Solubility, *In Vitro* Digestibility and Allergenicity of *Brassica juncea, Brassica napus* and *Sinapis alba* Proteins

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ABSTRACT

Canola (*Brassica napus, Brassica rapa* and *Brassica juncea*) and mustard (*Brassica juncea* and *Sinapis alba*) are economically important *Brassicaceae* crops in Canada. Distribution of proteins and antinutritive compounds in the seed coat and cotyledons and the solubility properties of the seed proteins were evaluated in six varieties of *Brassicaeae* [(*B. juncea* (oriental, brown and canola-quality mustard), *B. napus* (canola) and *S. alba* (yellow mustard)]. *In vitro* digestibility and potential allergenicity, as determined by pepsin digestibility and bioinformatic analysis, respectively, were conducted for the major seed storage proteins.

The major components in *Brassicaceae* oilseeds are oil and proteins. *B. juncea* and *B. napus* seed contain 40-42% oil; for *S. alba* seed the value is 30-32%. The seed coat percentage (by weight) of *S. alba* seed is higher than that of the other species; canola-quality *B. juncea* seed has a lower content of seed coat than *B. napus*. Glucosinolates, phytic acid and phenolic compounds are minor compounds present in *Brassicaceae* seed which may have negative effects on the nutritional value of seed meal.

The contribution of non-protein nitrogen to total seed N ranged from 3.1-10.8% depending on the seed type. In *Brassicaceae* seeds, 11S cruciferin and 2S napin were the major storage proteins. The solubility of storage proteins from all *Brassicaceae* seeds was dependent on pH. Napin protein in all species showed high solubility between pH 3 and 4, and the minimum total protein solubility of seed was observed within this range. Both napin and cruciferin proteins were soluble in strongly alkaline and acidic pH ranges. In all varieties, Na^{+} and Ca^{2+} increased overall protein solubility; however, the extent of protein solubility changes in relation to ion type varied among species. The combination of salt and pH in the media can be manipulated to maximize the solubility of seed storage proteins.

The *in vitro* digestibility values for the defatted meals were lower than those of the napin-free meals. Napin protein exhibited the lowest digestibility, and for the six seed types it was less than 10%. It was evident that the low digestibility of napin was related to the overall low protein digestibilities of the meals, and that low level of intestinal protease inhibitors, i.e., trypsin and chymotrypsin inhibitors, may have a negligible effect

on protein digestibility. At a high pepsin concentration, cruciferin was quickly digested and would be expected to show no or weak resistance to proteolytic cleavage under simulated gastric conditions. However, napin was resistant to pepsin digestion, and a large fraction of this protein remained intact within the gastric and intestinal digestion period, indicating the potential to remain as a gastrointestinal allergen.

The sequence homology assessment of napin from *B. juncea*, *B. napus* and *S. alba* revealed a high degree of homology among the napin protein of these species, and some isoforms exhibited almost 100% sequence identity to the known mustard allergens (Bra n 1, Bra j 1, Sin a 1) and a strong possibility of cross-reactivity among species. Cruciferin protein sequence alignment resulted in lower scores for other known allergens (from cashew, hazelnut, etc.). This implies that cruciferin protein has a lower possibility of cross-reactivity with these known seed allergens.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

AAFC	Agriculture and Agri-Food Canada
AOAC	Association of Official Analytical Chemists
APC	Antigen-presenting cells
BCN	β-conglycinin
BM	Brown mustard
BSA	Bovine serum albumin
BSB	Borate saline buffer
BTEE	N-benzoyl-L-tyrosine ethyl ester
BV	Biological value
ºC	Degrees of Celsius
CGC	Canadian Grain Commission
CP	Crude protein
CQM	Canola quality mustard
CRU	Cruciferin
CT	Chymotrypsin
Cys	Cystine
DBPCFC	Double blind placebo controlled food challenges
DMF	N, N, Dimethyl formamide
DNA	Deoxyribonucleic acid
DSC	Differential scanning calorimeter
FAO	Food and Agricultural Organization
GI	Gastrointestinal
GLM	General linear model
GM	Genetically modified crops
IgE	Immunoglobin E
IHRP	Horse radish peroxidase
Lys	Lysine
LYZ	Egg white lysozyme
ME	β-mercaptoethanol

Met	Methionine
MHC	Major histocompatibility complex
MSTFA	N-methyl-N-TMS-trifluoroacetamide
MTI	Mustard trypsin inhibitors
MWM	Molecular weight marker
NAP	Napin
NMR	Nuclear magnetic resonance
NPU	Net protein utilization
NR	Non reducing conditions
ns-LTP	Non specific lipid transfer proteins
OM	Oriental mustard
PBS	Phosphate buffered saline
PBST	PBS with Tween-20
*p*I	Isoelectric point
PSV	Protein storage vacuoles
RC	Reducing conditions
RNA	Ribose nucleic acid
RRF	Relative response factor
RTI	Rapeseed trypsin inhibitor
S	Svedberg number
S-ELISA	Sandwich-enzyme-linked immunosorbent assay
SGF	Simulated gastric fluid
SIF	Simulated intestinal fluid
SRP	RuBisCo protein
SPT	Skin prick test
SSP	Seed storage protein
STI	Soybean trypsin inhibitor
Thr	Threonine
TI	Trypsin inhibitor
TIU	Trypsin inhibitor unit
TMB	3, 3’, 5, 5’-tetramethylbenzidine

TMCS	Trimethylchloro-silane
Trp	Tryptophan
WHO	World Health Organization
YM	Yellow mustard
β-LG	Bovine β-lactoglobulin

1. INTRODUCTION

The *Brassicaceae* (*Cruciferae*) family contains over 370 genera and 3,200 species. Due to their survival ability in different environmental conditions, *Brassicaceae* plants are grown all over the world. Several members of the *Brassicaceae* family are cultivated globally and include oilseeds, condiment crops, vegetables and weeds. Mustard and canola are economically important *Brassicaceae* crops in North America.

Canola-quality *Brassica napus* is the major commercial *Brassicaceae* oilseed crop in Canada. In addition, *Brassica rapa* (canola), *Brassica juncea* (canola and brown and oriental mustard) and *Sinapis alba* (yellow mustard) of the same family are also economically significant crops in the prairies. These *Brassicaceae* members have special adaptation to western Canadian weather which includes heat and drought tolerance and resistance to a number of diseases (Kimber & McGregor, 1995). *Brassica napus* (canola), the predominant oilseed in Canada, has several similarities to, as well as differences from, *B. juncea* and *S. alba*. At present, both *B. juncea* and *S. alba* are grown primarily for the condiment and spice market. Canola-quality *B. juncea* has been developed and included in commercial canola cultivation. However, with respect to the oil-based biofuels, *B. juncea* is a preferred oilseed crop because of its high seed oil content and its desirable agronomic characteristics (Potts, Rakow & Males,1999). *S. alba* seed is higher in protein and lower in oil than other crucifers, and almost all Canadian yellow mustard production is used in food applications.

Mustard seed is used primarily for condiments because of the unique flavor it imparts to a variety of food products. The glucosinolates are the main flavor precursor of mustards; oil-extracted, air-dried meal of *B. juncea* and *S. alba* contains 150-200 μmol g^{-1} and ~200 μmol g^{-1} of allyl glucosinolate (sinigrin) and 4-hydroxybenzyl glucosinolate (sinalbin), respectively (Uppstrom, 1995). Yellow mustard seed contains a considerable proportion of mucilage. Mustard mucilage provides a thick consistency and texture to food products such as salad dressings and pasta sauces (Weber, Talllie & Stauffer, 1974).

Compared to canola, mustard oil use is very limited in Canada. The co-product mustard meal is a good source of protein similar to canola meal. Oil free-residue of *B. juncea* , *B. napus* and *S. alba* contain 45.0%, 38% and 47.8% protein, respectively, on a dry matter basis. In comparison to other industrially comparable protein-containing seeds such as soybean, and canola and mustard meal have a well balanced amino acid composition, as they are higher in sulphur containing amino acids and arginine, but lower in lysine (Applequist & Nair, 1977; Newkirk, 2009). Amino acid composition and protein digestibility are essential properties that directly influence the nutritional value for food and feed applications of oil-free *Brassicaceae* oilseed meal.

In the *Brassicaceae* family, mainly two types of seed storage protein (SSP) are present. A large, legumin type 11/12S globulin (cruciferin) is predominant and has a molecular mass in the range of 300-350 kDa. Napin, a smaller molecular size 2S albumin, has a molecular mass of 12 to 13 kDa. In mature *B. napus* seeds, the 11/12S globulin protein constitutes about ~60%, and napin ~20%, of total storage proteins (Crouch & Sussex, 1981; Lonnerdal & Janson, 1972). According to Malabat et al. (2003), the cruciferin content may vary from 32-52% of total proteins among European rapeseed cultivars.

Protein solubility properties are important factors that determine the quality of SSP for both food and feed industrial uses. The major implication of SSP solubility is for the recovery (extractability) of protein from seed or seed meal. Solubility of SSPs is dependant on their physicochemical characteristics such as size, shape, amino acid composition and sequence, and surface charges and affects technologically important functions that protein can impart in complex systems (e.g., ability to form emulsions, foams and gels). Therefore, most of the technologically important functional properties of proteins are directly affected by the solubility of the protein (Damodaran, 2008). Properties like thickening, foaming, emulsifying and gelling are especially important functionalities of proteins in the food industry and are related to solubility. The studies available on the solubility of *Brassicaceae* proteins have focused on the extrinsic factors that affect solubility of total proteins and do not differentiate the solubility properties of individual SSPs.

Protein digestibility is one of the important factors that determine protein quality. It is mainly related to the release and availability of amino acids for absorption in the small intestine. Since *in vivo* protein digestion is an enzyme catalyzed hydrolytic reaction, the soluble proteins are expected to have better protein quality parameters than insoluble proteins. The study of Bos et al. (2007) on human feeding of a *B. napus* protein product (36.8% globulin, 41% napin, 2.7% lipid transfer protein, 14.9% total nitrogen) showed that the digestibility values are between those of pulse and cereal proteins. The meal protein digestibility value may be related to the type of storage protein (e.g., cruciferin, napin), extent of each protein present, nature of the storage protein (e.g., association with other molecules), and the presence of enzyme inhibitors, phenolic compounds, glucosinolates and fibre in the seed.

It is considered that if proteins are not digested in the stomach, large protein molecules can activate the IgE (type E immunoglobulin) antibodies, and hence can trigger gastrointestinal (GI) allergic reactions (Mills, Madsen, Shewry & Wichers, 2003) in individuals who are susceptible to absorbing such proteins. According to Thomas et al. (2004), if proteins are resistant to digestion in an environment of high pepsin concentration, these proteins could be possible gastrointestinal (GI) allergens. The evaluation of *in vivo* digestibility is the most accurate method of evaluating protein digestibility but it is difficult to use human subjects in such studies and to challenge them with allergen containing foods. Therefore, several *in vitro* digestibility assessments and model systems are employed to mimic the gastric and intestinal phases of digestion (Thomas et al., 2004). The low molecular weight proteins present in mustard are considered allergenic to some individuals. The 2S albumin SSP Bra j 1 and Sin a 1 present in *B. juncea* and *S. alba*, respectively, are recognized allergenic proteins (Monsalve, Villalba, Martin & Eseverri, 2001; Sathe, Sathe, Kshirsagar & Roux, 2005). The structure of this protein is significant in eliciting allergenicity; 2S proteins have several inter-chain disulphide bonds, which may interfere with protein digestion in the GI tract.

Studies on the properties or potential uses of *B. juncea* or *S. alba* SSPs are scarce. An understanding of protein properties that are linked to protein types and structural

details will help in designing recovery methods for and uses of these plant proteins. The overall objectives of this research project were to compare the composition of nitrogenous components of the seed, protein solubility properties, *in vitro* GI digestibility and potential allergenicity of SSPs from *B. juncea* and *S. alba* with those of *B. napus*. The first phase of the project was focused on analysis of seed composition, solubility behaviour and thermal denaturation behaviour of the storage proteins of six varieties of *B. juncea, B. napus* and *S. alba*. The second part of the study involved evaluation of *in vitro* GI digestibility and gastric digestion stability of individual SSPs of these *Brassicaceae* seeds.

2. LITERATURE REVIEW

2.1 Oilseeds of *Brassicaceae* plants

The family *Brassicaceae* (*Cruciferae*) plants are grown in a wide variety of weather conditions all over the world. Several *Brassicaceae* family plants are economically important crops grown globally and include oilseeds, condiment crops, vegetables and weeds. Since historic times the edible value of *Brassicaceae* plant parts like inflorescences, leaves, root bulbs, roots, seeds and stems have been recognized. Species grown as oilseeds include *Brassica juncea* (L.) Czern, *Brassica napus* L. subsp. *napus*, *Brassica rapa* L. subsp. *oleifera* (DC) (previously known as *Brassica campestris* L.), *Brassica carinata* A. Braun and *Brassica nigra* (L.) W. D. J. Koch. The vegetable *Brassicaceae* plants include *B. napus* (rutabaga, kale), *B. rapa* (bok choy, broccoli, Chinese cabbage, Chinese mustard, and turnip), *B. oleracea* (broccoli, Brussels sprouts, cabbage, cauliflower, collards and kale) and *Raphanus sativus* (radish). (Downey & Robbelen, 1989) The condiment crops include *B. juncea* (L.) Czern (brown and oriental mustard), *Sinapis alba* L. subsp. *alba* (yellow mustard, formerly *Brassica hirta* Moench.), *B. nigra* (black mustard) and *Armoracea rusticana* (horseradish). There are some dominant weed species in this family and examples are *Sinapis arvensis* (wild mustard), *Raphanus raphanistrum* (wild radish) and *B. rapa* (wild rape). Recently, there is a growing interest to use oil of *Camelina sativa* (camelina, gold of pleasure or false flax) as feed stock for liquid biofuel production which may result in commercial cultivation of this *Brassicaceae* plant (Downey & Robbelen, 1989).

Brassicaceae plants are the earliest domesticated plants. In the Neolithic age, some *Brassicaceae* plants have been used as vegetables (Downey & Robbelen, 1989). According to Indian Sanskrit writing in 1500 to 2000 BC and Greek, Roman and Chinese writings in 2000 BC to 5000 BC, oilseed rape and mustard have been used for medicinal purposes. Commercially grown rapeseed were first recorded in Europe in the early 16th century; at that time, rapeseed oil was mainly used as lamp oil and production of

lubricants for steam engines. After World War II, breeding and production of low erucic acid and low glucosinolate rapeseed has been a major research interest in western countries (Downey & Robbelen, 1989).

2.2 Commercially cultivated *Brassicaceae* oilseeds

The *Brassicaceae* oilseed crops are grown in the temperate agricultural zones of the world. The ability to survive and produce seeds under low temperature and moisture conditions is a favorable characteristic of these plants and has made them adaptable to cool climate, high elevations and subtropical cultivation. Four closely related *Brassicaceae* species are grown all over the world for oil harvesting purpose. They are oilseed rape (*B. napus*) and turnip rape (*B. rapa*) mainly grown in temperate regions such as North America, Europe and China and tropical and semi tropical areas such as of Asia. The Indian mustard or Rai (*B. juncea*) are mainly grown in India, Pakistan and Bangladesh. Ethiopean or Abyssinian mustard (*B. carinata*) has originated in the sub Saharan region (Downey & Robbelen, 1989). Genomic relationship between these species is illustrated in Figure 2.1, and indicates several similarities and differences in the genetic composition among them.

In western Canada, *Brassicaceae* oilseed crops have significant economic importance. Low erucic and low glucosinolate germplasm (canola) has been derived from *B. napus, B. rapa* and recently from *B. juncea*, and are cultivated as canola. At present, herbicide tolerant *B. napus* comprises over 98% of Canadian canola production (Canadian Grain Commission, 2010). Varieties of *B. juncea* that fit canola's oil and glucosinolate profile (canola quality mustard; CQM) has been developed; usually *B. juncea* has good adaptation to the semi-arid regions of western Canada and is resistant to black-leg disease. Fatty acid profile of CQM is closely similar to *B. napus* (Potts, Rakow & Males,1999).

S. alba (yellow mustard, YM) and *B. juncea* (oriental and brown mustard) are mainly grown for the condiment mustard market and are economically significant crops in Saskatchewan. Mustard species have special adaptation to western Canadian soil and climate. They are heat and drought tolerant and resistant to a number of diseases that

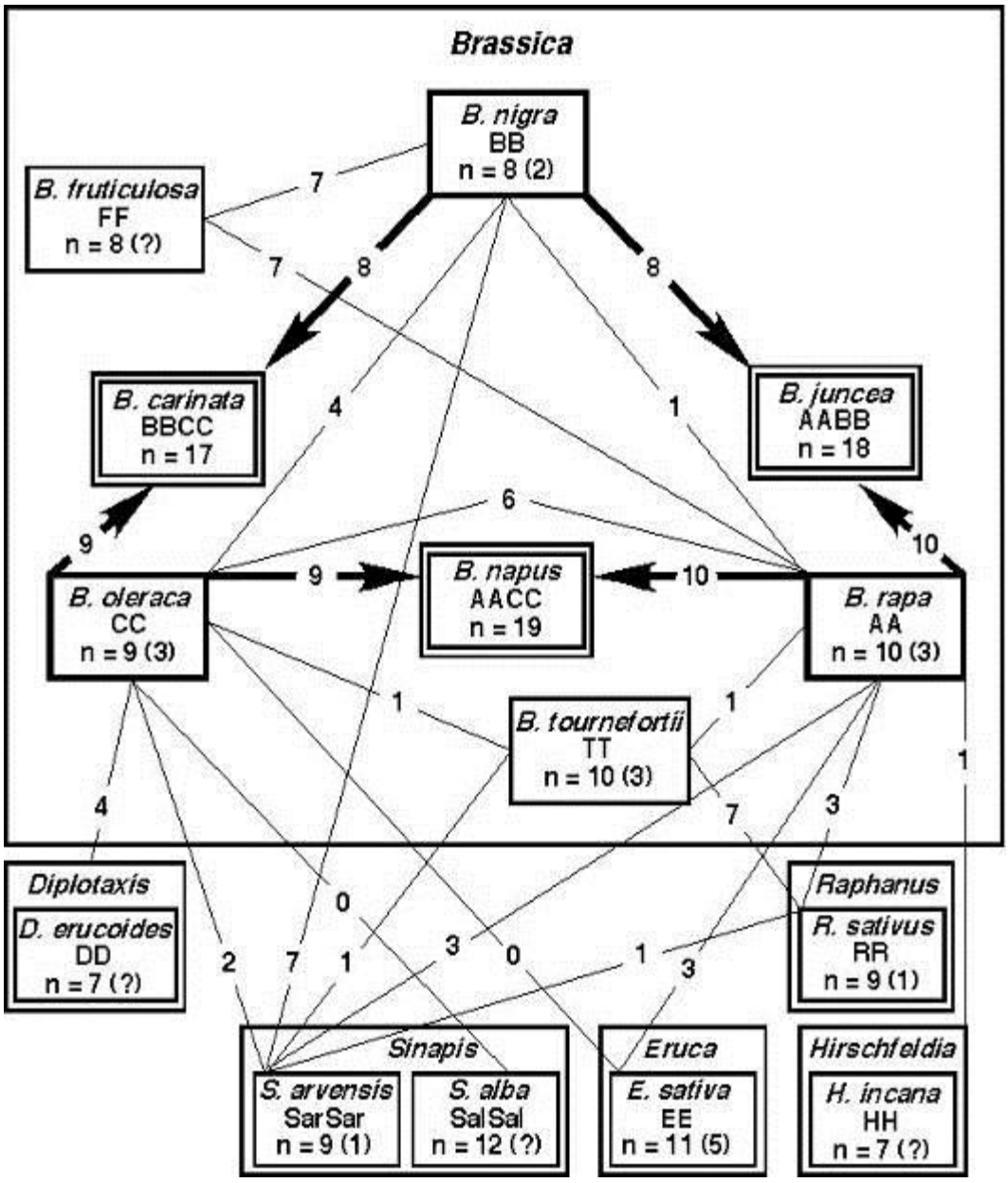

Figure 2.1 Genomic relationships of *Brassicaceae* species and allied genera. A, B and C are the genome symbols. The number in parenthesis following the haploid chromosome number (n) indicates the maximum possible number of autosyndetic chromosome pairs. The numbers within lines connecting two genomes give the maximum allosyndesis, i.e. the number of bivalents possible between the respective interspecific hybrids (Downey & Robbelen, 1989).

usually *Brassicaceae* species are vulnerable to. *B. napus* (canola), the predominant oilseed in Canada, has several similarities as well as differences when compared with *B. juncea*. With respect to oil-based biofuels, *B. juncea* is a preferred oilseed crop because of high seed oil content and desirable agronomic characteristics. Yellow mustard seeds are higher in protein than other crucifers and almost all Canadian YM production is used in edible applications.

Brassicaceae oilseeds are mainly grown for edible and industrial oil production purposes. In total, the *Brassicaceae* oilseeds comprise 9% of the world's edible vegetable oil supply and are the third most important source of edible oil after soy and palm (FAO, 2009). Residual canola meal after oil extraction is a good source of protein for animal feed and industrial applications (Newkirk, 2009).

2.2.1 *Brassica juncea* (brown and oriental mustard)

Brassica juncea is grown mainly in Canada, France, Germany, Indian subcontinent, Japan and the United States of America. In Canada, the prairie provinces of Alberta, Manitoba and Saskatchewan are the main producers of *B. juncea* and Saskatchewan leads the others. *B. juncea* has a longer history as a domesticated plant than *B. napus* and the species may be an interspecific cross of (*B. rapa* x *B. nigra*) (Uchimiya & Wildman, 1978) (Figure 2.1). The leafy parts of *B. juncea* are used in salads and a variety of prepared foods and the ground whole seed is a condiment. In countries such as Bangladesh, India and Pakistan, mustard (*B. juncea*) is used to obtain edible oil. Industrial uses of *B. juncea* seeds are also possible, for example, oil for biofuel and meal for bio-pesticides (Kimber & McGregor, 1995).

According to seed coat color, two market classes of *B. juncea* exists; brown mustard (BM) has dark brown seed coat and oriental mustard (OM) has golden yellow seed coat. Both BM and OM seeds are small in size (about 1.6 mm in diameter) and the seed coat contains low amount of mucilage (Kimber & McGregor 1995) compared to *S. alba*. Allyl glucosinolates is the main flavor precursor of *B. juncea* seed and the oil-extracted air dried meal contains 150-200 µmol g^{-1} of allyl glucosinolate (sinigrin). Sinigrin is non- volatile and the activity of myrosinase enzyme produces an aglycone that

can decompose to a number of compounds including allyl isothiocyanate contributing to hot pungent flavor of mustard. The crude oil and protein contents of *B. juncea* seed is about 33-45% and 23-29%, respectively. The content of seed oil and protein is affected by environmental conditions (Uppstrom, 1995). Predominant unsaturated fatty acids of *B. juncea* oils are oleic acid (18:1, ~20%), linoleic acid (18:2, ~10-15%) and linolenic acid (18:3). The oil-free meal of *B. juncea* is a good source of protein and contains ~45% (%N × 6.25) on a dry weight basis.

2.2.2 *Brassica napus* (canola)

Brassica napus is widely grown in Canada, China, Europe, and the United States of America. The origin of *Brassca napus* may be from the interspecific crossing between *B. rapa* and *B. oleracea* (Figure 2.1) in the Mediterranean or from the west coast of Europe where the two species have been grown close together (Andersson & Olsson, 1991). Two types of *B. napus* are found; the oleiferous rape and tuber bearing species. The oleiferous species is also divided into spring and winter forms (Robbelen, Downey & Ashri, 1989). At present, in Canada, >98% of canola comes from *B. napus* because of its superior yield, the popularity of the herbicide tolerance trait and availability of the hybrid varieties (Canadian Grain Commission, 2010).

The average oil content of *B. napus* is around 43.5% which may change slightly according to growing conditions. The oil of *B. napus* contains mainly oleic acid (C18:1), linoleic acid (C18:2) and linolenic acids (C18:3) in the amount of 61-62%, 19-21% and 10-11%, respectively. *Brassica napus* seed contains 21-23% of crude protein and the defatted meal contains 36-38% crude protein on a dry weight basis (Canadian Grain Commission, 2010).

2.2.3 *Sinapis alba* (yellow mustard)

Sinapis alba is referred to as “white mustard” in Europe and “yellow mustard” (YM) in North America (Kimber & McGregor, 1995). This spring-grown annual plant is primarily cultivated in the prairie provinces of Canada and seeds are destined mainly for spice and condiment markets in other countries.

The seed of *S. alba* is about 2.2 mm in diameter and larger than the seeds of *B. juncea* or *B. napus* (Kimber & McGregor, 1995). The protein and oil contents of *S. alba* seeds vary according to the growing conditions and average oil and protein values are between 25-30% and 27-32%, respectively (Canadian Grain Commisson, 2010). The fatty acid profile of *Sinapis alba* includes oleic acid (23%), linoleic acid (15%), linolenic acid (10%) and saturated fatty acids (7%). Presence of erucic acid (C22:1) at 41% is typical for YM seed. The predominant glucosinolates of *S. alba* seed is 4-hydroxybenzyl gluosinolate (sinalbin). Upon hydrolysis, sinalbin generates 4-hydroxybenzyl isothiocyanate that provides the typical sweet and warm mouthfeel of YM (Uppstrom, 1995).

The seed coat of *S. alba* is thicker than other mustard types and contains water soluble mucilaginous polysaccharides composed of glucose, arabinose, xylose, rhamnose, galactose, mannose and galactouronic acid (Cui, Eskin & Biliaderis, 1993). The mucilage increases liquid absorption when YM is an ingredient in food formulations.

2.3 Storage proteins of *Brassicaceae* oilseeds

The main nitrogenous compounds in *Brassicaceae* oilseed are the SSPs. Proteins are primarily stored in protein storage vacuoles (PSVs) of *Brassicaceae* seed embryo and cotyledons (Jiang et al., 2001). Protein accumulation occurs during seed development and the stored protein is mobilized to release N and S required by the germinating seed (Ashton, 1976; Muntz, 1998). In addition to storage protein, lytic enzymes are also stored in PSVs to assist in breaking down of SSP during seed germination (Herman & Larkin, 1999). The PSVs of *Brassicaceae* oilseeds are spherical organelles having an average diameter of 1.5 to 8 µm (Ashton, 1976). In addition to storage proteins, phytates (hexakisphosphate) and phosphorous-rich molecules which form insoluble crystals are also stored in PSVs of *Brassicaceae* oilseeds (Raboy, 2003).

In the *Brassicaceae* family, mainly two types of SSP are present. Large legumin type 11/12S globulin protein is called cruciferin. The 2S albumin napin has molecular mass of 12 to 16 kDa and found in less abundant quantities than cruciferin (Crouch & Sussex, 1981; Lonnerdal & Janson, 1972). Minor structural and functional proteins are

also present in *Brassicaceae* oilseed. Oleosin or oil body protein is a structural protein found in oil bodies which has a molecular weight of 20 kDa (Katavic, Agrawal, Hajduch, Harris & Thelen, 2006). Trypsin inhibiting protein of molecular weight 18 to 19 kDa and non-specific lipid transfer protein (ns-LTP) which is about 20 kDa are found as minor proteins in these seeds and may have functions other than as storage protein (Ostergaard, Hojrup & Knudsen, 1995).

Due to multigene involvement in the expression of cruciferin and napin proteins in the *Brassicaceae* family, a high degree of polymorphism is found among these proteins. No satisfactory method has been developed to quantify each type of protein and protein isoforms in *Brassicaceae* oilseeds.

2.3.1 Cruciferin

Cruciferin belongs to the cupin protein superfamily and is the most abundant storage protein in *Brassicaceae* species seeds. In mature *B. napus* seeds, the 11/12S globulin proteins constitute about 60% of total storage proteins and has molecular weight in the range of 300 to 350 kDa (Schwenke, Raab, Linow, Platz & Uhlin, 1981; Sjodahl, Rodin & Rask, 1991; Crouch & Sussex, 1981). The study by Malabat and group have found that cruciferin content may vary from 32-52% of total proteins among the European rapeseed cultivars (Malabat, Atterby, Chaudhry, Renard & Gueguen, 2003).

According to Bilodeau and group (Bilodeau, Lafontaine & Bellemare, 1994), 9 to 12 genes are involved in the expression of cruciferin (heterogeneous) in *B. napus*, however, in Arabidopsis, only 3 genes are involved. There are five major cruciferin subunit groups present in *B. napus*; CRU1, CRU2, CRU3 CRU2/3 (CRU A) (contain very similar subtypes CRU2 and CRU3) and CRU4 (Rodin, Ericson, Josefsson & Rask, 1990). Recently, CRU2/3 subunit is named as CRUA. Each subunit is around 50 kDa and is made of 30 kDa acidic (α) subunit and 20-25 kDa basic (β) subunit linked by disulphide bonds (DeLisle & Crouch, 1989). The cruciferin subunits of *B. napus* are composed of 443 to 486 amino acids. In *B. napus,* the 300-350 kDa cruciferin quaternary structure is composed of 6 subunits that could be a combination of CRU 1, CRU 2, CRU 3, CRU 2/3 (CRU A) and CRU 4. Cruciferin has an isoelectric point of pH 7.25

(Dalgalarrondo, Robin & Azanza, 1986; Schwenke et al., 1981). Cruciferin can be seen as several polypeptide bands in the 30 kDa and 20 kDa region with SDS-PAGE one-dimensional separation under reducing conditions because of microheterogeneity (Delseny & Raynal, 1999).

According to Aluko, McIntosh & Katepa-Mupondwa (2005), *S. alba* and *B. juncea* meals give polypeptide bands of 50, 55 and 135 kDa under non-reducing SDS-PAGE which may be originating from cruciferin. Under reducing conditions 16, 50, 55, and 135 kDa bands disappeared indicating the involvement of disulphide bonds in the parent proteins as low molecular weight polypeptides appeared.

The hexameric structure of cruciferin of rapeseed has exhibited reversible association and dissociation due to change of pH and ionic strength of the medium. When ionic strength is above 0.5, the hexameric structure of cruciferin is maintained. Further reduction of ionic strength leads to the dissociation of 11/12S hexamer into 7S trimers (Schwenke, Raab, Plietz, Samaschun, 1983). The dissociated 7S trimers can reassemble further when ionic strength is increased (Schwenke et al., 1983).

2.3.2 Napin

Napin is a small molecular weight 2S albumin protein found in *Brassicaceae* oilseed SSP and classified under the prolamin superfamily (Shewry, Napler & Tatham, 1995). Napin is the next abundant *Brassicaceae* oilseed storage protein after cruciferin; ranging from 15 to 45% of SSP depending on the variety and species. In *Brassicaceae* plants, multigene families are involved in the expression of napin. The identified genes in *B. napus* that are responsible for napin expression are napA, napB, BngNAP1, gNa and three cDND sequences pN1, pN2 and pNAP1 (later identified as napA) (Gehrig & Biemann, 1996) out of 10-16 multigenes. The napin precursor is 21 kDa and after the seed maturation stage two disulphide bonds connect the 4.5 kDa and 10 kDa fragments together (Schmidt, Renard, Rondeau, Richomme, Popineau & Axelos, 2004). *Brassicaceae* napins are strong basic proteins having an isoelectric point around pH 11 (pI ~ 11) due to the presence of a high content of amidated amino acids (*e.g.*, glutamine, asparagine) (Lonnerdal et al., 1972). The napins of *B. napus* are composed of two

polypeptides having isoelectric points of pH 8.8 and 9.4 (Schmidt et al., 2004). These polypeptides are linked by disulphide bonds (Krzyzaniak, Burova, Haertle & Barciszewski, 1998).

The napins of *B. juncea* have molecular weight ranging from 12 to 13 kDa (Venkatesh & Rao, 1988) and are composed of two polypeptides of 4 and 9 kDa. According to Menendez-Arias and group (Menendez-Arias, Monsalave, Gavilanes & Rodrigues, 1987), *S. alba* napin has a molecular weight of 14.6 kDa and consists of two polypeptide chains with molecular weights of 9.5 and 5 kDa. According to circular dichroism values, napin protein of *B. napus* contains about 25% α-helix and 38% β-sheets (Krzyzaniak et al., 1998), however *S. alba* napin containing 50% α-helix and 5% β-sheets in the molecule has been reported (Menendez Arias et al., 1987).

Disulphide bonds play a major role in three-dimensional molecular structure of napin. The number of cysteine residues and the location of cysteine in the polypeptide chain are the major conserved features of napin. A total of eight cysteine residues (8 Cys motif) have been identified in different napin isoforms; two in the short chain and six in the long chain (D'Hondt, Bosch, van Damme, Goethals, Vanderkerckhove & Krebbers, 1993). Because of the close proximity of the Cys residue in the polypeptide chain, a total of four disulphide bonds occur between chains and within the chains leading to a compact structure that is difficult to access by digestive enzymes (Gehring & Biemann, 1996). Due to this high number of disulphide bonds and the stable structure some of the napin isoforms of *Brassicaceae* family are considered as allergenic. Examples are Bra j 1 in *B. juncea* (Gonzalez, Menendez-Arias, Monsalve & Rodriguez, 1991), Bra n 1 of *B. napus* (Monsalve et al., 1997), and Sin a 1 of *S. alba* (Menendez Arias et al., 1987).

2.3.3 Other proteins

In addition to storage protein, structural and protective proteins are present in *Brassicaceae* oilseeds. Oleosins are structural protein found in the outer layer of oil bodies. These proteins provide binding sites for lipases during seed germination (Huang, 1992). It is believed that oleosin content is correlated with seed oil content of the seed

(Murphy, 1990). Oleosins of crucifers are not water soluble and they are produced after cruciferin and napin deposition in seed development (Huang, 1992).

Protease inhibitory proteins in *Brassicaceae* oilseeds are grouped as protective proteins that inhibit proteolytic enzymes of parasite microbes and insects (Menegatti, Palmieri, Walde & Luigi-Luisi, 1985). Most of the plant protease inhibitor proteins are single polypeptide chain proteins and contain an variable amount of disulphide cross links which gives resistance to enzymatic digestion and thermal decomposition. Several trypsin inhibitory proteins are reported in *Brassicaceae* oilseeds and a 18 kDa protein with trypsin inhibitory activity has been isolated from *S. alba* seed and found to contain 142 amino acid residues with high amounts of aspartate, glycine, lysine and serine (Menegatti et al., 1985).

Other minor proteins in *Brassicaceae* oilseeds are lipid transfer proteins (LTP) which are important in cutin layer synthesis, defense against pathogens and environment stress (Jenks, Tuttle, Eigenbrode & Feldman, 1995; Terras, Schofs, deBolle, van Leuven & Rees, 1992; Berot, Compoint, Larre, Malabat & Gueguen, 2005). The LTP are basic proteins and have pI between pH 9-10 and molecular weights of 7-9 kDa. The lipid transfer protein of *Brassicaceae* oilseeds are considered as allergenic and contain eight cystine residues which are engaged in forming four disulphide bonds (Berot et al., 2005).

2.4 Protein recovery from *Brassicaceae* oilseeds

As mentioned earlier, *Brassicaceae* oilseed proteins contain acceptable levels of all essential amino acids, including relatively high level of lysine and sulfur containing amino acids required in human nutrition (Sims, 1971). The glucosinolates, phytic acid, phenolic compounds and fibers limit the usage of protein containing *Brassicaceae* seed meal for food and feed (Bell, 1993; Sims, 1971). To obtain *Brassicaceae* protein rich products, either they need to be recovered from other non-protein components (as a protein isolate) or other components need to be removed from meal (as a protein concentrate).

Proteins of seeds can be recovered and separated by different ways such as ultracentrifugation, chromatographic methods, solubilization, precipitation and

electrophoresis (Mieth, Bruckner, Kroll & Pohl, 1983). Several factors determine the protein recovery process from seed meals. These include structural and physico-chemical properties of protein molecules (size, isoelectric pH, etc.), compounds around the protein molecules, proportion of proteins in the source, material conditions that protein source is exposed prior to processing, and properties of the solvents used in protein recovery (pH, ionic strength, polarity, etc.) (Mieth et al., 1983). Processing of oilseed for oil removal exposes seed proteins to a range of conditions such as temperature, pressure, pH and moisture, at levels which may affect the proteins and the interaction with other seed components. Therefore, not only genetic variation and plant growing conditions, but processing conditions also directly affect the protein extractability during the recovery of *Brassicaceae* oilseed proteins (Mieth et al., 1983).

Change in solubility of seed storage proteins according to pH lays within a minimum and maximum solubility range at specific pHs (Damodaran, 2008). The presence of ions will change the protein solubility due to salting-in or salting-out effect. The protein solubility, isoelectric precipitation, salting in and salting out properties can be applied in the protein recovery, purification and separation purposes.

Napin of *Brassicaceae* seeds are soluble in low ionic strength, neutral salt solutions and water (Monsalve & Rodriguez, 1990). Previous work (Bhatty, 1972; Bhatty & Finlayson, 1973; Gillberg & Tornell, 1976; Lonnerdal, Gilberg & Tornell, 1977; Raab & Schwenke, 1984) indicates that the albumin fraction can be separated by heat coagulation, precipitated by salting out (using NaCl, ammonium sulphate, etc.), ethanol treatment, heat coagulation at isoelectric point (pH 9.5-12) and complex formation with polyionic compounds (alginate, pectinate, carboxymethylcellulose, polyphosphate), floculating agents (polyacids, tanning agents), polyamides, polyalcohols and strong acids (trichloroacetic acid, sulfosalicylic acid).

Cruciferin, a globulin protein of *Brassicaceae* oilseed is soluble above or below their isoelectric pH (pH 7.2), and in solvents of low ionic strength, weak acids and aqueous electrolyte solutions of high ionic strength. Lonnerdal et al. (1977) and Mieth et al. (1983) have shown that the precipitation of cruciferin is facilitated by adjustment of pH to the isoelectric point, change of ionic strength to facilitate salting out, complexing

with polyionic compounds or by heat coagulation. According to Bhatty, Mackenzie & Finlay (1968), the 12S proteins of *B. napus* commercial meals extraction can be done in 10% (w/v) NaCl solution and protein precipitation with change of pH. Further purification of proteins can be done by dialysis and Sephadex G-100 gel filtration (Bhatty, MacKenzie & Finlayson, 1968). According to Schwenke et al. (1981), raw globulin precipitation occurs during dialysis of 5% (w/v) NaCl extract against water. The precipitated protein contained 1.7S (napin) and 12S (cruciferin) proteins. The low molecular weight 2S albumins of *B. napus* were first isolated by Bhatty et al. (1968) by extraction with 0.01M sodium pyrophosphate buffer at pH 7 and further chromatographic separation with Sephadex G-75 and Sephadex G-100.

Diosady and group (Diosady, Tzeng & Rubin, (1984) obtained a *B. napus* canola protein concentrate free of glucosinolates and non-protein nitrogen compounds (phenolic compound) using a two stage ultrafiltration (molecular weight cut off 5 and 100 kDa membranes) system and the product contained 80% protein on a dry matter basis. However, no differentiation between cruciferin and napin has been reported in their work. According to Murray and group (Murray, Myers & Barker, 1979; Murray, Maurice, Barker & Myers, 1980), *Brassicaceae* proteins can be solubilized by increasing the ionic strength (salting-in) in the aqueous medium. The proteins can be precipitated by lowering the ionic strength (salting-out) and the protein forms a micelle by hydrophobic association under low ionic strength. In the oilseed protein recovery processes, most of the procedures described aim to achieve maximum amount of protein removal from the seed meal without considering the protein type. Therefore, the published methods described for canola/mustard protein concentrates and protein isolate preparation mainly are used to produce protein products containing both cruciferin and napin.

Prapakornwiriya & Diosady (2004) have prepared a protein isolate of YM by microfiltration and isoelectric precipitation of a pH 12 seed extract. About 90% of defatted meal protein was extracted under alkaline condition (pH 12) and the extracted protein solution was subjected to microfiltration at pH 10. In this process, both retentate and precipitate contained low levels of glucosinolate and phytates. Alireza-Sadeghi and group (Alireza Sadeghi, Rao & Bhagya, 2006) have obtained a *B. juncea* protein isolate

by maintaining extraction medium pH at 11 with NaCl at 93°C (steam injection and heating). The isolated protein contained 95% of crude protein and was free of isothiocyanates, phytic acid and phenolic compounds. *Brassicaceae* oilseed meal contains tannins (proanthocyanidins) which mainly contribute to the dark, bitter and astringent taste of protein isolated under alkali conditions.

Protein recovery techniques are important in the improvement of *Brassicaceae* oilseed meal utilization and developing of a *Brassicaceae* protein industry. Efficient and economical *Brassicaceae* protein recovery technologies are limited; there are three companies in North America focusing on bringing *Brassicaceae* oilseed protein ingredients to the animal feed and human food market namely, BioExx Speciality Protein Ltd. (http://www.bioexx.com, Bioexx Speciality Proteins Ltd, Toronto, ON, Canada), the Burcon NutraScience (http://www.burcon.ca/, Burcon NutraScience Corporation, Vancouver, BC, Canada), and MCN Bio Products (www.mcnbioproducts.com, MCN Bio Products, Saskatoon, SK, Canada).

2.5 Other components of *Brassicaceae* oilseeds

In adition to seed storage proteins oil, glucosinolate and phytates are other compounds present in *Brassicaceae* seed kernals.

2.5.1 Oil

The oil content of oleaginous *Brassicaceae* seeds ranges from 38 to 44% of seed weight (Kimber & McGregor, 1995). The seed oil has a favorable fatty acid composition for nutritional and industrial use. The *Brassicaceae* oils are mainly composed of triacylglycerols (more than 90%) while phospholipids and glycolipids together constitute about 1% and monoacylglycerol, diacylglycerol and free fatty acids together are less than 0.5% (Uppstrom, 1995). Erucic acid (C22:1) is common among *Brassicaceae* family seed oil. Oils with low levels (<2%) of erucic acid are used in food applications and the high erucic acid oils are desired for industrial uses such as for plastics and biofuel (Robbelen et al., 1989). The behenic (docosanoic, C22:0), erucic (13-docosanoic, C22:1) and arachidic (eicosanoic, C20:0) acids are the predominant long chain fatty acids of

Brassicaceae oils and are economically important for the oleochemical industry (Kimber & McGregor, 1995). These >C20 fatty acids are used for the production of surfactants, plastics, photography materials, food additives, pharmaceuticals, ink, polish, paper, polish and wax (Kimber & McGregor, 1995). High level of oleic and linoleic acids content in *Brassicaceae* oils are nutritionally important in the human diet. According to Downey et al. (1987), a new mutant of *B. napus* which contain more than 20% linoleic acid has been developed, which is a nutritionally valuable omega-3 fatty acid. The level of each fatty acid in *Brassicaceae* oil varies according to genetic and environmental condition (Uppstrom, 1995). Recently, Dow Agro Sciences has developed a new canola variety (Nexera^{TM}) which has oil that is trans fat free, low in saturated fatty acid and containing omega-9 fatty acids. The oil of this new canola variety has high oxidative stability and desirable functional characteristics (Dow Agro Sciences, 2011).

The oil of *B. juncea* and *S. alba* contains high content of nutritionally undesirable erucic acid and saturated fatty acids. Feeding of high erucic oils to rats has resulted in significant numbers of myocardial lesions and lipid accumulation in the heart (Charlton, Davey, Kramer, Mahadevan & Sauer, 1975). According to Kako and group (Kako, Vasdev & Narbaitz, 1980) mustard oil-fed rats have shown fat deposits in heart and miocardiac lesions. It is believed that erucic acid could be a harmful fatty acid for human and animal health. Thus, canola has desirable level (<2%) of erucic acid which is recommended for human use (Robbelen et al., 1989).

2.5.2 Glucosinolates

Glucosinolates are sulfur-containing secondary metabolites (sulphonated oxime thioester glucose) widely found in *Brassicaceae* plants (Uppstrom, 1995) (Figure 2.2). About 90 glucosinolate types are found in *Brassicaceae* species including the oilseed (Fenwick, Heaney & Mullin, 1983). Glucosinolates are classified according to the precursor amino acid and the type of modification of the R group. Aliphatic glucosinolates have an R group derived from alanine, leucine, isoleucine, methionine, or valine. Those derived from phenylalanine or tyrosine are aromatic glucosinolates and the indole glucosinolates are derived from tryptophan (Fahey, Zalcmann, & Talalay, 2001).

The highest amount of glucosinolate accumulation is found in *S. alba* seed (Szmigielska & Schoenau, 2000) and 4-hydroxybenzyl glucosinolate (sinalbin) which gives a specific pungent flavor of mustard predominates (Uppstrom, 1995). In *B. napus* (canola) less than 30 $\mu molg^{-1}$ glucosinolate are found. In *B. juncea,* the major glucosinolate is 2-propenyl glucosinolate (allyl glucosinolate). Under natural conditions, glucosinolates are less likely to be in contact with the hydrolytic enzyme myrosinase which is present in the seed. When the plant material or seed is damaged during processing or harvesting and moisture is present, myrosinase catalyzes hydrolysis of glucosinolates to sulphates, isothiocyantes and thiocyanates. As an example, 3-butenyl glucosinolate is a major glucosinolate in mustard seed. Due to enzymatic (myrosinase) breakdown it produces 3-butenyl isothiocyanates. In YM 4-hydroxy-3-indolylmethyl glucosinolate is hydrolyzed into alcohol and free thiocyanates ions (Uppstrom, 1995).

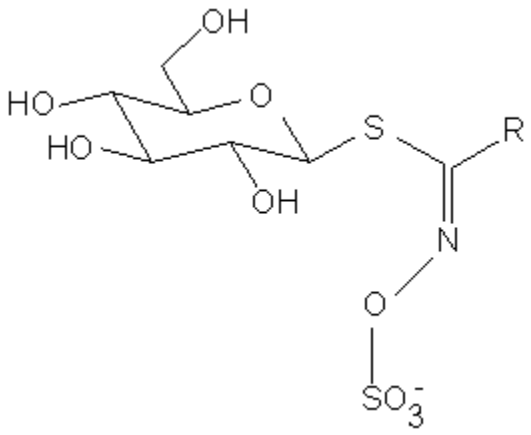

Figure 2.2 General structure of glucosinolate. (R group could be aliphatic or aromatic)

Glucosinolates and partially broken down glucosinolate products such as the isothiocyanates, thiocyanates and nitriles can pose physiological effects on animals. Feeding of high (> 1.43 μmol/g) glucosinolate containing rapeseed meal to poultry has caused reduction of blood plasma thyroid hormone concentration, enlarged thyroid (goiter), enlarged liver, hemorrhagic liver syndrome and reduced growth rate (Campbell, 1987; 1988). According to Bjergegaard and group (Bjergegaard, Eggum, Jacobsen, Otte & Sorensen, 1989) the highest recommended glucosinolate concentration in animal feed is 2.5 μmol/g or lower.

2.5.3 Phenolic compounds

Phenolic compounds contribute dark color, bitter taste, and astringency to the *Brassicaceae* meal (Spencer et al., 1988). The simple and complex oxidized phenolic compounds can form complexes with essential amino acids, enzymes and sugars (Kozlowska, Rotkiewicz, Zadernowski & Sosulski, 1983). Some phenolic compounds found in *Brassicaceae* seeds contain nitrogenous chemical attachments *e.g*., sinapic acid and choline ester (sinapine). Sinapine, the choline ester of 3,5-dimethoxy-4-hydroxycinamic acid (sinapic acid) is the main phenolic compound and *p*-hydroxybenzoic, vanillic, gentisic, protocatechuic, syringic, *p*-coumaric, cis- and trans-ferulic, caffeic, chlorogenic acids are other minor phenolic compounds of Brassica oilseeds. The phenolic compounds are mainly concentrated in seed cotyledon and small amounts are reported in seed hull (Kozlowska et al., 1983). During seed germination, sinapine is hydrolyzed to choline and sinapic acid. Choline is involved in plant metabolic functions of the methylation cycle and sinapic acid is involved in the biosynthesis of lignin and flavanoids (Kozlowska et al., 1983). According to Shahidi & Naczk (1992), in rapeseeds these phenolic acids are present as free (15%) and ester (80%) forms. Table 2.1 gives the concentrations reported for different phenolics in *Brassicaceae* species seed. In *B. napus* (canola), sinapic acid is the predominant free phenolic form (Robbelen et al., 1989). Esterified phenolic acids in rapeseed meal range from 9.82 to 15.38 g kg^{-1} and it is 70 to 90% of the total esterified phenolics of rapeseeds (Shahidi & Naczk, 1992). The concentration of insoluble bound phenolic acids in rapeseeds ranges from 0.96 to 1.01 g kg^{-1} and in white mustard (*S. alba*) it is 0.224 g kg^{-1} (Shahidi et al., 1992).

Phenolic compounds can bind with proteins of seed meal through hydrogen bonds and reduce the nutritional value of the meal. The products of non enzymatic and enzymatic oxidation of phenolic compounds can later react with $–NH_2$ group of lysine and CH_3S group of methionine of proteins (Milic, Stojanovic, Vucurevic & Turcic, 1968).

2.5.4 Dietary fibre

In plants, the dietary fibre includes the materials which are resistant to digestion by enzymes produced by monogastric animals. Based on water solubility, dietary fibre is

classified into soluble (pectins, gums, mucilage and some hemicellulose) and insoluble (celluloses, hemicelluloses and lignin) types (Brown, Rosner, Willett & Sacks, 1999). In *Brassicaceae* oil seeds, most of the dietary fibre components are present in the seed coat and cell walls of embryo and endosperm. In *S. alba*, the seed coat contains a high amount of mucilage (about 5% by weight), which is composed of soluble polysaccharides containing glucose, arabinose, xylose, rhamnose, galactose, mannose and galactouronic acid (Cui et al., 1993).

Table 2.1 Reported values of free, esterified and insoluble bound phenolic acid content of rape and mustard seeds.

Source	Phenolic acid content (g kg^{-1})			Reference
	Free	Esterified	Insoluble bound	
Flour				
Brassica napus, flour Var. Tower	0.982	9.820	ND	Krygier, Sousulski & Hogg (1982)
Brassica rapa, meal Var. Candle	0.845	11.964	ND	Krygier et al. (1982)
Brassica napus, seed				
Var. Tower	2.440	12.020	0.960	Naczk & Shahidi (1989)
Var. Altex	2.480	14.580	1.010	Naczk et al. (1989)
Var. Midas	1.445	15.240	0.687	Shahidi et al. (1992)
Var. Triton	0.615	12.120	0.513	Shahidi et al. (1992)
Sinapis alba, seed	1.081	15.38	0.224	Shahidi et al. (1992)

ND = Not determined

YM seed coat mucilage can be extracted using water at room temperature; it contains 80.2-94.1% carbohydrates (glucose 22-35%, galactose 11-15%, mannose 6-6.4%, rhamnose 1.6-4%, arabinose 2.8-3.2% and xylose 1.8-2.%), and 4.4% ash (Cui et al., 1993). Mustard mucilage gives a thick consistency and viscosity to salad dressings and pasta sauces (Weber et al., 1974). The mucilage of *S. alba* has an anti cancer effect in colonic cavities of rats, according to Eskin, Raj & Bird (2007). YM seed coat contains a considerably greater proportion of mucilage than *B. juncea* seed coat.

2.5.5 Phytates

Phytate (myo-inositol 1,2,3,4,5,6 –hexakis-dihydrogen phosphate) is one of the main phosphorous storage forms in plant seeds and may act as an antioxidant in plants (Graf, Empson & Eaton, 1987; Greenwood, 1990). In *Brassicaceae* oilseeds phytates are accumulated in protein storage vacuoles (Raboy, 2003). The phytate molecule is composed of a center ring of myo-inositol with six ester bonds to phosphate groups. Each phosphate group has the ability to form two ionic bonds with positively charged ions such as K^+, Mg^{2+}, Na^+, Zn^{2+} etc. and proteins. The *Brassicaceae* storage proteins, cruciferin and napin, may bind with phytates below their isoelectric points (napin ~ 10 and cruciferin ~ 7.2) and may produce insoluble compounds which tend to precipitate (Schwenke, Mothes, Marzilger, Borowska & Koslowska, 1987). At alkaline pH, phytates and cruciferins have net negative charges and are dissociated from each other in solution. The pH of the medium is a critical factor for phytic acid extraction from plant tissues. Phytic acid is also considered as an antinutrient because it can bind dietary minerals such as calcium, magnesium, iron and zinc (all in divalent form), through strong cation interaction and could lead to low mineral bioavailability in mammals (Cooper & Gowing, 1983). High salt concentration (0.7-0.9 M NaCl, 0.23 M $CaCl_2$) in combination with ultrafiltration/diafiltration can reduce phytic acid level in rapeseed meal leading to an increase in protein solubility (Thompson, 1990). Several groups have shown that microbial phytases can be used to reduce phytic acid level in canola meal (Ledoux, Broomhead, Firman & Bermudez, 1998).

2.6 Nutritional value of *Brassicaceae* oilseed proteins

Meal of *Brassicaceae* oilseeds is the major co-product of the oil extraction process and contains 36 to 44% of crude protein (includes all storage protein and other proteins) and has a balanced essential amino acid profile (Bell, Giovannetti, Sharby & Jones, 1976). The proteins of rapeseed meal have high level of lysine, methionine, cysteine, threonine and tryptophan (Bille, Eggum, Jacobsen, Olsen & Sorensen, 1983). According to a mice feeding study (Eggum, 1973), the biological value (BV) of rapeseed meal is higher (89.1%) than soybean meal (67.8%) (Table 2.2).

Table 2.2 Protein quality parameters and essential amino acid composition of rapeseed meal compared to soybean meal.

Parameter	Rapeseed meal (g/16 g N)	Soybean meal (g/16 g N)
Essential amino acid		
Arginine	6.54	7.19
Cystine	2.39	1.57
Histidine	2.71	3.38
Isoleucine	4.07	4.64
Leucine	7.18	7.49
Lysine	5.88	5.99
Methionine	2.13	1.60
Threonine	4.28	3.61
Tryptophan	1.47	1.18
Valine	5.17	5.07
Protein quality parameters	(%)	(%)
Protein (CP = N %* 6.25)	43.70	50.70
True protein digestibility %	83.30	93.40
Biological value (BV)	89.10	67.80
Net protein utilization (NPU) %	74.20	63.30
Utilizable protein (CP * NPU/100) %	32.40	32.10

Source: Eggum (1973)

The *in vitro* digestibility study of Savoie and group (Savoie, Galibois, Parent, & Charbonneu, 1988) has concluded that digestibility of rapeseed proteins by pepsin and pancreatin (gastrointestinal digestion) is at 83% which is a lower value than casein (97%). There are several studies that indicate positive and negative affects of

Brassicaceae oilseed meals in monogastric and ruminant nutrition. According to Mustafa et al. (2000) and Newkirk et al. (2003), feeding desolventized meal to chicken negatively affected their growth performances, protein digestibility, body weight gain and feed conversion efficiency consequently increasing the mortality. In commercial oil extraction, during the desolventization step, the meal is exposed to high heat (100-110ºC) for about 20-30 min which can cause Maillard browning reaction and lead to reduction of amino acid availability, especially lysine (Bell, 1993). According to the study of Piepenbrik et al. (1998), feeding dairy cattle with canola meal results in improved milk production. Canola meal can easily degrade in the rumen by the help of rumen microflora (Piepenbrik et al., 1998).

2.7 Gastrointestinal allergy

The gastrointestinal (GI) mucosa undergoes environmental changes due to exposure to a variety of food constituents such as food proteins, bacteria, chemicals and even pollens. The main functions of the GI track are digestion of ingested food and absorption of nutrients to the circulation for future metabolic and production mechanisms. However, during this process, the body may faces impairments in term of allergies and intolerance to ingested dietary substances (Holgate, Church & Lichtenstein, 2001). In monogastrics, protein digestion begins in the acidic environment of the stomach. First, pepsinogen is converted to pepsin in the presence of HCl of the stomach and presence of NaCl is necessary for its activity. It is believed that a protein tends to unfold its structure due to the low pH of the gastric fluid (Adibi, 1984). Pepsin has the ability to hydrolyse complex proteins to oligopeptides of smaller size (Adibi, 1984). In the small intestine trypsin, chymotrypsin and carboxy peptidases act on polypeptides that are generated from pepsin hydrolysis, and any unhydrolysed proteins from the gastric digestion (Savoie, 1993) can react as GI allergens. A GI allergy is a non-toxic, immune-mediated, abnormal reaction in response to ingested food protein or any other exogenous antigen causing variable GI disturbances such as pharyngeal pruritus, vomiting, abnormal pain, diarrhea, flatulence and malassimilation (Holgate, 2001). According to the Codex Committee of Food Labelling (1998) several groups of food are under the allergic food

category. They are cereals, fish, egg, legumes, nuts, milk, added sulfites, and others (animal fats and oils) (FAO/WHO Codex, 1999). The severity of allergic reactions caused by these food groups vary from person to person. However, the majority of the population is not allergic to many of these foods. According to Bocks (1987), 6-8% of young children (including infants) are affected by some kind of food allergy. The occurrence of these allergic reactions mostly disappear with the age; however, nut allergies and fish related allergies would persist throughout their lifetime.

2.7.1. Food related gastrointestinal allergy mechanism

Cells and sites involved in allergy sensitization reactions include the following (Mills et al., 2003; Morris, 2004; Davidson College website, 2006).

Macrophage: A type of white blood cells present in the skin and in mucosal tissues which involve immune response reactions.

Type E immunoglobin (IgE): These IgE molecules are a type of immunoglobulins in the body, however, they are specific for a given allergen, and are only found in subjects who are allergic to that particular substance.

IgE epitopes in proteins: The amino acid sequencees of protein, and that can be identified by antibodies are called epitopes. Two different kinds of epitopes have been identified according to their binding ability to antibodies; linear and conformational. In linear epitopes, it is the primary sequence of the polypeptide that is involved in antibody binding however, in conformational epitopes, a different number of amino acid segments present in the polypeptide chain are involved.

Mast cell: A type of white blood cells containing metachromatic granules which is important in inflammatory protein. A variety of inflammatory mediators are released by metachromatic granules including histamine and serotonin. Mast cells are found in the circulatory systems, as well as other tissues.

Allergic hypersensitivity reaction can be categorized into three stages (Figure 2.3). In all these phases antigens are involved. Initial allergic sensitization could be around 6 weeks, early phase reaction (immediate hypersensitivity) could be up to 2 hours and late phase reactions could be 2-24 hours after allergen exposure (Figure 2.3).

Sensitization: When the body comes in contact with a certain allergen by breath, skin, eyes or swallowing, the macrophage cells (a particular class of white blood cell) present in great numbers in the skin and mucosal tissues recognizes the allergen (Morris, 2004). Macrophages engulf antigen/allergen molecules through an endocytosis mechanism. Machrophage cells which have an antigen inside the cell with surface receptors are called major histocompatibility complex (MHC) type II which is a key functional cell unit for allergenicity (Mills et al., 2003). After taking antigen into the MHC type II cells, polymorphic reaction takes place inside this cell (Figure 2.4).

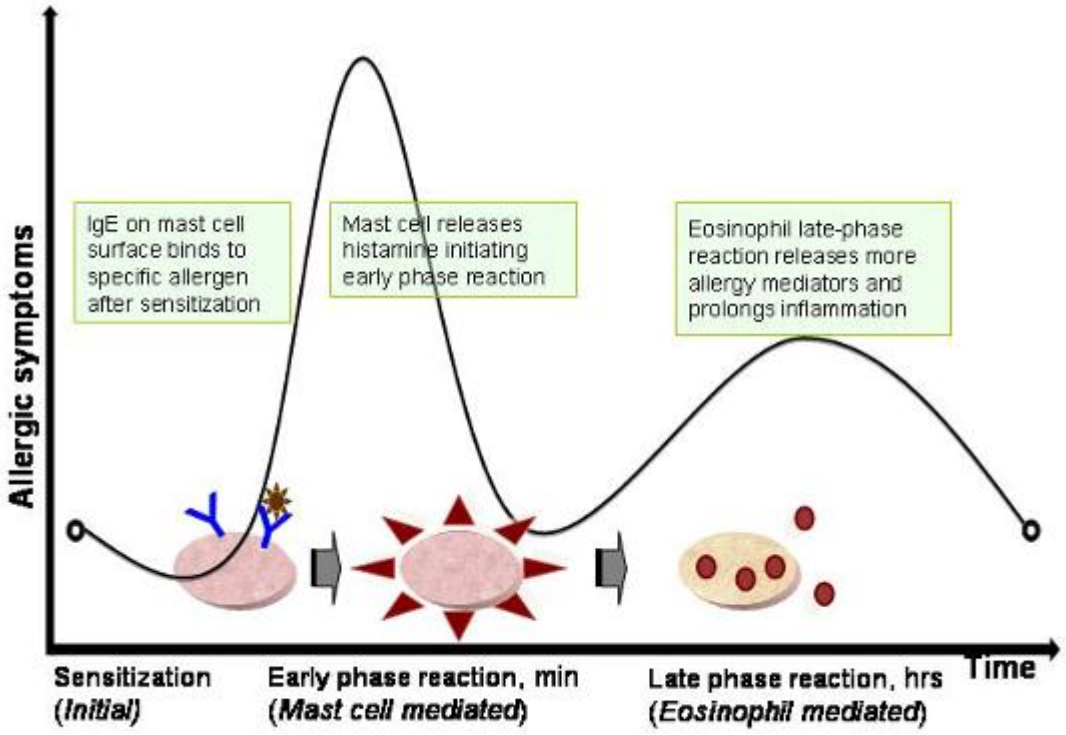

Figure 2.3 Three phases of allergy mechanism/reaction adapted from Morris (2004) with permission from Allergy Society of South Africa, ALLSA)**.**

The peptide fragment of an antigen selectively binds in the polymorphic groove of class II molecules, and transports it to the cell surface. Most of the time, the foreign

peptide with MHC (self-MHC) on the surface of the APCs complex is present inside the lymph nodes. The above complex molecule is recognised by the T-cell receptor of $CD4^+$ T helper cells (Brantzaeg, 2001). Consequantly, many other reactions such as maturation of B-cells (white blood cell, the B lymphocyte) into antibody secreting cells are activated. These reactions occur inside the body after the very first exposure to the antigen. The IgE production takes place inside the body of the antigen pre-exposed individual. The IgE is more specific than normal IgG to some sensitive individuals.

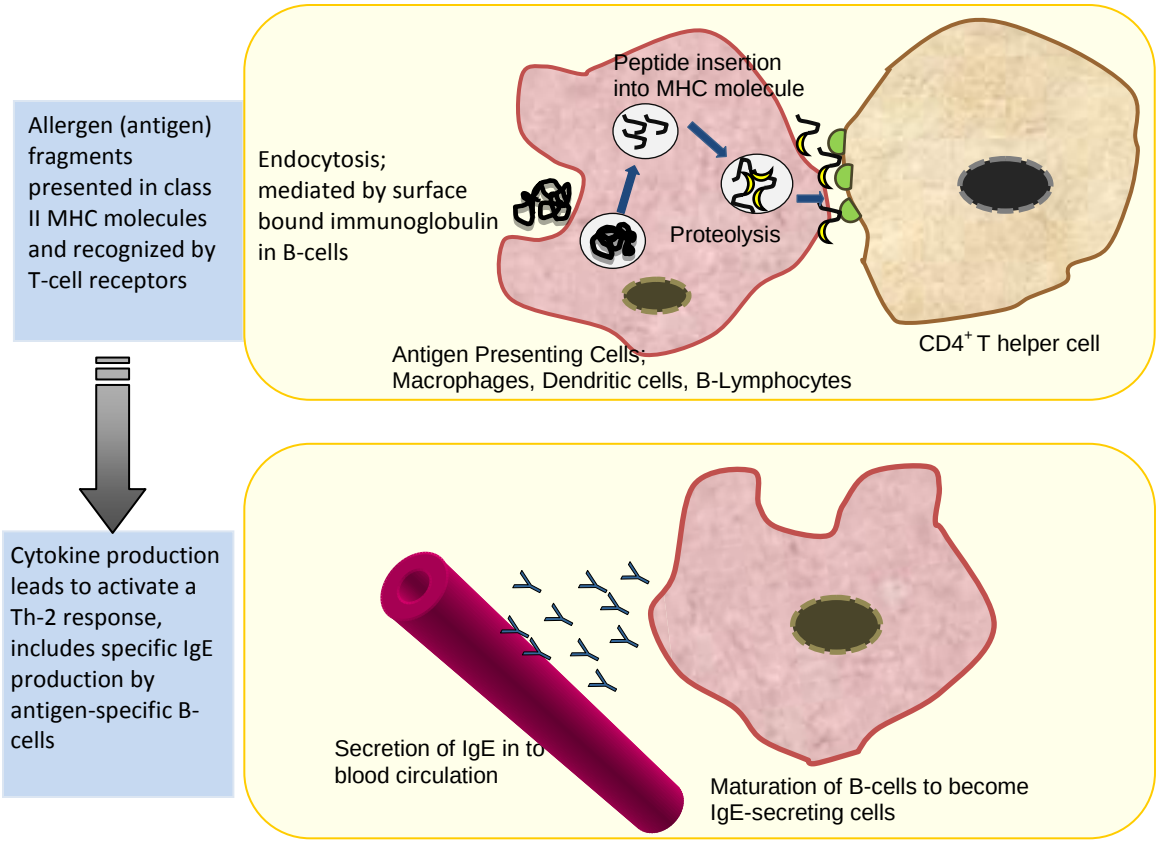

Figure 2.4 Allergic sensitization and the role of antigen presentation (adapted from Mills et al. (2003) with permission from Elsevier/Copyright Clearence Centre).

Normally it can take up to 6 weeks to develop an allergen-specific IgE. The sensitization phase is silent and the exposed person does not experience any allergic symptoms. Allergy symptoms such as runny nose, red and watering eyes, difficulty in

breathing, skin redness and itching, etc. only appear after a second exposure to the same allergen (which may not occur for a long time after the sensitization).

Early phase reaction: When a person who is sensitized during the first stage, their blood mast cell and blood basophile membranes bind with IgE through Fc receptors. The Fc receptor is a protein found on the surface of certain cells including natural killer cells, macrophages, neutrophils and mast cells that contribute to the protective functions of the immune system. Later, when this person is exposed to the above allergen again, the IgE antibodies, which are bound to the mast cells in the first phase initiate the damage. The allergen binds to these specific antibodies, which eventually triggers an intracellular response to undergo degranulation of mast cells; a process that results in the emptying of the small, intracellular granules into the extra cellular environment (Figure 2.5). These granules contain a variety of different chemical substances, including histamine, which is a key compound in allergies because it is directly responsible for increased vascular permeability, vasodilation, bronchial spasm and mucus secretions. The typical allergic symptoms are rhinitis, asthma, itching, etc. The chemicals released by mast cells act in different ways on the body systems (Figure 2.5). This phase can extend up to 2 hours. During this stage the sensitivity reactions take place at the peak level (Morris, 2004).

Late phase reaction: The late phase is usually 2 to 24 h after allergen exposure. In this stage, further mediator (leukotrienes, cytokines, histamine) production occurs by the mast cells (Figure 2.5). Furthermore, infiltration of inflammatory cells; the eosinophils releasing major basic protein, eosinophil cation proteins and more leukotrienes occurs. At the same time, TH2 lymphocytes are also released (Centner & de Weck, 1995). These cytokines further stimulate IgE production leading to an increase in mast cell numbers as well as the activity of the mast cells. This rapid reaction stage is referred to as an allergic inflammatory cascade. As a result of this cascade, mucosal damage and tissue remodeling happens.

The above gastrointestinal allergy sensitization and allergic mechanism theories are found in the literature. However, according to Stephen (2001), the understanding of most of the intestinal cells such as mast cells, and eosinophils are not well known, so most of the allergic mechanisms are still hypothetical.

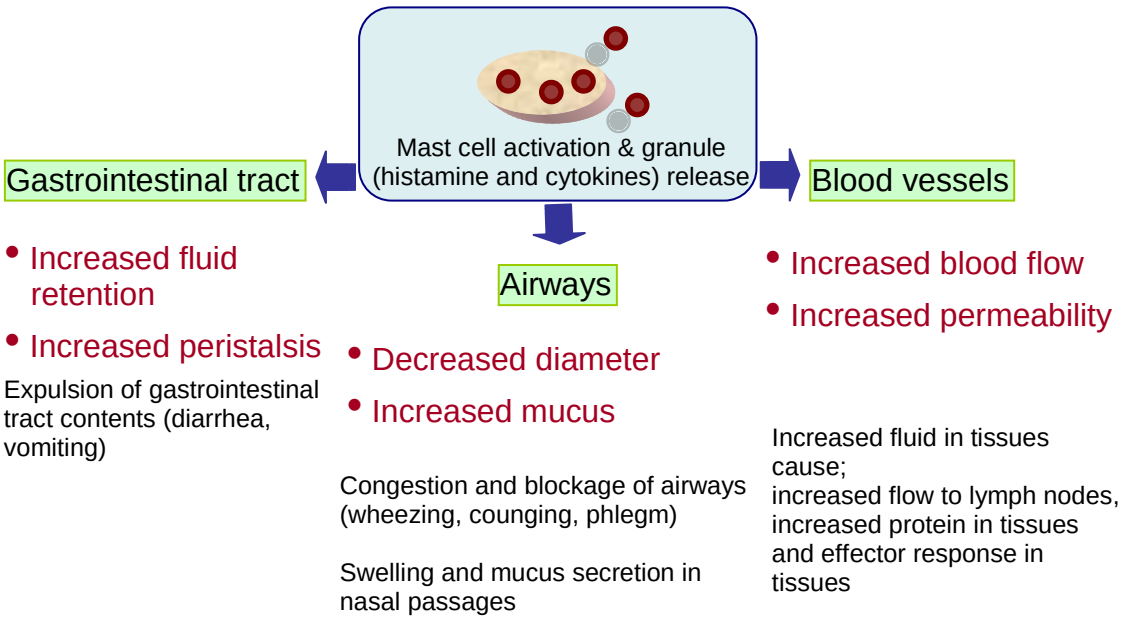

Figure 2.5 Effect of mast cell activation on different tissues (adapted from the Davidson College web page, 2006)

2.8 Allergenic proteins in *Brassicaceae* seeds

Most of the gastrointestinal allergenic SSPs belong to the prolamin and cupin protein superfamilies (Shewry & Casey, 1999). A case study carried out by Puumalainen and group (Puumalainen, Kautianen, Palosuo, Reunala & Turjanmaa, 2008) with 64 children subjected to a skin prick test for allergic foods (milk, egg, etc.) and Brassica oilseeds (*B. napus* and *B. rapa*) showed that the atopic dermatitis sensitive children exhibited asthma, allergic rhinitis and sensitization for oilseed rape and turnip rape.

Table 2.3 Molecules released by mast cells on activation (adapted from the Davidson College web page, 2006).

Class of product	Examples	Biological effect
Enzyme	Tryptase, chymase, cathepsin G, carboxypeptidase	Remodeling connective tissue matrix
Toxic mediator	Histamine, heparin	Toxic to parasites, Increase vascular permeability, Cause smooth muscle contraction.
Cytokine	IL-4, IL-13 IL-3, IL-5, GM-CSF	Stimulate and amplify TH2 cell response
	TNF-α (some stored performed in granules)	Promote eosinophil production and activation Promotes inflammation, stimulate cytokine production by many cell types and activate endothellium.
Chemokine	MIP-1 α	Attracts monocytes, macrophages and neutrophils
Lipid mediator	Leucotrienes C4, D4, E4	Cause smooth muscle contraction increase vascular permeability
	Platelet activating factor	Stimulate mucus secretion, Attract leucocytes, Amplifies production of lipid mediators, Activates neutrophils, eosinophils and platelets

The prolamin proteins have a specific feature, the presence of a conserved cysteine skeleton (Cys-X_n-Cys-X_n-Cys-Cys-X_n-CysXCys-X_n-Cys-X_n-Cys or 8 Cys motif) (Shewry & Casey, 1999). A range of small sulphur–rich proteins (15 kDa) that are present in seeds contain the 8 Cys motif. Some isoforms of *Brassicaceae* 2S albumin napin proteins are considered as allergens and has conserved 8 Cys motif. Reported allergenic 2S napins are Sin a 1 of *S. alba* (Menendez-Arias et al., 1987), Bra j 1 in *B. juncea* (Gonzalez et al., 1991), Bra n 1 of *B. napus* (Monsalve et al., 1997) and Bra r 1 of *B. rapa* (Dasgupta, Ghosh, Roy, & Mandal, 1995).

The allergenic napin Sin a 1 of *S. alba* has a molecular weight of 14.1 kDa; it is a basic protein composed of two polypeptide chains (light and heavy) which are joined by two interchain disulphide bonds. The light chain and heavy chain contains 39 and 88 amino acids, respectively (Gonzalez de la Pena, Menendez-Arias, Monsalve & Rodriguez, 1991). The Sin a 1 protein contains a high level of α-helical structure and is resistant to proteolytic cleavage and thermal treatments (Gonzalez de la Pena et al., 1991). The Bra j 1 of *B. juncea* is also a 2S albumin protein and consists of 2 polypeptide chains having 37 and 92 amino acids, which are linked by two interchain disulphide bonds. There are also two intrachain disulphide bonds found in the large polypeptide chain (Monsalve, Villalba & Rodriguez, 2001; Sathe, Kshirsagar, & Roux, (2005). According to Monslave et al. (2001), the amino acid sequence of Sin a 1 and Bra j 1 are closely related and contain common allergic epitopes that were detected in the large chain of both molecules.

In 2008, Health Canada completed a systematic review of allergenicity data available internationally on mustard containing foods (Canada Gazette Part 1, July 28, 2008) to evaluate allergenic potential of mustard. From this review, mustard satisfied all the requirements to be listed as an allergen according to the Canadian criteria which is similar to what is required in amending the Codex Alimentarius Commission. This report summarizes that even with the exposure of a small quantity of mustard-containing food, some individuals have shown mild to severe systemic reactions including anaphylaxis. In addition, mustard allergy is common in some regions of Europe. Due to this reason the European Commission has listed mustard as a priority allergen and foods containing mustard require clear labeling (EU Directive, 2005). According to the review, in the

Canadian situation, mustard used in cooking and in processed and pre-packed foods should be monitored because of the possible thermal and hydrolytic stability of the responsible allergenic molecules. Mustard is used as a spice and a flavoring agent in food processes and could be a “hidden" source of allergen in food (Health Canada, 2008).

This review recommends that mustard should be included in priority allergen list in Canada (Canada Gazette Part II). Mustard has been added to the list of foods in the definition of food allergens by the Canada Gazette, Part II (CGII, 2011). When the mustard containing foods come to market, these should be labeled with a allergen warning. The Canadian food industry have until August 04, 2012 to comply with the new changes of labeling (Canada Gazette II, February 16, 2011).

2.9 Allergenicity assessment of food proteins

Allergenic proteins enter into the food chain from natural sources. Genetically modified crops (GM) may contain new and not so common proteins that can be incorporated into the animal and human food chain and may pose allergenic risk. In order to avoid consumer exposure to serious allergens, it is necessary to identify and classify possible allergenic proteins.

A food allergen and specific IgE antibodies are the essential components of developing food allergies. A true food allergen (Class 1) is a food component, almost always a protein, which elicits both the sensitization and the effector phases of the IgE mediated food allergy. Class 2 food allergens cause allergic reactions to the individuals previously sensitized by inhalation or contact with other allergenic sources like pollen or latex. Homologous protein of the sensitizer source and allergenic food, sharing common IgE binding epitopes are the basis of these cross-reactions.

Characteristics such as abundance, resistance to digestion and processing conditions, relatedness to known allergen families, etc, that may be attributed to the allergenicity of allergic protein has been available through studying the respective purified molecules. However, no clear rules exist to predict potential allergenicity of a food protein. A combination of different *in vivo*, *in vitro* and *in silico* methods that evaluate different aspects of the sensitization and the development of clinical symptoms may be used to identify the allergenic risk of food proteins. Guidelines of the Codex

Alimentarius Food Standard Programme that recommends allergenicity evaluation of proteins of foods derived from biotechnology presents an integrated step by step approach which is developed based on the decision tree (Figure 2.6) of the Joint FAO/WHO Expert Committee on Allergenicity of Foods Derived from Biotechnology (FAO/WHO, 2001).

Among the methods utilized for food protein allergenicity assessment, the *in vivo* methods define the ultimate clinical relevance of food allergens. However, these methods have obvious ethical limitations (van Hage-Hamsten & Pauli, 2004; Sicherer & Teuber, 2004). *In vivo* methods include oral challenges, double blind placebo controlled food challenges (DBPCFC) and skin prick test (SPT). Currently, no validated animal model is available for predicting allergenicity to food proteins; however, many models have been applied to understand the mechanism of allergic responses and potential changes due to modification of the allergenic protein (McClain & Bannon, 2006). The rat, dog and swine models have been utilized by researchers and the piglet model is considered most relevant because of the physiological features that particular model mimics to those of the human newborn (Sanchez-Monge & Salcedo, 2005). *In vitro* assays are designed to detect and quantify specific IgE in the free form in serum or bound to blood cell surface. These methods can be used with purified natural protein or recombinant allergen and has been used to identify food allergens and also to assay the potential allergenicity of novel food proteins. The specific *in vitro* assays include; specific IgE immuno assays, allergen microarrays, SDS-PAGE immunoblotting, mediator release and basophil activation test and T-cell response assays.

The decision tree approach of FAO/WHO for the assessment of the potential allergenicity of protein starts with a sequence similarity search against known allergens (Figure 2.6). In this *in silico* method, the first step is the comparison of the primary sequence of the suspected protein with known allergenic protein sequences. A protein which shares > 35% sequence identity (over an 80 amino acid window) or at least six identical contiguous amino acids with a known allergen is considered allergenic. Only a limited number of epitopes has been identified so far for the food allergens. The T-epitopes are linear or continuous motifs of about 8 to 24 amino acid residues, where as B-epitopes can be conformational (Goodman, Hefle, Taylor & Ree, 2005). A cut off value

for the degree of sequence matching to divide allergenic and non allergenic is debatable in this bionformatic method (Goodman et al., 2005).

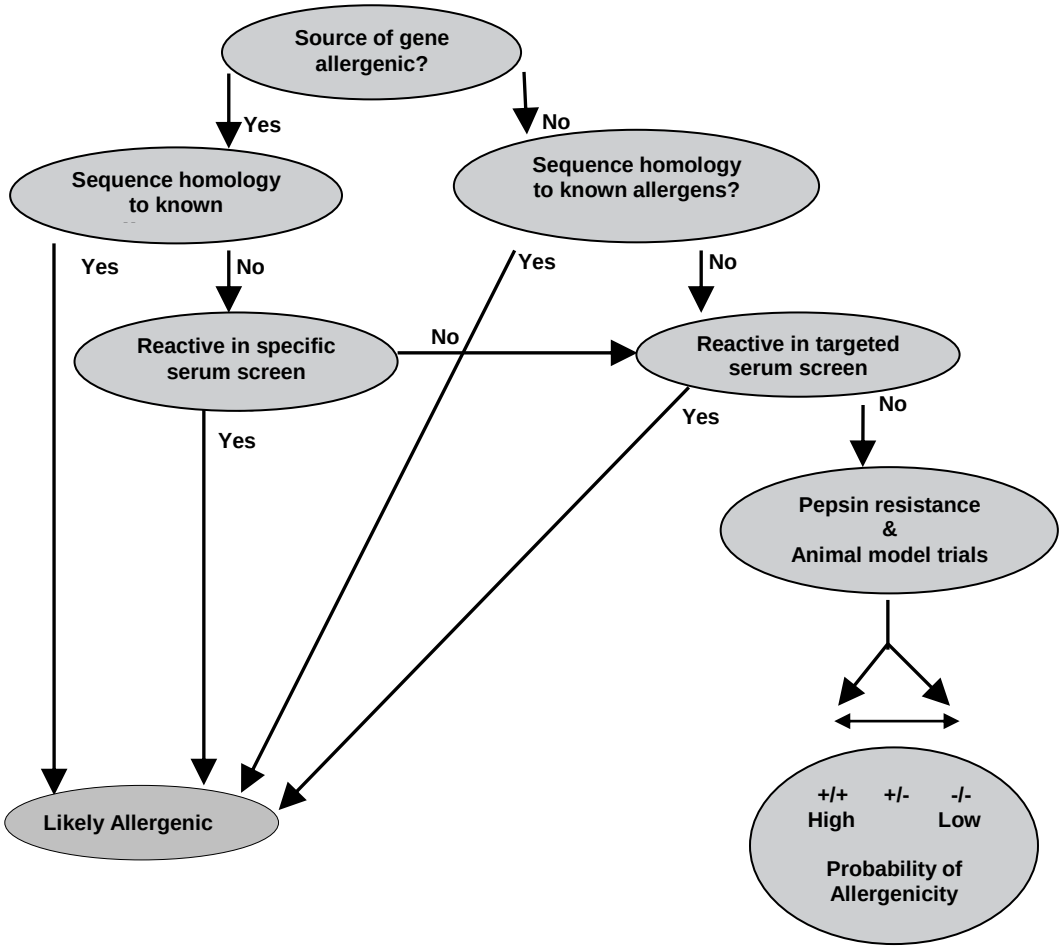

Figure 2.6 The decision tree for the evaluation of allergenicity of genetically modified foods accepted by FAO/WHO. (Re drawn from FAO/WHO, 2001)

2.10 Summary

Several *Brassicaceae* plants are important cultivated crops for their vegetative parts (leaves, stems and inflorescence) and storage organs (seeds and tubers) to use in our food. The oil bearing seeds producing *Brassica juncea, Brassica napus* and *Sinapis alba* are particularly significant in western Canadian agriculture. In general, the defatted *Brassicaceae* oilseed meal has crude protein content in the range of 40 to 50%. The proteins of these seeds have a nutritionally balanced amino acid profile. Currently,

Brassica seed meals are mainly used in animal feeds. The other nitrogenous constituents in *Brassicaceae* oilseeds are glucosinolates and N-compounds esterified phenolics. Seed storage proteins are the major N-storage form and in *Brassicaceae*, cruciferin (11S) and napin (2S) are the major protein types found. These proteins are the nutritionally valuable ingredients in canola meal for food and feed applications. The functional value of these proteins beyond their basic nutritive value and ability to provide essential amino acids is important in positioning Brassica seed proteins in the food ingredient market. Among these functionalities, solubility behaviour, thermal stability, surface active properties and safety such as reduced potential to pose allergic reactions are important. At present, Brassica seed protein recovery technologies are available to produce protein ingredients for food and feed uses. Therefore, comprehensive and detailed studies are needed to investigate storage proteins of *Brassicaceae* seeds with the aim to increase its competitiveness as an plant protein source for the food industry.

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3. STUDY 1: DISTRIBUTION OF PROTEIN AND OTHER NITROGEN-CONTAINING COMPOUNDS IN THREE *BRASSICACEAE* SPP. SEEDS AND THE SOLUBILITY PROPERTIES OF THEIR STORAGE PROTEINS

3.1 Abstract

The *Brassicacea*e family of plants that generate oil containing seeds play a major role in Canadian oilseed and oilseed biofuel industries. Six varieties of three *Brassicaceae* species; *Brassica juncea, Brassica napus* and *Sinapis alba* were studied for the seed composition related to non-protein nitrogen containing compounds (glucosinolates, nucleic acids, choline, betaine and sinapine) and storage protein, and the solubility properties of storage protein as pH and salt type and concentration changed.

The contribution of non-protein nitrogen to total seed N ranged from 3.1 to 10.8%. Cruciferin (11S) and napin (2S) were the major storage proteins found in these seeds. All species showed similar solubility pattern of their storage proteins with changing pH and ionic strength. Selective solubility of napin was observed when the pH was between 3 and 4 and this pH range overlapped with the minimum protein solubility of the seed meals. At basic pHs, both napin and cruciferin proteins became soluble. Presence of Na^+ and Ca^{2+} increased protein solubility at pH 4 and 7 significantly ($p<0.05$) for all varieties. Infusion of the Ca^{2+} resulted in a significant ($p<0.05$) increase in solubility of *B. napus* proteins at pH 3 and 7 compared to the buffer with Na^+. The effect of ionic strength and type was not significant when the medium of extraction was at pH 10. The solubility of *S. alba* proteins was low compared to other species and showed a reduction in solubility at alkaline pH when salt ions were present. Canola quality *B. juncea* proteins exhibited highest solubility values among these studied sseds.

The recoverable proteins of the studied seeds are mainly seed storage proteins. The type and the amount of protein that become soluble can be changed by changing pH and salt ion concentration.

3.2 Introduction

In Canada, the commercially produced oilseed-type *Brassicaceae* plants are *Brassica napus* (canola), *Brassica juncea* [brown mustard; BM, oriental mustard; OM and canola-quality mustard; CQM) and *Sinapis alba* (yellow mustard; YM). The widely grown canola is mostly *Brassica napus* and production of CQM is limited. The seeds of these plants are used in a variety of applications in relation to their composition. The mild flavored YM is widely used as a condiment in sauces, gravies and seasoning mixes and also as a functional ingredient in processed meat products. *B. juncea* is the hot mustard and mainly used as a condiment spice. In contrast to mustard, the whole seed of canola is not used in food applications and the food uses are limited to its oil fraction. *Brassicaceae* seed has two easily separable components; seed coat and embryo, cotyledons with endosperm (coteyledon). Although *B. napus* (canola) is the most widely grown *Brassicaceae* oilseed, its fractionation is limited to oil and meal.

Total protein content in *Brassicaceae* seed coteyledon or whole seeds is an important parameter during ration formulation, protein extraction, etc. Seed coteyledon protein determination is done based on total N content in the seed; glucosinolate, phenolic compounds and nucleic acids (RNA and DNA) are other non-protein nitrogen containing compounds in the seed. Quantification of total glucosinolates, phenolic compounds and nucleic acids are important to determine true protein in *Brassicaceae* oil seeds. Besides the protein, glucosinolates and sinapine are well documented as N-containing components of *Brassicaceae* oilseeds. Condiment-type mustard contains high content of glucosinolates while the canola-grade seeds contain very low amounts.

S. alba and *B. juncea* belong to the *Brassicaceae* (*Cruciferae*) family and it is expected that they may have similar protein profiles as *B. napus* (canola). Seed protein content of YM is usually higher (31% dry wt. basis) than brown and oriental mustard (25-

29% dry wt. basis) or canola (25.7% dry wt. basis) (Canadian Grain Commission, 2010). Storage proteins of *Brassicaceae* seeds are mainly composed of 11S (12S) and 2S (1.7S) proteins (Rao, Urs & Rao, 1978). Multiple genes are involved in expressing these proteins, therefore several isoforms of cruciferin and napin are found in *Brassicaceae* family seeds. The 11S cruciferins are of high molecular mass (300-350 kDa) and are composed of six subunits (Dalgalarrondo, Robin & Azanza, 1986a; Schwenke et al., 1983). In *B. napus* each subunit is made of two polypeptide chains (acidic 30-40 kDa and basic 20-25 kDa) that are linked by two disulfide (one interchain and another interchain) bonds. The other predominant storage protein is 2S napin, which also shows a high degree of polymorphism because of the multigene involvement in encoding of the protein (Scotfield & Crouch, 1987). Napin is small and has a molecular mass between 12 and 16 kDa (Venkatesh and Rao, 1988). In *B. napus*, napin has a 4.5 kDa small subunit and a 10 kDa large subunit (Gehrig, Krzyzaniak, Barciszewski & Biemann, 1998). The napin of *S. alba* is composed of 4.33 to 4.43 kDa and 1.02 to 10.19 kDa polypeptide chains (Neumann, Condron & Polya, 1996).

The physicochemical properties of seed storage protein (SSP) are important factors that determine the use of seeds as a source of protein for food, feed and industrial uses. Among these physicochemical properties, those that determine solubility of SSP are important when protein recovery from these seeds is concerned. In *Brassicaceae* oilseeds the residue of oil extraction (meal) is mainly composed of SSPs and carbohydrates (fibre of cell walls and seed coat). The ability of SSPs to come into solution enables the recovery of SSPs in highly concentrated form and further uses based on its nutritional value and functional properties. Most of the technologically important functional properties of proteins are directly affected by solubility (Damodaran, 2008) of these recovered proteins. Other properties such as the thickening, foaming, emulsifying and gelling are especially important functionalities of proteins in the food industry and are related to solubility. The solubility of a protein depends on intrinsic properties of the molecule such as its hydrophobic and ionic nature. The extrinsic properties such as the surrounding solvent and environmental conditions that influence the ionization state of side chain residues also affect the protein solubility. At the isoelectric point (*p*I) proteins

show minimum solubility. The salt concentration and type of the salt in the environment directly affects solubility of a protein (Damodaran, 2008)

The objectives of this study were to evaluate: a) the detailed chemical composition and distribution of major nitrogenous compounds in mustard (*B. juncea* and *S. alba*) in comparison with canola (*B. napus* and *B. juncea*) seed fractions, hulls and coteyledon (embryo and endosperm); and b) to study the solubility properties of SSPs with changes of pH and salt ion (NaCl and $CaCl_2$) concentration in the medium. Based on the objectives, the following hypotheses were assumed: 1) in *B. juncea*, *B. napus* and *S. alba* seeds, the contents of proteins and non-protein nitrogen components and thermal denaturation temperature are significantly different; 2) the pH of the medium affects the amount and type of protein solublilized from the seed meals of above mentioned species; and 3) the Na^+ or Ca^{2+} ions concentration affects the extent of SSP solubility and the effect of pH on solubility.

3.3 Materials and methods

3.3.1 Seed materials and their preparation

Seed of *B. juncea* (var. AC Vulcan, Duchess), *B. napus* (var. AC Excel) and *S. alba* (var. Andante, AC Pennant) were obtained from the Brassica breeding program of Agriculture and Agri-Food Canada Saskatoon Research Centre and producers in Saskatchewan. The canola quality mustard (CQM) (*B. juncea*, var. Dahinda) was from Viterra Inc. of Saskatchewan. Seeds were stored at ambient temperature in closed containers. First, the seeds were segregated according to size using #16 (1.18 mm) and #14 (1.4 mm) Tyler mesh (Tyler, Mentor, OH) and then cracked using a cracking stone mill (Morehouse-Cowles stone mill, Chino, CA). The seed coat of cracked seeds were separated from coteyledons (with endosperm and embryo) using an air classifier. Weights of each fraction, seed coat and coteyledon recorded. Separated fractions were ground using a home style coffee grinder and passed through a #40 Tyler mesh (425 μm) sieve and stored separately in air tight containers at 4^oC and used for all the studies.

3.4 Chemical analyses

3.4.1 Moisture, ash and crude protein

Each fraction was analyzed for moisture, ash, and total nitrogen (combustion-based protein) according to the AOAC methods 934.01, 942.05, 988.05, respectively (AOAC, 1997).

3.4. 2 Total oil content

The Swedish tube method as described in the AOCS method 2-93 (AOAC, 1995) was used to determine total oil content of each fraction.

3.4.3 Dietary fibre

Insoluble and soluble dietary fibre of the seed coat and defatted coteyledon were analyzed using the Megazyme (K-TDFR 01/05) kit, which was developed according to Lee, Prosky & DeVries, (1992).

3.4.4 Glucosinolate composition

Glucosinolate profiles of ground samples were determined according to the method of Landerouin & Ribailier (1987) as modified by the late Dr. J.P. Raney, of Agriculture and Agri-Food Canada, Saskatoon Research Center.

Ground samples (1.0 g) were weighed into 50 mL screw capped plastic bottles and one stainless steel ball was added to each vial. Then, 5 mL of absolute methanol, 400 μL of lead barium acetate (0.6 M) and 2 mL of internal standard solution (1 μmol mL^{-1} allyl glucosinolate for *S. alba* and *B. napus* and benzyl glucosinolate for *B. juncea*) were added and the sample were placed in a mechanical shaker for 1 h. After this step, the samples were centrifuged at 3100×g for 10 min. One mL of each supernatant recovered from centrifugation was added to a Sephadex A-25 column and each sample was washed with 1.5 mL each of 70% (v/v) methanol, 6% (v/v) acetic acid and distilled water, respectively. A 15 min interval was allowed between washing with each solution. Next, 1 mL of 0.02 M pyridine acetate was added to each sample. A purified sulphatase solution (50 μL) was added to each column, covered, and incubated at ambient temperature overnight. The desulphoglucosinolates produced by enzyme hydrolysis were then eluted

with 1 mL of purified water and collected into vials that were capped and sealed later. These eluates were freeze dried. Next 50 µL aliquots of MSTFA (N-methyl-N-TMS-trifluoroacetamide) and TMCS (trimethylchloro-silane) were added to the dried samples which were capped, mixed and heated to 40°C for 15 min for derivatization. The derivatized desulphoglucosinolates were separated using a gas chromatograph (Hewlett Packard 6890 Series Gas Chromatograph and an autosampler) equipped with a flame ionization detector. Chromatographic conditions were; column 15×0.32 mm, 1.0 µm film, DB-1, hydrogen flow rate at 1.0 mL/min for 9 min and ramp to 1-3 mL/min for 9 min, temperature program of 70°C for 2 min ramped at 5 °C/min to 300°C then maintained for 9 min, detector temperature 310°C. Relative response factor (RRF) was calculated from the area of the internal standard, allyl or benzyl glucosinolate. Area of each desulphoglucosinolate was converted to µmol glucosinolate/mL, using following equations.

Molecular weight of benzyl glucosinolate = 482.586

2 mL of 1 mM benzyl glucosinolate was added to each sample as internal standard.

So, 0.9652 mg benzyl glucosinolate / Extract

$$\text{Area ratio} = \frac{\text{area unknown}}{\text{area of internal standard}}$$

Area ratio of benzyl glucosinolate × 0.9652 mg = mg glucosinolate / unknown sample

$$= \frac{\text{mg/unknown sample}}{\text{weight of unknown sample (g)}} = \text{Total glucosinolates/g sample}$$

$$= \frac{(\text{total amount glucosinolate mg/g sample})/\text{g sample})/1000\ \text{mg})(1\text{x}10^{6})}{\text{molecular weight of glucosinolate, g/mol}} = \text{µmol glucosinolate/g}$$

3.4.5 Phytic acid content

Determination of phytic acid content of meal, hull and ground seed was performed according to Brooks (2001). For wet ashing, 0.1 g of ground sample was weighed into two digestion tubes (250 mL) and purified water and boiling beads added.

Two sets of tubes were prepared for each sample replicate. Then 1 mL of H_2SO_4 and 4 mL of 30% (v/v) H_2O_2 were also added. The samples were digested at 170ºC in a heating block. If the sample was turbid another 1 to 4 mL of 30% (v/v) H_2O_2 were added and the content were heated to 170^{o}C until the volume was down to 1 to 1.5 mL. The digested samples were cooled to ambient temperature, 8 mL of purified water was added, transferred to 25 mL volumetric flasks, and the volume was brought up to 25 mL. For the free P content analysis a 0.1 g of unashed sample was used. Weighed sample was added to nearly 90 mL purified water in a 100 mL volumetric flask, mixed thoroughly for 1 h and the volume was made up to 100 mL.

The standard curve for P was generated with serially diluted phosphate (potassium dihydrogen phosphate) solution (Caledon Laboratories, Canada). The P content of unashed, wet ashed and standard samples was determined as follows. A 100 µL of sample was transferred into eppendorf tubes which was added 420 µL of purified water and 40 µL of molybdate reagent (12.5 g ammonium molybdate dissolved in 200 mL of purified water and mixed with 50 mL of 10 N H_2SO_4 and made the volume up to 500 mL) and 420 µL of diluted sulphonic solution (1:1, by volume with purified water). The contents were mixed vigorously using a vortex and then incubated for 15 minutes at ambient temperature; absorbance of the reaction mixture was recorded at 660 nm. Based on the standard curve, sample P concentration was obtained.

Calculation of phytic acid based on the P concentration of the sample was as follows.

P_{free} = P content (g/g) in unashed sample

P_{total}= P content (g/g) of ashed sample

$$\text{Phytic acid, wt \%} = \frac{[P_{total} - P_{free}] \times \text{molecular weight of phytic acid (660 g/g mol)}}{\text{\# of mol P /1 phytic acid molecule} \times \text{molecular weight of P}}$$

$$= \frac{[P_{total} - P_{free}] \times 660 \text{ g/gmol}}{6 \times 30.9736 \text{ g/gmol}}$$

$$= [P_{total} - P_{free} \text{ (g/g \%)}] \times 3.5514$$

3.4.6 Nucleic acid (DNA and RNA) quantification

Commercially available TRIZOL reagent (Invitrogen, Carlsbad CA 92008, USA; Chomczynski et al., 1997) was used to extract DNA and RNA of samples. For each sample, the purity of DNA and RNA was determined as absorbance ratio at 260 nm and 280 nm, respectively using a Nano-drop spectrophotometer (Nano Drop Technology, Montchanin, DE, USA; Model Number ND-1000). Nitrogen content of the purified DNA and RNA samples was analyzed using combustion analysis.

3.4.7 Analysis of betaine, choline and sinapine

The contents of betaine, choline and sinapine of ground whole meal were determined according to the method of Li, Shen, Ratnapariyanuch, Thompson, Sammynaiken & Reaney (2009) and was developed by Dr Martin Reaney's research group at University of Saskatchewan. A defatted sample (500 mg) was weighed into a round bottom flask and 10 g of 99.8 % (v/v) methanol was added. The contents of the flask were stirred constantly up to 48 h at ambient temperature. The samples were filtered through cotton wool placed in 1×12 cm glass columns. The filtered methanol extract was evaporated under vacuum using a rotary evaporator. The concentrated sample was added (10-50 mg) along with internal standard; N,N, Dimethyl formamide (DMF) (EMD Chemicals, Gibbstown, NJ) and the weight of the round bottom flask was recorded. Then the sample and internal standard were re-suspended in 1-3 mL of deuterium oxide (Cambridge Isotope Laboratory Inc. 50, Andoner, MA). The proton NMR spectrometer of the samples were obtained and recorded at 500 MHz using a Bruker ^{1}H NMR (Avance, TXI, 5 mm, Bruker). The singlet peaks recorded at 3.25, 3.17 and 3.11 ppm were identified as phenylpropanoid ester (sinapic acid ester or sinapine), betaine and choline (-N $(CH_3)_3$), respectively. The contents of betaine, choline and sinapine in the sample were expressed on a weight basis.

3.4.8 Differential scanning calorimetry (DSC) study

Differential scanning calorimetry was employed to evaluate thermal properties (denaturation temperature of protein and enthalpy of denaturation) of meal proteins. Material was prepared as 10% (w/v) protein containing slurries in 20 mM phosphate

buffer at pH 7.2. Samples were heated from ambient temperature to 140°C at a heating rate of 10°C/min using a differential scanning calorimeter (DSC Q 2000, 143 TA Instruments, New Castle, Delaware, USA) equipped with an inbuilt cooling system and an auto sampler. Sealed, empty pans were used as reference. Thermal denaturation temperature and enthalpy of denaturation was obtained from the thermograms using curve integration software (TA Universal Analysis 2000). During preliminary experiments, whole ground seed, hull, undefatted cotyledons and defatted cotyledons were evaluated along with purified cruciferin and napin of *B. napus*. Later, for all varieties only defatted coteyledons were studied.

3.4.9 Polypeptide profiles of seed fractions

Polypeptide profiles of the proteins of each seed fraction were obtained by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing (with β-mercaptoethanol) conditions according to the Laemmli (1970). Samples and purified proteins were weighed into separate tubes with 300 µL of 0.1 M Tris-HCl buffer at pH 8 containing 10% (w/v) SDS added to each test tube. Then, 10 µL of β-mercaptoethanol was used to reduce S-S bonds. All the samples were heated at 99^{o}C for 10 min in an Eppendorf thermo mixer at 1300 rpm. The mixed samples were centrifuged for 10 min at 15,000×g. Molecular weight standards and samples so prepared were separated on a gradient (8-20% T) or 20 T% gel as required. Protein bands were stained by allowing binding to Coomassie blue R-350 and identification of the bands were by comparison with purified cruciferin and napin. Molecular weight of the polypeptide bands were estimated using the calibration curve developed using molecular weight standards and computed by Image Master Software (Amersham Pharmacia Biotech, Image Master ID Software Version Version 4.0).

3.5 Solubility properties of *Brassicaceae* seed storage protein

3.5.1 Nitrogen (protein) solubility at different pH

The solubility of coteyledon proteins with change of pH in the medium was studied using a series of solutions ranging from pH 2 to 10. Slurries (5%, w/v) of dehulled defatted seed meals were prepared in 100 mL and pH was adjusted using 0.1 or

1 M HCl and 0.1 or 1 M NaOH as needed to achieve each predetermined value. Slurries were stirred for 30 min while maintaining the required pH. Each extraction was centrifuged at 10,000×*g* for 20 min to separate soluble proteins from solids. The soluble containing extracts (supernatant) were filtered (Whatman #1) to remove any floating particles. The supernatant was analyzed for total N content using a combustion method. The total nitrogen value was converted to protein values by multiplying with 6.25 when needed.

3.5.2 Recovery of solubilized protein nitrogen

From the pH solubility study, it was confirmed that protein solubility value is at its maximum in highly basic solution (~ pH 10). Based on this finding pH 12 was selected as the pH with highest protein (N) solubility. The 5% (w/v) slurry of seed was prepared at pH 12 and extracted for 30 min and then filtered as described in section 3.5.1. The filtrate was aliquoted and the pH of the extract was changed using 0.1 or 1 M HCl and pH (pH 8.9 to 1.9) was noted. The precipitated proteins were removed by centrifugation (10,000×g) and the nitrogen content of the supernatant soluble protein was determined.

3.5.3 Nitrogen (protein) solubility under the effect of Na^+ and Ca^{2+} ions

Meal slurries (5%, w/v) were prepared in the pH 3 to 10 range as for the study 3.5.1 and Na^+ and Ca^{2+} ions were added to the slurry in the form of NaCl and $CaCl_2$. Ionic strengths of the solutions were adjusted to 0.25, 0.5, 0.75 and 1 M using either NaCl or $CaCl_2$. The pH of these slurries were maintained by addition of acid or base (0.01-0.1 M HCl or NaOH and stirring was continued for 30 min. Then solubles were recovered by centrifugation (10,000×*g*, 20 min). The meal extract were filtered (Whatman #1) to remove any floating particles. The supernatant was analyzed for total N content and then converted to protein values by multiplying with 6.25 as needed.

Polypeptide profiles of the meal extracts were obtained by SDS-PAGE as described in the section 3.4.9. Cruciferin and napin purified from *S. alba* (Andante) and *B. juncea* (AC Vulcan) according to the method of Berot et al. (2005) was used as reference proteins (from oilseed protein research program of AAFC).

3.6 Statistical Analysis

Protein solubility change with salt and pH data were statistically analyzed using General Linear Model (GLM) ANOVA (analysis of variance) and means were compared by Tukey test. Dependent variables and their interactions (salt concentration × ion type × veriety) were analysed in ANOVA for each pH level. The significant level was set at 0.05. All values are presented as means (and SD). All analyses were done using SAS v8.0 (SAS Insitute Inc., Cary NC) software for Windows.

3.7. Results

3.7. 1 Seed composition and nitrogenous compound distribution

The seeds of *S. alba* varieties AC Pennant and Andante seeds are relatively large in size compared to *B. juncea* or *B. napus* and yellow in color (Figure 3.1). The seed color of *B. juncea* AC Vulcan and Dahinda (CQM) were yellow-brown or tan and Duchess was dark brown (Figure 3.1). Percentage of hull and coteyledon (including germ and endosperm) fractions of each seed types on a as is are given in Table 3.1. In YM, per unit weight basis hull fraction was much larger than the other seed types. *B. juncea* Dahinda variety had the lowest weight of hull on percentage basis which was a reflection of seed size and thickness of the seed coat.

The levels of moisture, crude protein, oil, ash, fibre and phytic acid of seed, seed coat and coteyledon fractions are as in Table 3.2. It is clear that BM and OM contained higher seed oil levels (42.5 to 43.5%) than YM (30.4 to 33.5%). The seed coat fraction contained 7.4 to 15.6% oil with low levels found in YM. The protein content of coteyledon was 25.2% for *B. napus*, 28 to 29.9% for *B. juncea* and 33.5 to 39.0% for *S. alba* based on total nitrogen determination. According to the crude protein values of whole seed, *S. alba* contained higher protein content than *B. juncea* and protein was mainly concentrated in the embryo and endosperm. Seed coat of the all six seed varieties contained 14.7% to 17.5% crude protein. Ash content of seed coat was similar to that of the coteyledons. The seed coat of all seeds varieties contained more moisture than the coteyledon. The total dietary fibre content of the seed coat was in the range of 62.9% (AC Vulcan) to 73.5% (AC Pennant) and much higher in *S. alba* varieties than others. The coteyledon had lower total dietary fibre content (11.9% in AC Excel to 17.4% in

a). *Brassica juncea*

AC Vulcan `
(Oriental mustard, OM)

Duchess
(Brown mustard, BM)

Dahinda
Canola quality mustard, CQM)

(b). *Brassica napus*

AC Excel (Canola)

(c). *Sinapis alba* (Yellow mustard, YM)

Andante

AC Pennant

Figure 3.1 Macroscopic images of (a) *Brassica juncea* (AC Vulcan, Duchess, Dahinda), (b) *Brassica napus* (AC Excel) and (c) *Sinapis alba* (Andante, AC Pennant) seeds (Magnification × 4).

Table 3.1 Yield of coteyledon and seed coat fractions (weight basis as is)

Species	Variety	Coteyledon, %[1]	Seed coat, % [1]	Seed coat: coteyledon ratio
Brassica juncea	AC Vulcan	83.3 ± 1.4	16.7 ± 2.4	1 : 5.0
	Duchess	74.4 ± 2.7	25.6 ± 1.3	1 : 2.9
	Dahinda	86.1 ± 1.8	13.9 ± 1.8	1 : 6.2
Brassica napus	AC Excel	79.8 ± 1.2	20.2 ±2.2	1 : 4.0
Sinapis alba	AC Pennant	72.6 ± 2.2	27.4± 4.4	1 : 2.6
	Andante	72.2 ± 2.2	27.8 ± 2.5	1 : 2.6

[1] Mean ± SD; n = 3

Dahinda) than the seed coat fraction. Among both the seed coat and coteyledon , the insoluble fibre comprised most of the total dietary fibre (Table 3.2). The CQM (Dahinda) coteyledon contained comparatively high (16%) insoluble dietary fibre content while *B. napus* contained the lowest (10.2%) values. Significantly higher (5.5% to 7.7%) amount of soluble dietary fibre was recorded in *S. alba* seed coat than *B. juncea* and *B. napus* (1.9 – 3.7%).

The phytic acid level in seeds and seed components was below 2% in all the varieties (Table 3.2). The low content of phytic acid found in the seed coat indicated that phytic acid is mainly concentrated in the coteyledon. AC Excel canola seed recorded the lowest phytic acid content (0.52%) and the highest value was reported in the variety Duchess of *B. juncea* (1.48%).

The content of nucleic acid (DNA and RNA), glucosinolates and three major triamines were analysed to account for non-protein nitrogenous components of these *Brassicaceae* seeds. Total nitrogen originating from DNA and RNA in seeds was in the range of 0.022 to 0.038% (Table 3.3). The total contribution to seed nitrogen by nucleic acids was less than 0.1% for all the seeds analyzed.

Table 3.2 Composition of different fractions of mustard and canola seeds (as is).[1]

Sample (Species and Variety)		Moisture, %	Ash, %	Crude protein, %	Crude fat, %	Phytic acid, %	Dietary fibre, %[2]		
							Insoluble	Soluble	Total
Brassica juncea									
AC Vulcan:	Whole Seed	6.0 ± 0.1	4.1 ± 0.1	26.9 ± 1.5	42.5 ± 1.9	0.6 ± 0.1	ND	ND	ND
	coteyledon l	5.6 ± 0.1	4.1 ± 0.1	28.0 ± 0.5	48.3 ± 0.1	0.9 ± 0.3	15.0 ± 0.1	2.3 ± 0.1	17.3 ± 0.5
	Hull	10.0 ± 0.1	4.3 ± 0.1	17.3 ± 0.3	15.6 ± 0.3	0.7 ± 0.1	60.4 ± 0.0	2.5 ± 0.0	62.9 ± 0.3
Duchess:	Whole Seed	5.9 ± 0.1	3.5 ± 0.1	26.9 ± 0.0	43.5 ± 0.1	1.5 ± 0.0	ND	ND	ND
	coteyledon	5.7 ± 0.1	3.5 ± 0.1	29.9 ± 1.2	44.5 ± 0.1	1.6 ± 0.1	14.8 ± 0.0	2.6 ± 0.0	17.4 ± 1.2
	Hull	11.0 ± 0.1	4.3 ± 0.4	14.7 ± 0.9	9.2 ± 0.4	0.2 ± 0.0	65.5 ± 0.1	3.7 ± 0.4	69.2 ± 0.9
Dahinda:	Whole Seed	4.1 ± 0.2	4.0 ± 0.1	27.6 ± 4.9	46.9 ± 0.4	0.9 ± 0.1	ND	ND	ND
	coteyledon	4.4 ± 0.1	3.7 ± 0.1	28.2 ± 1.3	53.7 ± 0.8	1.1 ± 0.1	15.9 ± 0.1	1.5 ± 0.1	17.5 ± 1.3
	Hull	9.7 ± 0.1	6.2 ± 0.1	16.6 ± 0.9	12.3 ± 0.4	0.5 ± 0.1	63.8 ± 0.3	1.9 ± 0.0	65.7 ± 0.9
Brassica napus									
AC Excel:	Whole Seed	5.4 ± 0.2	4.0 ± 0.1	23.9 ± 0.6	49.2 ± 0.6	0.5 ± 0.0	ND	ND	ND
	coteyledon	4.4 ± 0.1	3.7 ± 0.5	25.2 ± 1.0	55.2 ± 1.6	0.6 ± 0.0	10.2 ± 0.1	1.8 ± 0.5	11.9 ± 1.0
	Hull	11.6 ± 0.2	6.2 ± 0.1	14.7 ± 2.5	14.3 ± 0.2	0.4 ± 0.2	63.6 ± 0.2	1.8 ± 0.1	65.3 ± 2.5
Sinapis alba									
AC Pennant:	Whole Seed	6.8 ± 0.1	4.4 ± 0.1	32.2 ± 0.7	33.5 ± 0.2	1.1 ± 0.2	ND	ND	ND
	coteyledon	5.9 ± 0.1	4.3 ± 0.1	33.5 ± 2.7	29.9 ± 1.5	1.3 ± 0.2	11.5 ± 0.1	2.9 ± 0.0	14.4 ± 2.7
	Hull	10.1 ± 0.1	4.6 ± 0.1	16.9 ± 0.2	9.4 ± 0.3	0.7 ± 0.2	65.9 ± 0.3	7.7 ± 0.1	73.5 ± 0.2
Andante:	Whole Seed	7.0 ± 0.1	3.9 ± 0.1	36.7 ± 0.1	30.4 ± 0.4	0.9 ± 0.2	ND	ND	ND
	coteyledon	6.1 ± 0.1	3.9 ± 0.1	39.0 ± 0.2	31.8 ± 1.4	1.2 ± 0.2	12.6 ± 1.1	2.0 ± 0.0	14.6 ± 0.2
	Hull	10.9 ± 0.1	4.4 ± 0.1	17.5 ± 0.3	7.4 ± 0.1	0.4 ± 0.1	60.9 ± 0.1	5.5 ± 0.1	66.4 ± 0.3

[1] Mean ± SD is provided ; n = 3
[2] ND: Not determined

Table 3.3 Nucleic acid nitrogen content in *Brassica* seeds (as is).

Species and Variety	Nitrogen, %		
	RNA[1]	DNA[1]	Total[2]
Brassica juncea			
AC Vulcan	0.01 ± 0.2	0.027 ± 0.1	0.037
Duchess	0.01 ± 0.1	0.012 ± 0.2	0.022
Dahinda	0.01 ± 1.8	0.015 ± 0.2	0.025
Brassica napus			
AC Excel	0.01 ± 0.2	0.026 ± 0.1	0.036
Sinapis alba			
AC Pennant	0.02 ± 0.1	0.013 ± 0.1	0.033
Andante	0.02 ± 1.2	0.018 ± 0.2	0.038

[1] Mean ± SD is provided; n = 3
[2] Mean values are used for calculation

The types of glucosinolates and their quantities found in these *Brassicaceae* oilseeds varied among the seed varieties (Table 3.4). In the seed of *B. napus* the predominant glucosinolates found were hydroxy butenyl, hydroxy indolyl and butenyl. Allyl glucosinolate was predominant in *B. juncea* AC Vulcan and Duchess. However in Dahinda, it was butenyl glucosinolate and the total glucosinolate content was much lower than condiment quality seeds. The *S. alba* seeds contained hydroxy benzyl and to lesser extent hydroxy butenyl glucosinolate. When the total content of glucosinolates is considered the canola quality seeds had the lowest content in the seeds. The glucosinolates were concentrated in the coteyledon fraction (Table 3.4) and hulls contained 5.1 µmol/g. Betaine, choline and sinapine distribution of seed fractions of *B. juncea* and *B. napus and S alba*, varieties showed these triamine containing phenolic compounds were mainly concentrated in the coteyledon (Table 3.5). The highest content (1.3 to 1.7%) of these triamines was found among *S. alba* seeds. The content of sinapine, betaine and choline in *B. juncea* was 0.58 to 1.0%.

Table 3.4 Glucosinolate content (µmol/g) of seed components of *B. juncea*, *B. napus* and *S. alba* (as is).

Glucosinolate and source	Glucosinolate content (µmol/g) [1]					
	B. juncea			*B. napus*	*S. alba*	
	AC Vulcan	Duchess	Dahinda	AC Excel	AC Pennant	Andante
Whole Seed						
Allyl	139.51 ± 1.2	109.63 ± 0.8	0.21 ± 0.0	0	0	0
Butenyl	2.73 ± 1.2	0.93 ± 0.1	5.42 ± 0.1	4.19 ± 0.1	0	0
Pentanyl	0.13 ± 0.0	0.14 ± 0.0	0.32 ± 0.0	0.9 ± 0.3	0	0
OH-Butenyl	0.20 ± 0.2	0.26 ± 0	0	7.54 ± 0.1	4.96 ± 0.1	6.71 ± 0.3
Benzyl	0	0	0	0	1.99 ± 0.0	1.98 ± 0
OH-Benzyl	1.58 ± 1.4	0.39 ± 0.0	0	0.08 ± 0.1	211.19 ± 0.3	220.86 ± 1.3
Indolyl	0.27 ± 0.2	0.08 ± 0	0.09 ± 0.0	0.29 ± 0.1	0.47 ± 0.0	0.31 ± 0.0
OH-Indolyl	1.83 ± 1.6	3.18 ± 0.1	3.80 ± 0.6	6.86 ± 0.8	0.70 ± 0.0	0.54 ± 0.1
TOTAL	**146.30**	**114.61**	**9.84**	**19.86**	**220.02**	**230.40**
Coteyledon						
Allyl	152.29 ± 1	122.14 ± 2.5	0.22 ± 0.0	1.23 ± 1.5	0	0
Butenyl	4.27 ± 1.4	2.86 ± 2.5	5.75 ± 0.1	4.68 ± 0.0	0	0
Pentanyl	0.15 ± 0.0	0.20 ± 0.1	0.29 ± 0.0	1.06 ± 1.9	0	0
OH-Pentanyl	0.34 ± 0	0.25 ± 0.1	0	8.04 ± 0.0	5.63 ± 0.6	7.22 ± 0.4
Benzyl	0	0	0	0	1.95 ± 1.2	1.97 ± 0.0
OH-Benzyl	1.75 ± 0.1	0.78 ± 0.0	0	0	254.9 ± 1.1	253.4 ± 2
Indolyl	0.47 ± 0.0	0.12 ± 0.2	0.11 ± 0.0	2.25 ± 0.5	0.55 ± 0.0	0.34 ± 0.0
OH-Indolyl	3.35 ± 0.1	3.52 ± 0.1	4.42 ± 0.1	57.6 ± 3.8	0.69 ± 0.1	0.47 ± 0.1
TOTAL	**166.62**	**129.87**	**10.79**	**74.86**	**263.72**	**263.40**
Hull						
Allyl	36.69 ± 0.9	21.61 ± 0.9	0.13 ± 0.0	0.39 ± 0.1	0	0
Butenyl	0.44 ± 0.1	0.49 ± 0.2	4.52 ± 0.8	0.86 ± 0.1	0	0
Pentanyl	0.09 ± 0.0	0.08 ± 0.0	0.24 ± 0.0	0.3 ± 0.0	0	0
OH-Pentanyl	0.16 ± 0.1	0.10 ± 0.1	0	2.92 ± 0.1	0	0.54 ± 0.4
Benzyl	0	0	0	0	0	1.95 ± 0.0
OH-Benzyl	1.56 ± 0.7	0.15 ± 0.1	0.07 ± 0.1	0.07 ± 0.1	40.2 ± 1	39.07 ± 33.8
Indolyl	0.13 ± 0.0	0.14 ± 0.0	0.11 ± 0.0	0.27 ± 0.2	0	0.08 ± 0.1
OH-Indolyl	0.09 ± 0.3	0.16 ± 0.0	0.03 ± 0.6	1.83 ± 1.6	0.33 ± 0.0	0.43 ± 0.0
TOTAL	**39.16**	**22.73**	**5.10**	**6.64**	**40.53**	**42.07**

[1] Mean ± SD is provided; n = 3

Table 3.5 Sinapine, betaine and choline percentage in defatted meals from *Brassicaceae* species (as is).

Species and Variety	Phenylpropanoid					
	Sinapine, %		Betaine, %		Choline, %	
	Whole seed	coteyledon	Whole seed	coteyledon	Whole seed	coteyledon
Brassica juncea						
AC Vulcan	0.426	0.865	0.514	0.547	0.062	0.125
Duchess	0.102	0.223	0.463	0.635	0.020	0.065
Dahinda	0.535	0.249	0.239	0.479	0.037	0.093
Brassica napus						
AC Excel	0.410	0.019	0.381	0.620	0.038	0.114
Sinapis alba						
AC Pennant	0.766	1.585	0.844	1.045	0.129	0.025
Andante	0.319	0.992	0.849	0.988	0.135	0.153

Table 3.6 summarises the nitrogen levels of various nitrogeneous components of *Brassicaceae* seeds. The total N content was the value determined for N freed by combustion of all organic matter and then detected as N. When protein values are derived based on total N content, N of glucosinolates, nucleotides, and other N-containing compounds such as sinapine are also included in protein content. The amino acid N of proteins is the main contributor to the total N content. In *B. napus*, observed low glucosinolate content (Table 3.6) was reflected in low N contribution from glucosinolates. In the variety Dahinda, the nitrogen contribution of glucosinolates was lower than AC Excel canola or other condiment quality *B. juncea* varieties. Among the 3 nitrogenous compounds studied, glucosinolates of condiment type seed coteyledons contributed mostly to the non-protein N content. Although it was assumed that other nitrogenous compounds have a significant contribution to the total N content, for these seeds, sinapine, betaine, choline, glucosinolate and nucleotide contributed <0.5%. The

highest was AC Pennant coteyledon which had 10.8% contributed mostly to the non-protein N content. This indicated that the N of SSPs are the major contributor to total N of these seeds.

Table 3.6 Nitrogen distribution in *Brassicaceae* seeds.

Name		Percent Nitrogen [1]				
		Total[2] N	Glucosinolate N	Nucleic Acid N	N of betaine, choline and sinapine	Contribution of non-protein N to the total N content, %
Brassica juncea						
AC Vulcan:	Whole Seed	4.30	0.18	0.03	0.09	6.90
	coteyledon	4.48	0.23	0.03	0.141	8.90
	Hull	2.77	0.01	ND	ND	ND
Duchess:	Whole Seed	4.30	0.13	0.02	0.06	4.90
	coteyledon	4.78	0.18	0.02	0.18	7.90
	Hull	2.33	0.01	ND	ND	ND
Dahinda :	Whole Seed	4.42	0.01	0.03	0.07	2.50
	coteyledon	4.50	0.02	0.03	0.09	3.10
	Hull	2.66	0.01	ND	ND	ND
Brassica napus						
AC Excel:	Whole Seed	3.83	0.03	0.04	0.08	3.90
	coteyledon	4.03	0.08	0.04	0.09	5.20
	Hull	2.35	0.01	ND	ND	ND
Sinapis alba						
AC Pennant:	Whole Seed	5.15	0.26	0.03	0.17	8.90
	coteyledon	5.36	0.32	0.03	0.23	10.80
	Hull	2.70	0.01	ND	ND	ND
Andante:	Whole Seed	5.90	0.28	0.04	0.14	7.80
	coteyledon	6.24	0.32	0.04	0.20	9.00
	Hull	2.80	0.01	ND	ND	ND

[1] Mean value of Tables 4, 5 and 6 was used for calculation of total N, ND – not determined
[2] Obtained from combustion analysis

The hulls of these seeds contained about 14.5 to 17.5% proteins (Table 3.2) and the polypeptide profiles of the hulls showed several stained bands (Figure 3.3) with varying molecular weights. There are bands similar to napin and cruciferin proteins (clear can see under reducing conditions) found among those (Figure 3.3).

Thermal properties of *Brassicaceae* meals, isolated cruciferin and napin were evaluated using differential scanning calorimetry (DSC). The DSC thermogram provides the maximum of each endothermic peak during the protein unfolding stage (Figure 3.4 and Table 3.7). Our observation was based on the purified cruciferin; *S. alba* has lower T_o (onset temperature) values 62.7 – 63.0ºC than the other two species (76.8 – 88.8ºC). Napins showed fairly high heat stability; T_P (peak temperature) values were around 100ºC or over for all three species.

3.7.2 Polypeptide profile and thermal properties

Figure 3.2 shows SDS PAGE polypeptide profile of purified cruciferin (A) and napin (B) of *B. juncea, B. napus* and *S. alba* under reducing and non reducing conditions. The cruciferin and napin isolated from *B. napus, B. juncea* and *S. alba* showed similar polypeptide pattern but subtle differences can be found (Figure 3.2 A). Polypeptide molecular weight ranging from 10 to 70 kDa was observed among these species. In all varieties the hull free coteyledon had polypeptide bands between 18 and 53 kDa corresponding to cruciferin under reducing conditions (Figure 3.3). In addition to these cruciferin bands, those observed in the 54 kDa region could be pro-cruciferins which were found in seeds. Cruciferin proteins resulted in 5 to 6 bands; under non-reducing condition *B. juncea B. napus and S. alba* showed bands between 45.5 to 58.0 kDa, 43.0 to 51.3 kDa and 46.3 to 53.3 kDa respectively. Under reducing conditions, these combined α and β (α-β) polypeptide bands separated into acidic (α) and basic (β) bands which appeared between 48 to 56 kDa regions. Purified napins under reducing and non-reducing conditions resulted in a single polypeptide band around 18 kDa region and two polypeptides around 6.5 kDa and 10 kDa, respectively (Figure 3.2).

The hulls of these seeds contained about 14.5 to 17.5% proteins (Table 3.2) and the polypeptide profiles of the hulls showed several stained bands (Figure 3.3) with varying molecular weights. There are bands similar to napin and cruciferin proteins (clear can see under reducing conditions) found among those (Figure 3.3). Thermal properties of *Brassicaceae* meals, isolated cruciferin and napin were evaluated using differential scanning calorimetry (DSC). The DSC thermogram provides the maximum of each endothermic peak during the protein unfolding stage (Figure 3.4 and Table 3.7). Our

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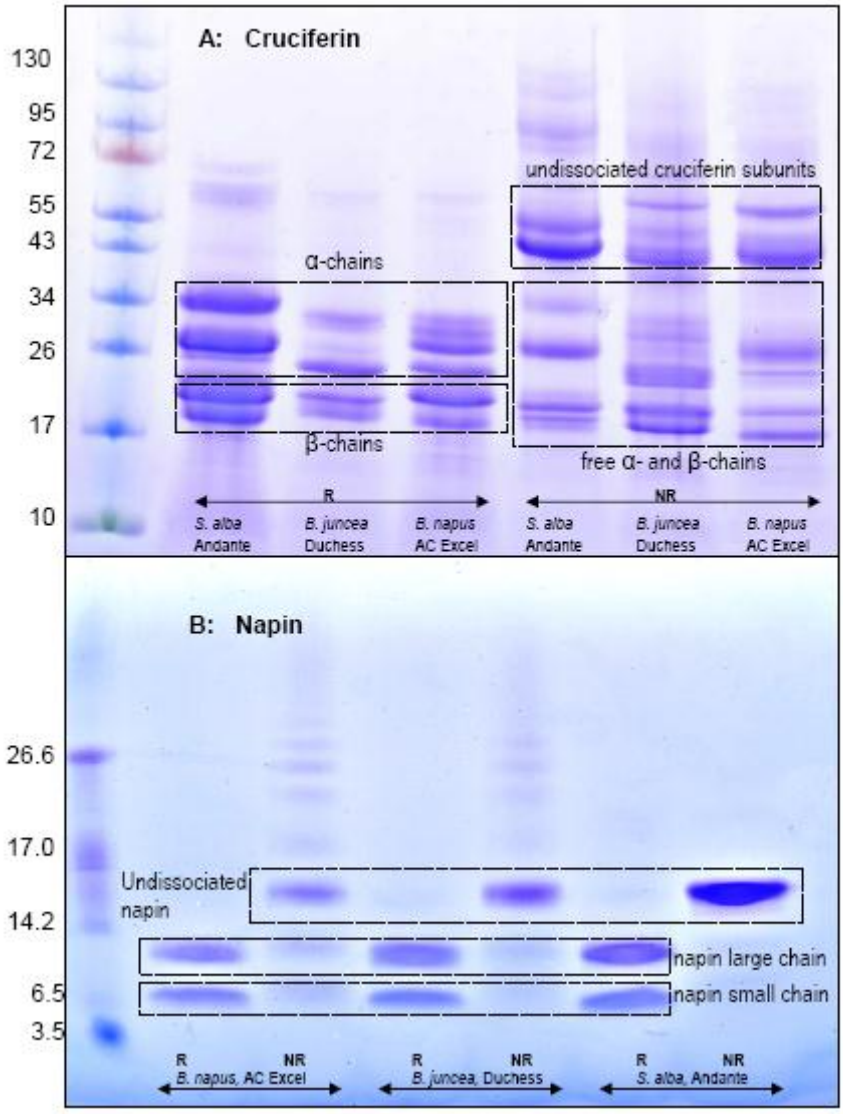

Figure 3.2 Polypeptide profiles of isolated cruciferin and napin from *B. juncea*, *B. napus* and *S. alba* along with molecular weight markers (MWM) (under reducing; R and non reducing; NR conditions).

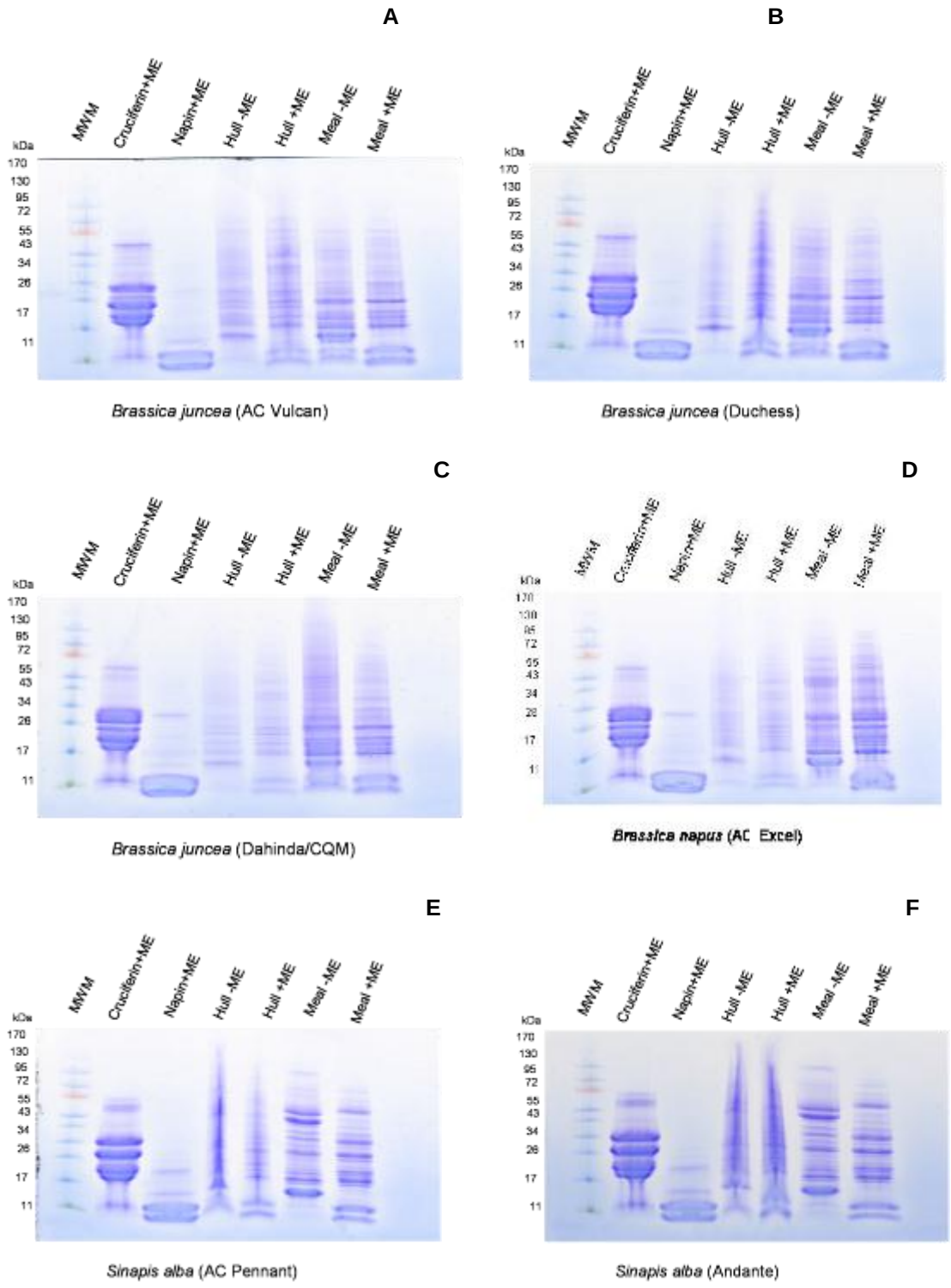

Figure 3.3 Polypeptide profiles (under reducing conditions) of seed coat and coteyledon of *B. juncea* (A, B and C), *B. napus* (D), *S. alba* (E and F). isolated cruciferin and napin from each species and molecular weight markers (MWM) are also presented.

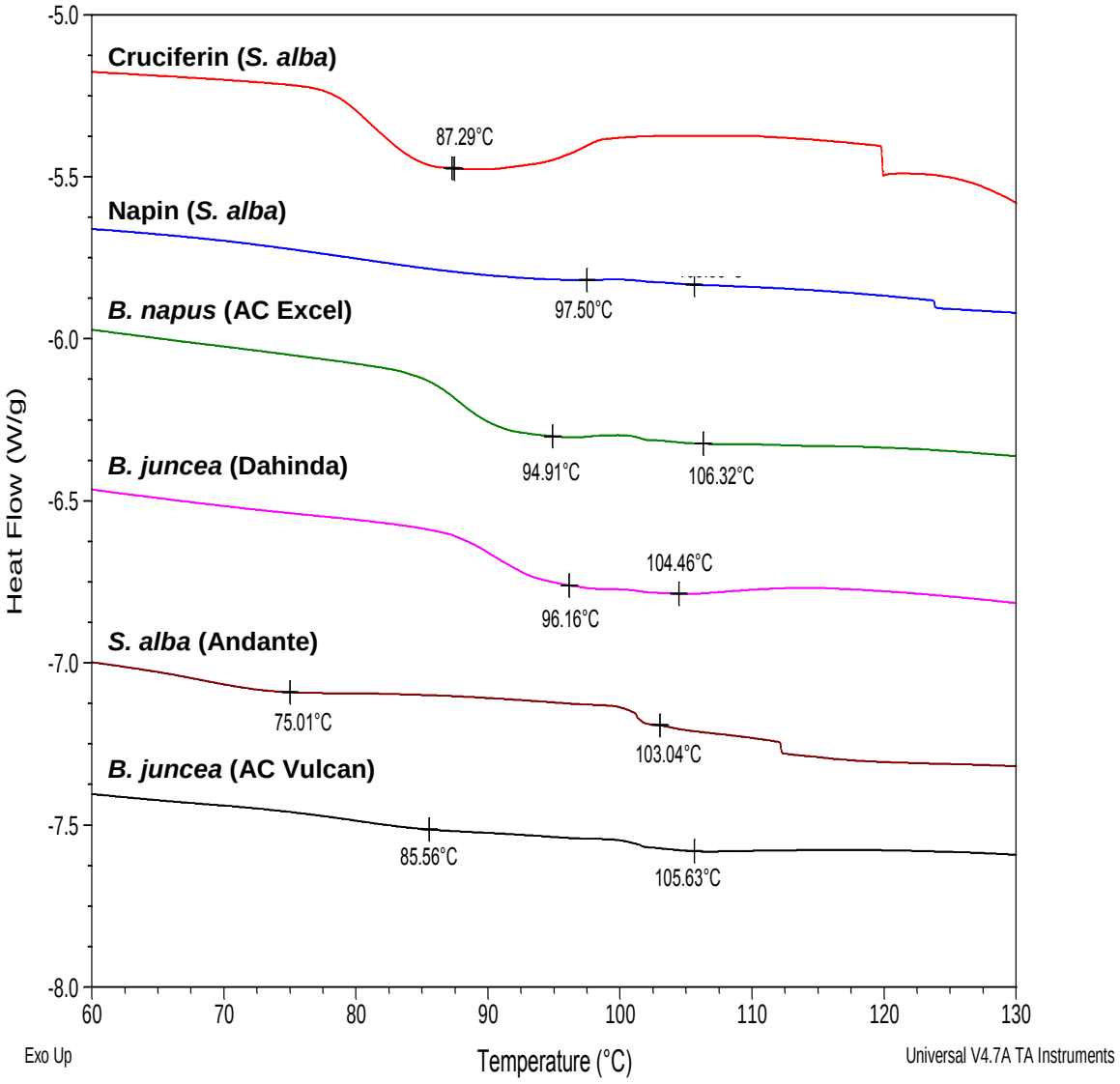

Figure 3.4 Endothermic curves of *B. juncea* (AC Vulcan and CQM), *B. napus* (AC Excel) and *S. alba* (Andante) defatted coteyledon and isolated cruciferin and napin of *S. alba*.

Table 3.7 Thermal denaturation temperatures (ºC) and enthalpy values (J/g protein) of denaturation for different mustard and canola varieties.

Species	Cruciferin			Napin		
	ΔH	T_o	T_p	ΔH	T_o	T_p
Brassica juncea						
AC Vulcan	1.4 ± 0.1	76.9 ± 0.5	85.2 ± 0.4	1.8 ± 0.1	100.7 ± 0.2	105.6 ± 0.1
Duchess	1.6 ± 0.3	79.4 ± 0.6	88.9 ± 1.4	0.6 ± 0.3	101.1 ± 0.2	105.7 ± 0.2
Dahinda	2.5 ± 0.1	88.8 ± 0.6	96.1± 0.1	0.3 ± 0.4	101.7 ± 0.3	104.5 ± 1.3
Brassica napus						
AC Excel	2.6 ± 0.5	86.2 ± 0.3	94.9 ± 0.4	0.7 ± 0.1	101.1± 0.1	105.6 ± 0.4
Sinapis alba						
AC Pennant	3.3 ± 0.3	63.1 ± 0.8	75.4 ± 0.2	4.9 ± 1.3	100.8 ± 0.1	103.6 ± 2.9
Andante	3.9 ± 0.3	62.1 ± 0.5	75.3 ± 0.1	2.0 ± 0.2	100.9 ± 0.1	104.1 ± 0.2

T_o = onset temperature T_p = peak temperature ΔH = enthalpy value

Mean ± SD is provided; n = 3

3.7.3 Solubility changes of nitrogenous compounds with pH

Figure 3.5 shows nitrogen solubility of *B. juncea*, *B. napus* and *S. alba* coteyledon meals with change of pH of the medium from 2 to 10. At pH 4, the nitrogen solubility of all the varieties was low and ranged from 18.8% to 30.9%; with the highest value recording for *B. juncea* Duchess (Appendix: A-T1). At pH 7, nitrogen solubility of *S. alba* was 33.9 to 35.9% which was the lowest and significantly different ($p > 0.05$) from that of *B. juncea* (43.1 to 47.2%) and *B. napus* (61.6%). At pH 10, about 70% of solubility was observed for nitrogenous compounds of Andante and AC Pennant while AC Excel and Dahinda recorded 74% and 84%, respectively. *B. juncea* AC Vulcan and Duchess had values lower than 70% (52.6% and 66.3%, respectively).

Examination of polypeptide profiles of soluble proteins at varying pHs showed bands corresponding to cruciferin and napin (Figure 3.6) when compared with the respective purified proteins (Figure 3.2). At pH 4, the soluble proteins were primarily napins for all the species showing that cruciferin remained insoluble at this pH. Both cruciferin and napin polypeptides were seen in the extracts obtained at neutral and basic

pHs. Polypeptide profiles of soluble nitrogen components of the extracts made above pH 8 were similar to what was observed in meal. It should be noted that the SDS-PAGE sample buffer was at pH 8.0. The extract obtained at pH 2 resulted in a single polypeptide band which was not observed for cruciferin or napin. Preparation of samples with more reducing agent and for longer reaction time (20 min incubation at 95 $^{\circ}$C) resulted in two polypeptide bands at 4 and 6.5 kDa, similar to the ones generated by napin (Figure 3.7).

The Figure 3.8 shows change of soluble nitrogen content of *B. juncea*, *B. napus* and *S. alba* coteyledon extract prepared at pH 12 when the pH of the medium was lowered to 1.9. The solubility of the proteins was decreased due to the pH change from 12 to 1.9; they started to aggregate and precipitate out from the solution. All three seed species showed similar changing pattern of solubility with higher soluble protein content for *B. napus* (AC Excel) as pH was lowered. The extracts showed a drastic decrease in soluble nitrogen as pH was below 9.8 and gave minimum solubility at pH 6.2 except AC Excel (*B. napus*) showed pH 5.2 as the pH of most aggregate formation. This minimum solubility pH value changed from what was observed for meals (compare Figure 3.5A and B with Figure 3.8) when extracts were made at the same pH. The content of N that remained as soluble at their lowest solubility (pH 6.2) was 36% for AC Vulcan and 20% for AC Pennant while AC Excel showed about 40% N that remained soluble at pH 5.2. for AC Pennant while AC Excel showed about 40% N that remained soluble at pH 5.2.

According to the polypeptide profiles of the soluble components pH 6.2, 4 and 12 (Figure 3.9), both cruciferin and napin proteins of *B. juncea*, *B. napus*, and *S. alba* species were in solution. Presence of cruciferin in the soluble fraction was a contrasting observation than the polypeptides profiles of pH 4 extracts (Figure 3.6) that contined only napin. At pH 6.2, the precipitate contained both cruciferin and napin proteins in higher concentration.

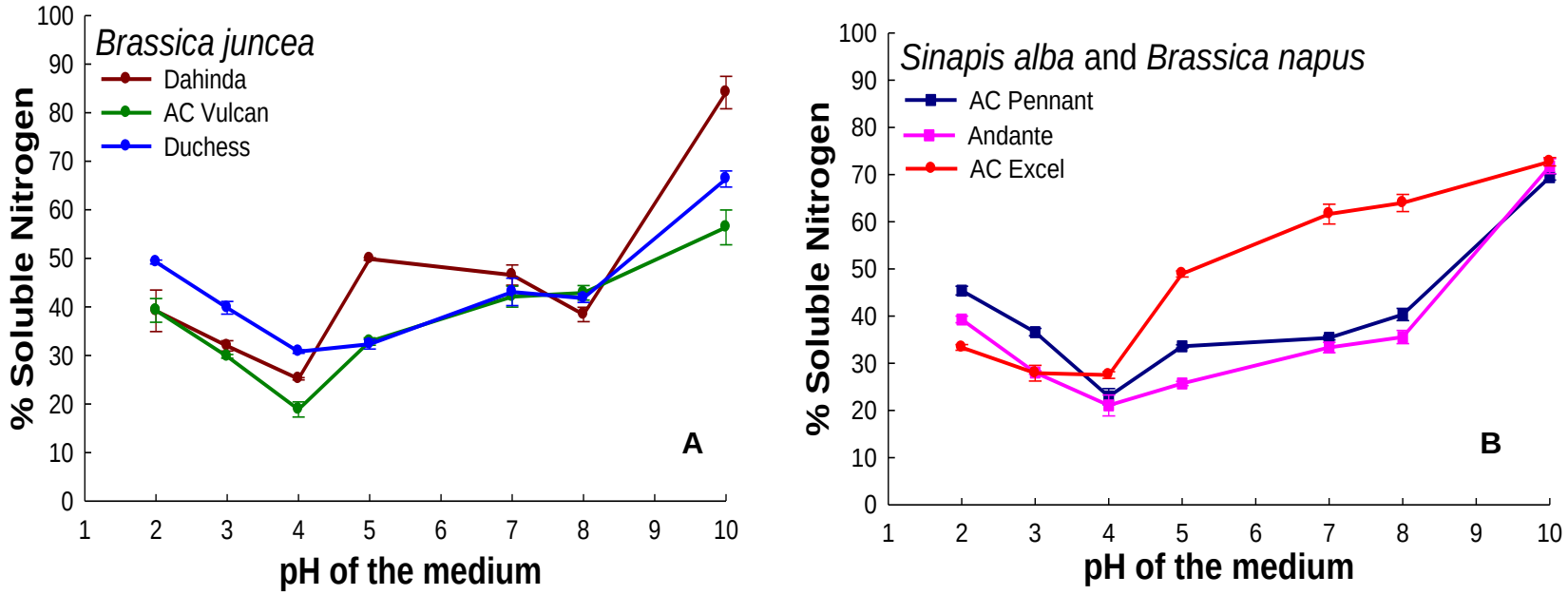

Figure 3.5 Changes of soluble nitrogen content of *B. juncea* (A), *B. napus* (A) and *S. alba* (B) coteyledon meal with changing pH of the extraction medium (meal to solvent 1:20, ambient temperature, 30 min extraction).

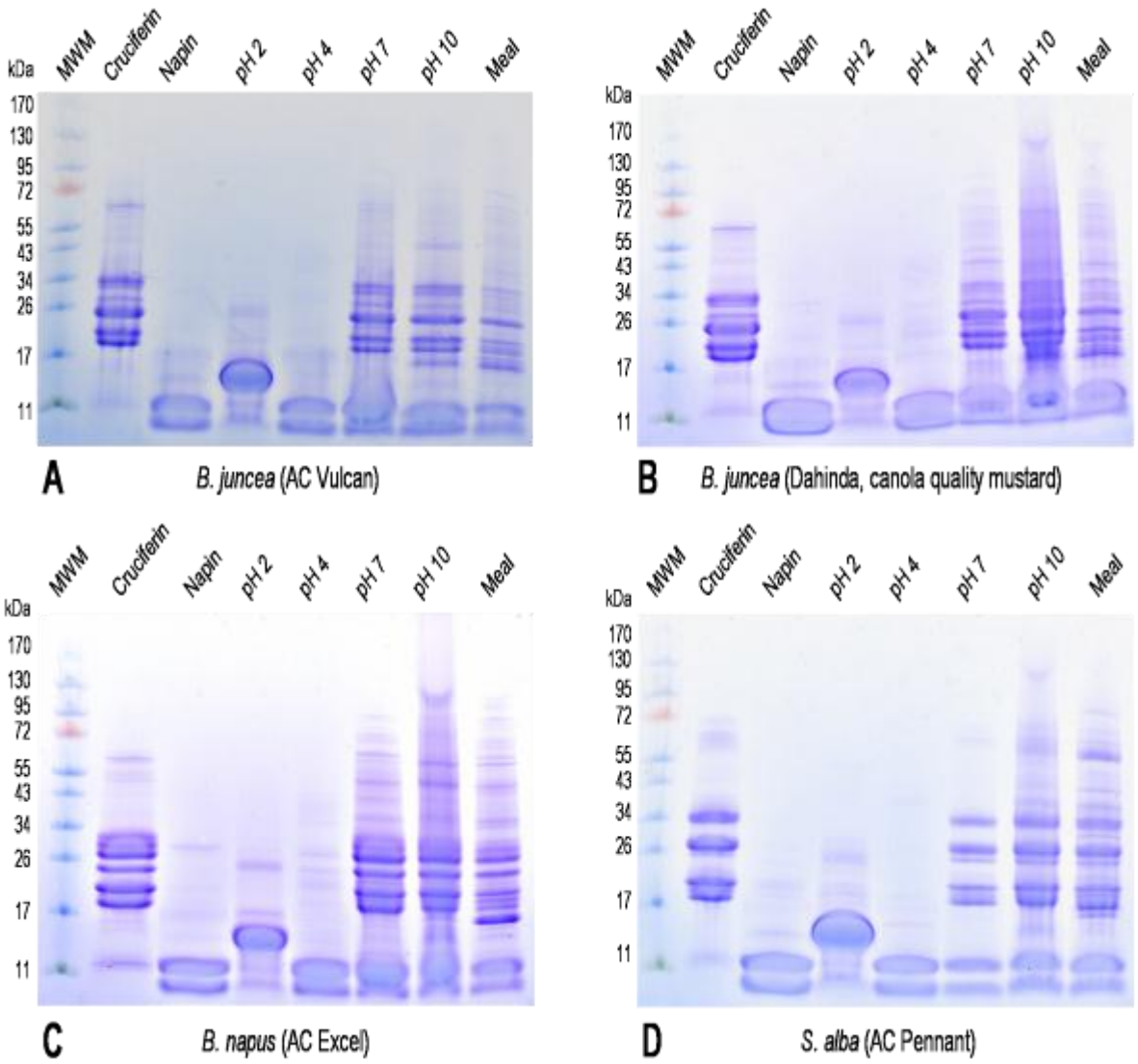

Figure 3.6 Polypeptide profiles *B. juncea* (A & B), *B. napus* (C) and *S. alba* (D) seed proteins soluble at pH 2, 4, 7 and 10. Isolated cruciferin and napin of each species and the molecular weight marker (MWM) are also separated under the same conditions.

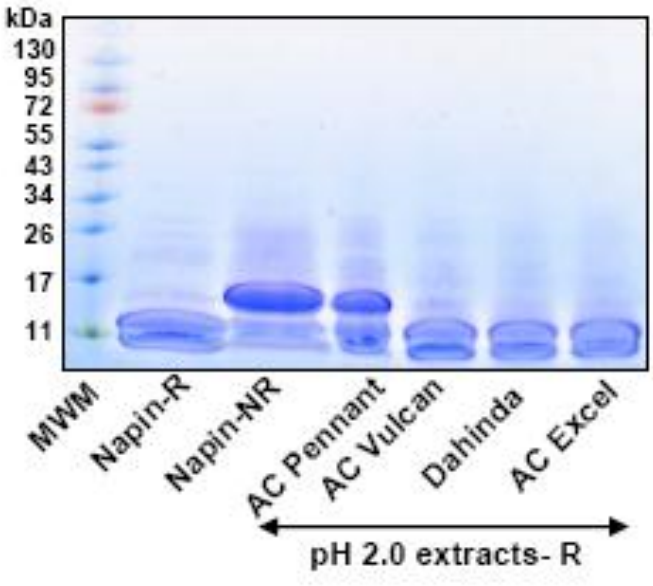

Figure 3.7 Polypeptide profiles *B. juncea* (AC Vulcan, Duchess, and CQM), *B. napus* (AC Excel) and *S. alba* (AC Pennant) seed proteins soluble at pH 2, under non reducing and reducing conditions

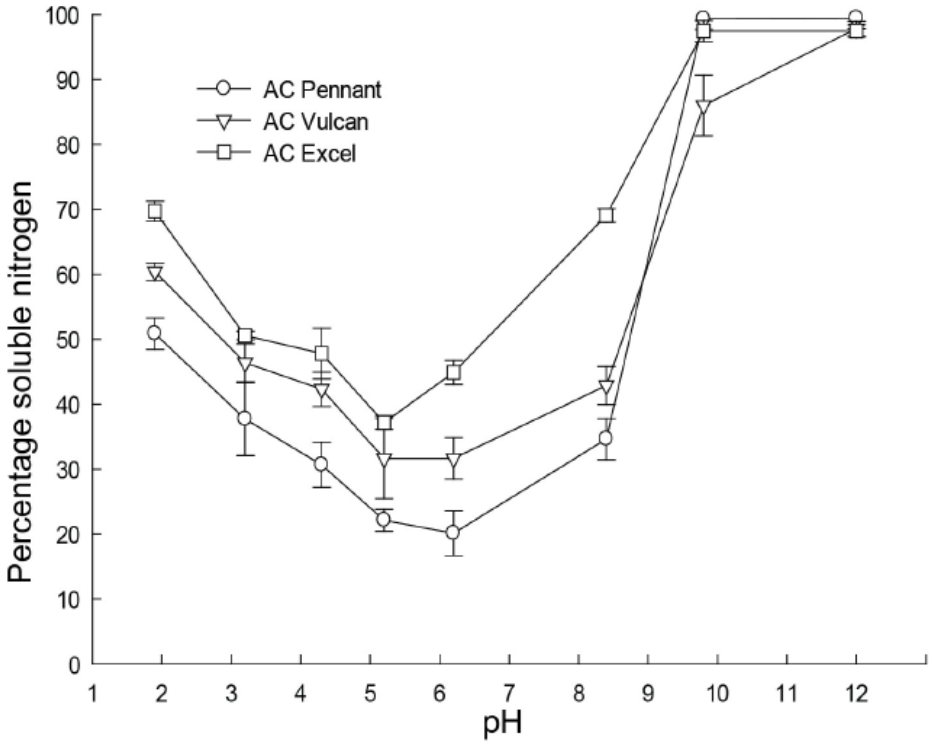

Figure 3.8 Soluble nitrogen content of *B. juncea* (AC Vulcan) *B. napus* (AC Excel) and *S. alba* (AC Pennant) coteyledon meal extract obtained at pH 12 as the medium pH was lowered.

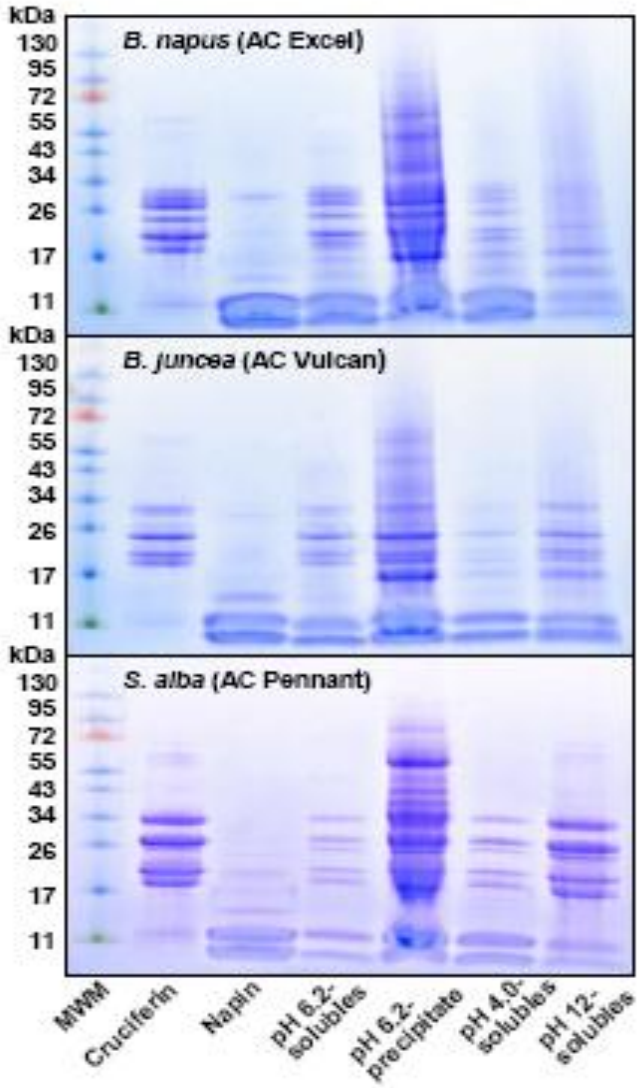

Figure 3.9 Polypeptide profiles of *B. juncea*, *B. napus* and *S. alba* seed proteins soluble at; pH 6.2 (precipitate and soluble), pH 4 (soluble) and pH 12 (soluble) as the medium pH lowered. Isolated cruciferin, napin from each species and molecular weight marker (MWM) are also separated on the same gel.

3.7.4 Solubility changes of nitrogenous compounds with salts and pH

The increase in ionic strength up to one with NaCl showed a positive influence on soluble N components than $CaCl_2$ (Table 3.8). In general, presence of Na^+ or Ca^{+2} in the medium enhanced the solubility of *B. juncea* and *B. napus* proteins irrespective of the pH of the medium. However, increase in ionic strength did not provide a linear increase for the solubility of N compounds. For varieties of *B. juncea* (AC Vulcan and CQM) at pH 4 and 7 both Ca^{2+} or Na^+ provided a significant ($p< 0.05$) increase in protein solubility. However, the Na^+ ion had a positive effect on protein solubility which was higher than Ca^{2+}. Overall, Na^+ and Ca^{2+} increased protein solubility at pH 7. The protein solubility of *S. alba* at pH 7 showed a significant increase with the presence of Na^+ but not with Ca^{2+}. A significant increase was observed at pH 4 for both Na^+ and Ca^{2+}. On the other hand the presence of these salts was significantly ($p< 0.05$) detrimental to protein solubility at pH 10.0 for *S. alba* (Table 3.8).

When pH was 7, presence of Ca^{2+} or Na^+ in the medium significantly enhanced the solubility of canola (*B. napus* and Canola quality mustard) proteins at the ionic strength of 0.75. The proteins of *S. alba* showed a different trend in solubility at this level of ionic strength. The effect of Ca^{2+} or Na^+ beyond 0.25 ionic strength at pH 10 was to decrease protein solubility.

3.8 Discussion

According to Kimber & McGregor (1995), seed coat color shows a wide variability within the *Brassicaceae* family as different proanthocyanidin types and levels are found in dark colored seeds (Marles & Gruber, 2004). The seed coat color differences observed among *B. juncea* is the basis for two market classes of condiment mustard: brown and oriental. Although the brown color seed coat is found in *S. alba*, only the less pigmented yellow mustard is grown in North America. The abundance of mucilage filled cells in the outer most epidermal layer of seed coat and makes YM hull heavier than *B. juncea* or *B. napus*. The mucilage is mostly soluble polysaccharides and the seed coat epidermal layer containing mucilage is found in the *Brassicaceae* family but not common in *B. napus* and *B. juncea*, while it is found in the *Sinapis* spp.

Table 3.8 Nitrogen solubility (%)of *B. juncea* (AC Vulcan and Dahinda), *B. napus* (AC Excel) and *S. alba* (AC Pennant) at different pHs and different ionic strengths provided by NaCl and $CaCl_2$.

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Seed and ion type	pH 4.0					pH 7.0					pH 10.0				
	0.0	0.25	0.50	0.75	1.00	0.00	0.25	0.50	0.75	1.00	0.00	0.25	0.50	0.75	1.00
B. juncea															
AC Vulcan															
NaCl	19.4^{a}	35.9^{b}	41.2^{d}	39.5^{c}	40.8^{cd}	45.9^{a}	50.3^{b}	64.5^{d}	54.8^{c}	63.9^{d}	54.8^{a}	83.6^{e}	74.4^{d}	68.6^{c}	64.6^{b}
$CaCl_2$	19.4^{a}	48.7^{b}	47.3^{b}	49.4^{b}	55.1^{c}	45.9^{a}	63.2^{b}	93.2^{c}	65.8^{b}	64.0^{b}	54.8^{a}	66.7^{c}	54.9^{a}	63.6^{b}	55.9^{a}
Dahinda															
NaCl	25.3^{a}	47.9^{e}	43.7^{d}	35.8^{b}	38.9^{c}	46.5^{a}	76.9^{c}	90.7^{e}	72.2^{b}	84.4^{d}	83.6^{b}	87.6^{c}	84.1^{b}	73.3^{a}	82.2^{b}
$CaCl_2$	25.3^{a}	55.8^{d}	47.8^{b}	50.0^{c}	48.6^{b}	46.5^{a}	88.7^{c}	82.0^{b}	80.6^{b}	78.4^{b}	83.6^{d}	86.0^{c}	69.3^{b}	73.6^{c}	66.3^{a}
B. napus															
NaCl	27.3^{a}	36.1^{b}	40.9^{c}	38.8^{c}	41.2^{c}	60.6^{a}	74.4^{b}	73.7^{b}	70.2^{b}	71.4^{b}	72.7^{a}	76.0^{b}	76.6^{b}	99.3^{d}	80.8^{c}
$CaCl_2$	27.3^{a}	45.6^{bc}	44.7^{b}	47.0^{c}	46.1^{bc}	60.6^{a}	81.4^{c}	76.5^{b}	81.0^{c}	79.4^{c}	72.7^{b}	78.3^{c}	73.0^{ab}	70.7^{a}	74.4^{b}
S. alba															
AC Pennant															
NaCl	23.7^{a}	32.5^{c}	31.3^{c}	29.6^{b}	31.7^{c}	35.4^{a}	52.0^{c}	53.9^{c}	46.0^{b}	56.5^{d}	68.8^{d}	57.8^{c}	55.1^{b}	52.0^{a}	68.2^{d}
$CaCl_2$	23.7^{a}	42.4^{d}	36.4^{b}	39.1^{bcd}	38.4^{bc}	35.4^{a}	39.3^{b}	42.9^{c}	42.6^{c}	42.7^{c}	68.8^{b}	44.2^{a}	37.5^{a}	38.3^{a}	39.3^{a}

$^{a, b, c, d}$ Means followed by the same letter within a column are not significantly differen. Mean ± SD is provided; n = 3

The comparatively high soluble fibre content of YM seed coat may be due to the soluble polysaccharides present. The YM seed coat mucilage can be recovered by water extraction at ambient temperature. The mucilage consists of 80.2-94.1% carbohydrates (glucose 22-35%, galactose 11-15%, mannose 6-6.4%, rhamnose 1.6-4%, arabinose 2.8-3.2% and xylose 1.8-2.%) and 4.4% ash (Cui et al., 1993). YM mucilage is found in several food applications that require thick consistency and texture such as in salad dressings and pasta sauces (Weber et al., 1974). According to Eskin et al. (2007) the mucilage of *S. alba* has anti-colon cancer effect in rats.

The canola grade *B. napus* (AC Excel) and *B. juncea (*Dahinda) are the varieties bred for high oil content which is of edible quality and it was reflected in the results giving 47-49% (as is) oil which was much higher than condiment grade mustard. YM is the low oil containing seed. These results confirm the values reported in literature; according to Katepa-Mupondwa, Rakow, & Raney (1999), oil free meal of *S. alba* has relatively high protein content (45-48%). The seed coat fraction contained 7.4 to 15.6% oil. Ash content of seed coat was not that different than the coteyledon, indicating there may not be a difference in mineral or inorganic constituents between these seed fractions. This study shows phytic acid is concentrated in seed embryo and endosperm rather than seed coat and supports the findings of Raboy (2003) that *Brassicaceae* oilseeds phytates are accumulated inside the protein storage vacuoles. The protein storage vacuoles are mainly concentrated in seed embryo. According to Cooper and Gowing (1983), phytic acid is considered as an antinutrtional compound because it can bind essential dietary minerals such as calcium, iron and zinc through strong cation interaction leading to deficiency of mineral bioavailability to monogastric animals. However, phytic acid is also considered as having antioxidant activities (Graf et al., 1987; Greenwood, 1990). Phytic acid and *Brassicaceae* oilseed proteins (napin and cruciferin) have exhibited phytic-protein complexation below the isoelectric point (*p*I of napin ~ 10, and cruciferin ~ 7.2) (Schwenke et al., 1987). Under high salt concentration (0.7-0.9 M NaCl, 0.23 M $CaCl_2$) in combination with ultrafiltration/diafiltration, phytic acid level can be reduced in rapeseed meal leading to an increase in protein solubility (Thompson, 1990). Several groups had shown that microbial phytases can be used to reduce phytate level in canola meal (Ledoux et al., 1998).

According to Kimber and McGregor (1995) protein values of whole seeds can vary from 27 to 32% (as is) for *S. alba* and 23 to 29% for *B. juncea* and may be affected by plant growing conditions. The method used for quantifying crude protein is based on total nitrogen values. The protein values are estimated by multiplying total nitrogen content by a conversion factor of 6.25. Nitrogen containing components such as glucosinolate, sinapic acid esters of choline (sinapine) and nucleic acids (DNA and RNA) can overestimate protein content. The condiment *B. juncea* mustard variety contained high amount of glucosinolate (115 to 146 µmol/g) which may cause 2.4 to 3.6 percentage over estimation but this value may be 0.8 to 1.3 percentage for canola-grade *B. juncea*.

Nitrogen in the nucleic acids (DNA and RNA) contributed less than 0.1% to the total N pool that was analyzed by combustion of material. The coteyledon of *Brassicaceae* seeds contains primarily protein storage vacuoles and oil bodies and low number of active cells are found contributing to low amount of nucleic acids present. Seeds are mainly storage organs therefore low levels of DNA and RNA are found; the low non protein nitrogen contribution through DNA and RNA does not have a significant effect on total non-protein nitrogen value in seed.

Glucosinolates are secondary metabolites in plants which are part of a plants chemical defence system against herbivores and insects. According to the definition of canola (Canola Council Canada, 2011), the glucosinolate amount in canola seed is mandated to be below 30 µmol/g of oil free meal. The total glucosinolate content observed for canola grade *B. napus* and *B. juncea* was les than < 15 µmol/g meal. The condiment mustard quality is characterized by the type and amount of major glucosinolates present. The type of glucosinolate found in the seed is species specific. The OH-benzyl glucosinolate (211-220 µmol/g) and allyl glucosinolate (110-140 µmol/g) are predominant in *S. alba* and *B. juncea*, respectively and primary precursors of the respective isothiocyanate that causes the typical pungent flavor of mustard. The present study reports contrasting results for mustard seed coat than what was reported by Chiba (1997). According to Chiba (1997), the seed coat of *B. juncea* mustard contain promising pesticidal properties due to the allyl isothiocyanates released from allyl glucosinolates present. However, the values we observed are low compared to the coteyledon and indicate *B. juncea* seed coat may not be a good source of allyl glucosionolate unless a

considerable amount of coteyledon contamination is found during bulk separation of the seed coat.

Phenolic compounds except the polymerized phenolics (tannins) were mainly concentrated in seed cotyledons. The highest amount of N-containing phenolic compounds was observed in *S. alba* variety (sinapine, 1.58%). Sinapine is the most abundant phenolic acid ester in canola and mustard seeds (Uppstrom, 1995) and primarily found in the embryo and endosperm. Due to the negative effects of sinapine such as bitter taste and pungency and fishy odour of eggs when fed to laying hens (Uppstrom, 1995), reduction of sinapine is always attempted through breeding and seed processing. According to Blair & Reichert (1984), sinapine is a sinapic acid choline ester and constitute 1-4 % of air dried oil-free canola meal.

The amino acid N of storage proteins is the main contributor to the total N content. However, any free peptides and amino acids which are non-proteins also will be detected for their N content. In all varieties, a higher amount of total N was concentrated in the seed coteyledon indicating the relationship to storage protein content. In *B. napus*, the observed low glucosinolate content in the coteyledon (25 µmol/g) was reflected in the low N contribution from glucosinolates. In the variety Dahinda, the nitrogen contribution of glucosinolates was lower (by 0.4 to 2.0%) than AC Excel canola. Although it was assumed that other nitrogenous compounds have a significant contribution to the total N content, for *B. napus* seeds, sinapine, betaine, choline, glucosinolate and nucleotides contributed 0.83%. This meant that the majority of the N is coming from seed storage proteins. However, the non-protein nitrogen content in seed meal can significantly affect the protein value based on total N content. For example taking 6.25 as the conversion factor the lowest value of 5.2 and 7.9% protein content was observed for *B. juncea* Dahinda variety and *B. napus* AC Excel, respectively. For the condiment mustards, this value was 8.9% and 9.0% for *B. juncea* and 10.8% for both *S. alba* varieties. Although the seed hull contained 4.5 to 17.5% crude proteins (Table 3.2), the polypeptide profiles showed different bands than the major storage proteins; cruciferin and napin. The proteins of the seed coat are mainly cell wall embedded proteins such as proteoglycans that are structural proteins rather than storage ones (Namasivayam, Skepper & Hanke, 2010). In all varieties polypeptide bands originating from cruciferin can be seen between

18 and 53 kDa region under non reducing conditions. According to Bilodeau and group (Bilodeau et al., 1994), 9 to 12 genes are involved in the expression of cruciferin (heterogeneous) in *B. napus* and five major cruciferin subunit groups are present in mature *B. napus* seed; CRU1, CRU2, CRU3 CRU2/3 (CRU A) and CRU4 (Rodin et al., 1990) and the hexameric cruciferin may be a combination of these subunits. Each subunit is around 50 kDa and is made out of acidic (α) 30 kDa subunit and 20-25 kDa basic (β) subunit linked by disulphide bonds (DeLisle et al., 1989) and this showed up cleanly in the cruciferin proteins under reducing condition (Figure 3.2).

The napin precursor is 21 kDa and after the seed maturation stage two disulphide bonds connect the 4.5 kDa and 10 kDa fragments together (Schmidt et al., 2004). Under reducing conditions, the two polypeptide bands (long and short) observed around 4 to 5 kDa region belong to napin proteins. Polypeptide bands of napin of these six seeds showed very little variation in molecular mass weight compared to cruciferin bands.

The thermal properties observed for these two proteins indicated different thermal stabilities for each protein. Napin shows fairly high heat stability; T_p values are around 100ºC or over for all three species. In napin protein, disulphide bonds play a major role in napin three dimensional structures (Schmidt et al., 2004). The number of cysteine residues and the location of cysteine in the polypeptide chain are the major conserved features in napin proteins (8 Cys motif). A total of 8 cysteine residues have been identified in different napin isoforms; two in the short chain and six in the long chain (D'Hondt et al., 1993). Because of the close proximity of the cysteine residue in the polypeptide chain, a total of four disulphide bonds occur between chains and within the chains leading to a compact structure that is difficult to access by digestive enzymes (Gehrig & Bienmann, 1996) and difficult to heat denature. Due to this high number of disulphide bonds and the stable structure, the denaturation of napin proteins occurs at a fairly high temperature. According to Jyothi, Sinha, Singh, Surolia & Appu Rao (2007), the thermal denaturation of napin molecules of *B. juncea* was observed at 82.7ºC. However, in the present study, the napin peak denaturation temperature is higher (100.5 - 105.7ºC) than the values reported by Jyothi et al. (2007). The napin protein that may exist could combined with other compounds in the seed matrices. For example, phenolics could bind with proteins forming more stable structure which hence would lead to

increased temperature required for the denaturation process. According to Milic et al. (1968), cruciferin and napin proteins can bind with other phenolic compounds which could lead to increased heat stability of these proteins in the matrix than purified proteins. The ability of phenolic compounds to bind with proteins of seed meal through hydrogen bonds could reduce the nutritive value of the meal. Especially, phenolic compounds either by non enzymatic or enzymatic oxidation can bind with $-NH_2$ group of lysine and CH_3S group of methionine and other proteins (Milic et al., 1968).

3.8.1 Solubility changes of N-containing compounds with pH

According to the observations of this study, major proteins of these crucifers are cruciferins and napins that differ in molecular mass, polypeptides and size. Therefore the solubility differences of these proteins are expected. All *Brassicaceae* varieties exhibited typical "U" shape protein pH solubility curves similar to seed meals of other oilseeds (e.g., soy, sunflower). The two solubility minima at pH 4 and 8 were observed for all seed varieties except AC Excel. Rao et al. (1978) also reported similar observation in *B. juncea*. This may be due to the different isoforms of cruciferin and napin present in a particular species or variety. In a previous section, the existence of several isoforms of cruciferin and napin was discussed.

Due to lack of information on Canadian grown mustard varieties, comparison can be made with *B. napus* as a botanically close species. In this study, it is clear that the phenolic compounds made of the condiment grade seed produced a maximum 10% N contribution to the total N content of the seeds which needs to be taken into consideration when interpreting soluble N values of these seeds. According to Xu & Diosady (2002) phenolic compounds of *B. napus* meal are mainly soluble at alkaline conditions (pH 7.5 to 12.5); in addition to above observation, Rauchberger, Mokady & Cogan (1979) have reported a water leaching effect of *B. napus* glucosinolate at pH 4, 6 and 8 at 25 $^{\circ}$C, therefore, it can be assumed that little possibility exists that sinapine contributes to soluble nitrogen content at pH 4. Besides that the very low glucosinolate containing canola quality varieties of *B. juncea* (Dahinda) and *B. napus* (AC Excel) did show higher soluble N content (25.2 to 27.6%) than the highest glucosinolate level containing *S. alba* varieties (21.1 to 22.9%). Analysis of the polypeptide profile of pH 4 solubles (Figure

3.6) showed primarily the polypeptide bands corresponding to napin. According to the Protein Data Bank information all the isoforms of napin of *B. napus*, *B. juncea* and *S. alba* have pI values ~11 (Crouch, Tenbarge, Simon & Ferl, 1983) and are basic in nature; therefore, solubilization at an acidic pH can be rationalized. Some isoforms of *Brassicaceae* napin are known allergens, for examples are Bra j 1 in *B. juncea* (Gonzalez et al., 1991), Bra n 1 of *B. napus* (Monsalve et al., 1997) and Sin a 1 of *S. alba* (Menendez-Arias et al., 1987). Knowledge that napin is soluble at pH 4 while cruciferin is not soluble may be advantageous for the selective removal of potentially allergenic napin by extracting these crucifer meals at acidic pHs such as 4.

Around pH 7 and 8 protein solubility of all *Brassicaceae* varieties (except AC Excel) dropped considerably and a possible explanation can be derived considering the isoelectic point of cruciferin and phytic acid interaction. At the isoelectric point the positive and negative charges on protein are equal and the molecule is electrically neutral. According to Schwenke et al. (1983), since the isoelectric point of cruciferin protein is around 7.25, the low solubility of some cruciferin isomers could decrease the protein solubility between pH 7 and 8. The second factor that may cause this solubility decrease could be the phytic acid in the seed matrix which can form insoluble protein-phytic acid complexes that could precipitate from solution (Gillberg & Tornell, 1976a) at alkaline pH. According to Gilberg et al. (1976), *B. napus* seed protein solubility reaches its maximum between pH 10 and 12 because of the ionization of basic amino acid residues of rapeseed protein at alkaline pH. The proteins of *B. napus* contain approximately equal amount of arginine and lysine; those amino acids would be positively charged around pH 10 where a higher solubility is expected for the protein. Polypeptide profiles of soluble nitrogen components of the extracts prepared above pH 8 were similar to the respective meal. It should be noted here that the SDS-PAGE sample buffer is at pH 8.0 and with SDS, extracts meal proteins that are soluble at pH 8.0.

Interestingly, the solubilized proteins at alkali pHs (pH 12) showed incomplete reversal of their solubility when pH was lowered (Figure 3.8). This information will be useful for designing protein recovery methods from crucifer seeds for extraction of protein at in basic pHs. Most of the studies in the literature on mustard and other crucifer proteins have used pH 4.5 as the precipitation pH (referred to as isoelctric precipitation)

to recover solubilized proteins at alkali pHs (Dendukuri & Diosady, 2003; Marnoch & Diosady, 2006). The pH 4.5 is based on the minimum solubility recorded for meal protein not for the basic pH extract. These authors have mentioned that a considerable amount of acid soluble proteins remained in the soluble fraction. The present study showed that recovery of alkali extracted protein by acid precipitation at a pH determined by nitrogen solubility such as isoelectric pH may not give maximum protein recovery for *B. napus, B. juncea* or *S. alba*. The polypeptide profile of the precipitate recorded at the minimum solubility pH showed both cruciferin and napin are precipitated. Therefore, no selectivity towards a single storage protein of the seed occurred. Also, the selectivity does not occur if these proteins were solubilized at basic pHs.

The single intensely stained polypeptide (between 11 and 17 kDa) band which appeared for the pH 2 extract of all the species indeed originated from napin bands. This was confirmed by the separation of this protein into two polypeptide bands when the amount of S-S bond breaking agent was increased in the reducing conditions (Figure 3.7). Due to the extreme acidic condition at pH 2.0 napin protein may have formed aggregates or dimerizatio may have happend. The suspicion of non-specific lipid transfer proteins of *Brassicaceae*, as described by (Berot et al., 2005) or oil body proteins were excluded by this confirmation.

3.8.2 Solubility changes of N-containing compounds with salts and pH

The presence of Na^+ or Ca^{2+} in the medium enhanced the solubility of *B. juncea* and *B. napus* proteins irrespective of the pH of the medium; however *S. alba* at pH 10 with presence of salt protein solubility did not increase. When the increase in ionic strength up to 1 is considered for both salts, NaCl definitely showed a more positive influence on soluble N components than $CaCl_2$. However, the increase in the ionic strength did not provide a linear increase for the protein solubility and the effect of ions was not significant on protein solubility at basic pH. According to Aluko et al. (2004) the isolated YM proteins showed significantly higher protein solubility and emulsifying ability than *B. juncea* or soybean in the presence of calcium chloride (0.75 M) at alkaline pHs.

Enhanced solubility of seed storage protein by added mono and divalent ions such as Na^+, K^+ and Ca^{2+} has been reported (Aluko et al., 2004). Proteins are amphiphilic molecules with positive and negative charges. The solubility of proteins is a thermodynamic equilibrium between protein-protein and protein-solvent interaction; hydrophobic and ionic nature of the protein mainly influence these interactions. The pH of the medium influences the ionization of the side chain residues of the proteins and proteins exhibit minimum solubility when net positive and negative charges are balanced. Typical "U" shape solubility curves are exhibited by most proteins and *B. juncea, S. alba* and *B. napus* species cotyledon proteins exhibited the same. Solubility of proteins in solution follows the relation; $\log (S/S_0) = \beta - K_S C_S$, where, S and S_0 are solubility of the protein in salt and water, respectively, K_S is the salting out constant, C_S is molar concentration of salt and β is a constant characteristic of the protein. Salts at low ionic strength (< 0.5) neutralize charges at the protein surface and this charge screening effect either decreases solubility of proteins that have high incidence of non-polar patches on the surface or increases it for those that do not have such patches (Damodaran, 2008). From the results of this study, both *B. juncea* and *S. alba* proteins may have low incidence of hydrophobic patches on their surfaces which may be denoted by the sharp increase in solubility observed at 0.2 ionic strength. According to Schwenke et al. (1983), rapeseed cruciferin proteins show reversible association and dissociation with change of pH and ionic strength of the media; when ionic strength of the media is above 0.5, cruciferin proteins formed 11/12S hexameric assemblies and with the lowering ionic strength the structure breaks into 7S structure form (trimeric form). However, dissociated 7S trimeric structures can re-assemble with increasing ionic strength in the medium (Schwenke et al., 1981). The 12S hexameric structure of cruciferin is unstable at low ionic strength and dissociates into 7S trimers and tends to precipitate. Low solubility values were observed at low ionic strength and high protein solubility at the ionic strength around 0.75 is a common trend for *Brassicaceae* proteins. At constant ionic strength the relative effectiveness of ions on protein solubility follows the Hofmeister series; the cations decrease solubility in the following order $NH_4^+ < K^+ < Na^+ < Li^+ < Mg^{2+}$ Ca^{2+}. The increasing effect on solubility by Na^+ is larger than Ca^{2+} and it was evident from our curent study as well.

3.9 Conclusions

The composition of nitrogenous compounds of *Brassicaceae* oil containing seeds varied between species *Brassica juncea*, *Brassica napus* and *Sinapis alba*. Proteins and lipids are the major components of these seeds and *B. juncea* and *B. napus* seeds contain more oil than protein. Proteins were concentrated in seed embryo and endosperm (coteyledon). The non–protein components namely, nucleic acids, glucosinolates, sinapine, choline and betaine contributed 2.5-8.4% to the total N content. Cruciferin and napin are the main seed storage proteins located in the coteyledon of these *Brassicaceae* seeds. Both these proteins are quite heat stable and have thermal denaturation temperatures above 73°C. Similar to other dicotyledons, seeds proteins of these *Brassicaceae* seeds showed a "U" shape curve for soluble protein over the range of pH 2 to 10. The minimum solubility was observed around pH 4; however, reversal titration of alkali solubles (at pH 12) showed minimum solubility around pH 6.2. About 20-40 % protein remained soluble at pH 4 depending on the species and these proteins are composed mainly of napin. The presence of Ca^{+2} or Na^{+} increased solubility of proteins at a given pH with exception of pH 10 of *S. alba*. The effect of calcium ions on enhancing solubility was higher than sodium ions at the same ionic strength at pH 4 and 7. The solubility differences of napin and cruciferin observed in all three species will be useful in obtaining these proteins separately for further studies and utilization.

3.10 Connection to Study 2

The contribution of non-protein nitrogen components to soluble N content and the differences of solubility charateristics of major storage proteins as the pH and ionic strength of the medium changed were studied in the Study 1. The Study 2 was carried out to investigate whether the two major storage proteins of these crucifer species are different in their ability to degrade in the presence of enzymes found in the mammalian digestive tract which may indicate their potential to become gastrointestinal allergens. This study was carried out using *in vitro* assays and bioinformatics analysis *in silico*.

4. STUDY 2: *IN VITRO* DIGESTIBILITY AND ALLERGENICITY ASSESSMENT OF *BRASSICACEAE* SEED STORAGE PROTEINS

4.1 Abstract:

This study focused on *in vitro* digestibility and allergenic potential of major seed storage proteins of *Brassica juncea*, *Brassica napus* and *Sinapis alba*. Dehulled, defatted meals, napin-removed meal, isolated napin and cruciferin of the six varieties of these three crucifer species were subjected to gastric (pH 2, pepsin to meal 1:250 [E]/[P], 2 h, 37ºC) and intestinal (pH 6.8, pancreatin 1:250 [E]/[P], 2 h, 37ºC) phases of digestion to evaluate digestibility of the containing proteins. The allergenic potential of these proteins were studied as the resistance to pepsin digestion in which higher pepsin concentration was used in the gastric phase digestion than regular *in vitro* digestibility studies. In addition, sequence homology of available cruciferin and napin isoforms of *B. juncea*, *B. napus* and *S. alba* was tested against known seed protein allergens using allergen databases. Across all the varieties, the resulting digestibility values for defatted meals were significantly lower than that of the napin-free meal. Napin protein exhibited the lowest digestibility and for all varieties, the values were below 10%. It is evident that the low digestibility of napin contributed to the overall reduction in meal protein digestibility. The presence of intestinal protease inhibitors, such as trypsin and chymotrypsin inhibitors, were negligible in these seeds and indicated their effect on protein digestion was minimal. Under high pepsin concentration, cruciferin proteins were quickly digested and showed no or weak resistance to proteolytic cleavage in the gastric conditions. However, napin was resistant to gastric digestion and a large fraction of the protein remained intact within the assay period indicating the potential to remain as a gastro-intestinal allergen. According to homology comparison, the similarity of the amino acid sequence of the known allergenic napin and *B. juncea* and *S. alba* napin was higher than 35%; however, cruciferin proteins showed low possibility to be allergenic.

4.2 Introduction

The essential amino acid composition and the ability to release these amino acids are primary properties that directly influence nutritional value of seed storage proteins (SSP) in food and feed applications. In comparison with soybean, canola and mustard meals possess well balanced amino acid profiles (Applequist and Nair, 1977). The proteins of rapeseed meal have high level of lysine, methionine, cysteine, threonine and trptophan (Bille et al., 1983). According to a mice feeding study (Eggum, 1973), the biological value (BV) of rapeseed meal is higher (89.1%) than soybean meal (67.8%).

In the *Brassicaceae* family mainly two types of SSP are present. The legumin type 11/12S globulin cruciferin is the predominant protein and has molecular weight between 300 and 350 kDa (Schwenke et al., 1981; Sjodahl et al., 1991). The 2S napins have molecular weight of 12 to 16 kDa (Crouch et al., 1981; Lonnerdal et al., 1972). In mature *B. napus* seeds, the 11/12S globulin protein constitutes about ~60% and the napin is ~20% of total storage proteins (Crouch et al., 1981).

The *in vitro* digestibility study of Savoie and group (1988) observed that digestibility of rapeseed proteins by pepsin and pancreatin (gastrointestinal digestion) was 83% which is a lower value than for casein (97%). According to Mustafa et al. (2000) and Newkirk et al. (2003), feeding of desolventized meal (by product from canola oil processing plant which contain defatted hull and coteyledon both together) to chicken has resulted in negative effects on their growth performances, protein digestibility, body weight gain, and feed conversion efficiency while elevating mortality. According to the study of Piepenbrik et al. (1998), feeding dairy cattle with canola meal has improved milk production. Canola meal can easily degrade in the rumen by help of rumen microflora (Piepenbrik et al., 1998). The study of Bos et al. (2007) on human feeding of *B. napus* protein product (36.8% globulin, 41% napin, 2.7% LTP, total nitrogen 14.9%) reported low digestibility values compare to cereal proteins and higher value than for a legume such as lupin. Using cruciferin-rich fraction of canola (Puratein® of Burcon Nutra Science), in a 13 week sub-chronic dietary toxicity study, Mejia et al. (2009) reported that the protein product can be included in the diets at the rate of 11.24 g/kg BW/day for male and 14.11 g/kg BW/day for female rats without observeable adverse effects.

The seeds of *B. napus*, *B. juncea* and *S. alba* are reported to contain proteins that trigger IgE-mediated reactions in children and adults (Morrisset et al., 2003; Monsalve et al., 2001; Poikonen et al., 2008; Puumalainen et al., 2006; Rance et al., 2000). The immune reactive proteins of these seeds have been identified as isoforms of cruciferin. They are Sin a 2 (CRU1_SINAL, P83908), 51 kDa, (Palomares et al., 2005) and napin Sin a 1 (ALLI_SINAL P15322) of *S. alba* (Menendez-Arias et al., 1987). Napin BnIII (napin nIII, napin 3, P80208, 2SS3_BRANA) and Napin BnIII (napin nIII, napin 3, P80208, 2SS3_BRANA) of *B. napus* (Monsalve et al., 1997).

The release and availability of amino acids for absorption in the small intestine relates to the digestibility of a protein. In monagastrics, protein digestion begins in the acidic environment of the stomach. The low pH (1.2 to 2.0) of the gastric environment tends to unfold large protein structures. Pepsin has the ability to catalyze hydrolysis of complex proteins to oligopeptides of smaller size (Adibi, 1984). In the small intestine, trypsin, chymotrypsin and carboxy peptidases act on polypeptides that are generated from pepsin hydrolysis, and also on any unhydrolysed proteins from the gastric digestion. Gastrointestinal digestion of protein produces small peptides and free amino acids which become available for absorption into the small intestinal enterocytes epithelial cells (Savoie, Galibois, Parent, & Charbonneu,1988). Some proteins if not degraded in the stomach can activate the IgE (type E immunoglobin) hence can trigger gastrointestinal allergic reactions (Mills, 2003). Based on this observation, several studies have been carried out to assess potential allergenicity of proteins, especially that of genetically modified ones (Thomas et al., 2004).

The protein digestibility of *Brassicaceae* seed meal may be related to the types of storage proteins present, their level of abundance and presence of enzyme inhibitors. Also pepsin digestion with high enzyme concentration may provide an indication of which proteins of these seeds are resistant to digestion, therefore having the potential to become gastrointestinal allergens. The objective of this study was to compare major SSP of three *Brassicaceae* species seeds for their *in vitro* digestibility, allergenic potential and cross-reactivity of *Brassicaceae* 2S proteins. The following hypotheses were tested in this study: 1) cruciferin and napin of *Brassicaceae* oilseeds are different in the digestibility and degradability catalyzed by pepsin and pancreatin; 2) trypsin and chymotrypsin

inhibitory activity of *Brassicaceae* seeds affect protein degradability; and 3) cruciferin and napin are different in their allergenic potential assessed by resistance to pepsin digestion and sequence homology.

4.3 Materials and Methods

4.3.1 Seed material and their preparation

Seed materials used and their preparation was the same as previously described in section 3.3.1.

4.3.2 Preparation of crude cruciferin, crude napin and napin-depleted meal.

The preparation of crude cruciferin, crude napin and napin-depleted meal were according to the sequential aqueous extraction procedure of defatted meals as described by Wanasundara and McIntosh (2008). First the defatted meal was dispersed with purified water (1:80, w:v) and pH was adjusted to 3.0. Then NaCl was added to bring the NaCl counter of the slurry to 2.0% (w/v). Extraction was continued for 2 h at 22°C while mixing. Solubilized proteins were recovered as the supernatant from centrifugation (10,000×*g*, 10 min) and further cleaned by filtration. Extraction of napin was repeated once more. The napin-removed meal was the resulting residue of two extractions of napin and mainly contained cruciferin and cell wall fiber. The extracted napins were desalted by dialysis in a 2 kDa MWCO membrane (Spectra/Por 6, Spectrum Laboratories, Houston, TX) against water for 12 h The napin-removed meal was used for extracting cruciferin as follows. The napin-depleted meal and 0.05 M NaOH in a 1:22 ratio were mixed for 2 h at room temperature (22°C). At the end of stirring, the mixture was centrifuged at 10,000×*g* for 20 min. The supernatant was filtered through #1(Whatman) filter paper. The remaining residue was re-extracted under the same conditions. The first and second extractions were combined and desalted by dialyzation against deionized water for 12 h, using 2 kDa MWCO dialysis tubes. The desalted cruciferin, napin and napin-removed meal were freeze-dried and stored at -18°C.

4.3.3 *In vitro* gastro-intestinal digestibility

Defatted meals (coteyledon), crude napin and napin-depleted meal (contains mainly cruciferin), bovine casein and defatted soybean meal were subjected to gastrointestinal digestion according to the described method of Gauthier, Vachon & Savoie (1986) and Vermeirssen, Camp, Decroos, Wijmelbeke & Verstaete (2002) with some modifications. Simulated gastric fluid (SGF; 0.084 N HCl, 35 mM NaCl, pH 2.0, 37°C) and simulated intestinal fluid (50 mM KH_2PO_4, 15 mM NaOH, pH 6.8, 37°C) were prepared according to the United States Pharmacopeia (USP, 2000). The gastric digestion was initiated by adding SGF to the sample, adjusting pH to 2.0 and equilibrating at 37°C. Then pepsin (Sigma Product No. 7012, 2,410 active units/mg solids) was added at an enzyme-to-protein ratio of 1:250 (w:w), and the final volume of the reaction mixture was adjusted to 30 mL with SGF. Gastric digestion phase was performed for 2 h at 37°C with continuous mixing at 169 rpm in a shaking water bath. At the end of the gastric phase, pH was adjusted to 6.8 using 10 M NaOH.

For the intestinal digestion phase, pH adjusted gastric phase digest was mixed with pancreatin (Sigma product. No. 7545-25G, Activity 8×USP, enzyme to protein ratio at 1:250, w:w), and the final volume was adjusted to 40 mL with SIF. Similar to the gastric phase, samples were incubated at 37°C for 2.5 h. At the end of intestinal digestion phase, samples were kept at 75°C for 2 min to inactivate enzymes. The undigested residue resulting in the mixture was removed by centrifugation at 12,000×*g* for 15 min. This residue (precipitate) was freeze-dried and used for analysis (polypeptide profile by SDS-PAGE) of undigested proteins. A 15 mL aliquote of the supernatant (digest) was mixed with a equal volume of 20% (w/v) trichloroacetic acid (TCA), and kept for 5 min at ambient temperature before centrifugation at 27,000×g for 10 min. The resulting supernatant was analyzed for the released nitrogen (or TCA soluble nitrogen) content due to enzyme activity after neutralizing the sample with Na_2CO_3. Each test sample with SGF and SIF without enzymes was included as sample blanks and SGF and SIF with enzymes but without test sample was carried out as an enzyme blank.

The *in vitro* digestibility of protein was calculated as following.

$$\text{*In vitro* protein digestion, \%} = \frac{\text{N in digest - (N in enzyme blank + N in sample blank)}}{\text{(N in meal sample - N in sample blank)}} \times 100$$

Except for analysis of the meal N by combustion, all other N values are TCA soluble nitrogen.

4.3.4 Assay of trypsin and chymotrypsin inhibitory activity

Sample preparation for the assay of trypsin and chymotrypsin inhibitory activity was similar. The dehulled and defatted coteyledon (1.00g) was stirred in 10 mL of double purified (deionized milliQ) water (meal to solvent ratio 1:10, w:w) and extracted for 2 h in a mechanical shaker. The samples were centrifuged at 12,000×*g* for 20 min. The supernatant was recovered by filtering through #1 filter paper (filtrate 1). Another 5 mL of water was added to the residue and it was extracted for another 2 h. Solubles were recovered by centrifugation and filtered as mentioned (filtrate 2) above. Filtrate 1 and 2 were combined and dialyzed using dialysis cassettes (Slide-A-Lyzer dialysis cassette, MWCO 3.5 kDa, Thermo Scientific, Rockford, IL). The dialysate was freeze-dried and reconstituted as needed for the assay. The extract so obtained represented water soluble components of the meals.

Trypsin inhibiting activity: The assay for trypsin and chymotrypsin inhibitory activity was performed according to Fritz, Trautschold & Werle (1974) and the technical bulletin (Sigma, 2008) of the Sigma trypsin and chymotrypsin inhibitory assay. The following solutions were prepared; triethanolamine (TEA) buffer (0.2 M TEA pH 7.8, 20 mM $CaCl_2$), trypsin (50 μg protein/mL, 50 mU/mL), 500 μg trypsin (dissolved in 10 mL of 1 mM HCl), and 2.2 mM substrate solution (50 mg N-a-benzoyl-DL-arginine-p-nitroanilide [BAPNA] dissolved in 50 mL of deionized water at 95^{o}C). The trypsin inhibitor (TI) assay was carried out as follows. Trypsin solution (0.2 mL) was added to 1.7 mL of TEA buffer and sample (0.05 to 0.1 mL) in a disposable cuvette, mixed and incubated at 25°C for 5 min. Finally, 5 mL of substrate solution was added and mixed. After 5 min of incubation at 25°C, absorbance of the inhibited mixtures (T_{TI}) were read at 405 nm using a spectrophotometer (light path: 1 cm, final volume 3 mL, at ambient temperature). Trypsin reference (uninhibited T_{UI}) assay was carried out with 0.2 mL trypsin solution and 1.8 mL of buffer incubated at room temperature for 5 min followed by addition of 1

mL of substrate solution, and absorbance at 405 nm (T_{UI}) was read. Trypsin inhibitory activity was calculated as:

One unit (U) of trypsin corresponds to an absorbance change (ΔE_{405}) of 3.32 per min in 3 mL.

$$\text{Units of trypsin inhibitory activity in sample (TI) U} = \frac{(\Delta E/\text{min})T_{UI} - (\Delta E/\text{min})T_{TI}}{3.32}$$

$$\text{Trypsin inhibitory units/g of soluble protein (pH 7)} = \frac{\text{TI (U)}}{\text{g protein in sample volume}}$$

Chymotrypsin inhibiting activity: The chymotrypsin inhibitory assay was done according to the colorimetric method of Wirnt (1974) and as described in the chymotrypsin inhibitory assay by Sigma (Sigma, 2008). The following solutions were used; 80 mM Tris-HCl buffer pH 7.8 at 25°C (A), 1.07 mM N-benzoyl-L-tyrosine ethyl ester (BTEE) in 50% (v/v) methanol as substrate (B), 2 M $CaCl_2$ (C), 10 mM Tris-HCl buffer, pH 7.8 at 25°C (D), 2 mg/mL α-chymotrypsin (Sigma C-4129) in reagent D (E), and *Brassicaceae* seed extracts which were assumed to have chymotrypsin inhibitory activity (F). Chymotrypsin inhibitory assay was carried out according to the reagent mixing as described in Table 4.1. Mixture 1 was prepared in seven separate test tubes and Mixture 2 was prepared in separate cuvettes according to the volumes indicated.

Table 4.1 Volumes (mL) of reagents mixed for chymotrypsin assay (prepared according to the Sigma technical bulletin, Sigma, 2008).

Reagent	Uninhibited	Test 1	Test 2	Test 3	Test 4	Test 5	Blank
Mixture 1							
Reagent F (Test extract)	0.0	0.05	0.08	0.1	0.15	0.2	0
Reagent E (Chymotrypsin 2 mg/mL)	0.5	0.5	0.5	0.5	0.5	0.5	0
Reagent D (10 mM Tris, pH 7.8)	9.5	9.45	9.43	9.4	9.35	9.3	0
Mixture 2							
Reagent B (BTEE in 50% (v/v) methanol)	1.40	1.40	1.40	1.40	1.40	1.40	1.40
Reagent A (80 mM Tris , pH7.8)	1.42	1.42	1.42	1.42	1.42	1.42	1.42
Reagent C (2 M $CaCl_2$)	0.08	0.08	0.08	0.08	0.08	0.08	0.08
Reagent D (10 mM Tris, pH 7.8)	0	0	0	0	0	0	0.1
Mixture 1	0.10	0.10	0.10	0.10	0.10	0.10	0

The reaction was started by mixing the contents and the breakdown of BTEE was monitored at 256 nm every 60 sec for 5 min. Maximum linear rate for the absorbance change was considered by calculating ΔA256 nm/min for each volume of the extract and uninhibited solution. Conversion of BTEE units by each assay sample was calculated as:

$$\text{BTEE units/mL} = \frac{[\Delta A_{256\ nm}/\text{min (test smaple)} - \Delta A_{256\ nm}/\text{min (blank)}]\times 3\times 0.5\times Df}{0.964\times(0.1\times 10)}$$

Where, Df is the dilution factor, 0.964 is the millimolar extinction coefficient of BTEE, 3 is the total volume of the assay in Mixture 2 in mL, 0.5 is the volume of enzyme used in the assay Mixture 1 in mL, 0.1 is the volume in mL of enzyme from Mixture 1 used in Mixture 2 of the assay in mL and 10 is the total volume of Mixture 1 in mL.

Then BTEE units/mL values and chymotrypsin inhibitor (test sample) volume was plotted separately for each extract (Appendix A-F6). The point at which the X axis was intercepted was taken to calculate mg of chymotrypsin inhibited by mg of proteins of the test inhibitor. Final calculation was as follows.

Y_0 = mg of chymotrypsin inhibitor/mL in Mixture 2 of the assay to complete inhibition of chymotrypsin = (X-intercept) (mg extract protein/mL in reagent F)

CT = mg of chymptrypsin/mL in Mixture 2 = (0.5)(mg chymotrypsin/mL in reagent E)

Therefore, mg of chymotrypsin inhibited by 1 mg protein of test inhibitor = CT/Y_0

4.3.5 *In vitro* pepsin digestion resistance assay for potential allergenicity

In vitro gastric digestion with high pepsin concentration was carried out on crude cruciferin and napin of *B. juncea*, *B. napus* and *S. alba*. The soybean trypsin inhibitor (minor allergen; Sigma, Cat. #T9003), bovine β-lactoglobulin-B (known allergen, BLG; Sigma, Cat. #L0130), β-conglycinin (minor allergen, BCN; Sigma, Cat. #C5868), lysozyme from chicken egg white (known allergen, LYS; Sigma, Cat. #T2011) and RuBisCo (non allergenic, SRP; Sigma, Cat. #R8000) obtained from commercial Sigma suppliers were also evaluated under the same experimental conditions. All the proteins

except napin were prepared at a concentration of 5 mg/mL in 50 mM Tris-HCl, pH 9.5. Napin was dissolved in gastric fluid control solution (0.084 N HCl, 35 mM NaCl, pH 1.2).

Each protein was subjected to digestion as described by Thomas et al. (2004). First, 1.52 mL of modified SGF (mSGF; 0.084 N HCl, 35 mM NaCl, pH 1.2, and 400U of pepsin to have pepsin: protein 1:13, w:w) was kept in a shaking water bath to equilibrate at 37^{o}C. Then 0.08 mL of protein solution (5 mg/mL) was added to mSGF and mixed well. The pepsin-catalyzed digestion of proteins was continued at 37^{o}C in the shaking water bath. Samples of 200 μL were withdrawn from the digestion mixture at 0, 0.5, 2, 5, 10, 20, 30 and 60 min of incubation. The pepsin activity of each sample was quenched by adding 70 μL of 200 mM $NaHCO_3$ (pH 11) and then 70 μL of Laemmli (1970) buffer [5 ×(40% glycerol, 5% (v/v) β-mercaptoethanol, 10% (w/v) SDS, 0.33 M Tris, 0.05% bromophenol blue)] at pH 6.8, were also added, and the mixture was heated to 75 ^{o}C for 5 min. A sample control (test proteins in SGF without pepsin) and pepsin control (pepsin in SGF) were also withdrawn at 0 and 60 min and incubated for polypeptide separation. The polypeptide profile of the digest was separated by SDS-PAGE electrophoresis under reducing conditions.

4.3.6 Quantification of allergenic proteins (Sin a 1 and Bra j 1) in seed meal using S-ELISA method

The estimation of Sin a 1 (*Sinapis alba*) content was done according to Shim and Wanasundara (2008) and Bra j 1 (*Brassica juncea*) was according to the unpublished method established in the Oilseed Protein Research Projects of AAFC. Defatted meal (0.25 g) was extracted with 10 mL of phosphate buffered saline (PBS) solution (10 mM Na_2HPO_4, 2 mM KH_2PO_4, 137 mM NaCl and 2.7 mM KCl, pH 7.4). Then 8% (v/v) of protease inhibitor (completely EDTA free, Roche, Germany) was added and stirred for 60 min at 4^{o}C. The mixture was centrifuged for 10 min at 12,000×*g* to recover soluble proteins. The supernatant was filtered through Whatman #1 filter paper. The residual pellet was re-extracted one more time under the same conditions. Finally the two supernatants were combined and the volume was recorded. The protein content of the combined extractions was obtained using Bio-Rad protein assay kit (Hercules, CA, based

on Bradford method). Analysis of Sin a 1 and Bra j 1 was by sandwich-enzyme linked immunosorbent assay (S-ELISA).

For the Sin a 1 analysis, bovine serum albumin (BSA) was dissolved in PBS and plate washing was done using borate saline buffer (BSB, 167 mM boric acid, 125 mM NaCl, pH 8.5). For Bra j 1 analysis BSA was dissolved in PBS and plate washing was done using PBS with Tween-20 (PBST). First, the micro-titre plate wells were coated with 100 µL of purified Sin a 1 (for *Sinapis alba*) or Bra j 1 (for *Brassica juncea*) and diluted with 10 µg/mL BSB (for *Sinapis alba*) or PBS (for *Brassica juncea*) (obtained from Oilseed Protein Research group at AAFC) and let stand overnight at 4^{o}C. After 24 h of incubation, the wells were washed three times using 150 µL buffer (BSB for *Sinapis alba* and PBST for *Brassica juncea*) for the allergen. The unbound sites of the plates were blocked with BSA in BSB (for *S. alba*) or (PBS for *B. juncea*) (1% w/v, BSA-BSB or PBS) by adding 150 µL of the solution and incubation of 1 h at room temperature. After incubation, 3 washings were done using BSB or PBST. A dilution series of purified protein standard (1/5000, 1/10000, 1/15000, Sin a 1 or Bra j 1) in PBS or BSB and sample 100 µL were added to wells and incubated at room temperature for 1 h and washed with BSB or PBST three times. Then 100 µL of goat anti-rabbit IgG-HRP (horse radish peroxidase) conjugate diluted with BSB or PBS (1:5000, v: v) was added as the secondary antibody to each well and incubated 1 h at room temperature. Finally, freshly made 100 µL of substrate solution (0.01%, v/v, 3,3', 5,5'-tetramethylbenzidine (TMB) in phosphate-citrate buffer (pH 5.0) with 0.002% (v/v) H_2O_2 was added to each well and incubated for 30 min at room temperature. HRP activity was inactivated by adding 50 µL of 2 M H_2SO_4. Absorbance values were measured at 450 nm using a micro plate reader. Values were graphed in log scale, and the linear region was used to calculate the level of allergenic protein in the extract and converted to a fraction of soluble protein recovered from seed meal.

4.3.7 SDS-PAGE separation of proteins

The remaining residue of gastrointestinal phase digestion and samples of high-pepsin digestion were separated by using 10-20%T Tris-Tricine SDS-PAGE gels (Bio-Rad Laboratories, Hercules, CA) to observe the changes in the polypeptide profiles. The

conditions used for SDS-PAGE separation was similar to what was described in section 3.4.9 of the previous chapter. The gel images were acquired by scanning and then analyzed using the ImageMaster software.

4.3.8 Nitrogen analysis

Analysis of nitrogen content of the meals, protein fractions and digests and their fractions were by a combustion based method as described in AOAC method 988.05 (AOAC, 1997) using a Flash N analyzer (Flash 4000, CE Instruments, Wigen, UK). When percentage protein content was needed a conversion factor of 6.25 was used.

4.3.9 Bioinformatic evaluation of *Brassicaceae* proteins for allergenicity and cross reactivity

Amino acid sequence data of cruciferin and napin of *B. juncea*, *B. napus* and *S. alba* was obtained from a database (UniprotKB, www.uniprot.org, access date 2011-06-06) search (FASTA search, Pearson and Lipman, 1988) for the search term "cruciferin" and "napin" and "Brassica" as the advanced search term. The results of the search was manually selected for *B. juncea, B. napus* and *S. alba* seed storage proteins and the sequences were aligned using the "align" tool (ClustalW multiple sequence alignment, Thompson, Higgins & Gibson, 1994) of the same website. These sequences were retrieved in FASTA format and manually submitted to the allergenonline database (www.allergenonline.org) for the "Full FASTA" and "80mer Sliding Window (80mer SW)" searches. The resulting scores for the each protein was analyzed according to the guidelines of FAO/WHO (2001) adapted by Codex Alimentarius Commission (2003) for similarity and cross reactivity. Proteins that scored over 50% identity (Aalberse, 2000) in the Full FASTA search and >35% in the 80mer Sliding Window search were selected and listed. The E score of $> 1\times e^{-30}$ was also considered in selecting matching allergenic proteins. The proteins that were submitted to this search are as shown in Table 4.2. The sequence alignments of these selected cruciferin and napins of these three species were obtained using the 'align" tool of Uniprot database.

Table 4.2 Proteins identified during cruciferin and napin searches in the protein databank (UniprotKB,www.uniprot.org/).

Accession	Entry name	Protein name	Organism	Gene name
Cruciferins				
P11090	CRUA_BRANA	Cruciferin	*Brassica napus* (Rape)	CRUA
P33522	CRU4_BRANA	Cruciferin CRU4	*Brassica napus* (Rape)	CRU2/3
P33523	CRU1_BRANA	Cruciferin BnC1	*Brassica napus* (Rape)	CRU4
P33524	CRU2_BRANA	Cruciferin BnC2	*Brassica napus* (Rape)	BnC1
P33525	CRU3_BRANA	Cruciferin CRU1	*Brassica napus* (Rape)	BnC2
Q39324	Q39324_BRANA	Cruciferin	*Brassica napus* (Rape)	CRU1
Q2TLV9	Q2TLV9_SINAL	11S globulin	*Sinapis alba* (White mustard)	-
Q2TLW0	Q2TLW0_SINAL	11S globulin	*Sinapis alba* (White mustard)	-
Q7XB52	Q7XB52_BRANA	Cruciferin	*Brassica napus* (Rape)	-
Q7XB53	Q7XB53_BRANA	Cruciferin	*Brassica napus* (Rape)	-

Table 4.2 Continued…

Accession	Entry name	Protein name	Organism	Gene name
Napins				
P01090	2SS2_BRANA	Napin-2	*Brassica napus* (Rape)	-
P01091	2SS1_BRANA	Napin-1	*Brassica napus* (Rape)	-
P09893	2SSE_BRANA	Napin embryo-specific	*Brassica napus* (Rape)	-
P15322	ALL1_SINAL	Allergen Sin a 1	*Sinapis alba* (White mustard)	-
P17333	2SS4_BRANA	Napin	*Brassica napus* (Rape)	NAP1
P24565	2SSI_BRANA	Napin-1A	*Brassica napus* (Rape)	-
P27740	2SSB_BRANA	Napin-B	*Brassica napus* (Rape)	NAPB
P80207	ALL1_BRAJU	Allergen Bra j 1, Bra j 1-E	*Brassica juncea* (Indian mustard)	-
P80208	2SS3_BRANA	Napin-3, Napin BnIII, Napin nIII	*Brassica napus* (Rape)	-
Q39344	Q39344_BRANA	Brassica napus napB napin	*Brassica juncea* (Indian mustard)	-
Q42413	Q42413_BRAJU	2S storage protein	*Brassica napus* (Rape)	-
Q42469	Q42469_BRANA	Napin	*Brassica napus* (Rape)	-
Q6PZE1	Q6PZE1_BRANA	Napin	*Brassica napus* var. napus	-
Q6PZE4	Q6PZE4_BRANA	Napin 1.7S (Fragment)	*Brassica napus* var. napus	-

4.3.10 Statistical analysis

Statistical differences between *in vitro* digestibility data (triplicate) were analyzed using General Linear Model (GLM) analysis of Varience (one way ANOVA) and means were compared by Tukey test. The level of significance was set at 0.05. All analysis were done using SPSS v14, 2006 (SPSS Inc. 2005).

4.4 Results

4.4.1 *In vitro* digestibility and trypsin and chymotrypsin inhibiting activities

The gastrointestinal digestibility values of *B. juncea*, *B. napus* and *S. alba* meals were calculated from the released TCA soluble N and values were between 20 and 34% (Table 4.3). Among *B. juncea* varieties, the canola-quality Dahinda variety exhibited statistically ($p<0.05$) higher digestibility values than other two varieties. The digestibility values for S. *alba* (Andante) and *B. napus* (AC Excel) were similar. When compared with soybean meal all *Brassicaceae* meals except the variety Dahinda of *B. juncea* exhibited low digestibility values. The *in vitro* digestibility values of napin-depleted meals were 7.8 to 21 percentage points higher than the unfractionated defatted meal. The highest (21.0%) increase in the digestibility was recorded for *B. napus* meal while *S alba* varieties showed 15.2% and 19.3% increrase and the condiment *B. juncea* varieties had 8.3% and 16.5% increase due to removal of napin from the meal (Table 4.3).

Among the napins of *B. juncea*, *B. napus* and *S. alba*, the values for napin gastrointestinal digestibility were comparatively lower than the values obtained for the respective meals and napin-depleted meals. The digestibility values were below 10% for napin of all three Brassica species. The highest digestibility value of napins was reported for the *S. alba* variety and no significant difference ($p<0.05$) in digestibility was found between Andante and AC Pennant (both had values around 9%). However, *B. juncea* napin had the lowest digestibility value and 4.4% was recorded for napin from the Duchess variety.

The polypeptide profiles of defatted meal and napin-depleted meal subjected to *in vitro* pepsin and pancreatic (protein:enzyme, 250:1, w:w) digestion showed polypeptide bands corresponding to cruciferin between 20 and 55 kDa region and the two polypeptide

bands of napin below 17 kDa (6.5 kDa and 10 kDa) (Figure 4.1 a and b) under reducing conditions.

It is clear for all the varieties that the polypeptide bands corresponding to cruciferin were either not present or reduced in their content after gastric and intestinal digestion. In *S. alba* varieties (AC Pennant and Andante), faint cruciferin bands can be seen after gastrointestinal digestion but cruciferin bands of other varieties have disappeared. In contrast to cruciferin, the polypeptide bands corresponding to napin (two bands <17 kDa) still remained after digestion in all the varieties and species. As expected, napin-depleted meals contained cruciferin bands (Figure 4.1c and d) and no bands corresponding to napin was observed. The remaining residue of napin-depleted meal after digestion did not contain polypeptide bands corresponding to cruciferin. New polypeptide bands having molecular masses below 12 kDa were resolved for these samples indicating possible digestion products of cruciferin polypeptides.

Figure 4.2 shows polypeptide profiles of napin proteins subjected to *in vitro* GI digestion. The SDS-PAGE profile of crude napin under reducing condition showed two clear polypeptide bands which were smaller than 17 kDa under reducing conditions and they had molecular masses between 6.5 kDa and 10 kDa. After GI digestion, the remaining residue retained these two polypeptide bands but with somewhat reduced intensity. Among the varieties studied, *B. napus* AC Excel showed loss of polypeptide bands corresponding to napin upon GI digestion (Figure 4.2 a, b) which was not observed in other varieties.

The trypsin and chymotrypsin inhibitory levels in soluble proteins of the seeds are shown in Table 4.3. The trypsin inhibitor level of the water extracts of these seeds was in the range of 15.6 to 51.6 TIU/g of soluble proteins. AC Pennant and Andante seeds recorded the lowest values (31.22 and 15.6 TIU/g, respectively) for trypsin inhibitor and the highest value was observed for oriental mustard AC Vulcan (52 TIU/g). The chymotrypsin inhibitory activity (Table 4.4) was not observable for most of the seed meals except *S. alba* Andante exhibited very low activity values.

Table 4.3 *In vitro* gastrointestinal digestibility (%)[1] of unfractionated meal, napin-depleted meal and crude napin compared to casein and soybean meal.

Species	Gastrointestinal digestibility, %[1]								
	Unfractionated meal			Napin-depleted meal			Napin		
	As-is	Compared to casein[2]	Compared to Soymeal[3]	As-is	Compared to casein[2]	Compared to Soymeal[3]	As-is	Compared to casein[2]	Compared to Soymeal[3]
B. juncea									
AC Vulcan	20.4 ± 0.2[a]	41.0	60.0	36.9 ± 0.7[b]	74.2	108	7.3 ± 0.8 [c]	14.7	21.4
Duchess	24.6 ± 0.3[c]	49.5	72.1	32.9 ± 1.4[a]	66.2	96.5	4.4 ± 0.2[a]	8.8	12.9
Dahinda	33.8 ± 0.5[d]	68.0	99.1	41.6 ± 0.0[bc]	83.7	122	5.6 ± 0.4[b]	11.2	16.4
B. napus									
AC Excel	22.9 ± 0.1[b]	46.1	67.1	44.2 ± 1.7[c]	88.9	129	6.5 ± 0.2[c]	13.0	19.0
S. alba									
AC Pennant	20.0 ± 1.0[a]	40.2	58.6	39.3 ± 1.2[b]	79.1	115	8.8 ± 0.4[d]	17.7	25.8
Andante	22.2 ± 0.1[b]	44.7	65.1	37.4 ± 2.2[b]	75.2	109	9.0 ± 2.0 [d]	18.1	26.4

[1]Mean ± SD are provided; n = 3 Values in a column with same superscript are not different at $p > 0.05$

[2] Digestibility of casein is 49.7% as-is [3] Digestibility of soybean meal 34.1% as is

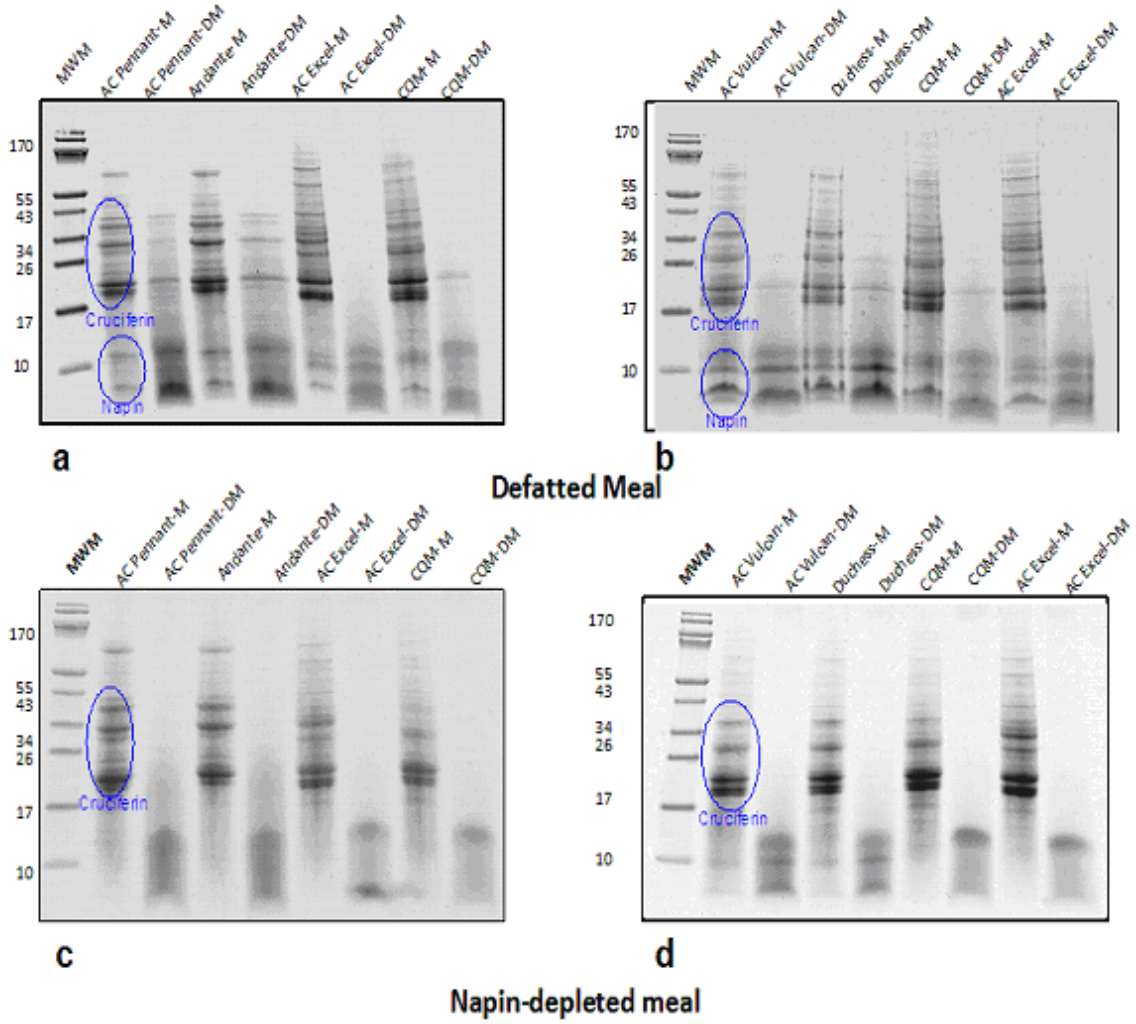

Figure 4.1 Polypeptide profiles of defatted meal (a and b) and napin-depleted meal (c and d) of *Brassicaceae* seeds subjected to *in vitro* pepsin and pancreatic (protein: enzyme, 250:1 w:w) digestion. M: before digestion, DM: remaining residue after digestion. The circles enclose polypeptides corresponding to cruciferin and napin. (3.5 µg protein was loaded per well).

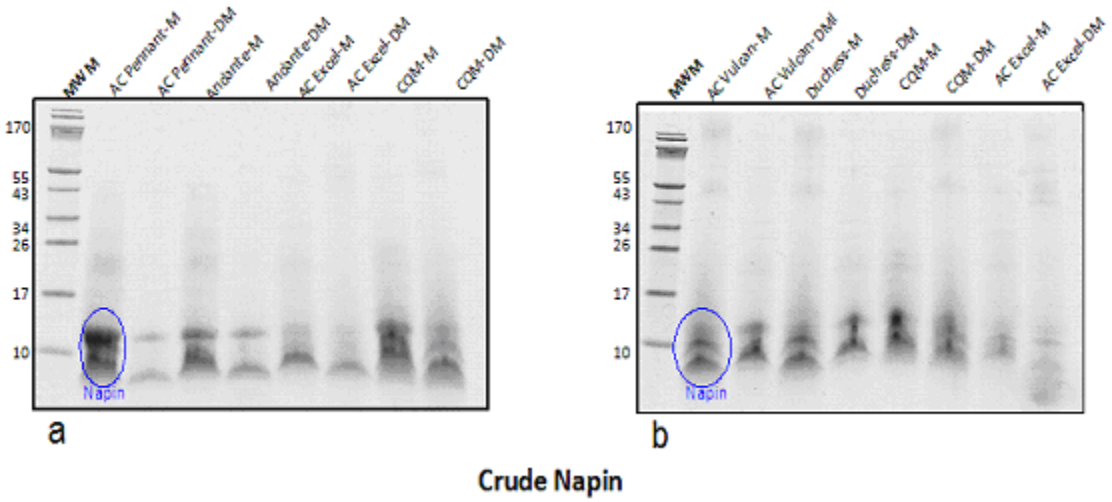

Figure 4.2 Polypeptide profiles of crude napin (a and b) of *Brassicaceae* meals subjected to *in vitro* pepsin and pancreatic (protein: enzyme, 250:1, w:w) digestion. M: before digestion, DM: remaining residue after digest. The circles enclose polypeptides corresponding to napin. (Protein concentration of prepared sample was 2.5 µg/mL and 3.5 µg protein was loaded per well).

Table 4.4 Trypsin and chymotrypsin inhibitor levels in defatted cotyledons (IU/g of soluble proteins at pH 7).

Species and Variety	Inhibitor level in pH 7 solubles (IU/g soluble proteins)	
	Trypsin inhibitor	Chymotrypsin inhibitor
Brassica juncea		
AC Vulcan	51.48 ± 0.68	0.0
Duchess	45.03 ± 1.63	0.0
Dahinda	37.14 ± 0.75	0.0
Brassica napus		
AC Excel	41.60 ± 2.12	0.0
Sinapis alba		
AC Pennant	31.22 ± 0.82	0.0
Andante	15.60 ± 0.74	7.4 ± 0.10

Mean ± SD is provided; n = 3

4.4.2. Resistance to pepsin digestibility

The ability to degrade cruciferin, napin and some other known allergenic and non-allergenic proteins under high pepsin level was assessed and the results were followed as disappearance of corresponding polypeptides of the proteins (Figures 4.3, 4.4 and 4.5). The degradation pattern of cruciferin of *B. juncea* varieties showed disappearance of corresponding polypeptide bands soon after digestion started (Figure 4.3a, b and c). There were no bands corresponding to cruciferin or its digestion products on the SDS-PAGE gels indicating most of the degraded fragments were <10 kDa and were not retained by the gel. However, napins of the same varieties were retained as the corresponding two polypeptide bands below 17 kDa even after 60 min of incubation under high pepsin levels (Figures 4.3d, e and f). Similar pattern of degradation was observed for cruciferin (Figures 4.4a, b and c) and napin (Figures 4.4d, e and f) of *B. napus* and *S. alba*. The added pepsin in the assay mixture corresponded to the polypeptide band of 39 kDa.

The soybean trypsin inhibitor (STI) (Figure 4.5a) and bovine β-lactoglobulin (β-LG) (Figure 4.5b) are known food allergens. The polypeptide bands corresponding to STI and β-LG were around 16 kDa and 17 kDa in mass, respectively. Both these polypeptide bands remained without change for up to 60 min of digestion under high pepsin concentration. The polypeptides of soybean β-conglycinin (BCN) (Figure 4.5d) bands gradually disappeared after 2 min of digestion. Egg white lysozyme (LYS) polypeptides remained visible for 5 min and then disappeared (Figure 4.5e). RuBisCo (SRP) protein is considered as a non-allergenic protein, and rapid digestion was observed as the polypeptide bands disappeared instantly upon adding pepsin (Figure 4.5c).

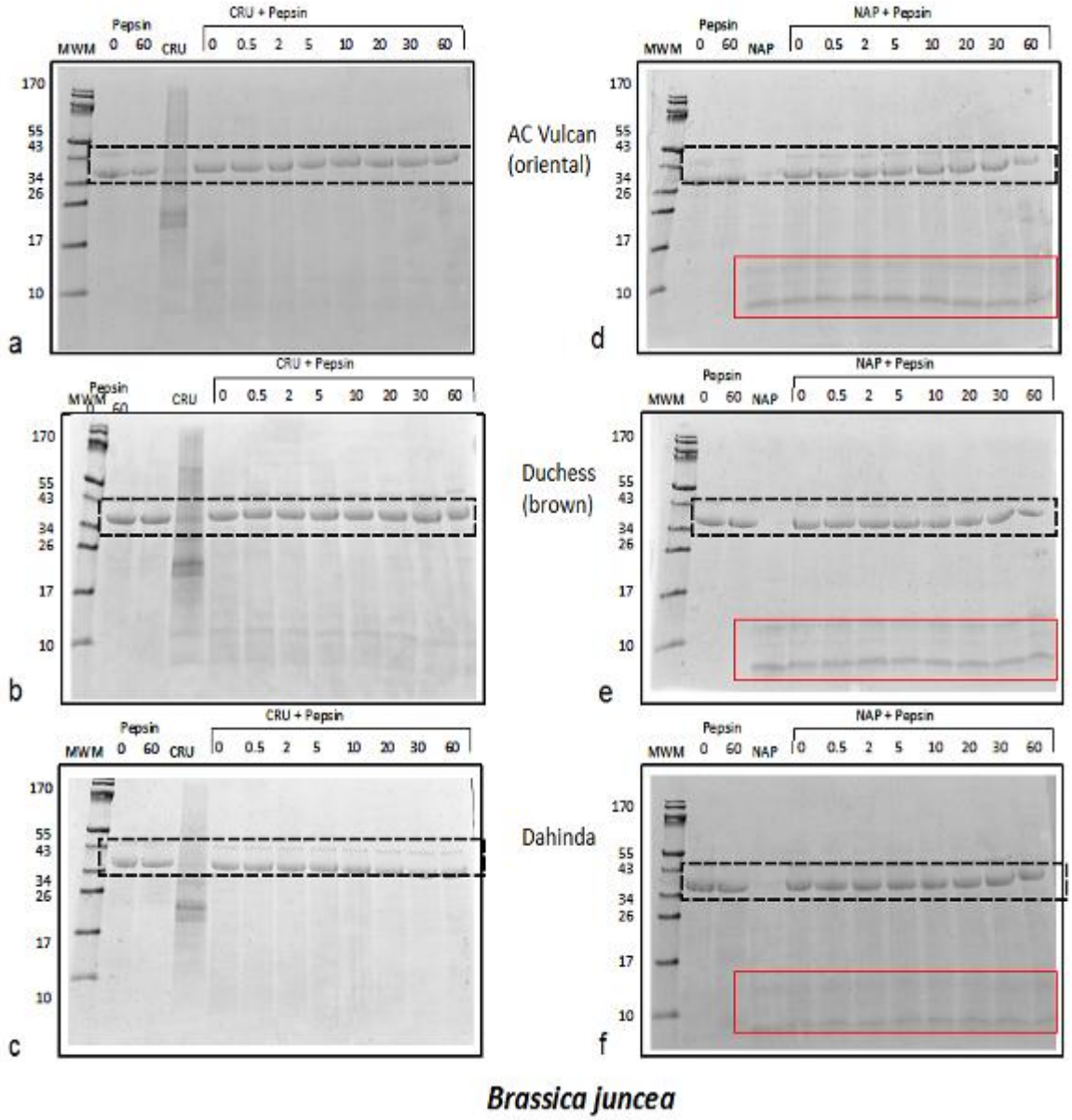

Figure 4.3 Digestibility of *B. juncea* cruciferin (CRU): AC Vulcan; a, Duchess; b and Dahinda; c and napin (NAP): AC Vulcan; d, Duchess; e and Dahinda; f under high pepsin concentration. MWM is molecular weight standards used. The dotted rectangle encloses the polypeptide band of pepsin and the solid rectangle encloses polypeptide bands that remained visible during the assay period.

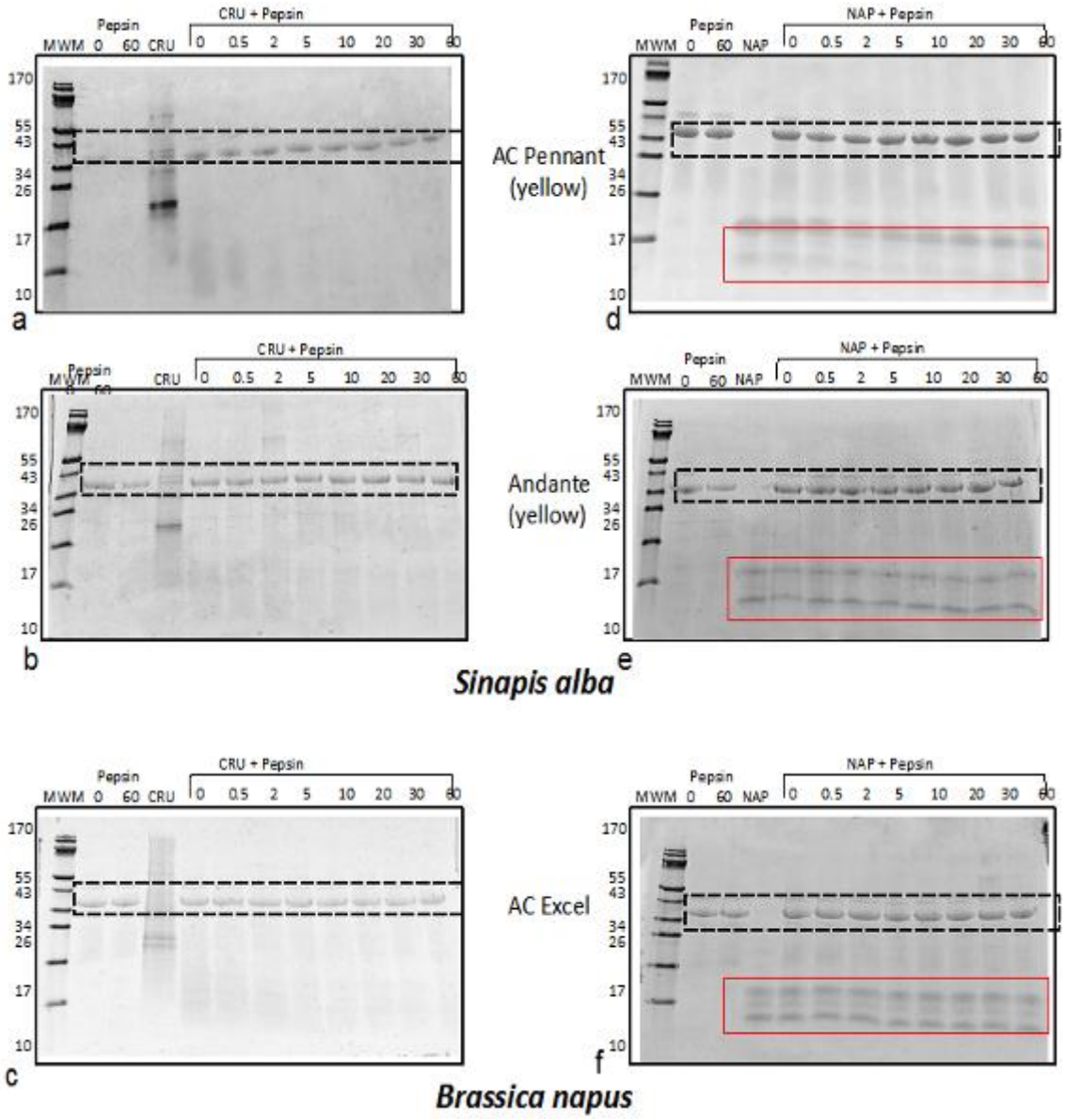

Figure 4.4 Digestibility of *S. alba* cruciferin (CRU): AC Pennant; a and Andante; b and napin (NAP): AC Pennant; d and Andante; e and *B. napus* cruciferin (CRU): c and napin (NAP): f under high pepsin concentration. MWM is molecular weight standards used. Dotted rectangle encloses polypeptides due to pepsin and solid rectangle encloses polypeptide bands that remained visible during the assay.

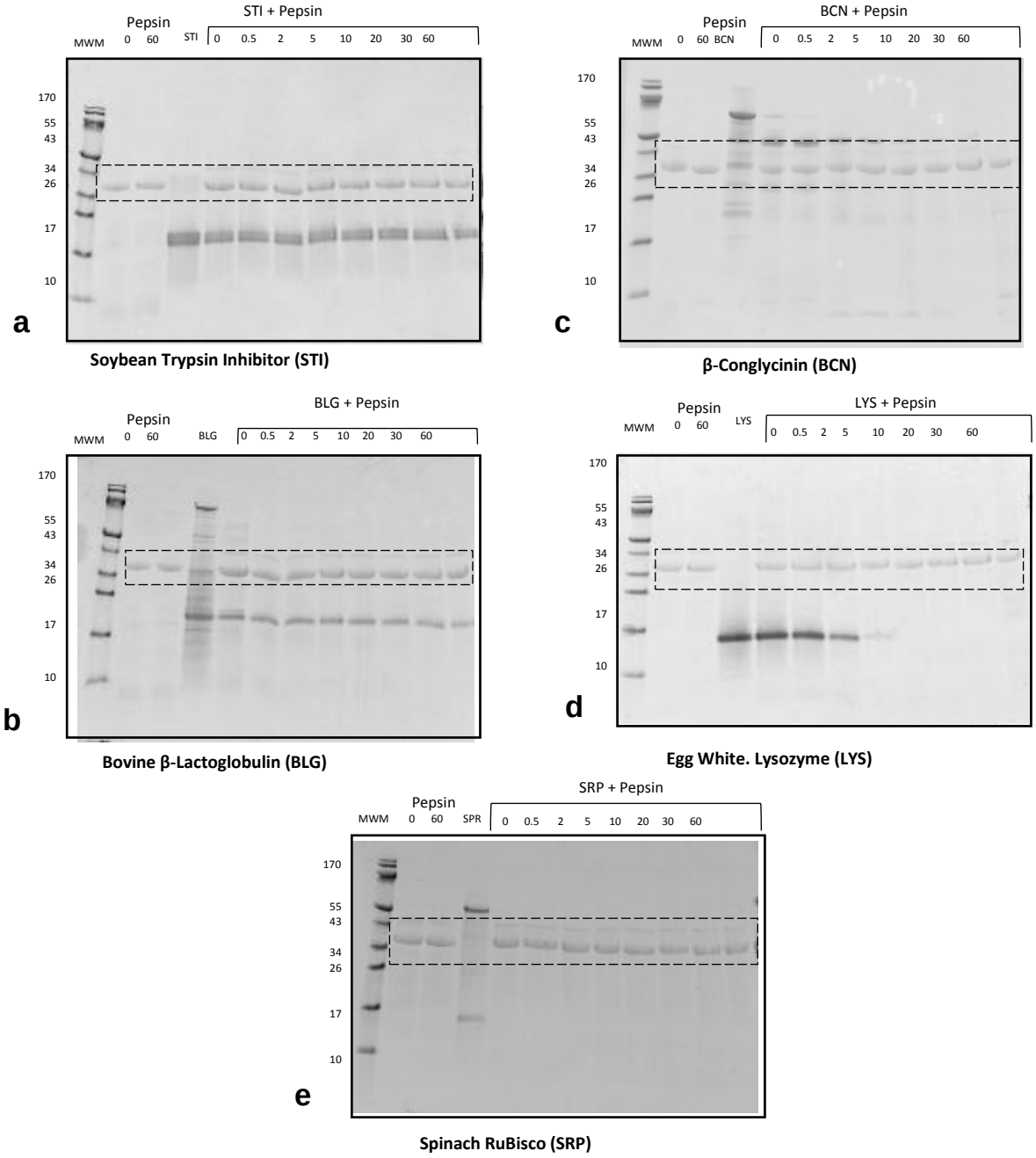

Figure 4.5 Digestibility of known allergenic and non allergenic proteins under high pepsin concentration. MWM is molecular weight standards used. Dotted rectangle encloses polypeptides due to pepsin.

Table 4.5 summarizes the presence and disappearance of polypeptide bands corresponding to cruciferin and napin as the time of digestion progressed. The resistance of napin polypeptides and the susceptibility of cruciferin to hydrolysis under these conditions were further confirmed by this analysis. Furthermore, the percentage reduction of polypeptide band intensity was calculated and is summarized in Table 4.6. Among *B. juncea* varieties napin protein of Duchess exhibited less than 10% reduction in the protein intensity and AC Vulcan and Dahinda has lost 15% and ~28% of napin intensity at 60 min digestion. Among YM, AC Pennant and Andante were at 28% reduction and for *B. napus* AC Excel recorded the highest level of 32% reduction in the band intensity. It is clear that cruciferin proteins for all species were digested quickly as the digestion started while napins remained stable to degradation throughout the 60 min digestion period. The allergenic proteins STI and BLG had higher loss of intensity at 60 min digestion than the napins. Cruciferin bands were not found and completely disappeared during the period of investigation.

The level of allergenic protein Bra j 1 of *B. juncea* ranged from 0.43-0.98% of soluble proteins at pH 7. In *Sinapis alba* the Sin a 1 was in the range of 0.39-0.53% (Table 4.7). These values remained less than 1% of the total soluble proteins at pH 7.0.

Table 4.5 Summary of the fate of polypeptide bands originating from cruciferin and napin and known allergenic and non-allergenic proteins.

Protein	Time (min) after initiation of digestion							
	0	0.5	2	5	10	20	30	60
Brassica juncea								
AC Vulcan: Napin	+	+	+	+	+	+	+	+
Cruciferin	-	-	-	-	-	-	-	-
Duchess: Napin	+	+	+	+	+	+	+	+
Cruciferin	-	-	-	-	-	-	+	-
Dahinda: Napin	+	+	+	+	+	+	+	+
Cruciferin	-	-	-	-	-	-	-	-
Brassica napus								
AC Excel: Napin	+	+	+	+	+	+	+	+
Cruciferin	-	-	-	-	-	-	-	-
Sinapis alba								
AC Pennant: Napin	+	+	+	+	+	+	+	+
Cruciferin	-	-	-	-	-	-	-	-
Andante: Napin	+	+	+	+	+	+	+	+
Cruciferin	-	-	-	-	-	-	-	-
Soybean Trypsin Inhibitor	+	+	+	+	+	+	+	+
Bovine β-lactoglobulin	+	+	+	+	+	+	+	+
β-Conglycinin	+	+	+	+	-	-	-	-
Lysozyme from chicken egg	+	+	+	+	-	-	-	-
RuBisCo from spinch leaf	-	-	-	-	-	-	-	-

[1] +; band present, -; band not present

Table 4.6 Percentage band intensity reduction of the polypeptides originating from cruciferin and napin with digestion time under high pepsin concentration.

Protein		Reduction of protein band intensity, % of original intensity		
		20 min	30 min	60 min
Brassica juncea				
AC Vulcan:	Napin	14.5	14.9	15.0
	Cruciferin	100	100	100
Duchess:	Napin	8.1	8.4	8.5
	Cruciferin	100	100	100
Dahinda:	Napin	14.7	21.5	28.3
	Cruciferin	100	100	100
Brassica napus				
AC Excel:	Napin	12.9	13.6	32.1
	Cruciferin	100	100	100
Sinapis alba				
AC Pennant:	Napin	28.2	28.4	28.6
	Cruciferin	100	100	100
Andante:	Napin	27.8	27.2	28.4
	Cruciferin	10	100	100
Soybean Trypsin Inhibitor		27.0	33.7	59.9
Bovine β-lactoglobulin		42.0	42.1	42.3
β-Conglycinin		100	100	100
Lysozyme from chicken egg		100	100	100
RuBisCo from spinch leaf		100	100	100

Table 4.7 Sin a 1 and Bra j 1 level of defatted meals of *B. juncea* and *S. alba* seed varieties, soluble protein extractable at pH 7.2.

Species and Variety	% of soluble protein content[1]	
	Sin a 1	Bra j 1
Brassica juncea		
AC Vulcan	ND	0.43 ± 0.11
Duchess	ND	0.46 ± 0.18
Dahinda	ND	0.98 ± 0.25
Sinapis alba		
AC Pennant	0.39 ± 0.11	ND
Andante	0.53 ± 0.06	ND

[1] Soluble protein at pH 7.2, ND – not detected

Mean ± SD is provided; n = 3

4.4.3 Bioinformatics analysis of cruciferin and napin for potential allergenicity

The multiple alignment of amino acid sequences of 10 cruciferin proteins (8 of *B. napus* 1 of *B. juncea* and 2 of *S. alba*) and 14 napin proteins (2 of *B. juncea*, 11 of *B. napus* and 1 of *S. alba*) retrieved from the protein database search showed very high similarity in the respective protein group (Figures 4.6 and 4.7). Two sequences were listed for Sin a 2 of *S. alba* that are different in the residue length (510 and 523) and molecular weight 56.510 and 57.889 Da. The allergenic protein database match for the cruciferins showed high scores with Sin a 2 allergen only (Table 4.8). The cut off values of >50 identity similarity for Full FASTA search and >35% identity similarity for 80mer Sliding Window search was used. The high scores resulting for these two evaluations indicate strong possibility of cross-reactivity of the *B. napus* cruciferins with Sin a 2. Among the other allergenic 11S proteins, mostly the ones of walnut, hazelnut, cashew and pistachio showed scores between 40 and 50% indicating low possibility of cross-reactivity of these protein antibodies with cruciferin. According to alignment results different isoforms of cruciferin showed different identity values within species. As an example, CRU3_BRANA showed high percentage of probability match (91%) with known *S. alba* allergen, CRU4-BRANA. However, CRUA_BRANA, CRU4_BRANA, CRU1_BRANA and CRU2_BRANA showed low probability match (53 to 59%) with the same allergen. This indicates that with different isoforms of cruciferin, the allergenic potential can be different.

The napins also indicated high degree of similarity between the species (Table 4.9). Except 2SSI_BRANA protein, all other napins gave >70% score for the allergenic napins of *B. juncea* (ALL1_BRAJU), *B. napus* (2SS3_BRANA, *B. napus* recombinant Ib pronapin precursor) and *S. alba* (ALL1_SINAL). The *B. napus* recombinant Ib pronapin precursor is not a naturally occurring protein in the seed but has similar structural and immunological properties as its mature protein counterpart in the seed.

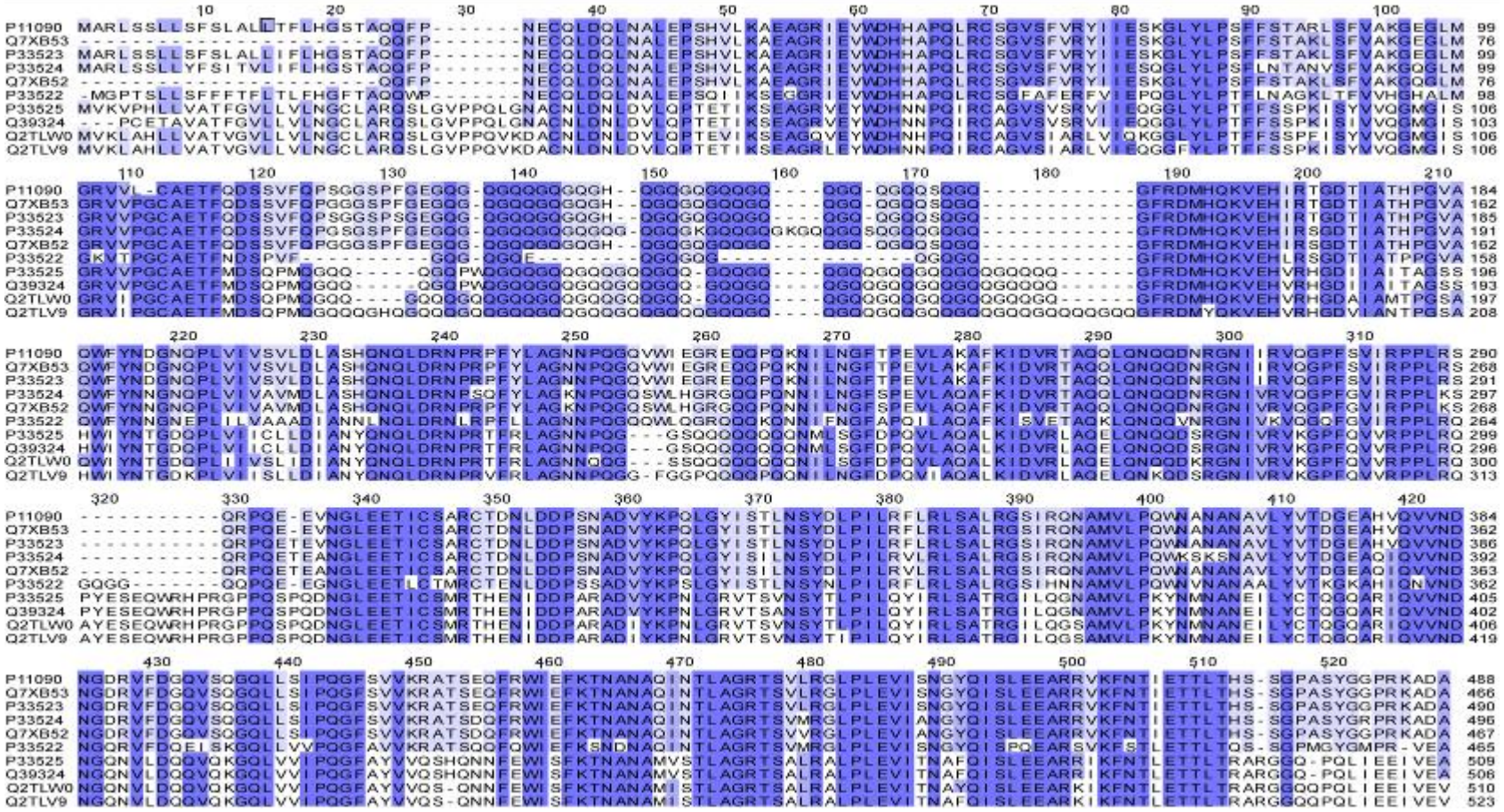

Figure 4.6 Multiple sequence alignment of cruciferin proteins of *B. juncea*, *B. napus* and *S. alba* of database search. Detailed description of these proteins is provided in Table 4.2 and blue color intensity relates to the degree of similarity of the amino acid residues.

Table 4.8 Summary of bioinformatics evaluation of cruciferin of *B. juncea*, *B. napus* and *S. alba* with known allergenic proteins.

Cruciferin	Search type	Allergenic protein (high probability match)		Allergenic proteins (low probability match)
		Q2TLV9_SINAL	Q2TLW0_SINAL	
Q7XB53	Full FASTA	59.2%	61.0%	Walnut legumin (Jug r 4),
Q7XB53_BRAJU	80mer SW	74.1%	76.5%	Cashew legumin (Ana o 2)
P11090	Full FASTA	56.5%	58.4%	Hazelnut 11S (Cor a 9)
CRUA_BRANA	80mer SW	74.1%	76.5%	
P33522	Full FASTA	53.3%	55.0%	Pistachio 11S (unassigned)
CRU4_BRANA	80mer SW	65.0%	70.0%	
P33523	Full FASTA	58.9%	57.2%	Hazelnut 11S (Cor a 9),
CRU1_BRANA	80mer SW	76.5%	74.1%	Cashew legumin (Ana o 2)
P33524	Full FASTA	57.5%	72.8%	Pistachio 11S (unassigned),
CRU2_BRANA	80mer SW	55.9%	70.0%	Cashew legumin (Ana o 2)
P33525	Full FASTA	91.6%	98.8%	Walnut legumin (Jug r 4),
CRU3_BRANA	80mer SW	90.1%	97.5%	Cashew legumin (Ana o 2)
Q39324	Full FASTA	89.9%	97.5%	Hazelnut 11S (Cor a 9),
Q39324_BRANA	80mer SW	91.4%	98.8%	Cashew legumin (Ana o 2)

Table 4.8 Continued…..

Cruciferin	Search type	Allergenic protein (high probability match)		Allergenic proteins (low probability match)
		Q2TLV9_SINAL	Q2TLW0_SINAL	
Q7XB52	Full FASTA	58.8%	61.1%	Hazelnut 11S (Cor a 9)
Q7XB52_BRANA	80mer SW	71.6%	75.3%	
Q2TLV9	Full FASTA	100%	92.2%	No close ones
Q2TLV9_SINAL	80mer SW	100%	100%	
Q2TLW0	Full FASTA	92.2%	100%	No close ones
Q2TLW0_SINAL	80mer SW	100%	100%	

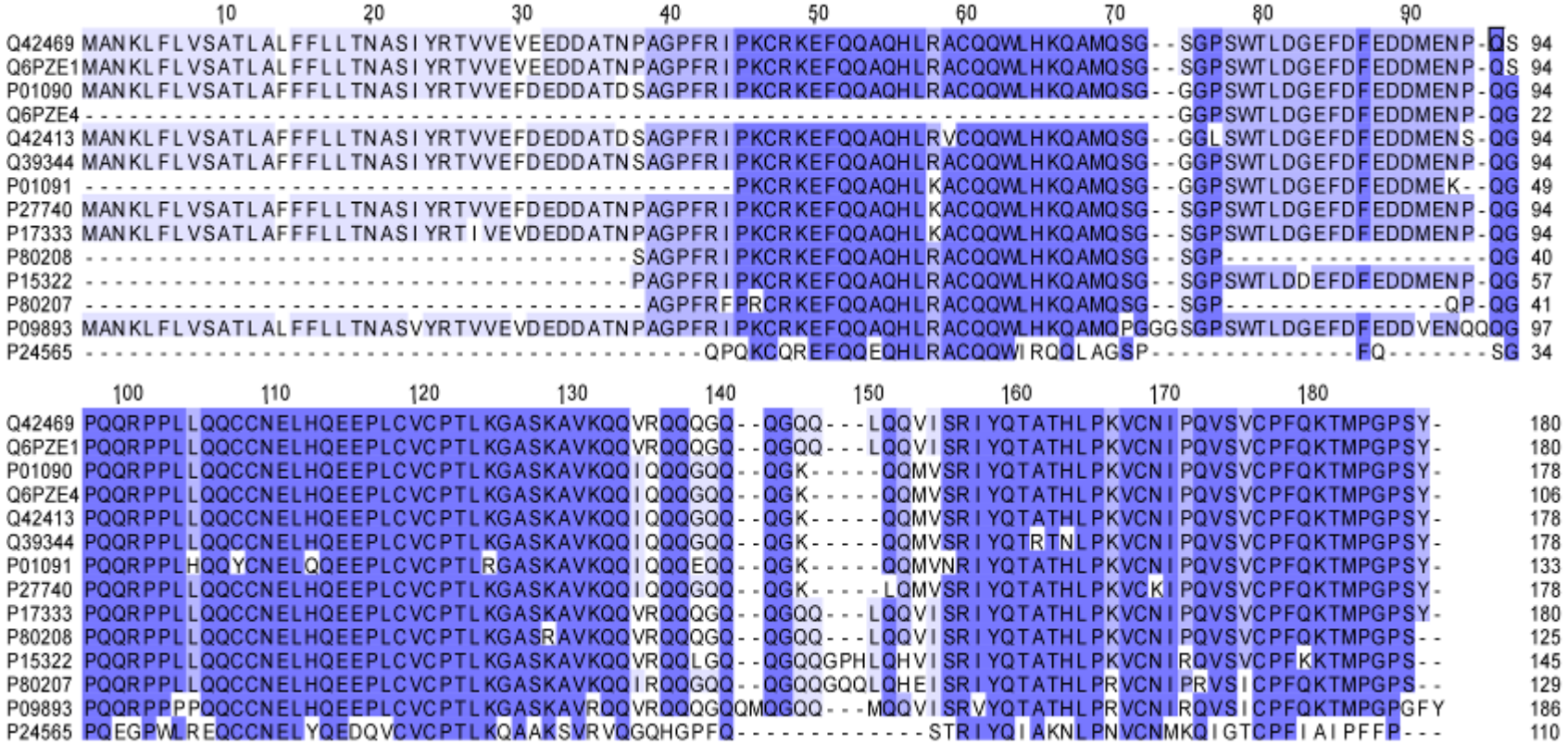

Figure 4.7 Multiple sequence alignment of napin proteins of *B. juncea*, *B. napus* and *S. alba* obtained from database search. Detailed description of these proteins is provided in Table 4.2 and blue color intensity relates to the degree of similarity of the amino acid residues.

Table 4.9 Summary of bioinformatics evaluation of napin proteins of *B. juncea, B. napus* and *S. alba* with known allergens available in databases.

Napin protein	Search type	Allergenic protein (high probability match)				Allergenic proteins (low probability match)
		ALL1_BRAJU	ALL1_SINAL	2SS3_BRANA	Recomb 1B pronapin	
P80207	Full FASTA	100%	80.6%	82.6%	50.4%	Ricinus 2S, Pistachio 2S, Wheat HMW gluten & Sesame 2S
ALL1_BRAJU	80mer SW	100%	88.7%	91.2%	53.7%	
Q42413	Full FASTA	76.4%	86.8%	78.9%	49.2%	Sesame 2S, Pistachio 2S, Cashew 2S & Walnut 2S
Q42413_BRAJU	80mer SW	85.9%	93.8%	87.8%	55.0%	
P01090	Full FASTA	78.5%	88.9%	80.3%	50.8%	Ricinus 2S, Pistachio 2S, Cashew 2S, Walnut 2S & Sesame 2S allergens
2SS2_BRANA	80mer SW	85.9%	97.5%	87.8%	55.0%	
P01091	Full FASTA	73.0%	82.6%	75.4%	47.2%	Pistachio 2S & Sesame 2S allergens
2SS1_BRANA	80mer SW	78.8%	88.9%	81.7%	49.9%	
P09893	Full FASTA	75.3%	85.0%	77.9%	47.3%	Sesame 2S, Hazelnut 2S, Wheat HMW, Pistachio 2S & Cashew 2S allergens
2SSE_BRANA	80mer SW	83.7%	88.7%	86.6%	52.5%	
P17333	Full FASTA	80.6%	93.1%	86.5%	50.0%	Pistachio2S, Sesamum 2S, Cashew 2S, Wheat LMW glutellin & Ricinus 2S
2SS4_BRANA	80mer SW	89.2%	97.5%	98.8%	53.7%	
P24565	Full FASTA	50.9%	48.8%	54.9%	94.5%	Ricinus 2S & Peanut 2S
2SSI_BRANA	80mer SW	56.3%	53.1%	56.3%	92.8%	
P27740	Full FASTA	77.8%	87.6%	80.9%	50.0%	Pistachio 2S, Sesame 2S & Cashew 2S
2SSB_BRANA	80mer SW	84.7%	97.5%	89.0%	55.0%	

Table 4.9 continued……

Napin protein	Search type	Allergenic proteins (high probability match)				Allergenic proteins (low probability match)
		ALL1_BRAJU	ALL1_SINAL	2SS3_BRANA	Recomb 1B pronapin	
P27740	Full FASTA	77.8%	87.6%	80.9%	50.0%	Pistachio 2S, Sesame 2S & Cashew 2S
2SSB_BRANA	80mer SW	84.7%	97.5%	89.0%	55.0%	
P80208	Full FASTA	89.1%	81.9%	100%	53.2%	Pistachio 2S, Ricinus 2S, Walnut 2S & Hazelnut 2S
2SS3_BRANA	80mer SW	92.7%	91.6%	100%	55.0%	
Q39344	Full FASTA	77.1%	87.5%	78.9%	50.8%	Pistachi 2S, Cashew 2S, Sesame 2S, Ricinus 2S & Walnut 2S
Q39344_BRANA	80mer SW	83.5%	97.5%	85.4%	55.0%	
Q42469	Full FASTA	80.6%	93.8%	86.5%	50.0%	Pistachio 2S, Cashew 2S, Sesame 2S & Wheat glutenin
Q42469_BRANA	80mer SW	89.2%	97.5%	98.8%	52.5%	
Q6PZE1	Full FASTA	80.6%	93.1%	86.5%	50.0%	Pistachio 2S, Cashew 2S, Sesamum 2S & Wheat glutenin
Q6PZE1_BRANA	80mer SW	89.2%	97.5%	98.8%	52.5%	
Q6PZE4	Full FASTA	86.8%	86.2%	88.6%	55.0%	Sesame 2S, Ricinus 2S & Pistachio 2S
Q6PZE4_BRANA	80mer SW	85.9%	84.7%	87.8%	55.0%	
P15322 ALL1_SINAL	Full FASTA	79.9%	100%	82.6%	50.4%	Sesame 2S, Wheat LMW glutellin & Pistachio 2S allergens
	80mer SW	87.5%	100%	91.2%	53.7%	

4.5. Discussion

4.5.1 *In vitro* gastric and intestinal digestion

The digestibility values for different *Brassicaceae* seed proteins were low compared to soybean proteins and only the variety Dahinda gave comparable values. In this study, seed coat-free meal was subjected to GI digestion, therefore the interference from seed coat fibre was eliminated. The digestibility of canola quality mustard was significantly ($p<0.05$) higher than canola quality *B. napus* or condiment quality oriental mustard (AC Vulcan). This may be a positive quality trait of this new canola quality Brassica seed. An increase in digestibility after removing napin (2S proteins) was observed for all meals and indicated the contribution of napin in lowering the digestibility values of whole meal protein. This is further confirmed by the <10% digestibility values that resulted in for the napin fraction for all species. Digestibility of meal proteins is directly related to the types of storage protein (in *Brassicaceae* cruciferin and napin), their content, and presence of enzyme inhibitors, phenolic compounds and glucosinolates. According to Crouch et al. (1981) and Lonnerdal et al. (1972), in mature *B. napus* seeds, the 11/12S globulin protein constitutes about ~60% and napins account for ~20% of total storage proteins. Malabat et al. (2003) reported that the cruciferin content may vary from 32-52% of total proteins among the European rapeseed cultivars and the cruciferin and napin vary in the ratio between 0.6 and 2.0. These authors postulated that the reduction of glucosinolate level and increase in oil content by intensive breeding of rapeseed has lead to a decrease in the content of proteins and increase in seed cruciferin level. No studies are available on the level of cruciferin and napin among Canadian varieties, therefore, relevance of digestibility to the level of protein type cannot be discussed. In general, low digestibility have been reported for rapeseed protein isolates consisting of 36.8% cruciferin and 41% napin compared to soy; when assayed in human subjects (Bos et al., 2007). Napin protein is known to have a low digestibility potential because of the compact nature of the molecule (Menéndez-Arias, Moneo, Dominguez, Moneo, & Rodriguez, 1988). The low digestibility of napin has been predicted as a result of the high number of internal S-S bonds of the molecule (Jyothi, Singh & Appu Rao, 2007b). According to the results of Chapter 3 (Study 1), mostly napin proteins are soluble at pH 3 and 4 (Figure 3.5) and this can be relateed to the high solubility of napin in the gastric

digestion phase which is around pH 2. Cruciferin remains mostly insoluble at this pH. As the predominant soluble protein at gastric pH, solubility of napin should not be a hinderance to access digestive enzymes during the gastric phase. The solubility of substrate protein is a factor that affects enzyme catalyzed hydrolysis. In addition, the structural features of the protein molecule may play a role in the accessibility of proteolytic enzymes (pepsin in the gastric phase) to the cleavage sites of the napin. Disulphide bonds play a major role in the three dimensional molecular structure of napin. The number of cysteine residues and the location of cysteine in the polypeptide chain are major conserved features in napin proteins (8 Cys motif). A total of eight cysteine residues have been identified in different napin isoforms; two in the short chain and six in the long chain (D'Hondt et al., 1993). Because of the close proximity of these residues in the polypeptide chain, a total of 4 disulphide bonds occur between chains and within the chains leading to a compact structure that is difficult to access by digestive enzymes (Gehrig et al., 1996).

According to Schwenke et al. (1983) in acidic pH and with change of ionic strength, the hexameric form of cruciferin molecule dissociates into trimers and this dissociation may facilitate enzyme accessibility to increase cruciferin digestibility in the stomach and intestinal phase. However, the structural changes of napin protein at low pH are not reported. The solubility data of Study 1 shows some polypeptide bands of cruciferin in the solution phase at pH 2 indicating only partial solubilization of cruciferin. However, the degradation of cruciferin was evident since the acidic and basic polypeptides were not in intact form after GI (pepsin) digestion. The presence of faint bands originating from cruciferin in the meal (Figure 4.2) showed the interference of other seed matrix components on digestibility compared to the polypeptide profiles of cruciferin only containing napin-depleted meal.

The level of proteolytic enzyme inhibitors, that is trypsin and chymotrypsin inhibitors, was very low in these meals compared to legumes such as soy and beans. The chymotrypsin inhibitory activity was negligible with the exception of *S. alba* variety Andante. This eliminates the possibility of lowering the proteolytic enzyme activity during the digestibility assessment. Trypsin and chymotrypsin inhibitors of mustard (mustard trypsin inhibitors, MTI) and rapeseed (rapseed trypsin inhibitor, RTI) are

different than the Kunitz- or Bowman-Birk type inhibitors found in legumes (Menegatti et al., 1985). Although the presence of RTI and MTI in rapeseed and mustard, respectively, is reported, no activity values are available. Trypsin and chymotrypsin are protease inhibitors which are considered as agents that offer protection from parasites during seed dormancy and are low molecular weight proteins containing sulphide cross-links making them stable towards thermal and acid decomposition (Menegatti et al., 1985). The 13 week sub-chronic dilatory toxicology study of the cruciferin-rich canola protein isolate Puratin® showed it was safe (without major antinutrients present in canola meal like glucosinolate, phytates and tannin) for monogastrics (Cooper & Gowing, 1983; Campbell, 1987; 1988). According to this research no significant histopathalogical changes in animal performances were observed up to 20% inclusion of isolate Puratin® in the diets of laboratory animals; confirming the safety of cruciferin-rich canola protein isolate (Mejia et al., 2009). The present study confirms high digestibility of cruciferins. All three *Brassicaceae* spp. of this study showed resistance of napin to degradation under pepsin and pancreatin catalyzed hydrolysis (meal:enzyme ratio 250:1). Cruciferin is a more digestible protein than napin and the most abundant protein in the seed therefore contributes more towards the nutritive value of *Brassicaceae* protein. Protein digestibility is an essential criterion that determines the availability of amino acids. According to the results of this study, meal protein digestibility can be improved by removing or separating the less digestible napin protein.

4.5.2 Predicting allergenicity of *Brassicaceae* proteins

Allergenicity is a vital quality trait of food products, especially novel foods originating from genetically modified organisms as well as new protein ingredients. There is no validated animal model available to assay allergenicity of Brassica proteins. Serum IgE binding tests for *Brassicaceae* proteins require serum samples of individuals who are allergic to mustard or rapeseed proteins which are not easily available in North America because mustard allergenicity is not highly prevalent. Regulation of mustard as allergen is just starting in Canada (Health Canada, 2010), therefore related clinical studies are not available in the published literature. The best available tool to assess new protein sources for allergenicity would be to adopt the same protocol that is described in the

consultation document for allergenicity assessment of foods derived from biotechnology (FAO/WHO, 2001). For cruciferin, the assessment showed few isomers of cruciferin have high degree of similarity to the known 11S allergen from *Brassicaceae* family; Sin a 2. Among the cruciferin isoforms, the allergenic cruciferin originates from *S. alba* (Sin a 2, CRU1_SINAL, P83908) and several cruciferins listed for *B. napus* showed identity resemblance to this protein at 53-73%. The CRU3_BRANA and Q39324_BRANA showed 90-99% sequence similarity for the Sin a 2 allergen indicting strong possibility of cross-reactivity. In addition to that lower scores (< 50%) were observed for the known 11/12S seed allergens from cashew, hazelnut, pistachio and walnut showed only a distant possibility of cross-reaction.

The sequence homology assessment of napin of *B. napus*, *B. juncea* and *S. alba* reveals that there is a high degree of homology among the napins of these species and some isoforms have almost 100% sequence identities to the known allergens of *B. juncea* (Bra j 1, ALL1_BRAJU, P80207), *B. napus* (2SS3_BRANA. P80208 and to lesser extent in Bra n 1, 2SSS4_BRANA, P17333) and *S. alba* (Sin a 1, ALL1_SINAL, P15322). The work of Puumalainen et al. (2006) showed that the proteins of 6 to 14 kDa in the *B. napus* and *B. rapa* extracts were recognized by the IgE immunoblotting of serum of 72 atopic children (mean age 3.3 years) having food allergies. These proteins are napins and this bioinformatics analysis also showed the high scores achieved by these napin isomers correspond with their findings. The isoform 2SSI_BRANA only showed <70% scores but all other *B. napus* napin isomers indicated strong possibility of cross-reactivity with mustard napin allergens. Some isoforms of *Brassicaceae* 2S albumin napin proteins which are considered as allergens have this conserved 8 Cys motif. The sequence alignment of napin found in the SWISS-PROT search of "napins" and sorted for *B. napus*, *B. juncea* and *S.alba* shows all these isoforms have the 8 Cys motif (Figure 4.6). According to Shewry et al. (1999) the prolamin proteins have a specific feature, the presence of a conserved cysteine skeleton (Cys-X_n-Cys-X_n-Cys-Cys-X_n-CysXCys-X_n-Cys-X_n-Cys or 8 Cys motif).

As proteins of same plant family, these cruciferins and napins have several conserved regions and has close relatedness among the isoforms of each protein group. According to the decision tree approach of FAO/WHO for the assessment, a protein

which shares >35% sequence identity (over an 80 amino acid window) or at least six identical contiguous amino acids with a known allergen is considered allergenic. The possible cross-reactivity with *B. napus* or *B. juncea* proteins is highly likely for individuals identified with *S. alba* allergenicity. According to Monsalve et al. (2001), amino acid sequence of Sin a 1 and Bra j 1 are closely related and contain common epitopes that were detected in the large chain of both molecules (Figure 4.6).

The pepsin digestibility resistance assay also showed napin is the most potent allergen. As outlined in the FAO/WHO 2001 decision tree, the pepsin resistance or stability to pepsin digestion at higher pepsin concentration (protein:pepsin, 13:1) than the usual *in vitro* GI digestion (protein:pepsin, 250:1) allows one to differentiate proteins that are highly resistant for proteolyic cleavage in the gastric phase. Under high pepsin concentration if a protein were to remain undigested more than 30 min; it is an indication of a very stable protein for hydrolysis. A protein that does not degrade during 5 to 30 min is considered as having intermediate stability and the disappearance of polypeptide bands by 2 min indicates a labile, rapidly digested protein. The proteins that are stable and of intermediate stability to this assay such as napins of *Brassicaceae* meals as found in this study could remain as molecules that can sensitize stomach mucosa thus a strong possibility exists that these may elicit allergenic reactions (Bannon, Goodman, Leach, Rice, Fuchs & Astwood, 2002). Although the *S. alba* cruciferin isoform CRU1_SINAL (P83908) is considered as an allergen (Sin a 2), supporting evidence was not found in the pepsin stability assay conducted. Peanut allergen Ara h 2 which is a 2S albumin protein has a conserved cystine motif and strong intra molecular disulphide bonds (Sen, Kopper, Pons, Abraham, Burks & Garry, 2002). These disulphide bonds are resistant to enzymatic digestion in the digestive track including chymotrypsin. The intra molecular disulphide bonds act as a physical barrier for the enzyme to reach the cleavage site as demonstrated in the Ara h 2. In addition to this resistance to proteolysis, 2S albumins appear to enhance interaction with lipids (Doke et al., 1989). This property greatly influences gut permeability of 2S albumin which can lead to an increase in allergic properties through GI track mucosal layer.

In the monogastric digestive tract, the epithelium layer contains enterocytes and macrophage cells. The intestinal epithelium layer acts as barrier layer to macro molecules

such as undigested foods, pathogens, etc. In some individuals the tight junctions of the gastrointestinal mucosa layer get loosened and let undigested proteins pass through by intracellular absorption or transcellular absorption (Moreno, Maldonado, Wellner, & Mills, 2005). Undigested proteins can sensitize the intestinal mucosa by transe-cellular absorption and can initiate allergic reactions. According to the present study, the stability of napin of *B. juncea*, *B. napus* and S. *alba* indicates that they can remain undigested at the pepsin levels of normal digestion and possibly can sensitize gastric and intestinal mucosa. In Chapter 3 (Study 1), napin showed high thermal stability and this study shows its high proteolytic stability.

4.6 Conclusions

Brassicaceae oilseed meal proteins showed lower *in vitro* gastrointestinal digestibility than soy meal protein except for canola quality *B. juncea*. Napin is the major contributor to low protein digestibility values. Cruciferin has higher digestibility than napin. The above conclusion supports the first hypothesis of this study that cruciferin and napin of *Brassicaceae* oilseeds are different in the digestibility and degradability catalyzed by pepsin and pancreatin. The low level of proteolytic enzyme inhibitors like trypsin and chymotrypsin inhibitors shows very weak or no influence on protein digestibility by inhibiting proteolytic enzymes. Cruciferin has less propensity to be allergenic compared to napin across the species studied. Napin is the major allergenic protein as indicated by the bioinformatics analysis and pepsin digestibility resistance assay. Separation of napin may enhance protein digestibility values and reduce allergenic potential of *Brassicaceae* meals.

5. GENERAL DISCUSSION

Brassicaceae seeds that are grown as canola and mustard have various seed coat colors according to species and variety. *S. alba* seed varieties are light yellow in color and *B. juncea* seed color varies from light brown to dark brown. *B. napus* canola seed is dark brown in color. According to Kimber and McGregor (1995), *Brassicaceae* seed coat color shows a wide variability within its family due to breeding procedures and it is believed that the tannin percentage of the seed coat is lower in lighter color seeds. According to the present study seed hull percentage is high in *S. alba* varieties and lower in *B. napus* and canola quality *B. juncea* varieties; this is in agreement with the work by Cui et al. (1993) on yellow mustard that the mucilage containing cells are in the outer most layer of the seed coat. The mucilage contains soluble polysaccharides (Cui et al., 1993) and the seed coat epidermal layer containing mucilage is not common in *B. napus* and *B. juncea*, while it is found in the *Sinapis* spp. (Cui et al., 1993). In this study, the content of soluble fiber of S. *alba* seed coat was higher (61% to 66%) than other species.

Brassicaceae seed protein and oil are mainly stored in the cotyledon; OM and BM contain high oil levels (42.5 to 43.5%) and YM contains low amounts (30.4 to 33.5%) in whole seeds on a dry weight basis. The protein content of cotyledons is 28 to 29.9% in *B. juncea*, 33.5 to 39% in *S. alba*, and 25.2% in *B. napus* based on the total nitrogen determination. Seed coat of these species contain 14.7% to 17.5% crude protein which may not contribute to the extractable proteins. With regard to the crude protein values, whole seed of *S. alba* contains higher protein content than *B. juncea*, and this protein is mainly concentrated in the embryo and cotyledons. Overall *Brassicaceae* seeds are good sources of protein and oil which could be incorporated into animal feed, human food and other industrial applications. In *Brassicaceae* seeds, compounds such as phenolics, glucosinolates and nucleic acids contain considerable amount of non-protein

nitrogen. These compounds may have certain adverse effects on animal and human nutrition. These non-protein nitrogen compounds of seed cotyledons cause significant problems in extracting seed storage protein, resulting as impurities in the final product.

The *B. napus* (AC Excel) and canola quality mustard (*B. juncea;* Dahinda) are varieties bred for high oil content which were reflected in their higher oil content than *S. alba* or other *B. juncea* varieties. *S. alba* (YM) is grown as a condiment crop. Similarly, *B. juncea* varieties are condiments.

Cruciferin and napin, the major storage proteins, showed different solubility behaviours with a change of solvent pH, salt concentration or salt type. The typical pH solubility curve for these proteins was "U" shaped and the solubility minima were shown at around pH 3 and 4; at this pH only napin was soluble. In other pH values both napin and cruciferin were soluble. This solubility difference of the constituent protein types can be utilized in *Brassicaceae* protein fractionation. As an example, by extracting at pH 4, napin proteins can be completely removed from the meal. As shown in Table 3.8, the protein solubility of the meal can be increased by adding monobasic or dibasic salts (Na^+ or Ca^{2+}) to the solvent during aqueous protein extraction.

Digestibility is one of the important factors that determine protein quality, and it is mainly related to the release and availability of amino acids for absorption in the small intestine. The digestibility study results are important to predict possible allergenicity of the proteins. The gastrointestinal digestibility values for seed meal protein varied among *Brassicaceae* species; degradability may be related to the type of storage protein (cruciferin and napin), their content in the seed meal, enzyme inhibitors, presence of phenolic compounds and glucosinolate levels. The gastrointestinal digestibility values for *B. juncea, B. napus* and *S. alba* meals calculated from the released TCA soluble N were between 20% and 34%. Among *B. juncea* varieties, the canola quality Dahinda variety exhibited higher digestibility values than did the other two varieties. Andante and AC Excel had similar digestibility values ($p<0.05$). The digestibility values for *S. alba* and *B. napus* also were similar. When compared with soybean meal, all *Brassicaceae* meals, except that of the variety Dahinda of *B. juncea,* exhibited low digestibility; the variety Dahinda had values comparable to soymeal. The *in vitro* digestibility values of napin-depleted meals showed an increase in compared to the unfractionated defatted meal. The

highest increase in the digestibility was recorded for *B. napus* meal (21.0%); and *S. alba* meal showed 15.2%, and 19.3% and condiment *B. juncea* varieties had 8.3% and 16.5% increase in gastrointestinal digestibility after removing napin. These results indicated that napin protein may limit the protein digestibility under monogastric gastrointestinal conditions. This confirms the allergenicity study results; in gastric digestion conditions of monogastrics under high pepsin concentration (pepsin:protein 13:1) napin proteins were resistant to digestion. However, cruciferin proteins were completely digested under gastric digestion conditions. The resistance of napin protein to digestion under gastric conditions indicates that it would be a possible allergen for monogastrics. Sin a 1 and Bra j 1 quantification results indicated that *S. alba* and *B. juncea* varieties contain allergenic proteins levels of less than 0.5% of proteins soluble at pH 7.0. The stable structure of the napin protein could be the reason for this digestibility stability. According to D'Hondt et al. (1993), napin protein has a conserved cysteine skeleton (8 Cys motif) which may lead to a stable and compact structure that is difficult to access by digestive enzymes.

According to the results of this work, *Brassicaceae* seed proteins are good sources of protein. However, napin in seeds may decrease the nutritional quality of the food or feed products. Extraction and fractionation of protein may improve the quality of *Brassicaceae* seed meals. This will improve the use of *Brassicaceae* oilseed meal proteins, and would allow one to utilize these proteins better in food and non-food applications.

6. OVERALL CONCLUSION

Brassicaceae oilseeds (*Brassica juncea, Brassica napus* and *Sinapis alba*) contain oil, protein and fibre as major components with variation found among species for the content and their composition. Protein and oil are concentrated mainly in the coteyledons of the seed. Seed coat contributes much of the fibre content and *S. alba* seed coat has a conderably different composition than the other species along with high content of soluble polysaccharides. These soluble carbohydrates could negatively affect protein extraction. Even though the *S. alba* protein content was higher than other species high seed coat content can reduce total protein yield and purity. In canola-quality *B. juncea* seed coat content was around 14% and could yield more protein content than the other varieties through aqueous extraction. The nonprotein components namely, nucleic acids, glucosinolates, sinapine, choline and betaine contributed 3.1% to 10.8% to the total nitrogen content which may reduce the amount of actual protein that can be recovered from these seed meals.

Cruciferin and napin are the main seed storage proteins located in the coteyledons of these *Brassicaceae* seeds and also are the extractable proteins. *Brassicaceae* oilseed meal proteins show lower *in vitro* gastrointestinal digestibility than soy meal protein except for canola quality *B. juncea* under *in vitro* monogastric digestion conditions. The digestibility of napin is lower than that of cruciferin and may contribute to low total meal protein digestibility. Both these proteins have high denaturaton temperatures (above 73°C) which indicates structural stability, and may be favorable under the conditions such as pasteurization, required during large scale protein extraction. Under a high pepsin concentration cruciferin was digested completely whereas napin showed resistance to gastric digestion conditions. According to the results of bioinformatics analysis and the pepsin digestibility resistance assay, napin proteins were identified as allergenic proteins in *Brassicaceae* seeds; however, cruciferin proteins have less potential to be allergenic. Therefore, separation or breakdown of napin in meal may improve meal protein quality

and digestibility. The minimum solubility of *Brassicaceae* seeds coteyledon proteins was observe around pH 4. The selective solubility of napin at acidic pHs, i.e. 3 and 4, is a good indicator that such conditions can be used to separate them from other proteins in *Brassicaceae* meal. Use of Na^{+} or Ca^{2+} salts at low ionic strengh may improve the extractability of these proteins. Most of the napin isomers found in these three seed species exhibited strong possibility to be gastrointestinal allergens through predictive assays. Therefore using conditions selective for napin protein solubilization may be a suitable way to improve *Brassicaceae* protein digestibility as well as reduce potential allergenicity. The solubility differences between napin and cruciferin that depend on pH and salt concentration as observed in this study, will be useful in separating these proteins, as well as in generating safer protein products for food and feed applications.

7. FURTHER IMPLICATIONS

According to this study, cruciferin and napin proteins of *Brassicaceae* seeds show different solubility properties, nutritional value and digestibility. At present, the distribution or content of cruciferin and napin proteins in crucifer seed species is not known. Because of the differences in structure, physico-chemical properties and biological activities of these two proteins, it is important to know their distribution in seed. As an alternative approach, since napin is resistant to monogastric digestive enzymes and leads to physiological implications such as becoming allergenic, further studies are needed to find possible biocatalysts that can assist in breaking down allergenic epitopes of napin there by reducing the allergenicity.

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9.0 APPENDIX

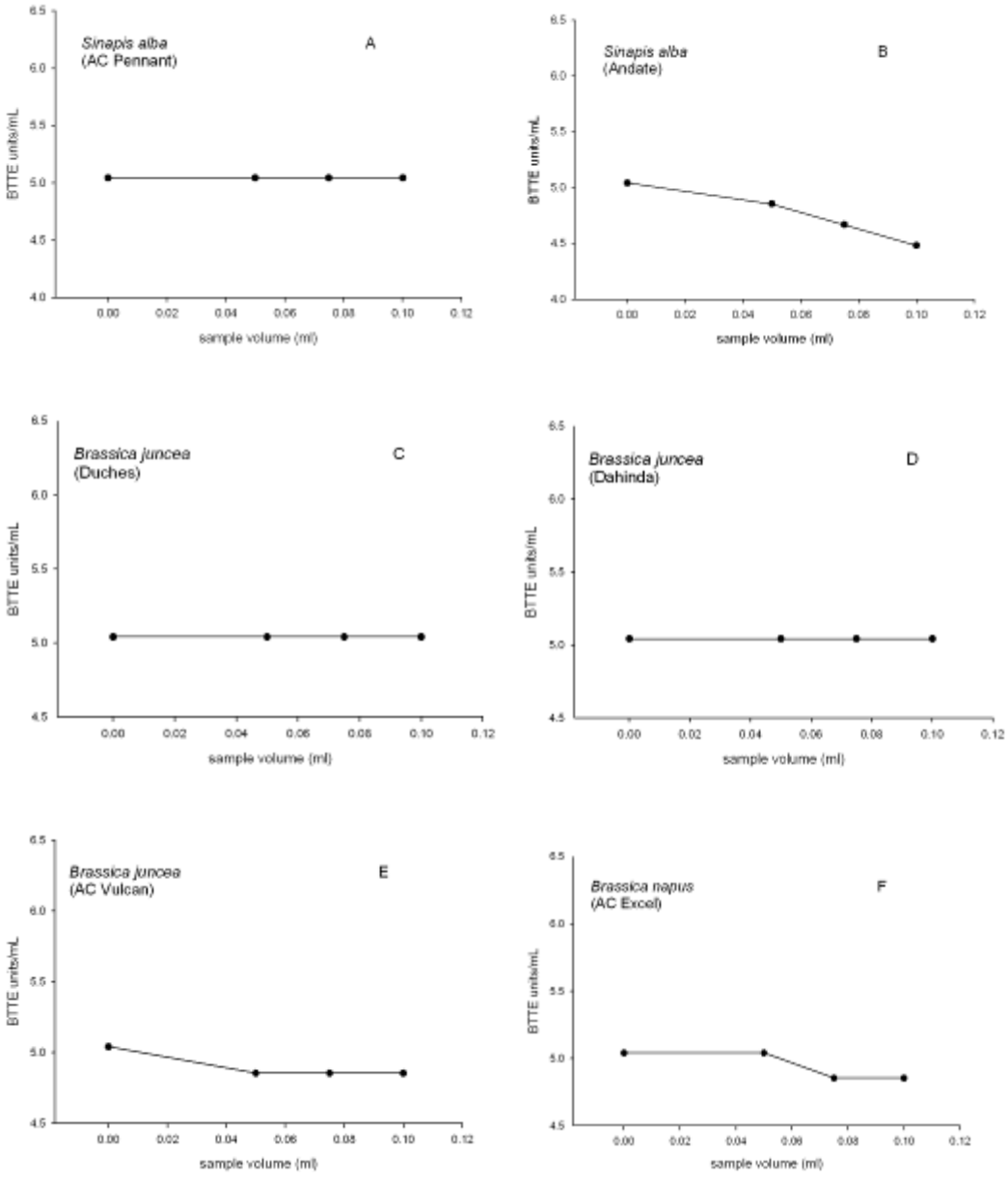

Figure 9.1. *B. juncea* (C, D and E), and *B. napus* (F) and *S. alba* (A, B), BTTE units/mL values against sample volume (mL) for chymotrypsin inhibitory activity calculation.