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Quantitative Detection of Allergenic Protein *Sin a* 1 from Yellow Mustard (*Sinapis alba* L.) Seeds Using Enzyme-Linked Immunosorbent Assay

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Allergy to yellow mustard (YM; *Sinapis alba* L.) seed proteins has been reported and is currently seen as a constraint that hampers expansion of YM protein utilization. The most predominant allergenic protein of YM seed has been recognized as *Sin a* 1. In this study, *Sin a* 1 was purified (*S. alba* var. Andante), rabbit polyclonal antibodies (pAb) specific to *Sin a* 1 were generated, and a sandwich enzyme-linked immunosorbent assay (S-ELISA) was developed to detect and quantify *Sin a* 1 from YM. The S-ELISA method using *Sin a* 1−pAb and its horseradish peroxidase conjugate resulted in a detection limit of 0.3 μg/mL for purified *Sin a* 1. The *Sin a* 1 contents of six YM lines were in the range of 0.82–2.94 mg/g when assayed by the developed S-ELISA method. The results showed that S-ELISA could distinguish *Sin a* 1 in YM seed-derived extracts rapidly and could be applied in controlling and/or monitoring of YM allergenic proteins.

KEYWORDS: 2S albumin; food allergens; mass spectrometry; napin; sandwich-ELISA; *Sin a* 1; yellow mustard

INTRODUCTION

Yellow mustard (YM, *Sinapis alba* L.) is frequently found as an additive in many food products and widely used in food formulations for its pungency, thickening and stabilizing abilities and other less-defined functionalities. Allergenic potential of mustards is well documented (*1*). Due to the ubiquitous use of mustard in product formulations, for example, sauces, dressings, marinades, seasonings, and processed meat, it could be a hidden allergen in many consumer products. Mustard is considered as the fourth most important food allergen for children in France, after eggs, peanuts, and cow's milk (*2*). Mustard allergy may account for up to 7% of food allergies among the patients where extensive studies were conducted (*3, 4*). The European Union includes mustard on its list of priority allergens which must be declared when present in foods (EU Directive 2003/89/EC). There is sufficient scientific evidence that the protein fraction of *Brassica juncea* (oriental/brown mustard) and *S. alba* possesses allergenic potential besides the skin sensitization by the oil fraction. Despite the extremely low number of reported cases of mustard allergy in Canada, many authors (*1, 5–8*) have emphasized the severity of allergic symptoms caused by mustard proteins including anaphylactic shock that need clinical intervention.

The major allergen in YM protein is *Sin a* 1, which is a napin (NAP) and also a 2S albumin storage protein (*9*). The NAP proteins of the Cruciferae (Brassicaceae) family belong to the prolamin protein superfamily similar to most other allergenic 2S seed storage proteins (*10, 11*). *Sin a* 1 is a fairly compact molecule that has a molecular mass of 14.18 kDa (*9, 11, 12*). This protein is composed of two polypeptide chains (large and small) linked by two interchain disulphide bridges. Structural details of *Sin a* 1 including the three-dimensional model are found in the following websites: http://ca.expasy.org/uniprot/P15322 and http://www.ifr.ac.uk/protall/images/Update_fig4.gif.

Immunological mapping of *Sin a* 1 with ten monoclonal antibodies (mAb) showed that two immunodominant regions can be found in the protein molecule (*13*). In these two regions most of the mAb were bound to a conformational antigenic determinant similar to IgE binding epitope. The most important allergenic determinants of the molecule are found to be the continuous epitope on the large chain of *Sin a* 1 and the hypervariable region of the molecule. NAP proteins are reported to resist both heat denaturation and in vitro trypsin digestion under simulated intestinal conditions (*12, 14*).

Numerous reports have indicated that mustard seed components possess various activities; whole seed is a herbal in alternative medicinal practices (*15*), mustard mucilage is active against sporadic and obesity-associated colon cancer (*16*), mustard isothiocyanates have anticancer properties (*17*), and phenolic compounds of mustard exhibit antioxidative properties in thermally processed meat (*18*). Compared to other Brassicaceae oil seed species such as *Brassica napus* L., *Brassica rapa* L., and *Brassica juncea*, *S. alba* L. has high content of seed proteins; 45.1 to 54.3% protein on an oil-free dry weight basis (*19*). Due to the high protein content, this protein rich

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seed could be further developed into a plant protein source. It has been reported that YM protein has unique solubility characteristics, i.e. a significant fraction of seed protein is soluble at high calcium ion concentration (*20*), which may enhance the utility of protein in a range of products. However, to our knowledge quantification of allergenic potential of YM seeds has not been reported previously.

The purpose of this study was to purify *Sin a* 1 and develop a detection method for *Sin a* 1 of YM using sandwich-ELISA (S-ELISA) approach. The sensitivity of the developed analysis method was compared with those of other immunoassays.

MATERIALS AND METHODS

Materials. Phosphate buffered saline with Tween 20 [PBST; 0.01 M phosphate buffer with 138 mM NaCl, 2.7 mM KCl, 0.05% (w/v) Tween 20, pH 7.4], phosphate−citrate buffer tablets (50 mM phosphate-citrate buffer, pH 5.0, 1 tablet/100 mL), hydrogen peroxide, 3,3′,5,5′-tetramethyl-benzidine (TMB), bovine serum albumin (BSA), horseradish peroxidase (HRP), Tween 20, goat antirabbit IgG-peroxidase conjugate, and Coomassie brilliant blue G-250 were purchased from Sigma (St. Louis, MO). For the purification of anti-*Sin a* 1 polyclonal antibody (pAb), a T-Gel absorbent column (T-Gel Purification kit, Pierce Co., Rockford, IL) was used. The columns, Hiprep 26/60, HiPrep phenyl Sepharose 6 and 15 S columns were from Amersham Biosciences (Pharmacia Biotech AB, Uppsala, Sweden). Microtiter plates (Maxisorp from Nunc Co., Roskilde, Denmark) and microplate reader (THERMOmax from Molecular Devices Co., Sunnyvale, CA) were used for the colorimetric assays. All chemicals were of analytical grade. A Milli-Q system (Millipore, Bedford, MA) was used to prepare deionized water for all mobile phases. MassPREP CHCA MALDI (α-cyano-4-hydroxycinnamic acid; matrix-assisted laser desorption ionization) matrix was obtained from Waters (Milford, MA).

Seed and Meal Preparation. Six genotypes of *S. alba* (AC Base, AC Pennant, Andante, HS3, HS4, and HS5; three commercially grown varieties and three breeding lines) seeds produced in 2004 at the Saskatoon Research Farm as part of the Brassica Oilseed Breeding Program of Agriculture and Agri-Food Canada were used in this study. The seeds were cracked in a stone mill (Morehouse Cowles, Chino, CA) and air classified to separate hulls and cotyledons using the protocol described by Lindeboom and Wanasundara (*21*). The cotyledons were then defatted using hexane (8:1, v/w) in Swedish extraction tubes (*22*) that were shaken at high speed for 20 min followed by filtration using a Whatman No. 1 filter paper (Whatman International Ltd., Maidstone, U.K.). The defatted meals were air-dried and ground in a coffee grinder to pass through a #40 mesh screen (425 μm, Tyler, Mentor, OH). The material so obtained has <3% (w/w) residual oil content when analyzed by AOCS official method (*22*).

Isolation and Preliminary Purification of *Sin a* 1. Protein extract prepared from the variety Andante (widely grown variety of YM containing high levels of protein and seed coat mucilage) was used to obtain purified *Sin a* 1. Protein extraction and separation were similar to the method described by Bërot et al. (*23*) with some modifications. Two grams of defatted hull-free YM flour was slurried in 20 mL of 50 mM Tris-HCl buffer, pH 8.5, containing 750 mM NaCl, 5 mM EDTA and 28 mM sodium bisulphite, mixed for 1 h and then centrifuged for 10 min at 15000*g*. The pellet was re-extracted under the same conditions. The supernatants of two extraction steps were combined, filtered through a Whatman no. 1 filter paper and referred to as the protein extract.

Protein separation and purification was achieved by a series of size exclusion (SEC), cation exchange (CEC) and hydrophobic interaction (HIC) chromatographic steps using an Åkta Explorer unit (Amersham Biosciences). Proteins were monitored as absorbance at 280 nm. Pigments of the extract were removed using Sephadex G-25 SEC (HiPrep 26/10 desalting column, 100 mm × 26 mm, 53 mL). The desalting column was equilibrated with 50 mM Tris-HCl buffer (pH 8.5) containing 1 M NaCl buffer, at a flow rate of 2.5 mL/min. The protein extract (19.8 mg of protein/mL as BSA equivalents, 13 mL per injection) was loaded on to the column and the depigmented protein extract was collected. The protein fractions (**Figure 1A**) were dialyzed 4 times against deionized water, using 2 kDa MWCO dialysis tubing and then lyophilized. Separation of NAP from cruciferin (CRU) in the depigmented extract (43.3 mg/mL as BSA equivalents, 8.75 mL per injection) was achieved by the CEC step using a cation-exchange Source 15 S column (92 mm × 16 mm, 18.5 mL) equilibrated in 50 mM Tris-HCl, pH 8.5, 5 mM EDTA, 28 mM sodium bisulphite (buffer A). The elution was performed at 0.5 mL/min by a linear gradient from buffer A to buffer B (buffer A with 1 M NaCl) in 10 min. The bound fraction (**Figure 1B**) that contained NAP was eluted when NaCl concentration of the elution buffer reached 0.25 M. Napin containing fraction so obtained was added with sodium sulfate to a 1 M final concentration and stirred overnight at 7 °C. After centrifugation for 10 min at 20000*g*, the supernatant was recovered and separated by hydrophobic interaction chromatography (HIC). Supernatant (26.8 mg of protein/mL as BSA equivalents, 3.75 mL per injection) was loaded on to a HiPrep Phenyl Sepharose 6 Fast Flow column (100 mm × 16 mm, 20 mL) equilibrated in 50 mM Tris-HCl, pH 8.5, containing 0.85 M sodium sulfate, 5 mM EDTA, 28 mM sodium bisulfite (buffer C). Subsequent elution of NAP was performed at 1 mL/min using a linear gradient of 0.85% (w/v) sodium sulfate and buffer A. Eluted protein fraction C2 (**Figure 1C**) was desalted on the HiPrep desalting column and lyophilized.

Further Purification of *Sin a* 1 for Immunogen and Reference Standard. The identity of purified NAP proteins was confirmed by electrophoresis separation using sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) using a Mini protean II system (Bio-Rad, Richmond, CA). Protein concentrations were adjusted to approximately 5 mg/mL before gel electrophoresis. The samples were diluted with sample buffer (1:4, v/v; pH 6.8; 0.25 M Tris-HCl; 4% w/v, SDS; 40% w/v glycerol; 0.005% v/v, bromophenol blue), maintained at 37 °C for 5 min, and then applied on to a polyacrylamide gel (15%, one 6 mm wide well in 1.5 mm thick gel) in the absence of β-ME. Electrophoresis was conducted for 160 min at a constant voltage of 80 V and then the gels were stained with Coomassie brilliant blue. The clear-cut bands with molecular mass between 12.5 and 14.5 kDa were excised and subjected to electroelution. Electroelution was performed to obtain further purification of these proteins. The gel slices were electroeluted at constant current of 40 mA for 5 h in an elution buffer containing 25 mM Tris, 192 mM glycine, and 0.1% (w/v) SDS, using 3.5 kDa MWCO membrane caps, and then electrodialyzed 4 h in the same buffer without SDS. A commercial electroeluter (model 422 Electro-Eluter, Bio-Rad) was employed according to the manufacturer's instructions. Gel slices were removed from the tubes, and the remaining aqueous fractions containing eluted proteins were combined and concentrated by vacuum centrifugation. Partially purified protein extracts obtained through these steps were dialyzed 4 times against PBS using 3.5 kDa MWCO dialysis cassettes and then lyophilized. Further purification was achieved by Sephadex G-25 (190 mm × 15 mm) column chromatography, and the protein fractions were monitored spectrophotometrically at 280 nm. Purity of the eluted *Sin a* 1 in the collected fractions was confirmed by SDS-PAGE. Concentration of proteins in the fractions was measured using Bradford Protein Assay. The fractions containing *Sin a* 1 were pooled and used as *Sin a* 1 reference standard (10 mg protein/mL), which was also the immunogen.

Peptide Mass Fingerprinting. *Sin a* 1 protein obtained as described above was separated on a 15% gel as described before. Protein bands of interest were excised using a stainless steel surgical blade (Sheffield, U.K.) and transferred to a 96 well polypropylene v-bottom microtiter plate (Corning Costar MTP, Corning, NY). Protein containing gel pieces were digested with trypsin using a MassPREP station robotic protein handling system (Waters/Micromass, Milford, MA), following the standard digestion procedure (*24*) that included destaining, reduction of cysteine with dithiothreitol, alkylation with iodoactemide, and digestion with trypsin treated with *N*-tosyl-L-phenylalanine chloromethyl ketone. The resultant peptides were extracted three times with 50% (v/v) acetonitrile into a microtiter plate. The plate was then dried under vacuum (DNA 120 Speed Vac, Thermo Savant), and redissolved in 1% (v/v) trifluoroacetic acid (TFA, 20 μL) with sonication for 30 min. Peptide mass fingerprinting (PMF) analysis of the digested peptide samples was carried out on an Matrix-assisted laser desorption

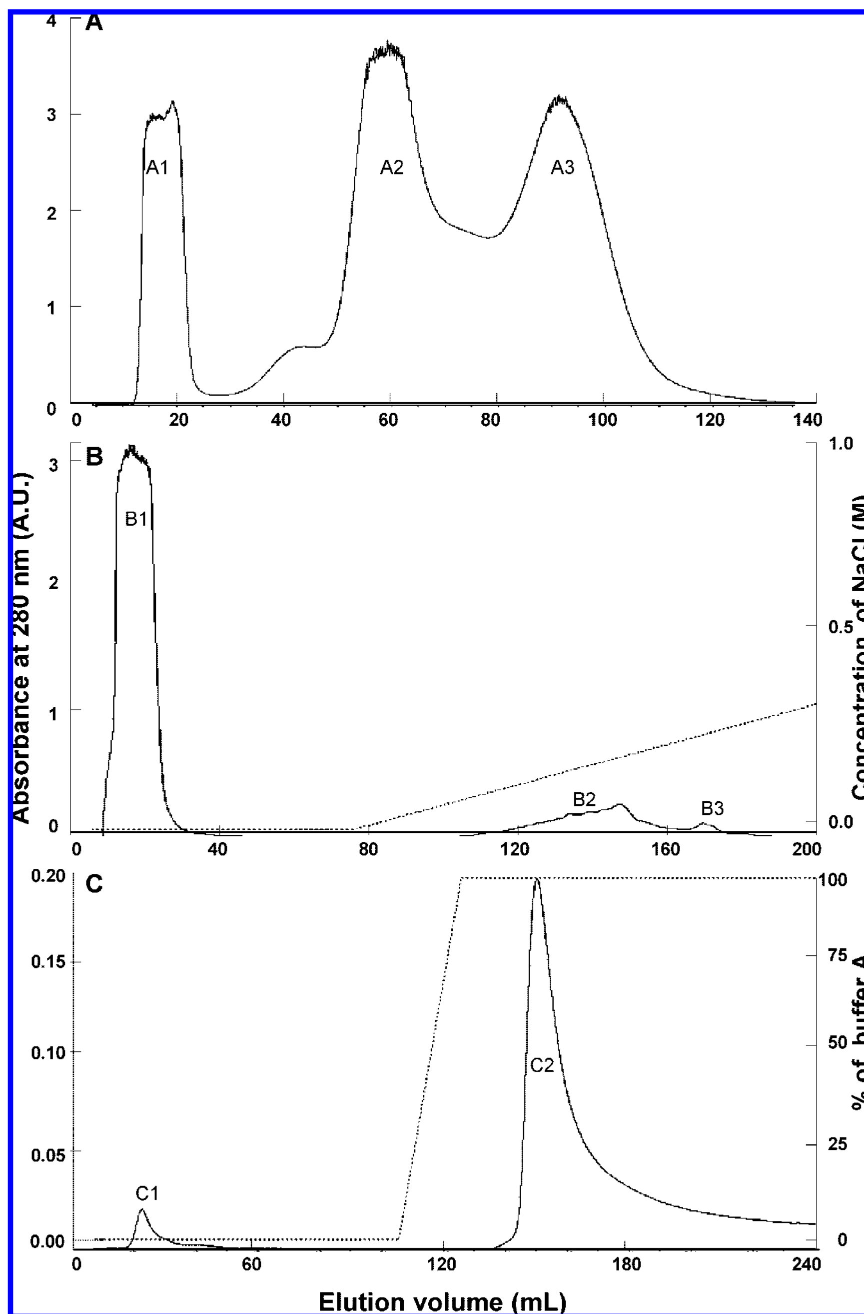


Figure 1. Elution profiles of YM protein extracts during purification of *Sin a* 1: (**A**) SEC on Sephadex G-25 column; (**B**) seperation of A1 fraction on a Resource S CEC column; (**C**) HIC separation of pooled fractions B2 and B3 on a HiPrep Phenyl Sepharose 6 Fast Flow column.

ionization time-of-flight mass spectrometry (MALDI-TOF-MS; Applied PerSeptive Biosystems Voyager DE-STR, Foster City, CA) equipped with a nitrogen laser operated at 337 nm, 3 ns pulse for ionization. The instrument was operated in positive ion reflectron mode. The samples were analyzed as follows; to the MALDI plate 0.75 μL of CHCA matrix (5 mg/mL) in 75% (v/v) acetonitrile containing 0.1% (v/v) TFA and 0.75 μL of protein digest was added, mixed on a 96-position of MALDI target plate and air-dried under a gentle stream of warm air. The MALDI plates had previously been mass accuracy optimized with the OptiPlate software in the Voyager 5.1 software (Applied Biosystems). The instrument was calibrated with a mixture of angiotensin I (MH^+ 1296.6853), adrenocorticotropic hormone (ACTH) 1-17 (MH^+ 2093.0867), and ACTH 18-39 (MH^+ 2465.1989). A total of 200 laser shots of the samples were converted into the monoisotopic values using Data Explorer (Applied Biosystems). The resulting peak lists were submitted to MASCOT (Matrix Science, London, UK) database engine (http://www.matrixscience.com/search_form_select.html).

Production of Polyclonal Antibodies. Immunization of rabbits with purified *Sin a* 1 was carried out at the Animal Care Unit, College of Veterinary Medicine under the animal care protocols approved by the Behavioral and Ethics Committee, University of Saskatchewan. First, the concentration of immunogen was adjusted to 1.0 mg of protein/mL in 10 mM PBS containing 138 mM NaCl and 2.7 mM KCl at pH 7.4. An aliquot of the protein solution containing approximately 150 μg of protein was then mixed with TiterMax Gold (CytRx, Norcross, GA) in a 1:1 ratio (v/v). Preimmune sera received from five New Zealand white rabbits were screened for crucifer protein exposure using YM extract by Western blot assay and those who were negative for exposure of napin was used for immunization. The selected animal (~3 kg) received the prepared immunogen through subcutaneous administration. Immunization boosts were given two times. Test bleeds were taken after 3 weeks of first immunization and 1 week after each boost. The antisera titer was determined by a noncompetitive indirect enzyme linked immunosorbent assay (NCI-ELISA) as described under ELISA. Blood from the animal (~80 mL/animal) was collected 1 week

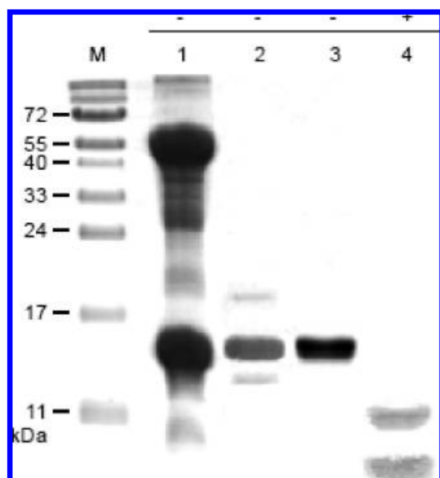


Figure 2. SDS-PAGE separation of napins from different steps of purification: lane M, broad range molecular marker; lane 1, YM proteins extract; lane 2, protein fractions containing NAP peaks (B2 and B3) from HIC; lane 3, NAP fraction after electroelution; lane 4, same protein as lane 3 after S−S band reduction with β-ME. Protein load in lanes 1–4 was 5 μg (− indicates no S−S bond reduction; + indicates S−S bonds are reduced).

after the last booster injection, which gave the highest antiserum titer. The antisera were isolated from the blood by centrifuging at 3000*g* for 20 min with NaN_3 to a final concentration of 0.02% (v/v) and stored at −70 °C until use.

Purification of Polyclonal Antibodies. The purification of immunoglobulin G (IgG) type antibodies was performed using a T-Gel purification kit and gel filtration chromatography (Sephadex G-25). Briefly, 1 mL of antisera was mixed with K_2SO_4 to a 0.5 M solution, centrifuged at 10000*g* for 20 min, and then filtered using 0.45 μm syringe filter (Sartorius Co., Goettingen, Germany). The filtrate was loaded onto a T-Gel column equilibrated with a pH 8.0 binding buffer (provided by the manufacturer) containing 0.5 M potassium sulfate and then washed with 50 mM sodium phosphate buffer at pH 8.0. The eluted IgG protein fractions (280 nm absorbing fractions) were pooled (2−10 mL). Then the fractions were desalted using a Sephadex G-25 column (190 mm × 15 mm) equilibrated with PBS. The fraction showing the highest 280 nm absorbing peak obtained through T-Gel column was found to be an IgG Ab according to the instructions provided. This purified pAb was used for HRP conjugate preparation. IgG concentration of 8 mg of protein/mL was recovered from 10 mL of antiserum through this purification.

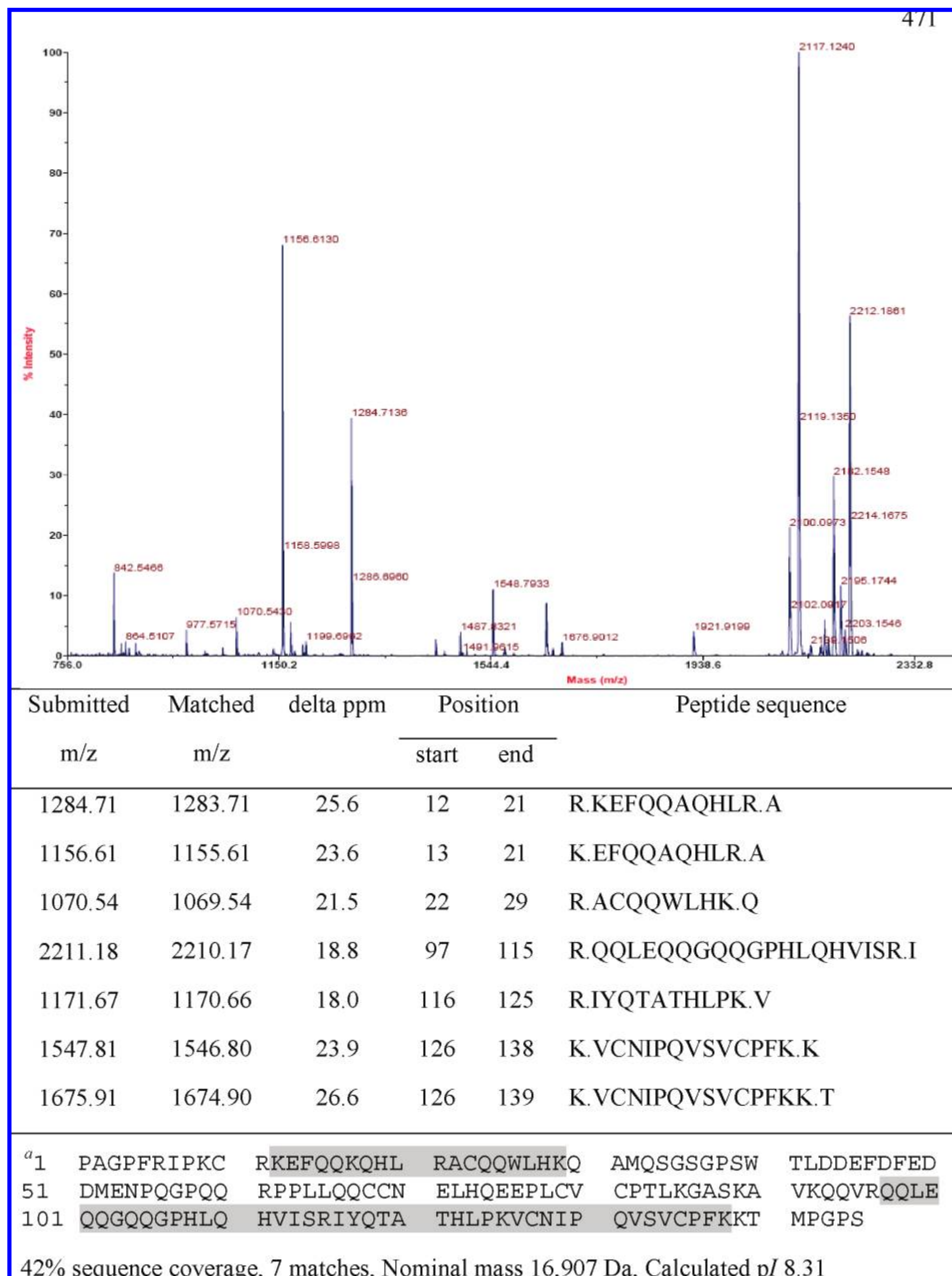
Preparation of HRP Conjugate. Conjugation of *Sin a* 1−pAb with horseradish peroxidase (HRP) was performed. To prepare periodate-activated HRP, equal volumes (0.6 mL each) of HRP (2 mg/mL) and $NaIO_4$ (21.4 mg/mL) were stirred at room temperature for 10 min and dialyzed against 5 mM of sodium acetate buffer (pH 4.0). The pH of the activated HRP was changed to 9.0 by adding 0.2 M of sodium carbonate buffer (pH 9.5) and then mixed with equal volume (1.0 mL) of the purified pAbs (8 mg of protein/mL) diluted with 0.01 M sodium carbonate buffer (pH 9.0). The mixture was stirred at room temperature for 2 h, and 0.1 M of $NaBH_4$ was added to the mixture (0.1× volume of the reaction mixture), further stirred at 4 °C for 2 h, and dialyzed against PBS. The pAb−HRP conjugates were purified by Sephadex G-25 chromatography and stored at 4 °C with 0.1% (v/v) thimerosal until used.

Sample Preparation for ELISA and Western Blot. Protein extraction of the defatted YM (AC Base, AC Pennant, Andante, HS3, HS4, and HS5) powders was carried out as described by Lindeboom and Wanasundara (*21*) using 0.25 g of material (1:20, w/v with 50 mM Tris-HCl buffer, pH 8.5, containing 750 mM NaCl, 5 mM EDTA, and 28 mM sodium bisulfite, mixed for 1 h, then centrifuged for 10 min at 15000*g* and re-extracted once more) unless specified otherwise. To assess the influence of extraction medium composition and pH on the assay, buffers of pH 7.4 (PBS or PBST) and pH 8.5 (0.05 M Tris-HCl) both containing 8% (v/v) protease inhibitors (Complete EDTA Free, Roche, Germany) were utilized to prepare protein extracts from meal. Extraction (similar to above) was performed at 4 °C for 1 h with stirring and then the mixture was centrifuged for 10 min at 10000*g* to recover soluble protein. The supernatant of the extraction step was filtered through a Whatman no. 1 filter paper. The pellet was re-extracted under the same conditions. The supernatants of the two extraction steps were combined (total volume ∼16 mL). One milliliter of the extract was centrifuged at 10000*g* for 10 min and the supernatant was divided for *Sin a* 1 analysis by ELISA and Western blot. YM extracts diluted by 10^0−10^2-fold with borate-buffered saline (BSB, 167 mM boric acid containing 125 mM NaCl, pH 8.5) was used for S-ELISA method. Protein content of the extract was assayed using the Bio-Rad Protein Assay Kit (Hercules, CA) with BSA as standard.

ELISA. ELISA assays were performed as described by Bhadra (*25*) and Acosta et al. (*26*). with some modification. The NCI-ELISA method was used to assess antibody titer. Each well of microtiter plate was coated with 100 μL of the purified *Sin a* 1 (10 μg/mL) diluted with BSB, and set at 4 °C overnight. After the incubation, wells were washed three times with BSB and unoccupied sites of the plastic were blocked with 150 μL of bovine serum albumin in BSB (1%, w/v, BSA−BSB) for 1 h at 25 °C, followed by three washings with BSB. One hundred microlitres of crude serum against *Sin a* 1, diluted with the BSB (dilution 1/5000, 1/10000, 1/15000) was added to each well, set at ambient temperature (∼22 °C) for 1 h and then washed with the BSB. One hundred microlitres of goat antirabbit IgG-HRP conjugates diluted with the BSB (1:5000, v/v) was added as secondary Ab to each well and incubated for 1 h. Finally, 100 μL of the substrate solution (0.01%, v/v, 3,3′,5,5′-tetramethylbenzidine; TMB, in phosphate−citrate buffer, pH 5.0 with H_2O_2 0.002%,v/v) was added and incubated for 30 min at room temperature. HRP activity was inhibited by adding 50 μL of 2 M of H_2SO_4. The absorbance of the mixture was read at 450 nm using a microplate reader. The serum dilution (1/10000) that gave optical density close to 1.000 was used for titer determination. In the case of competitive indirect ELISA (CI-ELISA), the method basically followed that of the NCI-ELISA, except that the step of diluted antiserum addition was replaced with 50 μL of antiserum (1/10000) and 50 μL of purified *Sin a* 1 standard (10 mg/mL, serial dilution up to 0.01 μg/mL) was added to each well. The *Sin a* 1 of the mixture competes for the pAb with the *Sin a* 1 of coated well. The plates were treated similarly as described for NCI-ELISA after this step. For S-ELISA, microtiter plate wells were first coated with purified *Sin a* 1−pAb diluted with BSB (10 μg/mL, 100 μL), incubated at 4 °C overnight, washed with BSB, blocked with BSA−BSB (1%, w/v), incubated at 25 °C for 1 h, and washed three times with BSB. Then *Sin a* 1 (100 μL, 10 mg/mL, serial dilution up to 0.01 μg/mL) or sample was allowed to react with pAb of the wells for 1 h at ambient temperature. After this incubation, 100 μL of pAb-HRP conjugates (dilution 1/2000, 1/4000, and 1/8000) were added to each. Subsequent assay steps for detection of HRP activity were similar to NCI-ELISA.

Cross-Reactivity and IC_{50} Value. The concentration of *Sin a* 1 in samples was calculated from the standard curve developed for S-ELISA and determinations were replicated minimum of three times. The IC_{50} value was determined using the standard curve generated with purified *Sin a* 1 (0−10^4 μg/mL) and *Sin a* 1−pAb−HRP (dilution of 1:4000, v/v). Relative affinity of the prepared pAb toward *Sin a* 1 was measured by determining 50% inhibition of antibody binding. The cross reactivity (%) of *Sin a* 1 pAb toward other Brassicaceae 2S proteins (purified NAP of *B. napus* and *Arabidopsis thalina*) was investigated using S-ELISA. The cross reactivity (%) was calculated using the concentration values of the test antigen required to produce 50% inhibition of pAb binding compared with IC_{50} of *Sin a* 1.

Western Blot Assay. Five micrograms of purified *Sin a* 1 or seed extracts were separated on SDS-PAGE gels (15%) in the absence of β-ME and electrophoretically transferred onto a nitrocellulose (NC) membranes (Mini Trans-Blot Module, Bio-Rad Co., Hercules, CA) according to the method described by Holbrook et al. (*27*) with the following modifications. The blots were blocked with TBS-Tween (20 mM Tris, pH 7.5, 150 mM NaCl, 0.05% Tween 20) containing 5% (w/v) dry skim milk powder overnight at 4 °C. The membranes were probed for 2 h at room temperature with antiserum diluted (1/5000)



Submitted m/z	Matched m/z	delta ppm	Position start	Position end	Peptide sequence
1284.71	1283.71	25.6	12	21	R.KEFQQAQHLR.A
1156.61	1155.61	23.6	13	21	K.EFQQAQHLR.A
1070.54	1069.54	21.5	22	29	R.ACQQWLHK.Q
2211.18	2210.17	18.8	97	115	R.QQLEQQGQQGPHLQHVISR.I
1171.67	1170.66	18.0	116	125	R.IYQTATHLPK.V
1547.81	1546.80	23.9	126	138	K.VCNIPQVSVCPFK.K
1675.91	1674.90	26.6	126	139	K.VCNIPQVSVCPFKK.T

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a1   PAGPFRIPKC RKEFQQKQHL RACQQWLHKQ AMQSGSGPSW TLDDEFDFED
51   DMENPQGPQQ RPPLLQQCCN ELHQEEPLCV CPTLKGASKA VKQQVRQQLE
101  QQGQQGPHLQ HVISRIYQTA THLPKVCNIP QVSVCPFKKT MPGPS
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42% sequence coverage, 7 matches, Nominal mass 16,907 Da, Calculated p*I* 8.31

Figure 3. MALDI-TOF-MS analysis results of peptide fragments from tryptic digest of purified *S. alba* protein fraction of C2 from HIC and the peptide match with the primary sequence of *Sin a* 1. Shaded areas in the primary sequence represent peptides identified by mass analysis.

with 5% (w/v) dry skim milk powder in TBS−Tween. Blots were washed three times with TBS−Tween and probed with the pAb−HRP conjugate at a 1/5,000 dilution in TBS−Tween for 2 h at room temperature. Subsequently, the blots were washed three times with TBS−Tween containing 5% (w/v) skim milk. The presence of *Sin a* 1 was detected by using the ECL Western blotting detection reagents (Amersham Biosciences Co., Piscataway, NJ) and enhanced chemiluminescence kit was employed for visualization.

Statistical Analysis. All measurements obtained were from minimum of three replicates. Mean, standard deviation and coefficient of variation

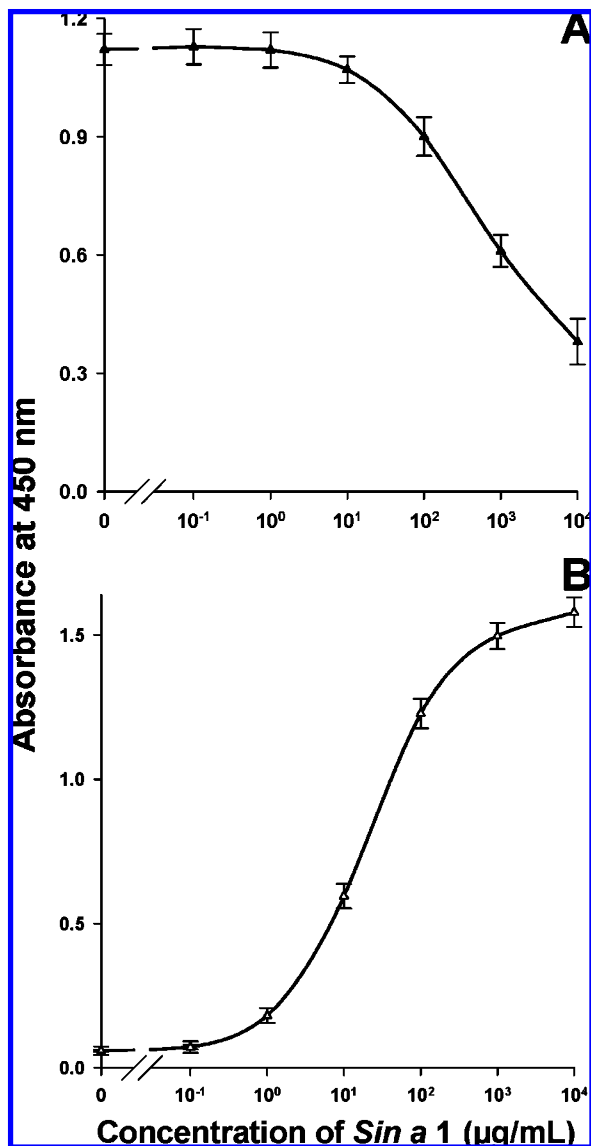

Figure 4. Standard curves for determination of *Sin a* 1 concentration by ELISA methods: (**A**) competitive indirect ELISA; (**B**) sandwich-ELISA.

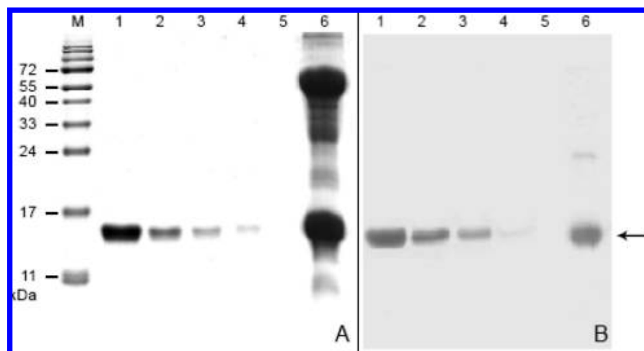

Figure 5. Detection of *Sin a* 1 by a Western blot analysis: (**A**) SDS-PAGE; (**B**) Western blot; M, broad range SDS-PAGE standards; lane 1, 5.0 µg of the purified *Sin a* 1 protein; lane 2, 4.0 µg; lane 3, 3.0 µg; lane 4, 2.0 µg; lane 5, 1.0 µg; lane 6, 5.0 µg pf protein from YM (AC Pennant) powder, respectively. Arrow indicates the position of *Sin a* 1 protein.

were calculated. Inter- and intra-assay coefficient of variation was calculated for S-ELISA using purified *Sin a* 1 (four concentrations, five determinations). Analysis of variance (ANOVA) was used to determine whether the protein of the extracts differed among lines. Least

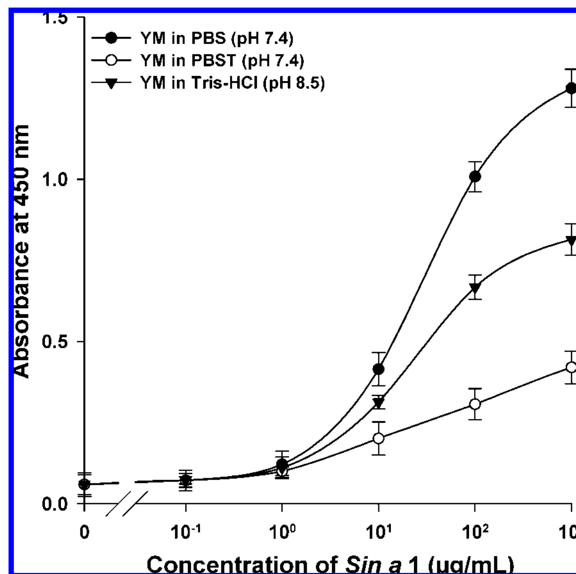

Figure 6. Effect of different sample extraction buffers on S-ELISA detection of *Sin a* 1 from YM. Supernatant of YM extract (AC Pennant, 0.3 g of protein/g of meal) was applied to sandwich-ELISA.

significant differences were calculated using the General Linear Model (GLM) procedure. SAS software (SAS Institute Inc., 1990) was used for statistical computations.

RESULTS AND DISCUSSION

Purification of *Sin a* 1. Isolation and purification of *Sin a* 1 involved three chromatographic steps of SEC, CEC, HIC (**Figure 1**) and electroelution. Defatted YM powder extract contained both CRU and NAP (**Figure 2**, lane 1) which were resolved together in the A1 fraction in the SEC (**Figure 1A**). Fractions A2 and A3 eluted from the SEC step were not selected for further purification due to their nonprotein nature as indicted by SDS-PAGE (data not shown). Most likely these two fractions are rich in low molecular weight components such as phenolics and pigments that give UV absorbance. Further separation of the A1 fraction by CEC resulted in three separate UV absorbing peaks (**Figure 1B**) that contained the two major proteins of YM, CRU (B1) and NAP (B2 and B3). Peaks B2 and B3 contained proteins below 20 kDa according to SDS-PAGE separation (data not shown) and resorbed by CEC between 0.01 and 0.3 M NaCl. The third chromatographic step HIC, further separated NAP and resulted in a small unabsorbed peak (**Figure 1C**, C1) and a large absorbed peak (**Figure 1C**, C2). The NAP-rich fraction (C2) was eluted after 0.85 M sodium sulfate containing in the buffer A was reduced to 0% by gradient elution. The recovery of purified NAP was about 4.3% of the total meal protein. The SDS-PAGE pattern of fraction C2 showed 3 protein bands between 11 and 24 kDa (**Figure 2**, lane 2). Following electro-elution, C2 was further purified and a single protein band having approximate molecular weight of 14.5 kDa (**Figure 2**, lane 3) was obtained. Upon reduction this protein band was dissociated into two polypeptide bands having approximate MW of 11 kDa and much less than 11 kDa (**Figure 2**, lane 4). According to Monsalve et al. (*28*) the allergen, *Sin a* 1 is composed of two polypeptide chains of 4.5 and 10 kDa linked by disulfide bonds. Although the MWs of lowest protein band could not be estimated for comparison, the purified protein of the C2 fraction may be the major allergen of *S. alba*. Separation and isolation steps utilized in this study have allowed purification of NAP and further *Sin a* 1.

Table 1. Total Protein and *Sin a* 1 Contents of Six Yellow Mustard Lines As Determined by Sandwich-ELISA[a,b]

YM	total protein[c] (mg/g)	*Sin a* 1[c] (mg/g)	*Sin a* 1 content as % of total protein[d]
AC Base	244.3 ± 11.8b	1.80 ± 0.29c	0.737
AC Pennant	324.5 ± 9.2a	2.32 ± 0.28b	0.715
Andante	388.7 ± 19.7a	2.94 ± 0.50a	0.756
HS3	176.4 ± 11.2d	0.82 ± 0.09d	0.465
HS4	181.8 ± 13.5d	0.82 ± 0.08d	0.451
HS5	209.6 ± 13.8c	1.08 ± 0.12d	0.515
range (mg/g)	176.4–388.7	0.82–2.94	0.451–0.756

[a] Sandwich-ELISA was performed similar to **Figure 4B**. [b] The defatted ground YM powders were slurried in PBS and used for the detection of *Sin a* 1. [c] All values are means of triplicate determinations. Values followed by the same letters are not significantly different at $p < 0.005$. [d] Mean values of total protein and *Sin a* 1 were used for calculation.

Protein Identification. To confirm the identity of the purified protein, mapping of its tryptic digested peptides to the primary sequence of *Sin a* 1 was carried out. Using MASCOT, the empirically determined mass-to-charge ratio of C2 fraction peptides were compared with the primary sequence of *Sin a* 1 protein. A number of abundant peaks from singly charged tryptic peptides in the range of 750 to 2400 Da were observed (**Figure 3**). Positions 12−29 (KEFQ QAQHLRACQQWLHK) and 97−139 (QQLGQQGQQGPQV QHVISRIYQTATHLPKVC-NIPQVSVCPFKK) as query sequence were matched with the sequence of *Sin a* 1 (*12*) in the database. Of 22 mass values submitted to MASCOT search, 7 showed significant homology to *Sin a* 1. The top four scores (all 98), all above the cutoff confidence limit of 95% ($p < 0.05$) belonged to *Sin a* 1, indicating that the purified protein of C2 is composed primarily of *Sin a* 1.

pAbs Raised against *Sin a* 1. The NCI-ELISA performed on sera obtained after the first immunization with *Sin a* 1, showed no significant changes in pAb titer of the rabbit, but the titer was remarkably increased after the second immunization (data not shown). After the second immunization, one of the sera that showed the highest titer (antiserum diluted 10000 times with BSB) was used as the antiserum for anti-*Sin a* 1 pAbs. The cross-reactivity tests for *Sin a* 1-pAb indicated 100% reactivity with *Sin a* 1, 30.3% with *B. napus* NAP, and very low reactivity of 2.1% toward *A. thaliana* NAP. This suggest that *Sin a* 1-pAb produced in this study has high degree of specificity toward YM allergen.

Standard Curves of Developed ELISA. The antibody and antigen concentration was optimized to use in S-ELISA to quantitate *Sin a* 1 in YM seeds. The standard curves of CI-ELISA and S-ELSA are as in the panels **A** and **B** of **Figure 4**, respectively. The S-ELISA was optimized using the antiserum having the highest titer and pAb-HRP conjugate (diluted 1/4000 with BSB; data not shown). The concentration of *Sin a* 1 that inhibited 50% of pAb−*Sin a* 1 binding (IC_{50}) was 20 ± 1.8 μg/mL. The theoretical detection limit (blank mean+2SD, $n = 6$) for S-ELISA was 0.3 μg *Sin a* 1/mL of sample and the 20% inhibition of antibody binding was 4.1 ± 0.3 μg/mL. Based on sample dilution and the detection limit, the assay should be able to detect 20 μg Sin a 1/g YM meal. The CI-ELISA had 2.0 μg *Sin a* 1/mL of detection limit indicating that the S-ELISA was more sensitive than CI-ELISA, which agreed with published work on less sensitivity of CI-ELISA than the S-ELISA or competitive direct ELISA (*29*). The intra- (within plates, five replicated determinations) and interassay (determinations between five plates) coefficients of variation (CV%) of the S-ELISA determined using 0.11, 1.02, 10.00, and 103.87 μg of *Sin a* 1 /mL were 8.51, 8.58, 10.49, and 9.11% for intra-assay and 15.14, 18.06, 10.69, and 11.33% for interassay, respectively. In general, CV values below 20% were achieved in the developed assay.

Comparison of Sandwich-ELISA and Western Blot. The pAb was employed to determine the IgG-binding protein bands with Western blot analysis (**Figure 5B**). Upon visualizing the IgG-binding protein bands at the level of 5.0, 4.0, 3.0, 2.0, and 1.0 μg, the purified *Sin a* 1 was able to be detected at the level of 2.0 μg, which was about 6.7 times less sensitive than of S-ELISA. As shown in **Figure 5B**, the *Sin a* 1 was clearly detectable at the level of 5.0 μg from YM seed when the pAb−HRP conjugates reacted with the proteins. In the case of prolonged exposure, a minor band appeared at about 24 kDa (**Figure 5B**, lane 6) which indicated either presence of proNAP, partial homodimerized product or aggregated proteins under certain denaturing conditions.

Due to high sensitivity, S-ELISA format was established against the anti-*Sin a* 1 pAb. The linear proportion of the curve was used to estimate the levels of undeclared *Sin a* 1 in a protein extract. The S-ELISA using the pAb produced in this study was suitable to estimate *Sin a* 1 content in YM seed extracts. ELISA methods have several benefits such as low cost and suitability to extend to a rapid field test as compared with DNA-based methods such as PCR. Therefore, the S-ELISA developed in this study could be useful in quantifying the targeted protein *Sin a* 1 as initial screening tool for YM containing material.

The study by Koppelman et al. (*30*) reports detection of *Brassica juncea* mustard protein in mustard oil. They were able to obtain 1.5 mg/kg (ppm) detection limit for the CI-ELISA using pAb raised against a mixture of mustard proteins obtained at pH 8.0. Although the lowest detection limit of the present study does not go to the low levels reported for the *B. juncea* study, the detection is very specific to *Sin a* 1 protein, which is the actual allergenic protein reported for YM.

Detection of *Sin a* 1 Protein of YM by S-ELISA. Figure 6 shows that the pH of the sample extraction buffer affects the absorbance values at 450 nm. The matrix effect and specific binding in antibody–antigen reaction caused by interfering substances in the sample can be avoided by diluting the extracts with PBS than other buffers. The interference effects of Tris-HCl buffer (pH 8.5) and PBST buffer on S-ELISA (1−100 times diluted) were considerable when used as the extraction buffer. Previously, Kwak and group (*28*) have also reported similar interferences of Tris-HCl and PBST buffers in S-ELISA when quantifying CP4EPSPS protein of glyphosate-tolerant soybean using mAb. Thus, PBS buffer at pH 7.4 was the suitable extraction buffer for quantitative determination of *Sin a* 1 by S-ELISA.

As shown in **Table 1**, the level of *Sin a* 1 for the six YM lines was in the range of 0.82−2.94 mg/g of meal ($p < 0.005$). There was a significant ($p < 0.05$) difference between the six genetic lines for the contents of extracted protein and *Sin a* 1. The highest protein and also the *Sin a* 1 content was resulted in for the Andante sample. When the protein contents of the extracts were compared with *Sin a* 1 content (**Table 1**),

0.451−0.756% of protein was estimated as the allergen, depending on the genetic line. This range of *Sin a* 1 was detected in the extracted proteins under the conditions used. It is not possible to extract all CRU or NAP proteins from YM seed material under these conditions (Wanasundara, unpublished data). Therefore, it has to be kept in mind that the values obtained here are for the *Sin a* 1 content of the extracted proteins with PBS at pH 7.4. The sample extraction procedure and conditions for full recovery of *Sin* a 1 need to be considered and studied for the declaration of absolute potential of allergenicity of YM due to *Sin a* 1.

These results indicate that the S-ELISA method developed in this study could be applied to detect *Sin a* 1 in seed extracts. The ELISA method developed to detect potentially allergenic proteins (*Sin a* 1) of YM could be adapted to screen YM genetic lines or YM-protein-containing products. So far, no reports are found in the literature on quantification of this nature. This analysis technique will help to screen and categorize YM products on the basis of potential allergenicity, which could be a safety and regulatory concern.

ABBREVIATIONS USED

BSA, bovine serum albumin; BSB, borate-buffered saline; CEC, cation exchange chromatography; CHCA, α-cyano-4-hydroxycinnamic acid; CI-ELISA, competitive indirect ELISA; CRU, cruciferin; ELISA, enzyme-linked immunosorbent assay; HIC, hydrophobic interaction chromatography; HRP, horseradish peroxidase; MALDI, matrix-assisted laser desorption ionization; NAP, napin; NCI-ELISA, noncompetitive indirect ELISA; SEC, size exclusion chromatography; S-ELISA, sandwich-ELISA; TMB, 3,3′,5,5′-tetramethylbenzidine.

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