Food Control 44 (2014) 233–241

Contents lists available at ScienceDirect

Food Control

journal homepage: www.elsevier.com/locate/foodcont

Quantification of major 2S allergen protein of yellow mustard using anti-Sin a 1 epitope antibody

Harsha K. Marambe, Tara C. McIntosh, Bifang Cheng, Janitha P.D. Wanasundara*

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

ARTICLE INFO

Article history:
Received 5 December 2013
Received in revised form
27 March 2014
Accepted 28 March 2014
Available online 18 April 2014

Keywords:
2S Allergen
Mustard
Sin a 1
Anti-epitope antibody
ELISA

ABSTRACT

The major allergenic protein in yellow mustard (YM, *Sinapis alba* L.) is the 2S napin Sin a 1. The level of Sin a 1 in YM seeds was quantified using anti-epitope antibody (AE-Ab) generated against the epitope sequence QGPHVISRIYQTAT as the capture antibody in a non-competitive enzyme linked immunosorbent assay (NCI-ELISA), and Sin a 1 containing napin purified from YM (var Andante) as the reference standard. The AE-Ab showed high specificity towards Sin a 1 epitope-containing napin long chain and showed no cross reactivity with other proteins of YM or other Brassicaceae proteins of similar genetic homology. The assay quantified Sin a 1 with a limit of detection and quantitation (LOD and LOQ) of 3.08 ppm and 3.23 ppm, respectively with acceptable recoveries and precision. The Sin a 1 content in YM varieties produced in 2006, 2010, 2011 was in the range of 2.65–4.68 g (AC Base); 3.81–5.98 g (AC Pennant) and 3.11–4.92 g (Andante) per 100 g of seeds. Sin a 1 composed more than 50% of napin protein fraction of YM seed. A trend of increased Sin a 1 level with the increased contents of total seed protein and napin was observed from this data.

1. Introduction

Mustard is consumed worldwide as a condiment and as a food ingredient. In several regions of Asia, mustard seed is a source of edible oil. In the global mustard production, Canada is the largest exporter (75–80%) and the second largest producer while the province of Saskatchewan produces 80% Canadian mustard (www.specialcrops.mb.ca). Among the different crucifer species that are collectively called as mustard, yellow mustard (YM) (*Sinapis alba*), brown and oriental mustard (*Brassica juncea*) are grown in Canada. Of these, *S. alba* seeds are larger, contain less oil (27% vs. 36%) and milder in pungency than *B. juncea*. YM seed has mucilage containing cells in the seed coat epidermis, higher protein content (30–33%) and less oil content compared to the other crucifer seeds. Therefore, YM finds applications beyond its use as a condiment or source of oil including use in the texture enhancement of food products. These applications include dry milled flour (fine powder from dehulled seeds) for salad dressings, mayonnaise, barbecue sauces, pickles and processed meat, wet milled mustard for mustard paste (e.g. with hot dogs) and whole ground seeds for use in spice mixes, as a seasoning, emulsifying and bulking agent in meat and other food products (www.specialcrops.mb.ca). Food industry also uses enzyme inactivated (deheated; primarily to inactivate myrosinase) YM flour for the functionalities provided by mustard protein and polysaccharides (Cui & Eskin, 1998).

Allergenicity of YM has been reported since 1980 and four different allergenic proteins have been identified and characterized in YM namely, Sin a 1 (14 kDa, a 2S albumin/napin), Sin a 2 (51 kDa, a 11S globulin/cruciferin), Sin a 3 (12.3 kDa, a non-specific lipid transfer protein/nsLTP) and Sin a 4 (14.2 kDa, a profilin) (Menéndez-Arias, Dominguez, Moneo, & Rodríguez, 1990; Palomares et al., 2005; Sirvent et al., 2009). Mustard is in the list of priority food allergens in Canada since 2012 (www.hc.sc.gc.ca) and in European Union it is listed among the 14 allergens to be declared on labels (The Commission of the European Communities, 2007). Mustard allergy accounts for 1–7% of all food allergies in the European regions (European Food Safety Authority, 2007). The prevalence of mustard allergy in Canada and other regions of the world is less known, but the reported severity of its allergy is very high. Ingestion of a minute amount of mustard is reported to cause anaphylaxis (Vidal, Diaz, Saez, Rodriguez, & Iglesias, 1991). According to a recent report of the VITAL expert panel, eggs and mustard were identified as the allergenic foods with lowest eliciting doses. The reported dose of mustard proteins to cause an allergenic reaction is 0.05 mg (Taylor et al., 2014). Patients with mustard allergy have shown a highly significant correlation with

* Corresponding author. Tel.: +1 306 385 9455; fax: +1 306 395 9482.
E-mail address: Janitha.Wanasundara@agr.gc.ca (J.P.D. Wanasundara).

http://dx.doi.org/10.1016/j.foodcont.2014.03.053
0956-7135/

specific IgE levels to mustard and skin prick test (SPT) for Sin a 1 ($r = 0.98$, P: 0.001), and a strong positive correlation with Sin a 2 ($r = 0.86$, P: 0.001). However, no significant correlation between rSin a 3 ($r = 0.15$, P:0.41) or rSin a 4 ($r = 0.07$, P:0.68) was found among mustard allergy patients with the SPT results and the level of allergen protein specific IgE levels of serum (Sirvent, Palomares, Cuesta-Herranz, Villalba, & Rodriguez, 2012; Verada et al., 2011).

Sin a 1 is a basic protein and believed to exist as a polymorphic dimer consisting of two polypeptide chains of 39 and 88 amino acids linked by disulfide (S–S) bonds; short chain and long chain, respectively. The Sin a 1 protein belongs to the 2S napin group and in the plants of Brassicaceae/Cruciferae family, napin is expressed by multigene families. Therefore, YM may contain other 2S napin isoforms besides Sin a 1 (Josefsson, Lenman, Ericson, & Rask, 1987). Sin a 1 is reported as highly stable to heat and retains immune reactivity after gastrointestinal (GI) digestion. There are at least two non-overlapping Ab binding sites (epitopes) in Sin a 1; one is conformational in nature whereas the other one is found in the large chain and suggested as a linear (continuous) epitope (Menéndez-Arias et al., 1990). The linear epitope that contains His is considered as the antigenic determinant of Sin a 1 immune response (Monsalve et al., 1993).

Currently, mustard allergen detection kits are commercially available and they detect the presence of mustard or quantify the total mustard seed proteins in food, based on polymerized chain reaction (PCR) (Mustorp, Engdahl-Axelsson, Svensson, & Holck, 2008) or ELISA techniques (Lee, Hefle, & Taylor, 2008). Although these detection methods may satisfy the labeling requirements of the food industry, they are not specific for the allergenic proteins of mustard and are therefore less relevant in the study of the allergenic potential of Sin a 1 or seed screening in YM breeding programs. The use of synthetic peptides containing the allergen epitope sequence to generate antibodies (anti-peptide or anti-epitope antibodies; AE-Ab) that specifically recognize the allergenic protein of interest may be efficient in capturing allergenic protein compared to the traditional antibodies raised against isolated protein. The aim of this study was to use Sin a 1 AE-Ab to detect and quantify Sin a 1 protein in YM varieties that are grown in Canada.

2. Material and methods

2.1. Materials

Seeds of YM varieties Andante, AC Pennant, and AC Base produced during 2006, 2010, 2011 and seeds of *B. juncea* (Centennial brown) and *Brassica napus* (AC Excel) were from Brassica breeding programs of Agriculture and Agri-Food Canada, Saskatoon Research Centre. The seeds were stored at 4 °C in air tight containers. The peptide synthesis and antibody preparations were provided by EZBiolab, USA. The bicinchoninic acid (BCA) assay kit was purchased from Pierce Thermo scientific, USA and the Clarity western ECL substrate for western blotting was from Bio-Rad (Canada). All the other chemicals used were of reagent grade and purchased from Sigma (Canada).

2.2. Methods

2.2.1. Development of Sin a 1 anti-epitope (AE-Ab) and anti-napin (AN-Ab) antibodies

The Sin a 1 AE-Ab was developed by immunizing rabbits with the synthesized peptide having the amino acid sequence of the allergenic epitope of Sin a 1 (Fig.1, Monsalve et al., 1993). The rabbit polyclonal anti-napin antibody (AN-Ab) was developed against chromatographically purified napin of *S. alba* (var. Andante). Antibodies were purified by affinity chromatography, dialyzed, and lyophilized. Antibodies were reconstituted (AE-Ab: 0.5 mg BSA equivalents/mL; AN-Ab: 9.33 mg BSA equivalents/mL) in borate buffered saline (BSB; 167 mM Boric acid, 125 mM NaCl, pH 8.5), and stored at −20 °C as aliquots until use.

2.2.2. Preparation of reference standards

YM seed (var. Andante) meal preparation, protein extraction and napin isolation were essentially similar to the procedures described by Shim and Wanasundara (2008). The napin purified using cation exchange chromatography (CEX-1) and hydrophobic interaction chromatography (HIC, Phenyl sepharose) was "unfractionated napin" and used for developing AN-Ab. This unfractionated napin peak of CEX-1 (peak B1 described by Shim & Wanasundara, 2008) was further separated by CEX (CEX-2) using 0–50% of 1 M NaCl gradient (5.5–20 column volumes) and the resulting napin peaks were collected. All protein peaks were dialyzed as needed, lyophilized and stored at −20 °C until further use.

2.2.3. Preparation of napin extracts

Because of the limited number of seeds and the selective solubility of napin (Wanasundara, Abeysekara, McIntosh, & Falk, 2012), special considerations were used in developing procedures for protein extract preparation. About 5–10 YM seeds were weighed into Polypropylene vials (5 mL) each containing a stainless steel ball (diameter: 1 cm) and pulverized in 0.5 mL hexane using a bench top homogenizer for 4 min (speed level 7) to extract oil. The steel balls were removed from the vials, rinsed with another 0.5 mL of hexane, and the meal slurries were centrifuged at 3074 × g for 10 min. The oil containing hexane supernatant was removed with a Pasteur pipette and the oil extraction was repeated two more times with 1 mL of hexane per extraction. The defatted meal was air dried in the same tube under a fume hood overnight. A 0.15 M $CaCl_2$ suspension (pH 3.0) of this defatted YM meal was made with 1 mL of water, 6 μL of 1 M H_2SO_4 and 0.5 mL of 0.066 g/mL $CaCl_2 \cdot 2H_2O$, homogenized for 10 min (speed level 7) and centrifuged as above. The clear supernatant containing napin was collected and the residual meal was extracted two more times sequentially with water (1.5 mL) and water (1 mL) + 0.066 g/mL $CaCl_2 \cdot 2H_2O$ (0.5 mL), the supernatants were recovered after centrifugation. The pooled napin extracts were filtered using 0.45 μm syringe filters, divided into aliquots and stored at −20 °C.

2.2.4. Protein content determination

The total N content of ground mustard seeds, meal and purified napin were determined by the combustion method using EDTA as the standard (AOAC, 1997) and converted to total N-based protein content using a conversion factor of 6.25. The protein content of seed extracts was determined using BCA assay (Smith et al., 1985) with the BCA assay kit and bovine serum albumin (BSA) as the standard. The seed extracts at pH 3 were filtered using AMICON centrifugal filters (3 kDa) prior to BCA assay to eliminate any interfering compounds.

2.2.5. Electrophoresis

The polypeptide profiles of the samples were obtained by SDS-PAGE under reducing (R, heated with β-mercaptoethanol; β-ME)

ALL1_SINAL

PAGPFRIPKCRKEFQQAQHLRACQQWLHKQAMQSGSGPSWTLDDEFDFED	50
DMENPQGPQQRPPLLQQCCNELHQEEPLCVCPTLKGASKAVKQQVRQQLG	100
QQGQ**QGPHLQHVISRIYQTAT**HLPKVCNIRQVSVCPFKKTMPGPS	145

Fig. 1. Primary sequence of allergenic napin isoform Sin a 1 (ALL1_SINAL) of *Sinapis alba*. Epitope sequence (17AA peptide) identified by Monsalve et al. (1993) is highlighted and used for synthesizing antigen peptide for Sin a 1 AE-Ab generation.