RESEARCH NOTE

IDENTIFICATION OF MAJOR ALLERGENS OF WILDFLOWER HONEY

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Abstract. The aim of this study was to identify the major allergens of wildflower honey in local patients with atopic disease. SDS-PAGE revealed ten protein bands of 25 to 110 kDa, with a heavy cluster in region of 40-75 kDa. Immunoblotting demonstrated seven IgE-binding bands of 39 to 110 kDa. The 60 kDa protein had the highest frequency of IgE-binding (100%) followed by 54 kDa protein (95%), thus identified as the major allergens of wildflower honey. Our findings indicate that the allergen extract used for diagnosis of honey allergy contains both the 54 kDa and 60 kDa proteins.

Keywords: allergens, wildflower honey, SDS-PAGE, atopic disease

INTRODUCTION

Honey is a carbohydrate-rich syrup produced by honey bees (*Apis mellifera*), primarily from floral nectars (Ruttner, 1988; Kücük *et al,* 2007). In cold weather or when food sources are scarce, bees use their stored honey as their source of energy (Ruttner, 1988). Fructose and glucose are the major components but a large number of other compounds are present (Helbing *et al,* 1992). The composition of honey is variable, owing to the differences in plant types, climate, environmental conditions and the contribution of the beekeeper (Anklam, 1998; Azeredo *et al,* 2003).

Honey has a long history as a comestible and is used in various foods and beverages as a sweetener and flavoring (Helbing *et al,* 1992). It also is used extensively in traditional medicine. There are numerous reports on the use of honey to treat ulcers, burns, surgical wounds, gastric ulcers and as a carbohydrate source in oral rehydration therapy (Postmes *et al,* 1993; Subrahmanyam, 1998; Molan, 1999; Al-Mamary *et al,* 2002; Orhan *et al,* 2003).

However, allergy to honey has been reported and can involve reactions varying in clinical signs, ranging from a local edematic swelling of the skin, up to a life threatening systemic anaphylactic shock (Helbing *et al,* 1992; Kiistala *et al,* 1995; Bauer *et al,* 1996). The allergenic components in honey may be derived from either

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bee or plant pollen proteins (Helbing *et al*, 1992; Florida-Lopez *et al*, 1995; Bauer *et al*, 1996; de la Torre *et al*, 1997). Several studies have shown that honey-sensitive subjects possess serum IgE antibodies to a number of honey components (Florida-Lopez *et al*, 1995; Kalyoncu, 1997; Ibero *et al*, 2002). Honey proteins mainly of 54, 46, 17 and 16 kDa have been detected as IgE-binding proteins (de la Torre *et al*, 1997). Other studies have also detected IgE-binding proteins of 54, 60, 72 and ~30 kDa (Florida-Lopez *et al*, 1995), 57 and 29 kDa (Ibero *et al*, 2002) as binding IgE.

In Malaysia, our earlier study among atopic patients demonstrated that honey sensitivity is present (unpublished data). However, study on characterization of local honey allergens has been limited. Our previous study on local rubber honey allergy had identified major allergens of 72, 60, 54 and 38 kDa (unpublished data). No study has been conducted so far on allergy to imported honey. Wildflower honey or also known as "multifloral" or "mixed floral" honey is derived from the nectar of many types of flowers. This type of honey, mainly imported from New Zealand, is among the most popular imported honey in Malaysia and is available in local stores. Its color ranges from very light to dark, while its flavor ranges from light to rich depending on the composition of different seasonal wild flowers. Thus, the aim of this study was to characterize the IgE-binding proteins and major allergens of wildflower honey imported from New Zealand in local patients with atopic diseases.

MATERIALS AND METHODS

Preparation of honey extract

Honey extract was prepared according to the procedures described by Bauer *et al* (1996) with slight modification. In brief, 100 g of honey was diluted in 100 ml of distilled water and was extracted overnight by agitation at 4°C before centrifuging at 6,196*g* for 30 minutes. Supernatant was filtered through 0.45 µm filter disc and dialyzed against 2,000 ml of distilled water for 48 hours. The honey solution then was lyophilized and stored at -20°C.

Patients' sera

Sera of 20 patients with a history of honey allergy and a positive skin prick test (SPT) to honey extract were used in this study. SPT was performed by a medical officer at the Ear, Nose and Throat (ENT) Clinic of Hospital Kuala Lumpur (HKL). This project was approved by Medical Research and Ethics Committee (MREC) of Ministry of Health of Malaysia.

Sodium dodecyl sulfate polyacrylamide-gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out in a 12% polyacrylamide separating gel with a stacking gel of 5%. Electrophoresis was performed using a Mini Protean 3 Apparatus (BioRad) at 120 mA for 45 minutes. Each sample was dissolved in Laemmli sample buffer (BioRad) in the presence of 5% 2-mercaptoethanol, heated at 97°C for 4 minutes and subjected to electrophoresis. Precision plus protein standards (BioRad) were used as reference molecular weight standards. Gels were stained with Coomassie brilliant blue R-250 and recorded using Imaging densitometer GS800 and Quantity One Software (BioRad).

Immunoblotting

The separated proteins were electrotransferred onto nitrocellulose membrane using a Mini Transblot System (BioRad) at 100 V for 70 minutes. Membrane was washed with Tris-buffered saline (TBS) containing 0.05% Tween 20 (TTBS), incubated with 5% non-fat milk in TBS and

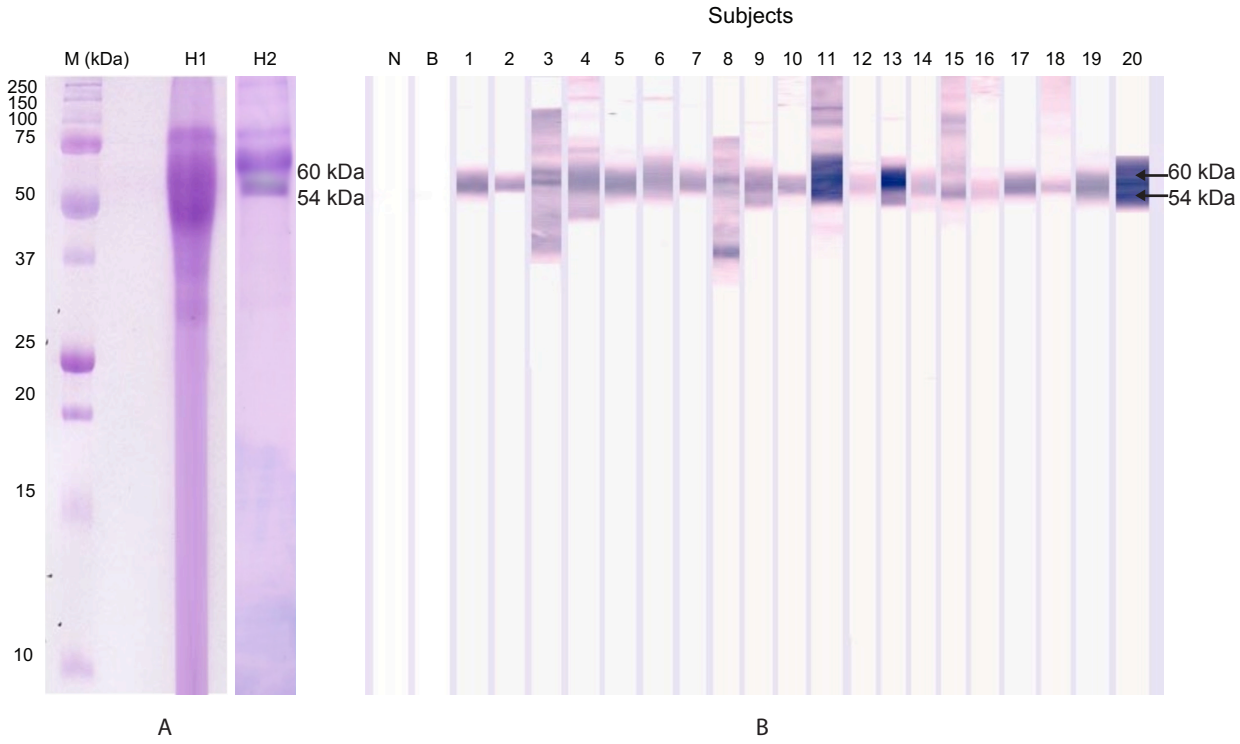

Fig 1–Protein profile of wildflower honey extract separated by SDS-PAGE (A) and immunoblots of binding of patients' serum IgE (B). Lane M, molecular mass markers; lanes H1 and H2, 50,000 µg and 10,000 µg of the honey extract, respectively; lanes 1-20, serum samples from patients with positive SPT to the honey extract; lane N, serum from a nonallergic individual; lane B, blank.

incubated with individual patient's serum diluted overnight at 4ºC. IgE-binding proteins were detected using biotinylated goat antihuman IgE-antibody (Kirkergaard and Perry Laboratories, Guildford, UK) diluted 1:1,000 in TBS with 5% nonfat milk, followed by incubation with streptavidin-conjugated alkaline phosphatase solution (BioRad) for 30 minutes at room temperature. Finally, Alkaline Phosphatase Conjugate Substrate Kit (BioRad) was used for color development according to manufacturer's instructions. Serum from a non-allergic subject was used as negative control.

RESULTS

SDS-PAGE of honey extract

SDS-PAGE of honey extract revealed at least 10 protein bands with molecular mass ranging from 25 to 110 kDa, with a heavy cluster in the region of 40 to 75 kDa (Fig 1).

IgE-immunoblot of patients' sera to honey extract

Patients with positive SPT to honey extract showed IgE-reactivity to seven honey proteins ranging from 39 to 110 kDa (Table 1). The majority of the patients' sera (>50%) exhibited IgE binding to 54 and

Table 1
Immunoblotting results of extracts of wildflower honey using 20 sera of honey-allergic patients.

Allergen (kDa)	Subjects 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	Frequency (%)
39			■					■													10
41			■					■			■										15
43			■					■			■										15
54	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■	95[a]
60	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	100[a]
72			■	■		■		■			■				■				■		35
110				■		■					■				■				■		25

[a]Major allergen

60 kDa proteins, thus these proteins were identified as the major allergens of this honey. None of the honey proteins showed binding with the negative control serum.

DISCUSSION

Allergy to honey can present a severe problem because systemic allergic reactions are not rare in person allergic to honey (Helbing *et al*, 1992; Kiistala *et al*, 1995; Bauer *et al*, 1996). Moreover, insidiously honey may be unlabeled in various foods and medicinal preparations (Helbing *et al*, 1992; Postmes *et al*, 1993; Subrahmanyam, 1998; Molan, 1999; Al-Mamary *et al*, 2002; Orhan *et al*, 2003).

In this study, ten protein bands in honey were identified by SDS-PAGE using Coomassie blue staining. Proteins of 54 and 60 kDa were detected as the major allergens of this honey by immunoblotting. The term major allergen is used to define proteins that elicit IgE-binding in the sera of half or more of patients with allergies to the specific source (Metcalfe *et al*, 1996). These 54 and 60 kDa proteins are similar in size with IgE-binding proteins reported in other studies (Florida-Lopez *et al*, 1995; de la Torre *et al*, 1997).

It has been postulated that IgE-binding could be caused by proteins from pollens contained in the honey, such as *Bet v 1* (17 kDa), *Bet v 2* (a profilin with 15 kDa), *Bet v 4* (8 kDa) and *Bet v 7* (a cyclophilin of 18 kDa) (Birnbaum *et al*, 1989; Florida-Lopez *et al*, 1995). However, our study demonstrated that wildflower honey only contains proteins between 25-110 kDa. These results agree with those of Kiistala *et al* (1995), Bauer *et al* (1996) and de Ia Torre *et al* (1997), who reported that allergic reactions to the pollen content of honey are possible but rare. Moreover, honey harvested by modern methods contains only 0.01-0.02% pollen (Helbing *et al*, 1992). Much higher pollen content is found in honey obtained by the local technique of pressing instead of centrifugation of combs (Helbing *et al*, 1992). It is worth

noting that the wildflower honey used in this study is commercial honey and was harvested by centrifugation.

Allergens derived from bee venom and enzymes from bee salivary and pharyngeal glands have been considered of second importance in cases of honey sensitization (Helbing *et al*, 1992; Bauer *et al*, 1996). To date, the best characterized bee venom allergens are phospholipase A2 (*Api m* 1), hyaluronidase (*Api m* 2), acid phosphatase (*Api m* 3), melittin (*Api m* 4) and CUB serine protease (*Api m* 7) (Allergen Nomenclature, Sub-committee, 2010). The IgE-binding bands may also be due to the presence of several enzymes from carbohydrate metabolism in the honey extract visualized as five proteins of 50-70 kDa identified as a diastase, α-amylase, invertase, glucose oxidase and glucosidase (de la Torre *et al*, 1997).

Major allergen measurements have relevance for the standardization of allergen extracts for immunotherapy and epidemiologic studies of allergic diseases. Thus, the isolation and further characterization of the two major allergens found in wildflower honey will facilitate the advancement of diagnosis and treatment of honey allergy.

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