-acid precipitation with 0 mM $CaCl_2$ (AAP-0).

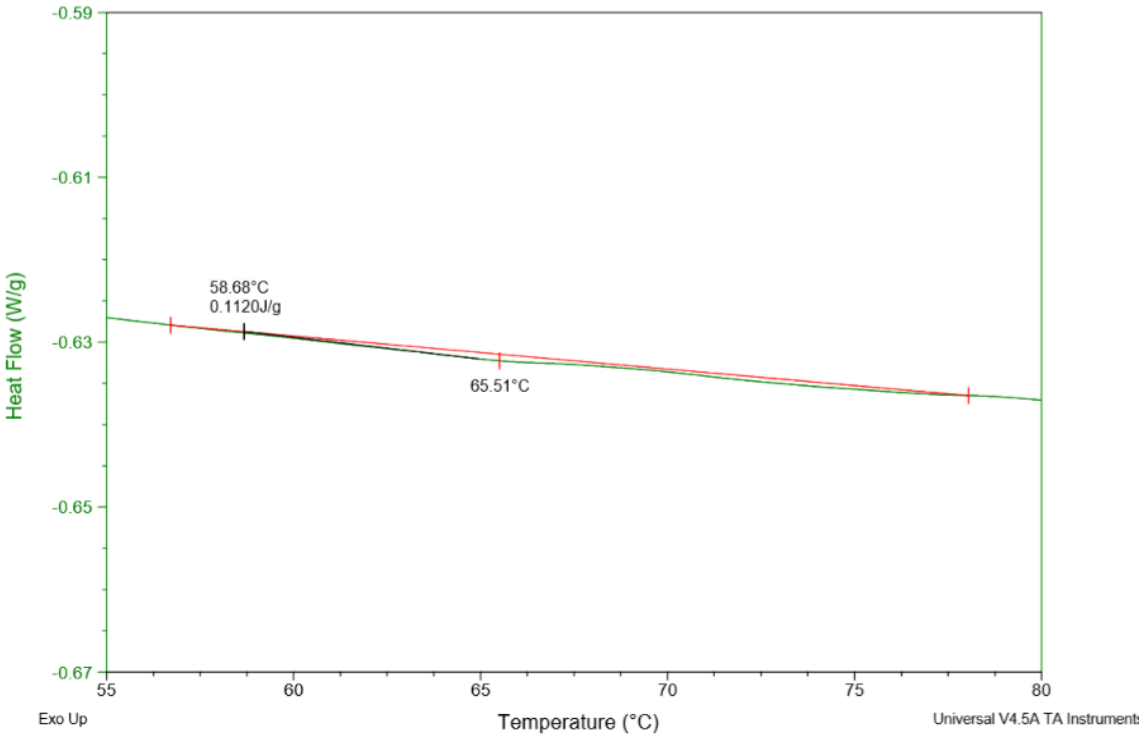


Figure 4.4: Thermal denaturation curve of leaf protein concentrate: Through heating at 50 °C, 15 minutes with 0 mM $CaCl_2$, followed by heat coagulation (HC-0).

Raw LPCs extracted through alkali-acid precipitation (AAP-0) and heat coagulation (HC-0) were subjected to DSC analysis to analyse denaturation temperature (T_d) and enthalpy. Figures 4.3 and 4.4 show the result of the DSC analysis of AAP-0 and HC-0, respectively, where the highest peak of the endothermic curve was determined to be T_d. As shown in Table 4.3, the triplicate result shows that the T_d of AAP-0 and HC-0 was 69.98 ± 0.14 ℃ and 65.26 ± 0.67 ℃, respectively. It indicates that the extraction methods affect the conformational change of proteins, resulting in proteins unfolding at different temperatures. Similarly, Kaur et al. (2021) observed that raw GPCs extracted through enzymatic-assisted extraction and acid precipitation were denatured at different temperatures. RuBisCo obtained from alfalfa and tobacco has T_d between 61.85 to 72.85 ℃ (Béghin et al., 1993). It can be seen that the T_d of both AAP-0 and HC-0 was in line with other plant sources; however, the difference could result from the conditions used during DSC analysis and plant type (Béghin et al., 1993).

The enthalpies of denaturation of both LPCs obtained in this study were 5.11 ± 0.66 J/g and 0.34 ± 0.59 J/g for AAP-0 and HC-0, respectively. However, these results were different from the value of 26.3 J/g obtained from alfalfa RuBisCo (Tomimatsu, 1980). The difference might relate to the presence of proteins other than RuBisCo (Privalov & Potekhin, 1986). Such as ATP synthase or protein kinase in plant leaves (Xue et al., 2015). Furthermore, the pH near the protein's PI might affect the enthalpy property. Béghin et al. (1993) stated that a pH lower than 7 decreased the enthalpy of alfalfa RuBisCo. This was due to the decreased electrostatic repulsion between protein molecules. Consequently, protein-protein interactions increased, causing an irreversible aggregation. The enthalpy property was affected (Privalov & Potekhin, 1986). The pH of raw AAP-0 was near pH 3.5, which was close to its pI range (3 to 5); hence, its enthalpy would decrease.

Leaf protein concentrate extracted by heat coagulation (HC-0) had lower T_d and enthalpy than the LPC extracted by alkali-acid precipitation (AAP-0), as shown in Table 4.3. This might involve the denatured proteins induced by heat coagulation used for concentrating proteins. Thermal treatment weakens the bondings that hold protein structures, such as hydrogen bonds, van der Waals, and electrostatic forces, causing protein aggregation (Pelegrine & Gasparetto, 2005). Differential scanning calorimeter (DSC) analysis measures the net change between the endothermic (conformation change and bond disruption) and exothermic processes (protein aggregation) (Farkas & Mohácsi-Farkas, 1996). Hence, the difference between the endo and

exothermic processes of the HC-0 sample might be less detected, resulting in a lower Td and enthalpy.

4.3.3 Protein molecular weight distribution

The molecular weight (MW) distribution of raw AAP-0 and HC-0 was investigated with Tricine-Sodium Dodecylsulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE), determining the protein type in the isolated LPCs.

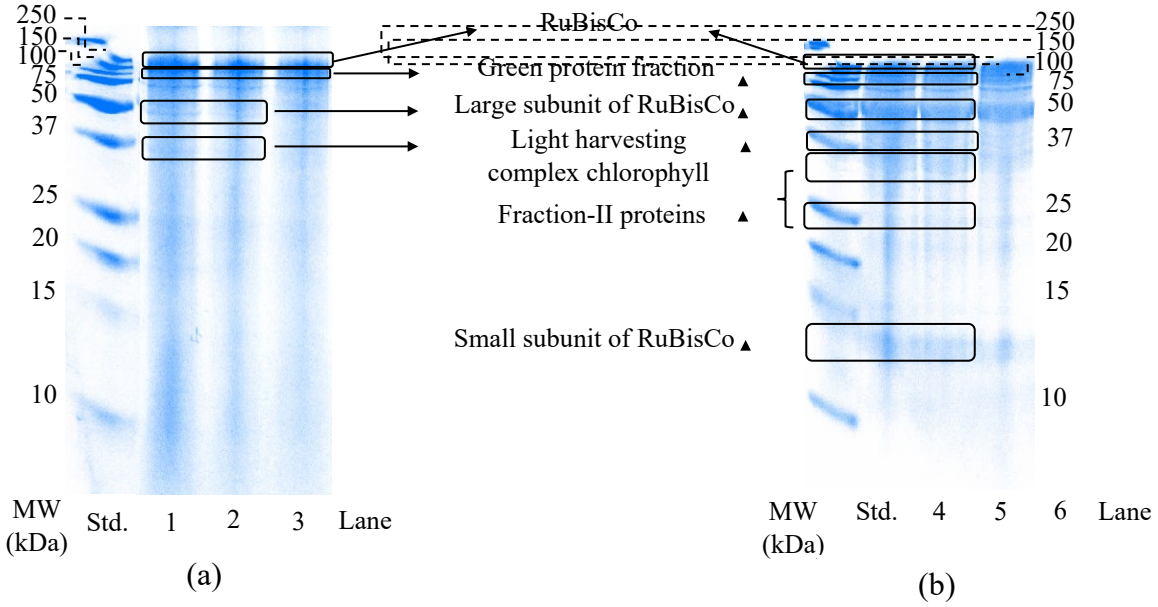


Figure 4.5: Tricine-SDS-PAGE results of leaf protein concentrate: (a) LPC extracted through alkali-acid precipitation with 0 mM $CaCl_2$ (lane 1 to 3); (b) LPC extracted by heating at 50 °C, 15 minutes with 0 mM $CaCl_2$, followed by heat coagulation (lane 4 to 6).

Figure 4.5 shows SDS-PAGE results in triplicates of raw LPCs extracted through alkali-acid precipitation (a) and heat coagulation (b), indicating that both LPCs contained various proteins. Similarly, Lamsal et al. (2007) reported that leaf proteins from alfalfa juice contained various proteins ranging between 6 kDa and about 550 kDa. This result could support that presence of other proteins affect the isoelectric point value, causing the different pI between both LPCs (pH 3-5) and a pure RuBisCo (pH 4-6), as mentioned in section 4.3.1.1.

The protein bands at MW above 250 kDa, 100 kDa, 50 kDa, and 37 kDa were detected in both LPCs, as shown in Figure 4.5. The band at the top of the gels indicates MW above 250 kDa, which could not migrate into the gels. It might represent the RuBisCo protein at a MW of 550 kDa (Barbeau & Kinsella, 1988). The protein band at 100 kDa was likely to be the green protein

fraction. This could be supported by Lamsal et al. (2007). They reported that a protein band at 107 kDa was detected in alfalfa juice, whereas it disappeared when the green protein fraction was removed. Therefore, they concluded that proteins at MW of 107 kDa were green proteins. Moreover, the protein band at 100 kDa of AAP-0 had higher intensity than HC-0. It might result from a darker green colour, indicating more green proteins being recovered in the AAP-0. The MW, about 37 kDa, also was observed in both samples, which might represent the light-harvesting complex chlorophyll. Sedigheh et al. (2011) and Ji et al. (1998) reported similar results: a light-harvesting complex of wheat and soybean leaves was detected at 34 kDa.

A protein band at 50 kDa observed in both samples was likely to be large subunits of RuBisCo as the MW of large subunits is 50-55 kDa (De Jong & Nieuwland, 2011; Udenigwe et al., 2017). Nevertheless, small subunits of RuBisCo at MW of 12 – 18 kDa were not found in the AAP-0, while it was detected in the HC-0. This indicates that the extraction process used for HC-0 isolation would isolate the RuBisCo with higher purity than the alkali-acid precipitation method. Lamsal et al. (2007) reported that clarified alfalfa juice (juice without green proteins) contained proteins at 52 and 12.5 kDa, representing the MW of large and small subunits of RuBisCo. In contrast, these two subunits were slightly observed in the whole alfalfa juice (green juice). The protein band between 25 and 37 kDa observed in only the HC-0 might be Fraction-II proteins (Lamsal et al., 2007) which are soluble proteins, except RuBisCo (Horwarth et al. 1973).

4.3.4 *In vitro* protein digestibility

The protein digestibility of the selected LPCs (AAP-0 and HC-0) was investigated by *in vitro* protein digestion under the simulated conditions of gastric and small-intestinal tracts. The protein digestibility of these LPCs was evaluated by measuring the content of soluble N, ninhydrin-reactive amino N, and protein MW distribution in the digested samples at different digestion times. As mentioned above, the different extraction methods affect protein structure and physico-chemical properties, which might impact protein digestibility. Thus, the study of protein digestibility was conducted by comparing the results of these two LPCs.

4.3.4.1 Soluble nitrogen content

The soluble N content of AAP-0 and HC-0 after *in vitro* protein digestion is shown in Figure 4.6. The solubility of both LPCs increased with the prolongation of digestion time. During the stomach phase of digestion, the soluble N content of both LPCs slightly increased after

digestion for 30 and 60 minutes, which was significantly different (p <0.05) compared to the starting point (0 minutes). However, the solubility of both AAP-0 and HC-0 increased significantly (p <0.05) during the small-intestinal digestion phase (Figure 4.6). This might be because prolonging time allowed pepsin to act the peptide bonds, causing protein hydrolysis and higher solubility to be detected.

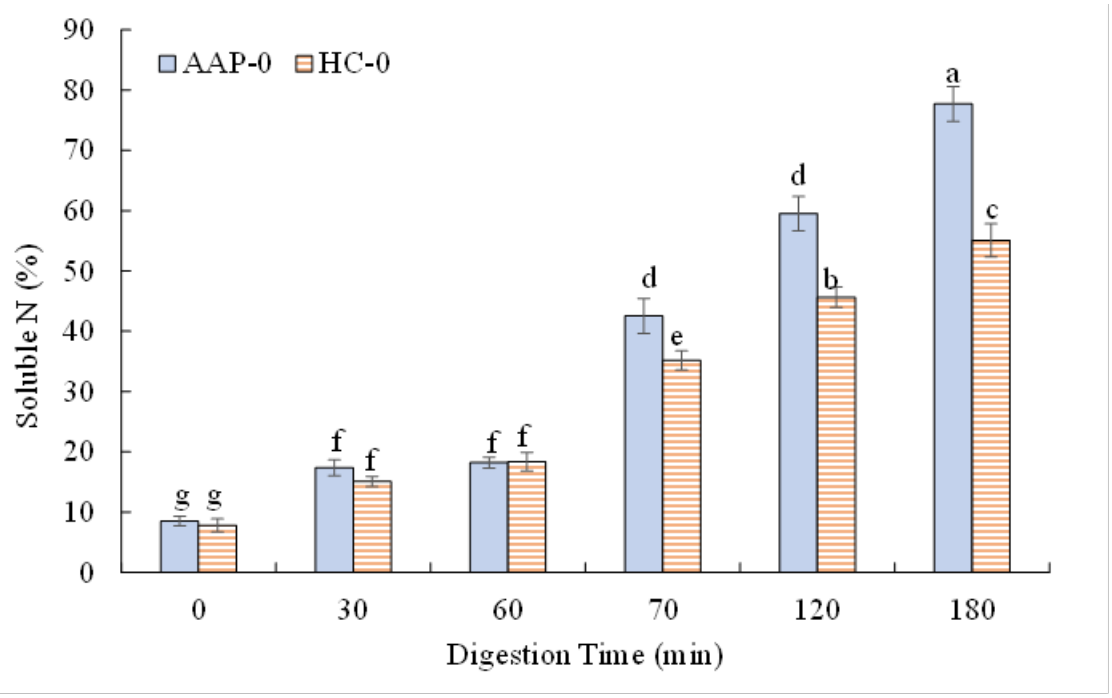


Figure 4.6: Soluble nitrogen content (%) of leaf protein concentrate after digestion at 0, 30, and 60 minutes of gastric digestion, and 70, 120 and 180 minutes of small-intestinal digestion.

Means (n = 3) that do not share a letter are significantly different (p < 0.05) (Tukey's HSD multiple comparisons and 95% confidence). HSD (Honest Significance Test).

AAP-0, LPC extracted through alkali-acid precipitation with 0mM $CaCl_2$.

HC-0, LPC extracted through heating at 50 °C, 15 minutes with 0 mM $CaCl_2$ and heat coagulation.

Nevertheless, a one-way analysis of variance shows that the solubility of HC-0 during the small-intestinal digestion was significantly lower (p <0.05) than AAP-0. A similar result was reported that the digestibility of cooked lentils and faba beans was impaired after thermal treatment. The low digestibility observed in these legumes might relate to protein aggregation due to the heat coagulation process (Carbonaro et al., 1997). Additionally, the lower solubility of the HC-0 sample, as shown in Figure 4.1, might associate with protein digestibility. Duodu et al. (2003) reported that low digestibility observed in cooked sorghum protein is potentially related to a disulphide crosslink that appears after cooking—resulting in less soluble kafirin

protein (a type of sorghum protein). Thus, irreversible protein aggregation and low solubility might result in a low soluble N content of HC-0.

4.3.4.2 Free amino nitrogen

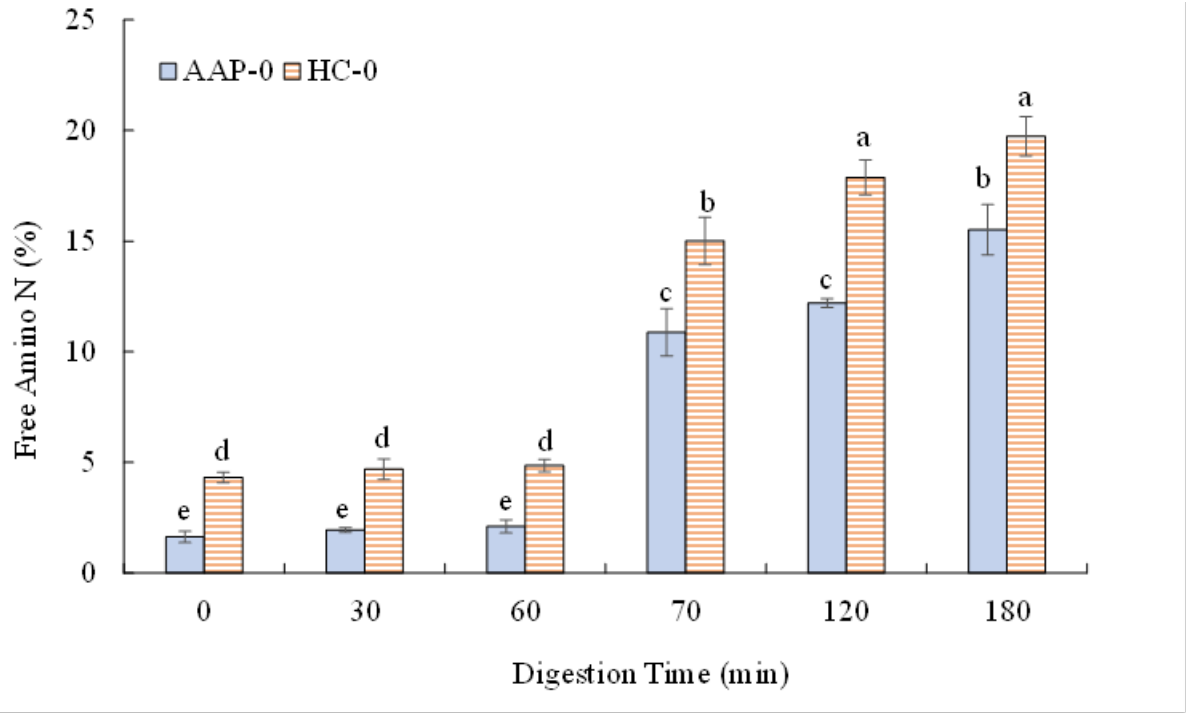


Figure 4.7: Free amino nitrogen content (%) of leaf protein concentrate after digestion at 0, 30, and 60 minutes of gastric digestion, and 70, 120 and 180 minutes of small-intestinal digestion.

Means (n = 6) that do not share a letter are significantly different (p < 0.05) (Tukey's HSD multiple comparisons and 95% confidence). HSD (Honest Significance Test).

AAP-0, LPC extracted through alkali-acid precipitation with 0mM $CaCl_2$.

HC-0, LPC extracted through heating at 50 °C, 15 minutes with 0 mM $CaCl_2$ and heat coagulation.

Figure 4.7 shows that leaf protein concentrate extracted through alkali-acid precipitation (AAP-0) had protein digestibility significantly lower (p <0.05) than HC-0 throughout the digestion, which is inconsistent with the result of soluble N, as shown in Figure 4.6. This result might be relevant to the purity of RuBisCo and the effect of the thermal process.

Many studies have reported that a purified RuBisCo has good digestibility (De Jong & Nieuwland, 2011; Ofori-Anti et al., 2008): it could be digested faster than β-lactoglobulin, bovine serum albumin, ovalbumin, jack bean concanavalin, and egg lysozyme (Ofori-Anti et al., 2008). As shown in Figure 4.5, large and small subunits of RuBisCo were found in HC-0.

In comparison, the small subunits were not detected in the AAP-0. This indicates that the purity of RuBisCo in HC-0 may be higher than in AAP-0. Furthermore, high protein digestibility depends on the ease of access of digestive enzymes to peptide bonds, which is enhanced by a lower amount of non-proteinous compounds (Aderinola et al., 2020). Therefore, the higher *in vitro* protein digestibility of HC-0 would be due to protein purity and low non-protein materials.

Moreover, the thermal process has been reported to increase the digestibility of legume seed proteins (Park et al., 2010). The thermal process leads to protein denaturation, enhancing pepsin to act on the susceptible sites of denatured proteins. Consequently, protein digestibility would be higher. Additionally, the heating process inactivates protease inhibitors (Carbonaro et al., 1997). Therefore, a higher protein digestibility observed in HC-0 might be attributed to the thermal process that denatures proteins and inactivates protease inhibitors, enhancing the accessibility of the enzyme.

The lower *in vitro* protein digestibility of AAP-0 might be due to non-proteinous components. Kaur et al. (2021) stated that a low digestibility of grass protein concentrate (GPC) was related to non-protein materials since a high amount of dietary fibre was detected in GPC extracted through the acid precipitation method. In agreement with Eggum (1995), dietary fibre affects the process in the gastrointestinal tract, decreasing protein digestibility. Hence, it may imply that the low protein digestibility observed in AAP-0 was attributed to the presence of non-protein materials.

4.3.4.3 SDS-PAGE result of digested leaf protein concentrate

The digests of AAP-0 obtained at 0, 30, 60, 70, 120, and 180 minutes are displayed from left to right, along with the protein standard marker (Figure 4.8). In addition, the digests of HC-0 obtained after the same digestion time are presented in Figure 4.9.

At 0 minutes of digestion, a protein band at MW above 250 kDa was observed in AAP-0 and HC-0, indicating that protein complexes could not migrate into the gels. This protein could be the main RuBisCo protein in which the MW is about 550 kDa (Barbeau & Kinsella, 1988). Proteins at 50 kDa representing large subunits of RuBisCo were detected in both samples, but a lower intensity was detected in AAP-0. Proteins with 25 and 14 kDa, which could be Fraction-II protein and small subunits of RuBisCo, were observed only in the HC-0. These results accord with the results observed in raw LPC, as shown in Figure 4.5. Thus, these results could suggest

that proteins might be undigested at the beginning of digestion, which was accordant with a low amount of soluble N (Figure 4.6) and free amino N (Figure 4.7).

The 250 kDa protein band intensity observed in AAP-0 and HC-0 decreased at 60 minutes of digestion. This indicates that proteins of both LPCs could be digested in the gastric phase and migrate into the gel when digestion time increased. The lower intensity of other proteins also was observed during gastric digestion. However, complete digestion of AAP-0 and HC-0 could not be obtained in the gastric phase as some proteins were detected in the intestinal digestion phase (70 to 180 minutes). Thus, it could suggest that proteins of both LPCs are partly broken down in the stomach.

The intensity of protein bands at MW of 100 and 250 kDa observed in AAP-0 was lighter as the intestinal digestion time increased; however, small MW proteins (10 kDa) were detected at 120 and 180 minutes (Figure 4.8). In comparison, the intensity of protein bands at the same MWobserved in HC-0 was lighter than in AAP-0 and disappeared at 180 minutes (Figure 4.9). These results suggest that both LPCs could be digested in the intestinal tract. However, HC-0 might have a higher digestibility than AAP-0 as some proteins (10 kDa) still were observed in AAP-0 at 180 minutes. This could be supported by a higher content of free amino N of HC-0 than the result obtained from AAP-0, as shown in Figure 4.7. Also, a higher digestibility of HC-0 might be due to the purity of RuBisCo and protein denaturation induced by thermal treatment.

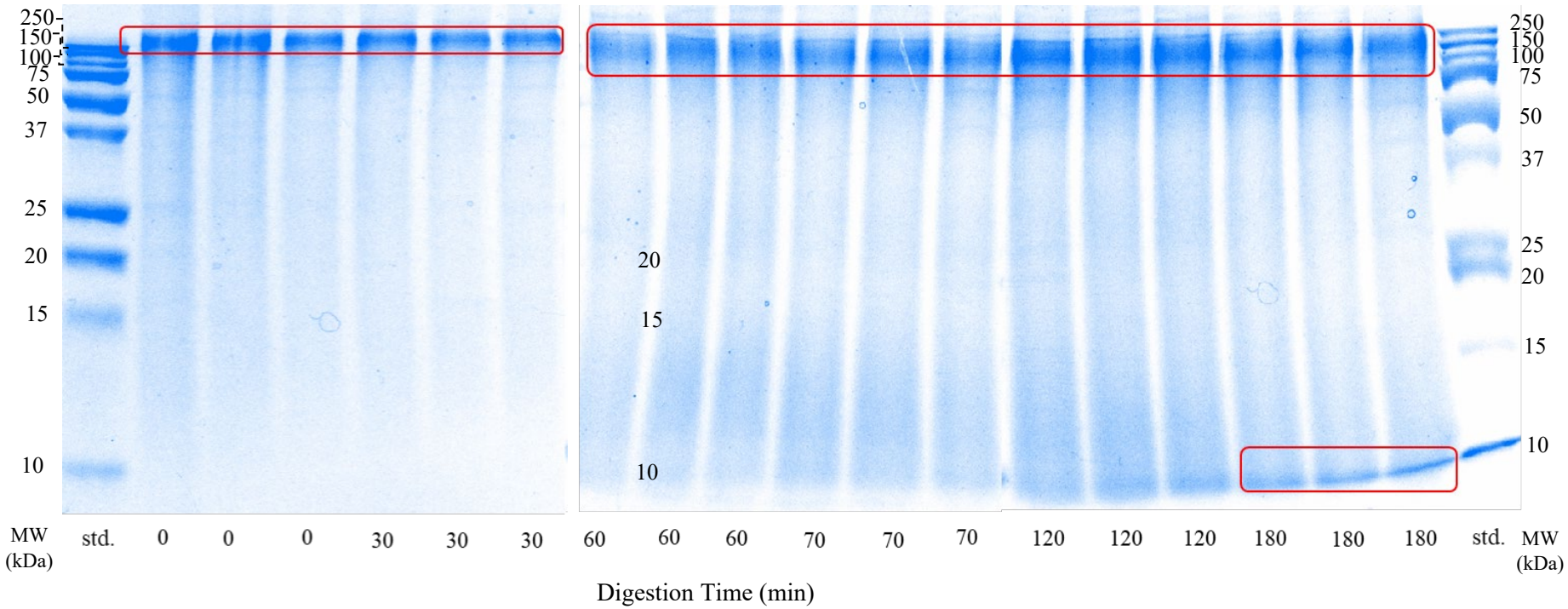


Figure 4.8: Tricine-SDS-PAGE result of leaf protein concentrate: Through alkali-acid precipitation with 0 mM $CaCl_2$ after digestion at 0, 30, and 60 minutes of gastric digestion, and 70, 120 and 180 minutes of small-intestinal digestion.

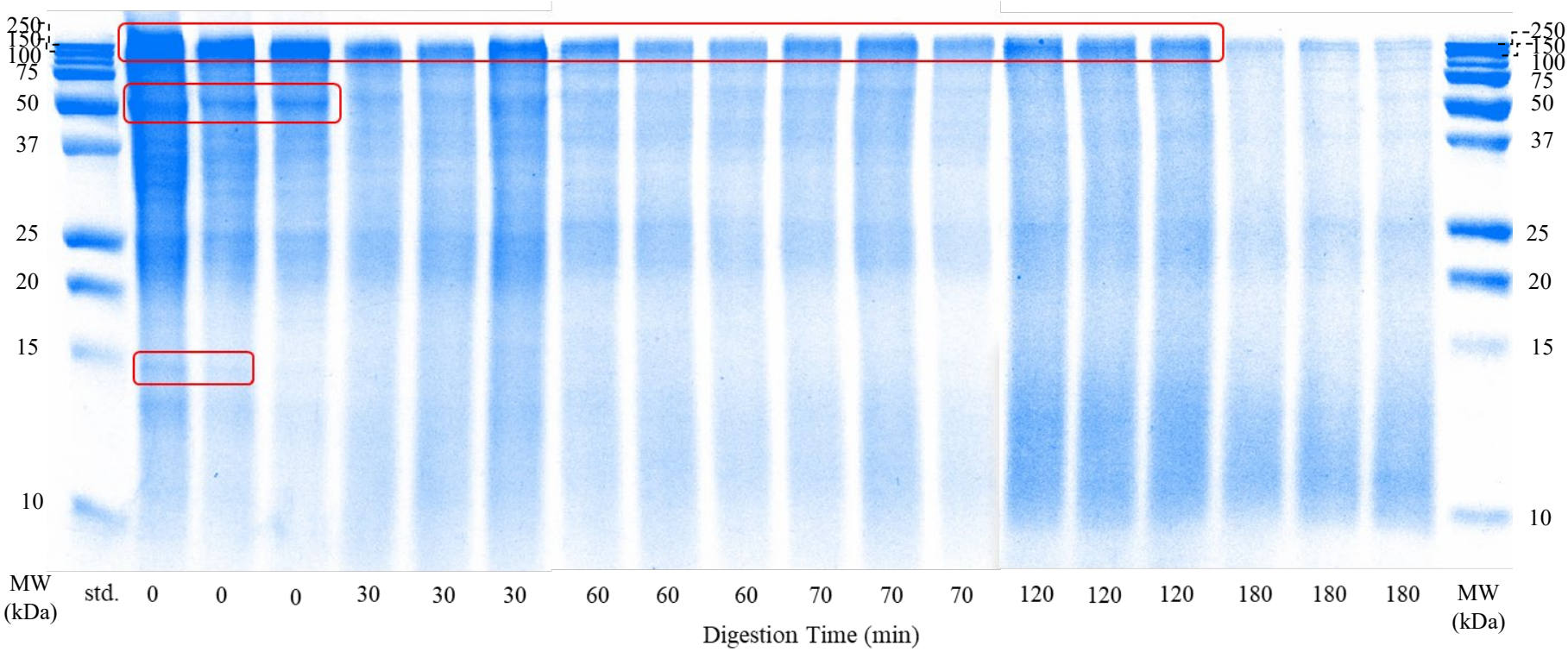


Figure 4.9: Tricine-SDS-PAGE result of leaf protein concentrate: Through heating at 50 °C, 15 minutes with 0 mM $CaCl_2$ and followed by heat coagulation after digestion at 0, 30, and 60 min of gastric digestion, and 70, 120 and 180 min of small-intestinal digestion.

4.4 Final summary

Overall, the protein solubility of both LPCs depends on the pH of the environment. Leaf protein concentrate extracted through heat coagulation (HC-0) had lower protein solubility than the LPC obtained by the isoelectric precipitation method (AAP-0). The lowest solubility of both LPCs was found at pH 3 to 5, which is accordant with the isoelectric point of isolated RuBisCo from other plant sources. High temperature could improve the protein solubility of AAP-0, and a significant improvement (p <0.05) was observed after high-temperature treatment combined with the pH-shifting process. However, less impact was observed in the HC-0 sample. Thermal denaturation temperature and enthalpy of HC-0 were lower than AAP-0 due to the effect of heat coagulation used for concentrating proteins. SDS-PAGE results have shown that both LPCs contain various protein types; however, higher purity of RuBisCo might be obtained through the heat coagulation method since both large and small subunits were found in the heat coagulated LPC (HC-0).

Protein digestion was determined by measuring the soluble N content, free amino N, and the protein MW distribution of digested samples. Overall, these three results have shown that the protein digestibility of AAP-0 and HC-0 increased as the digestion time increased. A lower solubility was observed in HC-0 than in AAP-0 due to the protein aggregation induced by thermal coagulation. Also, a lower protein solubility of HC-0 (section 4.3.1) may impair its protein digestibility. Nevertheless, the free amino N (%) of HC-0 was higher than AAP-0 through digestion, which is in accordance with the SDS-PAGE result, indicating that the heat-coagulated LPC had higher digestibility than AAP-0. This might be attributed to the purity of RuBisCo and protein denaturation. Therefore, the present results could confirm that the protein extraction method affects the physico-chemical of the isolated proteins.

CHAPTER 5

Conclusions and Recommendations

5.1 Conclusions

5.1.1 Assessment of extraction methods

The present study verifies that colourless and odourless LPC could not be obtained through the alkali-acid precipitation method and alkali-acid precipitation combined with activated carbon. Nevertheless, the high protein content and extraction yield were obtained. A suitable heating condition to fractionate chloroplastic proteins (green protein fraction) was 50 ℃ for 15 minutes to obtain the LPC without green colour. Subsequently, the clarified grass juice could be concentrated to enrich the protein content in the final LPC. Heat coagulation at 80 ℃ for 30 seconds could enrich the protein content in LPC at 48.16 % (dry basis). In comparison, ultrafiltration and chromatography used in this study were unsuitable for the protein purification method since the isolated proteins from both methods contained low protein and low recovery yield.

5.1.2 Assessment of relevant factors of isolated leaf protein concentrates

Various factors affect the quality and quantity of isolated proteins. $CaCl_2$ partially removed the green colour and decreased the grassy flavour; however, high concentration resulted in low protein content and yield. The high temperature (58 ℃ for 2 minutes) used for chlorophyll fractionation decreased protein recovery due to protein denaturation. Moreover, this study proved that combining activated carbon with the extraction process could reduce the grassy flavour of LPC. The pH of the solution is important as it relates to the charge of proteins. pH lower than 6 may lead to RuBisCo co-precipitation as this pH is near its pI. Alkali-aid extraction using NaOH increased the protein extractability, increasing protein recovery through acid precipitation; however, the green colour remained in the final product as the solubility of chlorophyll increased. In contrast, high protein content was not obtained when using alkali-aid extraction with heat coagulation since the green protein fraction was not recovered by heat coagulation. The long processing time and high temperature (room temperature) were speculated as the reasons for protein hydrolysis, causing protein loss in ultrafiltration and chromatography study.

5.1.3 Assessment of ethanol extraction

Ethanol extraction was conducted in the present study to remove the green colour and reduce the grassy flavour from the grass leaves and LPC. The results confirm that the green colour and grassy flavour of both samples decreased after ethanol treatment; however, brown colour was observed instead. The protein content of both samples increased, enhancing protein enrichment in the final products. Nevertheless, this method led to approximately a 50 % product yield loss. Although ethanol extraction can improve off-flavour and protein content, the final products with brown colour might be unsuitable for food application. Furthermore, a large amount of ethanol is required for this method, requiring a large-scale process.

5.1.4 Assessment of physico-chemical and functional properties of the selected leaf protein concentrates

Leaf protein concentrate (LPC) from alkali-acid precipitation (AAP-0) and heat treatment at 50 ℃ for 15 minutes, followed by heat coagulation (HC-0), were selected to represent the LPC with and without green pigment, respectively. The present study compared the protein solubility, denaturation temperature, protein types, and digestibility of both LPCs to evaluate the effect of extraction methods. Overall, the different extraction methods affected the properties of proteins. The AAP-0 had higher protein solubility than HC-0 as a function of pH. High temperature combined with pH shifting treatment significantly improved (p <0.05) protein solubility of AAP-0, while the solubility of HC-0 was rarely affected. It could suggest that high temperature combined with pH-shifting treatment would be a promising method to improve protein solubility.

Thermal properties (T_d and enthalpy) of both LPCs were in the range of RuBisCo from other leave sources; however, HC-0 had lower T_d and enthalpy. This was due to protein denaturation by thermal treatment (heat coagulation). SDS-PAGE results show that both LPCs contain various types of proteins. Nevertheless, large and small subunits of RuBisCo were detected only in the HC-0, indicating that the extraction method used for HC-0 isolation might isolate the higher purity of RuBisCo than the alkali-acid precipitation method. The *in vitro* protein digestibility of both LPCs was low in gastric digestion and increased during the small-intestinal digestion phase. The solubility (soluble N) of HC-0 was lower than AAP-0. This might relate to irreversible protein aggregation induced by heat and a lower protein solubility profile. However, a higher free amino N content was observed in HC-0, and the SDS-PAGE result of digested samples shows that HC-0 had a higher digestibility than AAP-0. These two results

show that a higher protein digestibility was observed in HC-0 than in AAP-0. This was due to fewer non-proteinaceous materials in HC-0.

5.2 Recommendations

The present study successfully isolated LPC without green colour and with less grassy flavour by thermal treatment. Green protein fractionation by thermal treatment is recommended to obtain the clear juice; however, the temperature and time may be varied depending on plant type. Protein concentration through heat coagulation affects the solubility of LPC, impacting the digestibility properties. Protein solubility is vital for food proteins as it is relevant to the ease of food application; thus, improvement of protein solubility would recommend for future studies, such as using surfactants. Since the amphiphilic molecules of surfactant can bind the proteins and shield the hydrophobic sites, resulting in protein solubility improvement. Using mild processes, such as ultrafiltration, would be another direction for protein concentration instead of heat coagulation. To be noted, time and temperature should be controlled as the proteases naturally present in plants may hydrolyse proteins, decreasing the recovery of target proteins. The addition of protease inhibitors may be applied to inhibit the enzyme activities. Lastly, innovative technologies (e.g., ultrasound and pulse electric field) or enzymatic-assisted extraction with traditional extraction methods could be explored to improve yield and enrich protein concentration in the final products.

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Appendix

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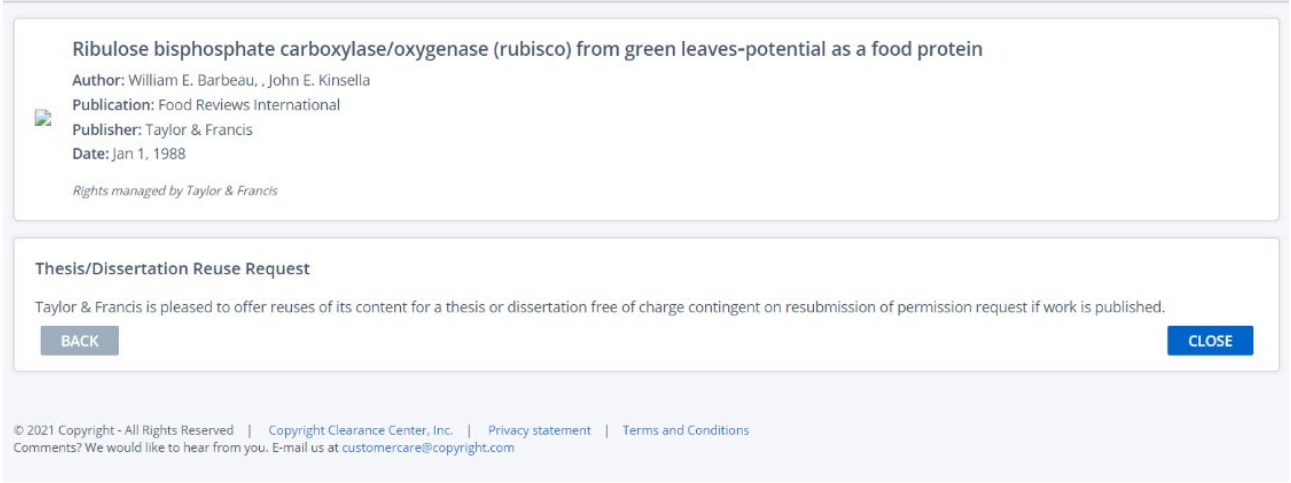
1. Table 2.3: Essential amino acids in RuBisoCo compared with other common protein sources and FAO/WHO reference.



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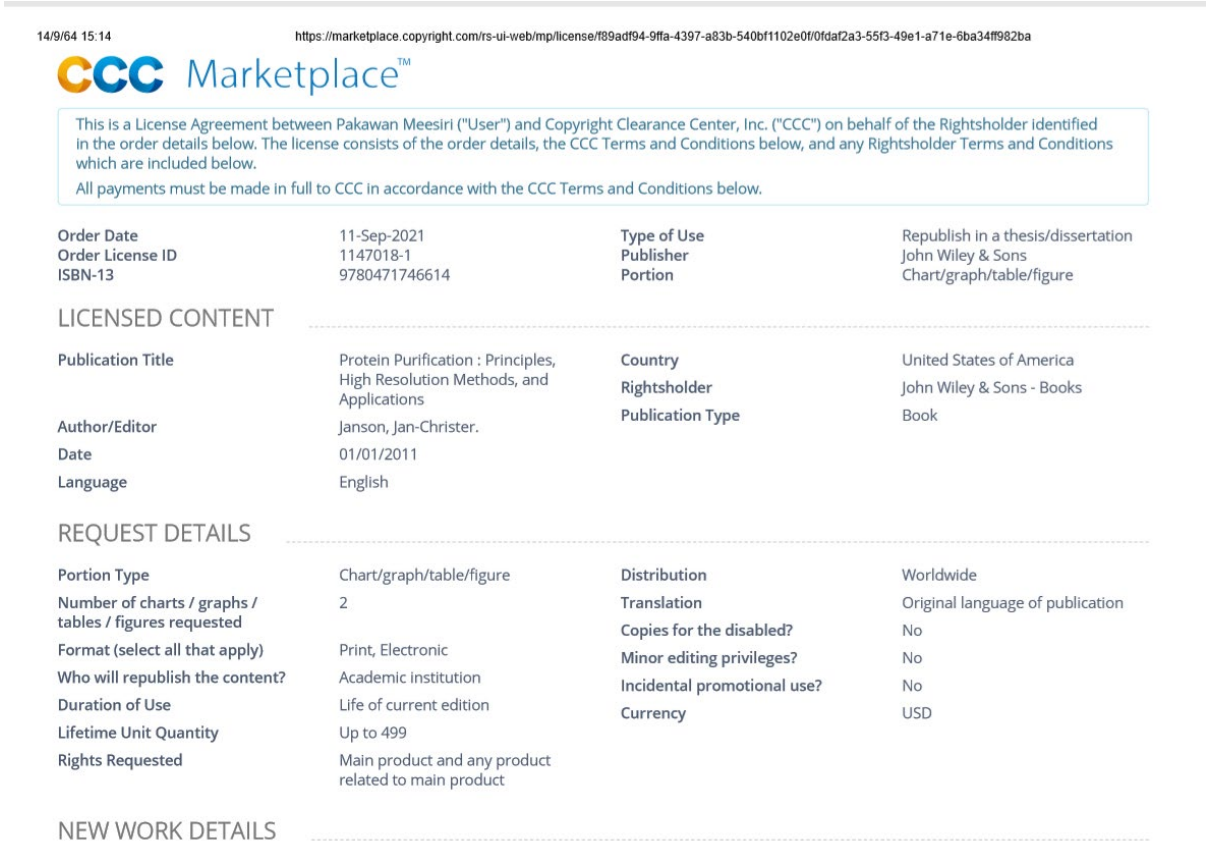
2. Figure 2.2: Schematic flow of tangential filtration (a) and static filtration (b).



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3. Figure 2.4: The Δnormalized RI detector response/Δactivated carbon volume represents the binding capacity of activated carbon as a function of dextran molecular weight

4. Figure 2.5: The effect of pH solution on the binding capacity of activated carbon

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5. Figure 2.6: PPO activities in the pH range 3 to 9 against methyl catechol in red clover (a) and perennial ryegrass (b)

6. Figure 2.7: Protein size distribution of protein extracts from perennial ryegrass and red clover during 24 hours of incubation, as analysed by SDS-PAGE.

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