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Isolation of a thiol-dependent serine protease in peanut and investigation of its role in the complement and the allergic reaction

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ABSTRACT

A serine protease activity was detected in aqueous peanuts seeds extracts, partially purified and characterized as a thiol-dependent serine protease. The potential role of this proteolytic activity on allergic reaction to peanuts was prospected through complement activation studies in human plasma and serum, and MDCK cells to investigate a possible occludin degradation in tight junctions. The peanut protease activity induced the production of anaphylatoxins C3a and C5a, and of the terminal membrane attack complex SC5b-9 whatever the complement activation pathway. The protease activity was also involved in the partial digestion of occludin within tight junctions, with for result, an increase of the epithelial permeability to antigen absorption.

1. Introduction

Peanut, *Arachis hypogaea*, causes a severe IgE-mediated disease that could affect up to 1.3% of the overall population in the U.S. (Liu et al., 2010) and in the EU (Nwaru et al., 2014), and from 1.4% to 1.8% of the children below five years old (Liu et al., 2010; Sicherer et al., 2010). Prevalence reports vary depending on the study type and the method (Sicherer, 2011). Allergic reactions to peanuts are known to be a Th2 mediated disease resulting from Th1/Th2 imbalance observed in young age (Vadas Peter et al., 2001; Vandenbulcke Liesbet et al., 2006). Sensitization remains unclear. The major peanut allergens were identified in the breast milk of lactating mothers who ingest peanuts (Vadas Peter et al., 2001). In contrast, genetic predispositions do not seem to be linked to peanut allergy (Dreskin et al., 2010; Sicherer and Sampson, 2007).

Peanut is one of the few allergens that can lead to particularly serious and sudden symptoms. Peanut proteins dose of 100 μg were reported to induce short lived symptoms of oropharyngeal itching and lip swelling within minutes after ingestion, and 5 mg are sufficient to provoke a systemic reaction (lip and tongue itching, nausea, vomiting, urticarial, shivering, wheeze). Dramatic anaphylactic shock (cutaneous, cardiovascular and respiratory disorders), with even a fatal outcome in the most severe cases were also reported (Hourihane et al., 1997b; Sampson et al., 1992). Why peanuts are able to induce so dramatic reactions while most allergens cause most limited clinical manifestations like asthma and eczema is not well understood.

Nine allergens (Arah 1 to Ara h 9) and two peanut oleosines (Arah 10 and Ara h 11) were described for *Arachis hypogaea* (Burks et al., 1995; Jin et al., 2009; Kleber-Janke et al., 1999; Pons et al., 2002; Stanley et al., 1997). More recently, two defensins produced by DNA cloning, Ara h 12 and Ara h13, were registered in the Allergome database and isolated from the lipophilic fraction of peanut seeds (Petersen et al., 2015). Classification of a peanut allergen is based on its capacity to induce a specific IgE production in sera of peanut-allergic patients. However, all allergens do not induce the same basophils histamine release (BHR) pattern or the same skin test reactions. The majors allergens being those for which 50% of the patients have the corresponding IgE and for which 90% of them present an immediate cutaneous reaction. Ara h 1, Ara h 2 and Ara h 3 constitute the three major allergens, while Ara h 2 and 6 were pointed as the most aggressive ones (Bernard et al., 2007; Koppelman et al., 2004; Palmer et al., 2005; Porterfield et al., 2009).

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Most peanut allergens belong to common seeds storage proteins present in various leguminous plants and are well characterized proteins (Barre et al., 2005; Dodo et al., 2004; Jin et al., 2009; Jin et al., 2007; Viquez et al., 2001), such as a 7S globulin vicilin (Arah 1), 2S albumin conglutin (Arah 2, Ara h 6 and Ara h 7) or 11S globulin glycinins (Arah 3 and Ara h 4).

The reasons why some proteins are allergic and others not may be due to the presence of a greater number of epitopes, an increased resistance to proteolysis, post-translational modifications like glycosylation, and an inhibition of enzymatic activities (Huby et al., 2000). Interestingly, aqueous peanuts extracts contain several proteins that have at least one of these characteristics: Ara h 1 includes 23 linear epitopes (Shin et al., 1998) and is glycosylated (Burks et al., 1998). Ara h 1, Ara h 2 and Ara h 6 resist to proteolysis, as shown by tridimensional epitope structure conservation (Maleki et al., 2000; van Boxtel et al., 2008), and their mediator release capacity (Eiwegger et al., 2006; Lehmann et al., 2006) and IgE binding conservation (Bernard et al., 2007) are preserved. Furthermore, the roasting of the whole peanuts extract improve the IgE reactivity to purified Ara h 1 and Ara h 2 (Mondoulet et al., 2005). Additionally, Ara h 2 shares also sequence homologies with trypsin and bifunctional trypsin/amylase inhibitors from ragi seeds (RBI), and could be a pancreatic trypsin inhibitor (Maleki et al., 2003).

Several proteases or their inhibitors are suspected to be responsible for inflammatory processes and allergies (Smith and Harper, 2006). The functional roles of protease have been suggested to be involved in the pathogenesis of allergic disease through IgE-independent innate immunity (Gunawan et al., 2008). Der p 1 and Der f 1 from the house dust mite, *Dermatophagoides pteronyssinus and D. farinae respectively*, were reported to exhibit proteolytic activity and to play a role in tight junctions disruption of the respiratory epithelial (Sakata et al., 2004; Takai et al., 2005; Wan et al., 2000). This could involve these proteases in sensitization or exacerbation of allergen effect due to the facilitating of allergen passage across tissues barriers. The presence of protease was reported in other insects such as cockroaches and in numerous pollens such as birch, cypress, cedar, juniper, ragweed and grass (Gunawan et al., 2008). Proteolytic enzymes of pollen from ragweed (*Ambrosia trifida*), birch (*Betula pendula*), blue grass (*Poa pratensis*) and Easter lily (*Lilium longifolium*) were showed to be able to cleave occludin, claudin-1 and ZO-1 within tight junction in a MDCK epithelial cell model and Calu-3 human airways epithelial cells (Runswick et al., 2007). Similarly, pollens from *Olea europaea*, *Dactylus glomerata*, *Cupressus sempervirens* and *Pinus sylvestris* showed proteolytic activity toward transmembrane adhesion proteins occludins, claudin-1, E-cadherin of Calu-3 cells (Vinhas et al., 2011).

Furthermore, proteases are known to show immunomodulatory and pro-inflammatory effects. The Der p 1 and Der f 1 proteases were also descripted in the cleavage of the CD23 on B-cells enhancing IgE response, and the CD25 on T-cells and reducing the Th1 cytokine response (Brown et al., 2003; Hewitt Colin et al., 1995). The cysteine proteases papain and Der f 1 induce human eosinophils degranulation and superoxide anion production *via* the protein-activated receptor (Miike and Kita, 2003; Reed and Kita, 2004). More recently, it was shown in a murine model, that cysteine protease papain directly activated naïve T-cells through G-coupled protein PAR-2 receptors to initiate CCL17, CCL22 and elicits IL-4 production in basophils and T-cells, leading to a Th2 orientation response and basophils trafficking (Liang et al., 2012).

Untill now, no protease were isolated from *Arachis hypogeae*. Peanuts were reported to provoke the activation of the complement system as demonstrated by the production of the C3a anaphylatoxin observed in response to peanut challenge in wild mice and not, or to a lesser extent, in complement factors deficient mices (Khodoun et al., 2009; Kodama et al., 2013). The underlying mechanism of the anaphylatoxin production, *i.e.* an activation of the "whole" complement system *via* one of the three pathways, and/or a direct proteolytic effect of a peanut protein on the C3 molecule is not clear. And as the responsible peanut's antigen is not yet identified, we hypothesized that peanuts extracts could have a such protease activity.

We report here for the first time, the detection of a proteolytic activity in aqueous extracts of *Arachis hypogaea* and the isolation and partial characterization of a thiol-dependent serine protease activity. We further show evidences that this newly described proteolytic activity could play a role in the pathogenesis of peanut allergy through direct anaphylatoxins production, complement activation and alteration of tight junctions.

2. Materials and methods

2.1. Materials

Proteins were extracted from commercial local market crude peanuts. *N*-[*N*-(L-3-*trans*-carboxirane-2-carbonyl)-L-leucyl]-agmatine (E64) and 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (PEFABLOC SC) were purchased from Roche (Mannheim, Germany). BOC-Gln-Ala-Arg-7-amido-4-methylcoumarine-HCl (BOC-Q-A-R-AMC) was provided by BACHEM AG (Switzerland). A commercial peanut lectin, Fluoromount Aqueous Mounting medium, benzamidine and lacto-agarose and α-lactose resins, Iodoacetamide, N-α-Tosy-L-phenylalanine chloromethyl ketone (TPCK), N-α-Tosy-L-lysine chloromethyl ketone (TLCK), dithiothreitol (DTT), S-methylmethanethiosulfonate (MMTS), phenylmethanesulfonate fluroride (PMSF), 1,10 phenanthrolin (orthophenathrolin) and benzamidine-HCl were purchased from Sigma-Aldrich (Steinheim, Germany). Culture media, nutrients and antibiotics were purchased from Gibco. Occludin rabbit antibodies and ABT146 were purchased from Millipore. All other chemicals were of analytical grade. HiTrap Benzamidine-Sepharose column, HiTrap Q-Separose column and Thiopropyl Sepharose 6 B powder were purchased from GE Healthcare (Uppsala, Sweden). Molecular mass standards were purchased from Bio-Rad Laboratories (Hercules, USA). All the other chemicals were of analytical grade.

2.2. Whole peanut proteins extract preparation

Proteins extraction was performed from commercial crude peanuts. Crude peanuts were peeled, ground in liquid nitrogen and defatted with diethylether in a Soxhlet at 40 °C before to be stored at −20 °C.

2.3. Proteins extraction

Four grams of defatted peanuts powder were solubilized for 1 h under stirring at 4 °C in 200 mL of the extraction buffer (Tris-HCl 65 mM at pH 8.3, containing EDTA 1 mM and NaCl 200 mM). The resulting suspension was filtered through six cheesecloth layers and clarified by centrifugation (67,000*g*, 4 °C, 30 min). Proteins from the clarified solution were fractionated by ammonium sulfate precipitation (adapted from Calowick and Kaplan, 1955). Solid ammonium sulfate was added up to 40% saturation. The pellet was discarded, after centrifugation (67,000*g*, 4 °C, 30 min), and additional ammonium sulfate was added to the resulting supernatant to reach 70% saturation. After centrifugation (67,000*g*, 4 °C, 30 min), the pellet was dissolved in a minimum volume of the suspension buffer (Tris-HCl 65 mM at pH 8.3, containing EDTA 1 mM), before being dialyzed (5 L of water, 4 °C, 48 h) and lyophilized. This frac-