Effect of food matrix on amandin, almond (*Prunus dulcis* L.) major protein, immunorecognition and recovery

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ABSTRACT

Amandin, the primary storage protein in almonds, contains key polypeptides recognized by almond-allergic patients. A variety of food matrices representing diverse categories of foods were analyzed to assess the effect of food matrix on amandin recognition and recovery using rabbit polyclonal antibody based immunoassays. Food matrices from dairy, nuts, and vegetables typically resulted in over-estimation of amandin. Some foods representing legumes and cereals resulted in over-estimation while others in under-estimation of amandin. The amandin recovery range was 116–198 μg/100μg (dairy) 110–292 μg/100μg (tree nuts), 43–304 μg/100μg (legumes), 106–183 μg/100μg (most cereals- with the exception of barley, whole-wheat flour, wild rice and raisin bran whole mix). Amandin recovery from spices was typically low (2–85 μg/100μg) with a few exceptions where higher recoveries were observed (121–334 μg/100μg). Salt (black and white), tea, confectionery (sugar, cocoa, dark chocolate), and fruits (1–83 μg/100μg) generally resulted in lower recoveries. Tested food matrices did not adversely affect amandin immunorecognition in Western blots. The pH and the extraction buffer type affected amandin recovery. The results suggest that food matrix effects as well as extraction conditions need to be carefully evaluated when developing immunoassays for amandin detection and quantification.

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1. Introduction

Globally, the US is the largest producer (50.35% of 2007 global production) and exporter (80.41% of the 2006 global export value) of shelled almonds (FAO Stats, 2007). Valued for their nutritional quality and sensory properties, almonds are eaten as snacks and are extensively used in confectionery and baked goods. Tree nuts are one of the eight food groups that account for the majority of food induced allergies and sensitive individuals are therefore susceptible to experiencing adverse reactions (Angus, 1998; Sicherer, Muñoz-Furlong, & Sampson, 2003). During 2001–2006, of the 31 confirmed fatalities due to food allergies, 17 were attributed to peanuts and 8 to tree nuts (Bock, Muñoz-Furlong & Sampson, 2007). At least one

out of these 8 deaths, that of a 29 year old male, was directly attributed to almond exposure upon candy consumption in an office, despite the ready accessibility of epinephrine. The Food Allergen Labeling and Consumer Protection Act (FALCPA) became effective in the US in 2006 (http://www.cfsan.fda.gov/~dms/alergact.html) and was designed to protect consumer from unintended exposure. FALCPA specifically requires declaration of the source of ingredients derived from common allergenic foods (milk, eggs, fish, crustacean shellfish, peanuts, soybeans, tree nuts, and wheat). However, the finding that "consumers with food allergy are increasingly ignoring advisory labeling" (Hefle, Furlong, Niemann, Lemon-Mule, Sicherer, & Taylor, 2007) is troubling. In the absence of a cure for treatment of food allergies, avoidance of the offending food, even if present at μg/g levels, therefore remains the prudent choice for sensitive individuals.

Bargman, Rupnow and Taylor (1992) using patient sera IgE and Western blotting identified several almond polypeptides recognized by the patient IgE. Sathe (1993) reported almond proteins to be highly soluble in aqueous buffers and that a single globulin, AMP or amandin, dominated the almond protein composition of all the major marketed varieties of almonds in the USA. Sathe, Wolf, Roux, Teuber, Venkatachalam, and Sze-Tao (2002) developed a column

chromatographic procedure to isolate and purify the major storage protein in almonds. Investigations by Wolf and Sathe (1998) using ultracentrifuge demonstrated that amandin accounted for ∼65 g/100 g of the soluble seed proteins. Roux, Teuber, Robotham, and Sathe (2001) demonstrated that amandin contained the key reactive polypeptides recognized by the patient sera IgE. For these and several other reasons, amandin was targeted as a useful marker protein to develop rabbit polyclonal antibodies (pAbs) with the intent of developing a sensitive immunoassay for detection of trace amounts of almonds. Development of a rabbit pAb-based inhibition Enzyme Linked Immunosorbent Assay (ELISA) with a sensitivity of 20 ng amandin/ml ensued (Acosta, Roux, Teuber, & Sathe, 1999). Further studies on molecular properties of amandin (Sathe et al., 2002), global applicability of amandin as a marker protein, regardless of almond cultivar/hybrid (Sathe, Teuber, Gradziel, & Roux, 2001), and the stability of amandin towards various food processing methods (Roux et al., 2001; Venkatachalam, Teuber, Roux, & Sathe, 2002; Su, Venkatachalam, Teuber, Roux & Sathe, 2004) suggested that amandin is a globally applicable marker protein that may be used as a target for the purpose of detecting trace quantities of almonds. Though useful, these studies did not evaluate possible interference from a variety of foods and food ingredients in the pAb-based ELISA targeting detection of trace amounts of amandin.

Scheibe, Weiss, Ruëff, Przybilla, and Görg (2001) investigated the effect of chocolate on almond protein detection and found that trace amounts (0.5 μg/g) of almonds could be detected using rabbit polyclonal sera and Western blotting. The rabbit polyclonal sera used in these experiments recognized both almond and hazelnut proteins. The authors stated that despite the observed cross reactivity, both protein sources could be easily distinguished based on polypeptide patterns. More recently, a competitive indirect ELISA capable of detecting peanut, hazelnut, almond, cashew and Brazil nuts in chocolate in a single run at <1 μg/g protein with good specificity has also been reported (Rejeb, Abbott, Davies, Cleroux, & Delahaut, 2005). Almonds are often used in a variety of foods, other than chocolate, and may also be present in a myriad of foods and food ingredients as contaminants in trace amounts. Consequently, sensitive individuals may be inadvertently exposed to trace amounts of undeclared almonds. Evaluating the potential influence of several food matrices and select environmental conditions on amandin immunorecognition by rabbit pAbs was therefore of interest.

2. Materials and methods

The foods/food ingredients used in the study were purchased from the local markets. Certain foods/ingredients were processed in the laboratory. Micro titer 96 well ELISA plates were from Costar (Cambridge, MA). BSA (bovine serum albumin), alkaline phosphatase labeled goat anti-rabbit IgG, phosphatase substrate [p-nitro phenyl phosphate, disodium (PNPP)], were from Sigma Chemical Co., St. Louis, MO. Electrophoresis chemicals and supplies were from sources described earlier (Sathe, 1993). Nitrocellulose and blotting papers were from Schleicher and Schuell, Inc. (Keene, NH). All other chemicals and supplies were of reagent or better grade and were purchased from Fisher Scientific, Pittsburgh, PA. Production of anti-amandin rabbit pAbs (6235) and the rabbit perimmune serum has been described earlier (Acosta et al., 1999).

2.1. Food matrix preparation

The food matrices and appropriate food ingredients (listed in Table 1) were ground in an Osterizer blender (at the "Grind" setting) and defatted for 8 h in a Soxhlet apparatus using petroleum ether (boiling point range 38.3–53.1 °C; sample to solvent ratio 1:10 w/v). The defatted samples were dried for 24 h under a fume hood at room temperature (RT, 25 °C) and further homogenized in an Osterizer blender to obtain a uniform powder (∼400 μm). The powdered samples were stored in airtight plastic containers at −20 °C until further use.

2.2. Amandin preparation

Amandin was prepared using isoelectric precipitation (pH 5.0) method as previously described (Sathe et al., 2002). Lyophilized amandin was stored in an airtight plastic bottle at −20 °C until further use.

2.3. Food spiking

Defatted foods and food ingredients (100 mg) were spiked with 100 μl antigen solution containing desired quantity of amandin or BSB soluble protein from defatted almond flour. Select food samples (1 g) were also spiked with known quantities of defatted almond flour to determine possible effects of food matrix on amandin immunorecognition when food was spiked with defatted almond flour instead of amandin.

2.4. Protein extraction and quantitation

The antigen spiked samples and the corresponding unspiked controls were extracted with 0.9 ml of borate saline buffer (BSB; 0.1 mol/L H_3BO_3, 0.025 mol/L $Na_2B_4O_7$, 0.075 mol/L NaCl, pH 8.45) with continuous vortexing for 1 h at RT, centrifuged (13,600 g, 10 min, RT), and the aliquots of the supernatants analyzed for soluble protein by the method of Lowry, Rosebrough, Farr and Randall (1951). The remaining supernatants were stored at 4 °C until further use. Appropriate blanks were used in all assays. Standard curve for BSA (0–200 μg/ml) was prepared in appropriate buffer for each assay.

2.5. ELISA

Competitive inhibition ELISA assays were performed as described previously (Acosta et al., 1999; Roux et al., 2001).

Amandin Recovery (μg/100 μg) = [(amandin in spiked sample − amandin in the corresponding unspiked control) × 100]/amandin used for spiking

2.6. Electrophoresis and immunoblotting

SDS-PAGE in the presence of β-mercaptoethanol (β-ME, 20 ml/L) was carried out according to the method of Fling and Gregerson (1986) and immunoblotting was done as described earlier (Acosta et al., 1999). The antibody dilutions, all diluted in Tris-buffered saline with Tween 20 (TBS-T; 10 mmol/L Tris, 0.9 g NaCl/100 ml, 0.05 ml Tween 20/100 ml, pH 7.6), used were: anti-amandin rabbit pAb = 1:10,000 (v/v), and rabbit preimmune serum = 1:10,000 (v/v). The blots were incubated at RT for 1 h with horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG (1:40,000v/v) diluted in TBS-T (the secondary antibody). The reactive bands were visualized using chemiluminescent substrates (ECL Plus, Amersham Pharmacia Biotech, Piscataway, NJ) as described by the manufacturer.

2.7. Effects of antigen extraction conditions

2.7.1. Buffer type
The antigen-spiked samples and the corresponding controls were extracted with the desired buffer type, buffer volume, and desired time. The buffers used were: 1) BSB, 2) antigen coating citrate buffer used in ELISA (48.5 mmol/L citric acid, 0.103 mmol/L