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Characterization of a Calcium-Soluble Protein Fraction from Yellow Mustard (*Sinapis alba*) Seed Meal with Potential Application as an Additive to Calcium-Rich Drinks

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A calcium-soluble protein isolate (CSPI) was prepared from the supernatant obtained after addition of 0.75 M calcium chloride to a pH 5.0 aqueous extract of yellow mustard (*Sinapis alba*) seed meal. Total amino acid analysis showed that the CSPI has significantly higher ($p < 0.05$) contents of glutamic acid + glutamine, cysteine, and proline when compared to the precipitated, calcium-insoluble proteins. Peptide mass fingerprinting of tryptic peptides of the major polypeptides by mass spectrometry indicated that the CSPI is composed mainly of cruciferin proteins with a contribution from napins (the major allergenic proteins of *S. alba*). The *S. alba* CSPI had significantly higher ($p < 0.05$) protein solubility and emulsion formation ability in the presence of 0.75 M calcium chloride when compared to similar isolates prepared from *Brassica juncea* (brown mustard) and soybean seed meals. We suggest that the *S. alba* CSPI could be used to prepare calcium-fortified high protein liquid products. However, the presence of allergenic proteins in this extract may limit its widespread food use.

KEYWORDS: Mustard; *Sinapis alba*; protein isolate; calcium; soybean; emulsion; glucosinolates

INTRODUCTION

Calcium is an important nutritional requirement in human and animal nutrition where it plays important roles in bone mineralization, blood clotting, and proper nerve and muscle functions, among others. Therefore, it is common practice to fortify foods with various levels of calcium to compensate for intrinsic deficiency or loss of calcium during food processing. Because of the high susceptibility of most food proteins to calcium-induced precipitation, very minimal levels of calcium can be tolerated in some food products. In fact, the susceptibility of proteins to calcium-induced precipitation is exploited in the manufacture of tofu, a soybean food product that consists of coagulated proteins (*1*). Moreover, calcium-induced precipitation of some food proteins is greater at high protein concentrations than at low concentrations. For example, emulsions stabilized by high casein concentrations are more susceptible to calcium-induced destabilization than those that contain lower concentrations (*2*). Therefore, calcium fortification of high protein diets poses a great challenge to food researchers.

Calcium-induced protein precipitation may be minimized in beverages by addition of hydrocolloids as stabilizers (*3*, *4*) and by a combination of calcium lactogluconate and potassium citrate (or hexametaphosphate) (*5*). However, addition of hydrocolloids is highly limited because it has a low threshold beyond which the flow properties and texture of the product is modified. Both polyphosphates and gluconates are made from nonrenewable resources, and their calcium binding capacity is very limited. Health and safety regulations also provide limits for the use of additives such as phosphates and gluconates in foods (*6*). Phosphorylated polypeptides such as caseinophosphopeptides have also been suggested as carriers for calcium fortification of foods (*7*, *8*). However, the increased cost associated with production of phosphorylated peptides has limited their use to laboratory experiments. Therefore, there is a need to find other types of biomolecules that could enhance calcium fortification of protein-enriched foods without adverse effects on organoleptic properties.

Sinapis alba is part of the mustard family and is commonly known as white or yellow mustard (*9*). Because of the high levels of glucosinolates, *S. alba* seeds are used mostly as a spice or seasoning in many foods in different parts of the world, although it is also used as a source of edible oil in parts of the Indian subcontinent (*10*). Research is being conducted at the Agriculture and Agri-Food Research Centre, Saskatoon in an effort to reduce the antinutrients in *S. alba* with the aim of increasing worldwide use of the seeds as a source of edible oil. Since the residual meal remaining after oil extraction is rich in proteins (40−48% dry weight basis), it may serve as a suitable

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raw material in the manufacture of protein ingredients for the food industry. Production of protein isolates from *S. alba* defatted seed meals with undetectable levels of glucosinolates have recently been demonstrated (*10*). However, *S. alba* seeds also contain the 2S albumin (napin) that has been confirmed as a major allergenic protein (*11*, *12*) and a trypsin inhibitor (*13*). Therefore, protein isolates that are prepared from *S. alba* seeds may contain these antinutritional factors.

In this study, we report that a subset of *S. alba* seed proteins are resistant to precipitation in the presence of high levels of calcium chloride. The aim of this work was to determine some of the physicochemical and functional properties of the protein fraction from *S. alba* that is resistant to calcium-induced precipitation. Similar protein fractions were also prepared from *Brassica juncea* (brown mustard) and soybean seed meals for comparison purposes.

MATERIALS AND METHODS

Materials. *S. alba* and *B. juncea* seeds were obtained from the Crop Breeding Section of Agriculture and Agri-Food Canada, Saskatoon Research Station. Defatted soybean meal was obtained from ADM Protein Specialties Division (Decatur, IL). Soybean lecithin and xanthan gum were purchased from Sigma Chemicals (St. Louis, MO).

Preparation of Calcium-Soluble Protein Isolates (CSPI). Mustard seeds were dried and ground in small samples in a coffee mill for 2−3 min to pass through a #40 mesh screen, and the resulting flours were defatted in a Soxhlet apparatus using hexane as the solvent. CSPIs were prepared according to the method of Aluko and McIntosh (*14*), which was modified as follows. Defatted seed meal (*S. alba*, *B. juncea*, or soybean) was extracted with a 0.1 M NaOH solution (flour/solvent, 1:10) for 20 min at 23 °C on a magnetic stirrer. The resulting slurry was centrifuged at 10000*g* for 30 min, and the supernatant was filtered through a Whatman no. 1 filter paper to remove particulate matter. The filtrate was adjusted to pH 5.0 with dilute HCl followed by gradual addition of solid calcium chloride up to a 0.75 M concentration. After stirring for an additional 20 min, the slurry was centrifuged at 10000*g* for 30 min, and the supernatant was dialyzed against water using a 6−8 kDa molecular weight cutoff membrane for 48 h at 4 °C with 4−5 water changes. The dialyzed fraction was freeze-dried and called CSPI.

Protein Solubility. The effect of an increase in protein concentration on solubility of the protein isolates in the presence of 0.75 M $CaCl_2$ was determined as follows. Protein slurries (5−25 mg/mL in 0.75 M $CaCl_2$ solution, pH 5.0) were prepared and mixed on an Eppendorf Thermomixer R at 1400 rpm and 25 °C for 30 min, followed by centrifugation at 10000*g* for 30 min. The supernatants were then analyzed for protein content using the modified Lowry method as described by Markwell et al. (*15*). Protein solubility was calculated as percentage ratio of protein concentration in supernatant to the protein content of the slurry.

Beverage Emulsion. The following beverage emulsion preparation protocol was adapted from the method of King et al. (*16*). Samples were prepared to contain 2% fat. For each sample, 1.0 g of lecithin (as emulsifier), 0.15 g of xanthan gum, and 10.0 g of partially hydrogenated soybean plastic fat shortening were weighed into a 15 mL beaker, and the mixture was heated on high in a microwave oven for 3.5 min. The oil mixture was swirled to ensure complete dissolution of lecithin. The oil mixture was added to 454 mL of water and 35 g of *S. alba* CSPI (or soy protein isolate as control) and blended for 1.5 min in an Ostersizer 10-speed household blender at high speed (setting = 7). For samples containing calcium, 3.0% (w/v) of calcium chloride was added to the water prior to mixing with the oil mixture and protein isolate. Particle size, $d_{3,2}$, of the beverage emulsions was determined in a Mastersizer 2000 with purified water as the dispersant according to the method of Aluko et al. (*17*).

Gel Electrophoresis. Reduced and nonreduced gel electrophoresis of protein samples on 8−25% gradient gels was performed as previously described (*14*) using the PhastSystem Separation and Control and Development Units according to the manufacturer's instructions (Pharmacia LKB, Montreal, PQ). Samples were prepared for nonreduced SDS−PAGE by mixing with a Tris-HCl buffer solution, pH 8.0 containing 10% SDS and 0.01% bromophenol blue. Sample solutions were placed in boiling water for 5 min, cooled to room temperature, and centrifuged at 16 000*g* for 10 min, and an aliquot (1 μL) of the supernatant was loaded onto the gel. Reduced samples were prepared by adding 5% (v/v) 2-mercaptoethanol (ME) to an aliquot of the supernatant from 10% SDS extraction, and 1 μL was loaded onto the gel.

Table 1. Glucosinolate Contents in *S. alba* Meal and Protein Products

	glucosinolates[a] (μmol/g)				
sample	HOBE[b]	TOTA	TOTMT	TOTI	TOTG[c]
defatted meal	190.83a	5.437a	0.120a	1.900a	199.15a
protein isolate (calcium precipitated)	0.14b	0.023b	0.000b	0.013b	0.18b
protein isolate (calcium soluble)	0.41b	0.190b	0.040b	0.000b	0.77b

[a] Mean of three determinations. For each column, means with different letters are significantly different ($p < 0.05$). [b] HOBE, hydroxybenzyl; TOTA, total aliphatics; TOTMT, total methylthios; TOTI, total indolyls; and TOTG, total glucosinolates. [c] Sum of all glucosinolates including minor components that are not indicated on the table.

Analysis of Glucosinolates. Glucosinolate content was determined according to the method of Heaney and Fenwick (*18*).

Amino Acid Analysis. Samples were hydrolyzed under vacuum at 110 °C for 24 h using 6 M HCl containing 1% phenol. Amino acid composition of the hydrolysate was determined by high-pressure liquid chromatography according to the method of Bidlingmeyer et al. (*19*). The cysteine and methionine content was determined using performic acid oxidation (*20*), and tryptophan content was determined by alkaline hydrolysis (*21*).

Peptide Sequencing. CSPI proteins were separated on a 16.5% Tris-tricine gel and stained with Coomassie Brilliant Blue G-250 as described by Schägger and von Jagow (*22*). Protein bands were cut out, destained, reduced, and processed for trypsin digestion following standard procedures (*23*). The tryptic peptides were recovered from the gel pieces by sonication in 5% trifluoroacetic acid (TFA)/50% acetonitrile (ACN) then dried down in a Speed Vac. The peptides were solubilized in the same TFA/ACN solution and then mixed with α-cyano-4-hydroxycinnamic acid in 0.1% TFA/50% ACN. The mixtures were applied to a Matrix-assisted laser desorption ionization (MALDI) plate, allowed to dry, then analyzed by MALDI time-of-flight (TOF) mass spectrometry analysis using a Voyager-DE STR mass spectrometer (Applied Biosystems, Bedford, MA) in the reflectron mode. Autocatalytic trypsin fragments were used as internal calibration standards. Proteins were identified by peptide mass fingerprinting using the *m*/*z* ratios of the tryptic peptides and the MS-FIT program of the ProteinProspector software (*24*).

Statistical Analysis. Each analysis was done in triplicate, and analysis of variance and Duncan's multiple-range test were carried out using the Statistical Analysis Systems software (*25*).

RESULTS AND DISCUSSION

Glucosinolates. Table 1 shows that the amount of glucosinolates, as a total or taken for individual group of glucosinolates, is significantly reduced ($p < 0.05$) in the protein isolates in comparison to the defatted *S. alba* seed meal. Therefore, the pungent hot flavor associated with yellow mustard seeds is not likely to be detected in food products containing the CSPI.

Amino Acid Content. The amino acid composition of *S. alba* seed meal, CSPI, and calcium-precipitated protein isolate (CPPI) are shown in **Table 2**. The most notable differences are the significantly higher ($p < 0.05$) levels of glutamic acid + glutamine, cysteine, and proline in the *S. alba* CSPI, when compared to the seed meal or CPPI. The increased levels of

Table 2. Amino Acid Composition of *S. alba* Defatted Seed Meal, Calcium-Precipitated Proteins (CPPI), and Calcium-Soluble Proteins (CSPI)

	concentration (g/100 g of protein)		
amino acid	meal[a]	CPPI[b]	CSPI[c]
Asx[d]	6.92	8.81	8.99
Glx[d]	13.29	12.29	21.01
serine	3.73	4.79	4.28
glycine	4.42	5.04	5.71
histidine	1.88	2.01	2.18
arginine	5.08	6.06	6.63
threonine	3.91	5.12	4.01
alanine	3.41	4.45	4.17
proline	4.78	4.39	6.06
tyrosine	2.94	4.34	2.59
valine	4.26	5.25	5.14
methionine	1.57	1.99	1.92
cysteine	1.59	1.10	1.80
isoleucine	3.11	4.18	4.18
leucine	5.71	7.39	7.66
phenylalanine	3.19	4.50	4.06
lysine	4.84	3.51	3.49
tryptophan	1.49	1.35	1.41

[a] Defatted seed meal. Protein content, dry weight basis (dwb), Kjeldahl N × 6.25 = 49.58%. [b] Precipitated proteins obtained after adjustment of a defatted seed meal aqueous extract to 0.75 M calcium chloride at pH 5.0. Protein content (dwb), Kjeldahl N × 6.25 = 84.93%. [c] Soluble (supernatant) proteins obtained after adjustment of a defatted seed meal aqueous extract to 0.75 M calcium chloride at pH 5.0. Protein content (dwb), Kjeldahl N × 6.25 = 89.47%. [d] Asx = Asp + Asn and Glx = Glu + Gln.

proline, cysteine, and glutamic acid may play a role in the calcium-resistance of the *S. alba* CSPI. This is because proline confers structural stability on polypeptides, while the negative charge of glutamic acid at pH > 5.0 favors interaction with the positively charged calcium ions. However, since the amino acid analysis result is for glutamic acid and glutamine, we cannot conclude that the difference observed in functionality is due to the differences in glutamic acid content of the samples. A decrease in proline content was shown to result from succinylation of canola 12S globulin, which had lower structural stability than native unmodified globulin (*26*). Similarly, replacement of proline with alanine led to decreased stability of ribonuclease T1 (*27*). Therefore, we suggest that the higher level of proline in *S. alba* CSPI may contribute to increased structural stability against calcium-induced protein precipitation. This is because binding of calcium to proteins can induce conformational changes (*28*, *29*) such as exposure of aromatic groups that favor increased protein−protein interactions. The higher structural stability of the CSPI could mean less conformational change and hence a reduced susceptibility to coagulation in the presence of calcium. The higher level of cysteine increases the potential for disulfide bond formation and hence higher structural stability of the CSPI.

Gel Electrophoresis. Results of the gel electrophoresis of proteins present in *S. alba* protein fractions are shown in **Figure 1**. In the absence of a reducing agent (disulfide-bonded polypeptides are present), the 15 (b), 28 (c), 50 (d), and 55 (e) kDa bands were the most prominent in the CSPI (lane 3). Proteins that were precipitated by calcium contained very small amounts of those proteins but contained a relatively high level of a 130 kDa protein band (a, lane 4) when compared to the CSPI. The 15 kDa polypeptide is part of the napin proteins and has been characterized as a trypsin inhibitor (*13*). Upon addition of β-mercaptoethanol (ME) to the CSPI, the intensity of the 15, 50, and 55 kDa bands was reduced, while that of the 28 kDa polypeptide was unaffected (lane 1). The results suggest

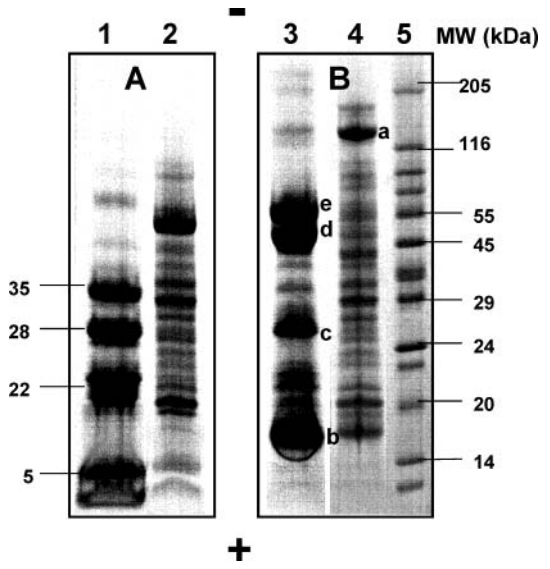

Figure 1. SDS−PAGE analysis of *S. alba* protein isolates. (**A**) With 2-mercaptoethanol: lane 1, calcium-soluble protein isolate (CSPI) and lane 2, calcium-precipitated protein isolate (CPPI). (**B**) Without 2-mercaptoethanol: lane 3, CSPI; lane 4, CPPI; and lane 5, standard molecular weight (MW) proteins.

that each of the three polypeptides (15, 50, and 55 kDa) contain smaller units of polypeptide chains held together by disulfide bonds. Covalent disulfide linkages in proteins contribute to structural stabilization, especially in conditions that lead to irreversible denaturation (*30*). The higher levels of the three disulfide-bonded polypeptides (15, 50, and 55 kDa) could have contributed to structural rigidity of the *S. alba* CSPI, which enhanced the ability of the proteins to resist calcium-induced precipitation, when compared to the calcium-precipitated protein isolate. The results are consistent with a previous report, which showed that the 15 kDa protein from *S. alba* seeds consists of two polypeptide chains held together by two disulfide bonds (*31*). The work of Neuman et al. (*13*) has also shown that the trypsin inhibitor activity of a *S. alba* 14 kDa subunit (estimated to be 15 kDa in the present work) was abolished upon treatment with dithiothreitol, which suggests that disulfide bonds are an integral part of the polypeptide structure and are required for proper function. The higher level of cysteine in the CSPI (**Table 2**) supports the higher incidence of disulfide bond formation that was observed in **Figure 1**.

Peptide Sequence. Polypeptides with masses of approximately 5, 22, 28, and 35 kDa were cut from a ME+SDS−PAGE Tris-glycine gel and digested with trypsin. The tryptic peptides were analyzed on a MALDI-TOF for accurate mass determination and peptide mass fingerprinting (**Table 3**). The masses were compared by database search with tryptic digest patterns of known peptides using the Protein Prospector software. The results indicated the presence of *S. alba* major allergen small subunit peptides in the 5 kDa band, while the 22, 28, and 35 kDa bands were composed of cruciferin peptides (**Table 3**). The amino acid sequence predicted for one of the tryptic peptides from the 5 kDa band is similar to the peptide corresponding to position 13-21 of the S1 polypeptide of *S. alba* (*13*). This polypeptide has been shown to be part of the napin group of proteins, which have allergenic properties. Therefore, incorporation of the *S. alba* CSPI into food drinks must recognize the potential for allergic reactions within the population.

Protein Solubility in the Presence of Calcium Ions. Figure 2 compares the protein solubility of different concentrations of CSPIs from *S. alba*, *B. juncea*, and soybean at pH 5.0 in the

Table 3. Amino Acid Sequence of Tryptic Peptide Fragments of *S. alba* Calcium-Soluble Protein Isolate

subunit mass[a] (kDa)	fragment sequence	calculated mass (Da)	actual mass (Da)	sequence assignment	position
5	EFQQAQHLR	1156.57	1156.53	napin	13–21[b]
22	GPPQSPQDNGLEETICSMR	2129.96	2130.20	cruciferin	311–329[c]
	VTSVNSYTLPILQYIR	1867.03	1867.06	cruciferin	350–365[c]
	YNMNANEILYCTQGQAR	2059.93	2060.00	cruciferin	383–399[c]
	FNTLETTLTR	1195.63	1195.61	cruciferin	485–494[c]
28	VQGQFGVIRPPLR	1466.86	1466.92	cruciferin	251–263[c]
35	GPFQVVRPPLR	1265.75	1265.86	cruciferin	288–298[c]

[a] Refers to **Figure 1A**, lane 1. [b] Ref *13*. [c] The ExPASy (Expert Protein Analysis System) proteomics server of the Swiss Institute of Bioinformatics.

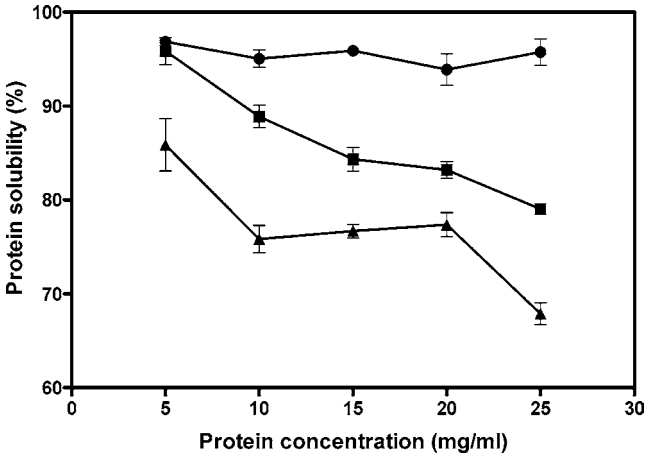

Figure 2. Effect of protein concentration on protein solubility of calcium-soluble protein isolates at pH 5.0 in the presence of 0.75 M calcium chloride: *S. alba* (●); *B. juncea* (■); soybean (▲).

presence of 0.75 M calcium chloride. The CSPIs from *S. alba* and *B. juncea* had higher solubility properties than the soy CSPI at all the protein concentrations used in this study. Furthermore, *S. alba* CSPI retained a constant high degree of solubility (>96%) over the protein concentration range, while solubility of both *B. juncea* and soybean CSPIs decreased with the increase in protein concentration. The results suggest that the *S. alba* CSPI would be resistant to calcium-induced protein−protein interactions within the protein concentration range of this work, while those interactions would gradually increase as the protein concentrations of *B. juncea* and soybean CSPIs increase. Since the pH that was used in this work (5.0) is close to the pH levels in some food drinks, it may be possible to use the *S. alba* CSPI to formulate functional beverages that contain high levels of calcium.

Particle Size ($d_{3,2}$) of Beverage Emulsions. The effect of the presence of calcium on the particle size and visual consistency of the beverages is shown in **Table 4**. In the absence of calcium, the soybean-stabilized emulsion is superior in quality to the *S. alba* CSPI -stabilized emulsion. No visible protein precipitate was present in either of the emulsions containing no calcium. However, upon addition of 3% (w/v) $CaCl_2$ there was an almost 20% decrease in the $d_{3,2}$ of the emulsion stabilized by the *S. alba* CSPI. This result demonstrates the ability of the *S. alba* CSPI to resist calcium-induced protein precipitation while maintaining the capacity to form an emulsion. In the absence of calcium, the emulsion stabilized with the soybean protein isolate had an excellent $d_{3,2}$ value when compared to a similar emulsion stabilized by the *S. alba* CSPI. There was also no visible protein aggregate (precipitate) in either emulsions in the absence of calcium (**Table 4**). On the other hand, the emulsion stabilized by soybean proteins had very poor qualities in the presence of calcium; $d_{3,2}$ increased substantially from 0.15 to 34.47 μm, while visible protein aggregates were present as clots in the emulsion. Such protein clots were not found in the emulsion stabilized by *S. alba* CSPI in the presence of calcium. The formation of visible protein aggregates in the soybean emulsion is in contrast to that obtained for the emulsion stabilized by *S. alba* CSPI and suggests that the soybean proteins are not capable of maintaining structural stability in the presence of high levels of calcium. The beverage emulsion results demonstrate the potential of the *S. alba* CSPI to form liquid food emulsions in the presence of high levels of calcium.

Table 4. Emulsion Properties of Model Beverage Formulations Stabilized by Calcium-Soluble *S. alba* and Soybean Proteins

protein sample	3% $CaCl_2$	$d_{3,2}$[a]	protein coagulation[b]
S. alba proteins	no	28.71	no
S. alba proteins	yes	23.52	no
soybean proteins	no	0.15	no
soybean proteins	yes	34.47	yes

[a] $d_{3,2}$ = particle size. [b] Protein coagulation = visible clots.

Conclusions. *S. alba* seeds contain a protein fraction (CSPI) that is resistant to precipitation in the presence of high levels of calcium chloride. The high levels of cysteine and proline coupled with the presence of disulfide-bonded polypeptides may be responsible for the structural stability of the *S. alba* CSPI. This extract provides an advantage over similar proteins from *Brassica* species and soybean in that it may be used to formulate food emulsions that are stable to calcium-induced destabilization. This CSPI has the additional advantage that it contains low concentrations of glucosinolates, meaning that such food emulsions would have low levels of the characteristic pungent flavor of *S. alba* seeds. However, the presence of napin proteins that have trypsin inhibitor activity, and allergenic properties may limit the extent to which CSPI can be incorporated into foods.

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