

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.

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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 (Ara h 1 to Ara h 9) and two peanut oleosines (Ara h 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 h 13, 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 conglutinin (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 described 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).

Until now, no protease were isolated from *Arachis hypogaea*. 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 mice (Khodoun et al., 2009; Kodama et al., 2013). The underlying mech-

anism 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 -Tosyl-*L*-phenylalanine chloromethyl ketone (TPCK), *N*- α -Tosyl-*L*-lysine chloromethyl ketone (TLCK), dithiothreitol (DTT), *S*-methylmethanethiosulfonate (MMS), phenylmethanesulfonate fluororide (PMSF), 1,10 phenanthroline (orthophenanthroline) 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-Sepharose column and Thiopropyl Sepharose 6B 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 (67,000g, 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 (67,000g, 4 °C, 30 min), and additional ammonium sulfate was added to the resulting supernatant to reach 70% saturation. After centrifugation (67,000g, 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-

tion constitutes the peanut protease enriched fraction or the 70% pellet fraction.

2.4. Protease activity measurements

The protease activity was measured by fluorescence with a Shimadzu RF-5301 PC spectrofluorimeter. Spectra were recorded from 350 to 500 nm with an excitation wavelength of 360 nm and a 3 nm excitation and emission slit width. Either 50 μ L active fraction or lyophilized fraction dissolved and adjusted to 1 mg/mL or lectin adjusted to 1 mg/mL or water (as control) were mixed with 100 μ L substrate Boc-Gln-Ala-Arg-7-amido-4-methylcoumarine-HCl 0.05 mg/mL (BOC-Q-A-R-AMC 2.5 μ M final concentration) and 2850 μ L of Tris-HCl 50 mM buffer at pH 8.0. Enzymatic activities were measured by monitoring the fluorogenic leaving group release, AMC, at 440 nm immediately after substrate addition and after 30 min incubation at 37 °C. All ELISA tests were performed with the 70% lyophilized pellet (4–7 nmol AMC produced/min/mg 70% pellet).

2.4.1. Inhibition measurement

The inhibitory activity measurements were performed after incubation, for 30 min at 37 °C, of the active fractions, or 5 μ g of the thiopropyl-Sepharose purified protein with the different inhibitors in Tris-HCl 50 mM pH 8.0 buffer: MMTS (1, 3 and 7 mM), PEFA-BLOC (1 mM), benzamidine (3 mM), iodoacetamide (1–5 mM), E-64 (10 μ M), phenanthroline (1 mM), pepstatin (10 μ M), EDTA (5 mM), EGTA (1 mM), TPCK (0.1 mM), TLCK (0.1 mM), DTT (3 mM), PMSF (1 mM), Hg₂Cl₂ (1 mM), Mn²⁺ (1 mM), Mg²⁺ (5 mM), Zn²⁺ (5 mM) and Ca²⁺ (5 mM). After the incubation, substrate was added and the residual proteolytic activity was measured as described above.

2.4.2. Temperature and optimum pH measurements

The effect of temperature on the enzymatic activity was tested at different temperatures ranging from 10 °C to 60 °C in Tris-HCl buffer 50 mM at pH 8.0, as described above. The pH effect on the proteolytic activity was examined in the pH range 3.0–10.0 using the following buffers, at a final concentration of 50 mM: glycine-HCl buffer for pH 3.0, sodium acetate buffer for pH 4.0–5.0, sodium phosphate buffer for pH 6.0–7.0, Tris-HCl buffer for pH 8.0–9.0 and sodium carbonate buffer for pH 10.0. The activity was measured at 37 °C, as described above.

2.5. Lectin extraction

A sample of 25–40 mL of the suspended 70% pellet fraction (6.5–10.5 mg/mL) was loaded on a 5 mL lacto-agarose column pre-equilibrated with a Tris-HCl 50 mM buffer at pH 8.0 containing 200 mM NaCl, using a flow rate of 1.5 mL/min. The unbound protein material was washed away with the equilibrating buffer and the bound proteins were eluted with the same buffer containing 250 mM α -lactose. Both, unretained and retained fractions were dialyzed overnight at 4 °C against 5 L water. To remove lactose, to be rinsed and concentrated, the Lactose-eluted fraction was centrifuged in a 15 mL Vivaspin device (5000 MW cut-off).

2.6. Protein purification

A volume of 25–50 mL of the suspended 70% pellet free from lectins (4.3–10.0 mg/mL), was loaded on a 5 mL Hi-TrapQ Sepharose ion exchange column previously equilibrated with 50 mM Tris-HCl at pH 8.0 and coupled to an ÄKTA Prime Plus apparatus. The unbound protein material was removed by the equilibrating buffer and the retained proteins were eluted by the same buffer with 100 mL of a linear concentration gradient ranging from 0 to

1 M NaCl. The flow rate was adjusted to 2.0 mL/min. The proteolytic active fractions were pooled and dialysed overnight at 4 °C against 5 L water and its pH adjusted to 8.0 with Tris before being chromatographed on a 5 mL Benzamidine-Sepharose affinity column pre-equilibrated with 50 mM Tris-HCl at pH 7.4 (flow-rate: 0.5 mL/min). Unbound proteins were washed with the equilibrating buffer and weakly bound proteins were eluted with the same buffer containing 0.5 M NaCl. Bound fractions were eluted with a 50 mM glycine buffer at pH 3.0. The activity of the retained fraction was controlled and the final purification step was achieved by batch thiopropyl affinity chromatography. To perform this, 500 mg of dry resin were directly mixed with 3 mL protein sample under moderate stirring at room temperature. After 15 min of incubation for binding, the mix was introduced on a 15 mL chromatography column coupled to a peristaltic pump (0.5–1 mL/min). The unbound fraction was washed with 50 mM Tris-HCl at pH 7.4. The column was rinsed with the same buffer (minimum 10 column volumes). Fraction sizes were adjusted to correspond to 1 column volume. Retained fractions were eluted with 50 mM Tris-HCl buffer at pH 7.4 containing 0.5 M NaCl and 5 mM DTT. Since the 2-thiopyridone released upon protein immobilization absorbs at 343 nm and 280 nm, the absorbance were measured at these two wavelengths. A chromatogram showing the difference Abs 280 nm–Abs 343 nm was used to detect the retained fractions. These fractions were pooled and concentrated by diafiltration with centrifugal filters (Amicon Ultra 0.5 mL, 3 kDa Cutt-off, Millipore) to eliminate the excess of DTT and the released 2-thiopyridone.

2.7. Protein characterization and quantitation

Protein samples were analyzed by electrophoresis in denaturing conditions on 12% polyacrylamide gel (SDS-PAGE) using the Bio-Rad Mini-Protein 3 Cell (running conditions: 120 V, 0.02 mA, 2 W at 4 °C). Protein bands were revealed by silver staining using the Dodeca Silver Stain kit according to the manufacturer's protocol. Proteins were quantified either in a 96 wells micro plate by Lowry's method using the DC Protein Assay kit (BioRad) with an Optimax microplate reader (Molecular Devices) according to the manufacturer's protocol or by the droplet technique using a Microview device (GE Healthcare) after BSA calibration.

2.8. Protein identification

2.8.1. MALDI-TOF mass spectrometry analysis

Protein bands of interest were excised from the electrophoresis gel before being reduced and alkylated. Proteins were destained with KCN and digested with trypsin before being analyzed by LC-ESI-MSMS coupled to MALDI-TOF mass spectrometry. Spectra were treated using data analysis 4.0 (Bruker). Databases search was performed on NCBI restricted to green plants taxonomy using the Mascot server 2.2.04 and Protein Scape 3.0 (Bruker).

2.8.2. N-terminal sequence analysis

Protein samples were concentrated on PVDF membranes before being submitted to N-terminal amino-acid micro sequencing, by gas-phase Edman degradation with an Applied Biosystems 476A protein sequencer (Foster City, CA).

2.9. Anaphylatoxin C3a, C5a, and SC5b-9 measurements

Anaphylatoxin C3a/C3a des-Arg and C5a/C5a des-Arg, SC5b-9 and Factor Bb were quantified using ELISA kits following manufacturer's instructions (MicroVue C3a Plus EIA, MicroVue C5a EIA, MicroVue SC5b-9 Plus EIA, MicroVue Bb Plus EIA, Quidel, San Diego, CA 92121). Briefly, human plasma collected on non-allergic

volunteers in EDTA tubes, or serum were kept at 4 °C until centrifugation at 2000g during 10 min at 4 °C. Volumes of 200 to 500 µL of serum/plasma were mixed with the lyophilized peanut extract (5 mg/mL final concentration) with or without PEFABLOC SC (2 mM final concentration). The reaction mixture was supplemented with EDTA in an Eppendorf (1 mM final concentration) and incubated at 37 °C during 3 h before ELISA procedure. To control the effect of inhibitors and lectins on the C3a production, the centrifuged 70% pellet adjusted to 5 mg/mL and previously inhibited with E64 and PEFABLOC, the commercial lectin (1 mg/mL) and the purified lectin (1 mg/mL) were added to plasma 1:1 (v:v), and incubated in a similar manner.

2.10. Cell cultures, treatments and immunostaining

Madin-Darby canine kidney cells (MDCK) were cultivated on flasks with DMEM media containing 10% FBS, 1% penicillin/streptomycin, 1% sodium pyruvate and 1% essential amino-acids at 37 °C under 5% CO₂ until confluence. Cells were suspended with 0.5% trypsin (w/v) during 15 min and centrifuged. The resulting pellet was rinsed and suspended in fresh media before being subcultured on microscopic sterile slices in 6 wells plates (2.5 mL/well). Treatments of cells were made after cultures reached confluence on slices. Proteolytic activity for the cellular experiments were assessed by the Azocoll degradation method, where 1 Unit activity was defined as 1000*Δ A₅₂₀/min mg protein (Chavira et al., 1984). Quantities of the proteolytic fraction were adjusted to 100 units/mL media (Winton et al., 1998). After overnight treatment with peanut protease-rich extracts, media were discarded and cells were washed with a stabilization buffer (PIPES 80 mM, MgCl₂ 1 mM, EGTA 1 mM, polyethylene glycol 4% at pH 6.9) before being fixed with an ice-methanol mixture (−20 °C) for proteins precipitation. After 5 min treatment, methanol was discarded and cells were rinsed with TBS (completed with azide (0.13%) as preservative). Staining was carried out by washing with 10 mM TBS – 0.3% Triton during 5 min. Three washes with TBS were used to rinse during 10 min. Cells were saturated by NGS 1/10 (v/v) in TBS during 30 min and incubated overnight in the dark, with occludin primary anti-antibodies (1/100 diluted) at 4 °C. After 3 washes with TBS, cells were incubated with alpha rabbit AP⁵⁹⁴ (1/100 diluted) during 30 min, before being rinsed again 3x with TBS and 1x with water and fixed with aqueous mounting medium. Pictures were captured

using a fluorescence microscope. Fluorescence data are treated with an Image J Software (Jensen, 2013). Occludin quantitation was performed with the total corrected cells fluorescence (CTCF). CTCF was determined as follows: CTCF = Integrated Density – (Area of selected cells X Mean fluorescence of background readings).

2.11. Data analysis

For experiments with n > 8, Student *T*-tests were carried out on activity/inhibition measurements, anaphylatoxins production and total corrected cells fluorescence. The significance criteria were set at p < 0.05 (* significant) and p < 0.01 (** highly significant).

3. Results and discussion

3.1. Peanut extracts proteolytic activity investigation

Proteolytic activity measurements have shown that the peanut crude protein extract presented amidolytic activity towards the Boc-Gln-Ala-Arg-AMC substrate and that this activity could be concentrated in the 70% pellet. Inhibitory activity tests on the 70% pellet revealed that PEFABLOC and benzamidine were able to inhibit this activity as well as partly MMTS (Fig. 1).

PEFABLOC and benzamidine are well-known inhibitors of serine proteases. Benzamidine is a competitive inhibitor of trypsin and thrombin (Renatus et al., 1998) and PEFABLOC binds the serine residue within the active site of a broad range of serine proteases. MMTS binds its CH₃-SH groups to easily accessible thiol groups under mild conditions (Valentine and Paglia, 1981). On the other hand, we demonstrated that other inhibitors, like iodoacetamide and E-64 which affect directly the cysteine residue of the active site (Matsumoto et al., 1999), have no inhibitory effect. Therefore, it was assumed that our extract (70% Pellet) contains a serine protease. In order to check if other classes of proteases could be present in peanut extract, orthophenanthroline (1 mM), 5 mM EDTA (5 mM) and 1 mM EGTA (1 mM) were tested for metallo-proteases, and pepstatin (10 µM) was evaluated for aspartic proteases. None of these inhibitors had a significant inhibitory effect (Fig. 1).

3.2. Protease fractionation and characterization

Prior to the affinity chromatography, an ion-exchange chromatography (IEX) was performed in order to separate the proteolytic fraction from the abundant non-proteolytic protein

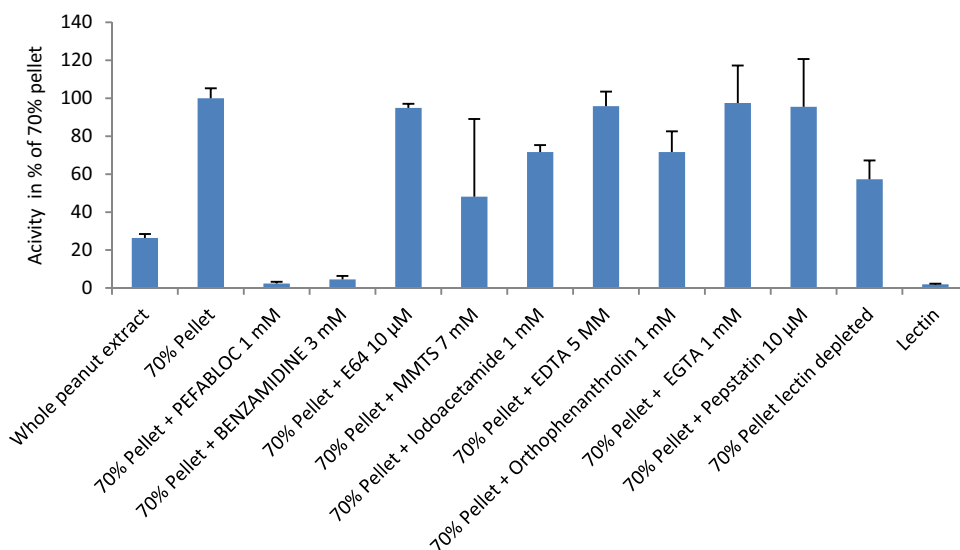


Fig. 1. Proteolytic activity of whole peanut soluble protein extract, commercial lectin, 70% NH₄SO₄ precipitated pellet (70% Pellet) and effect of inhibitors toward the 70% pellet. The Activity is expressed in% of the 70% pellet selected as reference. Results are representative of minimum 6 assays.

material in peanut. Proteolytic activity of all fractions was then assessed to detect fraction(s) of interest (see dots on the chromatogram) (Fig. 2a). The active fractions were pooled and the inhibitors behavior toward the active fraction was controlled before further chromatography.

As the protein material obtained after ion-exchange chromatography seemed to behave as a serine protease, an affinity chromatography on immobilized benzamidine was performed. Benzamidine interactions are well described with other serine and other trypsin-like serine proteases purifications (Sawada et al., 2000). The chromatogram of the active fractions loaded on this affinity column presented an unbound protein fraction, a protein fraction eluted with NaCl 500 mM and a retained protein fraction eluted with glycine-HCl buffer at pH 3.0. Interestingly, the proteolytic activity was exclusively concentrated in the protein fraction eluted at pH 3.0 (Fig. 2b).

This bound fraction was not pure as revealed by SDS-PAGE analysis (data not shown), though inhibitory activity data displayed a total inhibition by serine protease inhibitors such as benzamidine, PEFABLOC, PMSF and TLCK. On the other hand, cysteine proteases inhibitors such as iodoacetamide and E-64, also induced a ~40–50% decrease of proteolytic activity (Fig. 3). As both cysteine inhibitors affect partially the proteolytic activity of our extract, it was thought to possess a thiol group not directly implicated in the catalytic site. This hypothesis was investigated by the possible reaction of the fraction with a Thiopropyl-Sepharose support. The thiopropyl chromatography separated a not bound fraction and DTT-eluted fractions. As a proteolytic activity was measured in the DTT-eluted fractions, we concluded indeed that our protease contained a thiol group.

The Thiopropyl-Sepharose eluted active fraction (70%-TSF) displayed a single protein band with an apparent molecular weight of 30–35 kDa on silver stained SDS-PAGE gels (Fig. 4a). Unfortunately, the N-terminal sequencing of this eluted protein fraction failed to get a sufficient number of amino acids to be blasted in the database. Moreover purified fractions were often contaminated by protein bands of similar molecular mass and identified as Chain A peanut lectin. Although no proteolytic activity was ever associated to lectins, a Lactose-Agarose chromatography was performed to remove them from the 70% pellet. Interestingly, the Lactose-Agarose eluted fractions were also identified as Chain A peanut lectin, without proteolytic activity. Sequence analysis of lectins found in the ExPASy UniProt database revealed the presence of a cysteine residue not implicated in a disulfide bond formation. These free thiol groups could explain the binding of peanut lectin traces to the Thiopropyl-Sepharose support.

3.3. Protein identification

It is interesting to note that mass spectrometry and sequence homology analyses of the Thiopropyl-Sepharose protein fraction indicated a significant homology with Conglutin-7 (UniProt database). This protein is known to correspond to the Ara h 2 allergen, that behaves like a trypsin inhibitor in the roasted or reduced peanuts (Maleki et al., 2003) but lacks this inhibitory property in untreated peanuts. Ara h 2 is a 158–172 amino acids peptide (18.380–20.114 kDa depending on the isoform considered). In our case, the active 30 kDa protease corresponds to an intermediate size between the 38.9 kDa (with propeptide) of the cysteine protease papain from *Carica papaya* (O'Hara et al., 1995) and the 24.3 kDa of the serine protease trypsin (Mitsuru et al., 1986). Recently, a serine endopeptidase activity was associated to γ -conglutin enriched fractions of *Lupus alba* (lupine) which is also a member of the *Fabaceae* family (Magni et al., 2012). The γ -conglutin is a lupin non-storage protein composed of two subunits of 30 and 16 kDa respectively. Interestingly, Ara h 2 shows high sequence homology with this γ -conglutin. Sequence alignment with the UniProtKB database of the 172 amino acids of Cong-7 (Ara h 2) compared to the 452 amino acids of the γ -conglutin reveals the presence of 50 similar amino acids and 31 identical positions.

3.4. Characterization of the thiopropyl-sepharose fraction (70%-TSF)

3.4.1. Proteolytic activity/inhibitory measurements

The proteolytic activity/inhibitory measurements carried out on the 70%-TSF are presented in Fig. 4b. We can see from this figure that 70%-TSF activity is inhibited by PMSF, PEFABLOC and TLCK. PMSF, PEFABLOC and sulfonyl fluorides are well known alkylating agents of serine residues, forming a stable acylated enzyme, and TLCK is able to bind to histidine residues in the active site of serine proteases. It appears thus that these two residues are part of the active site of 70%-TSF, which is typical for a serine protease.

It is interesting to note that TPCK affected the activity of 70%-TSF to a lesser extent than TLCK. This indicates that the detected serine protease activity behaves like a trypsin-like rather than a chymotrypsin-like protease.

E-64 and iodoacetamide presented a partial inhibitory effect. E-64 is able to form a thioether bond with the thiol function of the active site, and iodoacetamide binds specifically to thiol groups and prevents the formation of a disulfide bond. The partial inhibition of these two highly selective cysteine inhibitors seemed to indicate that neither the thiol group nor a disulfide bond were absolutely required for the activity.

This is also in accordance with the fact that E-64 was shown to inhibit insect and bovine trypsin-like enzymes by a reversible competitive mechanism, while chymotrypsin-like enzymes did not

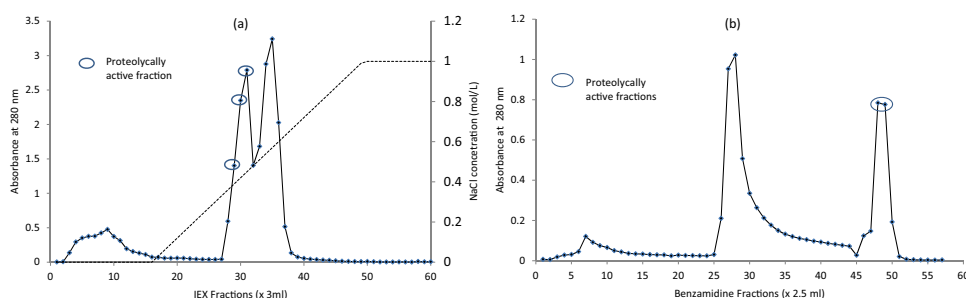


Fig. 2. Isolation of proteolytic fraction of 70% Pellet by Chromatography. (a) Ion Exchange Q-Sepharose HiTrap Chromatography of lectin-free 70% Pellet sample. Fractions 1–15: Binding and washing Tris-HCl 50 mM Buffer; DOT = Tris-HCl 50 mM + NaCl 0–1 M gradient. (b) Benzamidine Affinity Chromatography of selected active fractions from Q-sepharose Chromatography. Fractions 1–20: Washing Tris-HCl 50 mM pH 7.4; Fractions 21–44: Tris-HCl 50 mM pH 7.4-NaCl 0.5 mol/L; Fractions 45–60: Elution buffer Glycine 50 mM pH 3.0.

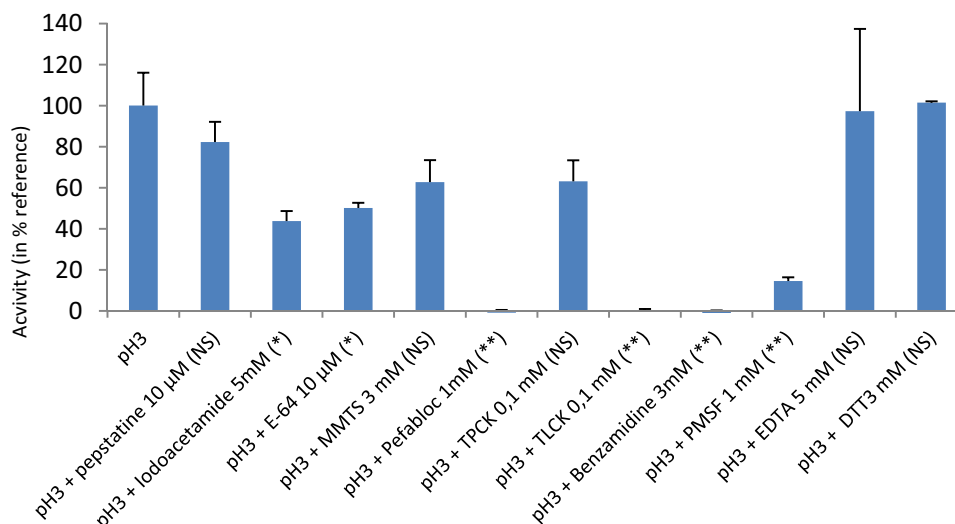


Fig. 3. Inhibitory activity profile of the protein fraction specifically retained on Benzamidine-Sepharose (pH3 eluted fraction). Activities are expressed in% of uninhibited pH3 eluted fractions selected as reference. (NS), (*) and (**): respectively non-significant, significant and highly significant vs uninhibited pH3 eluted fraction.

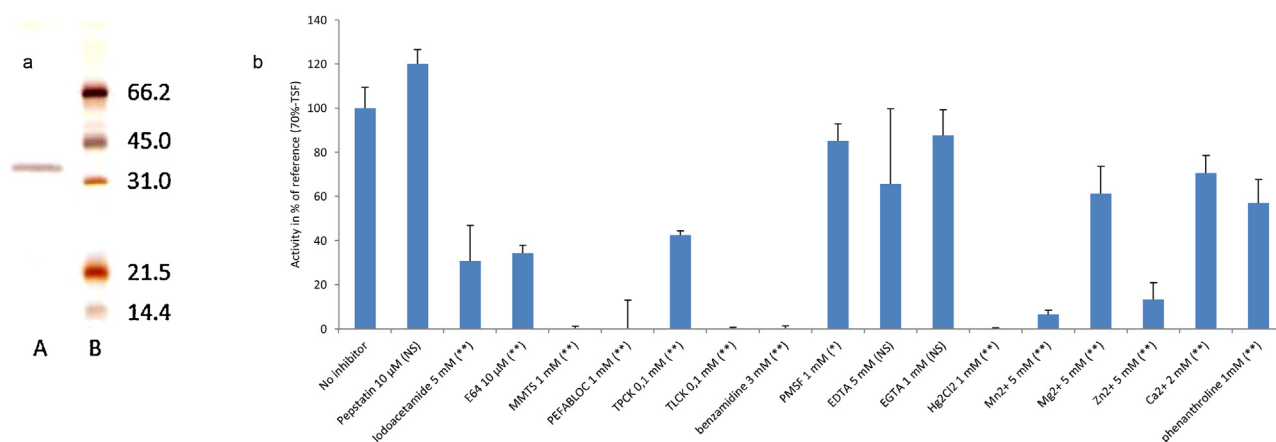


Fig. 4. (a) Silver Stained SDS-PAGE of the purified active protein fraction retained on Thiopropyl-Sepharose (70%-TSF). Lane A = 70%-TSF; Lane B = Molecular Weight Markers (kDa). (b) Inhibitory activity profile of the active protein purified on Thiopropyl-Sepharose (70%-TSF). Activity is expressed in% of 70% TSF uninhibited selected as reference. (NS): non-significant vs uninhibited 70%-TSF; (*) significant vs uninhibited 70%-TSF; (**) highly significant vs uninhibited 70%-TSF.

(Novillo et al., 1997). Moreover, SDS-PAGE analysis, in reducing conditions, of the purified and DTT pre-incubated fraction did not reveal smaller bands (data not shown). This indicated that the protease was not cleaved. Consequently, we concluded that the protease was not composed of different chains joined by disulfide bridges. Furthermore, the protease did not seem to need conformational disulfide bond to be active as also demonstrated by inhibition tests with DTT.

Iodoacetamide, beside its fixation to thiol groups, was also shown to react with histidine residues by the alkylation of the imidazole group as described in RNase (Fruchter and Crestfield, 1967) and in trypsin (Inagami, 1965). This is in accordance with its partial effect on the serine protease purified here. The histidine residue is part of the active site of both, serine protease triad and cysteine diad. This could thus explain the partial effect of iodoacetamide.

The variable effect of MMTS inhibitor on the heterogeneous proteins extract, at the early stage of the purification and in the pH3 benzamidine active fractions could be due to a steric congestion near the active site rather to a structure modification of the isolated protease. Moreover, MMTS can react with all easily accessible thiol groups, inducing variation in its ability to inhibit our extract.

Altogether, these elements lead us to consider the presence of a thiol group near the main active site of the protease.

It has to be noted that the protease did not seem to require metallic ion, its activity was indeed not significantly affected by EDTA, EGTA and orthophenanthroline. This finding also excluded the presence of metallo-protease activity. Moreover, the activity was not affected by pepstatin that is known to be a highly specific inhibitor of aspartic proteases. For all these reasons, we concluded that the proteolytic activity detected in peanut extracts did not belong to these two classes of proteases.

The effects of bivalent ions are variable. Likely due to their electrostatic charge influence in transition state of the protease reaction (Hedstrom, 2002).

3.4.2. Effect of temperature and pH on the 70%-TSF protease activity

The effect of temperature presented an optimum of activity at around 40 °C. This activity decreased at higher temperature values to reach 30% of the maximum activity at 60 °C (Fig. 5). This data is in accordance with the previously observed unfolding of trypsin above 60 °C (Gabel and Porath, 1972)

pH measurements showed that the optimal pH value for 70%-TSF was above neutrality, around pH 9. This finding is usual for a serine protease activity, while for cysteine proteases such as papain the optimal activity is around pH 6.0–7.0 (Fig. 5).

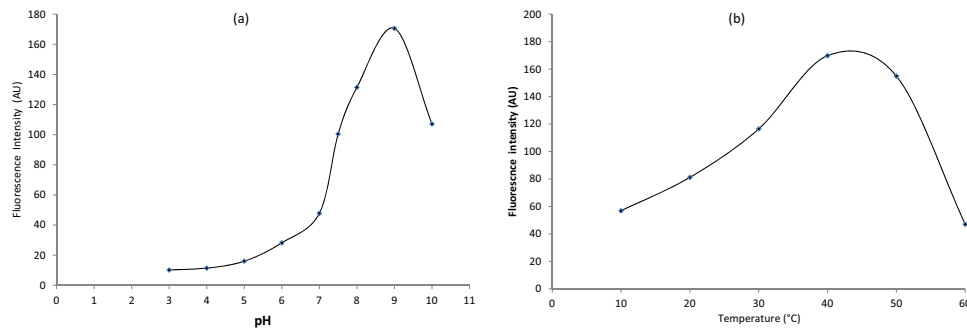


Fig. 5. Characterization of the purified 70%-TSF fraction. (a) Effect of pH on the proteolytic activity of the purified 70%-TSF Fraction. (b) Effect of temperature on the proteolytic activity of the purified 70%-TSF Fraction. Activity is expressed in arbitrary unit of fluorescence intensity emission at 440 nm.

Furthermore, we demonstrated that no peanut proteolytic activity could be detected at pH around 3.0 with the benzamidine pH3 eluted fraction obtained here, but, the activity could be recovered in neutral or slightly alkaline media. These findings suggested that the proteolytic activity could resist to the gastric transit and could be later recovered in the digestive tract.

Several serine proteases containing free cysteine residues, not implicated in catalysis, were described in different genders: the subtilisin-like thiol-dependent serine proteases of the thermitase sub-family contain one cysteine residue (Meloun et al., 1985) and the well-known serine proteinase K from *Tritirachium album* contains 5 cysteine residues that are not all involved in disulfide bonds (Betzel et al., 1988; Jany et al., 1986). In both cases, the free cysteine residues are located near the histidine residues of the catalytic triad. Moreover, proteinase K is inhibited by mercury ions (Müller and Saenger, 1993). More recently, other thiol-dependent serine proteases from *Bacillus mojavensis* (Beg and Gupta, 2003) and a subtilisin-like enzyme from *Paecilomyces lilacinus* (Kotlova et al., 2007) were also described. In those cases, the binding of the mercury ion, either covalently or by complexation of the cysteine residue located in the vicinity of the catalytic site of these proteases, affects their proteolytic activity. Similar behaviors could be implicated for the peanut protease. In addition, these proteases are active in alkaline pH and are similar in size to the protease described here.

Furthermore, sequence and evolutionary analyses of serine proteases of the human trypsin-like family revealed also the presence of 13 highly conserved cysteine residues that are not all involved in disulfide bonds (Rawlings and Barrett, 1994; Yousef et al., 2004).

Consequently, the results obtained for the peanut protease activity in the presence of cysteine proteases inhibitors and thiol-blocking reagents are in favor of the presence of a free thiol group in the close vicinity of the 70%-TSF active site of the serine protease.

3.5. Implication of peanuts proteolytic activity on complement activation

3.5.1. Anaphylatoxin C3a production

The 70% pellet and the 70% pellet depleted from lectin(s) samples both induced a significant increase of C3a concentration in plasma (Fig. 6). When these protein samples were pre-treated with a specific serine protease inhibitor, PEFABLOC, the C3a concentration was found comparable to that obtained with untreated plasma (control). Interestingly, addition of PEFABLOC, alone or in combination with the specific and irreversible cysteine protease inhibitor E-64, gave the same results, indicating that the observed C3a production was only due to a protease activity and not another factor present in 70% pellet. The C3a quantity produced in the present work was found to be 10 times higher than that previously described (Khodoun et al., 2009). The proteases of the complement

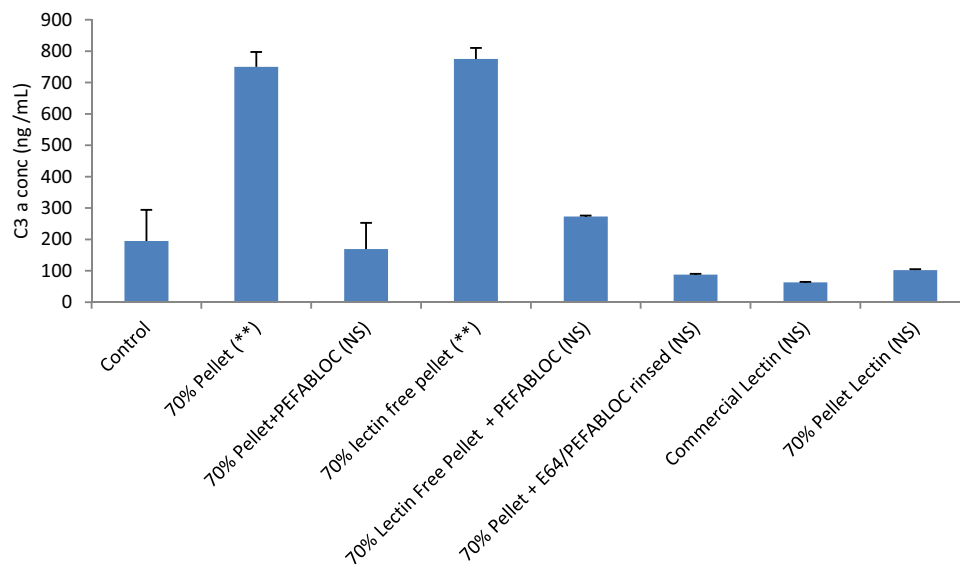


Fig. 6. Effect of proteolytic peanut extract on human plasma: ELISA quantitation of the anaphylatoxin C3a production in human plasma untreated (control) or incubated with 70% pellet, inhibited 70% pellet, 70% pellet with or without lectins or with lectins alone. Results are representative of minimum 8 assays. (**) and (NS): respectively highly significant and not significant vs control.

system are well characterized: these are serine proteases, belonging to the classical (C1qrs) and lectin pathways (Mannose-Binding Lectin (MBL)-Associated Serine Protease, or MASPs) requiring calcium ions, while those of the alternative pathway used magnesium ions for their interaction with factor B and C3b (Forneris et al., 2012). It is interesting to note that the serine protease activity detected in our peanut extract was not affected by EDTA up to 5 mM while this chelating agent is known to inhibit the proteolytic activity of the classical, the lectin and the alternative pathways, preventing thus *ex-vivo* activation of the complement (Pfeifer et al., 1999; Watkins et al., 1989). This indicates that the C3a production observed here on samples mixed with 70% pellet cannot be attributed to the classical and alternative pathway proteases.

Seeds peanut extracts are known to contain galactose-specific lectins (Pereira, 1983; Sun et al., 2011). It was thus reasonable to investigate the possible implication of peanut lectins in the C3 production following the 70% pellet treatment of the serum. Peanuts extracts were lectin-depleted by affinity chromatography although, as far as we know, seeds peanut lectins have never been reported to be implicated in the lectin activation pathway except in a murine model (Kodama et al., 2013). Furthermore, lectins alone were assessed for their own ability to cleave synthetic substrate and C3. Our results showed that no significant C3a production increase was observed when the plasma was supplemented with a commercially-available peanut lectin, or with the peanut lectin purified in our laboratory. This indicated that the lectin did not have any intrinsic proteolytic activity since, neither C3a production increase in lectin treated plasma (Fig. 6), nor synthetic peptide Boc-Q-A-R-AMC cleavage was observed (Fig. 1).

Our experiments demonstrated that the serine protease inhibitors, such as PEFABLOC and benzamidine, inhibited 100% of the activity of the 70% pellet sample, while the cysteine protease inhibitor E-64 showed low inhibitory effect. To exclude a potential effect of these inhibitors on the C3a variation, the 70% pellet sample was pretreated with these inhibitors and then exhaustively washed by diafiltration to discard the excess of inhibitors before being added to plasma. Results allowed us to ensure that the C3a increase, in treated plasma, could be attributed to a proteolytic effect of the 70% pellet fraction itself and not to any other substance that could be present in the plasma. Indeed, the natural self-degradation activity could be hidden by peanut extract activity or also inhibited by inhibitors.

3.5.2. Anaphylatoxin C5a production

As shown in Fig. 7a, the 70% pellet sample protease enriched fraction is also able to cleave the C5, leading to a second anaphylatoxin production. The C5 is cleaved in C5a and C5b between an arginine and a non polar and uncharged leucine residue, while C3 is cleaved in C3a and C3b between an arginine and a polar but also uncharged serine residue. This could indicate a partial selectivity of

the protease for uncharged residues, and could explain why Factor B, which is cleaved in Ba and Bb between an arginine and a positively charged lysine residue, was not significantly affected by the 70% pellet associated protease activity (data not shown).

3.5.3. Formation of the terminal membrane attack complex

The production of C3a and C5a by the peanut protease supposes *de facto* the production of C3b and C5b. As the formation of the membrane attack complex (MAC) and thus the final activation of the complement begin from these fragments, we evaluated the ability of the peanut protease to lead to a complete activation. For this purpose we quantified SC5b-9 complex (MAC associated to its natural inhibitor, the protein S) in the presence of the 70% pellet. Our results revealed a dramatic increase of the SC5b-9 levels in the presence of the 70% pellet, suggesting an activation of the complement until its final step. This increase was also observed, but to a lesser extent when the 70% pellet was previously inhibited (Figure 7b). Hence, it is highly probable that the cleavage of C3 and C5 by the peanut protease leads to the generation of C5b-9, which in turn might bind and destroy endothelial cells. In clinical situations where a physiopathological role of the complement is well documented like in the atypical hemolytic uremic syndrome, C5b-9 deposit on endothelial cells can be demonstrated from serum of patients in acute phase but not from patients in remission, suggesting a direct correlation between the circulating C5b-9 and the activity of the disease (Noris et al., 2014). This finding, *i.e.* the generation of C5b-9 by the peanut protease, would confirm a potential role of the complement in the allergy and inflammatory response to peanut, not only *via* the recruitment and activation of polynuclear cells by the C3a and C5a anaphylatoxins, but also by a direct cytopathic effect. Moreover, influence of C5b-9 in cell cycle rate (Rus et al., 2001) and inflammatory response such as cell lysis or tumor promoting activities were also described (Vlaicu et al., 2013). Therefore, the induced activation of complement observed here could influence these phenomena.

Our results are strengthened by other researches on molecular processes of peanuts allergy with MASP-1/3, MASP-2, immunoglobulin-deficient mice, and by C3 deposition tests (Kodama et al., 2013) and confirm those of Khodoun et al. (2009). Both demonstrated that peanuts extracts induced an increase of the C3a level *in vivo*. These previous works in murine model also showed that classical and lectin pathways are both involved *in vivo*, while neither the alternative nor the lectin pathway were implicated in the activation *in vitro*. These observations constitute an additional argument to exclude a potential role of contaminating lectins from the peanut extracts in human plasma.

The peanut antigen that activates the complement remains unknown. Natural antibodies of the IgM isotype, as well as MBLs and Ficolins (FCN) bind carbohydrates, and peanuts contain glycoproteins such as the Ara h 1 allergen recognized by IgM natural

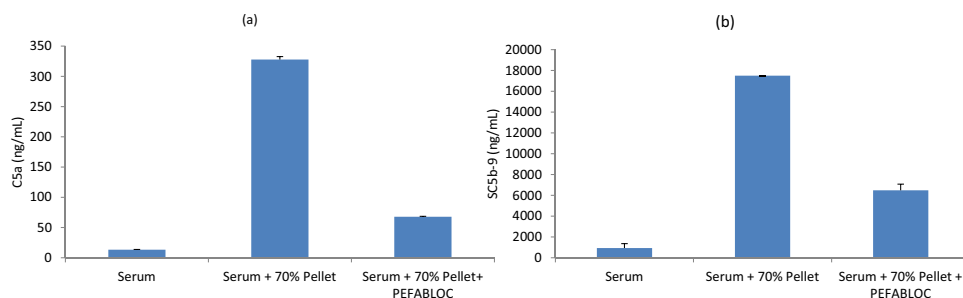


Fig. 7. Effect of proteolytic peanut extract on human serum: (a) ELISA quantitation of the anaphylatoxin C5a production in human serum untreated or incubated with 70% pellet or inhibited 70% pellet. (b) ELISA quantitation of the SC5b-9 complex production in human serum untreated or incubated with 70% pellet or inhibited 70% pellet. The data shown are representative of three independent experiments.

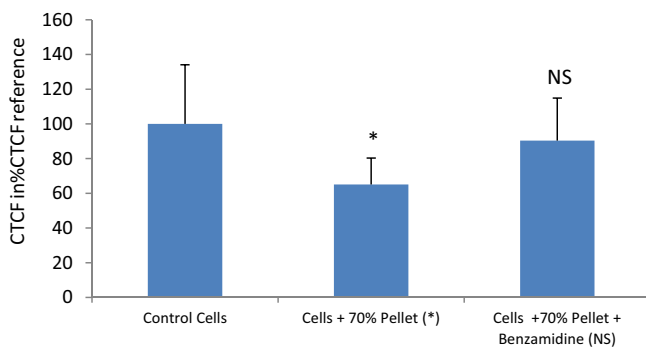


Fig. 8. Effect of proteolytic activity of peanuts extracts on tight junctions of MDCK cells. Quantitation of occludin is expressed in Corrected Total Cells Fluorescence (CTCF) of immunostained occludin; Data shown are representative of 8 trials. (*): significant vs Control cells; (NS) non-significant vs control cells.

antibodies and by lectins. Using deposition assays with mouse serum, Kodama et al. (2013) demonstrated that peanuts glycoproteins are recognized by IgM natural antibodies and by lectins. In our studies with non-allergic plasma or serum, the classical and alternative pathways are both inhibited by EDTA, the lectins are extracted, and the C3a increase does not take place in the presence of protease inhibitors. All these elements clearly indicate that peanuts play a direct role in the molecular process of complement activation by direct production of anaphylatoxin C3a and C5a. They also strongly suggest that the cleavage is only due to the proteolytic property of peanuts. The C3a and C5a anaphylatoxins induce the liberation by mast cells, basophils and macrophages, of different inflammatory mediators, like histamine, responsible of the contraction of smooth muscles and vascular permeability increase (Klos et al., 2009). Moreover, the C3a promotes a Th2 response (Hawlich et al., 2004) and modulates the inflammation by TNF- α and IL-1 β regulation (reviewed by Klos et al., 2009). Therefore, the direct C3a and C5a production and the total complement activation until the C5b-9 complex observed here could contribute to Th2 differentiation and act synergistically with the peanuts specific IgE, potentiating their biological effects, and could explain the severe clinical manifestations of peanuts allergy observed with extremely low dose of peanuts extract (Hourihane et al., 1997a).

3.5.4. Anti-occludin immunostaining in tight junctions

The enzymatic activity of peanuts was also investigated for its possible role in the penetration of other allergens. MDCK cells that express proteins of tight junctions (TJs) were used as epithelium model for studies of protease actions in allergy such as house dust mite proteinases (Desclozeaux et al., 2008; Wan et al., 2000, 1999; Winton et al., 1998) and pollen of ragweed, blue grass and white birch (Runswick et al., 2007). In all cases occluding degradation due to proteolytic activity was reported.

After digital processing of the picture and calculation of the corrected total cell fluorescence (CTCF), the occludin quantification displayed a significant decrease in cells treated with peanut extracts compared to untreated cells. Interestingly, inhibition of the 70% pellet sample with benzamidine canceled the decrease of occludin. These results suggest a direct link between peanut protease activity of the peanut 70% pellet and the occludin degradation (Fig. 8). Occludin is a transmembrane protein playing a role in adhesion and sealing of tight junctions (Furuse et al., 1994). In comparison with previous studies on Derp 1 proteases, (Wan et al., 1999) and pollen protease (Runswick et al., 2007) known to increase epithelial permeability by disrupting the seal, we noted a weaker effect in cell integrity so that no breaks could be detected between cells. However, our experiments obviously indicated a peanuts serine protease action toward occludin within the tight

junctions. This could therefore increase the permeability of the epithelium inducing an easier penetration of other peanuts compounds, which finally could explain the particularly severe allergic reactions observed in peanuts allergy.

4. Conclusions

Here we report, for the first time the existence and partial characterization of a proteolytic activity in peanut extracts. This activity can be concentrated in the 70% pellet sample. The proteolytic activity is likely due to a thiol-dependent serine protease, according to inhibitory activity analyses and optimum pH values. The finding that thiol-blocking reagents are able to partially affect the peanut protease activity is in favor of the location of the free thiol group in the close vicinity of the catalytic triad of the protease active site. MALDI-TOF mass spectrometry analyses revealed homology with Ara h 2, described as a trypsin inhibitor and with lupine γ -conglutin, exhibiting endopeptidase activity.

The C3 and C5 cleavage and the C5b-9 complex formation occur in a total independent way that does not require the initiation of one of the three complement cascades and which is not due to a protease of the complement system.

Moreover, the detected peanut proteolytic activity could partially affect the integrity of epithelium cells through degradation of the occludin within the intercellular seal, facilitating the penetration of other proteolytic incompetent peanut allergens. Taken together, these elements are clues for the understanding of the reported peanut allergy severity.

Further investigations are needed: complete sequencing, 3D-modelling and crystallization should provide lacking knowledges on its identification and its structure. Examination of the cleavage of peptides encompassing potential targets such as complement compounds or regulators, and further investigations on the potential influence of peanut protease towards PAR receptors signaling pathways and the subsequent Th2 orientation of CD4+, could provide insights into the mechanism underlying sensitization and the severity of peanut allergy.

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