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The effect of kiwifruit (*Actinidia deliciosa*) cysteine protease actinidin on the occludin tight junction network in T84 intestinal epithelial cells

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ABSTRACT

Actinidin, a kiwifruit cysteine protease, is a marker allergen for genuine sensitization to this food allergen source. Inhalatory cysteine proteases have the capacity for disruption of tight junctions (TJs) enhancing the permeability of the bronchial epithelium. No such properties have been reported for allergenic food proteases so far. The aim was to determine the effect of actinidin on the integrity of T84 monolayers by evaluating its action on the TJ protein occludin. Immunoblot and immunofluorescence were employed for the detection of occludin protein alterations. Gene expression was evaluated by RT-PCR. Breach of occludin network was assessed by measuring transepithelial resistance, blue dextran leakage and passage of allergens from the apical to basolateral compartment. Actinidin exerted direct proteolytic cleavage of occludin; no alteration of occludin gene expression was detected. There was a reduction of occludin staining upon actinidin treatment as a consequence of its degradation and dispersion within the membrane. There was an increase in permeability of the T84 monolayer resulting in reduced transepithelial resistance, blue dextran leakage and passage of allergens actinidin and thaumatin-like protein from the apical to basolateral compartment. Opening of TJs by actinidin may increase intestinal permeability and contribute to the process of sensitization in kiwifruit allergy.

1. Introduction

Together with its growing popularity kiwifruit (*Actinidia deliciosa*) has become an important cause of plant food allergy both in children and adults (Lucas et al., 2004; Popovic et al., 2013). Although the molecular basis of kiwifruit allergy has been associated with eleven IUIS kiwifruit proteins (www.allergen.org), the most abundant is a papain-like cysteine protease actinidin (Act d 1, EC 3.4.22.14) which constitutes up to 50% of soluble proteins in mature kiwifruit. Actinidin is the major IgE reactive protein (Aleman et al., 2004; Bublin et al., 2004; Pastorello et al., 1996) and a kiwifruit-specific allergen since no crossreactivity with birch or grass pollen has been observed (Pastorello et al., 1996). A link between IgE levels to actinidin and anaphylaxis has been found in some clinical subgroups of kiwifruit allergic patients (Aleman et al., 2004; Palacin et al., 2008).

Enzymatic activity of allergens has been proposed as a contributing factor to their sensitization potential. This idea is supported by the observation that the cysteine protease activity of major house dust mite allergens (Der p 1, Der p 3, Der p 6, Der p 9) has a pro-allergenic adjuvant effect by disruption of tight junctions, (Shakib et al., 1998; Wan et al., 1999; Robinson et al., 1997), inducing pulmonary epithelial cell detachment (Tomee et al., 1998) and production of proinflammatory cytokines (King et al., 1998; Tomee et al., 1998), resulting in the enhancement of bronchial epithelium permeability. No such properties have been reported for allergenic food proteases so far.

The physico-chemical properties of most food allergens confer stability to the proteolytic and acidic conditions in the digestive tract, which increases the probability of reaching the intestinal mucosa, where absorption and interaction with the immune system may occur (Bublin et al., 2008). The intestinal tract is covered by a continuous monolayer of intestinal epithelial cells responsible for maintaining the physical and functional barrier between the intestinal lumen and the underlying lamina propria (Groschwitz and Hogan, 2009). In the intestine, epithelial cells are connected to one another by a transmembrane protein complex of tight junctions (TJs), which govern the passive passage of ions, solutes, peptides and proteins from the lumen to the mucosa via the

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paracellular route (Sander et al., 2005; Camilleri et al., 2012). Various ethiological factors can cause defects in TJ proteins and increase intestinal epithelial permeability leading to disorders such as Crohn's disease, celiac disease, inflammatory bowel disease, food allergy, and acute pancreatitis (Catalioto et al., 2011). Proteolytic activity is also a factor contributing to intestinal barrier disruption resulting in increased amounts of antigen crossing into the lamina propria, driving further immune responses and sustaining the inflammatory process (Biancheri et al., 2013; Steck et al., 2011). The change of intestinal permeability is regarded as a risk factor for the development of food allergy as potential food allergens need to come into direct contact with the antigen-presenting cells in the submucosa of the intestine in order to induce sensitization (Chahine and Bahna, 2010; Groschwitz and Hogan, 2009; Liu et al., 2011). Although it has been shown that food allergens can permeate the intestinal barrier directly via intestinal epithelial cells (Perrier and Corthésy, 2011), the paracellular passage of food allergens has been less studied. Elucidation of the molecular mechanism responsible for epithelial barrier irregularities is of crucial importance for designing new therapeutic strategies for food allergy.

In the present study the direct action of kiwifruit allergen actinidin on T84 intestinal monolayer integrity was investigated by evaluating its effect on the tight junction protein occludin and by assesing monolayer barrier properties and the paracellular leakage of allergens.

2. Materials and methods

2.1. Cell culture

T84 colorectal adenocarcinoma cells (ATCC, Manassas, VA, USA) were grown and maintained in a 1:1 (vol/vol) mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium (DMEM/F12, Sigma–Aldrich, St. Louis, USA), supplemented with 10% fetal bovine serum and penicillin (100 IU)/streptomycin (100 μg/mL) (Sigma–Aldrich, St. Louis, USA) at 37 °C in 5% CO_2 humidified atmosphere. Cells were passaged using 0.25% trypsin solution in phosphate-buffered saline (PBS) without calcium and magnesium every 4–5 days. The confluency of T84 monolayers was monitored microscopically and by measuring the transepithelial resistance (TEER) across the monolayers using a volt ohm multimeter (Iskra, Ljubljana, Slovenia). Epithelial monolayers showing consistent high TEER values (>1000 Ω/cm^2) were obtained 5–6-day after seeding were considered confluent. T84 cells were stimulated in the plateau growth phase, and basic TEER values were comparable in all inductions (actinidin and controls). For transport studies, T84 cells (2×10^5 cells per well) were grown on polycarbonate cell culture inserts (0.4-μm diameter pores; Nunc, Roskilde, Denmark) in 24-well plates (Nunc, Roskilde, Denmark) at 37 °C in 5% CO_2. Each experiment contained at least two control monolayers, which allowed for normalization of the data and suitable statistical analysis to be conducted.

2.2. Actinidin preparations

Kiwifruit actinidin was isolated and purified as previously described (Cavic et al., 2012). About 20 mg of actinidin preparation was isolated from 100 g of fresh kiwifruit (approximate mass of one kiwifruit). Purity of the protein preparation was estimated to be >97% by SDS–PAGE and N-terminal amino acid sequencing.

Actinidin samples (1 mg mL^{-1}), dissolved in prewarmed (37 °C) serum-free cell culture medium, were used for the application to T84 cell monolayers. Prior to the treatment actinidin was (1) activated in 52 mM L-cystein (1 h at 45 °C) (treatment Act); (2) inactivated by thermal treatment (95 °C for 5 min) (treatment Act^{TI}); and (3) inactivated by P2714 Protease Inhibitor Cocktail Sigma–Aldrich, St. Louis, USA, 1:1; vol/vol) (treatment Act^{CI}). Serum-free cell culture medium was used as a negative control (treatment C). Proteolytic activity was quantified using a protease enzymatic assay with casein as a substrate. Both thermal treatment and addition of protease inhibitors diminished the enzymatic activity below 10% of the activity of actinidin (Grozdanovic et al., 2012). Before each treatment, the nutritive medium was removed, the plates washed with PBS, and the cells incubated in serum-free medium 1 h prior to actinidin exposure.

2.3. Transepithelial resistance measurement

T84 cells (2×10^5 cells per well) were grown on polycarbonate cell culture inserts (0.4-μm diameter pores; Nunc, Roskilde, Denmark) in 24-well plates (Nunc, Roskilde, Denmark) at 37 °C in 5% CO_2. Actinidin samples (1 mg mL^{-1}) were added to the apical surface of the monolayers and changes of TEER were monitored after 1 h and 4 h. The obtained results are expressed as % of pretreatment monolayer resistance (100%) corrected for values obtained for wells without plated cells. Results represent mean ± standard deviation (SD) of three independent experiments, and sample means were compared to corresponding non-treated controls.

2.4. Blue Dextran leakage assay

Blue Dextran 2000 (BD) dye (20 mg mL^{-1}, 2 000 kDa, Sigma–Aldrich, St. Luis, USA) was dissolved in serum-free cell culture medium and added (50 μL) to the apical surface (450 μL) of confluent T84 cell monolayers (2×10^5 cells per well), grown on 0.4-μm pore size culture inserts at the time of treatment with actinidin samples. Basolateral medium was sampled (50 μL) at appropriate time intervals and replaced by equal volume of serum free medium. The passage of BD in the basolateral compartment was measured after 1 h and 4 h at 610 nm using a Multiskan Ascent Photometric reader (Thermo Labsystems, Franklin, MA, USA). The obtained results are expressed as a percentage of BD added to the apical medium (100%) at the beginning of experiment. Results represent mean ± SD of three independent experiments, and sample means were compared to corresponding non-treated controls.

2.5. Occludin detection in Western blot

T84 cells (5×10^5 cells per well) were seeded onto 6-well plates (NUNC, Roskilde, Denmark) and grown until confluency. After 1 h and 4 h of actinidin treatment, whole-cell extracts were prepared by washing the cells twice with PBS and centrifugation (10 min at 420g, at 4 °C). Pellets were dissolved in 100 μL of lysis buffer containing 50 mM Tris–HCl pH 7.4, 50 mM NaF, 150 mM NaCl, 45 mM β-glycerophosphate, 1% Triton X-100, 20 mM phenylarsine oxide, 0.4 mM $NaVO_4$ and a protease inhibitor cocktail (Sigma–Aldrich, St. Luis, USA). The protein concentration was quantified by BCA. T84 cell lysates (20 μg) were separated under reducing conditions in SDS–PAGE (4/10%). After electrophoresis, the proteins were transferred to a nitrocellulose membrane (0.45 μm, Serva, Heidelberg, Germany), and after blocking with 5% wt/vol nonfat milk in 0.1% vol/vol Tween-20/20 mM Tris-buffered saline pH 7.6 (TTBS), for 60 min at RT, the membrane was incubated with rabbit polyclonal anti-occludin antibodies (1:2000, Sigma–Aldrich, St. Louis, USA) in 0.5% wt/vol nonfat milk/TTBS or with a mouse anti-β-actin antibody (1:2000, Sigma–Aldrich, St. Louis, USA) in 0.5% wt/vol nonfat milk/TTBS as a loading control. After a washing step (3 × 10 min, TTBS), the membrane was incubated with alkaline phosphatase-labeled anti-rabbit goat IgG (1:25,000, Sigma–Aldrich, St. Louis, USA) in 0.5% wt/vol nonfat milk/TTBS or anti-mouse goat IgG (1:30,000, Sigma–Aldrich, St. Louis, USA) in 0.5% wt/vol nonfat milk/TTBS, for 2 h at RT. Bromochloroindolyl phosphate/nitro blue tetrazolium (BCIP/NBT, Serva, Heidelberg, Germany) was used for the detection of antigen–antibody interactions. The molecular weight of the detected degradation product was calculated using the standard Rf determination method (Hames, 1998). Quantification of occludin degradation was performed using β-actin as a reference (Image Studio Lite v4.0, LI-COR Biosciences) and compared to nontreated controls. The results are presented as a graph corresponding to appropriate Western blot bands. Representative results of three independent experiments are shown.

2.6. In gel digestion

To test the direct proteolytic activity of actinidin, in-gel digestion of occludin was performed as outlined by Cleveland et al. (1977). In brief, T84 cell lysate was separated by SDS–PAGE and stained with Coomassie blue. Sections of the gel containing occludin (60–67 kDa) were cut out, trimmed to 5 mm slices, and equilibrated in 0.125 M TrisHCl, pH 6.8, 0.1% SDS, and 1 mM EDTA. Gel slices placed in the wells of the SDS gel were overlaid with 10 μL of 0.125 M Tris–HCl, pH 6.8 buffer containing 10% glycerol. Finally, actinidin samples (1 mg mL^{-1}) containing 10% glycerol were added into each well. Immunoblotting and detection of occludin was performed as described above. Representative results of three independent experiments are shown.

2.7. RT-PCR

Evaluation of occludin gene expression in T84 epithelial cells after 4 h of actinidin treatment was done by a reverse-transcriptase polymerase chain reaction (RT-PCR). For total RNA isolation epithelial cells were lysed using TRI REAGENT® BD kit (Sigma–Aldrich, St. Louis, USA). cDNA synthesis from total RNA (2 μg) was performed using random primers and MultiScribe™ Reverse Transcriptase from High-Capacity cDNA Reverse Transcription kit (50 U/μL, Applied Biosystems, CA, USA). PCR primers for occludin gene (GenBank ID: NM_002538.3) amplification were: forward 5′-CATTGCCATCTTTGCCTGTG-3′ and reverse 5′-AGCCATAACCATAG CCATAGC-3′ (150 bp PCR product) and for β-globin (GenBank ID: V00500.1) were: forward 5′-GAAGAGCCAAGGACAGGTAC-3′ and 5′-CAACTTCATCCACGTTCACC-3′ (268 bp PCR product). PCR was performed using the following program for occludin: 94 °C for 10 min, 40 cycles at 94 °C for 15 s, 60 °C for 1 min, 72 °C for 30 s; and for β-globin: 95 °C for 10 min, 39 cycles at 95 °C for 30 s, 55 °C for 1 min, 72 °C for 2 min, and 10 min at 72 °C. The presence, size and quantity of amplified PCR products was analyzed using an Agilent DNA 12,000 reagent kit on an Agilent