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Proteins of *Brassicaceae* Oilseeds and their Potential as a Plant Protein Source

JANITHA P. D. WANASUNDARA

Agriculture and Agri-Food Canada, Saskatoon Research Centre, 107 Science Place, Saskatoon, SK S7N 0X2, Canada

Among the commercially cultivated Brassicaceae (Cruciferae) plants, Brassica juncea, Brassica napus, Brassica rapa, and Sinapis alba store significant amounts of oil and protein in the seed. At present, Brassica seed proteins are primarily used for livestock feeding based on the nutritional value. The point of curiosity is whether the present knowledge on the protein structure, biochemical characteristics, nutritive value, and the recovery processes are inadequate to develop Brassica proteins into a usable plant protein source or these proteins are of substandard for uses beyond animal nutrition applications. Cruciferin (11S) and napin (2S) are the predominant storage proteins of Brassicaceae seeds that contribute to different properties and functions. A gamut of information is available on the chemistry, nutritional value, as well as the functionality in foods, and associated non-protein components of canola/rapeseed storage proteins. The intention of this article is to critically review what is known about the predominant storage proteins of commercially produced Brassicaceae seeds relative to the above aspects and identify the knowledge gaps.

Keywords canola, crucifers, cruciferin, mustard, protein value, napin, rapeseed

INTRODUCTION

Several species of *Brassicaceae* or *Cruciferae* family have become important agricultural crops around the world. Among these, canola or rapeseed (*Brassica napus* and *Brassica rapa*, formerly *Brassica campestris*), oriental and brown mustard (*Brassica juncea*), black mustard (*Brassica nigra*), and yellow mustard (*Sinapis alba* synonym *Brassica hirta*) are important in the global oilseed economy. Canola is the Canadian oilseed crop developed primarily for the purpose of edible oil. Seeds of *B. juncea*, *B. nigra*, and *S. alba* are used as condiments and spices. Among *Crucifers*, *Brassica carinata* (Ethiopian mustard), *Camelina sativa* (camelina, false flax, gold of pleasure), and *Crambe abyssinica* (Abyssinian mustard) are considered species that could be developed for commercial production primarily for oil directed to biofuel and other industrial uses in North America and Europe. These *Crucifer* seeds are rich in oil and contain considerable amount of protein that accounts for 20 to 35% of seed dry weight. The predominant storage proteins of these *Crucifers* are cruciferin (11 or 12S) and napin (1.7 or 2S).

These proteins are expressed during seed development as precursors, undergo co- and post-translational modifications, before being transported to membranous organelles (protein bodies) where they accumulate in large quantities and become a considerable fraction of the seed biomass. Details of historic use of the seeds, protein molecule structure, biological and biochemical properties, and current and potential applications of the storage proteins of *Crucifer* oilseed crops are focused in this review. The information found in this review refers mainly to canola or rapeseed proteins because of the abundance of information available.

HISTORIC AND ECONOMIC BACKGROUND

Economically, the crops of genus *Brassica* (Family *Brassicaceae*) are loosely categorized into oilseeds, vegetables, and condiments. *Brassica* crops are widely found in the cuisines of many cultures around the world owing to the variety of edible forms available in this genus. Many *Brassicaceae* vegetable crops found a place in our diet. The extreme morphological variation exhibited in *Brassica* crops is fascinating; for example, enlarged inflorescence of broccoli and cauliflower, stem of kohlrabi, root of turnip, twisted leaves of Chinese cabbage,

Address correspondence to J. P. D. Wanasundara, Agriculture and Agri-Food Canada, Saskatoon Research Centre, 107 Science Place, Saskatoon, SK S7N 0X2, Canada. Fax: 306 956 7247. E-mail: janitha.wanasundara@agr.gc.ca

Table 1 Commercially cultivated *Brassicaceae* family plant for seeds; nomenclature, chromosome number, and genomic descriptors (Vaughan, 1977; Labana and Gupta, 1993; Rakow, 2004)

Species	Subspecies/ variety	Chromosome number and genome descriptor ¹	Common name
<i>Brassica carinata</i>		17; BBCC	Ethiopian (Abyssinian) mustard
<i>Brassica juncea</i> ² L. (Czern and Coss)	oleifera	18; AABB	Indian mustard
<i>Brassica napus</i> ² L.	oleifera	19; AACC	Oilseed rape
<i>Brassica nigra</i> L. Koch	oleifera	8; B	Black mustard
<i>Brassica rapa</i> ²	trilocularis	10; AA	Turnip rape
syn. <i>B. campestris</i> L. Sensus lab		10; AA	Sarson (yellow/brown)
<i>Sinapis alba</i> L.		12; DD	Yellow mustard – North America
syn. <i>Brassica hirta</i> Moench			White mustard – Europe

¹ A, B, C, and D are genome symbols, chromosome number is provided.

² Canola includes genetic materials from these species that has similar compositional properties.

enlarged single epical bud of cabbage, curly leaves of kale, many axillary buds of brussels sprout, and seeds of condiment mustard. Seeds of some species of *Brassica* genus store considerable amount of lipids and proteins. Of the genus *Brassica*, *B. carinata*, *B. juncea*, *B. napus*, *B. nigra*, and *B. rapa* are rich in oil and cultivated worldwide (Table 1). Rapeseed (*B. napus* and *B. rapa* together) provides ~12% of the world edible oilseed supply (Labana and Gupta, 1993; FAO, 2008). Table 1 provides a list of *Brassicaceae* plants that are commercially cultivated for seeds.

Seeds of *Brassica* species have a long history of domestic use. They may be one of the earliest domesticated plants since some of the vegetable forms have been in common use during the Neolithic age. Carbonized seeds of *Brassica* or *Sinapis* have been located in several sites of Khafadje, Iraq (Mesopotamian civilization) that dates back to 3000 BC (Zohary and Hopf, 2000). Seeds of *B. juncea* have been found in the excavations of Chanhudaro sites, in the upper valley of Baluchistan which belongs to the Harappan civilization; ca. 2300–1750 BC (Allchin, 1969; Prakash, 1961). Presence of *B. rapa* remains is evident in several Neolithic and Bronze age lake-shore sites in Switzerland (Zohary and Hopf, 2000). The earliest written reference to these plants is found in the ancient Sanskrit texts of India (ca.1500–500 BC); the *Upanishads* and *Brahmanas* (*Veda* texts) have direct reference to mustard (*Rājika*) use as oil, spice, medicine, washing aid (white mustard), and in the scale of weight measures (Watt, 1889; Prakash, 1961; Agrawal and Shukla, 1984). In the teachings of the Gautama Buddha (ca.563–483 BC) mustard seed has been mentioned in several texts (e.g., Agama sutra, Dhammapada, Lotus sutra) to symbolize the minuteness and abundance which indicates the wide use of mustard among common people during that time. The medicinal value of the *Brassica* plant and its oleaginous seed has been described in Greek, Roman, and Chinese writings of 500 to 200 BC (Röbeelen et al., 1989). Mustard and rapeseed have also been mentioned by Gaius Plinius Secundus in his books which date back to 77 BC (Bostock and Riley, 1855; Fussel, 1955). Mustard finds mention in the Holy Bible (Luke13:19, Matt13:31, Mark4:31). Sufficient evidence is available to support that mustard seeds may have been a popular medicine, spice, and a source of oil since early civilization and indicates

that its oil, pungency elements, and therapeutic ability have been the dominant traits that drove the utility.

Literature compilations by Fussel (1955) and Baranyk and Fábry (1999) search back to trace the origin of *B. napus* and *B. rapa* because of the significant genetic enhancement of these species enabling them to become a successful plant in agriculture; however, it is often conflicting. It is proposed that wild *B. rapa* which originated in Europe from a common ancestor subsequently spread through Middle East to India and China. *Brassica juncea* may have originated anywhere between Eastern Europe and China or could be in the Middle East. *Brassica napus* is of recent origin and believed to be of southwest European or Mediterranean origin (Labana and Gupta, 1993). Regular cultivation of winter rape (*B. napus*) is reported as early as 1600 AD and rape has rapidly established as a crop throughout northwestern Europe (Fussel, 1955; Labana and Gupta, 1993). The Ethiopian mustard (*B. carinata* A. Braun) has been confined to the Ethiopian plateau, its probable origin. It is believed that *Sinapis alba* L. (yellow mustard in North America and white mustard in Europe) may have originated in the Mediterranean region (Tsunoda, 1980).

Cultivation of rape to obtain lamp oil can be traced back to 17th century in Europe (Appelqvist, 1972). Rapeseed was used primarily to obtain inexpensive oil for lighting and lubrication which was in high demand during 1920–1950. None of the *Brassica* vegetables are native to the American continent. During the 1920s to the late 1930s rapeseed (primarily *B. rapa*) was introduced to Canada, in the province of Saskatchewan, by immigrant farmers from Poland (Khachatourians et al., 2001). Commercial cultivation of rapeseed for oil production (lubricants for marine engines) was supported by Canadian federal government research since 1942. Rapeseed breeding programs have aggressively worked on changing the quality of seed oil and the meal. The term “canola” came to be registered as the trademark in 1978 to differentiate quality improved rapeseed genetic material with the composition of <2% erucic acid in oil in terms of 1996 standards and meal containing <30 μ mol of glucosinolates of any one or any mixture of 3-butenyl glucosinolate, 4-pentenyl glucosinolate, 2-hydroxy-3 butenyl glucosinolate, and 2-hydroxy-4-pentenyl glucosinolate per gram of air-dried,

Table 2 World production of major oilseeds (million tonnes)

Oilseed	2003/04	2004/05	2005/06	2006/07 (estimate)	2007/08 (forecast)
Soybeans	184.9	215.8	220.9	235.3	220.8
Rapeseed	38.9	46.0	49.1	47.4	48.0
Cottonseed	36.2	44.6	42.5	44.6	44.4
Groundnut (unshelled)	34.4	34.5	35.7	34.0	36.5
Sunflower	26.3	26.0	30.1	29.4	27.8
Palm kernel	8.2	8.8	9.7	9.9	10.8
Copra	4.9	5.1	5.0	4.9	5.3
Total	333.9	380.9	393.3	405.5	392.7

Source: FAO, 2008

oil-free solid at a moisture content of 8.5%. Canadian rapeseed or canola is bred from *B. napus* (summer rape) and *B. rapa* (turnip rape) (www.canola-council.org, 2009). With the development of herbicide tolerant varieties, >95% canola seeded in Canada is *B. napus* (CGC, 2008). Recently, *B. juncea* germplasm that fits into the oil profile and meal composition of canola definition has been recognized as “canola-quality juncea.” The glucosinolate profile and levels of *B. juncea* are different from *B. napus* or *B. rapa*. The start of commercial cultivation of mustard in North America has not been well documented as it has been for rapeseed. Canadian mustard production is dominated by yellow (*Sinapis alba*), oriental, and brown mustard (*B. juncea*) mainly for the condiment and spice market.

At present *Brassica* oilseeds rank second to soy among the world oilseed crops (Table 2). The oil of canola/rapeseed is recovered primarily by mechanical expression in combination with solvent extraction. Canola is a food grade oil consumed around the world. Edible use of *B. juncea* oil is limited to certain ethnic cuisines. Current interest on using canola/rapeseed oil in bio-based fuel and lubricant production ensures that its original value as an industrial oil is not lost. Generation of fatty acid methyl esters for biofuel and lubricants provides another avenue of utilizing *Crucifer* oil fraction. Presence of high erucic acid levels is a characteristic of *B. carinata*, *B. juncea*, *C. abyssinica*, and *C. sativa* oil fraction. These non-canola type *Crucifer* seeds are also rich in oil and protein.

Harvesting of oil as 40 to 45% of *Brassica* seed biomass generates dry matter residue amounting to about 55 to 60% of seed dry weight. The resulting high-volume, low-cost *Brassica* seed meal is primarily streamlined into the animal feed market. Canola meal is a competitive animal feed source owing to its high protein and energy values. The comparative protein production values of Canadian oilseed crops (Table 3) show that on average, in addition to oil, canola crop generates the largest volume of plant protein. At present canola meal (crude protein 35 to 40% by weight) is used in feed formulation for dairy cattle, poultry, swine, and farmed fish because of its high quality protein. However, canola meal is less competitive for high energy feed formulations because of its low energy availability (70% of soy meal; Hickling, 2007). Any meal volume that is generated from increased canola production will have to find

Table 3 Production and yield of oil and protein of major oilseed crops grown in Canada

Crop	Production (× 1000 MT/Yr) ^a	Oil content (%) ^{a,b}	Oil yield (× 1000 MT/Yr) ^c	Protein content (%) ^{a,d}	Protein yield (× 1000 MT/Yr) ^c
Canola	7139	47.0	3355	23.6	1685
Flax	828	44.5	368	23.0	190
Soybean	2672	21.1	564	40.8	1090
Mustard ^e	222	36.2	81	28.8	64

^a Values are average for 1997 to 2006 as provided by Canadian Grain Commission in 2008 (www.grainscanada.gc.ca) and Statistics Canada.

^b On moisture free basis, for soybean mean values of 2002–2006

^c Calculated using values for production and content of component

^d %N × 6.25, on moisture free basis

^e Oil and protein values are calculated based on a mixture of 45% yellow (30.5% oil, 31.9% protein), 30% brown (40% oil, 26% protein), and 25% oriental (42% oil, 26.5% protein) mustards.

alternative uses in order to balance the market price of oil and meal. Co-product development of meal biomass is a necessity for providing better economic competitiveness for the *Brassica* oil production industry. This will help to generate a wide array of uses for the natural products deposited in the seed other than the lipids. In this context it is important to understand the value of *Brassica* proteins to convert these natural polymers of amino acids into high value products and applications that can bring in another dimension to the *Brassica* oilseed economy.

STORAGE PROTEINS OF BRASSICA SEEDS

Similar to any other plant, *Brassicaceae* plants store reduced nitrogen predominantly in the form of seed storage proteins. Proteins are the long-term storage form of amino acids as the latter are stored in an osmotically inactive state by polymerization. In *Brassica* plants two classes of seed storage proteins are predominant; legumin-type globulins (11S or 12S or cruciferin, Schwenke et al., 1981; Sjödaahl et al., 1991) and napin-type albumins (2S or napins, Lönnerdal and Janson, 1972; Crouch et al., 1983). According to the empirical classification of Osborne (1924) based on the solubility, proteins that are soluble in dilute salt solutions are globulins of 11S or 12S and water soluble proteins are albumins having 1.7S or 2S.

The reviews by Shewry et al. (1995) and Müntz (1998) discuss and illustrate well how 11S and 2S proteins are synthesized, sorted, and arranged to form the final structure of the protein in dicotyledon seeds. During maturation, seeds act as strong amino acid sink when storage proteins are formed. Proteins are deposited in membrane-bound, dedicated, and highly specialized compartments; the protein bodies (PB) generated from the protein storage vacuoles (PSV). These cellular compartments may provide sufficient protection for accumulated proteins against premature breakdown by cytoplasmic enzymes (Müntz, 1998). The PSVs of *Brassica* seeds are roughly spherical organelles with an average diameter of 1.5 to 8 µm (Ashton,

1976). The accumulated proteins go through additional processing that modify and prepare proteins for dense packing. The vacuolar processing enzymes are localized in the PSVs to form mature hexamers for legumin type proteins (Jung et al., 1998). The PSVs in most seeds contain three morphologically distinct regions; matrix, crystalloid, and globoid (Rost, 1972; Neumann and Weber, 1978; Lott, 1980; Weber and Neumann, 1980). The matrix and crystalloids contain storage proteins, whereas the globoids are composed of phytic acid crystals (Lott, 1980; Weber and Neumann, 1980). However, in the family of *Brassicaceae*, PSVs lack visible crystalloids and have many small globoids dispersed throughout (Kuang et al., 2000; Gillespie et al., 2005).

The ratio of cruciferin to napin exhibits a great variability and is in the range of 0.6 to 2.0 for the European genotype pool of erucic acid- and glucosinolates-zero to high varieties. A significantly high value has been observed for the ratio of cruciferin to napin for double zero cultivars compared to both erucic- and glucosinolate-high varieties. The intensive breeding efforts that have been put into rapeseed to improve oil and to reduce glucosinolate levels have concomitantly induced a decrease in the total protein content and enrichment in the levels of cruciferin (Malabat et al., 2003).

The oleosins or oil body proteins are major structural proteins of *Brassicaceae* oilseeds. Several metabolic proteins such as lipid transfer proteins (LTP), protease inhibitors (Cecilian et al., 1994), Ca^{+2} dependant-calmodulin binding proteins (Neumann et al., 1996a; 1996b), and dehydrins (Svensson et al., 2000) have been reported in *Brassicaceae* seeds. The European *B. napus* cultivars contain 1.6 to 7.35% of total protein as LTP (Bérout et al., 2005). The proteins synthesized during late maturing stages such as late embryogenesis associated (LEA) proteins protect seed tissues from desiccation.

Cruciferin

According to Crouch and Sussex (1981) cruciferin constitutes about 60% of the *B. napus* seed protein at maturity. In double low rapeseed (several cultivars), cruciferin is about 32–53% of the total proteins and exists as the predominant seed storage protein (Malabat et al., 2003).

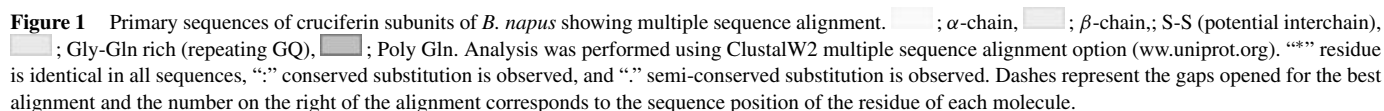
Cruciferin Structure

Cruciferin belongs to the cupin (*cupa* or small barrel) protein superfamily. Like any other 11 or 12S protein, cruciferin has well organized levels of primary, secondary, tertiary, and quaternary structure. Glycinin, the 11S protein of soy (*Glycine max*), has a known three-dimensional structure and is the most related protein to cruciferin known so far. In glycinin, the constituent subunits or protomers are synthesized as a single polypeptide precursor preprotein of which the signal sequence is removed co-translationally. The resulting preprotein assembles into trimers of ~8S in the endoplasmic reticulum (ER).

The proprotein trimers are transported from ER to PSV, then cleaved between Asn and Lys residues to form acidic and basic polypeptides that later get linked by S-S bonds (Dickinson et al., 1989). The mature proteins are assembled as hexamers having a sedimentation coefficient of 11S and stored. The stacking of trimers to form hexamers is not very clear; however, the cleavage of Asn-Lys is considered as the trigger to form a hexamer. Details of the synthesis of cruciferin subunit polypeptides, their processing in the vacuoles, assembly to form the final protein structure, and accumulation in the protein storage vacuoles are beyond the scope of this review. Shewry et al. (1995), Neuhaus and Rogers (1998), Müntz (1998), Herman and Larkins (1999), Jiang et al. (2001), Fujiwara et al. (2002), Gillespie et al. (2005) provide excellent reviews on these aspects of *Brassicaceae* seed storage proteins.

A search on the available protein database (UniProtKB/Swiss-Prot and UniProtKB/TrEMBL; www.uniprot.org) yields five cruciferin isoforms for *B. napus*. They are CRU1_BRANA (P33523; cruciferin BnC1 precursor; gene BnC1), CRU2_BRANA (P33524; cruciferin BnC2 precursor; gene BnC2), CRU3_BRANA (P33525; cruciferin CRU1 precursor; gene CRU1), CRU4_BRANA (P33522; cruciferin CRU 4 precursor, gene CRU4), and CRUA_BRANA (P11090; cruciferin precursor, gene CRUA syn CRU2/3), actually the protomers of cruciferin. The primary structure or the cruciferin polypeptide chain of these protomers is composed of 465 to 509 amino acid residues (Fig. 1) depending on the gene involved in expression. Each polypeptide consists of a heavy α - (acidic, M_r approx. 30 kDa, 254 to 296 amino acids) and a light β - (basic, M_r approx. 20 kDa, 189 to 191 amino acid residues) chain linked with one disulfide bond (Dalgalarondo et al., 1986). These α - and β -chains are generated by proteolytic cleavage of precursor polypeptides (51 to 56 kDa) that are synthesized on the rough endoplasmic reticulum (DeLisle and Crouch, 1989). Cruciferin sequences available in the databases are for the (α - β) polypeptides that form the subunits (protomers) of the 11S globulin molecule. Among the 5 different identified cruciferin subunits, the α and β chains differ from each other in terms of the number and type of amino acid residues; however, they conserve a particular degree of homology in certain areas of the sequence (Fig. 1). The primary structure contains a hydrophilic stretch of amino acids, mostly Gly-Gln repeats, and it is positioned in the middle part of the α -chain of cruciferin and is assumed to form a loop on the molecule surface (Simon et al., 1985). Perfectly conserved residues are found in cruciferins of *Arabidopsis thaliana*, *B. napus*, and *Raphanus sativus* when full length sequences are aligned (Delseny and Raynal, 1999).

About 9 to 12 genes have been identified in *B. napus* that encode nucleotide sequences for cruciferin (Simon et al., 1985; Sjö Dahl et al., 1991; Breen and Crouch, 1992; Bilodeau et al., 1994). The nucleotide sequences for cruciferin have been identified by the work of Simon et al. (1985), Shaw and Ryan (1989), Rödin et al. (1990); Sjö Dahl et al. (1991) and Inquello et al. (1993). The studies of Sjö Dahl et al. (1991) and Breen and Crouch (1992) have suggested that the precursors P1, P2, and



The mature cruciferin molecule has an isoelectric point of 7.25 (Schwenke et al., 1980). In a typical 1D SDS-PAGE separation, cruciferin gives several bands between 18 and 53 kDa range under reducing conditions. Under non-reducing conditions cruciferin α - β polypeptide chains may appear in the range of 48 to 56 kDa. Upon reduction, the polypeptides of α - β disappear and the free α (αf) and β (βf) polypeptides show high staining density. It is also common to observe polypeptide bands that remain at apparent molecular weight above 54 KDa, presumably from the precursor polypeptide or procruciferins of α - β which have not undergone regular *in vivo* processing (Dalgarrondo et al., 1986; Robin et al., 1991). Free polypeptides that are not covalently linked are common in *Crucifer* 11S globulin fraction

The hexameric form of 11S cruciferin of rapeseed exhibits reversible association and dissociation due to changes of pH and ionic strength confirming its multicomponent assembly. When the ionic strength of the medium is above 0.5, 11/12S hexameric assembly exists and dissociates into 7S trimers upon lowering the ionic strength (Schwenke et al., 1983). Dissociated trimers can reassemble when the ionic strength is elevated (Schwenke and Linow, 1982). Acidic conditions and the presence of 4 M urea cause irreversible dissociation of cruciferin. When 8 M urea

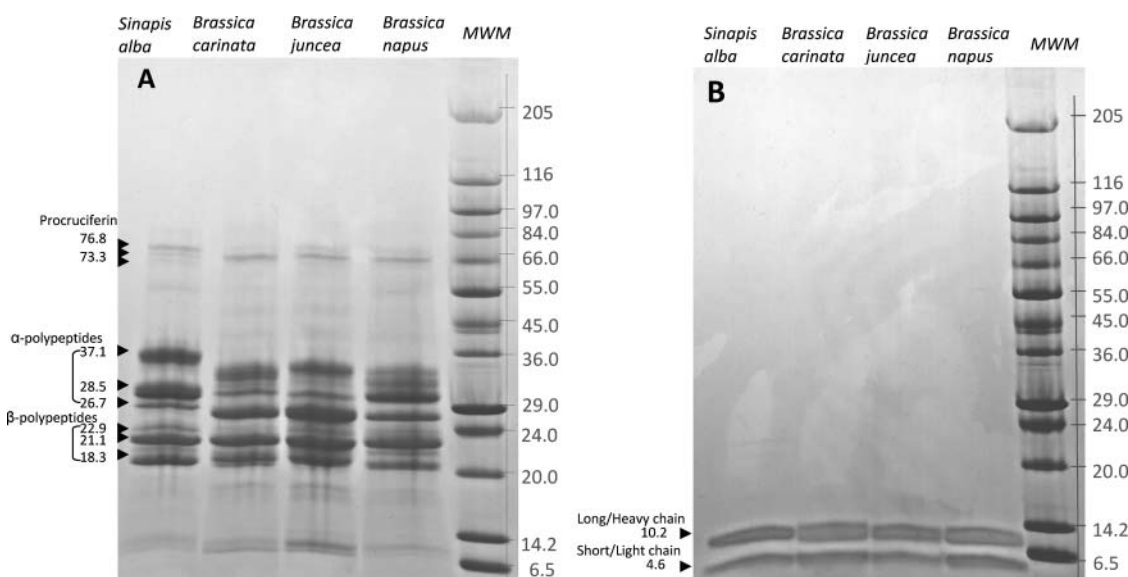


Figure 2 SDS PAGE (under reducing conditions) pattern of cruciferin (A) and napin (B) of four species of *Brassicaceae*. Proteins of *Brassica carinata* (Dodolla), *Brassica juncea* (AC Vulcan), *Brassica napus* (AC Excel) and *Sinapis alba* (AC Pennant) was extracted, isolated and purified according to the procedure described by Bérot et al. (2005).

is present rapeseed 11S molecules dissociate into its protomers (~50 kDa) and further dissociation into its monomeric peptides can happen when SDS is also present (Simard et al., 1979; Schwenke et al., 1983). Using *B. juncea* 12S globulins, Murthy and Rao (1984) have explained the unfolding of the cruciferin native structure between pH 5 and 3 and refolding beyond pH 3. This re-association is due to entropically driven hydrophobic interaction of non-polar residues of the protein.

Due to the molecular heterogeneity of cruciferin, obtaining crystals suitable for X-ray diffraction analysis is very difficult. This may be the reason cruciferin molecular structure is not available in any of the protein databanks besides the primary structure of subunits. Information about the crystal structure may enable to deduce the packing of protein in its tertiary and quaternary structure levels. Although it has not been clearly reported for cruciferin, the quaternary structure model developed for soybean proglycinin (11S protein) by Adachi and group (2003) may be the best existing to date for approximation of cruciferin structure. Therefore, it can be assumed that the secondary structure of cruciferin subunit is formed with 27 beta strands and 7 helices that are folded into two β -barrel (jelly-roll) domains and two extended helix domains containing 2 helices (Adachi et al., 2001) each similar to what has been observed in soy A3B4 protomer (Adachi et al., 2003).

Among the molecular assembly models proposed for the quaternary structure of cruciferin, the “universal model” by Badley et al. (1975) and Derbyshire et al. (1976) assumes 11S globulin is a 12-subunit structure composed of six α - β subunit pairs that form two hexagonal rings. A more plausible model that the α - and β -polypeptides making up the integral, single unit (protomer) and, 6 of these assembled as subunits to form a triangular antiprism (Plietz model) has been proposed by Plietz

et al. (1983) for cruciferin and helianthinin of sunflower. The “Plietz model” has been developed based on published primary sequences, predicted secondary structure using sequence data, and small-angle X-ray scattering data obtained from native *B. napus* cruciferin. In this model, the cruciferin protein molecule assumes almost spherical shape. The six structurally identical subunits are believed to have isologues and heterologues binding domains. The subunits are arranged as a triangular antiprism with the dihedral point group symmetry 32. According to the common structure model of Plietz et al. (1987) and immunological analysis of Robin and others (1991), the hydrophobic β -chain C-terminal should be located in the interior of the molecule while the strongly hydrophilic C-terminal region of the α -polypeptide is believed to be on the molecule surface. Therefore, two domains of the protein subunit are formed by the N-terminal regions of both α - and β -chains (Schwenke, 1990). The C-terminal of the α -chain is strongly acidic (owing to the acidic stretch of Gly-Gln residues, Fig. 1) and this region is assumed to be at the surface of the molecule and protects the domain from the solvent. According to the Plietz model, the more hydrophobic C-terminal of β -chain is located closer to the center of the molecule and stabilizes the oligomeric structure of 11S globulin by hydrophobic interactions with the same sequence regions of other 5 subunits. These non-specific interactions are related to the variability of the sequence of the region. Figure 3 provides the pseudoradial-symmetrical arrangement of subunits for 11S globulins of sunflower, rapeseed, faba bean, and sesame, as described by Plietz et al. (1987), which may represent a common structural model for 11S proteins.

The crystal structure (2.1 Å resolution) for soybean 11S glycinin A3B4 homohexamer reported by Adachi et al. (2003) shows that the homohexamer has 32-point group symmetry

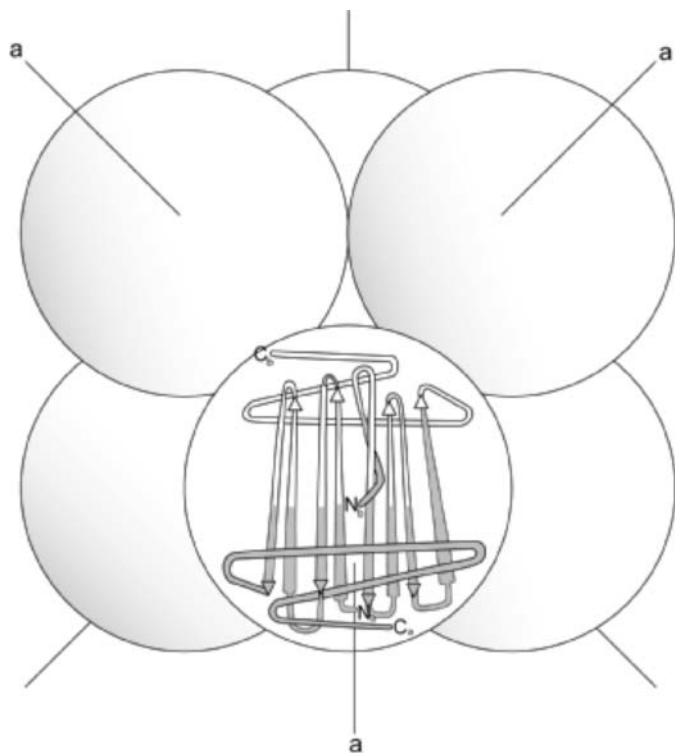


Figure 3 Pseudoradial-symmetrical arrangement of 11S globulin subunits in which the domains are arranged radial-symmetrically by Plietz and group (1987). Hypothetical arrangement of α and β chains in each subunit within the hexameric molecule is shown. N and C designate the N- and C- terminal regions, respectively, of α and β chains (e.g., $N\alpha$, $N\beta$, $C\alpha$, and $N\beta$). The twofold pseudosymmetry axes are indicated by "a" (adapted from Plietz et al., 1987).

formed by face-to-face stacking of two trimers. The interface buries the highly conserved inter- and intra-chain disulfide linkages. The two faces perpendicular to the 3-fold axis in the trimer is conveniently termed as the inter-chain disulfide containing (IE) and intra-chain disulfide containing (IA) faces based on the position of the inter- and intra-chain S-S bonds, respectively and believed to be conserved among members of the 11S globulin family (Adachi et al., 2001). Association of subunits to form a hexamer is mostly led by the hydrophobic interactions and short-range specific interactions (H-bonds, van der Waals, and H-bonded salt bridges), and primarily occurs in the PSV.

Only traces of sugars bound to cruciferin are found (Schwenke et al., 1981), indicating low probability of glycosylation and this corroborates with no glycosylation sites (Asn-X-Ser/Thr) found in the primary structure of cruciferin precursor (Simon et al., 1985). Phosphorylation has been found to be a possibility in *Arabidopsis thaliana* (wild and abi 1-1 mutant) cruciferin indicating that molecular polymorphism far exceeds than predicted on the basis of known isoforms which could arise from the phosphorylation site heterogeneity (Wan et al., 2007). Nineteen phosphorylation sites are found in *A. thaliana* cruciferin comprising 8 Ser, 8 Thr, and 3 Tyr residues, and the majority of the residues are on the inter-chain S-S containing IE face of the globulin trimer involved in hexamer formation (Wan et al., 2007). In the mature seeds of *Arabidopsis*, cruciferin is the main target for carbonylation during the initial stages of

germination. The acidic α -polypeptides are carbonylated to a much higher extent than the basic ones, corroborating the fact that the β -chains are buried in the molecule while the α -chains are exposed to the surrounding environment (Job et al., 2005).

Cruciferin Isolation and Purification

Bhatty and group (1968) were the first to report on isolation of 11S (12S) proteins from *B. napus* (var. Nugget) meal. Use of fairly high NaCl concentrations (>0.5 M) has been favored by most researchers to solubilize cruciferins from the seed matrix and to isolate and desorb from size exclusion chromatography. Table 4 provides a summary of the methods of extraction described in literature. The 4-step chromatographic method (Bérot et al., 2005) that separate and purify cruciferin and napin has been tested at pilot scale and proved to produce very pure cruciferin (Fig. 2) in our research program. No commercially viable large-scale isolation process of cruciferin is available yet.

Biological Activities of Cruciferin

Cruciferin is not widely reported for its biological activities besides the nutritional value of amino acids that could be available upon gastro-intestinal digestion. *Sinapis alba* cruciferin (CRU1.SINAL P83908, 51 kDa, Sin a 2) has been identified as a potential allergen among the individuals with mustard allergies (Palomares et al., 2005). *In silico* analysis of available primary sequences of cruciferin with the BioPep database (www.uwm.edu.pl/biochemia/biopep/) resulted in several matches to the known peptide sequences that have proven biological activities (Table 5) and is discussed under peptides with biological activities. In a practical situation it is difficult to obtain all these possible peptide fragments; however, cruciferin can be a substrate rich in peptides with a variety of bioactivities.

Napin

Napin proteins are 2S albumins, found mainly among *Brassicaceae* seed storage proteins and classified under prolamin protein superfamily (Shewry et al., 1995). About 20% of total rapeseed storage proteins are napin (Ericson et al., 1986; Höglund et al., 1992). The content of 2S proteins in *B. napus* varied between 25 and 45% among the European cultivars (Malabat et al., 2003). Because of the significance of amino acid composition, high content in the protein bodies, mobilization during germination, napin proteins are considered playing an important role in N and S supply of the seed (Müntz, 1998; Neuman et al., 1996a).

Napin Structure

Napins exhibit a high degree of polymorphism due to the coding by a multigene family and different proteolytic processing sites. In *B. napus*, the number of genes involved in coding of napin ranges from 10 to 16 (Scofield and Crouch, 1987;

Table 4 Summary of extraction, purification, and observed protein biochemical parameters of the globulin fraction of *Brassica* seeds

Extraction	Purification	Confirmation	References
Extraction of defatted <i>B. napus</i> (Nugget) with 10% NaCl (1:50 w/v) for 90 min at 5°C. Recovered soluble protein containing supernatant by centrifugation.	Dialysed supernatant against distilled water for 36 h. Dissolved precipitated protein in 5 mM phosphate buffer at pH 8.6 containing 5% (w/v) NaCl and separated on Sephadex G-100 using the same buffer as eluant.	The high molecular weight fraction contained 21% of total N in the salt extract. Proteins had $S_{20, \text{buffer}}$ (0.1M borate) of 12S which dissociated into 2.3S components in 6 M urea.	Bhatty et al., 1968
Extracted dehulled and defatted <i>B. juncea</i> (Varuna RT 590) and <i>B. campestris</i> (rapa) (yellow SarssonT151) with water (1:20, w/v) for 2 h at 28°C. Recovered supernatant rich in soluble protein by centrifugation.	Precipitated protein at 35% Ammonium sulfate saturation was dissolved in 1 M NaCl and dialysed against the same.	Estimated $S_{20, \text{w}}$ of <i>B. juncea</i> is 10.6 and <i>B. napus</i> is 10.2. Estimated M_r is 260 kDa by Sedimentation velocity-viscosity and Archibald method. Identified 8 protein bands between 70 and 11 kDa for SDS-PAGE under reducing conditions.	Rao et al., 1978
Extracted ground defatted <i>B. napus</i> (Sollux) (1:10, w/v) with 50 mM phosphate buffer at pH 8.0 (ionic strength maintained at 1.0 with NaCl) at room temperature for 60 min. Recovered supernatant by centrifugation.	Dialyzed protein extract against water for 2–3 days (4°C) and precipitated crude globulin was dissolved in the extraction buffer. Proteins were separated on Sephadex G-200 and protein fractions were dialyzed against 50 mM Tris-HCl, pH 8.0. Further purification was using DEAE Sephadex A-50 and elution with NaCl gradient.	Protein extracts had > 12S, 11.8S, and 2S components. After ion-exchange chromatography 11.8S protein devoid of phytates was obtained. This protein had pI of 7.5 ± 0.10 , molecular mass ~300 kDa, 704 amino acid residues, 13 disulfide bridges and 5 thiol groups all buried in the interior of protein.	Schwenke et al., 1981
Extracted defatted <i>B. napus</i> (Tandem) with 20 mM borate buffer, pH 8.0 containing 5% NaCl and 1 mM NaN_3 at 1:10 (w/v) ratio. Recovered supernatant by centrifugation.	Separation of protein fractions by Sephacryl S-300 equilibrated with the extraction buffer. Combined protein fractions were dialysed against 5 mM borate buffer at pH 8.0.	SDS-PAGE, IEF and 2D PAGE analysis revealed three major groups of polypeptides according to the separation characteristics. A: 60–52 kDa, B: B1 37–35 kDa and B2 31–29 kDa and C: 24–22 kDa which may form S-S bound subunits and free non-covalently associated polypeptides. Estimated M_r of the protein is 325 ± 10 kDa.	Dalgalarondo et al., 1986
Defatted <i>B. napus</i> was extracted (1:20, w/v) with 35 mM phosphate buffer (pH 7.6) containing 1M NaCl, 1 mM EDTA, and protease inhibitors.	Protein precipitate obtained at 10–20% $(\text{NH}_4)_2\text{SO}_4$ saturation was dissolved in and dialysed against the extraction buffer. Dialysate was separated on Sephacryl S-200 and protein fractions were screened for SDS-PAGE profiles.	Surface hydrophobicity is higher than 11S glycinin, T_m value is 84.6–85°C lower than glycinin. Insoluble between pH 3.5 and 5.7.	Salleh et al., 2002
Rapeseed meal (var Express) was extracted (1:10, w/v) with 50 mM Tris-HCl, pH 8.5 containing 750 mM NaCl, 5 mM EDTA and 0.3% NaHSO_3 for 1 h.	Pigments were removed by a desalting column. Cation exchange column elution separated cruciferin as unbound and napin as bound fraction. Further purification of cruciferin was through size exclusion (Sephacryl 300) chromatography of unbound fraction.	SDS-PAGE separation of obtained fractions showed polypeptide banding patterns of cruciferin. Sedimentation coefficient of 12 was confirmed for purified cruciferin.	Bérot et al., 2005

Josefsson et al., 1987). Four genomic sequences, napA (Josefsson et al., 1987), napB (Ericson et al., 1991), gNa (Scofield and Crouch, 1987), and BngNAP1 (Baszczynski and Fallis, 1990) and three cDNA sequences pN1, pN2 (Crouch et al., 1983), and pNAP1 (Ericson et al., 1986) have been revealed. Boutilier and others (1999) have listed all 2S protein encoding genes that are found in a variety of *Brassicaceae* plants. About 14 proteins are found in the UniProtKB/ Swiss-Prot and UniProtKB/TrEMBL (www.uniprot.org, July 2009) when “napin” is used as the search term which are originated from *A. thaliana*, *B. juncea*, *B. napus*, and *S. alba*.

According to the DNA sequences, the initial translation product of napin precursor is a polypeptide of ~21 kDa from which three short peptides are released during processing (Crouch et al., 1983; Ericson et al., 1986; Murén and Rask, 1995; 1996). The napin preprotein is synthesized on membrane-bound ribosomes, which is then translocated into the lumen

of the endoplasmic reticulum where the intrachain S-S bonds are formed. The proprotein is then transported via secretory pathway to the vacuole, where negatively charged prosequence is proteolytically cleaved and yield mature napin (Ericson et al., 1986; Murén et al., 1996). Five distinct domains have been identified in the napin precursor polypeptide; two glutamine-rich regions (within large and small subunits of mature napin) and three other domains that are different from the primary structure of mature napins (Crouch et al., 1983; Ericson et al., 1986; Murén et al., 1996). The N-terminus of napin precursor has a 21-amino acid long sequence rich in hydrophobic amino acids. First, the proprotein goes through N-terminal signal cleavage and subsequently in four other sites, to yield mature napin (Murén and Rask, 1996; Müntz, 1998). The processing of subunits passes through a cascade of proteolytic cleavage; first by endoproteases, then by amino peptidases, and finally by carboxypeptidases (Murén and Rask 1995; 1996). When the

Table 5 Peptide sequences and activities obtained from *in silico* analysis of *B. napus* cruciferin subunits (BioPep <http://www.uwm.edu.pl/biochemia> was used. Number in parenthesis indicates >1 time occurrence of particular sequence)

Activity	Protien and bioactive peptide	Sequences
Antiamnestic	P11090 (CRUA_BRANA): GP(3),PG P33523 (CRU1_BRANA): GP(2),PG(2) P33524 (CRU2_BRANA): GP(2),PG(3) P33525 (CRU3_BRANA): GP(2),PG P33522 (CRU4_BRANA): GP(2),PG(2)	
Antihypertensive	P11090 (CRUA_BRANA): AR(4),RL(3),SF(3),LA(4),LTF,HG,FP,LN(3),LKA,EA(3),AG(2),GR(3),IE(4),EV(4),VW(2),AP,PQ(7),SG(3),GV(2),VR(2),KG(2),GL(4),LY(2),YL(2),GE(3),EG(3),MG,VF(2),FQP,GG(2),GS(2),FG,GQ(12),QG(17),GH,GF(2),FR(2),QK(2),VE,HIR,IR(4),TG,GD(2),IA,HP,PG,FY(2),DG(2),PL(3),PR(2),RP(3),YL(2),NG(3),KA(3),AF,LQ,GP(3),IRP,PP,VY,YK,KP,LG,GY(2),SY(2),DLP,RF,VLP,IP,VK(2),RW,KR,RA,GLP,KF,GPA,YG,YGG,DA P33523 (CRU1_BRANA): AR(3),RL(2),SF(3),LA(4),IF,HG,FP,LN(3),LKA,KA(3),EA(2),AG(3),GR(3),IE(5),EV(4),VW(2),AP,PQ(7),SG(4),GV(2),VR(2),KG(2),LY(2),YL(2),KL,EG(3),MG,VP,PG(2),VF(2),FQP,GG(2),GS(2),GE(3),EG(3),GQ(15),QG(13),GH,GF(3),FR(2),QK(2),VE,HIR,IR(4),TG,GD(2),IA,HP,FY(2),DG(3),PL(3),PR(2),RP(3),AF,LQ,IR(4),GP(3),IRP,PP,TE,NG(4),GL(4),VY,YK,RR,KPLG,GY(2),SY(2),DLP,RF,VLP,DG(3),EA,AH,IP,VK(2),KR,RA,RW,KF,GPA,SY,YG,YGG,DA P33524 (CRU2_BRANA): AR(3),RL(2),LY(3),IF,HG(2),FP,LN(3),LKA,KA(2),EA(4),AG(3),GR(4),IE(4),EV(3),VW,AP,PQ(7),SG(3),GV(3),SF(2),VR(3),QG(19),YL(2),KG(3),MG,VP,PG(3),VF(2),FQP,GS(3),FG(2),GE(2),GQ(17),GK(3),GF(3),FY(2),NG(4),PL(3),VAV,LA(3),AF,LQ,GP(2),EG,IR(3),IRP,PP,RP(2),TE,FR(2),QK,VE,HIR,GD(2),IA(2),HP,GL(3),VY,YK,KP,LG,GY(2),SY(2),DLP,VLP,DG(2),IP,VK(2),KR,RA,RW,RR,KF,GPA,YG,PR,DA P33525 (CRU3_BRANA): VK(2),VP(2),PH,HL,HLL,FG,GV(3),LVL,LN,NG(3),LA(5),AR(5),LG(3),LQP,PT(2),TE,EA(3),AG(5),GR(4),VE(3),EY,PQ(7),IR(2),IE(2),QG(20),GL(2),YL,SY(2),MG,GM,GI(2),SG(2),PG,GQ(17),GF(3),FR,QK(2),HG,GD(2),IA(2),GS(2),IY,TG,PL(3),NY,FR(2),GG(3),VR(4),PP(2),HP,PR(2),GP(2),LQ(4),KG(2),VVRP,VRP,RP,PP(2),GPP,RA(3),VY,YK,KP,RL(2),VLP,KY,EI(2),LY(2),TQ,KG,IP,AY,NF,EW,SF,EV,AF,RR,KF P33522 (CRU4_BRANA): GP(2),PT(2),SF,LF,HG(2),LN(3),EG(2),GG(2),GR(3),IE(2),EV(2),VW,AP,PQ(8),SG(3),GE,AF(2),RF(2),IEP,QG(15),LY(2),YL,AG(3),KL(2),LTF,GH,MG(3),PG(2),VE(3),GQ(11),GF(5),FR,QK,HL,GD,IA(2),PP,GV(2),FY,NG(5),PL(3),VAA,AA(3),LRP,RP(2),LA(3),LQ(2),IF,VK(2),FG(2),IR,IRP,PP,GL(3),TE,VY,YK,KP,LG,GY(3),SY,RL,GS,VLP,KG(2),GK(3),KA,VF(2),KR,RA,EA(2),AR(2),RR,KF,EI,YG,GM	
Antithrombotic	P11090 (CRUA_BRANA): GP(3),PG, GPR, DGEA P33523 (CRU1_BRANA): GP(2),PG(2), GPR, DGEA P33524 (CRU2_BRANA): GP(2),PG(3),DGEA P33525 (CRU3_BRANA): GP(2),PG P33522 (CRU4_BRANA): GP(2),PG(2)	
Immunomodulating	P11090 (CRUA_BRANA): YGG,YG P33523 (CRU1_BRANA): YGG,YG P33524 (CRU2_BRANA): YG P33522 (CRU4_BRANA): YG	
Opioid	P11090 (CRUA_BRANA): YL(2),YG P33523 (CRU1_BRANA): YL(2),YG P33524 (CRU2_BRANA): YL P33525 (CRU3_BRANA): YL P33522 (CRU4_BRANA): YL,YG	
Immunostimulating	P11090 (CRUA_BRANA): LGY P33523 (CRU1_BRANA): LGY P33524 (CRU2_BRANA): LGY,LLY P33522 (CRU4_BRANA): LGY	
Regulating, Phosphoinositol mechanism	P11090 (CRUA_BRANA): LGY,GLY P33523 (CRU1_BRANA): LGY,GLY P33524 (CRU2_BRANA): LGY,GLY P33525 (CRU3_BRANA): GLY P33522 (CRU4_BRANA): LGY,GLY	
Regulating, Stomach mucosal membrane activity	P11090 (CRUA_BRANA): GP(3),PG P33523 (CRU1_BRANA): GP(2),PG(2) P33524 (CRU2_BRANA): GP(2),PG(3) P33525 (CRU3_BRANA): GP(2),PG P33522 (CRU4_BRANA): GP(2),PG(2)	

(Continued on next page)

Table 5 Peptide sequences and activities obtained from *in silico* analysis of *B. napus* cruciferin subunits (BioPep <http://www.uwm.edu.pl/biochemia> was used. Number in parenthesis indicates > 1 time occurrence of particular sequence) (Continued)

Activity	Protien and bioactive peptide	Sequences
Antioxidative	P11090 (CRUA_BRANA): LH,HH P33523 (CRU1_BRANA): LH,HH P33524 (CRU2_BRANA): LH(2),HH P33525 (CRU3_BRANA): HL P33522 (CRU4_BRANA): HH,HL	
Inhibitor, Neuropeptide	P11090 (CRUA_BRANA): GQ(12) P33523 (CRU1_BRANA): GQ(15) P33524 (CRU2_BRANA): GQ(17) P33525 (CRU3_BRANA): GQ(17) P33522 (CRU4_BRANA): GQ(11)	
Inhibitor, Dipeptidyl peptidase IV	P11090 (CRUA_BRANA): GP(3),PP P33523 (CRU1_BRANA): GP(2),PP P33524 (CRU2_BRANA): GP(2),PP P33525 (CRU3_BRANA): GP(2),PP(2) P33522 (CRU4_BRANA): GP(2),PP	
Inhibitor, Dipeptidyl aminopeptidase IV	P11090 (CRUA_BRANA): MA,LL(3),LA(4),FP,KA(3),HA,AP,VA(2),VV(3),LP(3),PA P33523 (CRU1_BRANA): MA,LL(3),LA(4),FP,KA(3),HA,AP,VA(2),VV(2),VP,LP(3),PA P33524 (CRU2_BRANA): MA,LL(2),LA(3),FP,KA(2),HA,AP,VA(2),VV(3),VP,LP(3),PA P33525 (CRU3_BRANA): VP(2),LL(3),VA,LA(5),LP(3),VV(5),PA,FA P33522 (CRU4_BRANA): LL(3),HA(2),AP,FA(3),LP(4),VV(3),VA(2),LA(3),KA	
Activate ubiquitin mediated proteolysis	P11090 (CRUA_BRANA): LA(4),RA P33523 (CRU1_BRANA): LA(4),RA P33524 (CRU2_BRANA): LA(3),RA P33525 (CRU3_BRANA): LA(5),RA(3) P33522 (CRU4_BRANA): LA(3),RA	

negatively charged prosequence that contributes to the neutral character of the precursor is removed a mature protein consisting of two subunits with a pI of ~11 is generated (Crouch et al., 1983). Calculated pI of napins based on amino acid sequence is 10.89 to 12.16 for the small chain and 10.25 to 11.30 for the large chain. Mature napin of *B. napus* (from BngNAP1; P17333 2SS4_BRANA) is composed of 1:1 disulfide-linked complex of ~ 4.5 kDa small (short) subunit and ~10 kDa large (long) subunit (Gehrig and Biemann, 1996; Gehrig et al., 1998). The seven napin complexes composed of large chain of 10.02 to 10.19 kDa and small chain of 4.33 to 4.43 kDa found in *S. alba* (Neumann et al., 1996a) exemplify the multiplicity of napin. A comprehensive review on the biosynthesis of napins of *Brassicaceae* plants is provided by Byczyńska and Barciszewski (1999).

A comparison of the primary structure of small and large napin chains indicates a high degree of homology differing only by few amino acids (Gehrig et al., 1998). When *B. napus* napin (napA, Napin 3, BnIII, Napin nIII, 2SS3_BRANA, P80208) is analyzed with other napin-type 2S albumins of *Arabidopsis*, *Bertholletica*, *Raphanus*, *Risinus communis*, and *Memordica charantia*, a common pattern of eight Cys motif ...C...C/...CC...CXC...C... (8CM, Fig. 4) can be identified (Rico et al., 1996; Neumann et al., 1996b). This particular motif occurs in other seed proteins which are not related to napin such as non-specific lipid transfer proteins (ns-LTP; Takishima et al., 1986) of plants and the hydrophobic protein from soybean (HPS; Odani et al., 1987; Terras et al., 1992; Polya, 1997). The

8CM proteins found in monocots (rice, maize, and barley) are primarily α -amylase and trypsin inhibitors but do not always function similarly in dicots (José-Estanyol et al., 2004). Superimposition of BnIb¹ (Monsalve et al., 1991) 3D structure with HPS shows 23 positively charged residues are located on the surface and these could be responsible for electrostatic interaction with the membrane proteins. It is believed that the 8CM protein scaffold has been evolved through modification in the region between Cys residues and provides necessary flexibility to accommodate different functionalities for 2S albumins. Examples are introduction of sequences that has inhibitory activity in cereal protease inhibitors and introduction of sequences that promote loop proteolytic cleavage in 2S albumins (José-Estanyol et al., 2004).

The secondary structure of *B. napus* napin has been observed as 25% α -helix and 38% β -sheet arrangement which is stable between pH 5.5 and 7.0 (Krzyzaniak et al., 1998). A mixture of *B. napus* (var Express) napin isomers 2SS3_BRANA(predominant isomer) and 2SS2_BRANA (P01090) or 2SSB_BRANA (P27740) (lesser extent) has exhibited 48.6 to 59.0% α -helix and 7.0 to 15.3% β -sheet structures in the pH range of 3 to 12 (Schmidt et al., 2004). Sufficient

¹This protein is similar to the protein database entry 2SSI_BRANA, P24565 (napin-1A, napin BnIa). It is one of the two low molecular weight *B. napus* proteins of BnI napin mixture described by Monsalve et al. (1991). Only differences are the variation at position 37, Glu is replaced by Gln and missing residues from position 107 to 110.



Figure 4 Primary sequences of napins of *B. napus* and allergenic proteins of *B. juncea* and *S. alba* showing multiple sequence alignment. Analysis was performed using ClustalW2 multiple sequence alignment option (www.uniprot.org). “*” residue is identical in all sequences, “:” conserved substitution is observed, and “.” semi-conserved substitution is observed. Dashes represent the gaps opened for the best alignment and the number on the right of the alignment corresponds to the sequence position of the residue of each molecule. 8CM is indicated in bold and the proposed epitope region of Sin 1 and Bra j 1 is identified in the box.

evidence exists to support high structural stability of napin in a wide pH range.

The 3D structure modelling of napin BnIb using NMR data (Fig. 5) shows a globular 4 helical motif with simple “up and down” topology with loops or turnlike regions (Rico et al., 1996). The small polypeptide forms two short helices separated by a few residues which is considered as a split helix; Helix Ia (H1a) and Ib (H1b) (Rico et al., 1996; Pantoja-Uceda et al., 2004). The large chain forms 3 helices, Helix II (HII), Helix III (HIII), and Helix IV (HIV). These four helices are arranged in a right-handed superhelix. Helix Ia and Ib run almost co-axial and connect by a non helical turn L1. The long non-helical loop L2 connects H1b and HII. The first helix of large chain HII runs antiparallel to H1a and H1b. The short interhelical loop L3 is found between HII and HIII. The HIII and HIV run almost antiparallel to each other and these two are connected by a segment of residues known as hypervariable region of 2S albumin (Krebbes et al., 1988; Raynal et al., 1991). A considerable variability in the type and number of residues is found in the members of 2S family in this hypervariable region. The HIV is followed by a long loop having no regular secondary structure. In BnI protein this folds over itself (Rico et al., 1996) and in recombinant napin BnIb (rpoBnIb) this loop packs against HIII (Pantoja-Uceda, 2004).

The two inter-chain disulfide bridges together with two intrachain bridges between Cys residues of the large chain are formed in the molecule (Rico et al., 1996). The protein core is formed by hydrophobic interaction between side chains of H1a, the interhelical loop L1, N terminus of H1b, C terminus of HII,

N terminus of HIII, and the C terminus of the tail. Another hydrophobic residue aggregate is found near the protein surface enclosing C termini of H1b and HII and middle of HIV. Three hydrophobic patches are found in the solvent accessible surface area of this molecule; 1- C terminus of H1b, N terminus of HIV and hypervariable loop, 2- in the channel between HIII and HIV, and 3- around one of the interchain S-S bond, interhelical loop L3 and C terminal tail. Most of the charged residues (7 negatively and 13 positively charged) are clustered in the molecule where negative charges are mainly located in HIII and HIV and the loop that links them. Positive charges are clustered on the loop between HII and HIII with participation of Glu residue form HI (Rico et al., 1996).

Although the global fold postulated for BnIb is similar to what is found in ns-LTP and HPS, a number of structural and functional differences are found among these proteins. Napin is derived from two polypeptide chains but ns-LTP and HPS are formed by a single polypeptide. Functionally, napin has no possibility of performing tasks of ns-LTP such as a carrier protein of lysolecithin or fatty acid (e.g., in wheat and maize; Shin et al., 1995). No apparent hydrophobic cavity has been identified in the BnIb structure or buried Tyr residues in the C-terminal which may help in forming lipid-protein complexes. Most of the Lys and Arg residues of BnIb project towards the solvent, acidic residues are located very close to each other in the proposed 3D structure, thus giving an overall positive charge to the molecule (Rico et al., 1996).

Napins show hydrophilic characters. This can be confirmed by the observation made on *B. juncea* that the napins exhibited

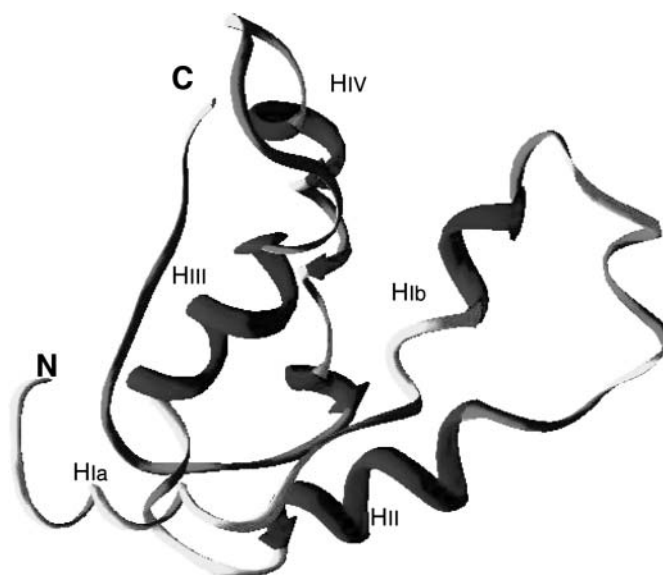


Figure 5 Schematic ribbon representation of *B. napus* napin-1A protein (P24565, 2SSI.BRANA). Three dimensional representation of P24565 primary sequence was modelled into 1sm7A template (94% identity) using SWISS-MODEL (<http://swissmodel.expasy.org/>) and the model was aligned and rendered in DeepView. The N indicates NH₂- and C indicates COOH terminal. The Hla and HII, HIII and HIV indicate Helix I, Helix II, Helix III and Helix IV, respectively.

low binding constants with the extrinsic fluorescence probes (~ 0.5 mol binding to 1 mol of protein with ANS, TNS, CPA, and PRODAN) and the binding constants decreased when 0.5 M NaCl is present (Jyothi et al., 2007a). The low hydrophobicity and net positive charge at neutral pH suggests that napin is a weakly folded molecule driven largely by its S-S bonds. Napin of *B. juncea* bears a stable structure up to 75°C, beyond that formation of napin aggregates with low stability occurs (Jyothi et al., 2007b). Ionization of side chains caused by increase in the pH may also lower thermal stability of napin. Napin monomers of 15 kDa is evident between 10 and 37°C and has a tendency to form dimers beyond 40°C as indicated by a thermal transition starting at 48°C. These states of *B. juncea* napin are reversible as the thermal transitions occur at 50.5 and 62.8°C are completely reversible. Complete dissociation unfolding of napin molecule may occur at after 75°C and at 83°C no change of free energy (ΔG) of the molecule is observed (Jyothi et al., 2007b). The limited total accessible surface area (7032 Å²) of the molecule, and considerably high ΔG_{H_2O} and ΔC_p of napin compared to the proteins having similar molecular weight and S-S bridges (e.g., RNase, lysozyme, and α -lactalbumin) may be due to the presence of both inter- and intra-disulphide bonds and internal repeats in the structure which also contribute to the overall stability of the molecule (Jyothi et al., 2007a; 2007b).

Isolation and Purification of Napin

Napins are soluble in water and could be recovered as water soluble seed components. Extraction of napin can be achieved with water or neutral pH buffers (phosphate or borate, pH 7–8; Lönnnerdal and Janson, 1972; Simard and Boulet, 1978; Monsalve and Rodríguez, 1990). Subsequent precipitation with ammonium sulfate (60 to 45% saturation; Simard and Boulet,

1978) has given high protein recovery but with possible pigment contamination. For further purification of protein, size exclusion (Sephadex G 50) and cation exchange (SE-Sephadex C25) chromatography (Lönnnerdal and Janson, 1972; Monsalve and Rodríguez, 1990) have been employed. Use of 1 M NaCl containing Tris buffer (50 mM, pH 8.5 with sodium sulfite and EDTA) for extraction and further purification through a series of size exclusion, cation exchange, and hydrophobic interaction chromatography have been described by Bérot et al. (2005) to obtain purified napin of *B. napus* (purity 84.6% and yield 5%; Schmidt et al., 2004). Table 6 summarizes the conditions used for isolation of napin by various research groups.

Allergenicity of Brassicaceae 2S Proteins

Proteins that trigger development of allergic response through the gastro-intestinal tract belong either to cupin or prolamins superfamilies. Almost all plant food allergens known are either protective or seed storage proteins. The 2S albumin is one of the three major groups of allergenic proteins of prolamins superfamily and found in *Brassicaceae* seeds. Napins of mustard (*B. juncea* and *S. alba*) have been reported in association with several allergic responses such as induction of celiac disease and baker's asthma (Monsalve et al., 2001). In general fatal anaphylactic reactions to mustard seeds is rare (Monreal et al., 1992); however, its allergenic potential has resulted in the European Union listing mustard as one of the allergenic food ingredients (EU Directive 2003/89/EC). Monsalve and group (2001) have reviewed clinical studies that confirm allergenicity development concerning mustard napins. Recently oilseed rape and turnip rape has also been reported to contain allergenic proteins in the 2S fraction (Puumalainen et al., 2006; Poikonen et al., 2008).

Table 6 Summary of extraction, purification and biochemical parameters observed for proteins of the *Brassica* albumin fraction

Extraction	Purification	Confirmation	References
Defatted rapeseed flour was extracted with water adjusted to pH 8.0 at 1:10 (w/v) and solubles were recovered by centrifugation.	Protein was precipitated by 90% ammonium sulphate saturation and 2S proteins were dissolved at 40 to 55% ammonium sulfate saturation at 24°C.	Sedimentation coefficient by ultracentrifugation.	Simard and Boulet, 1978
Defatted rapeseed flour (var Solloux) was extracted with phosphate buffer (50 mM, pH 8, 1:10 w/v) and ionic strength of 1 with NaCl. Solubles were recovered by fraction.	Protein was precipitated by 90% saturation of ammonium sulfate. Albumin of the precipitate was dissolved between 35% ammonium sulfate saturation. Soluble proteins were exhaustively dialysed against distilled water and lyophilization of soluble fraction.	PAGE and IEF.	Raab and Schwenke, 1984; Nitecka et al., 1986.
Crushed seeds (<i>B. rapa</i> , <i>B. napus</i> or <i>S. alba</i>) in liquid nitrogen was extracted with Na borate buffer (150 mM, pH 8, 1:10, w:v). Soluble fraction was recovered by centrifugation and lyophilization.	Dry powder was deoiled with an acetone (1:10, w/v) wash. Precipitated protein was dissolved in ammonium bicarbonate buffer at pH 8.0 and separated on Sephadex G-50 and eluted with same buffer. 2S proteins (2 nd protein peak) was dialyzed against 3 mM pyrophosphate -HCl buffer at pH 9.0 and purified by SP-sephadex C-25. Napin was eluted by pyrophosphate HCl buffer gradient of 3 to 50 mM at pH 9.	Molecular weight estimation by gel filtration and SDS -PAGE, 12.5 kDa.	Menendez-Arias et al., 1987; Monsalve and Rodriguez, 1990
Defatted rapeseed flour extracted with Tris-HCl (50 mM, pH7) containing NaCl at 1:20 (w/v) for 2h at ambient temperature, and centrifuged to recover soluble protein extract.	Extensive dialysis at 4°C to remove salt. Cruciferin precipitated and napin remained in the supernatant was recovered by centrifugation and lyophilized.	SDS-PAGE under non reducing conditions showed single band between 12–14.5 kDa.	Folawiyo and Owusu-Apenten, 1997
Rapeseed meal (var Express) was extracted (1:10, w :v) in 50 mM Tris-HCl, pH 8.5 containing 750 mM NaCl, 5 mM EDTA and 0.3% NaHSO ₃ for 1 h.	Pigments were removed by a desalting column. Cation exchange column elution separated cruciferin as unbound and napin as bound fraction. Further purification of the bound fraction containing napin was through hydrophobic interaction (Phenyl Sepharose) chromatography.	SDS-PAGE separation of obtained fractions showed polypeptide banding patterns of napin (<14 kDa).	Bérot et al., 2005
Defatted <i>B. juncea</i> meal extracted with phosphate buffer (20 mM, pH 7.0) containing 1M NaCl (1:10 w:v). Recovered soluble proteins By centrifugation.	Protein was precipitated at 15 to 40% Ammonium sulfate saturation. Precipitated protein was dissolved in same extraction buffer and dialyzed against the same buffer. Dialyzed protein separated on Sephadex G-50 and eluted with same buffer. Protein peak was dialyzed against phosphate buffer without NaCl and separated on CM-Sephadex and elution was with 0 to 0.5M NaCl gradient.	SDS-PAGE showed 14.5 kDa single band under nonreducing condition and 2 bands between 6.5 and 3.5 kDa upon reducing. MALDI-TOF analysis gave molecular mass of 14.724 kDa and identical residues (1 st eight residues of both chains) with the published sequence.	Jyothi et al., 2007a.

Four proteins of *Crucifer* 2S fraction have been identified as allergenic (<http://www.allergen.org/Allergen.aspx>, update 12 Feb, 2010). They are; Sin a 1 (P15322, ALLI.SINAL; UniProtKB/Swiss-Prot entry) of *Sinapis alba* (Menéndez-Arias et al., 1987; Menéndez-Arias et al., 1988), Bra j 1 (*Bra j* I; P80207, ALLI.BRAJU) of *Brassica juncea* (González de la Peña et al., 1991), Bra n 1 (Napin BnIII, napin nIII, napin 3; P80208, 2SS3.BRANA) (Monsalve et al., 1997) of *B. napus*, and Bra r 1 (Q42473, BRACM; UniProtKB/Swiss-Prot entry) of *B. rapa* (Dasgupta et al., 1995).

Allergenic napin of *B. napus* was first identified as a potential occupational allergen (Monsalve et al., 1997) apart from being for an individual with prolonged exposure to flours of cereals, rapeseed, soybean, and sunflower. A recent study carried out with children having IgE mediated food allergy and reacted to seeds of *B. napus* and *B. rapa* in skin prick tests led to confirm that the allergenic proteins are napin Bra n 1 and Bra

r 1, respectively (Puumalainen et al., 2006; Poikonen et al., 2008). The sequences of Bra j 1 and Bra n 1 show 86 and 92% identity and 91 and 94% similarity, respectively with Sin a 1 (Monsalve et al., 2001) after sequence alignment. When defined allergens such as peanut Ara h 2 and Ara h 6, brazil nut Ber e 1, and sunflower SFA 8 are matched for sequence homology and alignment, the major oilseed allergens SFA 8 and Ara h 2 (found in 90% of patients allergic to peanuts) show 14% (for both) identity and 29% and 31% of similarity, respectively to Sin a 1, which is borderline homologous to these two proteins (Monsalve et al., 2001).

Immunological mapping of Sin a 1 by competition and complementation assays using 10 specific monoclonal antibodies has revealed that it contains two immunodominant regions (Menéndez-Arias et al., 1990). A continuous epitope 2B3 (Menéndez-Arias et al., 1990) on the large chain of Sin a 1 has been recognized by monoclonal antibodies (mAb) raised against

Sin a 1 and this specific allergenic determinant has been found in Bra j 1 as well (Fig. 4). However, recognition of *B. napus* Bra n 1 (P80208, 2SS3_BRANA, BnIII) by this mAb has not been possible (Monsalve and Rodríguez, 1990). The region to which the mAb binds is in the hypervariable region and includes the only Tyr residue of the molecule which is found in the large polypeptide chain (Monsalve and Rodríguez, 1990). The variability of the hypervariable region of *Crucifer* 2S proteins is clear as long variable gaps in the sequences have been opened in this region to achieve the best alignment with Bra j 1 and Sin a 1 (Fig. 4). The variation in the primary structure of Sin a 1 and Bra j 1 compared to Bra n 1 particularly by the His residue in this region (Fig. 4) may be responsible for the difference in recognizing the continuous epitope. Comparison of 3D models of Sin a 1 and *B. napus* napin isomer BnIb (P24565; 2SSI_BRANA, napin BnIa or napin Ia, not reported as allergenic) shows that the hypervariable region of Sin a 1 is longer than the BnIb, indicating that the folding process of the core molecular structure is independent of the length or sequence variability of the loop and it is an important antigenic region of 2S albumins (Monsalve et al., 2001).

The presence of 8CM has been listed as another common feature of the allergenic 2S albumins. Using recombinant pro-BnIb, Pantoja-Uceda and group (2002; 2004) have explained that the existence of four S-S bonds allows tight packing and forming of a compact structure that give napin special resistance to proteolytic enzyme access and thermal unfolding. Limited unfolding that occurs, due to heat and resistance to degradation in the gastric fluid enzymes, indicate that this protein can reach gut immune system intact. Strong interaction of Sin a 1 with phospholipid vesicles (Oñaderra et al., 1994) also indicates a significant character of a food allergen that can result in increased cellular uptake, reduced neutralization by secretory antibodies, and lowered degradation in the blood stream. A recombinant precursor of Sin a 1 expressed in *Pichia pastoris* has retained the biochemical and immunological features of the native protein and has been recognized by 75% of the patients allergic to mustard (Palomares et al., 2005).

In addition to 2S proteins, 11S protein Sin a 2 (Palomares et al., 2005), nS-LTP Sin a 3 and profilin protein Sin a 4 (Sirvent et al., 2009) have been reported as allergenic proteins found in *S. alba* seed. Two proteins of *B. napus* and *B. rapa* have been identified as potentially allergenic and can be recognized by IgE of *Brassica* pollen allergic patients (Toriyama et al., 1995). These proteins carry amino acid sequences having two regions similar to Ca^{+2} -binding sites of Ca^{+2} -binding proteins such as calmodulin but with different flanking regions other than calmodulin or known Ca^{+2} -binding proteins. Such findings suggest that the allergenic protein of *Brassica* pollen is different from what has been identified as the aeroallergen of seed meal.

Antifungal Activity

Unlike cruciferin, napin-like proteins are involved in other biological functions besides the inactive storage routine. These

proteins exhibit significant antimycotic and antibacterial properties. They act synergistically with β -thionins, enhancing the antimicrobial activities of Gln-rich proteins and cause fungal membrane damage (Polya, 1997). The 2S albumins of *B. napus*, *B. rapa*, and *S. alba* have exhibited substantial antifungal activities (Terras et al., 1993a) against fungi such as *Alternaria brassicola*, *Pyricularia*, *Fusarium spp.*, and *Verticillium dahlia*. The antifungal activity of 2S proteins is believed to be due to direct interaction with the targeted structure and protein. Furthermore, together with Barley thionins, the large or small subunit of radish (*Rhaphanus sativus*) 2S protein acts synergistically to enhance antifungal activity (Terras et al., 1993b). The napins of mustard have exhibited strong interactions with phospholipids indicating that the permeability of fungal or bacterial membrane can be induced. The mechanism of this activity has been postulated as perturbation of lipid membrane due to the cationic surface character ($\text{pI} > 9.5$) of napin that neutralizes the negative charge of the phospholipids (Oñaderra et al., 1994).

Both large and small chains (subunits) of napin exhibit calmodulin-antagonist ability and are substrates for calmodulin dependant plant kinases (Neumann et al., 1996a; 1996b; 1996c). Calmodulin and small subunits of napin contains similar α -helix-hinge- and α -helix motif in their structures (Neumann et al., 1996a). The 2-sided (amphipathic) α -helix of napin in which basic (polar) residues are confined to one side similar to the calmodulin antagonist peptides such as melittin may be the functional feature of such activities. The competitive inhibition due to the binding of a small subunit of napin to kinases may also be responsible for this activity (Neumann et al., 1996b; 1996c). The Ser residue in the non-helical C-terminus of napin small subunit can be phosphorylated by calmodulin-dependant plant kinases as well as the Ser residue of C-terminus of amphipathic α -helix on the large napin subunit (Neumann et al., 1996a; 1996b). Such phosphorylation incidences may occur near the site of precursor processing; two Ser residues each on the short and the long chain (Neumann et al., 1996c; 1996d). The protein cleavage due to trypsin activity is affected by the phosphorylation of Ser residues (Ser⁶⁰) of the large chain. Prevention of proteolysis by trypsin due to the phosphorylation in these regions may allow napin to act as a protease/or protein kinase inhibitor which may also pose antifungal activity (Neumann et al., 1996a; 1996b; 1996c; Polya, 1997).

Trypsin Inhibitory Proteins of Crucifers

Napins of *Brassicaceae* species exhibit resistance to α -trypsin and chymotrypsin catalyzed digestion indicating the inaccessibility of the cleavage sites. In addition, serine protease inhibitors that belong to low molecular mass trypsin inhibitor class have been identified from *S. alba* (mustard trypsin inhibitor, MTI) and *B. napus* (rapeseed trypsin inhibitor, RTI). These proteins are homologous to one another and lack any apparent relationship to other trypsin inhibitor protein families such as Kunitz- or Bowman-Birk type. Three distinct classes of

P26780-1	DEF_SINAL	MAMAKKSVSSFTLIFILVLVIFEVPEIKAQDSECLKEYGGDVGFPPFCAPRIFFPTICYTRC	60
Q53WR2	Q53WR2_SINAL	MAMAKKSVSSFTLIFILVLVIFEVPEIKAQDSECLKEYGGDVGFPPFCAPRIFFPTICYTRC	60
Q2MD63-1	Q2MD63_BRANA	--MAIKPLSIFVVFVFFLVISDMPEIEAQDSKCLREYGGDVGFPPFCAPRIFFPTICYTRC	58
Q42328	DF195_ARATH	--MAKAIVSIFVVFVFFLVISDVPEIEAQGNECLKEYGGDVGFPPFCAPRIFFPTICYTRC	58
Q2MD61-1	Q2MD61_BRANA	MDMATKSVSSLAFLILFLVIFEMPEIEAQDSECLKEYGGDVGFPPFCAPRIYPSFCVQRC	60
O22867-1	DF194_ARATH	--MAMKSVSNFAIFLILFLVTSEISEIEAKDKECLKGY-IDAPLSYCMARIYPSLCYRNC	57
		** : * : . : : * : * * : : . * * : * : * : * : * : * : * : *	
P26780-1		RENKGAKGGRCIWGEGTNVKCLCDYCNDSPFDQILRGGI	99
Q53WR2		RENKGAKGGRCIWGEGTNVKCLCDYCNDSPFDQILRGGI	99
Q2MD63-1		RENKGAKGGRCRWGQGTNVTCLCDYCNDQP-----	89
Q42328		RENKGAKGGRCRWGQGSNVKCLCDFCDTTPQ-----	90
Q2MD61-1		RADKGALSGKCIWGGGNVKCLCNFCRHEPG-QILSG-I	97
O22867-1		RFYKGAKGGKCC--DG--LKCFCDFCSDKPF-----	84
		* * * * . * : * : * : * : * : * : *	

Figure 6 Comparison of mustard (P26780-1, DEF_SINAL and Q53WR2, Q53WR2_SINAL) and rapeseed (Q2MD63-1, Q2MD63_BRANA and Q2MD61-1, Q2MD61_BRANA) trypsin inhibitors with *Arabidopsis* (Q42328, DF195_ARATH and O22867-1, DF194_ARATH) trypsin inhibitors. Reactive site (Wilson, 1997) is indicated in the box. Multiple sequence alignment description is similar to Fig. 4.

inhibitors are found in *B. napus*; RTI-I and RTI-II are thermolabile, have molecular mass about 19 kDa, and selectively inactivate trypsin (Visintin et al., 1992), and RTI-III is thermostable, has molecular mass of 6.5 kDa, and inactivates both trypsin and chymotrypsin (Cecilian et al., 1994). In *S. alba*, a high molecular weight (~18 kDa) mustard trypsin inhibitor MTI-1 that inhibits β -trypsin but no activity toward α -chymotrypsin has been identified (Menegatti et al., 1985). MTI-1 is rapidly deactivated upon heating at 80°C. Another inhibitor, MTI-2, has molecular weight about 7 kDa and found in heat treated *S. alba* extract (Menegatti et al., 1992). MTI-2 isolated from non-thermally treated *S. alba* extract consists of a mixture of trypsin inhibitory peptides (at least 5) which act on both β -trypsin and α -chymotrypsin with 1:1 stoichiometry (Ruoppolo et al., 2000). MTI-2, RTI-III, and ATTI-1 (in *Arabidopsis thaliana* as transcribed genes) are highly homologous (Fig. 6), thermostable, and have molecular mass ~7 kDa. RTI-I, RTI-II, and RTI-III represent 4–10%, 13–33%, and 60–85% of the total serine protease inhibitory activity, respectively (Visintin et al., 1992). These proteins consist of single polypeptide chains with four intra-chain disulfide bonds. When the S-S bonds are disrupted, the trypsin inhibiting activity of *S. alba* napin fraction is diminished indicating small and large polypeptide subunits alone do not inhibit trypsin (Gehrig and Biemann, 1996; Neumann et al., 1996 a). RTI-II is found as isoinhibitors namely, 5-oxoPro1-Gly62-RTI-III, Asp2-Pro61-RTI-II, and Ser3-Pro61-RTI-III (Ascenzi et al., 1999).

7S Proteins of Crucifers

The 7S proteins have a trimeric structure and exclusively mentioned in legumes. These proteins are composed of three subunits of Mr between 50 and 70 kDa and have overall molecular mass of 150 to 200 kDa and lack S-S bonds (Shewry, 1998). Although there are no reports of the existence of 7S proteins in the *Brassicaceae* family, several partial complementary DNA (cDNA) sequences or expressed sequence tags (ESTs) have

been identified in *Arabidopsis* on the basis of similarities with pea vicilin and related legume 7S cDNA sequences (Delseny and Raynal, 1999). These available sequences are partial and correspond to incomplete clones; however, their high degree of matching with the sequences of other cotyledonous 7S vicilin-like proteins proves their identity. In *Arabidopsis* at least 1 to 2 expressed genes related to 7S proteins have been discovered (Delseny and Raynal, 1999; Shewry and Casey, 1999a; 1999b) although there are no reports on the presence of 7S proteins in mature *Crucifer* seed.

OTHER COMPONENTS ASSOCIATED WITH CRUCIFER SEED PROTEINS

Crucifer oilseeds have several other molecules stored besides lipids and proteins. Table 8 provides detailed breakdown of chemical components and their average levels found in oil removed drymass (meal) of Canadian canola.

Phytates

In protein storage vacuoles of *Brassicaceae* seeds, phytates (salts of Ca, Mg, and K of *myo*-inositol 1,2,3,4,5,6-hexakis-dihydrogen phosphate or phytic acid) are accumulated as one or few large insoluble crystals and referred to as globoids (Raboy, 2003). Phytic acid (PA) has six phosphate groups and 12 dissociable protons in the molecule; six strongly dissociate with pK of 1.92 (three), 2.38 (two), and 3.16 (one), three function as weak acids with pK_a of 5.20, 6.25, and 7.98, and three feebly dissociate with pK_a of 9.19 (one) and 9.53 (two) (Schwenke, 1994). Therefore, phytic acid undergoes strong electrostatic interactions with rapeseed proteins at pHs below their isoelectric points (pI of napin ≥ 10 , and cruciferin ~7.2). In alkaline pH both phytate and cruciferin have a net negative charge and become completely dissociated from each other. Phytic acid forms insoluble complexes with proteins and may act as a protein pre-

Table 7 Peptide sequences and activities obtained from *in silico* analysis of selected *Brassicaceae* napins using BioPep (<http://www.uwm.edu.pl/biochemia>). Number of occurrences (> 1) of the peptide sequence is in parenthesis

Activity	Protien and bioactive peptide	Sequences
Antiamnestic	P01092 (2SS2_BRANA): GP(3),PG,PGP P01091 (2SS1_BRANA): GP(2),PG,PGP P27740 (2SSB_BRANA): GP(3),PG,PGP P17333 (2SS4_BRANA): GP(3),PG,PGP P80208 (2SS3_BRANA): GP(3),PG,PGP P15322 (ALL1_SINAL): GP(5),PG,PGP P80207 (ALL1_BRAJU): GP(4),PG,PGP P24565 (2SSI_BRANA): GP(3)	
Antihypertensive	P01092 (2SS2_BRANA): NK,NKL,KL,LF,LA,AF,FFL,IY(2),VE,DA,AG,FR,IP(2),KE,HL(2),RA,HK,SG,GG(2),GP(3),GE,ME,PQ(3),QG((3),RP,PP,LQ,LQQ,PL,PT,KG,GA,KA,VK,GQ,RIY,PG,PSY,SY P01091 (2SS1_BRANA): KE,HL(2),KA(2),LKA,HK,SG,GG(2),GP(2),DG,GE,ME,EK,QG(2),GP(2),RP,PP,PL(2),LQ,LQQ,PT,VK,GK,RIYIY,IP,PQ,QK,PG,PSY,SY P27740 (2SSB_BRANA): NK,NKL,KL(2),LF,LA,AF,FFL,IY(2),VE,DA,TNP,AG,FR,IP(2),KE,HL(2),LKA,KA(2),HK,SG(2),GS,GP(3),GE,ME,PQ(3),QG(3),RP,PP,LQ(2),LQQ,PL,PT,KG,GA,VK,GQ,RIY,PG,PSY,SY P17333 (2SS4_BRANA): NK,NKL,KL,LF,LA,AF,FFL,IY,VE,EV,DA,TNP,AG,FR,IP,KE,HL(2),LKA,KA(2),HK,SG(2),GS,GP(3),GE,ME,PQ(3),QG(3),RP,PP,LQ(2),LQQ(2),PL,PT,KG,GA,VK,VR,GQ,QK,PG,PSY,SY P80208 (2SS3_BRANA): AG,GP(3),FR,IP(2),KE,HL(2),RA(2),HK,SG(2),GS,PQ(2),QG(3),RP,PP,PL(2),LQ(2),LQQ,PT,KG,GA,VK,VR,GQ(2),RIY,IY,QK,PG P15322 (ALL1_SINAL): AG,GP(5),FR,IP,KE,HL(3),RA,HK,SG(2),GS,ME,PQ(2),QG(3),RP,PP,PL(2),LQ(2),LQQ,PT,KG,GA,KA,VK,VR,LG,GQ,PH,RIY,IY,IR,P P80207 (ALL1_BRAJU): AG,GP(4),FR,RF,FP,PR(3),KE,HL(2),HK,SG(2),GS,PQ(3),RP,PP,PL(2),PT,KG,KA,IR,QG(3),GQ(2),VK,LQ(2),EI,IP,QK,PG P24565 (2SSI_BRANA): PQ(2),QK,HL,RA,IR,LA,AG,GS,SG,GP(3),EG,LY,PT,AA,VR,QG,GQ,HG,RIY,IY,IA(2),IAK,IG,GT,IAIP,AIP,IP,FP	
Antithrombotic	P01092 (2SS2_BRANA): GP(3),PG,PGP P01091 (2SS1_BRANA): GP(2),PG,PGP P27740 (2SSB_BRANA): GP(3),PG,PGP P17333 (2SS4_BRANA): GP(3),PG,PGP P80208 (2SS3_BRANA): GP(3),PG,PGP P15322 (ALL1_SINAL): GP(5),PG,PGP P80207 (ALL1_BRAJU): GP(4),PG,PGP P24565 (2SSI_BRANA): GP(3)	
Regulating, Stomach mucosal membrane activity	P01092 (2SS2_BRANA): GP(3),PG,PGP P01091 (2SS1_BRANA): GP(2),PG,PGP P27740 (2SSB_BRANA): GP(3),PG,PGP P17333 (2SS4_BRANA): GP(3),PG,PGP P80208 (2SS3_BRANA): GP(3),PG,PGP P15322 (ALL1_SINAL): GP(5),PG,PGP P80207 (ALL1_BRAJU): GP(4),PG,PGP P24565 (2SSI_BRANA): GP(3)	
Antioxidative	P01092 (2SS2_BRANA): HL(2),LH(2) P01091 (2SS1_BRANA): HL(2),LH P27740 (2SSB_BRANA): HL(2),LH(2) P17333 (2SS4_BRANA): HL(2),LH(2) P80208 (2SS3_BRANA): HL(2),LH(2) P15322 (ALL1_SINAL): HL(3),LH(2) P80207 (ALL1_BRAJU): HL(2),LH(2) P24565 (2SSI_BRANA): HL	
Inhibitor, Neuropeptide	P01092 (2SS2_BRANA): GQ P01091 (2SS1_BRANA): GQ P27740 (2SSB_BRANA): GQ P17333 (2SS4_BRANA): GQ P80208 (2SS3_BRANA): GQ(2) P15322 (ALL1_SINAL): GQ P80207 (ALL1_BRAJU): GQ(2) P24565 (2SSI_BRANA): GQ	
Inhibitor, Dipeptidyl peptidase IV	P01092 (2SS2_BRANA): GP(3),PP P01091 (2SS1_BRANA): GP(2),PP P27740 (2SSB_BRANA): GP(3),PP	

(Continued on next page)

Table 7 Peptide sequences and activities obtained from *in silico* analysis of selected *Brassicaceae* napins using BioPep (<http://www.uwm.edu.pl/biochemia>). Number of occurrences (>1) of the peptide sequence is in parenthesis (*Continued*)

Activity	Protein and bioactive peptide	Sequences
Inhibitor, Dipeptidyl aminopeptidase IV	P17333 (2SS4_BRANA): GP(3),PP	
	P80208 (2SS3_BRANA): GP(3),PP	
	P15322 (ALL1_SINAL): GP(5),PP	
	P80207 (ALL1_BRAJU): GP(4),PP	
	P24565 (2SSI_BRANA): GP(3)	
	P01092 (2SS2_BRANA): MA,LA,LL,VV,KA,LP,MP	
	P01091 (2SS1_BRANA): KA(2),MP	
	P27740 (2SSB_BRANA): MA,LA,LL(2),VV,PA,KA(2),LP,MP	
	P17333 (2SS4_BRANA): MA,LL,LL(2),PA,KA(2),LP,MP	
	P80208 (2SS3_BRANA): LL,LP,MP	
	P15322 (ALL1_SINAL): PA,LL,KA,LP,MP	
	P80207 (ALL1_BRAJU): FP,LL,KA,LP,MP	
	P24565 (2SSI_BRANA): LA,LP,FP	
Activate ubiquitin mediated proteolysis	P01092 (2SS2_BRANA): LA,RA	
	P27740 (2SSB_BRANA): LA	
	P17333 (2SS4_BRANA): LA	
	P80208 (2SS3_BRANA): RA	
	P15322 (ALL1_SINAL): RA	
	P24565 (2SSI_BRANA : RA,LA	

cipitating agent. The pH is a crucial factor that determines the extent of phytic acid extraction from plant tissue matrix and its complexation with proteins. Maximum precipitation of phytic-protein complexes happen with both cruciferin and napin at low pHs (Schwenke et al., 1987) although zero binding is possible at pH values corresponding to the isoelectric region of both proteins (Gillberg and Tornell, 1976a; Schwenke et al., 1987). When pH is between pH 6 and 8, negatively charged oligomeric napin-phytic acid association occurs predominantly as a “dimer” composed of two napin and four phytic acid molecules (Mothes et al., 1990). Rapeseed primarily contains *myo*-inositol hexakisphosphate (IP₆) and *myo*-inositol pentakisphosphate (IP₅) which are in the ranges of 15 to 21 mg/g and 1 to 2 mg/g, respectively for commercial oil meals (Matthäus et al., 1995).

Phytic acid has been considered as an antinutrient because of its strong cation binding ability that may cause binding of essential dietary minerals including calcium, iron and zinc as well as proteins and starch consequently reducing their bioavailability in the digestive tract. IP₆ is believed to be degraded by microbial phytase to a limited level in the colon of mammals (Cooper and Gowing, 1983; Wise et al., 1983). Removal of phosphate groups from the inositol ring decreases the mineral binding strength of IP_n and increases the solubility (Sandberg, 2002). Degradation of rapeseed meal phytates is affected by calcium levels in the diet (Sandberg et al., 1993). At low calcium levels (4.5 g/day) in the diet degradation of IP₆ could be at 97% level; however, high calcium level such as an amount of 15 g/day could lower degradation as low as 42%. This may possibly be due to the insoluble phytate-Ca complexation. The removal or reduction of phytate level in canola/rapeseed protein products can be reasonably argued based on the negative nutritional effects of mineral complexation. Ultrafiltration/diafiltration has

been effective in reducing phytic acid levels when high salt concentrations (0.7–0.9 M NaCl, 0.23 M CaCl₂) in the extraction medium weaken the electrostatic interaction of phytate with proteins (Thompson, 1990). Extraction of rapeseed meal at pH 4.0 followed by counter-current extraction of protein at pH 11.0 and subsequent precipitation of protein at pH 4.7 has eliminated most of PA in the protein isolates (0.04% PA); however, this resulted in a low protein extraction yield and a darker color product (Blaicher et al., 1983). Rapeseed protein acylation studies showed that low level of acylation such as at 21% can cause slight unfolding of the proteins and exposure of additional binding sites to the PA molecule and consequent increase in the protein-mineral-PA complexation (Thompson, 1987). Use of phytases and phytic acid degrading enzymes (e.g., from microbial sources or over expression in seeds) has been used by few groups to reduce phytate levels in canola meal or protein products (Ledoux et al., 1998; Maenz et al., 1999; 2004).

Despite the antinutritional properties reported for phytates, beneficial effects have also been reported. IP₆ acts as a natural antioxidant due to its chelating properties and reduction of catalytic activities of divalent transition metals (Graf et al., 1987; Thompson, 1990; Thompson and Zang, 1991). The chelation activity of PA is linked to the suppression of iron-mediated oxidation reactions in the colon which may help in reducing the colon cancer risk (Graf et al., 1987; Graf and Eaton, 1993; Shamsuddin, 1995). IP₆ can provide anticancer function by exerting antineoplastic activity via entering into a cellular inositol phosphate pool and affecting common cellular signal transduction pathway thereby decreasing cell proliferation as described by Graf and Eaton (1993). Therefore, the dephosphorylation and rephosphorylation of IP₆ and inositol are important functions that facilitate signal transduction. The ability to bind

Table 8 The chemical composition of meal and amino acid content of protein of Canadian canola. All values are% on 10% moisture basis unless specified. Amino acids are% of crude protein. (Adapted from Bell, 1993 and Newkirk, 2009)

Component	Content
Crude protein (%N × 6.25)	34.0
Ala	4.36
Arg	5.78
Asp+Asn	7.25
Cys	2.39
Glu+Gln	18.14
Gly	4.92
His	3.11
Ile	2.30
Leu	7.06
Lys	5.56
Met	2.06
Met+Cys	4.44
Phe	3.83
Pro	5.97
Ser	4.00
Thr	4.39
Trp	1.33
Tyr	3.22
Val	5.47
Ether extract	3.8
Ash	4.8
Crude fiber	12.0
Acid detergent fiber	17.2
Neutral detergent fiber	21.2
Total dietary fiber	33.0
Oligosaccharides	2.3
Sucrose	6.9
Non-starch polysaccharides	
Soluble	4.6
Insoluble	1.4
Tannins	14.7
Sinapine	1.5–3.0
Phytic acid	0.6–1.8
Glucosinolates (total)	3.0–6.0
Aliphatic	14.1 μ moles/g
Indole	4.2 μ moles/g

into biopolymers, especially to dietary starches affects the solubility, digestibility, and absorption of the carbohydrate thus affecting the colonic environment and the products of digestion and fermentation. For example trapped carbohydrates stimulate the production of beneficial short chain fatty acids (Thompson et al., 1987). In the light of these beneficial effects, the association of phytates and PA with *Crucifer* proteins warrants further investigation.

Phenolic Compounds

Crucifer seeds store a wide variety of phenolic compounds including phenolic acids in the free and esterified form and condensed tannins (proanthocyanidins). The total extractable content of phenolics ranges from 1592 to 1837 mg/100 g of defatted canola meal and varies from 624 to 1281 mg/100 g of

flour (Dabrowski and Sosulski, 1984; Nacz and Shahidi, 1989; Nacz et al., 1998).

Phenolics of rapeseed flour occur as hydroxylated and/or methoxylated derivatives of benzoic and cinnamic acids, constituents of dietary fiber, and esters of glucose, sucrose, malate, and choline. Sinapic acid (3,5-dimethoxy-4-hydroxy cinnamic acid) is the abundant phenolic acid in rapeseed and constitutes over 73% of free- and 99% of esterified- (released from esters and glucosides) phenolic acids (Dabrowski and Sosulski, 1984). Free sinapic acid also exists and contributes about 16% of the total phenolics. Sinapine, the choline ester of sinapic acid, is widely found in canola and mustard. In *B. napus* seed, sinapine constitutes about 80% of the total phenolics (Dabrowski and Sosulski, 1984) and it is found to be in the range of 6.8–10 mg/g seed in European cultivars (Matthäus, 1998) and 13–15 mg/g defatted meal for Australian cultivars (Mailer et al., 2008). A number of other phenolic-choline esters such as, feruloyl choline, isoferuloyl choline, coumaroyl choline, 4-hydroxy benzoyl choline, and 3,4-dimethoxy benzoyl choline are also found in the seeds of the *Brassicaceae* family (Larsen et al., 1983). Glycosidic ester of sinapic acid, glucopyranosyl sinapate has also been reported for rapeseed (Wanasundara et al., 1994; 1995).

The phenolic compounds in the cotyledons and seed coat are the major contributors to the dark color and bitter, astringent taste of the meal, which remains in the alkali extracted protein products. The phenolic-protein complexes of rapeseed can change physicochemical and functional properties of the protein (Spencer et al., 1988; Schwenke and Dabrowski, 1990). Both phenolic acids and condensed tannins are easily extractable with proteins under alkaline conditions (pH 7.5 to 12.5). Interaction of phenolics with proteins is predominantly by ionic bonding (>50% of extractable phenolics) and hydrophobic interactions. The H-bound or covalently bound proportion of phenolics are <10% of the extractable amount, and the protein-phenol complexes can create a dark color in canola protein products (Xu and Diosady, 2000). A low seed coat lignin concentration, reduced proanthocyanidin concentration, and light seed coat weight are associated with the yellow-seeded phenotype of *B. juncea*, *B. napus*, and *B. rapa*. Low lignin content is strongly associated with the unpigmented seed coat trait of *Brassicaceae*. The content and tissue location (in seed coat) of lignin are important features that determine the concentration of proanthocyanidins and their extent of extractability (Marles and Gruber, 2004).

Binding of free sinapic acid to 12S canola globulins is high when pH is 4.5 and no NaCl is present (Rubino et al., 1996a). When free sinapic acid is exposed to alkaline conditions (similar to alkali extraction of proteins) thomasidioic acid is formed and the presence of oxygen enhances this conversion, which can be controlled by antioxidants such as ascorbic acid. When pH is above 7.0 and NaCl is present, globulins are highly soluble and thomasidioic acid binding is not favored (Rubino et al., 1996b). Binding of either sinapic or thomasidioic acid negatively influences the functional performance of canola globulins especially heat induced gel formation and properties of the formed gel (Rubino et al., 1996a).

Carbohydrate and Fiber Fraction

The presence of low molecular weight carbohydrates in rapeseed protein products (pH 11 extracted and recovered at pH 6) has been reported, especially, the high ribose content probably resulting from RNA (2%), then arabinose and glucose as the predominant sugars followed by mannose, galactose, and traces of xylose, rhamnose, and fucose (Åman and Gillberg, 1977). Fiber components contribute much to the carbohydrate fraction of *Brassicaceae* oilseeds and are found in the seed coat, endosperm, and cell walls of the cotyledon cells. Several approaches have been considered to lower the fiber content in seed by *Brassica* breeders as fiber has been negatively correlated with oil and protein content in the seed (Simbaya et al., 1995). Fiber fraction is also associated with low bioavailability of meal protein in animal feeds. In *B. napus*, seed coat (hull) contains 15–18% protein in which Lys and Thr content is higher than cotyledon (including endosperm) proteins. The proteins of hull are considered unavailable for monogastrics (Finlayson, 1974; Bell and Shires, 1982; Jensen et al., 1995) or laboratory animals (Ward and Reichert, 1986; Bjerregaard et al., 1991; 1997a; Grala et al., 1998) and pose a negative influence on nitrogen digestibility. Hull protein may be partly responsible for the low digestibility values of canola meal compared to soy; 67–84% energy digestibility and 82–90% protein digestibility. Total digestibility of meal shows a negative correlation with the hull and the lignin content of seed (Jensen et al., 1995). Rapeseed carbohydrates (cellulose, hemicelluloses, and pectin) and lignin may absorb amino acids released during protein hydrolysis; therefore, more amino acids of both exogenous and endogenous origin pass into the large intestine (Grala et al., 1998).

Most of the data available for canola/rapeseed meal fiber have used the official methods for feed analysis (crude fiber, neutral detergent fiber, acid detergent fiber) thus providing high values. The dietary fiber (DF) content of canola/rapeseed meal is at high levels compare to soy and of low digestibility. DF isolated from rapeseed cotyledon has a more negative effect on the digestibility of proteins than DF isolated from hulls (Bjerregaard et al., 1991). Cellulose and lignins are primarily found in the insoluble dietary fiber (IDF) and pectins, hemicellulose, mixed β -glucans, gums, and mucilage are in the soluble dietary fiber (SDF) fraction. Minerals, nitrogen, and phenolic compounds are associated with both these fractions. In spring and winter type rapeseed, 27.5 to 33.0% IDF and 3.1 to 5.2% SDF have been reported using enzymatic gravimetric methods of analysis (Ochodzki et al., 1995a). Although the fermentable SDF stimulates microbial growth with increasing microbial N excretion, low energy and protein digestibilities are reported for rapeseed meal fiber. Proteins are associated at levels of 2.4 to 6.7% in SDF and 11.8 to 14.0% in IDF but are not susceptible to digestion by pepsin-pancreatin combination. The amino acid composition of the proteins associated with IDF is distinctly different from those with SDF (high content of Lys and Asn), and more close to the amino acid pattern of the rapeseed meal. More tannin is associated with IDF (0.8–1.1%) than SDF (0.3–0.4%).

The IDF fraction is primarily responsible for decreasing the digestibility of rapeseed protein in rats (Ochodzki et al., 1995a). Treatment with cell wall degrading enzymes has reduced IDF and increased SDF contents and has caused an improvement in protein utilization in poultry but not in rats (Ochodzki et al., 1995b; 1995c).

The indigestible components of rapeseed fiber other than cell wall proteins, phenolic and carboxylic acid derivatives, oligosaccharides, and tannins are categorized as non-starch polysaccharides (NSP). The non-cellulosic polysaccharides of IDF are about 7.8 to 10.6% and 1.6 to 2.3% in SDF with arabinose and galactose as the dominant sugars. Xylose is the next dominant sugar of IDF and glucose is in SDF (Ochodzki et al., 1995a). Arabinose and galactose of SDF may exist as constituents of acidic pectic substances. In IDF, the presence of xylans suggests that branched polysaccharides such as xyloglycans, amyloids containing xylose, glucose and possibly galactose are present. Fucoamyloids (Aspinall et al., 1977) and galactomannans are also present in rapeseed (Siddiqui and Wood, 1977; Slominski and Campbell, 1990). These components modify the functions of dietary fiber ranging from its ability to hold water, binding of organic molecules, macronutrients, micronutrients, xenobiotics, and minerals (Bjerregaard et al., 1997b). The major non-cellulosic polysaccharides of *B. rapa* cotyledon cell walls are found to be arabinans, arabinogalactan proteins, rhamnogalacturonan I, and hemicellulosic polymers (Ghosh et al., 2004). Delignification accentuates the extractability of cell wall polysaccharides and arabinans of the major non-hemicellulosic polymer. Arabinogalactan proteins are made of arabinose and galactose (primarily) and also with xylose and mannose residues as sugars and Glu/Gln, Gly and Pro residues (mostly) in the proteins. The hemicellulosic fraction is made of xyloglycans and xylans and the *B. rapa* xyloglycans are mostly G, XXFG, XLFG, and XXLG subunits whereas xylan has β -(1 $\rightarrow$ 4)-linked-D-xylopyranose backbone substituted with 4-O-MeGlcA residues similar to many other higher plants (Ghosh et al., 2004). Nonhemicellulosic polymer components such as arabinogalactan proteins found in *B. rapa* cell walls may have many pharmacological and food industry applications.

The yellow seed character is associated with significantly lower lignin and polyphenol content than brown seed color (Slominski et al., 1994). The NSP of yellow seeded canola is digestible than brown seeds when assessed in poultry diets. Yellow-seeded canola that is developed in Canada using interspecific transfer of genes from related yellow-seeded *Brassica* species has low contents of total fiber and lignin. Yellow color, thinner seed coat, and low acid soluble lignin content are this traits that are specific in the new *Brassica napus* genetic material to generate reduced fiber and improved meal energy and protein availability than regular brown seeded canola (Slominski et al., 1999; G. Rakow, personal communication).

PROTEIN PRODUCTS FROM CRUCIFER OILSEEDS

Oil has a long history of being the prime, high value product of *Brassicaceae* oilseeds. Commercially produced Canadian

canola meal contains 35 to 36% of crude protein on weight basis (Newkirk, 2009). Protein is the most valuable component of canola meal for animal nutrition due to the acceptable essential amino acid profile, biological value and net protein utilization values, which are also the indices of protein quality measurement as a food protein. A typical chemical composition of the meal obtained for Canadian canola is provided in Table 8.

The commercial oil extraction process is optimized to recover the maximum amount of oil for edible and other applications. Meal is the low cost byproduct of the oil extraction process, thus nutrient conservation of meal is not built into the oil extraction process. The value of meal protein is reduced due to the limitations such as negative nutritional effects of glucosinolate breakdown products, seed coat associated phenolic compounds, and detrimental effects of solvent extraction and removal caused on proteins. The desolventizer-toaster step of commercial oil extraction process is the most harmful step for meal proteins (Mustafa et al., 2000a). In the commercial canola oil extraction, desolventizer-toasting is the last step of meal processing before storage, which includes injecting steam into wet meal coming from hexane extraction (MARC meal). Temperature of 103°C to 107°C is maintained for 30 to 40 min during this operation to ensure the residual hexane level of meal is lowered below 50 ppm. The traditional practice of combining gum residues of the degumming step of oil extraction increases the residual oil content of the meal. Increased oil content is advantageous for feed formulation; however, it limits other uses of the meal such as starting material for protein product preparation.

To obtain the full potential as a plant protein ingredient, isolation and concentration of the canola protein fraction has been attempted since the time commercial oil processing has been in practice. Utilization of *Crucifer* oilseed proteins in human nutrition applications has not yet been completely materialized despite its comparable biological value with other major plant proteins such as soy. Majority of the protein recovery processes described in literature are direct adaptations of soy protein processing operations. Two *Brassica* protein products are described in literature; protein flour/concentrates and protein isolates. The reviews compiled by Mieth and group (1983a; 1983b) describe developments in the rapeseed protein product technologies up to 1983.

Protein Concentrates

According to soy protein industry standards, soy protein concentrates are prepared by removing most of the oil and water soluble non-protein constituents from clean dehulled seeds and must contain not less than 65% protein on a moisture-free weight basis (Golbitz, 2008; Association of American Feed Control Officials; AAFCO). The same definition has been used to describe protein products of canola/rapeseed. However, *Brassicaceae* seeds are much smaller than soybean, dehulling is not practiced in the commercial oil mill process, and the seed coat is a considerable fraction of the meal. Therefore, achieving protein levels of commercial protein concentrates require extensive processing. In the very early studies, non-toxic light colored, bland

rapeseed flour containing traces of isothiocyanates and protein content of ~53% has been obtained by several steps of water washing; FRI Process (Eapen et al., 1968; 1969). In order to reduce high fiber levels that arise from the seed coat and to improve protein levels, the FRI-71 process has been suggested by Jones and Holme (1979) as an alternative to commercial oil mill meal preparation. In this process, first the seeds are cracked and then air classified to remove hulls. The resultant meal (cotyledons and endosperm only) has reduced fiber content. After inactivation of myrosinase enzyme by heat treatment, several water extraction steps are included to recover or to remove water soluble components. The resulting washed meal is dried and hexane extracted to recover oil and the meal to generate protein concentrate. The final protein product has 66% protein and 24.5% of whole seed dry matter. The glucosinolates-rich *Brassica* oilseed meal, when extracted with aqueous alcohol (70%, v/v isopropanol) containing SO₂ from potassium metabisulphite (suppresses oxidation reactions of phenolics), has removed 36% solid weight mainly comprised of glucosinolates, phenolic, and nitrogen-free components such as low molecular weight carbohydrates and sugars (Jones and Holme, 1979). The remaining residue of this extraction contains 89% of N-containing components of the original material. Extension of this process by Jones and McGinnis (1991) has provided an integrated process that can generate both oil and protein rich material low in glucosinolates and phenolic compounds using non-aqueous solvents such as hexane, methanol, ethanol, isopropanol, and acetone. A cascade of liquid cyclones has been applied in this process and the products are; oil recovered from non-aqueous solvent, protein rich flour from the micelle plus overflow, and hull-rich insoluble solids from the underflow. The product (flour) contained about 70% protein and hull particles to a much lesser extent than commercial meal. Aqueous enzymatic processing of rapeseed to recover oil and protein is possible (Sosulski and Sosulski, 1990; Ohlson, 1992) and can be extended to obtain high value bio-products through a more environment friendly, solvent-free process (Bagger et al., 1998). A "Green Chemistry" biorefining process to maximize utilization of *Brassica* seed components is a further improvement of the aqueous enzymatic process. The basic processing steps involved are; dehulling, cold pressing, and/or enzyme-catalyzed cell wall degradation, with separation processes comprised of decanter centrifugation, ultrafiltration, and flash chromatography. The final products of the process are oil (no degumming required), protein enriched (40–55%, w/w) meal, lipophilic proteins in complex with amphiphilic lipids, hydrophilic and amphiphilic compounds, glucosinolates for biocide precursors, and hulls rich in dietary fibers. The advantage of this process is the separation and recovery of almost all the biopolymers and phytochemicals of the seed which is a desirable alternative to the current commercial rapeseed crushing process.

Protein Isolates

A protein isolate is the most enriched protein product obtained by isolating proteins from other non-proteinaceous

components. According to the definition provided by Soya and Oilseed Bluebook (Golbitz, 2008), isolated soy protein has at least 90% protein on a moisture-free weight basis. This level of protein is adapted to define many other plant protein isolates. From the time *B. napus* and *B. rapa* has become a commercial oilseed crop, preparation of concentrated and purified protein in isolated form has been investigated. Among the several processes described in literature to release proteins from the *Brassica* seed matrix, changes of solubility characteristics due to ionic strength or pH of the medium has been widely employed. Recovery of solubilized proteins can be through any form of solute concentration method, such as drying or membrane separation. Because of the amphiphilic nature, change of pH (isoelectric pH), or ionic strength (salting out) in the medium can render proteins in concentrated aggregates that are not soluble under the prevailing conditions. Two basic processes have been utilized to achieve protein isolates from rapeseed/canola and mustard.

Alkali Extraction and Isoelectric Precipitation

The first reported preparation of rapeseed protein isolate is by alkali mediated dissolution of proteins, removal of insoluble material, and recovery of solubilized protein by acid precipitation (Sosulski and Bakal, 1969; Kodagoda et al., 1973a). Loss of Cys in the macropeptide portion of whey (soluble proteins at pH 3.6) has been observed in this process (Kodagoda et al., 1973b) and actually it is the loss of Cys rich 2S protein. Use of polyacids and/or sodium hexametaphosphate (SHMP) can improve protein yield and color simultaneously lowering glucosinolates and PA levels in the final isolate (Gillberg and Tornell, 1976a; 1976b; Thompson et al., 1976; Mansour et al., 1992; Mahajan and Dua, 1994). From rapeseed protein extracts prepared at pH 11, mainly high molecular weight proteins are precipitated at pH 6.6 (Gillberg and Tornell, 1976a; 1976b). Carboxymethyl cellulose and hexametaphosphate can be precipitation aids to recover more proteins from the solubilized state. Stepwise precipitation of rapeseed alkaline (pH 11.1) extract, first at pH 6.6 then the remaining supernatant at pH 4.8 has been able to recover >50% of meal nitrogen (Gillberg and Tornell, 1977). Compared to soy, peanut, or sunflower, *Brassicaceae* proteins have high solubility of nitrogen compounds in the pHs where minimum protein solubility (pH 3 to 4) is observed. Since the canola-type *B. napus* and *B. juncea* has also exhibited a similar trend (Wanasundara and McIntosh, 2008), the soluble nitrogen at minimum solubility pH may not be totally attributed to glucosinolate nitrogen.

Membrane separation (ultrafiltration) of rapeseed alkaline extract produces protein product containing 76% protein by weight (Maubois et al., 1976). Using counter-current extraction of protein alternatively at pH 9.5 and 12.0, and successive precipitation at pH 6.0 and 3.6 can recover up to 71% of meal protein (Blaicher et al., 1983). Extraction of meal at pH 4.0 can dissolve about 70% of meal phytate. Addition of insoluble polyvinyl pyrrolidone to the meal before alkali extraction or use of sodium bisulphite in the extraction medium can produce

lighter color protein products (Blaicher et al., 1983). As much as 94% meal nitrogen (*B. napus*, Var Erglu, and Lesira) can be recovered using counter-current extraction with 0.02 N NaOH and successive precipitation at pH 6.0 and 3.6. The resulting product has protein content over 93% and polypeptides ranging from 12.5 to 68 kDa (El Nockrashy et al., 1977). Removal of glucosinolate breakdown products (3-pentenyl isothiocyanate, 4-pentenyl isothiocyanate, 5-vinyl-2-oxazolidinethione) of pH 10 extracted and isoelectrically precipitated *B. rapa* protein isolate can be achieved by incubating the protein material with myrosinase at pH 7.2 and then by passing (pH adjusted to 10) through an activated charcoal bed (Woyewada et al., 1978). The polyacids present in rapeseed meal such as phytic acid, RNA, acidic carbohydrates, and acidic polyphenols tend to precipitate with the proteins when isolated at acidic pHs (Åman and Gillberg, 1977).

Alkali extraction with SHMP of laboratory prepared rapeseed meal and ultrafiltration, isoelectric precipitation, and diafiltration of the extract has produced a protein isolate containing 87.4% ($N\% \times 6.25$) protein. This process has recovered 42.8% of meal N (Tzeng et al., 1988b; 1990a; 1990b). The soluble protein that cannot be recovered by isoelectric precipitation results in an isolate containing 96.1% protein ($N\% \times 6.25$) representing 32.6% of starting meal nitrogen. A marked improvement in the protein content of the final products (97.3% in the isoelectric precipitate and ~100% in the soluble fraction) can be achieved by dehulling of the starting seed material but without improving meal N recovery. When commercial canola meal is the starting material, meal N recovery is low; 33% in both isoelectric precipitate and soluble fraction opposed to 75.6% from the laboratory prepared meal (Tzeng et al., 1988b), as a result of low protein solubility of the commercial meal (Diosady et al., 1989; 2005). Alkali extraction tends to give a dark color to the final products as the seed coat polyphenols polymerize and co-extract with proteins. Use of activated carbon pretreatment and ultrafiltration of protein extract with a 30 kDa MWCO membrane in combination with mixing of the extract with strong basic ion exchange resin results in dark color reduction of the final product and low levels of contaminated phytates and glucosinolates (Tzeng et al., 1988a). Lighter color, bland tasting protein products from canola can be developed using different approaches such as alkanol/ammonia treatment (Xu and Diosady, 1994a; 1994b). In canola protein isolates, about 50% of extractable phenolics and tannins are in the unbound state. When alkaline pH is used for extraction, diafiltration can remove a considerable fraction of unbound phenolics (Xu and Diosady, 2000). A further reduction can be achieved when the alkaline extract is treated with 0.05 M NaCl and 0.1% SDS to weaken ionic bonds between proteins and phenolics (Xu and Diosady, 2002). After adjusting pH of the alkaline protein extract to 3.5, the soluble proteins at this pH is found highly contaminated with phenolic acids but treating with polyvinylpyrrolidone can reduce the level of phenolics in the final product (Xu and Diosady, 2002).

The process described by Newkirk et al. (2009) extracts canola white flakes (non-desolventized or MARC meal) with

an aqueous solution containing CaO and acid with pH of mild acidic to basic. Solubility of proteins is further improved by heating. Separation of hulls is achieved by sieving the meal slurry. Addition of more CaO to the soluble fraction maintains pH at 10.5 to 11.5 and coagulation of protein in the liquid fraction is further facilitated by heat treatment. Phytases can be introduced at this stage to reduce phytate level and to increase the soluble *myo*-inositol and inorganic phosphates (Maenz et al., 2004). This process will soon be used in commercial canola protein ingredient production in Saskatchewan, Canada and the products are targeted for animal and aquaculture feed markets.

In producing *S. alba* protein isolates dehulling of yellow mustard seed facilitates protein isolation by reducing the interference of hydrocolloidal mucilage in the seed coat. Use of methanol/ammonia/water-hexane treatment can help in reducing antinutrient levels of the yellow mustard protein isolates prepared by alkali extraction, acid precipitation, and ultrafiltration combination (Xu et al., 2003). When dehulled full-fat yellow mustard flour is used, precipitated protein isolate and soluble protein isolate (extracted at pH 11, membrane filtered, then pH adjusted to 2.0) having >86% protein content that composes at least 71% meal nitrogen can be obtained (Dendukuri and Diosady, 2003). Alternatively, a fraction of proteins of the microfiltered protein extract can be precipitated at pH 5.5 (Prapakornwiriya and Diosady, 2004) resulting a 94% protein (oil and moisture free basis) containing isolate which recovers 64% meal protein. When *B. juncea* defatted meal is the substrate, 96% protein containing precipitate isolate and 72% protein containing soluble protein isolate can be obtained by alkali extraction, membrane filtration, and precipitation at pH 5.0 (Marnoch and Diosady, 2006). Inclusion of NaCl to the extraction medium of pH 11 and maintaining the temperature at 93°C (with steam injection), a protein isolate containing 95% crude protein that is devoid of isothiocyanates, phytic acid, or phenolics can be obtained from *B. juncea* meal (Alireza-Sadeghi et al., 2006).

Isolation of *B. carinata*, *B. juncea*, *B. napus*, and *S. alba* (several varieties) meal proteins using alkali solubilisation (0.2 M NaOH) and precipitation at pH 4 has resulted in products containing 81–85%, 75–81%, 80–81%, 76–78%, and 75–81% protein, respectively (Aluko and McIntosh, 2001; 2004; Aluko et al., 2005). Addition of CaCl₂ (0.75 or 1.0 M) to the *Crucifer* seed meal alkali extract (after adjusting to pH 6) has generated Ca-soluble protein and Ca-precipitable protein isolates (Aluko and McIntosh, 2001). The presence of cruciferin and napin in the protein isolates of acid precipitated, Ca-precipitated, and Ca-soluble has been confirmed by 1D SDS-PAGE and mass spectroscopic analyses (Aluko and McIntosh, 2001; 2004; Aluko et al., 2004; 2005). The protein levels of Ca-precipitated isolates are lower than the acid precipitated isolates, and in the range of 73–76% for *B. carinata*, 76–80% for *B. juncea*, and 69–75% for *S. alba*. Only trace levels of glucosinolate breakdown products are found in the protein products of *S. alba* (Aluko et al., 2001). When the meal of *B. carinata* is the substrate of protein recovery, 50.1% of total nitrogen can be obtained as protein

isolate by extracting at pH 12 and protein precipitation at pH 5.0 and 3.5 (Pedroche et al., 2007).

All these protein isolation methods require extraction at highly alkaline pHs. Exposure of canola meal to 0.1 N NaOH for 2 h at 75°C is reported to generate 14.4 mg lysinoalanine per g of meal as an artifact of the process (Deng et al., 1990), showing that a careful control of alkali concentration and temperature is needed to protect Lys of the recovering protein.

Protein Micelle Formation

Brassicaceae seed storage proteins are soluble in dilute salt solutions such as 0.1 M NaCl. Owen (1973) has patented a process that utilizes change of ionic strength of neutral salts to enhance solubility of rapeseed meal protein between pH 7.0 and 8.5 and to recover proteins by isoelectric precipitation. Using the salting-in and salting-out phenomena of seed proteins, a non-covalent approach (protein micellation) to recover *Brassica* seed proteins has been devised by Canadian scientists (Murray et al., 1981a,b; Ismond and Welsh, 1992). The protein micellation process includes first providing conditions suitable to solubilize (salting-in) seed meal protein by modifying the ionic environment (increase ionic strength using salt), then proteins of the extract are precipitated by a hydrophobic-out mechanism via lowering the ionic strength (e.g., dilution with water). An amorphous, glutaneous mass which is the “micelle” is produced by *Brassica* proteins and this phenomenon has been observed for proteins of other seeds as well (e.g., flax, soy). The commercial process of this approach has been modified to bring the pH of the extraction medium (meal slurry 5 to 15, w/w, 35°C) to alkaline (pH 6.8 as the original process) at the same time providing ionic strength between 0.15 and 0.6 using a food grade salt such as NaCl. The recovered solubles may go through ultrafiltration and the retentate may be diluted to ionic strength of 0.2 (Schweizer and Greene, 2005). Several patents cover technological developments of this protein recovery process (Murray, 1997; 1999; Murray et al., 1979; 1980; 1981a; Schweizer and Greene, 2005). This process is utilized in developing two protein products, Puratein® and Supertein™, by the commercial venture *Burcon Nutrascience Co*, Vancouver, Canada. According to Logie and Milanova (2005), the protein micelle mass (Puratein®) is mainly composed of 7S globulins (88–98%) and the supernatant protein (Supertein™) composition to be predominantly 2S (70–95%). The 7S proteins are not the native form of assembly for *Brassicaceae* globulins. However, changes of ionic environment can bring about conformational changes of the hexameric subunit assembly (Schwenke and Linow, 1982; Schwenke et al., 1983; Wright, 1987) and may generate proteins with 7S in a NaCl-assisted protein isolation process.

Ultrafiltration

Protein isolates can be prepared from commercial canola meal using water as the extraction medium in combination with

ultrafiltration (50 kDa MWCO membrane) to recover most of the proteins in the retentate while leaving glucosinolates and their breakdown products in the filtrate (Diosady et al., 1984). With this process protein isolate containing 80% protein can be recovered as 10% of meal dry matter. The remaining residue of water leaching is the protein concentrate having 41.6% protein and 75% of seed dry matter. Kroll et al. (1991) have also described the possibility of using ultrafiltration and diafiltration to obtain rapeseed protein products.

Amino acid destruction and reactions between non-protein compounds and rapeseed proteins are highly favored at high temperatures. In addition to the technological hurdles to conserve protein quality deterioration, reduction of protein associated non-protein components such as phytates, phenolics, and glucosinolates that lower organoleptic properties, biological value, and nutritional quality of protein is a challenge. To be viable and successful, the developed technologies should overcome such issues and the resulting protein products should provide competitive economical advantages over existing plant protein ingredients. It is important to note that the processes moved up to commercial scale use MARC (non-desolventized) meal as the starting material.

Table 9 summarizes the processes employed for obtaining concentrates and isolates of *Brassica* seed proteins. All these described methods focus on obtaining as much protein from seed cellular matrix and removing non-protein components to enrich proteins in the final product. None of the methods recognize obtaining cruciferin and napin separately. The scalable methods to obtain cruciferin and napin have been described by Berot et al. (2005) and Wanasundara and McIntosh (2008).

NUTRITIONAL VALUE OF CRUCIFER OILSEED PROTEINS

Nutritional value of *Brassicaceae* oilseed proteins plays an important role in determining their value as a food or feed protein source. Soy, wheat, and corn are the predominant edible plant protein sources and animal protein sources include eggs, fish, meat, and milk. To date canola/rapeseed proteins are exclusively used in animal nutrition applications, but can be of immense potential in human food applications once the technological hurdles are surpassed to obtain suitable protein ingredients. Most of the studies available for mining nutritional value of *Brassicaceae* proteins have dealt with intact proteins (as meal) or protein products containing both cruciferin and napin. No studies have substantiated the nutritional value of the two major proteins of *Brassicaceae* seed separately.

In Vitro Digestion Models

Rapeseed protein concentrate (52%, %N $\times$ 6.25) has exhibited lower *in vitro* digestibility values (83%) than casein (97%) (Savoie et al., 1988). These authors have attributed the structural

rigidity of canola proteins that resist acid induced (optimum activity of pepsin is pH 1.3–2.0) denaturation and unfolding is responsible for the slow N release in the early digestion phase. The amino acids Leu, Phe, and Tyr are released in free form while His, Thr, and Val exist as peptides upon digestion of rapeseed protein (Savoie et al., 1988). When isolated crude cruciferin and napin of *B. napus*, *B. juncea*, and *S. alba* are studied under *in vitro* gastro-intestinal digestion conditions, napin shows higher resistance to degrade than cruciferin (Wanasundara unpublished data). Primary structure modifications that can enhance cruciferin digestibility have been described by Tandang et al. (2004a) and are discussed under molecular engineered proteins.

The ratio of α -helices to β -sheets of a protein is important in determining digestible protein value (Yu, 2008). Different ratio values for α -helix and β -sheet components have been resulted in for intact proteins of yellow *B. rapa* and brown *B. napus* seeds when studied under synchrotron-based FTIR microscopy indicating the possibility of observing differences in the gastro-intestinal digestibility of constituent proteins (Yu, 2008).

In Experimental Animal Models

According to early rodent model studies, rapeseed protein isolate (86.9% crude protein; %N $\times$ 6.25) has resulted in higher feed intake, weight gain, energy gain, protein efficiency ratio (PER), and apparent biological value compared to its original meal (Sarwar et al., 1973). Absence of fiber and glucosinolates is one of the advantages that protein isolates have when compared to the meal. In a weaned piglet model study, toasted rapeseed cake obtained from double-low varieties has exhibited net absorption of total N in the terminal ileum as 74% of intake and net reabsorption of endogenous N at 69%. In the same study commercial soy protein concentrate has resulted in 83% and 75% net absorption of total N and net reabsorption of endogenous N, respectively (Grala et al., 1998). A 13-week rat feeding study carried out for sub-chronic dietary toxicity assessment of Puratein® (cruciferin rich protein isolate) has reported “no observed effect level” of protein in the diet is at 10%. This level is equivalent to 11.24 g/kg body weight/day for male and 14.11 g/kg body weight /day for female animals (Meja et al., 2009).

When rapeseed protein isolate is included as the protein source in experimental diets high in sucrose and saturated fat, prevention of early onset of insulin resistance has been observed in rats (Mariotti et al., 2008). Mechanism of such activities is linked to the high cysteine availability from rapeseed proteins (Table 8). Rapeseed protein is also rich in arginine (Table 8), a precursor of nitric oxide which is an *in vivo* signalling molecule and a regulator of nutrient metabolism (Wu, 2009). Insulin resistance is central to the onset of metabolic syndrome and this study points out dietary protein, especially those from plant sources such as from *Brassicaceae* can be a factor besides dietary

Table 9 Processes employed to prepare *Brassica* seed protein products

Summary of the process	References
Preparation of rapeseed (<i>B. napus</i> & <i>B. campestris/rapa</i>) meal protein isolate by aqueous saline extraction (NaCl solution 2–10% w/v, pH 7.0–8.5, 5–60°C, ratio of 1:5–1:10, meal:aqueous medium, ~1 h), recovering of solid-free liquid and acidification to pH 2.0–4.0 to precipitate protein, washing of protein rich precipitate with water (×5) and forming a slurry having pH of 5.5–6.0 before drying. Final product has 80.8–84.2% protein (N% × 6.25) by weight.	Owen, 1973
Cracking and dehulling of <i>Brassica</i> species seeds to obtain hull free meat fraction (75–77% DM yield) and de-oiling by pressing. Removal of soluble components including glucosinolates in the press cake by heat treatment (80–100°C) at wet state and water leaching (counter-current process, starting from 42:1, 60–80°C, pH 5.5–6.0). Drying (50–70°C) of recovered solid or protein concentrate having 64–67% protein (N% × 6.25).	Anjou et al., 1976; 1978
Solubilization of rapeseed meal proteins using ultrasonic energy (~300W), refluxing of treated meal with N,N, dimethylformamide (DMF, up to 75% by wt), precipitation of protein by addition of methyl chloride, acetone or di-methyl ether to the refluxed condensate and drying the protein precipitate. About 75% of seed protein can be extracted and dry matter yield is 55–60% containing 95–96% protein (N% × 6.25).	Goodwin, 1977
Dehulling of <i>Brassicaceae</i> seed by cracking and air classification, defatting of hull-free meat with hexane, repeated aqueous lower alcohol extraction to remove phenolics and glucosinolate breakdown products, recovering of solids by screen separation and drying at 60°C. The protein concentrate has minimum of 66% protein and 24.5% of seed dry matter and up to 92% of seed N can be recovered.	Jones and Holme, 1979
Extraction of rapeseed meal or flour with aqueous alkaline medium at pH 8.5–10.5, adjusting pH of the extract to 3.6–4.0 to precipitate protein and recovering of precipitate. Protein so obtained can be treated with myrosinase (from <i>S. alba</i>) to hydrolyze glucosinolates after dispersing in water at neutral pH or to remove water by evaporation, osmosis or ultrafiltration, and treatment with activated carbon (45–60°C) to remove glucosinolate breakdown products and pigments in the protein solution. Protein yields are not disclosed.	Woyewoda et al., 1978
Extraction of commercial rapeseed meal with a food grade salt solution (μ = 0.2–0.8, pH 5.3–6.2, 15–35°C) to solubilize protein. Separation of extracted residual oil by chilling. Concentration of protein extract while maintaining the same ionic strength using membrane techniques and then dilution with chilled water to bring ionic strength <0.2 to favour formation of protein micelles. Preferred pH is 5.0–5.5 and temperature is <25°C. Aggregated proteins form an amorphous, sticky, gelatinous, gluten-like protein micelle mass (PMM) that can be recovered from the aqueous phase (dialysis or membrane separation) and dried to obtain a protein powder.	Murray et al., 1979; 1980; Murray, 1997; 1999
Dissolution of proteins of rapeseed meal using continuous or discontinuous countercurrent extraction with water at pH 7.0 or with aqueous NaOH or KOH (pH 7.5–10.0) that recovers ~95% meal N. Two protein fractions were recovered by stepwise precipitation at pH 5.7–6.0 and 3.6 which together make up ~90% of meal N.	El-Nockrashy et al., 1977
Rapeseed meal is mixed with myrosinase (preferably from mustard meal, in the ratio naturally present), water (1.5: 1 to 3:1 w/w) and ascorbic acid (0.1–1.0% w/w) and allowed to hydrolyse glucosinolates. The appropriate proportion of organic polar solvent is added (20–40%, v/v) to extract glucosinolate breakdown products. Remaining residue is a protein concentrate free of glucosinolate or their break down products.	van Megan, 1981
Process to obtain protein isolates free or low in phytic acid from <i>Brassica</i> or <i>Crambe</i> seed is described. Alkali extraction of meal (pH 10.5–12, up to 70°C), separation of dissolved components from residue, precipitation of protein in one- or two-step process. First pH is adjusted with acid to 3.5, separate precipitate and recover unprecipitated protein by adjusting pH to 6.5 with a precipitation aid; an acid polyelectrolyte or a salt of polyvalent acid (e.g., carboxymethylcellulose, polyphosphates, hexametaphosphates, alginates, pectic acid, carageenan, furcellaran, starch or starch derivative).	Gilberg and Tornell, 1976 a,b; 1977
Preparation of protein isolate from rapeseed (pea, sesame or cottonseed) by extracting protein with tap water (weakly acidic, pH 5.8), dilution of resulting suspension with cold water to form a dispersion of protein aggregates. Protein aggregates grew under cold temperature (4°C) was recovered by centrifugation or natural settling. Final dry product is substantially undenatured and contains >90% crude protein.	Cameron and Myers, 1982; Cameron, 1983
Preparation of two protein products from canola (<i>B. napus</i> ?) meal (commercial or methanol/ammonia/water & hexane extracted) was described. Extraction of proteins with alkaline aqueous solvent [NaOH, KOH, Ca(OH) ₂] at pH 10–12.5, recovery of fraction of soluble proteins by adjusting pH to 3.5 using aqueous acid solution (HCl, H ₂ SO ₄ , H ₃ PO ₄ , Acetic acid) and recovery of remaining soluble protein (22.7% N yield) that is not precipitated by membrane separation (MWCO 5 to 50 kDa). The precipitated protein (19.5% N yield) was washed and dried. Further improvement can be done to obtain a lighter colour and bland protein isolate. Initial extraction solution to contain 0.1% (w/v) Na ₂ SO ₃ and at pH 12, ultrafiltration of the extract after addition of SDS and NaCl and diafiltration with water containing Na ₂ SO ₃ and low concentration of NaCl, adjustment of the retentate pH to 3.5, recovering precipitate, ultrafiltration of remaining liquid after addition of polyvinylpyrrolidone and ultrafiltration and diafiltration to recover protein in the soluble form. Precipitated protein is washed and dried.	Diosady et al., 1984, 1988; 1989; 2005
A continuous process to obtain protein rich material and oil from <i>Brassica</i> seeds was described. Whole seeds are macerated with a solvent suitable for extracting oil then passed on to a liquid cyclone to obtain micelle (lipid rich), endosperm flour (protein rich) and seed coat meal (fiber rich). Use of multi-cyclone cascade series ensures efficient separation and the endosperm rich flour has protein content ~57%.	Jones and McGinnis, 1991
A green chemistry approach to recover several products from rapeseed. A second generation aqueous processing of rapeseed as a biorefinery platform. Rapeseed is milled treated with hot water to inactivate myrosinase and Fryma milled. Meal slurry was pH adjusted and reacted with cell wall degrading enzymes. Centrifugation and decantation of products; oil (30–40% Dm), protein rich meal (18–22% DM, 40–55% protein), hulls for fiber (13–18% Dm, 25–35% protein), and solubles (25–34% DM, 30–40% protein). Ultrafiltration and flash chromatography result in protein concentrates, protein isolates, emulsifiers, phospholipids, glucosinolates and other low molecular weight compounds.	Bagger et al., 1998
Extraction of defatted canola meal with water (mild acidic or basic) and recovery of solubles, pH adjustment >9 to separate out solids, recovery of soluble proteins either by heat-induced or isoelectric precipitation and/or ultrafiltration, phytase treatment to reduce phytate content in the final protein product. One or more protein products can be obtained.	Maenz et al., 2004; Newkirk, et al., 2009

(Continued on next page)

Table 9 Processes employed to prepare *Brassica* seed protein products (*Continued*)

Summary of the process	References
Describes improvements and modifications to PMM process to obtain food-grade protein products from canola meal. Preparation of meal by dehulling, using myrosinase inactivated seed, desolventization of meal <50°C, solvent extraction to recover residual oil of meal and/or washing with alcohol is described. Protein solubilization medium contains; protein meal 5–15% by weight, food grade salt solution maintaining ionic strength 0.15–0.8, pH 5–6.8 or 6.8–9.9, temperature up to 65°C, presence of ascorbic acid or sodium sulfite (0.01–1% by wt) and use of membrane separation to maintain protein concentration and ionic strength is covered. Use of PVP, activated charcoal for removal of oxidized phenolics and free phenolics is covered. Extraction process could be batch, semi-continuous, or continuous. Lowering ionic strength down to 0.1 of the protein solution (200–250 g protein/L) with chilled water (<15°C) at pH 5.3–6.2 to facilitate micellation process, use of suitable membrane techniques at this stage and allowing settling is described. Recovering of PMM by decanting or centrifugation and recovery of supernatant proteins using membrane techniques, recovery of two protein products having different proportion of 2S/7S/12S molecules is described. PMM contains predominantly (>90% by wt) 7S proteins and the other product containing 90–85% of 2S proteins from supernatant is described.	Schweizer and Green, 2005; Murray and Westdal, 2005; Barker et al., 2005
Describes protein (cruciferin and napin) fractionation of <i>Brassicaceae</i> oilseeds. Napin is solubilized at low pH with Na or Ca ions and concentrated using ultrafiltration and then diafiltration to obtain protein values >90%. Cruciferin of the remaining residue can be isolated by solubilizing at alkaline pHs (then concentrated) or concentrated by removing cell wall polysaccharides using suitable enzymes. Minor products such as mineral concentrates, fiber concentrates or soluble sugar concentrates can also be obtained.	Wanasundara and McIntosh (2008)

fats and carbohydrates that can be manipulated in preventing metabolic syndrome.

Studies of toxicological evaluation of rapeseed/canola and mustard proteins indicate that most of the adverse effects are associated with the glucosinolates and their break down products that are isothiocyanates (ITC) such as 5-vinylloxazolidine-2-thione (VOT, goitrin), butenyl-ITC, pentenyl-ITC, phenylethyl-ITC in addition to phytates and phenolics. When rapeseed protein concentrates of the FRI-71 process (Jones, 1979) is tested in weanling rats to assess the effect of residual glucosinolates and their breakdown products associated with the protein, increase in VOT level has resulted in depressed growth and thyroid enlargement of the animals. However, the increase in ITC levels of the protein product alone may not be responsible for such adverse effects. Thyroid enlargement has occurred at 69 mg/kg of dietary goitrin level, and above 260 mg/kg level, depressed food consumption and growth has occurred. Evaluation of rapeseed protein concentrates at subacute levels in Beagle dogs and rats has suggested that goitrin levels in the range of 0.02 to 0.29 mg/g in the protein products may not produce antithyroid effect or any other abnormalities. These levels are comparable with the goitrin levels found in fresh cabbage (0.02–0.03 mg/g) and rutabaga (0.15 mg/g). The glucosinolate breakdown product, VOT of rapeseed meal exhibits thyrotoxic effect and decrease in the level of T₄ and synthesis of T₃ hormones in rats. Such effects are not seen when protein isolates which are low in glucosinolate breakdown products are fed (Kroll and Przybilski, 1991; Plass et al., 1992). A slight increase in liver weight and decrease in kidney weight has been observed when rats are provided with 10% rapeseed protein isolate in a 4-week feeding study, with achieving similar performance for casein at same inclusion level. On feeding rapeseed isolate, a slight impairment of thyroid hormone metabolism has been found but without antithyroid effects (Plass et al., 1992). Eklund (1973) has reported that use of detoxified (glucosinolates and breakdown products content is reduced) rapeseed concentrate as the sole source of dietary protein may cause toxic effects on pregnant rats with negative effects on the litter size. These effects can be circum-

vented when the rapeseed protein of the diet is replaced with casein having added methionine. The compounds in rapeseed meal that may be responsible for the effects of pregnant rats are soluble in water and methanol at 65°C and 0°C or at pH 6. Heating of rapeseed to 100°C makes these components less soluble in water but not in 65°C methanol. The high solubility of these components in methanol and insolubility at pH 1 excludes PA among the responsible components for the observed toxic effects of rapeseed proteins (Lieden and Hambraeus, 1977).

The phytates present in rapeseed and *S. alba* protein concentrates have been considered as the factors that hamper zinc nutrition in pregnant rats causing heavy stress, increase in still-born pups, and low levels of Zn in serum and femur (McLaughlan et al., 1975; 1977; Shah et al., 1976). These conditions could be alleviated by zinc supplementation (McLaughlan et al., 1977; Shah et al., 1979). Rats fed on rapeseed and *S. alba* protein concentrates have exhibited similar symptoms as animals fed with zinc depleted diets as described in the studies by Apgar (1970, 1972). When rapeseed protein concentrate supplemented with Zn is provided, high protein efficiency ratio, relative nitrogen utilization, and relative protein value has been observed in young weanling rats. The phytic acid of rapeseed protein concentrate may be the possible cause that hinders Zn nutrition in young (Lieden and Hambraeus, 1977) and pregnant rats (Jones, 1979). When canola protein isolate prepared from SHMP-assisted extraction is included at 5% and 10% levels in rat diet, higher protein-efficiency ratio (PER) has been reported than casein at the same inclusion level; however, at 20% level, a slight enlargement of kidneys has been observed probably due to the high P content in these isolates (Liu et al., 1982).

In Humans

Little or no evidence can be found on seed of *B. napus* and *B. rapa* as a direct food ingredient. However, since historic times *B. juncea* and *S. alba* seeds have been passed through the food chain as spices and condiments. In northern African cuisines,

Table 10 Comparison of relative ileal digestibility (RID), postprandial biological value (PBV) and net postprandial protein utilization (NPPU) of rapeseed protein in human with other dietary protein sources

Protein source/Reference	RID%	PBV%	NPPU%
Lupin (Mariotti et al., 2002)	91	81.5	74
Milk (Gaudichon et al., 2002; Bos et al., 2003)	95	82	78
Pea (Marriotti et al., 2001)	90	79	71
Rapeseed (Bos et al., 2007)	84	84	70.5
Soy (Gaudichon et al., 2002; Bos et al., 2003)	91.5	80	73.5
Wheat (Bos et al., 2005)	91.5	73	66

B. carinata (Ethiopian or Abyssinian mustard) is directly incorporated into foods. The presence of glucosinolates and their breakdown products in canola/rapeseed are perceived as a safety concern that hinders human thyroid function (Jones, 1979; Mieth et al., 1983a; 1983b). Available scientific evidence points out mostly the negative nutritional effects of associated components of rapeseed proteins rather than the details of nutritional value of protein. In regulatory perspective, protein meal of *B. napus*, *B. rapa*, or *B. juncea* (canola/rapeseed) has not received GRAS status to date. Since October 2008, canola protein products of *Burcon Nutrascience Corp*, Puretein® and Supertein™ have received GRAS status.

Human studies that exhibit the efficiency of *Brassica* protein products is extremely limited. An elaborative human feeding trial conducted by Bos et al. (2007) has used ¹⁵N labelled rapeseed protein obtained by fertilizing the plants with ¹⁵N labelled ammonium nitrate. The ¹⁵N labelled-protein isolate (36.8% globulin, 41% napin, 2.7% lipid transfer protein, total nitrogen 14.9%) has been included into the test meal (27.3 g protein, 700 kcal total energy) of a healthy human feeding trial (n = 7). During the postprandial period, a real ileal digestibility of 84.0% ± 8.8 with a net postprandial utilization (NPPU) of 70.5%, and 83.8% postprandial biological value has been observed for rapeseed proteins. The NPPU values observed for rapeseed protein can be placed between legume and wheat proteins. A mean ileal dietary and endogenous N fluxes of 68 and 79%, respectively, observed for the individuals is actually a higher value than pea protein and breast milk (43 and 57%). After 8 h of ingestion, the protein bands of cruciferin, cruciferin α- and β-polypeptides, and napins remained intact upon electrophoresis separation of the ileal digest supporting the hypothesis that some rapeseed proteins resist proteolysis. A very low deamidation rate of rapeseed protein compared to lupin and wheat proteins indicates that once absorbed, catabolism of derived amino acids is minimal. High postprandial biological value of rapeseed proteins may be associated with high content of indispensable amino acids especially higher levels of Met and Cys (39.0 mg/g Met + Cys, 1:1 ratio) than the limiting value (2.3 mg/g), which is comparable with egg proteins. The high protein biological value and high levels of S-amino acid of *B. napus* protein is very much of interest in human nutrition, and the digestibility can possibly be improved through partial exogenous hydrolysis (Bos et al., 2007). Table 10 provides rapeseed protein utilization indices in human compared with other food proteins.

Assessment of the same rapeseed protein product in weaned piglet model has revealed that data closely relates with the human feeding study conducted by Bos and group (2007). The correlation values of 0.98 for true ileal digestibility of nitrogen (over 3 × 2, *P* = 0.11) and 0.87 for amino acids digestibility (over 26 × 2 data, *P* < 0.0001) have been reported (Deglaire et al., 2009). Rapeseed proteins exhibit atypical nutritional properties in humans and is characterized by low bioavailability which is counter-balanced by high postprandial biological value.

In Farmed Animals

According to Canola Council of Canada, canola meal protein is suitable for both ruminant and monogastric farm animals and the emphasis is on the suitable amino acid profile of the protein. Numerous studies discuss negative and positive effects of canola protein in livestock, aquaculture fish, and sports animals.

Low meal protein degradability, reduced body weight gain, reduced feed conversion efficiency, and high mortality has been observed for regular desolventized meal fed broiler chicken compared to the feeding of meal obtained from desolventizing at 100°C using indirect heat opposed to traditional toasting (Mustafa et al., 2000a; Newkirk et al., 2003). The studies in poultry, rodents, and swine concerning the protein quality of canola meal emphasize the essentiality of minimizing heat treatment to the meal. Excessive heat causes Maillard browning and other complexation reactions that lead to reduced bioavailability of amino acids, especially Lys of meal protein. Lysine is often the limiting amino acid in animal feeds (Bell, 1993) thus any reduction in Lys availability affects the competitive positioning of canola meal protein based ingredients for monogastric nutrition. As mentioned in an earlier section, the fiber fraction of canola whether it originates from hull or cell walls exerts a negative effect on protein availability in monogastrics (Bjergegaard et al., 1991; Jensen et al., 1995).

At present canola protein meal is optimally used in dairy cattle nutrition. Improved milk production has resulted in dairy cows being fed canola meal included rations compared to soybean meal or other protein meals (Piepenbrink and Schingoethe, 1998; Maesoomi et al., 2006). Rapeseed protein degrades more readily in the rumen where protein is degraded by ruminant microflora and excess ammonia may be absorbed through the rumen wall, converted to urea, and excreted. Various treatments have been used to improve the “by pass” value of canola protein. Therefore protein will be digested in the abomasum and ileum after passing through the rumen. In ruminant nutrition, ways to reduce ruminal undegraded protein (RUP) is a major concern and heat treatment has been considered the best way to reduce RUP (Mustafa et al., 2000b) in canola meal.

In Cultured Finfish

With the declining supply of fish meal, feed cost accounting for about 35–60% of the production cost of farmed salmonids, and the high energy-high protein feed requirement of the farmed

carnivores (Higgs et al., 1995), the aquafeed industry seeks suitable plant protein sources as alternatives. When protein values and amino acid profiles are compared with soy, canola protein should provide similar or better nutritional advantages to the fish. Direct use of canola/rapeseed meal in aquaculture diet has limitations arising from fiber, glucosinolates, and phytates (Burel et al., 2000; Gatlin et al., 2007). The phytate-reduced canola protein concentrates (63–70% protein) perform similar to fish meal (Forster et al., 1999) when used with a feed attractant in farmed rainbow trout diets (Thiessen et al., 2004). A significant improvement in specific growth and feed conversion response of trout and salmon could be obtained when a combination of ethanol and an acid wash is employed for the commercial meal (McCurdy and March, 1992; Mwachireya et al., 1999). This observation emphasizes the need of removing non-protein components or acid soluble proteins to improve protein utilization by aquatic fish. Reduction of fiber level and more refined protein is needed for cultured fish, especially for the cold water species such as salmon, charr, and rainbow trout.

TECHNOLOGICALLY VALUABLE FUNCTIONALITIES OF CRUCIFER PROTEINS

Much of the published research on functional properties of canola/rapeseed proteins compare with commercial vegetable protein ingredients in which the properties are dictated by the protein processing procedure used, protein composition, and the presence of other compounds. McCurdy (1990) provides a concise review of some functional properties of canola/rapeseed protein products. Prakash and Narasingha Rao (1986) have reviewed physico-chemical properties including N-solubility of high and low molecular weight protein fractions of mustard and rapeseed. However, due to the discrepancies between the protein ingredient preparation processes and the methods of assessment employed for each property, it is difficult to compare data available in literature. Therefore, the functionalities described for canola/rapeseed products that have defined composition of proteins only will be discussed here.

Concentrated or Isolated Protein

Solubility

Intact proteins in the PSV may possess the native form of assembly and any method of recovery from the stored organelle may bring about changes to the molecules. Solubility properties are of tremendous value to recover storage proteins from seed cellular matrix. In general 11S globulin preparations have good solubility in a wide range of pH. For isolated cruciferin, the extent of solubility and the pH range that exhibit minimum solubility is different than glycinin, possibly related to the high Gln residues in the variable region of cruciferin compared to acidic residues found in glycinin (Salleh et al., 2002).

It is known that seed globulins undergo reversible association/dissociation under non-denaturing conditions. Under extreme acid or alkali conditions, dissociation of multiunit globulin into constituent subunits may occur with concomitant denaturation of the protein. Ionic strength of the medium is critical on the type of globulin molecular species that exists between pH 4 to 10. As a globulin, cruciferin also shows a stable hexameric form at pHs other than the isoelectric pH. When ionic strength is low, cruciferin tends to precipitate at the isoelectric pH. Isoelectrically precipitated glycinin at low-ionic strength can be redissolved by titrating to neutral pH in the absence of added salt; however, this is not clearly proved for cruciferin. The protein solution so obtained predominantly contains 7S form; trimer or half the 12S molecule (Wright, 1987). When ionic strength is increased subunits re-assemble (Lillford and Wright 1981) and 12S form reappears indicating the hexameric form of glycinin is unstable at low ionic strength. Similar behavior has been observed in rapeseed 11S protein (Schwenke et al., 1983). Logie and Milanova (2005) claim that canola protein obtained from solubilization at high ionic strength and precipitated by lowering the ionic strength predominantly contains 7S proteins. According to the observation by Schwenke and group (1983) and Wright (1987), the 11 or 12S form of cruciferin is unstable at low ionic strength therefore dissociation into 7S components is possible. It can be reasonably argued that the 7S protein claimed by Logie and Milanova (2005) is merely a product of the process. There is no other supporting evidence that 7S proteins (or vicilin-type) are the predominant proteins in *Crucifers*. Wright (1987) has reported an interesting behavior of arachinin from peanut which has two types of 11S proteins. Both these proteins are stable as 11S at high ionic strength above pH 7. One of these types dissociates into 7S form when the ionic strength is reduced while the other transforms to its 7S form when the pH is lowered below 7, therefore no single explanation that can be devised for the diverse association/dissociation behavior of globulin whereas no rule can be formed. The possible mechanism involved in this association and dissociation with increase in ionic strength is definitely beyond simple charge-screening. The nature of the variable regions which are hydrophilic and located on the surface of the subunits may play a role in the association. Also, the degree of glycosylation and phosphorylation of the molecule may be the factors that can affect solubility behavior.

The albumin fraction of rapeseed is a heterogeneous mixture of proteins with differing pH dependant solubilities. When pH 8 preparation of albumin fraction is titrated, maximum solubility has been observed at pH 4.5 and a shift to pH 5.0 can occur if protein preparation is titrated up to pH 8.0 from 2.0 (Schwenke et al., 1991). The hydrophilic nature of the molecule and its subunits and the absence of hydrophobic core greatly dictate the solubility behavior of napins as observed in *B. juncea* (Jyothi et al., 2007b). Salts such as NaCl tend to stabilize napin structure by compacting. At low concentration of monoalcohols such as trifluoro ethanol, ethanol, and methanol, napin has a propensity to form α -helix structure. Selective solubility of *B. napus* and *S.*

alba napins has been observed in 70–80% (v/v) ethanol and 30% (v/v) iso-propyl alcohol (Wanasundara and McIntosh, 2008). The basic nature of napins is exhibited as different solubility behavior than cruciferin. Having a pI close to 11, napins get ionized in more acidic pHs and remains soluble in pHs as low as 3 to 4 (Wanasundara and McIntosh, 2008). However, due to the presence of isoforms, some napins are soluble at alkaline pHs as basic as 12.

Most of the solubility studies on crude protein isolates of *Brassicaceae* seed storage protein are mixtures of cruciferin and napin of unknown ratio therefore difficult to reproduce and interpret. In general, the solubility of seed meal proteins is highly pH dependant and affected by ionic strength and type of salt. Rapeseed proteins have relatively high solubility in the acidic pH range (Quinn and Jones, 1976). At pH 4.0 soluble nitrogen content of rapeseed meal depresses to a minimum of 40% but dissolution of total and phytic acid P is at their maximum (>80% of total) which can be revealed by following the dissolution pattern of meal N and P (Gillberg and Tornell, 1976a). Increase in pH beyond 10 can solubilize N over 80% of total with a reduction of phytate P. Added phytates (1 to 5%) can drastically change the solubility of isolated rapeseed protein with a shift in the minimum solubility pH from 7 to 4.5 (Kroll, 1991). Such behavior of proteins may be due to the binding of PA to the basic amino acid residues that are protonated at low pH and formation of insoluble protein-PA complexes.

The Ca-soluble proteins of *S. alba* (Aluko et al., 2004) resist aggregation when protein concentration increased from 5 to 25 mg protein/ml in the presence of 0.75 M CaCl₂. Under these conditions Ca-soluble proteins of *B. juncea* loose solubility and form aggregates. Although there is no plausible explanation for this activity, Ca-soluble protein of *S. alba* is much higher in Gln, Pro, and Cys and lower in Tyr than the Ca-precipitable proteins of the same source.

Emulsifying and Foam Forming Properties

Adsorption at the interface and surface denaturation are necessary qualifications to perform surface activities to assist in forming protein emulsions and foams. Adsorption of proteins is mainly driven by hydrophobic interactions. Proteins are denatured at the interface to adopt a stable conformation and to minimize the interfacial free energy. Therefore, protein structure, extent of protein-protein, and protein-solvent interaction affect adsorption and surface denaturation of protein at the interfaces (Damodaran, 2008).

Unmodified *Brassica* 2S and 11S proteins exhibit excellent foam forming and stabilizing properties; 1% (w/v) protein levels at pH 7.0 has given 440% foam capacity and 90% foam stability lasting for 10 min (Nitecka et al., 1986; Nitecka and Schwenke, 1986). Satisfactory foaming ability has been observed for the 12S protein obtained by protein micellation (Gruener and Ismond, 1997a). The differences in the interfacial activity observed for rapeseed 11S and 2S protein in air-protein dispersions, solid phases, and emulsions are related to the molecular

size (Krause and Schwenke, 2001). The 11S proteins exhibit low emulsifying ability because of the globular conformation maintained at the interface contributing to the low surface activity. Albumins are highly surface active in stabilizing oil-water interfaces compared to globulins. A high initial surface coverage in the monolayers is generated by albumins favoring more intramolecular short-range interactions. The presence of PA in rapeseed protein isolate (e.g., 70% globulin, 30% albumin and ~1% phytic acid) may cause stable electrostatic complexes of protein-PA which thus reduces the number of surface active molecules (Krause and Schwenke, 2001).

Heat Induced Gelation

Heat induced gelation of globular proteins involves partial unfolding of the native structure with consequent release of buried domains of the protein which then interact intermolecularly to form a 3D matrix or network. The network provides the structure and rigidity of the gel. The formed gel network is stabilized through H-bonding, hydrophobic interactions, and covalent cross links such as S-S bonds (Damodaran, 2008). Thermally induced gelation is a significant factor that marginalizes proteins with weaker gelation properties to use in products such as processed meat containing emulsions and bake mixes.

A decrease in maximum gelation temperature as low as 80°C has been observed for napin when pH is at 10, attributing to the basic isoelectric pH of the protein (Schwenke et al., 1998). A weak napin gel network is formed at pH 10 with intensive syneresis. The presence of a high degree of helical structure and structural stabilization by disulphide bonds tend to renature napin molecule after denaturation. This partial renaturation of heat-induced denatured napin upon cooling may be the reason for instability and hysteresis of the napin heat-induced gels. Napin has exhibited resistance to heat induced modification between pH 4 and 8 (Folawiyo and Owusu-Apenten, 1997). Below pH 4.0, napin undergoes structural modifications upon heating that lead to a significant change in surface hydrophobicity (S_0) indicating that its heat stability is pH dependant. In the calorimetric studies, abnormally low values of apparent heat capacity resulted in for napin and the negative slope of the curves are considered as indications of the non-protein ligands (lipids and nucleic acids) bound to the protein which need to be corrected by using purified napin (Krzyzaniak et al., 1998). Purified napin exhibits denaturation accompanied by exothermic heat effect of aggregation at pH 6 making it difficult to obtain values for transition enthalpy. At this pH napin denaturation is irreversible and the formed aggregates are mostly hydrophobically associated. Napin shows resistance to unfolding at low pH (Murén et al., 1996; Krzyzaniak et al., 1998). Unfolding of napin into two structural domains is evident at pH 3.0. At this pH, two excess heat capacity curves can be observed in napin; one at 84.3°C and another at 88.3°C with enthalpy of transition values 39.4 and 60.6 kCal/mol, respectively indicating the unfolded domains (Krzyzaniak et al., 1998).

Below the least gelation concentration, cruciferin exhibits increased surface hydrophobicity (S_0) when temperature is above 65°C and ionic strength of 0.1. Increasing the ionic strength of the medium up to 1 has caused a decrease in the S_0 irrespective of the temperature (Folawiyo and Owusu-Apenten, 1996). Under iso-ionic ($\mu = 0.1$) conditions a 10-fold increase in S_0 has been observed with change of pH supporting the argument that cruciferin tertiary structure is susceptible to acid unfolding below pH 3, most likely the dissociation of subunits as described by Schwenke and group (1983). A two-stage model has been postulated for the heat induced changes of cruciferin by Folawiyo and Owusu-Apenten (1996), in which the native protein goes through a reversible unfolded state before coming to an irreversibly denatured state. Soluble protein aggregates and incorrectly refolded structures may predominate at the irreversible denatured state. A reversibly unfolded state may have high incidence of exposed non-polar amino acid residues and partial refolding (incorrectly) of these may cause the irreversible denaturation. Acid treatment alone can cause reversible changes to the cruciferin structure which may cause changes into reversibly unfolded state only (Folawiyo and Owusu-Apenten, 1996).

Rapeseed protein isolate composed of 70% globulins and 30% albumins has exhibited a gelation temperature of 69°C (15% w/v protein slurry, pH 9.0) (Schwenke et al., 1998) which is a lower value than the denaturation temperature reported for micelle isolate (88°C, Murray et al., 1985), isoelectrically precipitated isolate (78.5°C, Murray et al., 1985), or 11S globulin (80°C and 81.3°C for pH 6 and 10, respectively, Léger and Arntfield, 1993). Gelation temperature of rapeseed protein isolate is concentration dependent. Increased protein concentration of cruciferin can lead to an earlier onset of gelation upon heating as observed for 72°C for 7.5% and 70°C for 20% w/v dispersions. Similarly 7.5% (w/v) napin dispersion has formed gels at 86°C and 20% (w/v) dispersion at 82°C. The decrease of onset temperature is more pronounced in the mixtures of cruciferin (70%) and napin (30%) (79.5°C for 7.5% dispersion and 66°C for 20% dispersion) (Schwenke et al., 1998). The gelation temperature of cruciferin has exhibited pH dependence. For a 12.5% (w/v) dispersion of cruciferin maximum gelation temperature of 72°C at pH 7 (isoelectric pH) has been observed. Napin has exhibited maximum gelation temperature of 95°C at pH 7. Rapeseed globulin gels are stronger than napin gels with maximum gel strength observed around pH 7 (Krause and Schwenke, 2001). Both mixed protein isolate and individual proteins develop opaque gels. When an isolate containing globulin and napin is heated, stronger gels are formed around pH 9 indicating that the poor gelling properties of napin can overcome by interacting with high molecular weight cruciferin (Krause and Schwenke, 2001).

When alkali extracted and acid precipitated protein isolate (74.9% crude protein, 320 to 13 kDa MWT profiles, from *Var* Tower) dispersions (11.4% w/v) are heated at 72°C for 30 min (to simulate cooking of comminuted meat products) opaque gels are formed at pH ≥ 9.5 without NaCl (Paulson and Tung, 1989b). At pH 11, translucent gels are formed. The major bonds

involved in forming and stabilizing canola protein isolate gels have been identified as hydrophobic interactions and hydrogen bonds (Paulson and Tung, 1989b). Canola (*Var* Tobin) protein isolate obtain by micellation forms much stronger gels at alkaline pH (≥ 8) than at acidic conditions when 6% (w/v) suspensions are used (Léger and Arntfield, 1993). The presence of sodium salts promotes protein molecule stability and negatively affects gel structure formation. Establishment of canola globulin protein gel network is primarily by hydrophobic forces and electrostatic interactions and gel stabilization and strengthening is attributed to disulfide bonding, electrostatic interactions, and hydrogen bonding (Léger and Arntfield, 1993).

Canola protein isolate prepared by micellation (2S:11S/7S, 3:97, 87% protein) can generate stable gels in protein-hydrocolloid hybrid systems composed of up to 20% protein and 3% guar gum or κ -carrageenan by weight (Uruakpa and Arntfield, 2004; 2005; 2006a; 2006b). Addition of κ -carrageenan can improve protein gel strength and structure and help in generating more elastic gels from canola micella protein (Uruakpa and Arntfield, 2004). Possible structural changes of the protein are evident in this hydrocolloid-protein mixture as the enthalpy of denaturation (ΔH) is decreased when κ -carrageenan concentration is increased at pH 6. Hydrocolloids such as guar gum has produced less elastic gels and has generated values of rheological indicators directing to incompatibility between the polymer and the protein (Uruakpa and Arntfield, 2005). Structure formation and stabilization of the mixed polysaccharide-canola protein gels are mediated by hydrophobic, noncovalent, and covalent interactions (Uruakpa and Arntfield, 2006a). In general, both polysaccharides are able to increase incidence of surface hydrophobic patches indicating the potential of greater and rapid adsorption of oil/water interface which is required in emulsion formation (Uruakpa and Arntfield, 2006b).

Modified Proteins

Acylation of Proteins

Modification with acyl groups such as acetyl or succinyl has been widely studied for *B. napus* proteins as a means of improving technologically valuable functional properties of cruciferin and napin and their mixtures. Acylation blocks reactive amino residues of protein with an acyl residue. Lysine is the most reactive amino acid towards acylation due to the high nucleophilic character of the ϵ -amino group. Often, the extent of acylation of protein is expressed as the percentage of blocked lysyl amino groups or free amino groups.

Extensive succinylation can block 98% and 83% of free amino groups of rapeseed albumin and globulins, respectively (Schwenke, 1990). Decrease in free lysyl residues has been observed after acetylation of 12S canola proteins and succinylation has reduced both free lysyl and prolyl residues. Overall decrease in the isoelectric point of 12S protein indicates the changes in ionizable groups (Gruener and Ismond, 1997a). It has been suggested that critical modification (by acylation) level of 60–70%

exists for rapeseed 11S protein. Beyond the critical modification level, the protein is completely dissociated into its subunits with unfolding of subunits. Dissociation of protein structure due to acetylation or succinylation (modification >60%) is evidenced by decreased molecular weight and enthalpy of denaturation (Gruener and Ismond, 1997a). Introduction of negatively charged succinyl groups to cruciferin induces progressive dissociation into smaller units. Cruciferin dissociation is proposed as $11S \rightarrow 7S \rightarrow 3S$, and unfolding of the molecule may happen only at very high degree of modification (Gueguen et al., 1990). At 19.4% degree of succinylation, 7S and 3S components become noticeable. The 3S components become predominant when amino groups are succinylated above 66% (Schwenke et al., 1998).

Structural changes occurred due to amino group modification are manifested in the changes of functionality such as reaggregation tendency and improvement of gelation properties. For a canola protein isolate containing 11S and 2S proteins, succinylation up to 84% has made the protein isolate more soluble in the pH range of 3.5 to 8.0 (Paulson and Tung, 1987). The degree of succinylation >17% has completely inhibited aggregation of 11S rapeseed proteins in the temperature range of 25 to 90°C (Nieteka et al., 1986). Acetylation of both napin and cruciferin has resulted in a different type of gelling mechanism. Partial unfolding of the globulin molecule as indicated by increased hydrophobicity may have contributed to the formation of translucent and even transparent gel structures at pHs above 6. Such structures are formed by aggregation of unfolded polypeptides in an orderly fashion and may be true for the transparent weak gels generated by napin at pHs between 7 and 9 as indicated by Schwenke and group (1998). Succinylation of 11S canola proteins tend to form stable gels between pH 5 and 11 compared to the unmodified protein isolate forming a stable gel structure when pH is >9.5. Unmodified protein and the succinylated proteins at pH 5 produce particulate and opaque gels opposed to translucent gels formed at alkaline pHs (Paulson and Tung, 1989b).

Even at 61% succinylation, properties of the 11S globulin that manifest stabilization and generation of air-water and oil-water interfaces may not change (Guéguen et al., 1990). At low levels of succinylation, the induced unfolding and dissociation may cause changes in the distribution of hydrophilic and hydrophobic groups in the sequence areas exposed. The observed increase in viscosity and potential changes of hydrophobic and hydrophilic residue distribution if achieved under controlled succinylation may actually benefit in improving foaming and emulsifying properties of rapeseed cruciferin. The increase in surface hydrophobicity and electronegativity due to increased number of succinylated carboxyl groups may facilitate diffusion of canola 11S protein molecules on to the oil-water interface and rearrangement within the interfacial film (Paulson and Tung, 1988a; 1988b). Increase in both aromatic and aliphatic hydrophobicity can occur due to acetylation of canola proteins. Improvement of aliphatic hydrophobicity of canola protein due

to succinylation is directly reflected in elevated fat absorption capacity and surface active properties (Gruener and Ismond, 1997b).

To make the rapeseed protein more hydrophobic, long aliphatic chains and arylsulfonyl groups can be grafted on to the lysyl residues by reacting with respective anhydrides and chlorides (Gerbonaski et al., 1999). Both sulfamidation and acylation (with chains of C_2 to C_6) increase the S_0 of 11S proteins. Dramatic increase in the S_0 of 11S protein upon grafting of aromatic chains indicates unmasking of hydrophobic residues, expansion of the molecule and drastic hydrophobization. Grafting of long aliphatic or aromatic chain induces wider perturbation of secondary and tertiary structure than grafting a short aliphatic chain but dissociation of 11S protein may not occur (Gerbanowski et al., 1999). Both acylation and sulfamidation are considered as efficient ways of hydrophobizing protein molecule (both 11S and 2S) which may in turn induce new functionalities as a result of improvement in the ability to reduce surface tension in air/water interface and wettability with water of thin protein films (Krause, 2002).

Although napin exhibits remarkable temperature stability owing to the compact rigid structure stabilized by several inter- and intra-chain disulphide bridges, reported high foaming properties (activity and stability) suggests the flexible nature of napin and potential for interfacial adsorption. In relation to the high hydrophilic character of the 2S proteins, succinylation can further increase the hydrophilicity. The lysyl residues of napin are exposed at the molecule surface (Rico et al., 1996) and are accessible to modification. Three of the lysyl residues of napin molecule are in the α -helix region. Therefore, the decrease in α -helix content due to acylation (Gerbanowski et al., 1999) may be a result of secondary structure perturbation. Grafting of aliphatic (< C_4) chains even at high levels of addition cannot change intrinsic fluorescence of napin which is an indication that tertiary structure is stable. However, grafting of a bulkier group such as aromatic chains makes the napin molecule more apolar as indicated by the changes in the tryptophan residue environment (a blue shift in intrinsic fluorescence). Aliphatic acyl chains increases S_0 of napin depending on the length; however, grafting of aromatic chains does not result in increased S_0 indicating that the grafted hydrophobic groups may be oriented towards the hydrophobic interior of the napin molecule (Gerbanowski et al., 1999).

High degree of acetylation (up to 87% modification) and succinylation (up to 63% modification) of free amino groups of canola meal proteins *in situ* lowers the alkali extractability of protein and the extractability of phytic acid in contrast to low levels of acylation such as 21% succinylation and 55% acetylation. Protein products obtained from acylated meal exhibit high nitrogen solubility, emulsifying and fat absorption properties (Thompson and Cho, 1984a; 1984b). Deamidation of Gln and Asn groups of canola meal proteins using sodium dodecylsulfate (SDS) can improve the protein recovery from the seed matrix (Igor et al., 1993).

Hydrolyzed Proteins

Enzyme catalyzed hydrolysis has been utilized to improve technologically important functions of canola proteins and to generate peptides with bioactivities. The latter is described under bioactivities. Pepsin-assisted hydrolysis of rapeseed concentrate has generated material with high solubility between pH 4 and 7.0. Acid hydrolysis has improved emulsion stability at pH 5.5 and 7.0 and has also provided high foam capacity (Hermansson et al., 1974). Hydrolysis of rapeseed globulins by Alcalase® is more efficient than albumins (Chabanon et al., 2007). This observation confirms the compact, enzyme resistant structural features of albumins. The extent of peptide bonds broken (degree of hydrolysis or DH) influences functional properties of the resulting hydrolysate; for rapeseed globulins (DH of 5 to 15%) solubility at pH 4.5 and fat absorption can be improved, and for the albumin fraction foaming capacity can be improved but not the fat absorption. Low DH values (5%) are beneficial for improving emulsifying activity of both protein types (Chabanon et al., 2007). Hydrolysates that have average peptide chain length (PCL) of 7.5 (from 11S) and 11 (from 2S) can be obtained with pepsin (for 11S) and pepsin + trypsin (for 2S) assisted hydrolysis of rapeseed proteins (Malabat et al., 2001). For the peptides released from 2S proteins and having PCL of 8 and hydrophobic amino acid content of 36%, sulfamidation can be used to improve interfacial properties but butylation cannot (Malabat et al., 2001). Foaming properties of Alcalase hydrolysed rapeseed meal protein can be modified by attaching acyl groups of C₆ to C₁₆ (Sanchez-Vioque et al., 2001). Attaching C₁₀ to C₁₂ acyl chains can provide better foaming properties than C₆, C₈, C₁₄, or C₁₆ chain attachments. A more stable foam texture, with low amounts of incorporated liquid and homogeneous bubble density is characteristic of the foams of rapeseed peptides having C₁₀ and C₁₂ attachments compared to sodium dodecylsulfate (SDS) or bovine serum albumin (BSA) foam. The new foaming agents generated via this modification of rapeseed protein exhibit properties in between proteins and surfactants.

Limited hydrolysis (up to 7.7% DH) of rapeseed protein isolate (alkali extracted and precipitated at pH 5.0, 97.8% protein) using Alcalase has improved whippability, foam capacity, and water and oil absorption compared to the unhydrolysed protein (Vioque et al., 2000). Controlled hydrolysis of canola micelle protein with ficin and/or trypsin has resulted in reduced elasticity and hardness of the formed heat-induced gels (Pinteritas and Arntfield, 2007). Enzymatic cross-linking with microbial transglutaminase that catalyses cross-linking of ϵ -(γ -Glu)-Lys can improve both elasticity and hardness of the gels generated from hydrolyzed micelle protein of canola (Pinteritas and Arntfield, 2007). Hydrolysis of *B. carinata* meal protein with Alcalase has increased soluble N content of meal at pH 9 (from 40% to 67% at 1.5% DH) resulting in protein hydrolysates with solubility values above 52% in the pH range of 2 to 12, without improving interfacial properties of the hydrolysate (Pedroche et al., 2004a).

Phosphorylated Proteins

Endogenous phosphorus content of rapeseed protein may be from the phytates and phospholipids associated with proteins besides actual phosphorylation of proteins. Cruciferin may be naturally phosphorylated (Wan et al., 2007). Phosphorylation of proteins *in vitro* by covalently attaching an anionic phosphate groups to the molecule can increase net electronegativity leading to protein cross-linking to certain extent (Schwenke et al., 2000) and causing changes in the protein molecule surface properties such as surface hydrophobicity, microstructure, and solubility. It is also expected that deliberate phosphorylation of reactive groups will provide characteristics of highly phosphorylated proteins such as casein. When phosphorylated, rapeseed protein isolate obtained by alkali extraction and isoelectric precipitation has exhibited pH shift of minimum protein solubility and overall low solubility in the acidic pH range (Krause, 2002). Phosphorylation cannot decrease time-dependant surface tension of air-water (buffer) interface as succinylation; however, the cross-linking favors the formation of strong cohesive protein film around the air bubbles that can stabilize the foam consequently improving foam capacity and stability of rapeseed proteins. Foaming properties depend on rapid adsorption and unfolding of the protein at interface, especially foam stability is favored by a strong flexible cohesive film around the air bubbles (Kinsella, 1976; Dickinson, 1998). Succinylation or phosphorylation of rapeseed proteins results in high net negative charge and increases the polar component of the free surface energy. As a result of this change the wettability of protein-water film on hydrophobic glass surface is improved. Distinct high molecule surface areas observed for acetylated and phosphorylated (largest) proteins indicate that modified rapeseed proteins can form large molecular structures (Krause, 2002).

Molecular Engineered Proteins

The changes that have been observed for the *in vitro* digestibility and thermal properties of molecular engineered procruciferin will lead directions to generate cruciferins with improved functionalities. Using the most suitable structure model available for cruciferin, six disorder regions can be designated similar to the ones identified in the proglycinin crystal structure (Adachi et al., 2001), in which 5 of the regions have been identified through alignment of various 11S globulins of legumes and non-legumes as described by Wright (1987). Modified procruciferin² cru2/3a has been obtained using cDNA constructs (Tandang et al., 2004a; 2004b) altered for the variable regions II (Glu-rich and the longest variable region) and IV (shortest variable region of cruciferin), and replacing the additional Cys²⁸⁷ residue with Thr (Tandang et al., 2004b). The cleavage site of α -chymotrypsin on the trimeric procruciferin lies

²The protein sequence of this cDNA sequence is equally similar to P33523 of BnCI (99.4%) and P11090 of CRUA (99.1%).

within the variable region II which has been confirmed based on the N-terminal sequence (QPGGGSP) of the major digestion product after insertion. The location of the cleavage site in the interchain disulfide bond containing IE-face of the native hexameric protein (Adachi et al., 2003) may cause the resistance to α -chymotrypsin attack. Introduction of the acidic region can make the variable region IV of cruciferin more susceptible to the α -chymotrypsin; however, removal of the additional Cys²⁸⁷ residue at the β -chain makes it less susceptible.

The presence of additional Cys²⁸⁷ has been implicated in generating SH/S-S exchange and causing release of basic polypeptides (Inquello et al., 1993). In native and wild type cruciferin this release of polypeptides may result in precipitation of protein without forming a gel when heated to $>70^{\circ}\text{C}$ (Tandang et al., 2004b). This behavior is unfavorable in forming soluble aggregates. Generation of good quality thermally induced gels require soluble aggregate formation rather than heat induced precipitation. Replacement of the additional Cys residue of the cruciferin β -chain with Thr has no effect on the thermal stability of the molecule. As a consequence of deleting variable region II together with replacing the additional Cys residue with Thr, destabilization of the molecule can happen. This has been indicated in the reduction of the T_m from 74.7°C (of wild type procruciferin) to 71.7°C for the modified protein. Provision of a highly charged region (e.g., insertion of acidic residues of Gly) as well as removal of free Cys residue (reduces possibility of S-S bond formation) is very effective in forming soluble aggregates hence the gel formation of cruciferin. The removal of additional Cys residue also prevents SH/S-S exchange reaction and causes favorable association that leads to oligomerization before heat denaturation to form soluble aggregates. According to this argument, the CRU3_BRANA (P33525, Fig.1) subunit which does not have this additional Cys residue should perform similar to the modified cruciferin subunit described by Tandang and group (2004b; 2005). Insolubilization of cruciferin upon heating is further induced due to deletion of variable region II probably due to the exposure of hydrophobic regions. The absence of region II may not support the formation of folding intermediates. The exposure of hydrophobic region may cause loss of solubility in the lower pH ranges (pH 4 to 6, $\mu = 0.08$). Insertion of charged and hydrophilic region into the molecule has helped the protein to form soluble aggregates upon heat denaturation. Having a charged region (in the variable region IV) also facilitates the formation of a lesser amount of insoluble aggregates. None of the destabilizing and hydrophilization effects brought out by these molecular modifications have facilitated emulsifying properties of cruciferin (Tandang et al., 2005).

EDIBLE PRODUCT DEVELOPMENT WITH CRUCIFER PROTIENS

The first reported study on rapeseed protein isolation by Sosulski and Bakal (1969) and a later study by Sosulski et al. (1976) have concluded that protein products are brown in color, therefore, have limited use in incorporating to foods despite

the superior functionalities exhibited over soy protein isolates. Later, with improved *Brassica* protein concentrates and isolates replacement of meat proteins in emulsion-type meat products have been studied by many groups. Emulsified processed meat products (e.g., bologna, wieners) are suitable systems to incorporate plant proteins to replace meat proteins and lipids. Only few studies are available showing the utility of *Brassica* proteins in baked foods.

Possibility of adding rapeseed protein isolate and concentrate obtained from successive water, HCl and NaOH extraction processes into bread dough to replace wheat flour at 5% (w/w) level has been reported (Kodagoda et al., 1973b). A decrease in the loaf volume by 10–15% and 20% for concentrate and isolate, respectively have been observed and this could be restored by addition of 0.5% emulsifier. With the emulsifier, 10 to 15% larger loaf volume than wheat flour containing control has been obtained when water extracted or HCl extracted protein isolate is used. Replacement of egg white protein in meringue with water extracted rapeseed isolate at 3% level has resulted in no drip. The HCl extracted protein at the 3% addition level has provided 10% larger specific volume and both these protein products at the same level have improved the foam stability. Incorporation of canola protein isolate (92.2% crude protein) and concentrate (89.4% crude protein) into bread dough to replace wheat flour protein up to 18% has not caused any detrimental effect on dough and loaf quality (Mansour et al., 1999). At this level of inclusion, Val and Tyr become the first limiting amino acid for the concentrate and isolate, respectively. A pasta product containing *B. juncea* mustard protein isolate (95% protein) that replace semolina at 2.5, 5.0, and 10.0% levels has exhibited decrease in cook weight loss, protein loss, and stickiness. Incorporation of mustard protein has enriched the Lys, Cys, Met, and Arg levels of this pasta product (Alireza-Sadeghi and Bhagya, 2008).

Use of rapeseed protein concentrate of SHMP-assisted extraction has resulted in improved peelability of the wiener (3.8% level of addition) casing, more liquid retention in beef patties (3% level of addition), and enhanced whipping properties in the meringue (9% dispersion and 1:1 ratio with egg white) formulations (Thompson et al., 1982). Changes in the color, flavor, and texture have been noted for all these three products. Mixing with egg white protein (1:1, 9% dispersion) has helped in alleviating some of the color and flavor problems of meringue with rapeseed concentrate. Replacement of meat content of bologna formulation up to 3% weight by canola protein isolate and concentrate has resulted in high water holding capacity and cook yield compare to the control product containing 55% lean beef, 20% tallow, 20% water, 2% salt, 1.5% mixed spices, 0.3% sodium tripolyphosphates, 0.2% pickling mix (Mansour et al., 1996). The products containing concentrates are darker than the products with the isolate. The flavor and taste are the most significant ($p < 0.5\%$) attributes that sensory panelists have noted for the products with rapeseed protein products. In model products containing 10.5% beef protein, 29% fat, 57.5% water, and 2.5% NaCl, and replacement of 1/3 and 2/3 of meat protein with canola protein isolate has generated improved cook stability of the product obtained at

high temperature (70°C vs 95°C) and long time (25 min vs 50 min) thermal treatment (Paulson and Tung, 1989a). This may be due to the high water holding and fat adsorption capacities of the plant protein which usually muscle proteins lose under such conditions. High values for hardness and springiness (by texture profile analysis) can be achieved in canola protein containing products when treated at 95°C for 50 min compared to the products containing soy protein isolate. Examination of the microstructure of the canola protein-substituted cooked meat emulsions has revealed a tighter and less lacey protein matrix mixed with small fat particles. In such systems, by the time and temperatures that the canola proteins contribute to structure formation, the meat proteins are already denatured and the gel matrix is already formed, therefore, protein-lipid interactions such as fat absorption, hydrophobicity, and oil emulsification properties contribute to the final performance of the ingredient rather than protein gelation alone. A sausage formulation containing rapeseed protein concentrate (dehulled meal washed with isopropanol and alkali) replacing casein (2% by weight) while maintaining the same meat protein and fat content has scored better aroma and taste attributes but poorer texture and color for the cooked product than the control containing casein (Yoshie-Stark et al., 2006). Canola meal proteins hydrolyzed with Alcalase and Flavourzyme® have exhibited potential antioxidative activity and its hydrolyzed product has provided improvement in water binding ability of cooked meat, thus the cook yield (Cumby et al., 2008). The canola protein products of micelle process show a broad spectrum of functional properties that can compete with non-plant proteins such as egg white, egg yolk, gelatine, and milk proteins when incorporated into various prototype food products (Murray, 2005; Hiron et al., 2006; Hiron, 2007).

Yellow mustard flour prepared by dehulling and heat inactivation of myrosinase (deheated mustard flour) is widely used in processed meat products as a binder (<http://www.gsduunn.com>; <http://www.minndak.com>). The liquid retention abilities, emulsifying properties, and contribution to the structure formation upon thermal treatment are the functionalities of yellow mustard flour components (mainly proteins and to certain extent mucilage) that are valuable in such applications. The possibility of developing a stable emulsion-type beverage with Ca-soluble *S. alba* protein isolate has been reported by Aluko and group (2004). This protein product can provide physicochemical stability to the beverage-type product formulated with CaCl₂ up to 3% (w/v) level of addition, which is a distinguishable property of yellow mustard protein. Current food use of *Crucifer* protein products other than yellow mustard protein containing flour is limited to experimental or proto-type products.

VALUE IN NON-FOOD USES

Peptides with Biological Activities

Primary amino acid sequences of seed storage proteins have been carefully analyzed to find peptide sequences that have

proven biological activities (Tessier et al., 2005). *In silico* analysis of available primary sequences of *Brassica* cruciferin and napin with BioPep (<http://www.uwm.edu.pl/biochemia>; Tables 5 and 7) provides potential peptide sequences that can be generated from these proteins and the respective bioactivities. These activities include antiamestic, antihypertensive, antithrombotic, antioxidative, anorectic, etc. which has potential health benefits as well as pharmaceutical value. Cruciferin and napin of *B. napus* contain peptide sequences that could present unique biochemical functions; however, releasing of these sequences from the intact proteins poses a technical challenge. Tables 5 and 7 provide potential peptide sequences that can be generated from cruciferin protomers and napin proteins of *B. napus*, respectively.

Among the bioactivities of plant protein derived peptides, angiotensin I-converting enzyme inhibiting (ACEI) ability has been studied due to the relative importance of lowering elevated blood pressure levels under renin-angiotensin mediated hypertensive conditions. Peptides with ACEI activity have been obtained from *B. napus* (Pedroche et al., 2004b; Wu et al., 2008) and *B. carinata* (Pedroche et al., 2007) seed protein hydrolysis. Peptides having IY, RIY, VW, and VWIS sequences have been isolated as ACE inhibiting molecules that can be generated from rapeseed proteins (extracted with 1 M NaCl and extensively dialyzed) upon hydrolysis with subtilisin (enzyme Carlsberg-type III from *Bacillus licheniformis*) (Marczak et al., 2003). Among these peptides RIY has exhibited high potency as an antihypertensive peptide in spontaneously hypertensive rat models. The potential of generating ACEI peptides from rapeseed protein hydrolysed with Alcalase (Pedroche et al., 2004b) has been confirmed to produce two peptides having sequences of VSV and FL (Wu et al., 2008).

Oral feeding of RIY peptide has resulted in anorectic effects on fasting ddY male mice (Marczak et al., 2006). The peptide RIY is capable of blocking cholecystokinin-1 (CCK1) receptor antagonist lorglumide and decreasing gastric emptying rate by blocking lorglumide. Although no affinity towards the receptor is found, the peptide can decrease food intake and gastric emptying by stimulating CCK release indirectly. The RIY peptide sequence is exclusive to napin protein and can be verified by *in silico* analysis (Table 7).

Hydrolysis of *B. napus* proteins (alkali extracted and acid precipitated) with endoprotease Alcalase has generated peptides capable of inhibiting human immunodeficiency virus (HIV) protease (Yust et al., 2004). The ethanol soluble peptides of rapeseed meal protein possess antioxidant activities manifested as reducing power, hydroxyl, and DPPH radical scavenging activity and ferrous-induced phosphatidyl choline oxidation inhibition, and antithrombotic activity (Zhang et al., 2008). Table 11 summarizes the bioactivities and respective peptide sequences identified from hydrolyzed *Brassica* seed storage proteins showing the potential of using protein derived molecules in performing biochemical functions related to important diseases in human.

Rapeseed protein hydrolysate produced using Orientase 90N® from *Bacillus subtilis* (32% DH, 1.2% free amino acids, and size distribution of 74% < 1 kDa, 23% of 1–10 kDa, 3%

Table 11 Reported biological activities and respective peptides obtained from hydrolyzed *Brassica* seed storage proteins

Reference	Protein source	Biological activity and test model	Peptide sequence	Relation to major seed storage protein
Marczak et al., 2003	Rapeseed meal protein extracted into 1 M NaCl and dialyzed and digested with subtilin.	• ACEI – In vitro, HHL substrate • Antihypertensive activity – SHR model	IY, RIY, VW, VWIS	Cruciferin (IY, VW) Napin (IY, RIY)
Marczak et al., 2006	Synthesized RIY peptide (identified from Marczak <i>et al.</i> 2003 study)	• Food intake and gastric emptying using male ddy mice model	RIY	Ribosomal protein (VWIS) Napin
Pedroche et al., 2004b	Alkali extracted and pH 4.3 precipitated rapeseed protein, Alcalase hydrolyzed up to ~23% DH	• ACEI – In vitro, HHL substrate	No peptide sequences reported	Not identified
Wu et al., 2008	Aqueous slurry of canola meal was hydrolyzed with Alcalase and unhydrolyzed protein was removed.	• ACEI – In vitro, HHL substrate	VSV, FL	Cruciferin (VSV, FL) Napin (VSV)
Yoshie-Stark et al., 2006	<i>Brassica napus</i> (Express) protein concentrate of heat treated, hexane extracted meal with an isopropanol wash. Hydrolysed with pepsin and pancreatin	• ACEI – In vitro, HHL substrate • Bile acid binding – In vitro binding of Na cholate • Radical scavenging – In vitro DPPH radical scavenging	No peptide sequences reported	Not identified
Yust et al., 2004	Alkali extracted and pH 4.3 precipitated rapeseed protein, Alcalase hydrolyzed up to ~23% DH	• In vitro pepsin assay and Cell assay with <i>E. coli</i> containing plasmid PT₅ with cDNA coding for HIV-1 protease	No peptide sequences reported	Not identified
Zhang et al., 2008	Aqueous rapeseed slurry treated with pectinase, cellulase and beta glucanase at pH 5 and then with Alcalase at pH 9.0. Recovered aqueous phase fractionated into ethanol (25% and 55%) soluble peptides	In vitro – • Reducing power assay with K ferricyanide • Radical scavenging assay with DPPH radical • Hydroxyl radical scavenging assay with 2-deoxy-D-ribose • Inhibition of ferrous induced phosphatidylcholine oxidation liposome system • Inhibition of thrombin catalyzed coagulation of fibrinogen	No peptide sequences reported	Not identified

>20 kDa on weight basis) has enhanced the growth of insect cell Sf9 line in serum-free media more effectively than bovine lactalbumin hydrolysate without affecting the general metabolism of the cells (Depris et al., 2003). Supplementation of serum-free media for CHO C5 cell line with rapeseed protein hydrolysates containing peptides of 500–5000 Da and <500 Da also has enhanced cell growth rate (Farges-Haddani et al., 2006) indicating that ability of hydrolyzed rapeseed proteins to replace animal proteins in the cell culture media.

Industrial Adhesives, Foams, and Plastics

Proteins can adsorb at air-water interface and form a continuous viscoelastic film around air bubbles and stabilize foams. The surfactant stabilized foams have well ordered orientation of molecules at the interface, with the polar head placed in the aqueous phase and the hydrophobic chain towards the apolar phase. Generation of a “green surfactant” using rapeseed

protein hydrolysed products (Alcalase-assisted hydrolysis of de-fatted meal) having PCL of 5.4 amino acid residues has been described by Sanchez-Vioque and group (2001). Acylation of peptides with C₁₀ and C₁₂ chains (68–94% amino group modification) has enhanced the foam properties such as reduced foam drainage, foam decay, and liquid incorporation into foams. The acylated peptide foams contain homogeneous bubble size with uniform texture and delayed aging indicating that grafting of aliphatic chains of 10 and 12 carbon atoms to amino groups of rapeseed protein peptides is an excellent approach to generate protein-based foaming agents.

Canola proteins are also natural thermoplastic polymers having various amino acid residues available for a wide range of molecular interactions. A plasticizer such as glycerol can disrupt protein polymer-polymer interaction and enhance lubricity of canola protein polymers thus the flexibility and brittleness of the resulting thermoplastic material. Such canola protein/polyester blends can generate plastic-type material through extrusion and injection moulding process (Manamperi et al., 2009).

SUMMARY

Oleaginous *Brassicaceae* plants are efficient in storing proteins of 11S globulin and 2S albumin groups in their seeds. Cruciferin and napin proteins of *Brassicaceae* are not well understood for their utilization and conversion into useful molecules or products. Although much is known about the structure of predominant proteins of *B. napus*, the major Crucifer oilseed, the current portfolio for their applications is very short. Crucifer 11S proteins share a high degree of similarities with the molecular structure and properties of soy 11S legumin. Cruciferin and napin exhibit different physico-chemical and technologically valuable functionalities. Napin exhibit more hydrophilic protein properties than cruciferin. The extent of cruciferin and napin levels and their variation in the commercial *Brassica* oilseed cultivars are not available. The products of current *Brassica* protein recovery technologies are mixtures of unknown levels of these two proteins and this leads to unpredictable functions and activities of the products. Convincing evidence exists on the nutritional value, biological activities, and routes of improvement of cruciferin and napin. The technologically valuable functionalities and the nutritional value of cruciferin and napin can be maximized if they are separately utilized. As a food protein source, *Brassicaceae* proteins have different essential amino acid levels, digestibility profiles, and associated component profiles than soy protein. Other than using as a bulk plant protein ingredient, Crucifer protein-derived molecules can be employed in nutrition, disease therapy in humans as well as plants (as antimicrobials), and new material and structure generation, etc. A comprehensive understanding of the proteins structure, structure-function relationships, and genetic control of structure-function will lead to improved utilization of *Brassicaceae* proteins and development of new protein-based applications.

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