THE EMULSIFYING PROPERTIES OF CRUCIFERIN-RICH AND NAPIN-RICH PROTEIN ISOLATES FROM *BRASSICA NAPUS* L.

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ABSTRACT

The influence of pH (3.0, 5.0 and 7.0) and ionic strength (0, 50 and 100 mM NaCl) on the physicochemical and emulsifying properties of cruciferin-rich (CPI) and napin-rich (NPI) protein isolates were examined. Specifically, the surface characteristics (charge and hydrophobicity), solubility, interfacial tension and emulsifying activity (EAI) and stability (ESI) indices were measured.

In the case of the cruciferin-rich protein isolate, surface charge was found to be negatively and positively charged at pHs above and below its isoelectric point (~4.6-4.8), respectively, ranging in potential from -33 mV at pH 8.0 to +33 mV at pH 3.0. In the presence of NaCl, the overall magnitude of charge became reduced at all pHs. In contrast, hydrophobicity, solubility and the ability for CPI to reduce interfacial tension all were found to be dependent upon both pH and NaCl concentration. Solubility was found to be lowest at pH 5.0 (~11%) and 7.0 (16%) for CPI without salt, but was significantly improved with the addition of NaCl (>80%). Interfacial tension was found to be lowest (10-11 mN/m) for pH 5.0 – 0 mM NaCl and pH 7.0 – 50/100 mM NaCl. Overall, the presence of salt reduced EAI with increasing levels of NaCl at pH 5.0 and 7.0, but not at pH 3.0. In contrast, ESI became reduced with the addition of NaCl (regardless of the concentration) from ~15.7 min at 0 mM NaCl to ~12 min with 50/100 mM NaCl, from ~14.7 min at 0 mM NaCl to ~11.5 min with 50/100 mM NaCl and from 15.1 min at 0 mM NaCl to ~11.7 min with 50/100 mM NaCl for pH 3.0, 5.0 and 7.0, respectively. ESI also was found to be unaffected by pH.

In the case of a napin-rich protein isolate, surface charge for the NPI in the absence of NaCl ranged between ~ +10 mV to ~ -5 mV depending on the pH, becoming electrically neutral at pH 6.6. The addition of NaCl acted to reduce the surface charge on the NPI and caused a shift in its isoelectric point to pH 3.5 and 3.9 for the 50 and 100 mM NaCl levels, respectively. Overall, surface hydrophobicity for the NPI was reduced as the pH increased, whereas as NaCl levels were raised the hydrophobicity declined. In contrast, NPI solubility was found to be high (~93-100%) regardless of the solvent conditions. The ability of NPI to reduce interfacial tension was enhanced at higher pHs, however the effect of NaCl was pH dependent. Overall, EAI values were similar in magnitude at pH 3.0 and 5.0, and lower at pH 7.0. The effect of NaCl on EAI was similar at pH 3.0 and 7.0, where EAI at the 0 mM and 100 mM NaCl levels were similar in

magnitude, but increased significantly at 50 mM NaCl. However, the EAI values at pH 5.0 were reduced as the level of NaCl increased. Overall, the stability of NPI-stabilized emulsions degraded rapidly and the addition of salt induced faster emulsion instability.

In summary, CPI and NPI were very different in terms of their physicochemical properties. However, the emulsifying properties were similar in magnitude indicating that they had similar emulsifying potential under the solvent conditions examined.

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LIST OF ABBREVIATIONS AND SYMBOLS

CPI	Cruciferin-rich protein isolate
NPI	Napin-rich protein isolate
SPI	Soy protein isolate
W/O	Water-in-oil
O/W	Oil-in-water
O/W/O	Oil-in-water-in-oil
W/O/W	Water-in-oil-in-water
FAO	Food and Agriculture Organization of UN
UN	United Nation
SHMP	Sodium hexametaphosphate
EAI	Emulsifying activity index
ESI	Emulsifying stability index
ES	Emulsion stability
ANOVA	Analysis of variance
HPLC	High performance liquid chromatography
CFC	Critical flocculation concentration
IP	Isoelectric precipitation
PMM	Protein micellar mass
SDS	Sodium dodecyl sulfate
PAGE	Polyacrylamide gel electrophoresis
ANS	8-anilino-1-naphthalenesulfonic acid
PA	Phytic acid
GRAS	Generally recognized as safe
pI	Isoelectric point
S	Svedberg Unit
U	Separation rate (mm/day)
R	Radius of droplet
$\Delta\rho$	Difference of density
η	Viscosity

ζ	Zeta potential
ε	Permittivity
nm	Nanometer
µm	Micrometer
cm	Centimeter
mm	Millimeter
m	Meter
^{o}C	Degree Celsius
µmol	Micromole
mM	Millimolar
M	Molar
N	Normality
min	Minute
h	Hour
kDa	Kilodalton
p.s.i.	Pascal per square inch
mg	Milligram
µg	Microgram
g	Gram
kg	Kilogram
mL	Milliliter
L	Liter
rpm	Revolution per minute
g	Gravitational acceleration
mS	MilliSiemens
w/v	Weight to volume
v/v	Volume to volume
w/w	Weight to weight
U_E	Electrophoretic mobility
µL	Micro liter
s	Second

mN	Milli-Newtons
w.b.	Wet basis
mV	Millivolt
A.U.	Arbitrary units
mol	Mole

CHAPTER 1: INTRODUCTION

Canola was originally bred from rapeseed varieties (e.g., *Brassica napus* L.*)* to have low levels of erucic acid (<2%) and glucosinolates (<30 μmol/g) defatted meal for use as an edible healthy oil (Canola Council of Canada, 2011). Canola meal, the co-product of oil processing, is rich in protein (36- 39%) and crude fibre (~12%), and to- date is commonly used in low cost livestock feed for its nutritional value (Khattab and Arntfield, 2009; Newkirk, 2009). The meal also contains high levels of phenolic compounds and phytic acid which can lead to poor protein functionality depending on the extraction method used (Wu and Muir, 2008; Aider and Barbana, 2011). However, research surrounding adding value to the under-utilized and under-valued meal has intensified recently, particularly as it relates to the protein fraction. Despite its well-balanced amino acid profile (Ohlson and Anjou, 1979), the utilization of canola protein by the food industry has been limited due to its poorer functionality compared to animal-derived protein ingredients. Depending on the canola variety used, processing practices and methods of extraction, protein functionality can vary considerably (Aluko and McIntosh, 2001; Khattab and Arntfield, 2009; Can Karaca et al., 2011). Successful processing innovations and product characterization could lead to the development of a new plant sourced protein food ingredient.

Canola proteins are dominated by two types of protein: cruciferin and napin. Cruciferin (12 S (Svedberg Unit); molecular mass ~240-300 kDa) is a salt-soluble globulin with a isoelectric point (pI) of ~ 7.2 (Schwenke, 1994), and is comprised of high level of β-sheets (~50%) and low level of α-helices (~10%) (Zirwer et al., 1985). Napin (1.7-2.0 S; molecular mass ~12-14 kDa) is a water soluble albumin and comprised of higher level of α-helices (~45%) than β-sheets (~12%) (Crouch et al., 1983; Schwenke, 1994). Cruciferin and napin constitute roughly 70% and 30% of the total storage protein, respectively (Krause and Schwenke, 2001; Dong et al., 2011; Tan et al., 2011). Over the past few decades, there have been a number of studies looking at means to improve the functional attributes of canola protein isolates through chemical modifications (Gruener and Ismond, 1997a,b), enzymatic cross-linking (Pinterits and Arntfield, 2008) or through controlled protein-polysaccharide interactions (Klassen et al., 2011).

However, success has been limited. Most of these studies primarily looked at mixed cruciferin/ napin isolates rather than the individual fractions. The present research takes a structure-function approach to understand the emulsifying properties of two separate protein isolates, one rich in cruciferin and one rich in napin type proteins.

Emulsions consist a mixture of two (or more) immiscible liquids formed after an input of mechanical energy (e.g., homogenization), where one liquid becomes dispersed as small droplets within a continuous phase of the other (Hill, 1996; McClements, 2005). The stability of protein-stabilized emulsions is dependent upon protein characteristics (e.g., globular vs. fibrous, conformational entropy, molecular weight), surface properties (e.g., hydrophobic and hydrophilic residues), processing (e.g., shear) and solvent properties (e.g., temperature, pH and salts). During emulsion formation, soluble proteins diffuse towards the interface, then re-arrange and re-organize at the interface to orient hydrophobic amino groups towards the non-polar oil phase and the hydrophilic amino groups toward the aqueous polar phase in order to reduce interfacial tension and form a viscoelastic film (Dalgleish, 1997). The viscoelastic film typically induces an electric charge on the emulsion droplet, which depending on the pH may lead to attractive or repulsive forces between neighboring droplets (Tcholakova et al., 2002; McClements, 2004). At solution pHs close to the pI of the protein, emulsion droplets would exert little to no repulsive charge leading to flocculation and/or aggregation due to hydrophobic interaction, followed by partial or complete coalescence (Xu et al., 2005). In contrast, at pHs away from the pI, proteins in the interface may exert a repulsive force between neighboring droplets to keep the emulsions stable. The addition of NaCl or other salts can cause shielding of the repulsive charge on the droplets, inducing droplet flocculation even if the solution pH is away from the pI. To date, mixed information is found in the literature regarding the emulsifying properties of napin. Krause and Schwenke (2001) reported napin to be highly surface active and capable of forming emulsions, whereas Malabat et al. (2001) reported neither native nor chemically modified napin fraction (acylation and sulfamidation) can form stable emulsion even though the hydrophobicity of napin was increased in the process.

The overall goal of this research was to better understand structure-function relationships driving the stability/instability of oil-in-water emulsions prepared using both a cruciferin-rich and napin-rich protein isolate as a function of pH and NaCl. Findings could lead to improved

utilization of canola protein products in the food industry, as their performance as food ingredients would be more understood.

1.1 Objectives

In order to achieve the overall goal of this research, the following objectives will be tested on a CPI and NPI separately.

- To investigate changes to the physicochemical properties (surface charge, surface hydrophobicity, solubility and interfacial tension) of a CPI and NPI in response to medium pH (3.0, 5.0 and 7.0) and NaCl concentration (0, 50 and 100 mM).
- To investigate changes to the emulsifying properties (emulsifying activity and stability indices) of a CPI and NPI in response to medium pH (3.0, 5.0 and 7.0) and NaCl concentration (0, 50 and 100 mM).

Similarities and differences between the two isolates will then be discussed.

1.2 Hypotheses

- It is hypothesized that the surface charge of NPI will carry a positive charge over the tested pH range (3.0-7.0), and has an overall higher net charge than CPI. In contrast, the net charge of CPI should become reduced at pHs closer to its isoelectric point.
- It is hypothesized that the overall surface hydrophobicity of CPI will be greater than NPI, however the effects of pH and salt on the surface hydrophobicity on each isolate is unpredictable since the effect of solvent condition to the molecular conformation is yet to be determined.
- It is hypothesized that protein solubility of NPI will be high over complete pH range, whereas CPI will be lower in magnitude and be more influenced by pH. The addition of NaCl will improve the solubility of both NPI and CPI.
- It is hypothesized that NPI will be more surface active (charge and hydrophobicity) (than CPI, allowing it to be a more effective emulsifier, leading to higher emulsification activity and stability indices than CPI. The addition of NaCl is hypothesized to improve the emulsifying properties for both proteins.

CHAPTER 2: LITERATURE REVIEW

2.1 Food emulsions

Emulsions consist of a mixture of two (or more) immiscible liquids formed after an input of mechanical energy (e.g. homogenization) where one liquid becomes dispersed as small droplets within a continuous phase of the other (Hill, 1996; Schultz et al., 2004; McClements, 2005). Depending on the ingredient formulation and processing conditions, various structures, flavor release and textural attributes can be achieved in foods by forming emulsions, leading to improved organoleptic quality for consumers. Physicochemical and sensory attributes of the emulsion product are controlled by tailoring characteristics of the dispersed droplets, such as concentration, size distribution, surface charge and level of interactions (e.g., flocculation, aggregation, and coalescence) (McClements, 2007). In food emulsions, droplet sizes typically range between 0.1 to 100 μm in diameter, and are classified either as water-in-oil (W/O) (e.g., margarine and butter) and oil-in-water (O/W) (e.g., salad dressings, ice cream, milk, soups, dips/sauces and beverages) emulsions based on their continuous phase. Emulsion-based technology is also important in terms of drug or bioactive molecule delivery where multiple emulsions (e.g., O/W/O or W/O/W) or nanoemulsions are used for carrying, protecting and releasing sensitive bioactive ingredients (Garti and Bisperink, 1998; Cho and Park, 2003).

Emulsions are typically formed under high shear conditions using homogenizers, high pressure valve homogenizers, microfluidizers or high shear mixers (McClements, 2005, 2007). However, they are inherently unstable and tend to separate over time due to various mechanisms of instability. The rate of which depends on many factors, including droplet characteristics (size distribution, surface charge and level of interactions), continuous phase viscosity, the presence of emulsifiers and solvent conditions (e.g., presence of salts, temperature and pH) (Dickinson and Stainsby, 1988; Wu and Muir, 2008; Can Karaca et al., 2011). The Stoke's law has commonly been used to describe the rate of gravitational separation of dispersed droplets within the continuous phase. Stoke's law is as follows (eq. 2.1),

$$U = -2gr^2\Delta\rho / 9\eta \qquad \text{(eq. 2.1)}$$

where, U is thecreaming rate (mm/day), g is the gravitational acceleration (9.81 m/s^2), r is the radius of droplet, $\Delta\rho$ is the density difference between the continuous and dispersed phase and η is the viscosity of the continuous phase for oil in water emulsion.

Emulsifiers are surface active molecules or macromolecules that absorb to the surface of the dispersed droplets forming a viscoelastic film or coating that prevent aggregation (Dalgleish, 1997; McClements, 2007). Examples of emulsifiers include low molecular weight surfactants (e.g., Tween, Span), phospholipids (e.g., lecithin), and biopolymers (e.g., polysaccharides and proteins) (Friberg et al., 2004). Emulsifiers act to reduce interfacial tension between the oil and water phases by positioning their hydrophobic amino acid group within the oil phase and hydrophilic amino acid groups within the polar phase. Emulsifiers act by reducing the energy needed to form an emulsion, and then delay the likelihood of phase separating into two immiscible phases. Macromolecules such as polysaccharides and proteins can act to raise the continuous phase viscosity or form a gel network, significantly inhibiting Brownian motion and emulsion droplet interactions (McClements, 2007). Proteins, are either filamentous (e.g., casein and gelatin) or globular in nature, and comprise of both hydrophobic and hydrophilic amino acid residues at their surface making it capable of interacting with both the aqueous and lipid phases. Protein at the interface sometimes form “loops” and “tails” that can also provide steric hindrance which physically prevent the emulsion droplets come into close proximity of each other depending on the conformation, protein molecular size and amino residues of the protein. (Damodaran, 2005).

2.2 Mechanisms of emulsion instability

As previously discussed, emulsions are inherently unstable comprised of two (or more) thermodynamically incompatible phases, which overtime moves towards a more energetically favorable state that minimizes Gibb’s free energy within the system (Gupta and Muralidhara, 2001; Tolstoguzov, 2003). In this state, oil and water phases are completely separated into distinct layers to give the least amount of contact surface area, rather than having one dispersed within the other. Thermodynamic equilibrium in emulsions is maintained with the addition of

emulsifiers, which act to minimize the driving energy to phase separation (i.e., reduces interfacial tension between the two phases) (Damodaran, 2005).

Food emulsions become unstable due to various interconnected processes including gravitational separation (i.e., creaming/sedimentation), flocculation, coalescence (or partial coalescence), Ostwald ripening (also called disproportion) and phase inversion (McClements, 2007). These mechanisms are each summarized in Figure 2.1. In brief, gravitational separation refers to either an upward migration of droplets to the surface to form a cream layer, in the case of creaming, or the downward migration of droplets to form sediment, in the case of sedimentation. Migration rate and separation depends on density differences between the two phases, and the level of droplet aggregation, whereby larger droplets either cream or sediment at much faster rates than smaller droplets (McClements, 2007). Aggregation of droplets may be reversible in the case of flocculation, where adjacent droplets stick together but remain distinct as their viscoelastic membranes which encase the droplets. In the case of coalescence or partial coalescence, membranes surrounding adjacent droplets become ruptured leading to complete or partial exchange of dispersed materials and the formation of one larger irregular sized droplet (McClements, 2007). Ostwald ripening (more common in foams) occurs based on the diffusion of the dispersed phase from a small droplet through the continuous phase to merge into larger droplets. The process is also known as disproportion. In the case of phase inversion, an oil-in-water emulsion will invert into a water-in-oil emulsion or vice versa (McClements, 2007).

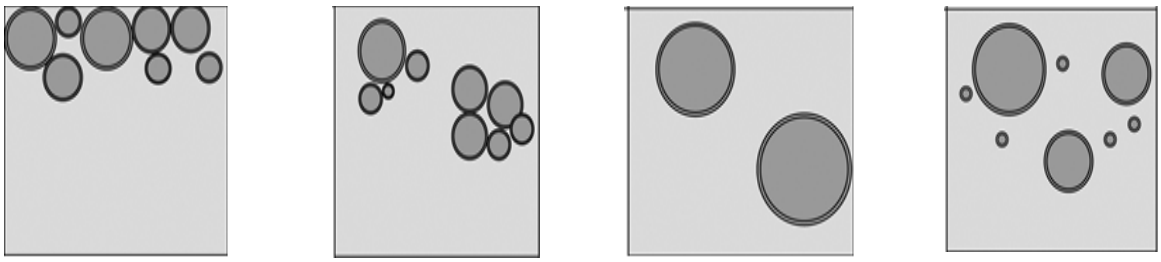

Figure 2.1 Schematic diagram describing mechanism for emulsion instabilities (modified from Robins and Wilde, 2003).

2.3 Protein-stabilized emulsions

The stability of protein-stabilized emulsions is dependent upon the type of protein (e.g., globular vs. fibrous, conformational flexibility, molecular weight) and surface (e.g., hydrophobic and hydrophilic residues) characteristics, processing conditions (e.g., shear and heat) and solvent properties (e.g., temperature, pH and salts). During emulsion formation, soluble proteins diffuse towards the interface, then re-arrange and re-organize at the interface to orient hydrophobic amino groups towards the non-polar oil phase and the hydrophilic amino groups towards the aqueous phase forming a viscoelastic film (Dalgleish, 1997) (Figure 2.2a). This process is highly depended on the molecular flexibility and packing of the protein (Freer et al., 2004). Film strength is then enhanced via protein-protein interactions, hydrogen bonding and electrostatic interactions and van der Waals attractive forces. Moreover, the addition of macromolecules such as protein would increase the overall viscosity of the medium and restrict random movements of the oil droplets. In some studies cross-linking agents (e.g., transglutaminase) is added to improve stability (Fñrgemand et al., 1998; Dickinson et al., 1999) of O/W emulsions.

Although the majority of the hydrophobic groups are buried within the interior of the 3-dimensional structure, some remain on the surface amongst the hydrophilic residues as hydrophobic patches. As such, a prerequisite to achieving good emulsion stabilization is partial or complete denaturation or unraveling of the protein to expose reactive non-polar sites (Damodaran, 2005). Depending on the primary structure, and the spatial arrangement of the protein at the interface, tails or loops comprised of protein chains may extend into the aqueous solution leading to steric forces or interactions. In contrast, low molecular weight surfactants tend to form micelles in the aqueous phase, and diffuse towards the interface at a much faster rate (Figure 2.2b). Alignment at the interface tends to result in complete coverage rather than having irregular intermittent breaks in the viscoelastic films due to the presence of loops or tails, or from incomplete absorption to the oil-water interface.

Protein absorbed to the interface often forms a thin film around the dispersed droplet. The viscoelastic film typically provides an electric charge to the droplet, which depending on the pH, may lead to attraction or repulsion between neighboring droplets (Friberg et al., 2004). The concentration of protein absorbed at the interface could also affect the film formation, and could have detrimental effects on emulsion stability. For instance, insufficient protein at the interface could lead to a thinner film, which is more susceptible to film rupture or incomplete coating of

the droplet (Tcholakova et al., 2002; McClements, 2004). In turn, this could increase the chance of coalescence when droplets are of close proximity. When there is sufficient protein absorbed at

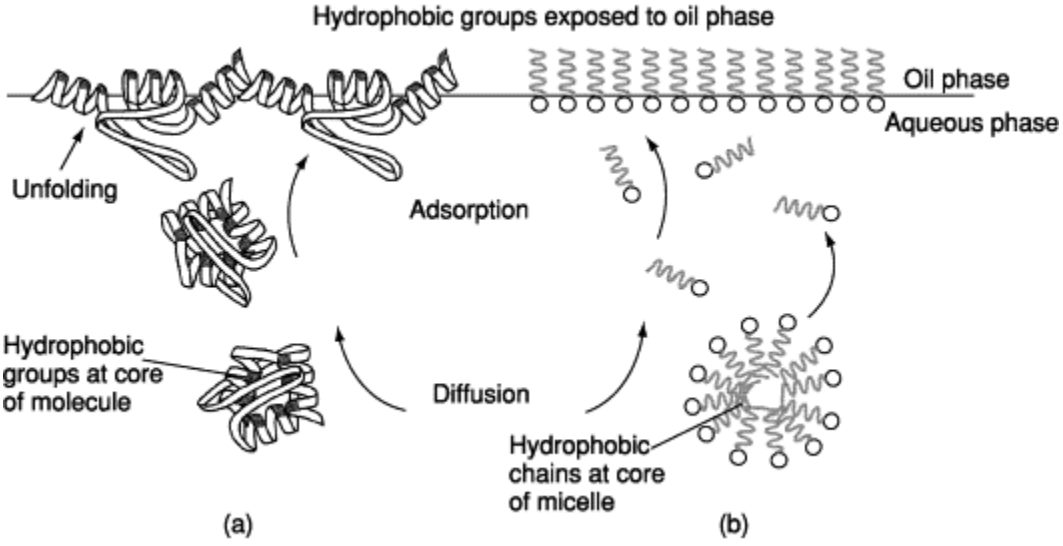

Figure 2.2 Schematic diagram of protein (a) and low molecular weight surfactant (b) absorption to an oil-water interface (reproduced with permission from Robins and Wilde, 2003).

the interface, the stability of emulsion is mainly affected by the mechanical force input to form smaller droplets which could reduce the density differences of the dispersed phase and the continuous phase and delay gravitational separation (McClements, 2004). The relationship of mean droplet size and protein as emulsifier concentration can be illustrated mathematically (eq. 2.2).

$$r_{\min} = \frac{3\Gamma_{sat}\phi}{cs} = \frac{3\Gamma_{sat}\phi}{cs'(1-\phi)} \qquad \text{(eq. 2.2)}$$

Where, Γ_{sat} is the excess surface concentration of the emulsifier at saturation (in kg m^{-2}), ϕ is the disperse phase volume fraction, *CS* is the concentration of emulsifier in the emulsion (in kg m^{-3}) and *CS'* is the concentration of emulsifier in the continuous phase (in kg m^{-3}) (McClements, 2004). Excess protein or protein that cannot be absorbed at the interface could cause depletion flocculation due to competition of solvent around and between the droplets similar with the "salting out" phenomenon (McClements, 2000). A critical flocculation concentration (CFC) has

to be reached before depletion flocculation occurs and CFC value reduces as droplet size increases and protein volume fraction increases (McClements, 2000). Proteins rich in sulfur containing amino acids such as rapeseed proteins, could form disulfide bonds with other protein molecules as the protein unravel at the interface (Wu et al., 2011). Disulfide bonds formed between proteins at the same interface could enhance emulsion stability (Tcholakova et al., 2002). However, disulfide bonds formed between two different interfaces might lead to flocculation and followed by coalescence and it is also known as bridging flocculation (Joshi et al., 2012; Wang et al., 2012). Joshi et al. (2012) also suggested reducing inter and intra disulfide bonding all together could improve overall emulsion stability in a lentil protein stabilized emulsion.

Protein stabilized emulsions are most stable at pHs away from its pI value because of the presence of an electric charge on the oil droplet's surface which acts to repel neighboring droplets. In contrast, when solution pH is close to the pI of the protein, electrostatic repulsive forces are minimal between droplets enabling them to flocculate or undergo partial or complete coalescence (Xu et al., 2005; Foegeding and Davis, 2011). Larger droplets are then more prone to gravitational separation. Furthermore, protein solubility tends to be reduced near the pI of the protein, also leading to flocculation and/or partial or complete coalescence and subsequent reduced absorption to the oil-water interface (Kinsella, 1979). Often low protein solubility is associated with poor emulsifying properties (Dickinson, 2003; Can Karaca et al., 2011).

Protein stabilized emulsions are also very sensitive to ionic strength, which when exceed certain concentration, emulsion stability can be reduced (McClements, 2004). Multivalent ions such as Ca^{2+}, Mg^{2+},Fe^{2+} or Fe^{3+} are more prone to cause emulsion instability than monovalent ions such as Na^{+}, Cl^{-} or K^{+} because they are more effective at screening electrostatic repulsive forces between surfaces to reduce the zeta potential (ζ), which is a measure of the protein's surface charge (Keowmaneechai and McClements, 2002). Demetriades and co-workers (1997) found that an oil-in-water emulsion stabilized by 2% whey protein was unstable when pH was close to pI of whey protein (pH 4.6). The authors also reported the addition of NaCl up to 100 mM resulted in large droplet sizes, and high levels of flocculation and creaming. Kulmyrzaev et al. (2000) found emulsions prepared with diluted whey protein isolate (0.5% w/w) showed that the addition of only 20 mM of $CaCl_2$ resulted in 3 times reduction in the zeta potential around the droplets both below and above the isoelectric point of whey protein. The authors also found

emulsion stability was relatively insensitive to $CaCl_2$ (<20 mM) when pH was below the pI of whey protein, however creaming occurred at pH above the pI of whey protein at levels > 5 mM and above $CaCl_2$ (Kulmyrzaev et al., 2000). Solubility and zeta potential of canola protein isolate was also found lowest near the isoelectric point (pH 4-5) and reduced substantially with the addition of 350 and 700 mM NaCl by Paulson and Tung, (1987).

According to the Stokes' Law (eq. 2.1), creaming rate has a reciprocal relationship with bulk phase viscosity. Increasing bulk phase viscosity could reduce the chances of droplet-droplet collision which might induce coalescence (McClements, 2004). Previous studies have shown the addition of sucrose was able to improve the thermal stability of milk protein stabilized emulsions (Kim et al., 2003). The authors also found the addition of sucrose before thermal treatments prevented extensive droplet aggregation, however if the sucrose was added after thermal treatment, it promoted droplet aggregation. The author speculated that sucrose affects emulsion stability mainly by stabilizing the conformation of the adsorbed protein rather than changing the properties of the bulk phase condition since the results showed dependency on the order of addition of sucrose before or after thermal treatment (Kim et al., 2003).

The other important aspect to the emulsifying properties of plant protein is the extraction methods because it can impact the purity, quantity and the conformational structure of the protein extracted (Aider and Barbana, 2011; Can Karaca et al., 2011). For oilseed proteins, the defatting process used to create an oil free meal involves the use of both heat and chemicals, and often leads to partial or complete denaturation of the protein (Khattab and Arntfield, 2009). Table 2.1 provides some brief methodology for extracting proteins from various oilseeds found in literature, along with their emulsifying properties, using emulsifying activity index (EAI) and emulsion stability (ES) as indicators. EAI indicates the area of interface covered per one gram of protein, whereas ES is the measure of creaming after a standard period of time to quantify the ability of the protein film to stabilize the emulsion to delay droplet aggregation.

Many plant protein materials contain undesirable compounds which will affect the organoleptic and/or functional properties of the protein. For instance, oilseed proteins often contain phenolic compounds and phytates that make them undesirable as a human food ingredient because of the inferior organoleptic properties or poor functional properties (Schwenke, 1994; Krause et al., 2002; Wanasundara, 2011). Fortunately, with proper extraction, these undesirable compounds could be reduced to safe levels suitable for human consumption

(Ismond and Welsh, 1992). Krause and co-workers (2002) extracted flaxseed protein isolate with conventional isoelectric precipitation (IP) and protein micellar mass (PMM) methods and found although 11S globulin was the main fraction in both isolates, the isolate produced by IP had lower solubility and EAI compared with the isolate produced by the PMM method. In the same study, isolates produced by the PMM method also achieved much lower phenolic and phytic acid levels. The authors stated that PMM method preserved the protein's native form with minimal amount of undesirable compounds, whereas IP produced isolate might have undergone partial denaturation and irreversible protein aggregation (Krause et al., 2002).

2.4 Protein extraction methods

Canola oil production ranks second only to soy bean among the oilseed crops (Food and Agriculture Organization of UN, 2012). Canola represents a significant economic value to Canada, especially Saskatchewan, which is the major canola growing province along with Alberta. Canola meal, the co-product of oil processing is rich in protein (36-39%) and crude fibre (~12%), which to date is commonly used in low cost livestock feed for its nutritional value (Khattab and Arntfield, 2009; Newkirk, 2009). The meal contains high levels of phenolic compounds and phytic acid which can lead to poor protein functionality depending on the extraction method used due to the interaction between the protein and phenolic compounds and phytic acid (Wu and Muir, 2008; Aider and Barbana, 2011). Depending on the canola variety used, processing practices and methods of extraction, protein functionality can vary considerably (Aluko and McIntosh, 2001; Khattab and Arntfield, 2009; Can Karaca et al., 2011). Successful processing innovations and product characterization could lead to the development of a new plant sourced protein food ingredient.

Table 2.1 Summary of various extraction processes for oilseed protein isolates, and their emulsifying properties reported in literature (modified from Moure et al., 2006).

Protein in oilseeds	**Extraction Solvent/pH/time(h)/temperature ^{o}C**	**Purification**	**EAI (m^2/g)**	**ES (%)**	**Reference**
Almond	20 mM Tris-HCl/8.1/1/25	Dissolve defatted meal. Filter through glass wool, followed by centrifugation. Supernatant is then filtered to remove debris, and dialyzed against 5 L of distilled deionized water. Supernatant is then freeze-dried.	51.77	-	Sze-Tao and Sathe, 2000
Canola	0.1 M NaOH/–/0.33/23	Dissolve defatted meal. Filter with filter paper, adjust to pH 4.0, centrifuge, wash to remove salt, and then centrifuge again to recover the pellet.	28.27	71.0	Aluko and McIntosh , 2001
Canola	0.1 M NaOH/–/0.33/23	Dissolve defatted meal. Filter with filter paper, adjust to pH 6.0, add $CaCl_2$ up to 1 M, and centrifuge. The supernatant is diluted in 200 volume of water to remove salt, and then recover protein after centrifugation.	32.34	26.9	Aluko and McIntosh, 2001
Canola	0.3 M NaCl/-/4/23	Dissolve defatted meal. Centrifuge, filter the supernatant, further concentrate the supernatant by ultrafiltration, and then dilute the supernatant with 6x volume of water, and recover protein micelle by centrifugation.	39.80	68.0	Gruener and Ismond, 1997a,b

Table 2.1 Summary of various extraction processes for oilseed protein isolates, and their emulsifying properties reported in literature (modified from Moure et al., 2006).

Flaxseed	0.5 M NaCl/5.5-6.5/1/25	Dissolve defatted meal and collect clear supernatant. Concentrated supernatant by ultrafiltration, dilute with 5x volume of cold water, and then centrifuge to recover the protein micelle.	2550	80.0	Krause et al., 2002
Flaxseed	Water/8.5/–/25	Dissolve defatted meal. Adjust pH to 4.5 to precipitate the protein, and then centrifuge to obtain protein material.	2100	81.5	Krause et al., 2002
Sesame	1 M NaOH/9.5/1/50	Dissolve defatted meal. Sample is centrifuged, the supernatant liquid is adjusted to pH 4.9, and then stirred for 1 h at 50−55 °C and again centrifuged, the solid residue is collected and dried.	114.33	35.5	Bandyopadhyay and Ghosh, 2002
Soybean	20 mM Tris-HCl/8.1/1/25	Dissolve defatted meal. Filter through glass wool, and then centrifuge. The supernatant is adjusted to pH 4.5 and centrifuged to precipitate the proteins. Proteins were dialyzed against distilled deionized water.	11.61	–	Sze-Tao and Sathe, 2000
Soybean	Acetic acid-acetate buffer/4.5/–/25	Dissolve defatted meal. Protein is fractionated by ultrafiltration with 10, 30 and 50 kDa cut off (centrifugation prior to ultrafiltration is optional) and concentrated using a 5 kDa membrane.	106.7	27.6	Moure et al., 2005

As mentioned previously, extraction and purification methods can cause great variations in the physicochemical and functional properties of the protein isolates. In general, canola protein extraction found in literature could be generalized to be either, alkali extracted followed by acid precipitation (Mieth et al., 1983; Aluko and McIntosh, 2001; Can Karaca et al., 2011) or using the PMM method developed by Murray et al (1980). In the case of the former, NaOH is often used to bring the solvent pH to strongly basic conditions (pH 11-12) in order to have high protein recovery rate (Tan et al., 2011). Sodium hexametaphosphate (SHMP) at pH 7.0 is another alkaline medium used to extract canola proteins followed by acid precipitation (Thompson et al., 1976). Tzeng et al. (1988) found that a canola protein isolate produced by SHMP had better color and taste, but lower protein recovery than if extracted using NaOH. Once the proteins are dissolved, solutions are acidified to bring the pH closer to the isoelectric point to allow the protein to precipitate with HCl or CH_3COOH in the presence or absence of NaCl (Klockeman et al., 1997; Aluko and McIntosh, 2001). Ghodsavali et al. (2005) reported that a range of pHs between 4.5 and 5.5 was the optimum precipitation pH for canola proteins.

In the case of the PMM approach, defatted meal is often stirred with NaCl to achieve an ionic strength at least 0.2 M and then diluted with 6-10 parts of cold water to reduce ionic strength to 0.06-0.1 M in order to precipitate the salt soluble proteins in the form of protein micelles (Murray et al., 1980). The PMM approach first provide conditions to solubilize protein with elevated ionic strength (salting-in), and then reduce the ionic strength to promote hydrophobic interactions between protein molecules by diluting the solution with cold water to form protein micelle. PMM method tends to be less harsh on the native protein than other extraction means, possibly leading to the production of a higher quality (i.e., non-denatured) protein. However, the PMM method was found to have relatively lower protein yield (~71.3%-78.5%) in comparison to alkali extraction methods (Ismond and Welsh, 1992). Some extraction methods found in literature using the alkali extraction/acid precipitation and PMM methods as summarized in table 2.2.

Table 2.2 Summary of alkali extraction/acid precipitation and PMM methods for rapeseed protein found in literature.

(a) Alkaline extraction (followed by acid precipitation)	
Aluko and McIntosh, 2001	Defatted meal is dissolved in 10 volumes of solution of 0.1 M NaOH, stirred at room temperature for 20 min. Acid adjustment to pH 4.0 by 0.1 M HCl.
Pedroche et al., 2004	Defatted meal is dissolved in 10 volumes of 0.2% NaOH, stirred at room temperature for 1h twice. Acid adjustment to pH 2.5- 6.0 in 0.5 increments by 0.5 N HCl.
Klockeman et al., 1997	Defatted meal is dissolved in 0.4% NaOH, stirred in room temperature for 1 h. Acid adjustment to pH 3.5 by acetic acid.
Tzeng et al., 1988	Defatted meal is dissolved in 1.0% aqueous SHMP in room temperature for 30 min. Acid adjustment to pH 3.5 by 6 N HCl.
(b) PMM method extraction	
Gruener and Ismond, 1997a,b	Defatted meal is stirred in 0.3 M NaCl (1:10 meal: solvent) for 4 h in room temperature. Supernatant is ultrafiltrated and concentrated through 10 kDa molecular weight cut off spiral ultrafiltration, pressure maintained at 20 psi., the volume of the supernatant is reduced 8 times and then diluted with 6 times volume of cold water.
Ismond and Welsh, 1992	Defatted meal is stirred in buffer (NaH_2PO_4) in room temperature with either 0.01 or 0.1 M NaCl ranging from pH 5.5-6.5. Supernatant is concentrated through ultrafiltration with 100 kDa molecular weight cut off, pressure maintain at 60-70 psi., the volume reduced 4 times, and then diluted with 15 times volume of cold water.
Ser et al., 2008	Defatted meal is stirred in buffer (NaH_2PO_4) with 0.5 M NaCl with pH 5.5-6.5. Supernatant is concentrated with ultrafiltration with 10 kDa molecular weight cut off, pressure maintain at 60-70 psi and diluted with 15 times volume of cold water.

2.5 Canola proteins

Canola protein is dominated by a salt soluble 12S globulin cruciferin, representing up to 60% of total protein. The remaining composition comprises of water soluble albumin (Napin, 2S) and alcohol soluble prolamins (Hoglund et al., 1992). The exact ratio of these two proteins varies among cultivar and extraction processes used. A significant variation of globulin: napin ratios have been reported, ranging from 0.7 to 2.0 (Raab et al., 1992; Aider and Barbana, 2011).

2.5.1 Cruciferin proteins

Cruciferin is a hexamer (molecular mass of ~300 kDa) with each monomer comprising of two polypeptides; an α- chain (~30 kDa) and a β- chain (~20 kDa) stabilized by a disulfide bridge (Schwenke et al., 1983) (Figure 2.3). The reversible dissociation of 12S subunits into 7S trimmers has been reported depending on the ionic strength (<0.5) (Schwenke and Linow, 1982). It was also found that 12S cruciferin can further dissociate into 2-3S components irreversibly after dialyzing the protein solution against 6 M urea (Bhatty et al., 1968). Similarly, the 12S cruciferin can dissociate into a 7S trimer at low pH (<3.6). Cruciferin has a neutral pI (~pH 7.2), and its secondary structures composed of high level of β-sheets (~50%) and low level of α-helices (~10%) (Zirwer et al., 1985).

The emulsifying properties of 12S *Brassicaceae* protein were investigated (Krause and Schwenke, 2001; Wu and Muir, 2008). Krause and Schwenke (2001) found higher concentrations of cruciferin was needed to form a stabilized viscoelastic film around an oil droplet than napin, indicating the need for high packing density of the protein at the interface and cruciferin was able to have more intermolecular interaction at the interface due to its lower surface charge compares with napin molecules. In the same study, cruciferin was found with much lower emulsifying activity index (168 m^2/g) compared with napin (418 m^2/g) (Krause and Schwenke, 2001). Wu and Muir (2008) found cruciferin prepared emulsions resulted in smaller droplet sizes(<1μm) compared with napin prepared emulsions (>10 μm) as well as higher emulsion stability (97.74% compared with 77.41% for napin).

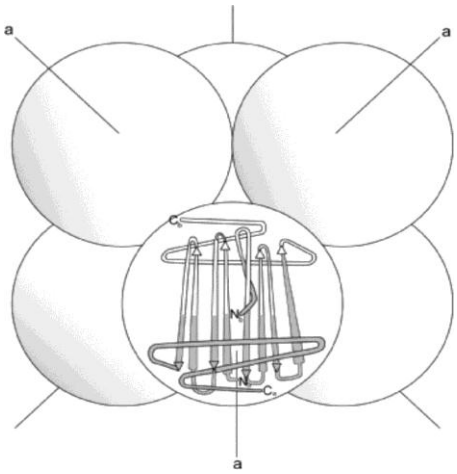

Figure 2.3 Hypothetical structure of cruciferin where "a" denoted the twofold pseudosymmetry axes where the molecules split into trimers during reversible dissociation. The hydrophobic β-chain C terminal located in the interior of molecule and the hydrophilic C terminal of α-chain is on the surface of the molecules. N terminal of both β chain and α chain therefore formed the two domain of the subunit (adapted from Wanasundara, 2011).

Schwenke (1994) reported unmodified cruciferin is more surface active than napin, this could be due to the higher hydrophobicity of cruciferin. Surface hydrophobicity was found positively correlated with emulsifying properties in soy protein, sunflower protein and rapeseed protein (Nakai et al., 1980; Townsend and Nakai, 1983).

2.5.2 Napin proteins

Napin in *Brassicaceae* seed has a homologous structure and similar to a group of closely related low molecular weight 2S albumin proteins in many plants such as Brazil nuts, mustard, sunflower seeds, etc. (Lönnerdal and Janson, 1972). Napin is a basic protein with a calculated pI > 10 and molecular weight 12-14 kDa (Bhatty et al., 1968; Schwenke et al., 1988). Napin is comprised of one small (~4.5 kDa) and one large subunit (~10 kDa) stabilized by two disulfide bonds (Schwenke, 1990; Gehrig et al., 1998) (Figure 2.4). It was reported that chemical modification such as acetylation and succinylation would not change the secondary or tertiary

structure of napin unless the disulfide bonds are broken such as under S-S bond reduction conditions, indicated napin is a highly stabilized structure (Schwenke et al., 1988; Schwenke, 1994). Structural stabilization of napin by disulfide bond would reduce the molecular flexibility of napin and could become a disadvantage for napin during the formation of emulsion when the molecules need to rearrange and realign at the interface (Schwenke, 1994). The secondary structure of napin is characterized by high content of α- helix (40-46%) and low content of β-sheet (12%) at pH range from 3-12 (Schmidt et al., 2004). Positively charged amino acids accumulate on the surface of napin small and large chains makes them highly reactive towards chemical modification, however, this could be a disadvantageous because it is easy to form insoluble complexes with phytic acid and/ or phenolic compounds through electrostatic attractive forces (Schwenke, 1990).

Crucifer 2S napin is considered the main allergen in mustard seed and was identified as structurally homologous with 2S albumin in many mono- and di-cotyledonous plants such as cotton seeds, Brazil nuts, sunflower seeds and castor bean, etc. (Monsalve, 1991; Moreno and Clemente, 2008). Thus far four proteins of *Brassicaceae* 2S fraction have been identified as mustard seed allergens: Sin a 1 from *Sinapis alba*, Bra j 1 from *Brassica juncea*, Bra n 1 from *Brassica napus* and Bra r 1 from *Brassica rapa*. (Wanasundara, 2011). Because of the highly stabilized molecular structure of 2S albumin, it was found that 2S protein is able to cross the gut mucosal barrier to sensitize the mucosal immune system to trigger an allergic response (Monreno and Clemente, 2008). There were attempts of transferring 2S albumin coding gene from Brazil nut, which is homologous with napin, into soybean (Nordlee et al., 1996). It was found that the transgenic soybean retained the 2S albumin allergenicity and triggered allergic reactions on skin-prick tests.

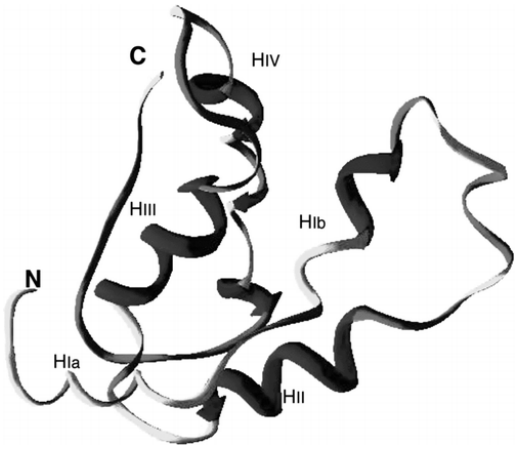

Figure 2.4 Schematic diagram of napin. The N indicated the NH_2 terminal and C indicated COOH terminal; HIa, HIb (on the small chain), HII, HIII and HIV (on the large chain) indicated helix structures in the molecule (adapted from Wanasundara, 2011).

Functionality of native and modified napin has been investigated (Schwenke, 1990; Krause and Schwenke, 2001; Wu and Muir, 2008). Krause and Schwenke (2001) reported that napin has a higher diffusion rate and is highly surface active with higher emulsifying activity index (EAI) than its globulin counterpart. The authors also found that a smaller concentration of napin was needed to form a saturated protein film on droplets; however the film was not as protein packed compared with cruciferin which could be due the electrostatic repulsive force between napin molecules that prevent close stacking on the film (Krause and Schwenke, 2001). On the contrary, Wu and Muir (2008) reported a napin prepared emulsion had lower stability (77.41%) compared with cruciferin (97.74%) and a canola protein isolate (89.95%), and concluded napin content in canola protein is a major factor contributing to the inferior functionality of canola protein. Wu and Muir (2008) suspected high level of basic amino acid residues on napin might favor electrostatic interaction which might be responsible for the inferior emulsifying properties of canola protein. Protein solubility of *Brassicaceae* meals was investigated by Wanasundara and others (2012) and showed that napin has high solubility across pH 2.0 to 10.0 in many of the crucifer oilseeds, this could explain the findings of Krause and Schwenke (2001), where napin has higher diffusion rate compared with cruciferin due to its high protein solubility at neutral pH. Jyothi et al. (2007) found napin has low binding constant to extrinsic fluorescence probes which indicated napin molecule may have lesser number of hydrophobic sites on the surface. Nitecka et al. (1986) found that upon succinylation and

acetylation, surface hydrophobicity of napin increased linearly with the level of reactions and the modified napin shown aggregation at pH below its pI value. The authors also found acetylation reduced the emulsifying activity of napin simply due to the linear increasing of surface hydrophobicity. Schwenke (1994) stated that based on the studies done on native and modified canola main storage protein, napin shown excellent foaming ability comparable with egg white, however, napin has poorer emulsifying properties than cruciferin. Chemical modification of napin such as succinylation and acetylation resulted in poor foaming and emulsifying properties due to reduced solubility and higher surface hydrophobicity (Nitecka et al., 1986; Schwenke, 1990).

2.6 Anti-nutritional properties

2.6.1 Phytates

Phytic acid (PA) is found as salt of calcium, magnesium and potassium in crystal form inside the storage protein bodies of *Brassicaceae* seed (Yiu et al., 1983). Phytic acid content was reported to be 2.0-4.0% in whole seed and the level of phytic acid increases to 5.0-7.5% in protein concentrate to 1.0-9.8% in protein isolates depending on the protein extraction methods (Ismond and Welsh, 1992). Phytic acid has six phosphate groups and 12 protons that are dissociable in pH range from 1.92 to 9.53 (Schwenke, 1994). As a result, phytic acid could undergo attractive electrostatic reaction with the storage proteins when pH below their isoelectric point (Wanasundara, 2011). Phytic acid-protein complexes are often insoluble during solvent extraction of protein; therefore the presence of phytic acid could reduce the overall protein yield if the extraction pH is under the isoelectric point of the protein. Fortunately, the addition of salt such as NaCl is able to reduce the level of phytic acid effectively during extraction (Ismond and Welsh, 1992). Ismond and Welsh (1992) found the addition of 0.01 M and 0.1M NaCl reduced total phytic acid level in the protein isolate to 0.96% and 0.49% representatively by limiting the electrostatic attraction between the PA and protein. Phytic acid could potentially cause changes in the physicochemical and functional properties of the canola protein. Krause and Schwenke (2001) shown the protein isolate had slightly different interfacial behavior which the authors speculated might be due to complex formation of protein with phytic acid. PA is also known as one of the antinutritional compounds present in rapeseed due to its ability to reduce the bioavailability of essential dietary minerals (Wanasundara, 2011).

2.6.2 Phenolic compounds

Phenolic compounds in *Brassicaceae* are considered to be the major contributor of the poor organoleptic properties of rapeseed flour or protein products (Schwenke, 1994; Aider and Barbana, 2011). Level of phenolic compounds in rapeseed meal were found 5 times higher than soybean meal and thus has become one of the limiting factors to utilize rapeseed meals and rapeseed protein concentrate or isolate in food or feed industry (Ismond and Welsh, 1992; Aider and Barbana, 2011). Phenolic compounds exist in many forms in rapeseed meal, the predominant type of phenolic compound in rapeseed meal and its derivative products is sinapic acid, which could constitute 70-85% of total phenolic compounds present in rapeseed meal (Naczk et al., 1998). Similar to phytic acid, phenolic compounds are also capable of altering the physicochemical and functional properties of rapeseed proteins (Schwenke, 1994). Spencer and others (1988) suggested that phenolic compounds might be binding protein through hydrophobic interaction with aromatic groups or hydrocarbon side chain, and then the binding was reinforced by hydrogen bonds between phenolic residues and polar groups of the protein. The binding of free sinapic acid is high when pH is lower than 7.0 without NaCl, however, Ismond and Welsh (1992) found the addition of 0.1M NaCl to NaH_2PO_4 buffer at pH 5.5 reduced total phenolic compounds in the protein isolate by 85.3%.

2.6.3 Glucosinolates

Glucosinolates are a group of compounds commonly but not exclusively found in the plants of the family *Cruciferae* which includes many economically valuable crops such as the *Brassicaceae* genus for edible oil and animal feeds from meal (McDanell et al., 1988). All glucosinolates have a fundamental backbone comprises of a β-D-thioglucose group, a sulphonated oxime moiety and a variable side-chain derived from methionine, tryptophan or phenylalanine (Mithen et al., 2000). Glucosinolates undergo enzymatic hydrolysis to produce variety of breakdown products that catalyzed by indigenous enzyme myrosinase which co-exists with glucosinolates in the seeds but in separate compartments (Fahey et al., 2001). Upon the crushing of the seeds and/or other physical injuries occurred to the seeds, myrosinase could be released and initiate the hydrolysis process of glucosinolates. Some of the major glucosinolates breakdown products such as isothiocyanates, are responsible for the pungent aroma and hot/

bitterness of mustard seeds and mustard products (McDanell et al., 1988). Another major glucosinolates degradation product thiocyanate ion is considered goitrogenic that reduces the bioavailability of iodine and causing goiter in extreme cases (Fenwick and Heaney, 1983). The level of total glucosinolates in *Brassicaceae* seeds are depending upon the species of the plants and agronomic factors, often researchers are also interested in the level of specific types of glucosinolates because of their distinct physical, chemical and physiological properties of their break- down compounds (McDanell et al., 1988; Fahey et al., 2001). Problems associated with glucosinolates in canola meal were limited due to the low total glucosinolates level in the seeds.

CHAPTER 3: MATERIALS AND METHODS

3.1 Materials

Canola seed (Brassica *napus*/ variety VI-500) was kindly donated by Viterra (Saskatoon, SK, Canada). All chemicals used in this study, unless otherwise stated were purchased from Sigma-Aldrich (Oakville, ON, Canada).

3.2 Canola meal preparation

Prior to use, canola seed were stored in containers at 4^{o}C. Seed preparation and defatting procedures were according to Wanasundara et al. (2012) with slight modifications. At room temperature (21-23^{o}C), small seeds were first removed using a #12 (1.7 mm mesh size) Tyler screen (Tyler, Mentor, OH, USA) in order to maximize the cracking efficiency of the screened seeds. The screened seed was then placed in a -40^{o}C freezer overnight to aid in the dehulling processes. Frozen seed was then cracked using a stone mill (Morehouse-Cowles stone mill, Chino, CA, USA), followed by separation of the seed coat and cotyledons based on density difference by using an air blower (Agriculex Inc., Guelph, ON, Canada). The dehulled seeds were then pressed using a continuous screw expeller (Komet, Type CA59 C; IBG Monforts Oekotec GmbH & Co., Mönchengladbach, Germany) to physically remove the majority of the oil. The screw expeller was operated at speed 6.5 using a 3.5 mm choke and resulted in a meal temperature of ~75^{o}C. The meal was then ground into a powder. Residual oil was then reduced using hexane (1:3 meal: hexane ratio) at room temperature for 16 h (final fat content <3%). The meal was then left in a fume hood overnight to allow residual hexane to evaporate. The hexane extraction was repeated an additional time.

3.3 Canola protein fractions preparation

3.3.1 Cruciferin-rich protein isolate (CPI) preparation

A cruciferin-rich protein isolate was prepared based on the method described by Murray and co-workers (1980) with slight modifications. In brief, 20 g of defatted ground meal was dispersed in 200 mL of Milli-Q^{TM} water containing 0.2 M NaCl, and then maintained at pH 5.8-6.3 with continuous stirring (500 rpm) for 90 min at room temperature (21-23^{o}C). The dispersion was then centrifuged at 17,700 × *g* for 20 min at 4^{o}C using a Sorvall RC-6 Plus centrifuge (Thermo Scientific, Asheville, NC, USA). The supernatant was then collected via vacuum filtration using Whatman No. 1 filter paper (Whatman International Ltd., Maidstone, UK). Afterwards, the filtered supernatant was diluted with prepared cold Milli-Q^{TM} water (< 4^{o}C) to 2000 mL and allowed to settle overnight. The clear upper layer was decanted and the precipitated protein micelle was pooled and collected with a separatory funnel. The concentrated protein liquid was then dialyzed (Spectro/ Pro$^{®}$ tubing, 6-8 kDa cut-off, Spectrum Medical Industries Inc., Rancho Dominguez, CA) at 4^{o}C for 48 h against Milli-Q water with several changes of water until the conductivity reached 2.0–2.5 mS/cm. Desalted protein micelles were then freeze dried and crushed to obtain a CPI powder. Protein content was determined according to the Association of Official Analytical Chemists method 920.87 (AOAC, 2003), using a micro-Kjeldhal digestion and distillation unit (Labconco Corp., Kansas City, MO) with a nitrogen conversion factor of 6.25 (% Protein = 6.25 x % Nitrogen).

3.3.2 Napin-rich protein isolate (NPI) preparation

A napin-rich protein isolate was prepared based on the method described by Wanasundara and McIntosh (2008). Briefly, 100 g of defatted ground meal was dispersed in 1 L of Milli-Q^{TM} water containing 0.75% (w/v) NaCl, adjusted to pH 3.0 using 1.0 M HCl, and then allowed to stir continuously at 500 rpm for 90 min at room temperature (21-23°C). The dispersion was centrifuged at 17,700 × *g* for 20 min at 4°C using a Sorvall RC-6 Plus centrifuge (Thermo Scientific, Asheville, NC). The supernatant was collected via vacuum filtration using Whatman No. 1 filter paper (Whatman International Ltd., Maidstone, UK). Afterwards, the filtered supernatant was adjusted to neutral pH (6.8-7.0) using 1.0 M NaOH, followed by centrifugation at 17,700 × *g* for 20 min at 4°C using the same centrifuge to separate precipitate. The supernatant was then diafiltered with a Pellicon-2 Tangential flow membrane filtration

system through a 5 kDa regenerated cellulose membrane (using three membranes with a surface area of 0.1 m^2 each) (Millipore Corporation, Milford, MA) to remove salt and small molecular weight substances in the protein extract (Wanasundara and McIntosh, 2008). The concentrated supernatant was stored at -30°C, freeze dried and crushed to yield a free flowing powder. The protein content of the resulting powder was determined according to the Association of Official Analytical Chemists method 920.87 (AOAC, 2003), using a micro-Kjeldahl digestion-distillation unit (Labconco Corp., Kansas City, MO, USA) with a nitrogen conversion factor of 6.25 (% Protein = 6.25 x % Nitrogen).

3.4 Characterization of CPI and NPI

3.4.1 Amino acid composition analyses

The amino acid composition analysis was performed by POS (POS BioSciences Corp., Saskatoon, SK, Canada). Amino acid profiles of CPI and NPI were determined using a pico-tag amino acid analysis system (Waters Corporation, Milford, MA, USA) and by high performance chromatography (HPLC). In general, fifteen amino acid residues were quantified according to the method developed by Bidlingmeyer et al. (1987), which involved adding 15 mL of 6 N HCl to the CPI or NPI samples (~20 mg) to hydrolyze the proteins into individual amino acids prior to HPLC separation. The amount of sulfur-containing amino acid residues was determined according to AOAC official methods 985.28 (AOAC, 2003) where 10 mL cold performic acid was added to oxidize cysteine and methionine prior to hydrolysis with 15 mL 6 N HCl. The quantity of tryptophan was determined according to AOAC method 988.15 (AOAC, 2003) in which 10 mL of 4.2 M NaOH was added to samples to hydrolyze tryptophan prior to HPLC separation.

3.4.2 SDS-PAGE

The CPI and NPI were subjected to sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) to observe the molecular weight band profile, according to the Laemmli procedure (Laemmli, 1970) and using the PhastSystem equipped with separation and development capabilities (Amersham Pharmacia, Uppsasla, Sweden). The NPI material was analyzed under both reducing and non-reducing conditions to verify the disulfide bond which bonds the two subunits, whereas CPI was only measured under reducing conditions. In brief,

samples were prepared under reducing conditions by dissolving 2 mg of protein with 1 mL in a 0.1 M Tris-HCl buffer (containing 5% SDS (w/v) at pH 8.0) with addition of β-mercaptoethanol (5%, v/v), followed by heating at 99^{o}C (Incu Block model 285, Denville Scientific Inc., South Plainfield, NJ) for 10 min to unravel and disassociate the protein, followed by cooling the solution to room temperature (21-23^{o}C). Mixtures were then centrifuged at 16,873 x *g* for 10 min to remove any insoluble materials (Marcone et al., 1998). NPI was also produced under non-reducing conditions using the same method, with the exception of adding β-mercaptoethanol. One microgram of protein solution was applied into each well and standard proteins (Sigma wide range molecular weight markers) of 170, 130, 95, 72, 55, 43, 34, 26, 17 and 10 kDa were applied to a separate well. Gradient mini gels (resolving 8–25%T (T, denotes the total amount of acrylamide present) and 2%C (C, denotes the amount of cross-linker), stacking zone 4.5%T and 3%C, 43 mm × 50 mm × 0.45 mm, polyacrylamide gels cast on GelBond® plastic backing, buffer 0.112 M acetate, 0.112 M Tris, pH 6.4) were used to separate proteins. Following the separation, the proteins were fixed and stained using PhastGel blue R (Coomassie R-350) and developed to obtain suitable background colour. Molecular weight of each band and relative percentage were estimated using the ImageQuant® (Ver. 3.0; Amersham Pharmacia Biotech, Piscataway, NJ, USA) software based on the darkness intensity of each band (Marambe et al., 2013).

3.5 Physicochemical properties

3.5.1 Protein solubility

Protein solubility of CPI and NPI was investigated as a function of pH and NaCl content according to Can Karaca et al. (2011) with slight modifications. In brief, protein solutions were prepared by dispersing isolate powder in Milli- Q^{TM} water (0.25%, w/w) with NaCl solution (0, 50 or 100 mM) that has adjusted pH to 3.0, 5.0 or 7.0. Protein solutions were then stirred at 500 rpm for 1 h at room temperature (21-23^{o}C). For each solution, 12 mL was then transferred to a 15 mL centrifuge tube and centrifuged (VWR clinical centrifuge 200, VWR International, Mississauga, ON, Canada) at 4,180 × *g* for 10 min at room temperature. Protein content was determined by measuring the total nitrogen levels in ~5 g of supernatant using a micro-Kjeldahl digestion and distillation unit (Labconco Corp., Kansas City, MO, USA) with a 6.25 conversion factor (% Protein = 6.25 x % Nitrogen). Protein solubility (%) was determined as a percentage of

dividing the total amount of protein within the supernatant by the original amount in the sample. All measurements were reported as the mean ± one standard deviation (n = 3).

3.5.2 Surface charge

The surface charge (or zeta potential) of 0.05% (w/w) CPI or NPI solutions as a function of pH and NaCl concentrations were determined by measuring the electrophoretic mobility (U_E) using a Zetasizer Nano-ZS90 (Malvern Instruments, Westborough, MA, USA). Samples were prepared by stirring the CPI overnight at 4°C to ensure complete solubilisation, or for 1 h at room temperature in the case of NPI after being dispersed in Milli-Q water at their respective NaCl concentration (0, 50 and 100 mM NaCl) and then pH adjusted to 3.0, 5.0 or 7.0. Zeta potential (ζ; units: mV) was calculated by applying Henry's equation:

$$U_E = \frac{2\varepsilon \times \xi \times f(k\alpha)}{3\eta} \qquad \text{(eq. 3.1)}$$

where, ε is the permittivity (units: F (Farad)/m), $f(\kappa\alpha)$ is a function related to the ratio of particle radius (α; units: nm) and the Debye length (κ; units: nm^{-1}), and η is the dispersion viscosity (units: mPa·s). The Smoluchowski approximation $f(\kappa\alpha)$ was set as 1.5. All measurements were reported as the mean ± one standard deviation (n = 3).

3.5.3 Surface hydrophobicity

The average surface hydrophobicity of CPI and NPI as a function of pH and NaCl concentrations was estimated using a FluoroMax-4 spectrofluorometer (Horiba Jobin Yvon Inc., Edison, NJ) by the ANS (8-anilino-1-naphthalenesulfonic acid) fluorescent probe method based on the original work by Kato and Nakai (1980) and later modified by Can Karaca et al. (2011). Stock solutions (0.1% w/v) of CPI and NPI were prepared with NaCl solution (0, 50 or 100 mM) that has adjusted pH to 3.0, 5.0 or 7.0 and diluted into concentrations of 0.1%, 0.08%, 0.06%, 0.04% and 0.02% (w/v) with Milli-Q water containing the desired NaCl and pH level. Stock solutions of CPI were stirred overnight at 4°C, whereas NPI solutions were stirred for 1 h at room temperature, as previously described. Four mL of diluted CPI or NPI solutions were transferred into two 15 mL glass test tubes. One tube was left as is, whereas in the other, 20 µL of 8 mM ANS solution (in Milli-Q water containing 0, 50 or 100 mL NaCl) was added. Tubes were then vortexed for 10 s and stored in the dark for 15 min at room temperature (21-23°C)

prior to measuring. Fluorescence intensity at 390 nm excitation and 470 nm emission wavelengths were measured for: (a) an ANS blank (containing only the ANS probe); (b) a protein blank (containing only the protein solution); and (c) the protein solution containing the ANS probe. The net fluorescence intensity was obtained by subtracting the ANS (a) and protein (b) blanks from the sample (c) at equivalent protein concentrations. The net fluorescence intensity was then plotted as a function of protein concentration, where the slope (as determined by linear regression) of the initial rise was taken (arbitrarily divided by 10000) as an index of average protein surface hydrophobicity. All measurements were reported as the mean ± one standard deviation (n = 3).

3.5.4 Interfacial tension

The interfacial tension between canola oil and an aqueous protein solution (CPI or NPI) as a function of pH (3.0, 5.0 and 7.0) and NaCl (0, 50 and 100 mM) content were measured using a semi-automatic tensiometer (Lauda TD2, GmbH & Co., Lauda- KÖnigshofen, Germany) according to the Du Noüy ring method. CPI and NPI (0.25% w/w) samples were prepared as previously described in Section 3.3.3 (protein solubility). Interfacial tension for a water (without protein)-canola oil system served as a control. Interfacial tension was calculated from the maximum force *(F_{max}; units: milli-Newtons; instrument measures mg x gravity)* using the following equation:

$$\gamma = \frac{F_{max}}{4\pi R\beta} \qquad \text{(eq. 3.2)}$$

where, γ is the interfacial tension (mN/m), R is the radius of the ring (20 mm), β is a correction factor that depends on the dimensions of the ring and the density of the liquid involved (McClements, 2005). All measurements were reported as the mean ± one standard deviation (n = 3).

3.6 Emulsifying properties

Emulsification activity (EAI) and stability (ESI) indices were determined according to Pearce and Kinsella (1978). In brief, 5.0 g of the CPI or NPI solutions (0.25%, w/w) were homogenized with 5.0 g of canola oil using an Omni Macro Homogenizer (Omni International, Marietta, GA) with a 20 mm saw tooth generating probe at speed 4 (~7,200 rpm) for 5 min.

Samples were prepared as previously described in Section 3.3.3 (protein solubility) as a function of pH (3.0, 5.0 and 7.0) and NaCl (0, 50 and 100 mM) contents. Fifty microliters of the formed emulsion was immediately taken from the bottom of the tube and diluted in 7.5 mL of 0.1% sodium dodecyl sulphate (SDS) solution, followed by vortexing for 10 s. A Genesys 10 UV-visible spectrophotometer (Thermo Scientific, Madison, WI, USA) was used to determine the absorbance of the diluted emulsion samples at 500 nm using a plastic cuvette (1 cm path length). A second absorbance reading was taken from the dilution after 10 min. EAI and ESI were calculated by following equations.

$$EAI\left(\frac{m^2}{g}\right) = \frac{2 \times 2.203 \times A_0 \times N}{c \times \varphi \times 10000} \quad \text{(eq. 3.3)}$$

$$ESI(\text{min}) = \frac{A_0}{\Delta A} \times t \quad \text{(eq. 3.4)}$$

where, A_0 is the absorbance of the diluted emulsion immediately after homogenization, N is the dilution factor, c is the weight of protein per volume (g/mL), φ is the oil volume fraction of the emulsion, and ΔA is the difference in absorbance between 0 and 10 min ($A_0 - A_{10}$) and t is the time interval (10 min). All measurements were reported as the mean ± one standard deviation (n = 3).

3.7 Statistical analyses

All statistics were performed using SPSS Ver. 20.0 software (SPSS Inc., 2012, Chicago, IL, USA). A two-way analysis of variance (ANOVA) was used to test the significance of the main effects (pH and NaCl) and associated interaction on the physicochemical (surface hydrophobicity, solubility and interfacial tension) and emulsifying (EAI and ESI) properties. A simple Pearson correlation analysis was conducted to examine the relationship between physicochemical and emulsifying properties of the isolates, however only significant correlations were reported in the discussion section.

CHAPTER 4: RESULTS AND DISCUSSION

4.1 Effect of pH and NaCl on the physicochemical and emulsifying properties of a cruciferin-rich protein isolate

4.1.1 Characterization of the CPI material

The protein content of CPI was determined to be 90.35% (d.b.). Ismond and Welsh (1992) obtained a 78.5% (d.b.) protein content using a similar extraction procedure. Figure 4.1 shows an SDS-PAGE profile of CPI under reducing conditions with bands occurring between ~17 and ~65 kDa accounting for 85.6% of the total bands as determined by densitometry (lane 1, CPI). The results are presumed to correspond to the molecular mass of the individual subunits of cruciferin (~50 kDa), along with their α- (~30 kDa) and β- (~20 kDa) chains (Dalgalarrondo et al., 1986), aligning with the results of Wu and Muir (2008). The amino acid composition of CPI was found to be high in glutamine (+ glutamic acid) (17.90%), along with asparagine (+ aspartic acid) (9.49%), leucine (7.21%) and arginine (6.97%) (Table 4.1). Chabanon and others (2007) and Schwenke (1990) reported cruciferin to be rich in glutamine (+ glutamic acid) and arginine, accounting for ~20 and 10% of the total amino acids, respectively. The most abundant amino acid group, glutamine has a pK_a of 4.1; leaving its reactive side group negatively charged at pHs > 4.1. In contrast, arginine has a very high pK_a at 12.5, and its reactive side group assumes a positive charge at pHs less than this pH.

4.1.2 Surface characteristics

Surface charge on the protein is highly dependent upon on the amino acid composition, protein conformation and solvent conditions (e.g., pH and salt content). Figure 4.2 shows the change in zeta potential (mV) for CPI solutions as a function of pH and salt concentration. For CPI in the absence of added NaCl, zeta potential rose from ~-33 mV at pH 8.0 relatively linearly to pH 4.8 (isoelectric point) where it became 0 mV, then increased further to +33 mV at pH 3.0, before declining slightly to ~+22 mV at pH 2.0. In contrast, the addition of both 50 and 100 mM NaCl resulted in only a slight charge ranging between ~-10 mV at pH 8.0 and ~+10 mV at pH

2.0, the pI values (pH 4.6) remained relatively similar to the sample without added NaCl. The addition of NaCl acts to screen charged amino groups on the protein's surface, functioning to reduce the relative thickness of the electric double layer and the charge exerted out into solution (McClements, 2004). Paulson and Tung (1987) reported similar findings with the addition of 350 and 700 mM NaCl for unmodified CPI where the zeta potential was reduced from -18 mV to -5 mV at pH 5.0 and from -40 mV to -20 mV at pH 7.0. Schwenke (1990) and Mieth et al. (1983) both reported pI values of ~7.2 for cruciferin extracted from *B. napus*. However, cruciferin rich isolates has been recorded to have pIs ranging from pH 4-10 depending on the extraction method (Paulson and Tung, 1987; Can Karaca et al., 2011; Wanasundara et al, 2012).

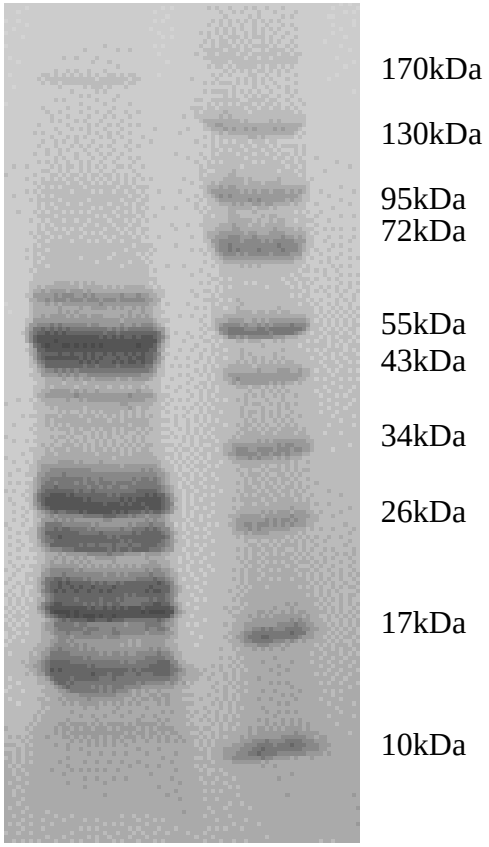

Figure 4.1 SDS-PAGE (reducing) (1 μL of 2 mg mL^{-1} CPI solution) applied to gradient 8-25% PhastGels. Lanes: (1) CPI and (2) standard.

Table 4.1 Amino acid profile for the cruciferin-rich protein isolate.

Amino acid	**Percent**
(Aspartic acid + Aspargine)	9.49
(Glutamic acid + Glutamine)	17.9
Alanine	3.76
Arginine	6.97
Cysteine	1.33
Glycine	5.18
Histidine	2.19
Isoleucine	4.06
Leucine	7.21
Lysine	3.37
Methionine	1.52
Phenylalanine	4.28
Proline	5.00
Serine	5.00
Threonine	4.07
Tryptophan	1.32
Tyrosine	2.89
Valine	4.65
Total:	**90.19**

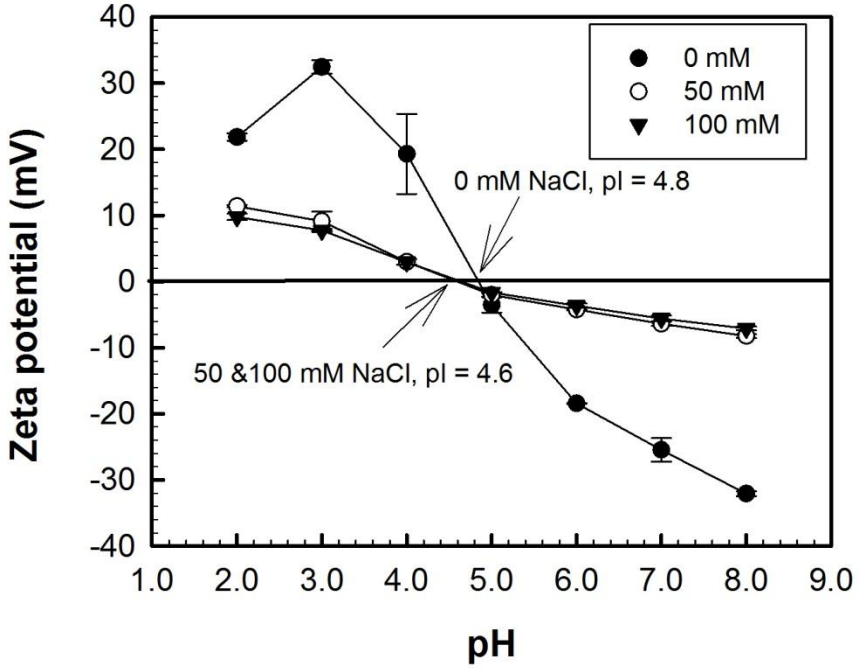

Figure 4.2 Zeta potential (mV) of CPI in the function of pH and NaCl (mM) content. Data represent the mean ± one standard deviation (n = 3).

Surface hydrophobicity plays an important role in driving protein-protein aggregation and the protein's interfacial activity (Dickinson, 2003). Higher amounts of protein-protein interactions may also have a negative impact on protein solubility in aqueous solutions and alignment at the interface if hydrophobic moieties become re-buried (Jung et al., 2005; Avramenko et al., 2013). Gerbanowskia and co-workers (2003) also reported that hydrophobicity can impact the rate of interfacial tension reduction in terms of protein diffusion, adsorption, conformational change and molecular rearrangement at the interface. In the present study, the effects of pH and NaCl on surface hydrophobicity was shown to be significant ($p<0.001$). Overall, surface hydrophobicity was found to be at the highest level at low pH, however the effects of salt level were different at each pH (Figure 4.3). At pH 3.0, surface hydrophobicity was similar for solutions with 0 and 50 mM of added NaCl (100 and 98.4 arbitrary units, A.U.); however surface hydrophobicity was significantly reduced with the addition of 100 mM NaCl (68.8 A.U.). At pH 5.0, hydrophobicity was found to increase linearly with NaCl content from 45.3 A.U. at 0 mM NaCl to 74.0 A.U. at 100 mM NaCl. At pH 7.0, hydrophobicity was found to

increase from 16.4 A.U. at 0 mM NaCl to 25.0 A.U. for 50 mM NaCl; and then declined to 5.04 A.U. as NaCl content increased to 100 mM. At pH 3.0, it was hypothesized that hydrophobicity was overall higher than the other pHs due to possibly dissociation of protein subunits which would expose hydrophobic moieties. At pH 5.0, the CPI carried a relatively neutral charge (~ 0 - -5 mV), where the addition of NaCl most likely resulted an increase in conformational entropy allowing for greater mobility of the proteins in solution. It was hypothesized that because of these conformational changes, a greater amount of aromatic groups became exposed and available for interaction with the ANS probe. In contrast, at pH 7.0 the rise and fall of hydrophobicity with increased NaCl content is thought to be due to conformational change of the cruciferin molecules which affect the binding efficiency of ANS probes. Paulson and Tung (1987) have also reported similar trends for canola salt soluble globulins where the effect of salt was opposite at pH < pI and pH> pI. Alizadeh-Pasdar and Li-Chan (2000) reported that hydrophobicity results obtained by ANS probes need to be treated with caution because the charges carried by the probes might affect the ability of the probe to bind to the protein surface especially at pHs above the pI value of the protein.

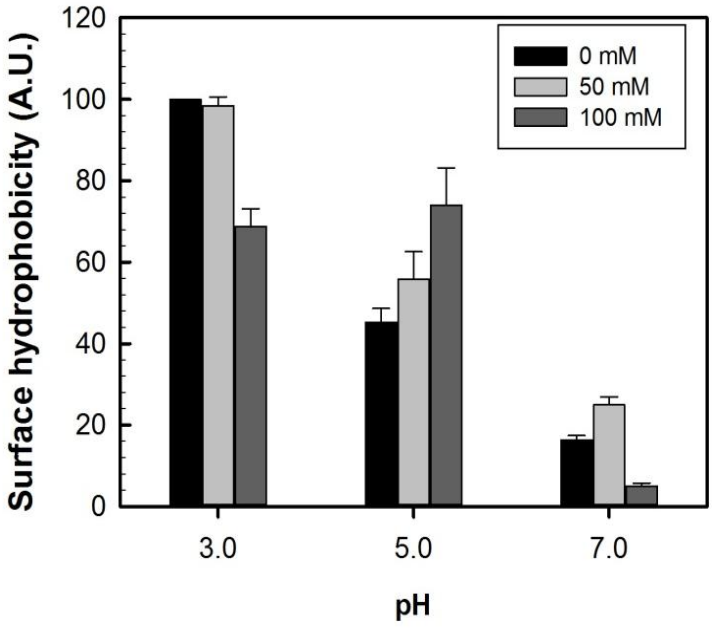

Figure 4.3 Surface hydrophobicity for CPI solutions as a function of pH and NaCl (mM) content. Data represent the mean ± one standard deviation (n = 3).

4.1.3 CPI solubility

The solubility of CPI as a function of pH and NaCl concentration is shown in Figure 4.4. A two-way analysis of variance found the main effects of pH ($p<0.001$) and NaCl concentration ($p<0.001$) along with their associated interaction ($p<0.001$) to be significant. Overall solubility was found to be highest at pH 3.0 regardless of the concentration of NaCl (~91%), whereas at pH 5.0, solubility increased from 10.7% at 0 mM NaCl to 77.4 % and 88.2% with the addition of 50 mM and 100 mM NaCl, respectively. A similar trend was reported for pH 7.0, where solubility increased from 15.7% at 0 mM NaCl to 86.6 % and 90.4% with the addition of 50 mM and 100 mM NaCl, respectively. Findings from the present study indicated that NaCl had a salting in effect on the CPI, in which Na^+ ions contributed to the ordering of the hydration layer to improve protein-water interactions; resulting in relatively high solubility (Dickinson, 2003). For CPI solutions in the absence of added NaCl, solubility was good at pH 3.0 due to a sufficient amount of electrostatic repulsive forces between proteins in solution to keep them dispersed. In contrast, at pH 5.0 (near the pI) and at pH 7.0, electrostatic repulsion was less leading to protein-protein interactions and aggregation.

Can Karaca et al. (2011) found a CPI solution at pH 7.0 showed very poor solubility at a ~5% level. Paulson and Tung (1987) reported poor solubility of cruciferin at pH 5.0 (~5%) in the absence of NaCl, which was increased to ~20% with the addition of 0.35 and 0.7 M NaCl. The authors also reported that solubility was improved at pH < pI with the addition of NaCl due to a salting-in process, however was adversely affected at pH > pI due to a salting-out process. Although this pH-salt dependence contradicts our findings, Paulson and Tung (1987) used much higher NaCl levels than in the present study. The overall lower solubility found by the authors versus the present study is also presumed to be attributed to the much higher protein concentration used (11.4% vs 0.25%).

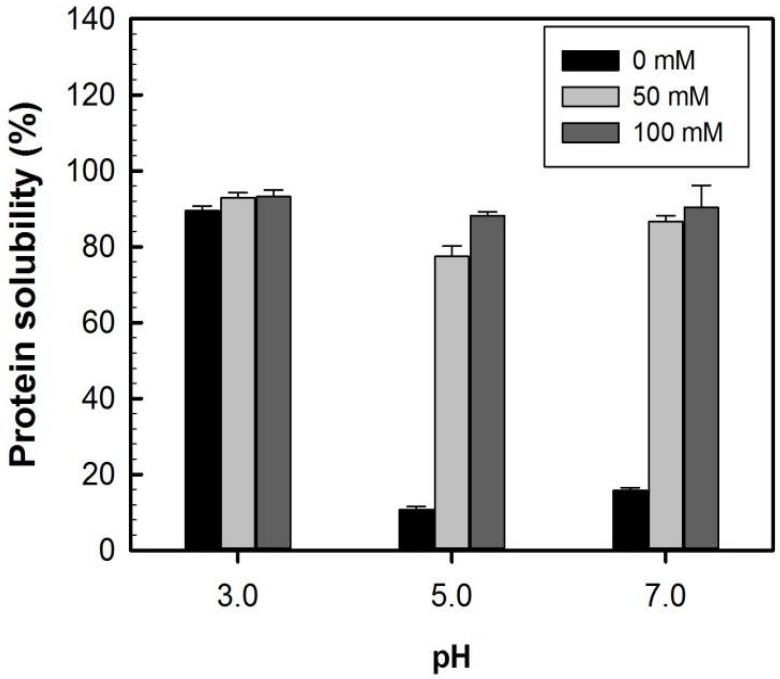

Figure 4.4 Percent protein solubility for CPI solutions as a function of pH and NaCl (mM) content. Data represent the mean ± one standard deviation (n = 3).

4.1.4 Interfacial tension

Interfacial tension is defined as the work required creating a unit area of interface at a constant temperature, pressure, and chemical potential (Drelich et al., 2002). The ability for CPI to reduce interfacial tension at an oil-water interface was investigated as a function of pH and salt content, and is given in Figure 4.5. A two-way analysis of variance found that the main effect of pH and (salt x pH) interaction term to be significant ($p<0.001$), whereas the effect of salt alone was not significant ($p>0.05$). The addition of CPI to the aqueous phase at all pHs and NaCl concentrations was found to reduce the interfacial tension at an oil-water interface from 22.5 mN/m (control, no protein) to 10-17 mN/m. Overall, interfacial tension declined from ~16.7 mN/m at pH 3.0, to 14.0 mN/m at pH 5.0, and then to 12.0 mN/m at pH 7.0, however the influence of salt was different at each pH. At pH 3.0, interfacial tension remained similar regardless of the salt content at 16.5-17.3 mN/m. At pH 5.0, the addition of salt had a negative effect on the ability of CPI to lower interfacial tension, where values were found to be 15.3 and 15.6 mN/m for solutions with 50 and 100 mM NaCl, respectively relative to that without NaCl at

11.1 mN/m. Furthermore, at pH 7.0 the addition of NaCl showed a positive effect on reducing interfacial tension by lowering values to 10.3 and 11.0 mN/m for the 50 and 100 mM NaCl levels, respectively relative to that solution without added NaCl (14.8 mN/m). A simple Pearson correlation shown that interfacial tension is positively correlated with surface hydrophobicity ($r= 0.765$, $p<0.01$) which indicated higher hydrophobicity might promote protein- protein interaction and have negative impacts on the CPI molecules ability to migrate and realign at the interface due to reduced structural flexibility. Krause and Schwenke (2001) found that although cruciferin had lower diffusion rate compared with napin, cruciferin achieved greatest decrease in interfacial tension. Previous study also found the surface hydrophobicity has significant effect to the interfacial tension where the sample with modified cruciferin through succinylation (at 66.0%) had the lowest interfacial tension (Gueguen et al., 1990).

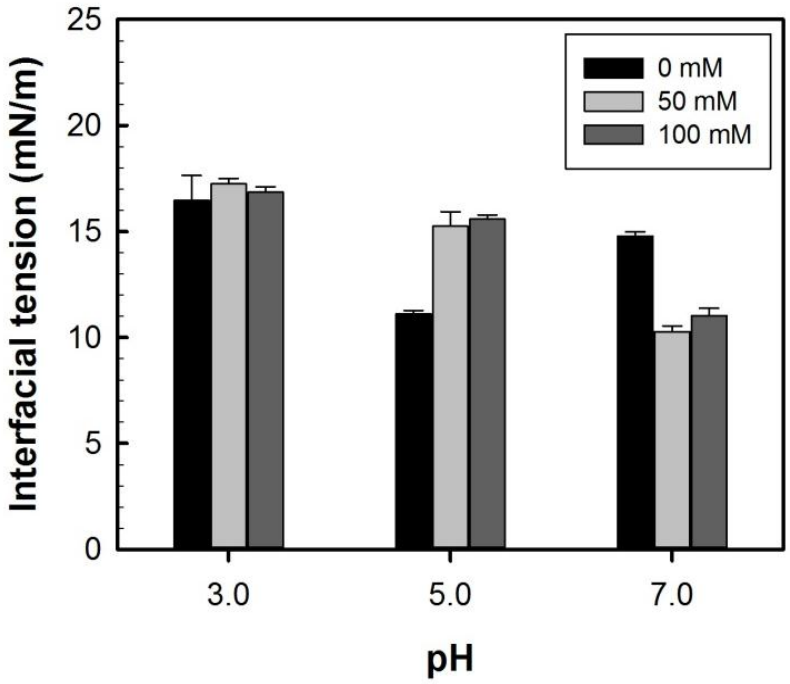

Figure 4.5 Interfacial tension (mN/m) for CPI solutions as a function of pH and NaCl (mM) content. Data represent the mean ± one standard deviation (n = 3).

4.1.5 Emulsifying properties

The emulsifying activity index (EAI) gives a measure of interfacial area coated by an emulsifier such as protein during the formation of an emulsion and is a good predictor for protein surface activity (Pearce and Kinsella, 1978). The EAI for CPI under the influence of pH and NaCl was investigated, and was found that the main effects of NaCl and pH were significant ($p<0.001$), along with their associated interaction ($p<0.01$). Overall, NaCl had no significant effect on EAI at pH 3.0 where EAI values ranged between 18.8 and 19.4 m^2/g. The effect of NaCl was more pronounced at pH 5.0 and pH 7.0 in which the addition of NaCl reduced the EAI values (Figure 4.6A). For instance, at pH 5.0, EAI values declined from 21.1 m^2/g to 18.8 m^2/g and then to 12.8 m^2/g as NaCl levels increased from 0 mM to 50 mM and then to 100 mM, respectively. A similar trend was observed at pH 7.0, where the addition of NaCl reduced EAI values from 14.9 m^2/g at 0 mM NaCl to 5.2 m^2/g at 100 mM NaCl. A simple Pearson correlation analysis found that EAI was positively correlated with surface hydrophobicity ($r = 0.642$; $p<0.01$). It is believed that having high hydrophobicity leads to greater alignment and integration of protein into the oil-water interface, allowing interfacial tension to be reduced and greater to occur (Kato and Nakai, 1980; Zayas, 1997). Paulson and Tung (1987) and, Wanasundara and Shahidi (1997) suggested that the ratio of hydrophobic to hydrophilic sites on the protein's surface impacts the ability for a viscoelastic film to form. Krause and Schwenke (2001) reported that under neutral conditions, EAI for rapeseed globulin was relatively lower than the albumin fraction, and that of a mixed rapeseed protein comprised of 30% albumin and 70% globulin. The larger molecular mass of proteins of CPI may have a negative effect during the formation of emulsion due to the lack of conformational changes at the interface in its globular undissociated state (Wanasundara, 2011).

The emulsifying stability index (ESI) provides a measure of the stability of the diluted emulsion over a fixed period of time (Can Karaca et al., 2011). ESI for CPI as a function of pH and salt content was investigated and is given in Figure 4.6B. An analysis of variance found that only the effect of salt was significant ($p<0.001$). Overall, ESI was reduced with the addition of NaCl from ~15.1 min at 0 mM NaCl to ~11.6 min and ~12.0 min for the 50 and 100 mM NaCl levels, respectively. In this study, the ESI results were corresponded with the surface charge where the addition of NaCl reduced overall surface charge which would lead to droplets flocculation and coalescence due to lack of electrostatic repulsion between droplets. ESI values

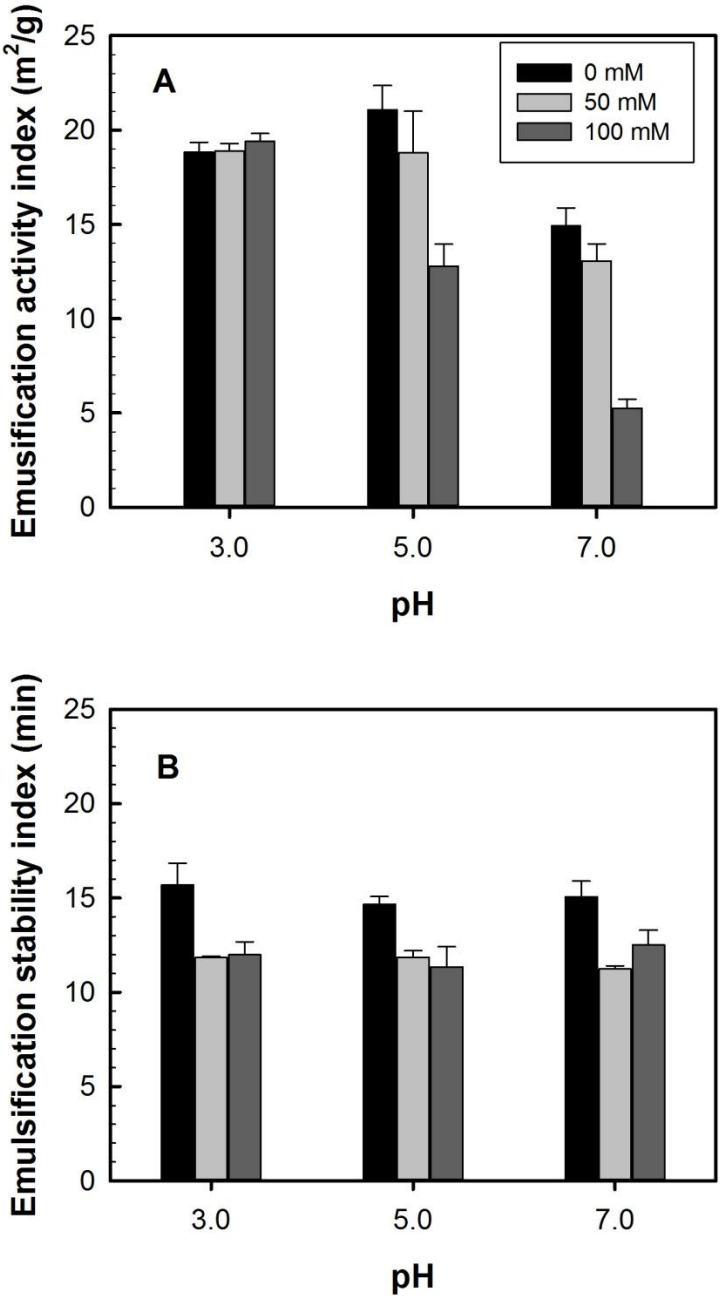

Figure 4.6 Emulsification activity (m^2/g) (A) and stability (min) (B) indices for CPI solutions as a function of pH and NaCl (mM) content. Data represent the mean ± one standard deviation (n = 3).

were also found to be negatively correlated with solubility ($r = -0.582$, $p<0.01$), where it was thought that reduced solubility was important for additional proteins to precipitate and adhere to the viscoelastic film surrounding the droplets. Solubility was higher for CPI solutions with NaCl present than without added NaCl, possibly due to a salting-in effect which kept a greater amount of protein in solution, despite pHs near its pI value (5.0, 7.0). Zhang et al. (2009) reported the effect of NaCl on the emulsion stability of chickpea proteins by measuring changes in mean droplet size over time, to find greater instability with the addition of NaCl as a result of coalescing droplets. In the study of Can Karaca et al. (2011), there were no significant differences of ESI between CPI extracted by different methods which was believed to be dominated by globulin or globulin/albumin mixed fraction. The authors found ESI of canola protein isolates around 10.5-15.5 min at pH 7.0 which was similar to the ESI values in the present study.

4.1.6 Summary

In general, physicochemical properties of CPI varied with increasing ionic strength depending on the pH level. For instance, the addition of NaCl greatly improved solubility at pHs 5.0 and 7.0 but had no effect at pHs 3.0. In contrast, surface hydrophobicity was found to decline with increasing ionic strength at pHs 3.0 and 5.0. Increasing ionic strength increased interfacial tension at pH 5.0 but had the opposite effect at pH 7.0. Overall, the emulsifying properties of CPI were strongly influenced by the physicochemical properties of the protein, pH and NaCl content. For instance, EAI was positively correlated with the protein's surface hydrophobicity and ability to reduce interfacial tension, whereas ESI was negatively influenced by the solubility of the protein. EAI was reduced with the addition of NaCl at pH close to pI value and at pH 7.0. Emulsion stability was also reduced with the addition of NaCl at all tested pH levels.

4.2 Effect of pH and NaCl on the physicochemical and emulsifying properties of a napin-rich protein isolate

4.2.1 Characterization of the NPI material

The NPI was determined to be comprised of 97.41% (d.b.). Figure 4.7 gives an SDS-PAGE polypeptide profile under non-reducing (lane 1) and reducing conditions (lane 2). Major bands under non-reducing conditions were found at ~24.5 kDa, ~14.4 kDa and ~12.3 kDa,

representing ~12%, ~66% and ~20% of the total bands, respectively as determined by densitometry (lane 1, Figure 4.7). Under reducing conditions, major bands were found at ~24.5 kDa, ~21.7 kDa, ~16.5 kDa, ~11.2 kDa and ~ 9.1 kDa, accounting for ~6%, ~3%, ~11%, ~43% and 34% of the total bands, respectively (lane 2, Figure 4.7). The halo surrounding the 14.4 kDa and 11.2 kDa bands under non-reducing and reducing conditions, respectively, is thought to be associated with a high protein loading onto the gel. Based on the SDS-PAGE analysis, the NPI appears to be rich in napin, since predominant polypeptide bands have typical molecular weight of small and large chains of *Brassica napus* 2S proteins (Schwenke, 1990). The amino acid composition of NPI was found to be high in glutamine (+ glutamic acid) (22.50%), along with proline (8.25%), lysine (6.46%), leucine (5.97%) and arginine (5.91%) (Table 4.2). Chabanon and others (2007) also reported napin to be rich in glutamine (+ glutamic acid) and arginine, accounting for ~30 and 8.6% of the total amino acids.

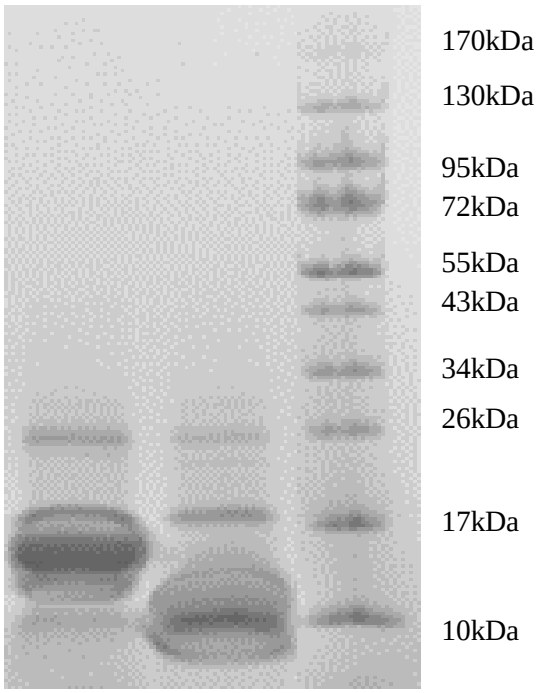

Figure 4.7 SDS-PAGE (reducing and non-reducing)(1 μL of 2 mg mL^{-1} NPI solution) applied to gradient 8-25% PhastGels. Lanes: (1) NPI (non-reducing); (2) NPI (reducing) and (3) Standard.

Table 4.2 Amino acid profile for the napin-rich protein isolate.

Amino acid	**Percent**
(Aspartic acid + Aspargine)	2.98
(Glutamic acid + Glutamine)	22.50
Alanine	3.63
Arginine	5.91
Cysteine	4.38
Glycine	4.21
Histidine	3.34
Isoleucine	3.09
Leucine	5.97
Lysine	6.46
Methionine	1.82
Phenylalanine	2.97
Proline	8.25
Serine	4.26
Threonine	3.33
Tryptophan	1.27
Tyrosine	1.58
Valine	3.82
Total:	**88.77**

4.2.2 Surface characteristics

Protein surface charge depends on both the amino acid composition and conformation of the protein molecules in solution (McClements, 2004). In general, highly charged proteins tend to have better solubility in aqueous systems due to the large amount of electrostatic repulsion (Can Karaca et al., 2011). Strong repulsion also fosters improved emulsifying properties of protein-stabilized emulsions, greater reactivity to cross linking formation during gelation and improved water hydration properties of the protein (McClements, 2005). Figure 4.8 shows the zeta potential (mV) for NPI solutions as a function of pH and salt concentration. A two-way

analysis of variance found the medium pH (5.0) and NaCl concentration had a significant effect ($p<0.001$) on zeta potential of the protein so as the combination effect of these two factors. At acidic pH (i.e. pH 3.0), the addition of salt reduced the overall positive surface charge of the protein molecules. At medium pH (i.e. pH 5.0), the addition of salt shifted the overall surface charge from positive to negative and also greatly reduced the magnitude of the surface charge. At higher pH, which the native protein exhibited negative surface charges, the addition of salt slightly increased the magnitude of the overall surface charge.

For all materials, surface charge was low; having zeta potentials ranging between ~-5 mV to ~10 mV suggesting that the protein carried little net charge in the pH range of 2.0 to 8.0. NPI in the absence of added NaCl, showed a pI (zeta potential = 0 mV) at pH 6.6, where at pH > pI and pH < pI proteins assumed a net negative and positive charge, respectively. Figure 4.8, also showed that as pH declined from 8.0 to 4.0, zeta potential increased relatively linear up to pH 4.0, and then dropped to ~1 mV at pH 3.0, before rising again to ~3 mV at pH 2.0. It was suggested napin molecule structure remains stable at the pH range of 5.5 to 7.0 (Krzyzaniak et al., 1998). The zeta potential behavior of NPI in the present study between pHs 3.0 and 4.0 is thus presumed to be due to structural changes in the NPI molecule. Jyothi and others (2007) showed that with the addition of 0.5 M NaCl, the napin became more compact indicating that there might be changes to the level of exposed amino acid groups on the surface that could lead to changes of surface charge.

The addition of NaCl to the NPI solutions resulted in a gradual and steady change in zeta potential as pH declined from pH 8.0 to pH 2.0. From pH 8 to 3.5, negative values were observed and between pH 3.5 and 4.0, NPI reached zero zeta potential. It is believed that NaCl at low concentration <0.5M, charge screening is prominent and may have shielded the charged sites of NPI molecule to reduce the thickness of its electric double layer. This effect was more pronounced at the 100 mM NaCl level, were the zeta potential values were closer to neutrality over the entire pH range, than at the 50 mM level (Figure 4.8). The addition of NaCl also acted to shift the pI of NPI from 6.6 to 3.5 and 3.9 when 50 mM and 100 mM NaCl was present, respectively. Wanasundara et al. (2012) reported at low ionic strength (<0.2 M NaCl), ions can non-specifically bind to the protein's surface to increase the thickness of the electric double layer. Consequently, molecules can adopt greater charge at its normal pI (i.e., without added NaCl) and a shift in the pH where net neutrality occurs. In the present study, the addition of 100

mM NaCl caused less of a shift in the pI value since its electric double layer was reduced more in size. In the literature, napin has been reported to have pI values >10.0 from *B. napus*, however this was typically based on theoretical values determined from its amino acid composition (Aider and Barbana, 2011). However, depending on the extent of various napin isoforms present, ionisable amino acid residues on protein surface may change. According to Yoshie-Stark et al. (2008), protein extraction conditions may affect the isoform composition in the final protein isolate. The present study used napin solubilized at low pH (3.0) leading to selectivity towards more basic isomers, however it seems all napin was extracted as no napin originated polypeptides were detected in the remaining meal residue (Wanasundara and McIntosh, 2008). However, further purification was not carried out for this NPI and the final protein product may contain some contaminants such as soluble fibre and non-napin protein (Figure 4.7 shows some other polypeptide bands) which can modify napin protein surface charges.

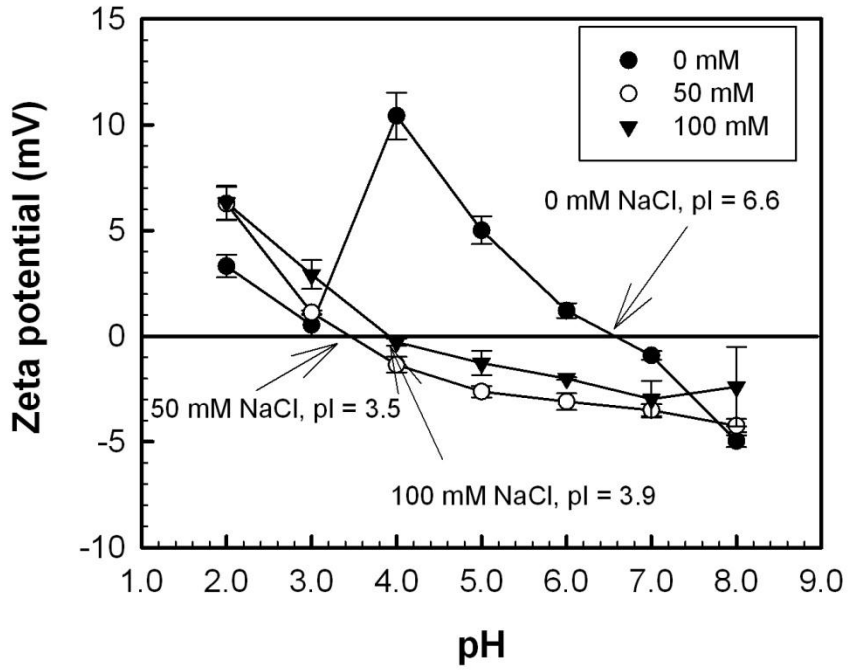

Figure 4.8 Zeta potential (mV) for NPI solutions as a function of pH and NaCl (mM) content. Data represent the mean ± one standard deviation (n = 3).

Surface hydrophobicity plays an important role in terms of protein solubility, protein-protein interactions (via hydrophobic interactions) and interfacial activity. The latter plays an

active role in stabilizing the oil-water interface within emulsions by hydrophobic moieties orientating inwards towards the oil phase, and hydrophilic moieties towards the aqueous phase to lower interfacial tension (Stuart et al., 1991; Krause and Schwenke, 2001). In the present study, pH and NaCl concentration along with their combined effect were found highly significant ($p<0.001$) on changing surface hydrophobicity of napin. Figure 4.9 shows surface hydrophobicity for NPI solutions as a function of pH and salt concentration. Overall, surface hydrophobicity was found to be highest at low pH and 100 mM NaCl content. At pH 3.0 and 5.0, surface hydrophobicity increased with increasing NaCl content and the effect of NaCl was found greater under acidic conditions. Possibly as the screening of charge sites on the NPI increased, the protein molecule gained greater conformational entropy or freedom (i.e., chain flexibility); allowing for the partial unraveling and exposure of previously buried hydrophobic sites. It is presumed that the greater rate of change in hydrophobicity with salt content at pH 3.0 is due to the overall slightly higher surface charge. At pH 7.0, surface hydrophobicity declined from 2.5 arbitrary units (A.U.) for NPI in the absence of added NaCl to 1.4 A.U. in the presence of 50 mM NaCl, and then increased to 3.5 A.U. with 100 mM NaCl present. At pH 7.0, surface charge on the native NPI is minimal since its close to its pI value (pH 6.6). It was presumed that the addition of NaCl content caused fluctuations to occur in the NPI conformation leading to slight changes in surface hydrophobicity. Jyothi et al. (2007) reported a low binding constant of various extrinsic fluorescence probes including ANS (~0.5 mol of probes binding to 1 mol of protein), which indicated napin is hydrophilic in nature. However in contrast to the present study, Jyothi et al. (2007) reported a decline in the hydrophobicity of napin with the addition of 500 mM NaCl. The authors proposed that the high concentration of NaCl lead to the stabilization of a more compacted NPI molecule in solution.

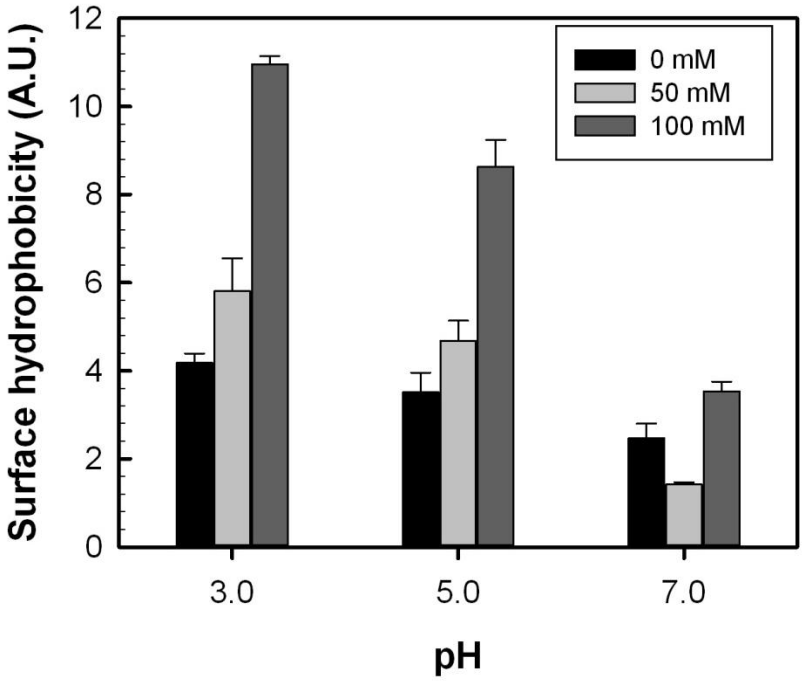

Figure 4.9 Surface hydrophobicity for NPI solutions as a function of pH and NaCl (mM) content. Data represent the mean ± one standard deviation (n = 3).

4.2.3 NPI solubility

A two-way analysis of variance found the main effects of pH ($p<0.001$) and NaCl concentration ($p<0.05$) to be statistically significant, whereas their associated interaction was not ($p>0.05$). Solubility was reported between 93.4% - 100% and only slight changes in values where observed due to the addition of NaCl. Overall solubility was found to be similar at pHs 3.0 (98.2%) and 5.0 (99.2%), however was slightly reduced at pH 7.0 (96.3%). Solubility of NPI was also found to be similar at NaCl levels of 0 mM (96.8%) and 100 mM (96.5%), however was found to be completely soluble with the addition of 50 mM NaCl. Although significant differences were found among the treatments, caution should be taken in terms of interpreting differences among treatments, as solubility for all NPI solutions remained high (>93.3%).

In a previous study conducted by Wanasundara et al. (2012) it was found that napin protein has high solubility at acidic pHs; when Brassica seed meals were extracted at pH between 3.0 and 4.0, low molecular weight proteins (<17 kDa) were found in the soluble fraction. Schwenke (1990) also showed that native napin isolate was completely soluble in the pH range of 1.0-10.0. Protein with good solubility is often associated with high surface charge

and low hydrophobicity (McClements, 2004). Napin is known to have very basic pI (>10.0) and is hydrophilic in nature (Schwenke, 1990; Jyothi et al., 2007). Although the NPI in the present study exhibited low surface charge, NPI used in this study showed a relatively low hydrophobicity value, which may be the main factor for its excellent solubility across the tested pH levels. Having good solubility represents an important attribute for proteins to be used as an emulsifier, as the protein is required to diffuse to the oil-water interface from the bulk aqueous solution to reduce interfacial tension (Kinsella et al., 1985; McClements, 2005; Can Karaca et al., 2011). Proteins with lower surface charge or if salts are added to screen charged sites on the protein's surface, then protein- protein interaction dominates and proteins have a tendency to associate into larger aggregates and fall out of solution (McClements, 2004). Similarly, proteins with high surface hydrophobicity tend to aggregate via hydrophobic interactions to form larger aggregates, which then fall out of solution (Damodaran, 1989). In the present study, napin was found with relatively low surface hydrophobicity which reduced the chance of protein aggregation due to hydrophobic interaction between droplets.

4.2.4 Interfacial tension

The addition of NPI to the aqueous phase (regardless of the solvent conditions) was found to reduce the interfacial tension at an oil-water interface from 22.5 mN/m to 10-17 mN/m. Figure 4.10 shows interfacial tension for NPI solutions as a function of pH and salt concentration. A two-way analysis of variance found pH, along with the combination effect of pH and salt, to have significant effect on the interfacial tension ($p<0.001$). Overall, interfacial tension declined from ~16.7 mN/m at pH 3.0, to 14.3 mN/m at pH 5.0 and further declined to 10.6 mN/m at pH 7.0, the major effect of salt was not significant and only caused slight changes of interfacial tension at each pH. At pH 3.0, interfacial tension declined slightly from 17.3 mN/m to 16.1 mN/m as NaCl levels increased from 0 mM to 100 mM; at pH 5.0, interfacial tension was relatively constant at ~14.3 mN/m as NaCl increased over the same range; and at pH 7.0, interfacial tension increased slightly with increased NaCl levels. When surface hydrophobicity and interfacial tension values are taken into consideration, the comparatively high surface hydrophobicity values of napin at pH 3.0 may have contributed to their reduced ability to lower interfacial tension at oil-water interface. It is hypothesized that at pH 7.0, NPI had low hydrophobicity and negative zeta potential which together have contributed to the lowest

interfacial tension. As stated previously, the low hydrophobicity and surface charges allowed the NPI to become better solubilized and reduced the chance of protein- protein interaction before aligning at the interface, a protein's ability to reduce the interfacial free energy is essential for its use as an emulsifier and it is a good predictor of its emulsifying properties (Stuart et al., 1991).

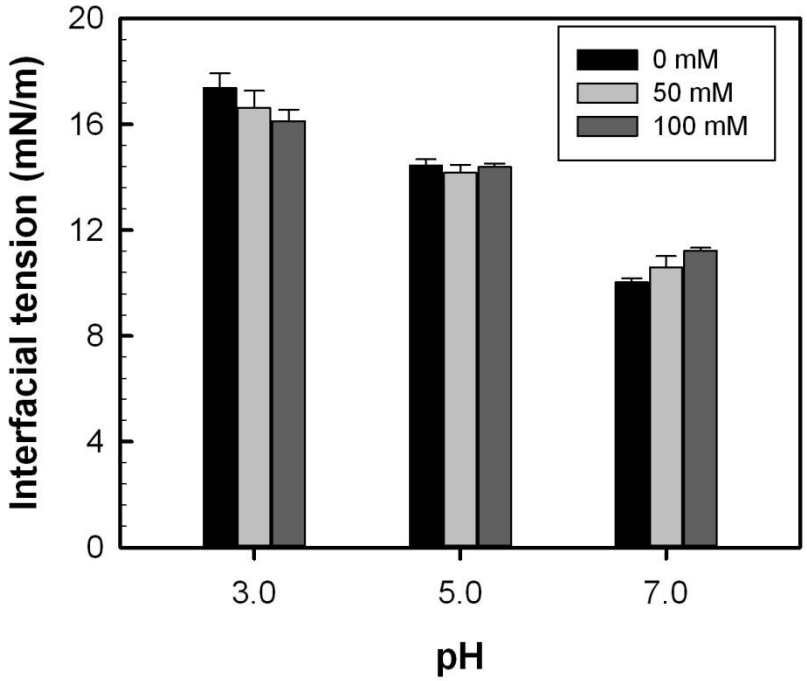

Figure 4.10 Interfacial tension (mN/m) for NPI solutions as a function of pH and NaCl (mM) content. Data represent the mean ± one standard deviation (n = 3).

4.2.5 Emulsifying properties

Emulsifying activity index (EAI) is the measure of interfacial area coated by a surfactant such as protein as explained by Pearce and Kinsella (1978). Figure 4.11A shows EAI for NPI solutions as a function of pH and salt concentration. A two-way analysis of variance found that the main effects of salt and pH to be significant ($p<0.001$), along with their associated interaction ($p<0.01$). Overall, EAI values were similar in magnitude at pH 3.0 (19.4 m^2/g) and pH 5.0 (18.7 m^2/g), and lower at pH 7.0 (12.8 m^2/g). The effect of NaCl on EAI was similar at pH 3.0 and 7.0. For instance, EAI at the 0 mM and 100 mM NaCl level were similar in magnitude, but increased significantly at the addition of 50 mM NaCl. However, the EAI values at pH 5.0 reduced as the level of NaCl increased. Proteins with good emulsifying properties are often found with high

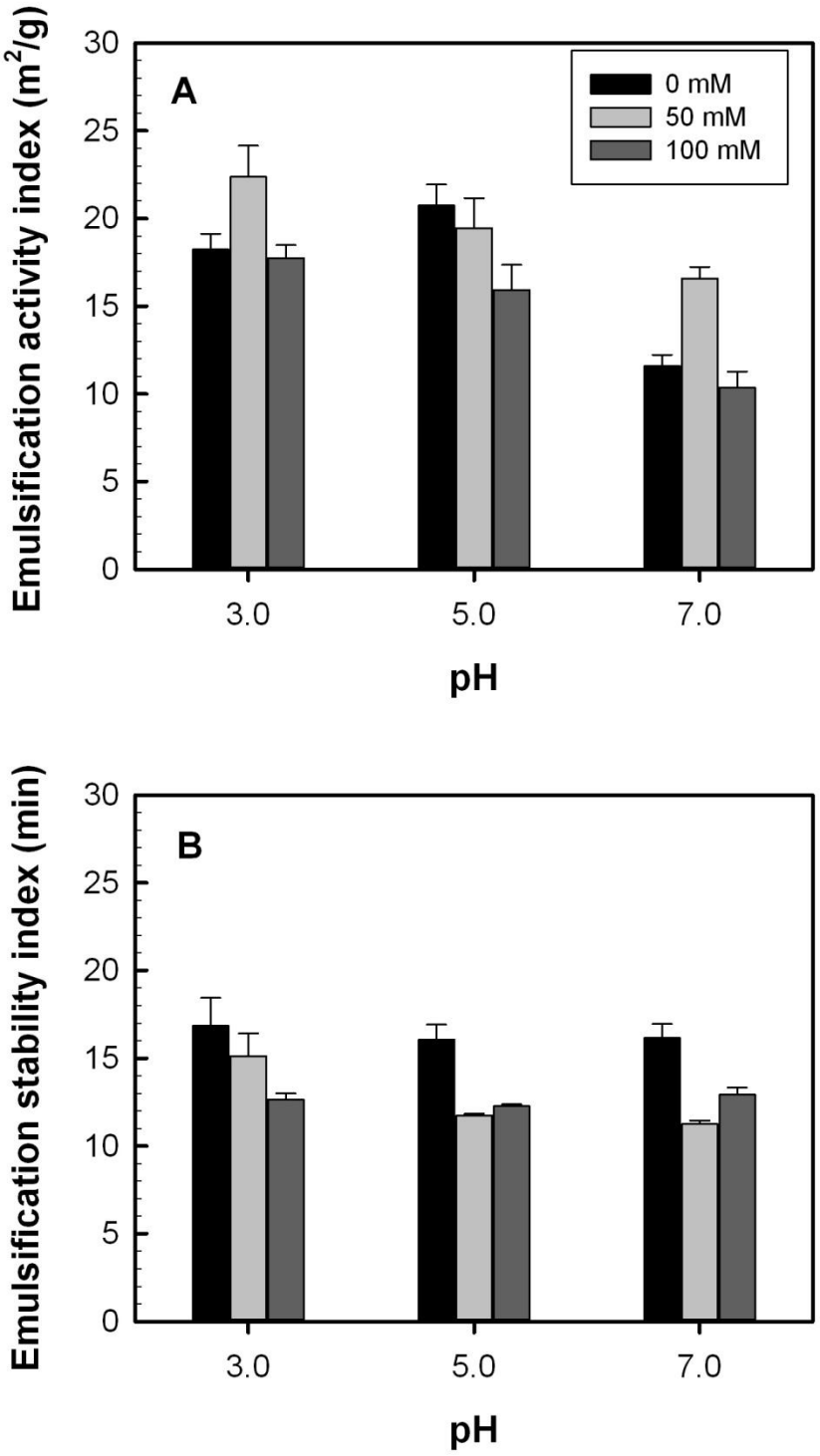

Figure 4.11 Emulsification activity (m^2/g) (A) and stability (min) (B) indices for NPI solutions as a function of pH and NaCl (mM) content. Data represent the mean ± one standard deviation (n = 3).

solubility and high surface charge (Dalgleish, 2004; Can Karaca et al., 2011). In the present study, solubility of NPI was excellent across the pH and NaCl levels tested which indicated there are other factors contributed to the variation of EAI values of NPI. For instance, similar trends were observed between the surface hydrophobicity and EAI of NPI where the values of NPI reduced as pH increased. It was hypothesized that surface hydrophobicity value alone was not a good predictor of emulsifying properties, but rather the overall distribution of hydrophobic and hydrophilic moieties on the protein molecule (Zayas, 1997). Kato and Nakai (1980) observed that a protein often has good emulsifying properties if the protein has more than 30% of nonpolar amino acid residue in its total amino acid profile. NPI in the present study show ~35.5% of total amino acid composition was composed of non-polar amino acid residues. Moreover, in this study, it is believed that the effects of pH and NaCl as well as their combined effects had greater impacts on the EAI values of NPI than the physicochemical properties of NPI. In fact, Krog and Sparsø(2004) stated that the emulsifying process and the final distribution of oil droplets are mainly affected by the energy input during homogenization and the influence of emulsifiers is limited. Krause and Schwenke (2001) reported ~4X higher surface coverage by napin isolate compared with cruciferin isolate indicated that napin is very surface active.

The emulsifying stability index (ESI) provides a measure of the stability of the diluted emulsion after homogenization (Can Karaca et al., 2011). Figure 4.11B shows ESI for NPI solutions as a function of pH and salt concentration. A two-way analysis of variance found that the main effects of pH ($p<0.01$) and salt ($p<0.001$), along with their interaction ($p<0.01$) were significant. According to ESI, NPI stabilized emulsion degraded rapidly and the addition of salt induced faster emulsion instability. At pH 3.0, ESI declined relatively linear from 16.8 to 12.6 min as NaCl content increased from 0 mM to 100 mM. In contrast, at pH 5.0, ESI declined from 16.1 min to 11.7 min as NaCl increased from 0 to 50 mM, and then increased slightly at 100 mM NaCl. A similar trend was also reported for pH 7.0. In all cases ESI was relatively the same in the absence of added NaCl, ranging between 16.0 to 16.8 min and then declined with the addition of NaCl. A charged viscoelastic surface can lead to increased electrostatic repulsion between droplets to help keep the emulsion stable (Damodaran, 1989). This is also verified by the statistic results of the present study which indicated higher surface charge lead to higher ESI values. This effect however can be reduced through the addition of salts, which act to screen charged sites and reduce the thickness of the electric double layer leading to droplet

flocculation/aggregation (McClements, 2005). In this study, however the addition of salt only caused reduction of the overall surface charge at pH 5.0 and did not significantly reduced overall charges of NPI at pH 3.0 and 7.0, thus it is believed other factors might be affecting the reduced ESI value of NPI beside surface charge. Kulmyrzaeva and Schubertb (2004) studied the effect of potassium chloride and pH on whey protein induced emulsions and found the addition of KCl at more than 10 mM negatively affected the stability of the emulsion system due to the lowering of overall zeta potential from pH 2.5-7.0. Another study compared the effect of NaCl on flaxseed protein and soy protein induced emulsions and found that the addition of 50 mM NaCl and 100 mM effectively reduced oil droplet flocculation in flaxseed protein and soy protein induced emulsions, respectively, at isoelectric pH (4.2) (Wang et al., 2010). McClements (2004) also studied the effect of both monovalent salt (NaCl) and divalent salt ($CaCl_2$) on oil droplet size of soy protein stabilized emulsions and found oil droplet size remained < 1µm at 200 mM NaCl. However droplet size increased dramatically from 1.5 µm to 10 µm when $CaCl_2$ was added at levels > 4.0 mM.

4.2.6 Summary

Surface charge and the isoelectric point for NPI were found to be much lower than expected. It was speculated that this could be due to the presence of impurities such as phenolic compounds and/ or phytic acids. It was found that NPI is hydrophilic in nature which could be associated with the high solubility across tested pHs. Overall, the emulsifying properties of NPI showed a relationship to the protein's surface characteristics (i.e., charge and surface hydrophobicity) which influenced their ability to lower interfacial tension. The medium factors such as pH and NaCl also had impacted NPI's emulsifying abilities. The emulsion forming properties of NPI appeared to be better at pH 3.0 and 5.0 than at a higher pH (7.0), with slight variations in response to NaCl. In contrast, the stability of these formed emulsions was less dependent on pH, and more influenced by the presence of NaCl which lead to greater instability.

CHAPTER 5: CONCLUSIONS

Canola proteins, because of their nutritional and functional properties, could emerge as a potential alternative choice to soy in the plant protein ingredient industry to soy, once launched into the marketplace. However, more information is needed to understand how the various protein fractions behave from a functional stand point in order to optimize breeding programs, extraction technology, and ingredient performance in foods and/or in non-food industrial applications. Canola proteins are dominated by two main proteins, a salt-soluble cruciferin protein and a water-soluble napin protein. Each protein is different in terms of their structure, size and surface properties, all of which could lead to differences in their functional performance as ingredients, depending on the relative composition of commercially produced mixed isolates. The goal of this research was to examine similarities and differences in the surface properties of cruciferin- and napin-rich protein isolates, and then relate this to their emulsifying properties under different pHs (3.0, 5.0 and 7.0) and salt concentrations (0, 50 and 100 mM NaCl).

Both proteins differed considerably in size. The result of SDS-PAGE under reducing conditions showed cruciferin to have much larger sub-units, ranging in molecular mass from 17 to 150 kDa, whereas napin proteins were significantly smaller ranging between 12 and 17 kDa. Amino acid composition indicated that both proteins were high in glutamic acid and glutamine; however, napin had slightly more (22.5 vs. 17.9%). Glutamine has pKa values of 2.17 and 9.13 for the α-carboxyl and α-amino sites, respectively, whereas glutamic acid has pKa values of 2.10, 9.47 and 4.07 for the α-carboxyl, α-amino and side chain groups, respectively. At the pKa values, 50% of the respective sites (i.e., α-carboxyl group) are protonated. In the present study, surface characteristics and functionality was measured at pH 3.0, 5.0 and 7.0. Since napin proteins contained higher levels of glutamine + glutamic acid than found in cruciferin, the overall charge should be less, especially at pH 3.0 and 5.0 where more sites would be protonated. Zeta potential values overall for the napin protein isolate as a function of pH were substantial lower than those of the cruciferin isolate. For instance, in the absence of NaCl, the napin protein isolate showed a zeta potential value between -5 mV and +10 at pH 8.0 and 3.0, respectively,

whereas the cruciferin protein isolate had values ranging between -30 mV to +35 mV at corresponding pHs. The addition of NaCl acted to shield the electric surface charge of both proteins through a counter-ion screening effect. As such, the electric double layer was thought to decline in both cases. The isoelectric point of napin and cruciferin protein isolates (in the absence of salts) was found to occur at pHs 6.6 and 4.8, respectively. Values were lower than those reported in the literature where the pIs of cruciferin and napin proteins have been reported as 7.2 and ~10.0-11.0 in the literature (Schwenke, 1988, 1994). Interaction of the protein isolates with phenolic compounds and phytic acid also might have altered the chemical and physical properties of the protein isolate (Aider and Barbana, 2011; Wanasundara, 2011). However, since cruciferin was extracted with a method reported to produce products low in phenolic and phytic acid (Krause et al., 2002), there is little concern for the presence of phenolic compounds and phytic acid with the cruciferin-rich isolate. In the case of napin, at pHs<pI, proteins might interact with non-protein compounds such as phenolic compounds and/ or phytic acid, leading to variations in surface properties (Wanasundara, 2011).

Overall, the average hydrophobicity at the surface of cruciferin was also much higher than that of napin, suggesting that more hydrophobic moieties (alanine, valine, isoleucine, leucine, methionine, phenylalanine, tyrosine and tryptophan) were present (~30% vs. 24%) and exposed at the surface. Furthermore, the effects of NaCl and pH on surface hydrophobicity were found also to be different between the two proteins. In the case of napin, hydrophobicity declined as the pH increased from pH 3.0 to 7.0 however, hydrophobicity was raised in the presence of NaCl. It is hypothesized that the screening of charged sites along the protein's surface lead to increased conformational entropy (flexibility) allowing for a higher amount of hydrophobic groups to become exposed. In contrast, for cruciferin, the effects of pH and salt on hydrophobicity were less clear. The highest hydrophobicity was found at pH 3.0 without NaCl, present, whereas the lowest was found at pH 7.0 in the presence of 100 mM NaCl. Overall, hydrophobicity declined as pH was raised from 3.0 to 7.0.

Napin protein isolate was almost completely soluble regardless of the pH and NaCl content. In contrast, the solubility of the cruciferin protein isolate ranged between ~80 and 90% under all solvent conditions with the exception of pH 5.0 and 7.0 in the absence of NaCl in which solubility was <20%. The presence of NaCl showed a 'salting-in' effect on the cruciferin where protein-water interactions were enhanced, resulting in greater structuring of the hydration

layers surrounding the protein to lead to high solubility. This effect was presumed to be more dominant than the screening effect of NaCl on the surface charge of the protein, which would have had an adverse effect on solubility, as was the case seen at pH 5.0 and pH 7.0.

During emulsion formation, soluble proteins migrate or diffuse towards the oil-water interface from the bulk aqueous phase where they then re-arrange and re-orient to position their hydrophobic moieties towards the oil phase and the hydrophilic moieties towards the bulk phase. The ability of napin and cruciferin proteins to reduce the interfacial tension was similar between the two proteins, despite minor differences seen in response to changes in pH and ionic conditions. Findings suggest that slight differences in protein solubility for both napin and cruciferin at the various solvent conditions did not impact its ability to reduce interfacial tension, nor did there appear to be a relationship with surface charge or average surface hydrophobicity.

The emulsifying properties of both cruciferin and napin proteins were both influenced by pH and ionic strength, however overall they had EAI and ESI values similar in magnitude indicating that they had similar emulsifying potential under the solvent conditions examined. For cruciferin proteins, no clear trend was evident with pH or NaCl level. EAI values were found to be similar at pH 3.0, regardless of the NaCl levels, whereas at pH 5.0, EAI values declined with increasing levels of NaCl. At pH 7.0, EAI values declined with the addition of 50 mM NaCl then remained constant. As for napin, the addition of 50 mM NaCl resulted in higher EAI values at pH 3.0 and 7.0 however at pH 5.0, the addition of NaCl reduced the EAI as NaCl increased. The ability for both cruciferin and napin proteins to stabilize the emulsion was reduced with the addition of NaCl. The stability of an emulsion is depended on the electrostatic repulsion between droplets in order to delay coalescence and flocculation (McClements, 2005). Addition of NaCl in this study resulted in reduced zeta potential for both protein isolates which lead to reduced emulsion stability.

Overall, this research found that despite cruciferin-rich and napin-rich protein isolates having quite different surface characteristics and solubility, the emulsifying forming and stabilizing effects were similar. Furthermore, separation of the two proteins from the isolate ingredient may not be necessary if emulsification is the only functional role the proteins are being used for, from a commercial stand point.

CHAPTER 6: FUTURE STUDIES

Stemming from this research, recommendations for future studies include: examining more closely the effects of protein extraction on the functionality of resulting ingredients; studying the interactions between canola proteins and other ingredients (e.g., sugars, polysaccharides and lipids); and looking at enzymatic modification as a means to improve their emulsifying properties.

Within the literature, there is a huge variation in extraction protocols for canola proteins combined with non-standardized functionality testing making comparing and contrasting results difficult. Many extraction methods, especially for napin involve the use of chromatography to achieve much greater purity than seen in the current work. The impact of cross-contamination of the other protein within the isolate products also remains unknown, and would be interesting to explore their impact on ingredient performance. Experiments may involve examining differences in functionality of isolate products of varying degrees of purity after running the crude isolate materials through various size exclusion columns. Methods of extraction found in the literature tend to be more at the bench top level of product, where the resulting products may be quite different from large scale commercial products that will soon hit the market. A greater comparison of the magnitude of the extraction process may also be important to better connect the bench top research to emerging industrial products.

A greater understanding of ingredient interactions involving cruciferin and napin proteins and other food ingredients (e.g., sugars, polysaccharides and lipids) is also of significant importance. For instance, Klassen and others (2011) reported that both physicochemical and functional properties (e.g., solubility) of a canola protein isolate differed depending on whether ι-carrageenan or alginate was present. Effects of sugar (sucrose) on the physicochemical and functional properties were also reported and it shown added sugar (10-40%) was able to delay droplet flocculation by increasing the solvent viscosity and stabilizing globular conformation of α-lactoglobulin, which reduces exposure of hydrophobic moieties (Kim et al., 2003). Furthermore, limited enzymatic modification of plant proteins have been shown to have both a

positive and negative impact on protein functionality. For instance, Zhao and others (2011) found peanut protein isolate partially hydrolyzed with alcalase showed improved protein solubility, thermal stability and gelling capacity, however the partial hydrolysis impaired emulsifying activity index. Similarly, Avramenko et al. (2013) also found partially hydrolyzed lentil protein isolate with trypsin resulted in inferior emulsifying properties compared with native lentil protein isolate. As such, further investigation in the level of hydrolysis and modification of conditions as a means for improve canola protein functionality should also be explored.

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