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Actinidin, a protease from kiwifruit, induces changes in morphology and adhesion of T84 intestinal epithelial cells

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ABSTRACT

Actinidin belongs to the papain-like family of cysteine proteases and is a major kiwifruit allergen. In this study, the effect of actinidin on cellular morphology and adhesion of T84 intestinal cells was investigated. Both rounding and detachment of T84 cells were observed upon actinidin treatment. The morphological changes and cell desquamation was protease-dependent, as well as time- and concentration-dependent. Changes of intercellular adhesion and adhesion of epithelial cells to collagen upon actinidin treatment could be responsible for the cell rounding and give rise to discontinuous breaches in the epithelial monolayer observed in this study. Actinidin's action on cell morphology, adhesion and monolayer integrity were not due to compromised viability of T84 epithelial cells, as confirmed by MTT assay and flow cytometric analysis of the cell cycle. Damage to the epithelial monolayer of the intestine induced by actinidin should be further evaluated as an important factor in the development of kiwifruit allergy and other intestinal disorders.

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1. Introduction

Actinidin (EC 3.4.22.14) is an abundant cysteine proteinase present in various kiwifruit (*Actinidia* sp.) cultivars. It belongs to a family of plant cysteine proteases which includes enzymes such as papain (from papaya), ficin (from fig) and bromelain (from pineapple stem), sharing similarities in sequence and the mode of action (Kamphuis et al., 1985). Although its physiological roles in kiwifruit have still not been clarified, it is possible that it acts as a storage protein and contributes to defense from pests, diseases and various forms of stress (Malone et al., 2005). Using an *in vitro* digestion model it has been shown that the proteolytic activity of actinidin enhances protein digestion in the stomach (Kaur et al., 2010a) and in the small intestine (Kaur et al., 2010b). In addition, the contribution of actinidin to the digestion of food proteins was confirmed *in vivo* in the growing rat (Rutherford et al., 2011). It has also been used in the food industry for tenderizing meat and as a milk coagulating enzyme (Christensen et al., 2009; Alirezai et al., 2011). Along with its beneficiary effect on human health, in the last 30 years kiwifruit has become one of the most common food allergy sources, leading to clinical symptoms ranging from localized oral allergy syndrome (OAS) to life-threatening anaphylaxis (Lucas et al., 2003). Actinidin (Act d 1) has been reported as an important

kiwifruit allergen in 1998, involved in inducing allergic symptoms, such as OAS, laryngeal edema, and even anaphylactic shock (Pastorello et al., 1998). Actinidin is considered as a marker allergen for genuine sensitisation to kiwifruit (Bublin et al., 2011). The endogenous protease activity of actinidin may cause rapid protein degradation after tissue disruption (Bublin et al., 2008, 2010).

Inhalatory allergen proteases from house dust mites, fungi and pollen with enzymatic properties have been shown to induce changes in morphology, adhesion and permeability of the pulmonary epithelium contributing to the pathogenesis of asthma (Kauffman et al., 2006; Robinson et al., 1990; Tomee et al., 1997; Runswick et al., 2007). It is possible that proteolytically active food allergens, as actinidin, might exhibit similar activity on the intestinal epithelium, thus contributing to food allergy sensitisation. The epithelial lining of the gastrointestinal (GI) tract performs the necessary absorption of fluids and digested nutrients, but also serves as a barrier that prevents unspecific intrusion of nutrients that have not been made harmless by GI digestion to the underlying tissue. There have been many reports about substances that affect the epithelial integrity of the intestine, ranging from alcohol, nonsteroidal anti-inflammatory drugs, to pathogens and various forms of cellular stress (Groschwitz and Hogan, 2009). Changes in morphology and adhesion of intestinal epithelial cells (EC) can lead to intestinal barrier dysfunction characteristic for many disorders including inflammatory bowel disease, Crohn's disease, and ulcerative colitis (Laukoetter et al., 2008). Changes in epithelial permeability of the

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intestine are also regarded as a risk factor for the development of food allergy, along with the abundance of a potential allergen in the allergen source and its relative resistance to degradation by GI digestive processes (Chahine and Bahna, 2010). Actinidin is very abundant in kiwifruit and accounts for over 50% of the solvent protein component. Some studies reported on actinidin precipitation in simulated gastric conditions and its slow partial digestion in the intestine (Bublin et al., 2008). However it has been shown in an *in vivo* model that ingestion of pectin-rich fruits, such as kiwifruit, may have a protective action on pepsin-labile allergens and prolong their survival in both gastric and duodenal juices, enabling efficient uptake and presentation to the immune system (Polovic et al., 2010). Also, actinidin is a cysteine protease belonging to the same family as house dust mite allergen Der p 1, which has been shown to affect the lung epithelial barrier, contributing to the appearance of clinical symptoms of asthma (Wan et al., 1999).

The aim of this study was to investigate if kiwifruit allergen actinidin can induce protease-dependent changes in morphology and cell adhesion of T84 intestinal epithelial cells. Immortalized cell line T84 of the intestinal epithelium grows to confluence as a monolayer with the basolateral surface attached to the glass, plastic, or collagen-coated wells retaining the structural polarity typical for differentiated intestinal epithelial cells (Dharmasathaphorn et al., 1984).

2. Results

2.1. Evaluation of actinidin activity

Prior to application on T84 cells, the activity of treatments (Act d 1, Act d 1 inactivated by thermal treatment (95 °C, 5 min), Act d 1 inactivated with a cocktail of protease inhibitors, papain) dissolved in serum-free cell culture medium DMEM/F12 needed to be assessed. Enzymatic activity of treatments (1 mg/mL) was quantified using the caseinolytic assay. In Table 1 the percentage of caseinolytic activity of the treatments compared to activated actinidin (1 mg/mL, 100% activity) in DMEM/F12 cell culture medium is presented. Results represent mean $\pm$ SD of three independent experiments, each consisting of two replicates. Thermally inactivated actinidin preparation showed around 7% activity in DMEM/F12 medium when compared to activated actinidin. Activity of around 9% in DMEM/F12 medium was detected for actinidin inactivated with a cocktail of protease inhibitors. It is evident that both thermal treatment and addition of protease inhibitors successfully diminished the enzymatic activity of actinidin. For commercial papain values over 500% of caseinolytic activity were obtained when compared to activated actinidin.

2.2. Effect of actinidin on cell morphology

The concentration-dependent change of cell morphology after 4 h of treatment was assessed on an arbitrary scale and results are summarized in Table 2. Actinidin exerted no effect up to a

Table 2

Concentration-dependent effect of treatments on T84 cell rounding and detachment (in μ g/mL). Treatments: 1 – Act d 1, 2 – Act d 1 inactivated by thermal treatment (95 °C, 5 min), 3 – Act d 1 inactivated with a cocktail of protease inhibitors, 4 – Papain.

Treatment	No effect	Rounding	Desquamation
1	10–250	250–750	750–1000 ^a
2	10–1000	n.o.	n.o.
3	10–1000	n.o.	n.o.
4	10–50	50–250	250–1000 ^a

^a Viability of cells $\geq$ 95%; n.o. – not observed.

concentration of 250 μ g/mL, and induced rounding of cells at concentrations from 250 to 750 μ g/mL. At applied concentrations of 750 μ g/mL to 1 mg/mL actinidin led to further rounding and mild desquamation of confluent T84 cells. Actinidin inactivated thermally and actinidin inhibited with a cocktail of protease inhibitors neither induced cell rounding nor desquamation after 4 h of treatment at applied concentrations up to 1 mg/mL. Papain induced both cell rounding and mild desquamation at concentrations lower than 250 μ g/mL, while higher concentration up to 1 mg/mL induced desquamation of the monolayer.

Representative images of the time-dependent effect of treatments (1 mg/mL) on cell morphology from three independent experiments are shown in Fig. 1. Under control conditions, when cells were treated only with serum-free cell culture medium, a confluent monolayer of T84 cells was formed (Fig. 1A-0). Untreated T84 cells were mainly oval to polygonal and closely packed which is characteristic for colonic epithelial cells. After treatment with activated actinidin, the cohesive appearance of the epithelial cells became obviously changed. Only mild cell rounding was observed 1 h after treatment (Fig. 1A-1). This effect was more pronounced after 4 h, accompanied by partial cell detachment and localized dissociation of the cell monolayer (Fig. 1B-1). No such effect was observed for neither thermally inactivated actinidin (Fig. 1A-2 and B-2) nor actinidin inactivated with a cocktail of protease inhibitors (Fig. 1A-3 and B-3) after 1 and 4 h of treatment. Papain induced cell rounding and detachment after only 1 h (Fig. 1A-4), with the effect growing stronger after 4 h and leading to dissociation of the cell monolayer (Fig. 1B-4).

2.3. Effect of actinidin on cell adhesion and monolayer integrity

To quantify the observed changes in cell adhesion to matrix after treatment with actinidin, a cell-adhesion test followed by SRB-assay measurement of the remaining cell protein component was performed on plastic (uncoated bottom of the plates), and collagen. Results are given in Fig. 2 and represent mean $\pm$ SD of three independent experiments, each consisting of three replicates, with the adhesion of untreated control samples taken as 100%. After 1 h of treatment with actinidin, the percentage of cell adhesion of T84 cells grown on plastic (Fig. 2A) and collagen (Fig. 2B) significantly decreased to around 82% and 81%, respectively, compared to untreated control samples ($p < 0.01$). After 4 h of treatment with actinidin, this effect was more pronounced both on plastic and collagen, the percentage of cell adhesion significantly decreasing to 48% and 53%, respectively, compared to untreated control samples ($p < 0.0001$). Cell adhesion after treatment with actinidin inhibited thermally and with a cocktail of protease inhibitors was not significantly changed ($p > 0.05$). Papain induced a significant decrease of T84 cell adhesion to 42% (plastic) and 24% (collagen) after 1 h, and to 8% (plastic) and 10% (collagen) after 4 h compared to untreated control samples ($p < 0.0001$).

Confocal microscopy analysis of the integrity of the epithelial monolayer was performed after staining T84 cells with anti- β -actin antibody. Actinidin was applied at a concentration of 1 mg/mL for

Table 1

Relative proteolytic activity of treatments compared to activated actinidin. Treatments: 1 – Act d 1, 2 – Act d 1 inactivated by thermal treatment (95 °C, 5 min), 3 – Act d 1 inactivated with a cocktail of protease inhibitors, 4 – Papain. Results represent mean $\pm$ SD of three independent experiments, each consisting of two replicates.

Treatment (1 mg/mL)	% of activity
1	100 $\pm$ 5
2	7 $\pm$ 2
3	9 $\pm$ 2
4	514 $\pm$ 56

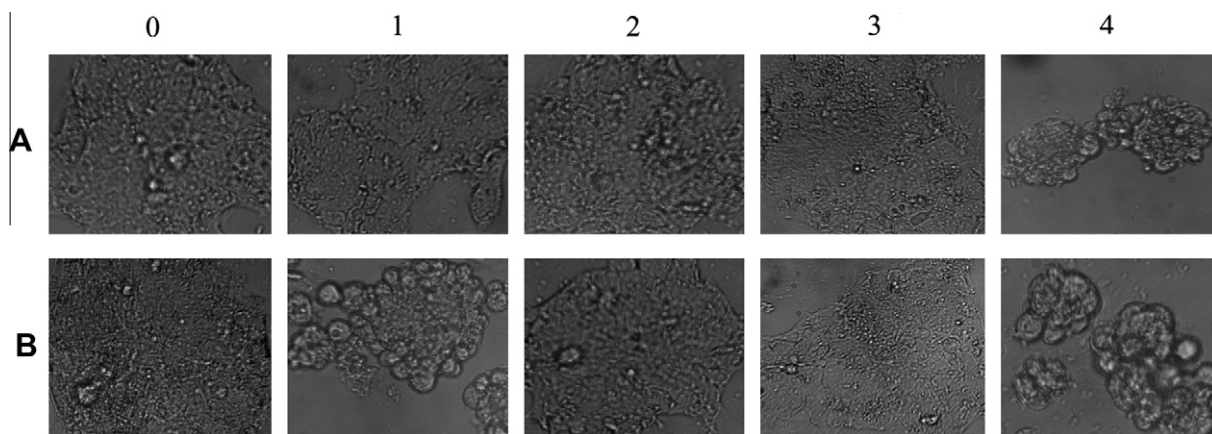


Fig. 1. Time-dependent effect of treatments on T84 cell rounding and detachment. Representative microscopic images after 1 (A) and 4 h (B) of treatment from three independent experiments are shown. Treatments: 0 – cell culture medium, 1 – Act d 1, 2 – Act d 1 inactivated by thermal treatment (95 °C, 5 min), 3 – Act d 1 inactivated with a cocktail of protease inhibitors, 4 – Papain.

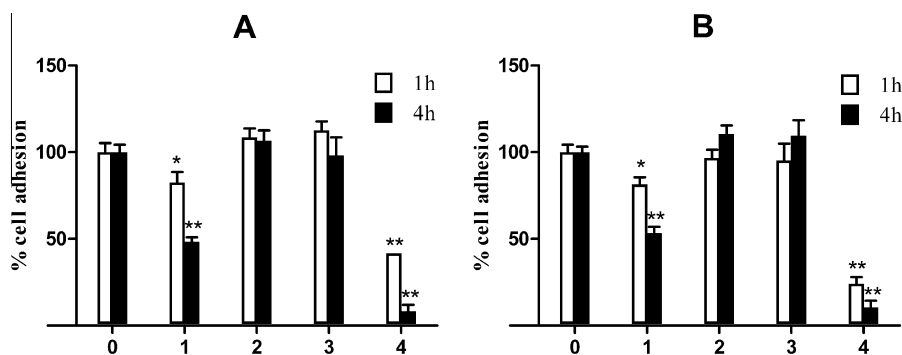


Fig. 2. Effect of actinidin on T84 cell adhesion on plastic (A) and collagen (B) after 1 and 4 h of treatment. Treatments: 0 – cell culture medium, 1 – Act d 1, 2 – Act d 1 inactivated by thermal treatment (95 °C, 5 min), 3 – Act d 1 inactivated with a cocktail of protease inhibitors, 4 – Papain. Results represent mean $\pm$ SD of three independent experiments, each consisting of three replicates. * $p < 0.01$, ** $p < 0.0001$.

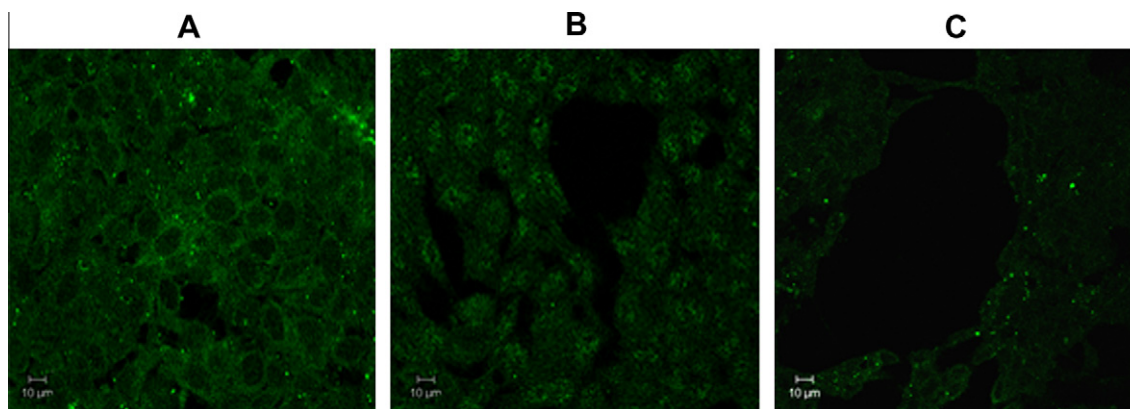


Fig. 3. Effect of actinidin on the integrity of T84 epithelial monolayer. Representative confocal microscopy images after β -actin staining from three independent experiments are shown. A – non-treated cells, B – cells treated for 1 h, C – cells treated for 4 h.

4 h, and representative images from three experiments performed in duplicate are given in Fig. 3. In non-treated cells, actin staining revealed a dense, punctate pattern in the cytoplasm and a dense network of actin at the cell boundaries (Fig. 3A). After 1 h of treatment, actin staining appeared less sharply defined at the cell boundaries and the cells were moving apart which led to mild desquamation of the monolayer (Fig. 3B). After 4 h of treatment, further detachment of cells was observed, creating discontinuous breaches in the epithelial monolayer (Fig. 3C).

2.4. Effect of actinidin on cell viability and cell cycle

To assess the viability of T84 epithelial culture after treatment, an MTT assay was employed. Varying concentrations of the treatment were used, from 0.1 to 1 mg/mL. A dose-dependent effect of actinidin on T84 cell viability is presented in Fig. 4. Results represent mean $\pm$ SD of three independent experiments, each consisting of three replicates, with viability of non-treated cells taken as 100%. After 1 and 4 h of treatment the viability of cells was not

significantly changed ($p > 0.05$) over the concentration range, varying normally from 95% to 106% of control cell viability.

To determine whether actinidin induced changes in proliferation of T84 cells, and confirm that no significant damage of cells occurs that would lead to apoptosis, DNA content after propidium iodide staining was analyzed by flow cytometry. Representative histograms of cell cycle phase distribution before and after treatment with actinidin are given in Fig. 5. The percentage of cells in G_0/G_1 , S and G_2/M phases of the cell cycle are also presented on the histograms. After treatment of T84 cells with 1 mg/mL of actinidin, no alterations in the distribution of cell cycle phases were detected after 1 h (Fig. 5A) and 4 h (Fig. 5B) of treatment. In cells treated with actinidin for 1 and 4 h the percentage of cells in G_0/G_1 phase remained around 60%, in S phase around 25% and in G_2/M phase around 15%, as in non-treated cells. Also, the presence of sub- G_1 peak in the histograms of treated cells was not detected, confirming that actinidin did not induce fragmentation of DNA characteristic for cells undergoing apoptosis.

3. Discussion

In order to induce sensitisation, potential food allergens present in the gut lumen need to come in contact with antigen-presenting cells in the underlying tissue. The mechanisms enabling this contact are still not clear, but it is possible that disturbed intestinal barrier could be one factor contributing to allergic sensitisation in the gut and subsequent appearance of clinical symptoms of food allergy.

In this study, we have shown that kiwifruit cysteine protease actinidin induces changes in morphology and adhesion of T84 intestinal epithelial cells, which could be a significant factor for the process of sensitisation and subsequent allergic reaction to kiwifruit.

In T84 cells grown to confluency, cellular morphology has been changed upon incubation with actinidin. Rounding and detachment of cells were not observed when actinidin was inactivated by thermal treatment or a cocktail of protease inhibitors. It can be concluded that the morphological changes and cell desquamation was protease-dependent, as well as time- and concentration-dependent. Actinidin dose-dependently induced morphological changes, with lower concentrations exerting no effect after 4 h of treatment, and higher concentrations (up to 1 mg/mL) inducing cell rounding and partial cell desquamation of the confluent T84 cell monolayer. Light microscopy showed that the cells started to become circular after 1 h of treatment with 1 mg/mL of activated actinidin, and that after 4 h this led to partial dissociation of the epithelial monolayer. With respect to intestinal barrier breach, it is hard to say what extent of epithelial cell detachment could be significant for the sensitisation to food allergens to occur, but even partial dissociation of the epithelial monolayer may be sufficient for allergens to induce the immune response.

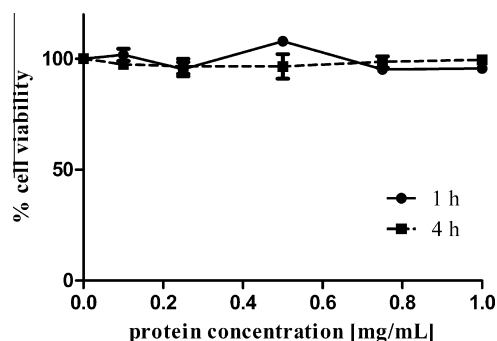


Fig. 4. Dose-dependent effect of actinidin on T84 cell viability after 1 and 4 h of treatment. Viability of non-treated cells is represented as 100%. Results represent mean $\pm$ SD of three independent experiments, each consisting of three replicates.

Quantification of the non-detached cells by SRB assay allowed a better appreciation of the anti-adhesive effect of actinidin. It is known that T84 cells attach to and grow more quickly on collagen-coated dishes than on uncoated ones (Murakami and Masui, 1980). Collagen is an important component of the basal lamina, a layer of extracellular matrix that facilitates the formation of cell–substrate adhesion essential for correct proliferation and differentiation of tissue cells. Therefore, we performed the adhesion assay on both uncoated plates (plastic) and plates coated with collagen to verify if the interaction of T84 cells with collagen makes them less likely to detach. However, we did not observe any difference in the level of cell detachment after actinidin treatment of cells grown on plastic and collagen-coated plates. It has previously been shown that actinidin possesses the ability to degrade collagen fibrils and can be efficiently used for isolation of different cell populations from various solid tissues (Mostafaie et al., 2008). Therefore, the detachment of T84 cells from collagen-coated plates could be a consequence of actinidin's action on both cell–cell and cell–collagen interactions. Inactivated forms of actinidin had no effect on cell detachment, confirming that the process is protease-dependent.

By confocal microscopy it was also shown that actinidin induced mild rearrangement of cellular actin making the cells move apart from each other and detach. Changes of intercellular adhesion and adhesion of epithelial cells to their basal lamina upon actinidin treatment could be responsible for the cell rounding and appearance of discontinuous breaches in the epithelial monolayer observed in this study. Which cell–cell and cell–matrix adhesion proteins could be the potential targets of actinidin's proteolytic action on human epithelial cells of the intestine still needs to be determined. Understanding how the epithelial barrier changes upon contact with plant proteolytic allergens may allow the development of therapeutic agents that prevent the breakdown or enhance recovery of normal barrier function (Catalioto et al., 2011).

To ensure that there was no cytotoxic effect of the treatments which would breach the integrity of the T84 monolayer, the viability of cells upon treatment was tested by MTT assay. Our results showed that activated and both inactivated forms of actinidin, as well as papain, in concentrations up to 1 mg/mL exerted no cytotoxic effect on T84 intestinal cell line. The cell cycle phase distribution of actinidin-treated cells analyzed by flow cytometry was performed to confirm that actinidin did not induce changes in proliferation of T84 cells, or significant pro-apoptotic damage. The analysis confirmed that there was no reduction of the number of viable cells after treatment compared to non-treated (control) population of cells, and that there was no perturbation of cell cycle phase distribution. Also, the absence of sub- G_1 peak in the histograms of treated cells confirmed that actinidin treatment did not induce fragmentation of DNA, characteristic for cells undergoing apoptosis. Taken together with the MTT viability results, it can be concluded that the observed effects of actinidin on cell morphology, adhesion and monolayer integrity were not due to compromised viability of T84 epithelial cells. Damage to the epithelial monolayer of the intestine induced by actinidin should be further evaluated as an important factor in the development of kiwifruit allergy and other intestinal disorders.

There is a general hypothesis that food allergens must exhibit sufficient structural integrity in the gastrointestinal tract to be able to reach the intestinal mucosa. Ingestion of pectin-rich fruits may have a protective effect on pepsin-labile allergens and prolong their survival in both gastric and duodenal juices (Polovic et al., 2007, 2010). Pectin gel protected actinidin from pepsin digestion in an acidic environment and dissolved slowly in the slightly basic environment of the intestine, allowing the survival of the fruit allergen for an additional time and enabling a possible interaction with the gut immune system (Polovic et al., 2009). Therefore, it

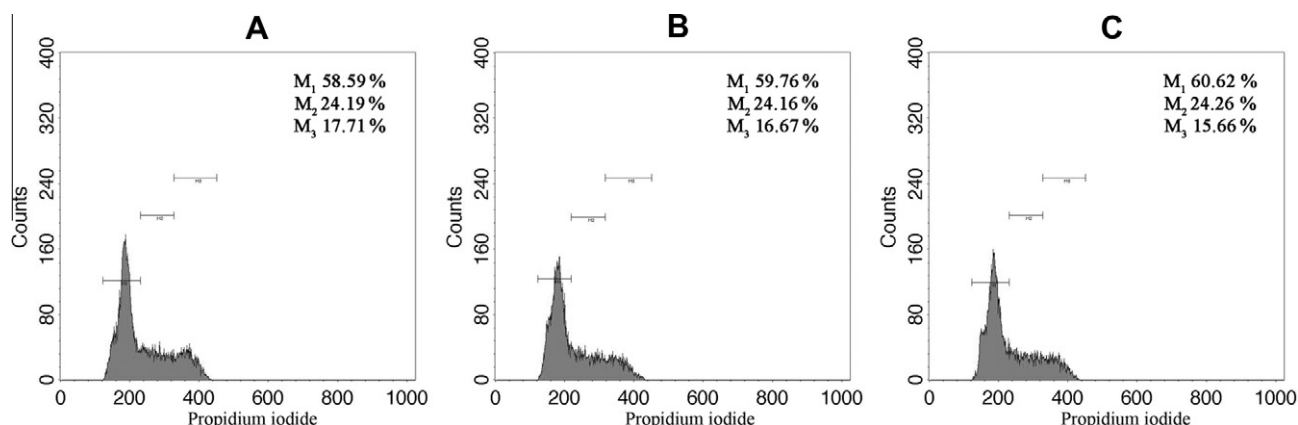


Fig. 5. Effect of actinidin on T84 cell cycle progression. Cells were treated with actinidin (1 mg/mL), fixed in ethanol, stained with propidium iodide, and DNA content was determined by flow cytometry. A – non-treated cells, B – cell treated for 1 h, C – cells treated for 4 h. M₁ – cells in G₀/G₁ phase of the cell cycle, M₂ – cells in S phase of the cell cycle, M₃ – cells in G₂/M phase of the cell cycle. The percentage of cells in each phase of the cell cycle is indicated.

seems that actinidin is able to compromise the integrity of gut epithelium and in that way contribute to the process of sensitisation.

4. Conclusions

The findings in this study indicate that actinidin, a major kiwifruit allergen, induces protease-dependent morphology changes of T84 intestinal cells, leading to cell rounding and desquamation of the epithelial monolayer, without affecting cell viability. Those events could be a significant factor for the process of allergic monosensitisation to kiwifruit.

5. Experimental

5.1. Cell culture

T84 human 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 1% penicillin–streptomycin (Sigma–Aldrich, St. Louis, USA) at 37 °C in 5% CO₂ humidified atmosphere. Cells were maintained in high confluency and were passaged using 0.25% trypsin solution in phosphate-buffered saline without calcium and magnesium every 4–5 days.

5.2. Activity of treatments

Kiwifruit actinidin (Act d 1) was isolated and purified from the fresh fruit (*Actinidia deliciosa*, Hayward cv). For the preparation of protein extract kiwifruit pulp was homogenized in 100 mM sodium citrate buffer, pH 5.0. After extraction (2 h at 4 °C), the slurry was centrifuged (1000g, 5 min) and the obtained supernatant was extensively dialyzed against the extraction buffer. Actinidin was purified from the kiwifruit extract which was subjected to ion-exchange chromatography on a DEAE-Sephadex A-50 column (145 mm × 27 mm, GE Healthcare, Uppsala, Sweden), pre-equilibrated with 100 mM sodium citrate buffer, pH 5.0. The unbound protein fraction was concentrated by ultrafiltration, and dialyzed against 20 mM TRIS–HCl buffer, pH 8.0 for 48 h. The protein sample (125 mL) was applied on a SP-Sephadex C-50 ion-exchange column (125 mm × 15 mm, Pharmacia-Biotech, Uppsala, Sweden) equilibrated in the dialysis buffer and the column was eluted by a pH step gradient ranging from pH 5 to 2.5 with eight column volumes. Collected fractions were analyzed by SDS–PAGE, and those containing actinidin

were pooled and concentrated by ultrafiltration. About 20 mg of actinidin preparation was isolated from 100 g of kiwifruit. The purity of the protein preparation was estimated to be >97% by SDS–PAGE and N-terminal amino acid sequencing. Commercial papain (P 4762, Sigma–Aldrich, St. Louis, USA), a cysteine protease from papaya latex (*Carica papaya*) with similar structure and specificity as actinidin, was used as a positive control in this study. All preparations were dissolved in prewarmed (37 °C) serum-free cell culture medium DMEM/F12 and used as treatments for application to T84 cells. For activation of Act d 1, 52 mM L-cysteine was added to the sample and the preparation incubated for 1 h at 45 °C. Inactivation of Act d 1 was achieved either by thermal treatment (95 °C for 5 min) or by addition of P2714 Protease Inhibitor Cocktail (Sigma–Aldrich, St. Louis, USA) to the protein sample (1:1; vol/vol).

Proteolytic activity of the treatments was quantified by using a protease enzymatic assay with casein as a substrate. Briefly, a 2% casein solution was prepared in 0.1 M potassium phosphate buffer, pH 7.6. A 400 µL aliquot of casein was added to 40 µL of 250 mM L-cysteine, 120 µL 1 M KH₂PO₄ pH 7.0, 40 µL of 250 mM EDTA, 40 µL of 250 mM NaOH, and 80 µL of distilled water. The reaction mixture was equilibrated to 37 °C, after which 80 µL of treatment (1 mg/mL) was added and the assay mixture was incubated for 1 h at 37 °C. The enzyme reaction was stopped by addition of 1.2 mL of 5% trichloroacetic acid (TCA) and the reaction mixture was incubated for an additional hour at 37 °C. Following centrifugation (13,000g for 15 min) absorption of the supernatant was measured at 280 nm against a blank. The blank was prepared by first mixing the enzyme with TCA and then adding the casein solution to the enzyme–TCA mixture. Results are presented as relative proteolytic activity of samples calculated as % of caseinolytic activity of the sample compared to activated Act d 1 (100% activity) in DMEM/F12 cell culture medium. Results represent mean ± SD of three independent experiments, each consisting of two replicates.

5.3. Cell treatment with actinidin and microscopic analysis

T84 cells were seeded onto 6-well plates (NUNC, Roskilde, Denmark) at a density of 5 × 10⁵ cells per well and grown for approximately 4–5 days at 37 °C in 5% CO₂ humidified atmosphere until they reached confluency. Nutritive medium was removed, the plates washed with PBS, and the cells incubated in serum-free medium 1 h prior to treatment. The treatments were applied to the cells in concentrations 10 µg/mL, 50 µg/mL, 250 µg/mL, 500 µg/mL, 750 µg/mL and 1000 µg/mL, with serum-free cell culture medium as a negative control. Morphology of the cells was monitored microscopically (Carl Zeiss GmbH, Jena, Germany) in

three independent experiments. After 4 h of treatment the changes induced by actinidin were analyzed and expressed in an arbitrary scale (no effect – same morphology as non-treated cells; rounding – visual changes in morphology predominated by cell shrinking and partial cell desquamation ($\leq 10\%$ of detached cells); desquamation – $>10\%$ cells have detached and are floating around) (Kauffman et al., 2006). To observe the time-dependent effect of the treatments, cell morphology was monitored microscopically after 1 and 4 h of treatment, and photographed with Camedia 4040 digital camera (Olympus, Tokyo, Japan). Representative microscopic images from three independent experiments after 1 and 4 h of treatment are presented.

5.4. Confocal microscopy

T84 cells were grown on coverslips (22×22 mm, Isolab, Wertheim, Germany) in 6-well plates (NUNC, Roskilde, Denmark) at a density of 5×10^5 cells per well for approximately 4–5 days at 37°C in 5% CO_2 humidified atmosphere until they reached confluency. Nutritive medium was removed, the plates washed with PBS (3×1 min), and the cells incubated in serum-free medium 1 h prior to treatment with actinidin (1 mg/mL). After 4 h, treatments were removed, cells washed three times with PBS (3×1 min) and fixed at room temperature in 4% paraformaldehyde in PBS (1×20 min). The cells were washed with PBS (3×1 min) and then permeabilized with PBS containing 0.1% Triton X-100 (1×10 min). Blocking was performed in 1% BSA in PBS (1×30 min). Subsequently, the cells were incubated overnight at 4°C with primary mouse anti- β -actin antibody diluted $1:200$ in PBS (Sigma–Aldrich, St. Louis, USA). After washing the cells with PBS (3×10 min), Alexa Fluor® 488-conjugated secondary antibody diluted $1:200$ in PBS was applied for 2 h (goat anti-mouse IgG; Molecular Probes, Eugene, OR). After washing the cells with PBS (3×10 min), the coverslips were mounted to glass plates with Mowiol based mounting medium (Calbiochem, Merck GmbH, Darmstadt, Germany). The samples were examined using LSM 510 confocal laser scanning microscope (Carl Zeiss GmbH, Jena, Germany). Fluorescence images were acquired with a plan-neofluar oil-immersion objective ($40\times$, 1.4 numerical aperture). Images were assembled and analyzed using Zeiss LSM 510 software V3.2. The final images were prepared using Adobe Photoshop V9.0. All images were processed identically.

5.5. Cell adhesion assay

Cell-adhesion test was performed on plastic (uncoated bottom of the plates), and collagen. Wells of 96-well plates (NUNC, Roskilde, Denmark) were coated with collagen ($20 \mu\text{g/mL}$ in serum free cell culture medium, $50 \mu\text{L}$ per well, Sigma–Aldrich, St. Louis, USA) by leaving the plate at 37°C in 5% CO_2 humidified atmosphere for 4 h. Before cell seeding, each plate was washed with sterile PBS (2×1 min). T84 cells were seeded at a density of 2×10^4 cells per well and incubated at 37°C in 5% CO_2 humidified atmosphere. After 4–5 days when cell confluency was reached, nutritive medium was removed, the plates washed with PBS (3×1 min) and the cells incubated in serum-free medium 1 h prior to treatment. Treatments (1 mg/mL) dissolved in serum-free medium were added to the wells and incubated for 1 h and 4 h at 37°C in 5% CO_2 humidified atmosphere. The supernatant was discarded, plates were washed with PBS (3×1 min), and nondetached cells were fixed with 10% (w/v) cold trichloroacetic acid (TCA, $200 \mu\text{L}$ per well, Sigma–Aldrich, St. Louis, USA) at 4°C for 1 h. After fixation, TCA was removed and wells washed with distilled water (3×1 min). Plates were left to dry at room temperature for a few minutes.

To verify the remaining cell number in the plate wells, adherent cells in each well were dyed with a colorimetric assay based on quantification with sulforhodamine B (SRB) of the cell protein com-

ponent (Skehan et al., 1990). SRB solution (0.4% , w/v, in 1% acetic acid, Sigma–Aldrich, St. Louis, USA) was added to the wells containing cells treated and fixed as described above, and the plates were allowed to stain for 30 min at room temperature. Unbound SRB was removed by washing with 1% acetic acid (3×1 min). Plates were air-dried, bound stain was dissolved with 10 mM Tris base (Sigma–Aldrich, St. Louis, USA) at pH 10.5 and the optical density was read at 570 nm in Multiskan Ascent Photometric reader (Thermo Labsystems, Franklin, MA, USA) using a 630 nm reference wavelength. The modification in cell adhesion was expressed as percentage of adhesion in treated samples compared to untreated control samples (taken as 100%) corrected for values obtained for wells without plated cells. Results represent mean $\pm$ SD of three independent experiments, each consisting of three replicates, and sample means were compared to corresponding non-treated controls.

5.6. Cell viability assay

T84 cells were seeded on 96-well plates (NUNC, Roskilde, Denmark) at a density of 2×10^4 cells/well, and grown for 4–5 days until they reached confluency. Nutritive medium was removed, the plates washed with PBS, and the cells incubated in medium without serum 1 h prior to treatment. Triplicate wells were treated with varying concentrations of enzyme preparations (0.1 , 0.25 , 0.5 , 0.75 and 1 mg/mL dissolved in cell culture medium) in $100 \mu\text{L}$. After 1 and 4 h of incubation at 37°C in 5% CO_2 humidified atmosphere, $10 \mu\text{L}$ of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution (MTT 5 mg/mL, Sigma–Aldrich, St. Louis, USA) was added and incubated for 4 h at 37°C in 5% CO_2 humidified atmosphere. The MTT assay is based on the reduction of yellow tetrazolium salt to water insoluble purple formazan by metabolically active cells, based on their mitochondrial activity, therefore reflecting the metabolic activity of the living cells (Supino, 1995). After incubation, $100 \mu\text{L}$ of 10% SDS in 0.01 M HCl was added to dissolve the purple blue sediment overnight. The relative optical density (OD)/well was determined at a test wavelength of 570 nm in Multiskan Ascent Photometric reader (Thermo Labsystems, Franklin, MA, USA) using a 630 nm reference wavelength. The modification in cell viability was expressed as percentage of viability in treated samples compared to untreated control samples (taken as 100%) corrected for values obtained for wells without plated cells. Results represent mean $\pm$ SD of three independent experiments, each consisting of three replicates, and sample means were compared to corresponding non-treated controls.

5.7. Flow-cytometric analysis of cell cycle phase distribution

Quantitative analysis of cell cycle phase distribution was performed by flow-cytometric analysis of the DNA content in fixed T84 cells, after staining with propidium iodide (PI) (Ormerod, 1994). Cells were seeded at a density of 5×10^5 cells per well in 6-well plates (NUNC, Roskilde, Denmark) in duplicates and grown for 4–5 days until they reached confluency. After 1 and 4 h of treatment with actinidin (1 mg/mL), cells were collected by trypsinization, washed with ice-cold PBS (2×1 min), and fixed for 30 min in 70% EtOH at 4°C . Fixed cells were washed with PBS (2×1 min), and incubated with RNase A (1 mg/mL, Sigma–Aldrich, St. Louis, USA) for 30 min at 37°C . Just before the flow-cytometric analysis cells were stained with PI ($100 \mu\text{L}$, $400 \mu\text{g/mL}$, Sigma–Aldrich, St. Louis, USA). Cell cycle phase distribution was analyzed using a fluorescence activated sorting cells (FACS) Calibur (Becton Dickinson) flow cytometer and Cell Quest computer software.

5.8. Statistical analysis

GraphPad Prism Version 5.04 (GraphPad Software, San Diego, CA) was used to compare means by two way analysis of variance

(ANOVA). When an overall statistically significant difference was seen, post-tests were performed to compare pairs of treatments, using the Bonferroni method to adjust the p -value for multiple comparisons. Differences were considered significant if $p < 0.05$. P -values for each analysis are indicated in figure legends.

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