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10 **Macadamia nut allergy in children: clinical features and cross-reactivity with walnut**

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34 To the editor:
35 Macadamia nuts, the seeds of *Macadamia tetraphylla* or *Macadamia integrifolia* of family
36 *Proteaceae*, are a familiar food item. Previous case reports of macadamia nut allergy demonstrated
37 the possibility of false negative IgE antibody test results and cross-reactivity of macadamia nut with
38 hazelnut.¹⁻⁵ However, the clinical features of childhood macadamia nut allergy and the cross-
39 reactivity of macadamia nut with foods other than hazelnut remain understudied. The present study
40 aimed to demonstrate clinical features in seven children with macadamia nut allergy and the cross-
41 reactivity of macadamia nut with peanut, other tree nuts, and seeds. Our study was approved by the
42 independent review board of the Tokyo Metropolitan Children's Medical Center (H28-57b), and
43 informed consent was obtained from the subjects' guardians.

44 Food allergy was defined as either of the following: (1) a positive open oral food challenge
45 result or (2) a history of an objective reaction consistent with an IgE-mediated allergy after definite
46 exposure to an allergen together with evidence of IgE sensitization from ImmunoCAP (Thermo Fisher
47 Scientific, UK) and/or a skin prick test (SPT).

48 Inhibition enzyme-linked immunosorbent assay (ELISA) and IgE immunoblotting were
49 performed to examine the cross-reactivity of macadamia nut with peanut, other nuts, and seeds.
50 Roasted samples (macadamia nut, peanut, almond, walnut, cashew nut, pistachio, pine nut, hazelnut,
51 sesame, and buckwheat) were pulverized and mixed in Tris-HCl buffer (pH 8.0). The supernatants
52 were then aspirated and filtered for extraction. Nunc MaxiSorp plate (Thermo-Fisher Scientific, UK)
53 was coated with macadamia nut extract in PBS buffer (20 µg/ml) overnight at 4°C. In parallel,
54 appropriately diluted serum samples were pre-incubated with protein extracts, added to the plates, and
55 incubated overnight at 4°C. IgE-binding to macadamia nut extract in the wells was detected using a
56 mouse anti-human IgE antibody conjugated with horseradish peroxidase (HRPO, Southern
57 Biotechnologies Associates, Birmingham, AL, USA) in combination with HRPO substrate, 3,3',5,5'-
58 Tetramethylbenzidine (TMB, BD biosciences, MD, USA). The macadamia nut extract was separated
59 by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE, Bio-Rad, CA, USA) and
60 stained with Coomassie brilliant blue or transferred to nitrocellulose membrane (GE Healthcare Life
61 Sciences, Little Chalfont, UK). After blocking, the membrane was cut into strips and incubated with

appropriately diluted serum samples overnight at 4°C. For blotting inhibition, a protein extract was added to a serum sample and incubated overnight at 4°C. IgE binding to the separated extracts was detected using alkaline phosphatase (AP)-labeled rat anti-mouse IgE-specific antibodies (BD biosciences, MD, USA) in combination with AP substrate reagent kit (Bio-Rad, Munich, Germany).

Table 1 shows the characteristics of the seven subjects (three males, four females). The age of onset ranged from 2 to 5 years. All seven subjects had lip swelling and facial erythema as part of their allergic reaction symptoms. General erythema occurred in five subjects (2, 4, 5, 6, and 7). Respiratory and gastrointestinal symptoms occurred in three (2, 6, and 7) and two (5 and 6) subjects, respectively. Subject 2 experienced anaphylactic shock after ingesting 300 mg macadamia nut powder in an oral food challenge. Other food allergies were present in four subjects, three (2, 3, and 6) of whom had hazelnut and/or walnut allergy. Clinical allergic reactions to almost all the foods examined for cross-reactivity with macadamia nut were evaluated in each subject, except walnut allergy in subject 2, which was not evaluated because consent for an oral food challenge was unable to be obtained. Four subjects (2, 3, 5, and 7) had eczema. Wheal size on a SPT with roasted macadamia nut extract ranged from 8 to 21 mm while the results of a specific IgE test against macadamia nut ranged from 0.14 to 28.4 UA/ml.

Inhibition ELISA was performed using serum samples from five subjects (2 - 6) except subjects 1 and 7, in whom macadamia nut-specific IgE was not detected on direct ELISA. The results showed that IgE reactivity to the macadamia nut extract was inhibited in a dose-dependent manner when walnut and hazelnut extracts were pre-incubated with serum samples from subjects 3 and 4 (Fig. 1). However, there was almost no inhibition by peanut, almond, pistachio, cashew nut, pine nut, sesame or buckwheat extracts when these were pre-incubated with the serum samples from the five subjects (data not shown).

SDS-PAGE analysis showed that macadamia nut had protein bands ranging from 5 to 50 kDa with the bulk of proteins being of low molecular weight (Fig. S1). Immunoblot analysis using serum samples from six subjects revealed common IgE binding in protein bands between 15 and 20 kDa in all six subjects. Serum samples from subjects 2, 5, and 6 reacted with proteins between 35 and 50 kDa in molecular weight while those of subjects 3, 4, and 5 reacted to bands of a relatively low molecular weight of 10 kDa (Fig. S2-A). However, a serum sample from subject 7 reacted with no macadamia

nut protein bands on the same immunoblot analysis. Further immunoblot inhibition assay of IgE binding to macadamia nut protein revealed that pre-incubating a serum sample (subject 3) with walnut and hazelnut protein extract inhibited binding of serum IgE to macadamia nut on the membrane (Fig. S2-B) in line with the inhibition ELISA findings, indicating clear cross-reactivity of macadamia nut with walnut and hazelnut.

The present study demonstrated the cross-reactivity of macadamia nut with walnut on inhibition ELISA and IgE immunoblotting while also confirming the findings of a previous report by Sutherland, et al.¹ of the cross-reactivity of macadamia nut with hazelnut. A prospective, multicenter, European study evaluating challenge-proven, co-existent nut allergy showed a higher rate of coexistence of hazelnut and walnut allergies with macadamia nut allergy (OR, 56.2 and 20.4, respectively), followed by the coexistence of cashew nut and pistachio allergies (OR, 585.2), then of walnut and pecan allergies (OR, 150.6)⁶. Together with the results of the present study, the coexistence rate of walnut and hazelnut allergies with macadamia nut allergy in some patients might be explained by the cross-reactivity of these items with macadamia nut.

This study has some limitations. Food allergy was not diagnosed using a double-blind, placebo-controlled, oral food challenge, and the sample size was small. In addition, co-allergy and co-sensitization to pecan and walnut have been reported⁷, but the clinical allergic reaction and sensitization to pecan and its cross-reactivity with macadamia nut were not evaluated in this study. Further clinical and laboratory studies are warranted to clarify the allergen components associated with severe symptoms and cross-reactivity.

In conclusion, macadamia nut often induces severe symptoms with objective oral manifestations. Some children with macadamia nut allergy might also be sensitized for both walnut and hazelnut due to cross-reactivity and should therefore be tested carefully for walnut and hazelnut allergies if they are allergic to macadamia nut.

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Table 1 Characteristics of children with macadamia nut allergy

Subject	1	2	3	4	5	6	7
Age (years)	4	6	6	4	4	5	2
Sex	F	F	F	M	M	F	M
Age at onset (years)	2	3	2	2	2	5	2
Symptoms after ingestion							
Lip swelling	+	+	+	+	+	+	+
Facial erythema	+	+	+	+	+	+	+
Generalized erythema	-	+	-	+	+	+	+
Respiratory	-	+	-	-	-	+	+
Gastrointestinal	-	-	-	-	+	+	-
Cardiovascular	-	+	-	-	-	-	-
Comorbidity							
Food allergy besides macadamia nut	-	Hazelnut, cashew nut, salmon roe	Hazelnut, walnut	-	Hen's egg	Walnut	-
No intake for sensitization	-	Walnut	-	-	-	-	-
Asthma	-	-	-	-	-	+	-
Eczema	-	+	+	-	+	-	+
Allergic rhinitis	-	-	-	-	-	-	-

SPT (wheal diameter: mm)							
Macadamia nut	12	21	16	8	13	14	10
Total IgE (IU/ml)	21.7	1480	24.7	23.8	234	424	39.2
Specific IgE (UA/ml)							
Macadamia nut	0.58	6.68	4.14	0.83	28.4	10.3	0.14
Hazelnut	<0.10	3.99	4.57	0.51	0.27	4.44	<0.10
Walnut	0.47	18.7	8.92	0.81	<0.10	22.2	0.20
Almond	N.D.	1.41	0.48	N.D.	0.29	0.95	<0.10
Cashew	<0.10	11.7	0.27	0.31	0.21	2.53	<0.10
Peanut	<0.10	2.73	<0.10	0.44	0.15	0.40	<0.10
SPT: skin prick test							

Figure Legends

Figure 1. Dose-dependent inhibition of IgE binding to macadamia nut extract on ELISA using macadamia nut, hazelnut, and walnut extract

IgE inhibition in individual sera (Subjects 2 - 6) was performed after preincubation with 0, 0.01, 0.1, 1, and 10 nmol/L nut extract.

Figure 1

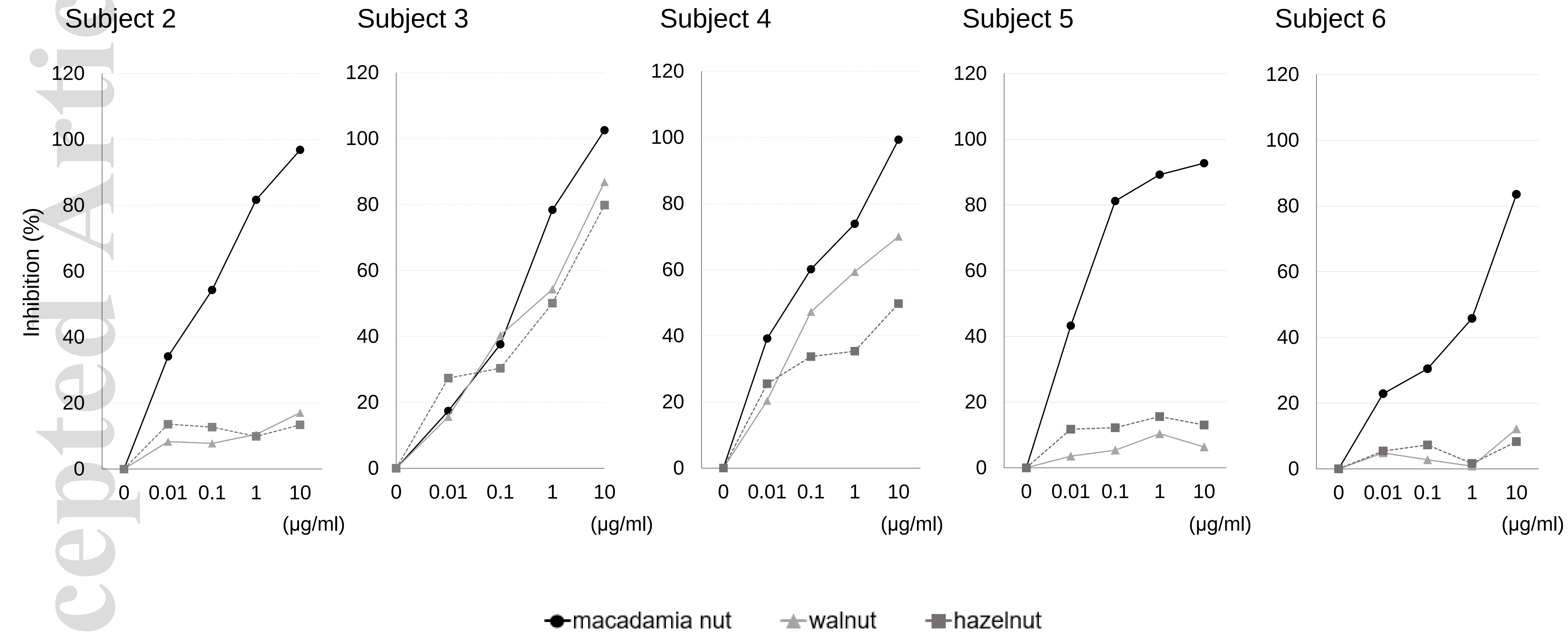


Figure S1

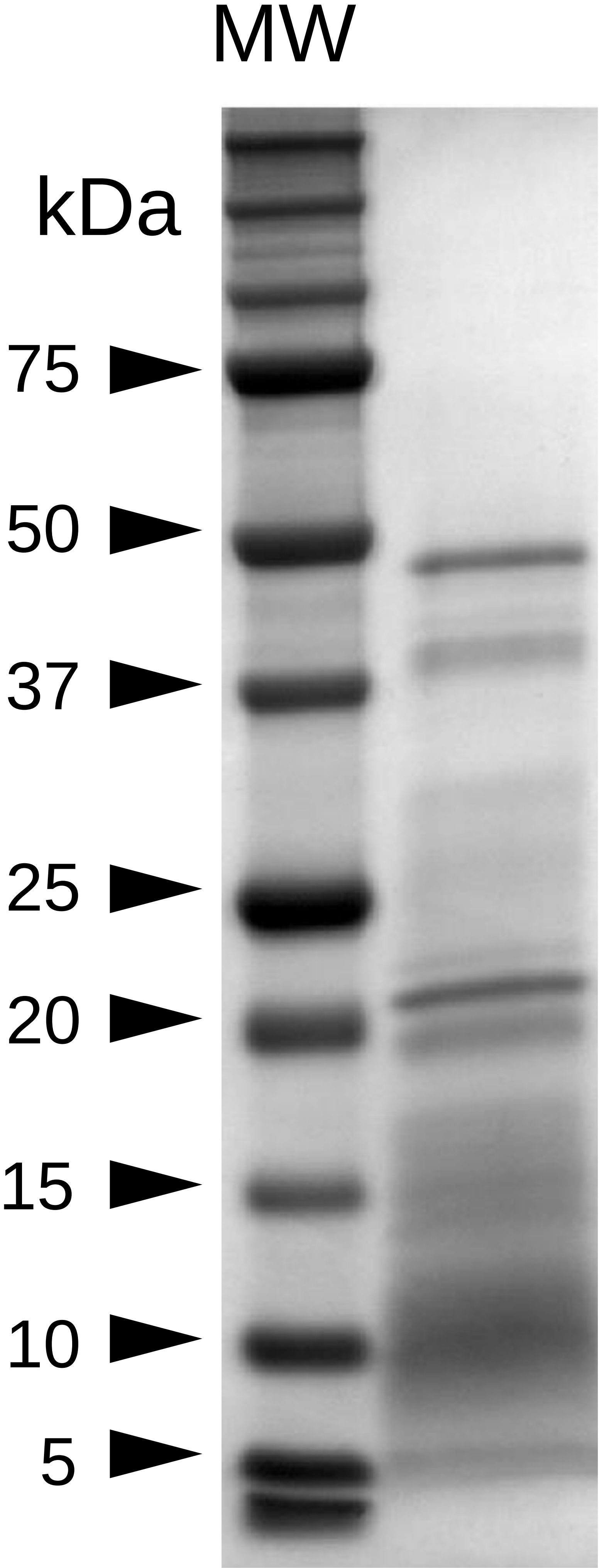
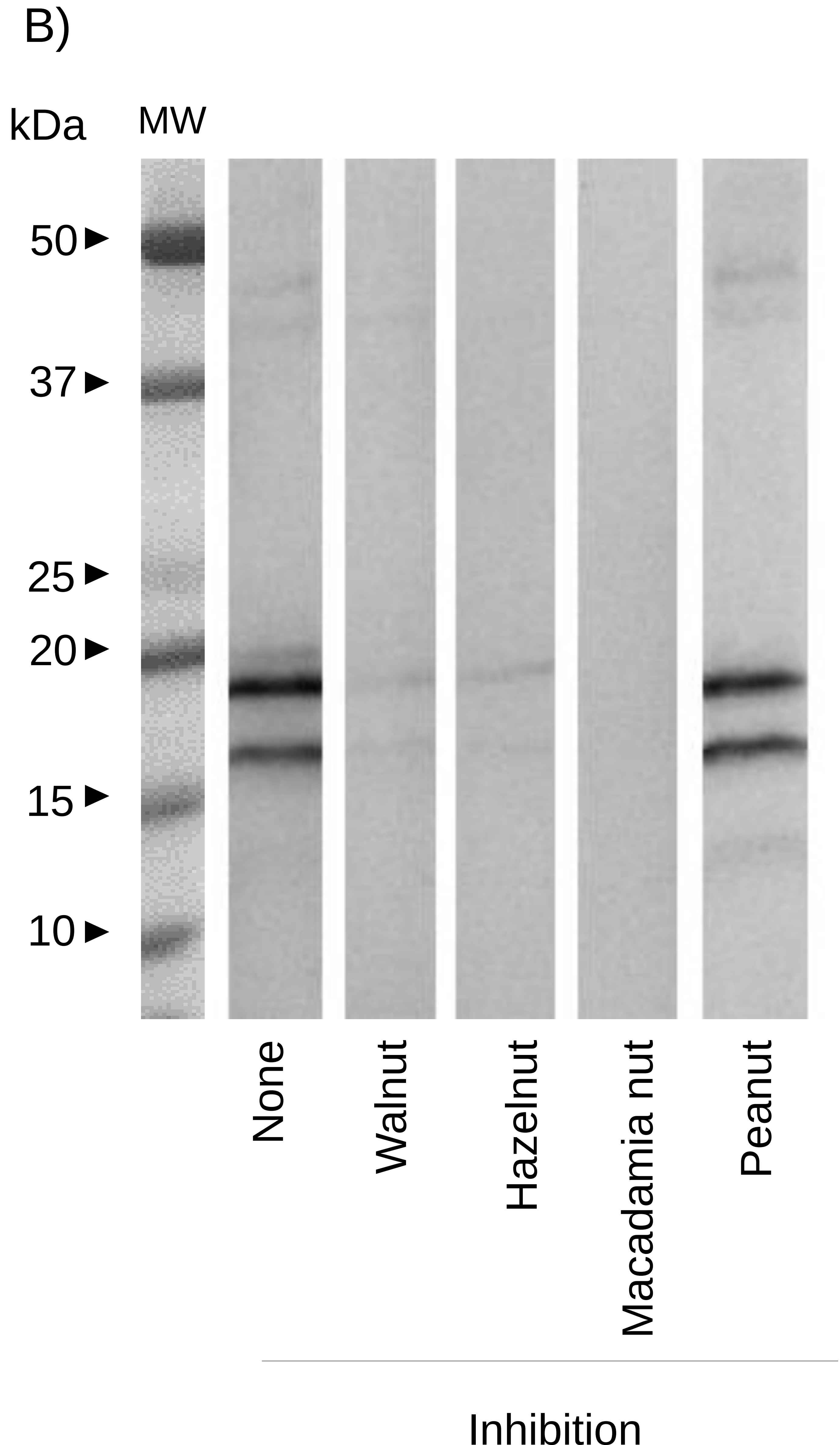
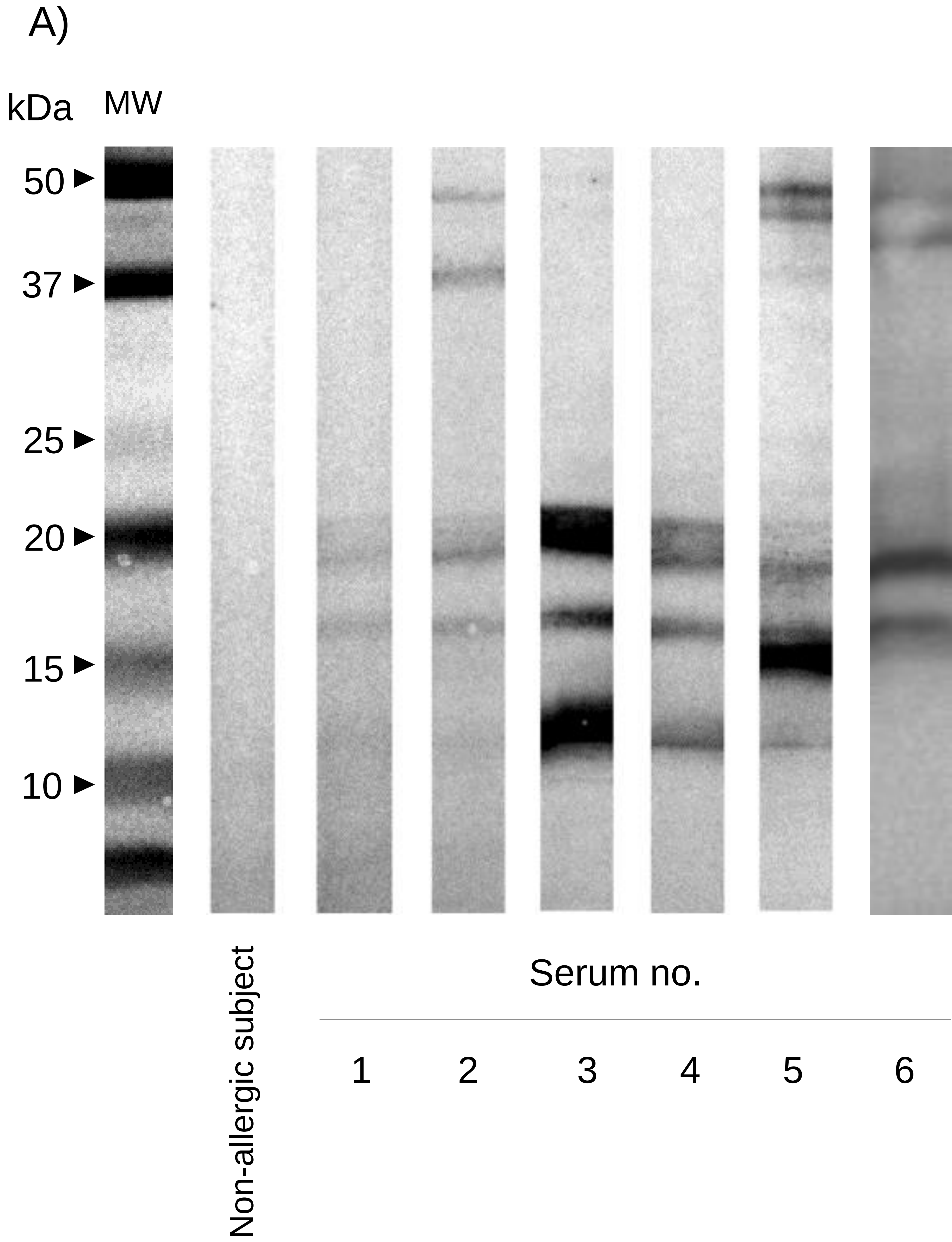


Figure S2



1 **Supporting Information**

2 **Figure S1. SDS-PAGE (4-20%) of macadamia nut protein extract**

3 MW: molecular weight marker

4

5 **Figure S2. Results of IgE immunoblotting**

6 **A: IgE immunoblotting of macadamia nut using serum samples from six patients (see**
7 **Cases 1-6 in Table 1) and a non-allergic subject**

8 **B: Immunoblot inhibition of macadamia nut with walnut, hazelnut, macadamia nut,**
9 **and peanut using serum from subject 3**

10 Subject 3 had clinical symptoms of allergy to hazelnut, walnut, and macadamia nut.

11 MW: molecular weight marker