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# Kiwifruit cysteine protease actinidin compromises the intestinal barrier by disrupting tight junctions

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ABSTRACT

*Background:* The intestinal epithelium forms a barrier that food allergens must cross in order to induce sensitization. The aim of this study was to evaluate the impact of the plant-derived food cysteine protease — actinidin (Act d1) on the integrity of intestinal epithelium tight junctions (TJs).
*Methods:* Effects of Act d1 on the intestinal epithelium were evaluated in Caco-2 monolayers and in a mouse model by measuring transepithelial resistance and *in vivo* permeability. Integrity of the tight junctions was analyzed by confocal microscopy. Proteolysis of TJ protein occludin was evaluated by mass spectrometry.
*Results:* Actinidin (1 mg/mL) reduced the transepithelial resistance of the cell monolayer by 18.1% (after 1 h) and 25.6% (after 4 h). This loss of barrier function was associated with Act d 1 disruption of the occludin and zonula occludens (ZO)-1 network. The effect on intestinal permeability *in vivo* was demonstrated by the significantly higher concentration of 40 kDa FITC-dextran (2.33 μg/mL) that passed from the intestine into the serum of Act d1 treated mice in comparison to the control group (0.5 μg/mL). Human occludin was fragmented, and putative Act d1 cleavage sites were identified in extracellular loops of human occludin.
*Conclusion:* Act d1 caused protease-dependent disruption of tight junctions in confluent Caco-2 cells and increased intestinal permeability in mice.
*General significance:* In line with the observed effects of food cysteine proteases in occupational allergy, these results suggest that disruption of tight junctions by food cysteine proteases may contribute to the process of sensitization in food allergy.

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## 1. Introduction

The gastrointestinal tract is the largest organ of the immune system and is under constant exposure to an array of exogenous antigens, such as ingested food proteins and commensal bacteria [1]. Upon ingestion, dietary proteins undergo enzymatic hydrolysis in the harsh acidic environment of the stomach. A single epithelial cell layer separates this potential antigenic load from lymphocytes, antigen-presenting cells, and other immune cells of the lamina propria that together constitute the mucosa-associated lymphoid tissue (MALT) [2]. The proper function of this epithelial barrier is dependent on the intactness of the apical plasma membrane of epithelial cells, as well as on the intercellular tight junctions (TJs). TJs are macromolecular protein assemblies composed of integral membrane proteins (claudins, occludin, tricellulins, and junctional adhesion molecules), cytoplasmic cytoskeletal linker proteins zonula occludens (ZO)-1, ZO-2, and ZO-3, and associated signaling molecules and cell cycle regulators [3]. The disruption of intestinal epithelium tight junctions can cause increased permeability, which enables normally excluded molecules to cross the mucosal epithelium by paracellular permeation. This, in turn may cause inflammatory conditions in the intestinal mucosa such as Crohn's disease, celiac disease, inflammatory bowel disease, acute pancreatitis, and food allergy [4].

In the context of food allergy it is hypothesized that intestinal barrier dysfunction might contribute to both antigen sensitization and the IgE/mast cell mediated effector phase of allergic disease, however no concluding data has been published to date [5]. An

*Abbreviations:* TJs, tight junctions; ZO, zonula occludens; LDH, lactate dehydrogenase; TEER, transepithelial electrical resistance; MALT, mucosa-associated lymphoid tissue; EL, extracellular loop, mmu — milli-mass unit.

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understanding of the mechanism of primary sensitization to allergens is paramount for determining the pathogenesis of allergic diseases and for their eventual prevention [6]. Several studies have shown that inhalant allergens which posses proteolytic activity can disrupt the airway epithelial barrier and skin barrier by direct activity against TJ occludin [7–11]. It has been recently shown that papain, a cysteine protease from papaya, can cause epidermal barrier impairment in human primary keratinocytes and thereby, like house dust mite allergen Der p 1 [7], might facilitate the sensitization to secondary allergens [12].

Actinidin (Act d 1) belongs to the papain-like family of cysteine proteases and is a major allergen from kiwifruit, where it constitutes up to 50% of soluble proteins [13–15]. Interestingly, active Act d 1 retains its primary structure, proteolytic activity, and immunological reactivity under conditions of simulated gastric followed by simulated intestinal digestion [16], thus indicating that it is probably capable of reaching the intestinal mucosa in a proteolytically active and immunogenic state. Furthermore, in a set of recent *in vitro* studies it was demonstrated that Act d 1 induces changes in the morphology and adhesion of T84 human colonic crypt epithelial cell monolayers [17], and breaches the occludin network [18]. Therefore, we hypothesize that the proteolytic activity of Act d 1 disrupts the integrity of tight junction protein occludin and enables passage of this kiwifruit allergen through the intestinal barrier. Our specific aims were: (*i*) to evaluate the impact of proteolytic activity of Act d 1 *in vitro by* measuring parameters of permeability in human adenocarcinoma enterocyte-like Caco-2 cell monolayers, (*ii*) to delineate specific human occludin degradation and map out the putative cleavage sites, and (*iii*) to demonstrate *in vivo* in a mouse model that Act d 1 is capable of increasing permeability of the intestinal barrier. The answer to these questions will provide a platform for future studies on the mechanism of food allergy sensitization, a yet unresolved fundamental aspect of food allergy pathogenesis.

## 2. Materials and methods

### 2.1. Purification of actinidin

Actinidin was isolated from fresh kiwifruit (*Actinidia deliciosa*, Hayward cv) according to Grozdanovic et al. [19]. Kiwifruit was peeled and the pulp was homogenized in 600 mL of 50 mM sodium-citrate buffer, pH 5.0. Extraction of total soluble proteins was performed for 2 h at +4 °C under constant agitation. The slurry was centrifuged (3000 ×*g*, 30 min) and loaded onto a QAE-Sephadex A-50 column (200 mm × 27 mm) (GE Healthcare, Uppsala, Sweden) pre-equilibrated in 50 mM sodium-citrate buffer, pH 5.0. Bound proteins were eluted by 1 M NaCl and extensively dialyzed against 20 mM TRIS–HCl buffer, pH 8.0. In the second step the protein sample (200 mL) was applied onto a SP-Sephadex C-50 ion-exchange column (150 mm × 15 mm) (GE healthcare, Uppsala, Sweden) equilibrated in the dialysis buffer and the column was eluted by a salt step gradient ranging from 0.1 M to 1 M NaCl with 10 column volumes. A total of 125 mg of actinidin was isolated from 300 g of kiwifruit. Purity of the obtained actinidin preparation was estimated to be >97% by N-terminal amino acid sequencing and SDS-PAGE.

Proteolytic activity of purified actinidin was quantified by using a protease enzymatic assay with casein as a substrate as previously described by Čavić et al. [17]. In brief, 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 17 mM L-cystine, 120 μL 1 M $KH_2PO_4$ pH 7.0, 40 μL of 17 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 actinidin (0.1 mg/mL or 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 trichloroacetic acid (TCA, 10%) and the reaction mixture was incubated for an additional hour at 37 °C. Following centrifugation (13,000 ×*g*, 15 min) absorbance 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. One unit of activity was determined as that amount of enzyme which releases acid-soluble fragments equivalent to 0.001 $A_{280}$ per minute at 37 °C under the specified conditions. Proteolytic activity of actinidin dissolved in prewarmed (37 °C) serum-free DMEM cell culture medium was 0.74 IU (1 mg/mL) and 0.62 IU (0.1 mg/mL), respectively.

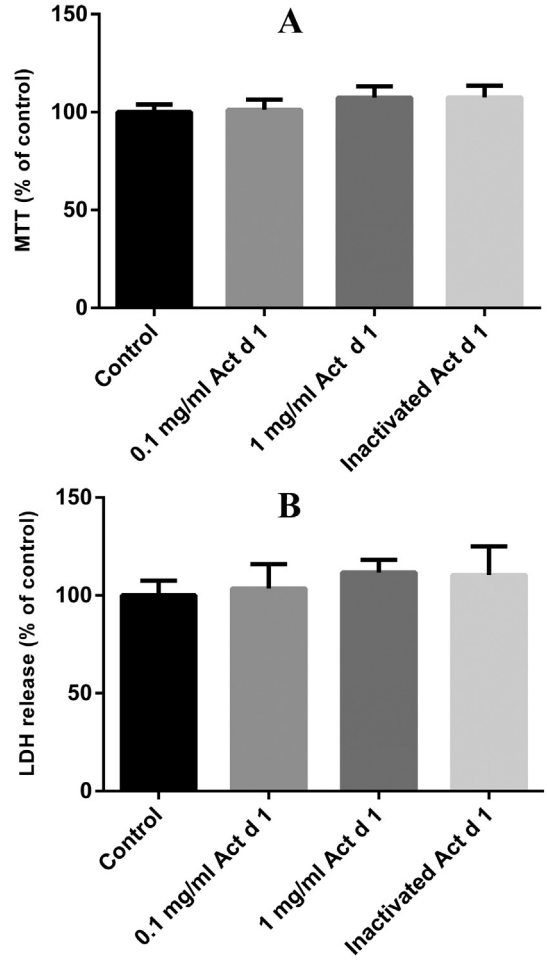


**Fig. 1.** The metabolic activity of control and actinidin-exposed cells was determined by a MTT reduction assay (A). The cytotoxic effect of actinidin on Caco-2 cells was evaluated by measuring lactate dehydrogenase (LDH) leakage (B). *$P > 0.05$.

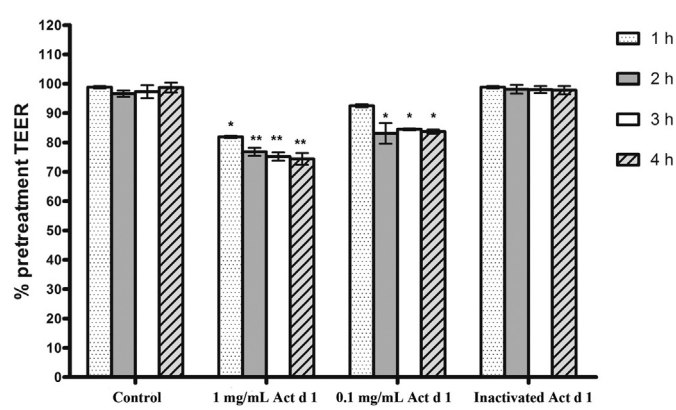


**Fig. 2.** Relative TEER of Act d 1-treated Caco-2 cell monolayers: Control- untreated cells, 1 mg/mL and 0.1 mg/mL — cells treated with Act d 1 samples, Inactivated-cells treated with 1 mg/mL of heat inactivated Act d 1. Values are expressed as % of pretreatment monolayer resistance measured after 1–4 h of treatment application. *$P < 0.01$, ** $P < 0.001$.

### 2.2. Caco-2 cell culture

Caco-2 cells were cultured in a medium composed of DMEM containing 4.5 g/L glucose (Gibco, Invitrogen, Carlsbad, USA) supplemented with 10% FCS, 1% non-essential amino acids, 1% (v/v) 200 mM L-glutamine (Gibco, Invitrogen, Carlsbad, USA), 1% Pen/Strep. Cultures were maintained at 37 °C in an atmosphere of 5% $CO_2$ at 37 °C. For transport studies, Caco-2 cells were seeded at $6.8 \times 10^4$ cells/cm$^2$ on polycarbonate membranes with a pore size of 0.4 μm in 12-well plates (Transwell, Costar, Corning, NY, USA). Cells at passage of 59–68 were used in experiments at the age of 21 days and the cultures were fed three times a week. Prior to the transport studies, TEER was measured using Millicell-ERS Volt-Ohmmeter (Millipore, Massachusetts, USA).

### 2.3. Cytotoxicity assays

Caco-2 cells were seeded in flat-bottom 96-well plates (Costar, Corning, NY, USA) at a cell density of $0.1 \times 10^5$ cells/well. After 15–16 days, confluent and differentiated Caco-2 cells were exposed for 6 h to 0.1 mg/mL and 1 mg/mL of active actinidin as well as to 1 mg/mL of heat inactivated actinidin (95 °C, 5 min). The cytotoxic effect of actinidin on Caco-2 cells was evaluated by measuring lactate dehydrogenase (LDH) leakage. LDH leakage was measured in the culture media using the CytoTox 96 Non-radioactive Cytotoxicity Assay Kit (Promega Corporation, WI, USA), according to the manufacturer's instructions. In addition, the metabolic activity of control and actinidin-exposed cells was determined by a MTT (tetrazolium) reduction assay (Sigma-Aldrich, St. Louis, Mo, USA). Briefly, 20 μL MTT (1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan) (3 mg/mL PBS) was added to the cultures and incubated for 4 h at 37 °C. The medium was removed and the produced formazan crystals were dissolved in 100 μl of 0.04 M HCl in isopropanol. After dissolution, absorbance was recorded at 595 nm using a microplate reader (Bio-Rad, Japan).

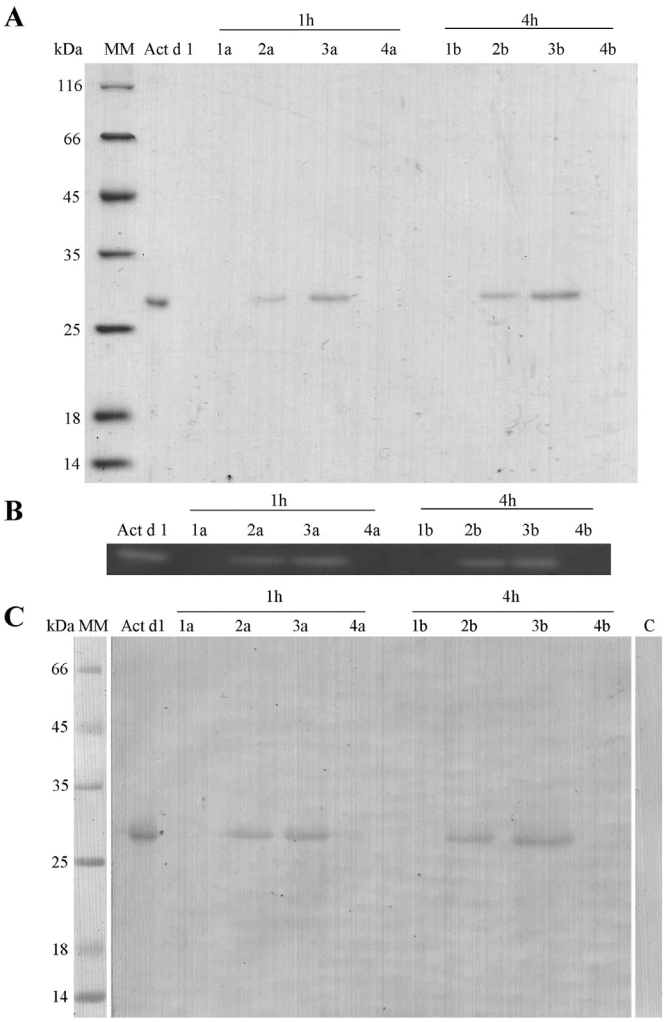


**Fig. 3.** Detection of Act d 1 leakage in the basolateral medium after 1 h and 4 h of treatment: A) SDS-PAGE, B) Zymography of basolateral samples, C) Immunoblot of basolateral samples. Act d 1) apically applied actinidin, 1) untreated cells, 2) 0.1 mg/mL Act d 1, 3) 1 mg/mL Act d 1, 4) 1 mg/mL of heat inactivated actinidin, c) control of secondary antibodies.

### 2.4. Monolayer resistance assay

Only intact polarized Caco-2 cell monolayers with an initial net resistance >1000 Ω/cm$^2$ were used. Actinidin was dissolved in cell culture medium to a final concentration of 1 mg/mL or 0.1 mg/mL. For preparation of the inactive enzyme, actinidin was inhibited by thermal inactivation (95 °C, 5 min) and dissolved in cell culture medium to a concentration of 1 mg/mL. Actinidin preparations (350 μL) were applied onto the apical surface of monolayers and transepithelial electrical resistance (TEER) displayed by filters containing cells in cell culture medium was measured after 1, 2, 3, and 4 h of actinidin treatment using a Millicell-ERS Volt-Ohm Meter (Millipore, Billerica, Massachusetts, USA). Untreated Caco-2 cell monolayers were used as control. Measurements were corrected for the background resistance of membranes without cells and are expressed as percentage of initial monolayer resistance (100%). The results represent mean ± SD of three independent experiments.

### 2.5. Translocation of actinidin across the cell monolayer

Treatments consisting of 350 μL of active actinidin (1 mg/mL, or 0.1 mg/mL) or heat inactivated actinidin (1 mg/mL) in cell medium were added to the apical surface of confluent Caco-2 cells. Untreated Caco-2 cell monolayers were used as control. Aliquots of the basolateral medium were collected after 1 and 4 h. The passage of actinidin from the apical to the basolateral compartment was detected by SDS-PAGE and Western blot and proteolytic activity of actinidin was confirmed by zymography with gelatin [20].

### 2.6. Actinidin cleaves occludin in Caco-2 cell monolayer

Caco-2 cells ($5 \times 10^5$ cells per well) were seeded onto 6-well plates (NUNC, Roskilde, Denmark) and grown until they reached 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 420 ×g, 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 and a protease inhibitor cocktail (Sigma-Aldrich, St. Luis, USA). The protein concentration was quantified by BCA. Cell lysates (25 μg) were separated under reducing conditions in SDS-PAGE (4/12%). After electrophoresis, the proteins were transferred

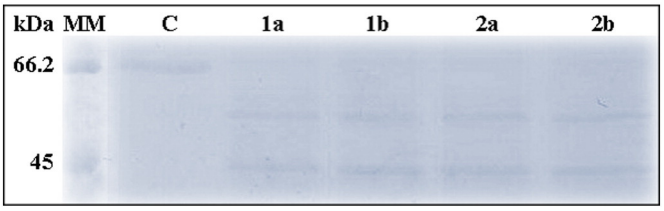


**Fig. 4.** Effects of Act d 1 on occludin from Caco-2 cells detected in immunoblot: C) untreated cells; 1) cells treated with 0.1 mg/mL Act d 1(0.62 IU) a) for 1 h, and b) for 4 h and 2) cells treated with 1 mg/mL Act d 1(0.74 IU) a) for 1 h, and b) for 4 h.

to a polyvinylidene fluoride (PVDF) membrane (0.45 μm, Bio Trace, PALL Gelman Laboratory, MI, USA), 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 occludin antibody (1:3000, ABfinity™ Rabbit, Thermo Scientific, USA) in 0.5% wt/vol nonfat milk/TTBS or with mouse anti-Claudin-1 monoclonal antibody (3 μg $mL^{-1}$, Novex Life Technologies, MD, USA) in 2.5% wt/vol nonfat milk/TTBS, overnight at 4 °C. After a washing step (3 × 10 min, TTBS), the membrane was incubated either with Horseradish Peroxidase-labeled goat anti-rabbit antibody (1:10,000, Santa Cruse) or with biotin-labeled anti-mouse IgG (1:100,000, Sigma-Aldrich St. Louis, USA) and extrAvidin peroxidase (1:4000, Sigma-Aldrich St. Louis, USA) in 0.5% wt/vol nonfat milk/TTBS for 1 h at RT. 3,3′-Diaminobenzidine (DAB, Sigma-Aldrich, Germany) was used for the detection of antigen–antibody interactions. Representative results of three independent experiments are shown.

### *2.7. Visualization of cell culture treatments, occludin, and ZO-1 by confocal microscopy*

Only intact polarized Caco-2 cell monolayers with an initial net resistance >1000 Ω/$cm^2$ were used for immunofluorescence microscopic analysis. Treatments consisting of 350 μL of active actinidin (1 mg/mL or 0.1 mg/mL) or heat inactivated actinidin (1 mg/mL) dissolved in cell medium were applied to the apical surface of the monolayer. Control Caco-2 cell monolayers were treated with cell culture medium for 4 h. After 1 h or 4 h the chambers were washed three times with PBS and the cells were fixed for 20 min at room temperature in PBS containing 4% paraformaldehyde. After fixation, the chambers were washed three times with PBS and the cells were incubated for 1 h at room temperature with rabbit anti- ZO-1 (1:100 dilution in PBS, Invitrogen, Carlsbad, USA) or rabbit anti-occludin (1:80 dilution in PBS, Invitrogen, Carlsbad, USA) antibodies. After washing with PBS, the inserts were

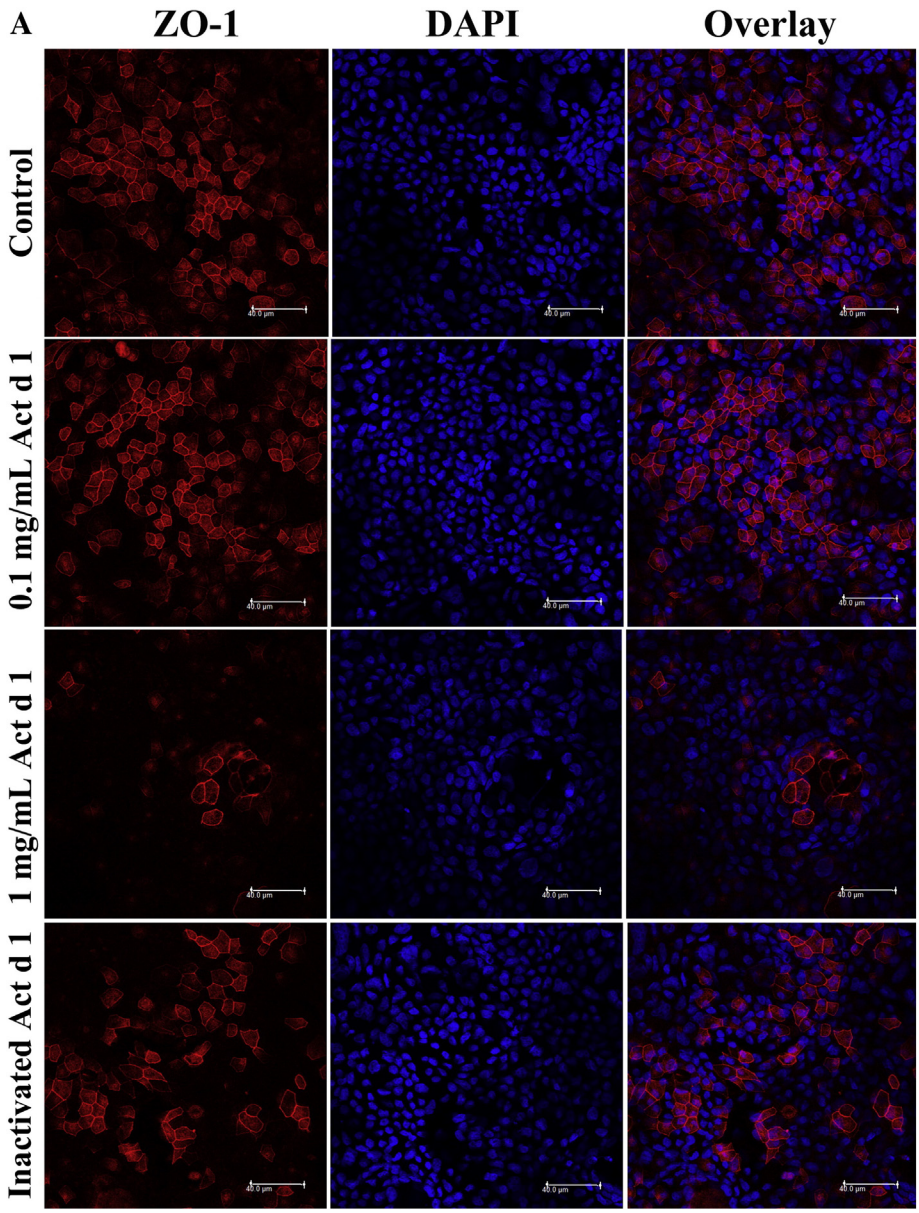


**Fig. 5.** Confocal microscopy images of ZO-1 and occludin distribution in Caco-2 epithelial cell monolayers after 1 h of treatment with actinidin: A) ZO-1 staining, B) Occludin staining. ZO-1 and occludin staining is represented in red. Nuclear signal was obtained by epifluorescence of DAPI staining and superimposed over confocal images of ZO-1 or occludin staining to create an overlaid image.

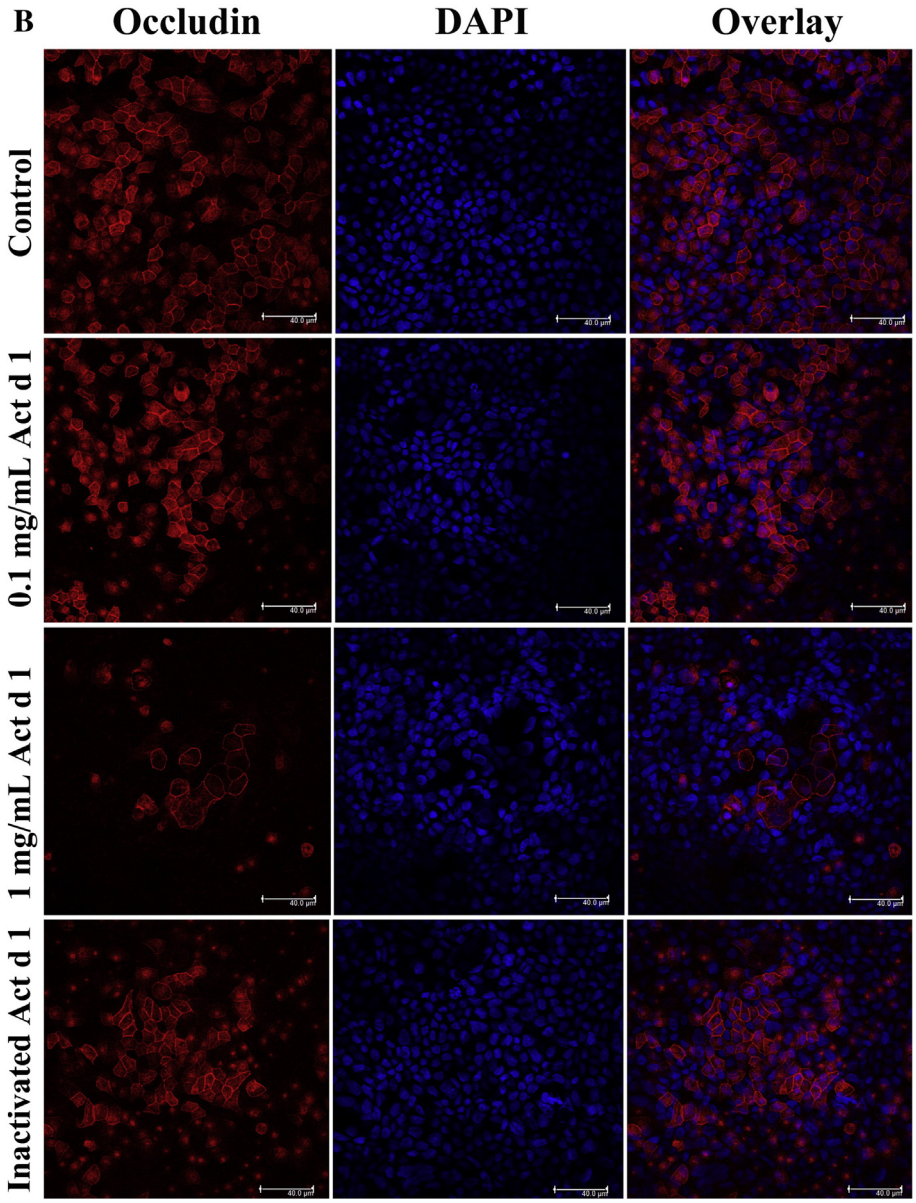


**Fig. 5** (*continued*).

incubated with goat anti-rabbit IgG antibodies conjugated with fluorescent dye (dilution 1:80 in PBS, Invitrogen, Carlsbad, USA) for 1 h at room temperature. To visualize DNA, cells were stained for 2 min with 4′,6-diamidino-2-phenylindole (DAPI) (350 nM in PBS, Invitrogen, Carlsbad, USA). After washing three times with PBS, the filter inserts were sliced from the chambers and mounted on glass slides. The slides were examined by Leica TCS SPE-II confocal laser scanning-microscope on a DMI4000. Images were acquired with an oil-immersion objective (40×), and assembled using LAS AF 3 software (Leica Microsystems, Wetzlar, Germany).The final images were prepared using Adobe Photoshop CS5 (Adobe Systems Incorporated, CA, USA).

### 2.8. Animal studies

Five to eight weeks old BALB/cAnNCrl mice from Charles River Laboratories (L'Arbresle, France) were used. Mice were specific pathogen free and maintained under barrier conditions in filter-topped Makrolon cages with wood chip bedding at a mean temperature of 23 °C, 50–55% relative humidity and a 12 h light/dark cycle. Drinking water and standard laboratory food pellets were provided *ad libitum*. The experiments were conducted according to guidelines and permission from the animal experiments committee of Utrecht University (Utrecht, Netherlands).

### 2.9. In vivo permeability measurements across intestinal mucosa

*In vivo* permeability measurement across intestinal mucosa was performed by measuring the translocation of FITC-dextran (MW 40,000; Sigma-Aldrich, St. Louis, USA), as previously described [21]. For the activation of actinidin, an equimolar amount of L-cysteine (0.2 mM) was added to the enzyme preparation. The first group of mice (n = 6) received 350 μL of active actinidin (5 mg/mL in PBS) by oral gavage. The control group (n = 6) received 350 μL of PBS with 0.2 mM L-cysteine. After 30 min, mice from both groups received 10 mg (500 mg/kg of body weight) of FITC-labeled dextran by oral gavage. Blood samples (~150 μL) were drawn from the cheek vein 4 h after FITC-dextran administration. Following centrifugation (3000 ×*g*, 10 min), sera were collected and diluted 1:1 (vol/vol) in PBS. Fluorescence intensity of each sample was measured (excitation: 485 nm; emission: 530 nm; FLUOstar OPTIMA, BMG Labtech, Offenburg, Germany) and FITC-

dextran concentrations were determined from a standard curve generated by serial dilution of FITC-dextran in untreated mouse serum diluted 1:1 (vol/vol) in PBS.

### 2.10. Human recombinant occludin degradation

Full length human recombinant occludin (Abnova, Taipei City, Taiwan) was dissolved to a concentration of 0.1 mg/mL in a buffer suggested by the manufacturer (50 mM TRIS, 10 mM glutathione, pH 8.0). For the digestion assays, an equimolar amount of active or heat inactivated actinidin was added to occludin (1.2 μL of 0.5 mg/mL actinidin in 50 mM TRIS, 100 mM L-cysteine, pH 8.0, was added to 20 μL of occludin preparation) and incubated at 37 °C for 4 h. The digestion control sample consisted of occludin with no actinidin added. Following separation under reducing conditions in SDS-PAGE (4/12%), the proteins were transferred to a nitrocellulose membrane (0.45 μm, Serva, Heidelberg, Germany) by semi-dry blotting (2 mA/cm$^2$, 40 min). For immunoblot development, following an 1 h incubation step with blocking solution consisting of 5% (*w*/*v*) nonfat dry milk in TTBS buffer (50 mM Tris–HCl, pH 7.4, and 150 mM NaCl containing 0.1% Tween 20), membranes were incubated with rabbit polyclonal anti-occludin antibodies (1:2000, Sigma-Aldrich, St. Louis, USA) in 0.5% wt/vol nonfat milk/TTBS for 1 h at room temperature (RT). Afterwards the membrane was incubated for 1 h at RT with secondary antibody (alkaline phosphatase-labeled anti-rabbit goat IgG (1:30,000, Sigma-Aldrich, St. Louis, USA) in 0.5% wt/vol nonfat milk/TTBS). Bromochloroindolylphosphate/nitro blue tetrazolium (BCIP/NBT, Serva, Heidelberg, Germany) was used for the detection of antigen–antibody interactions.

### 2.11. Detection of occludin fragments by MALDI TOF/TOF

Two synthetic 15-mer peptides corresponding to sequences found in the two extracellular loops of human occludin (UniProt Q16625), EL1 (AWDRGYGTSLLGGSV) and EL2 (GVNPTAQSSGSLYGS) were purchased from GenScript (Piscataway, New Jersey, USA). Each peptide (40 μg)

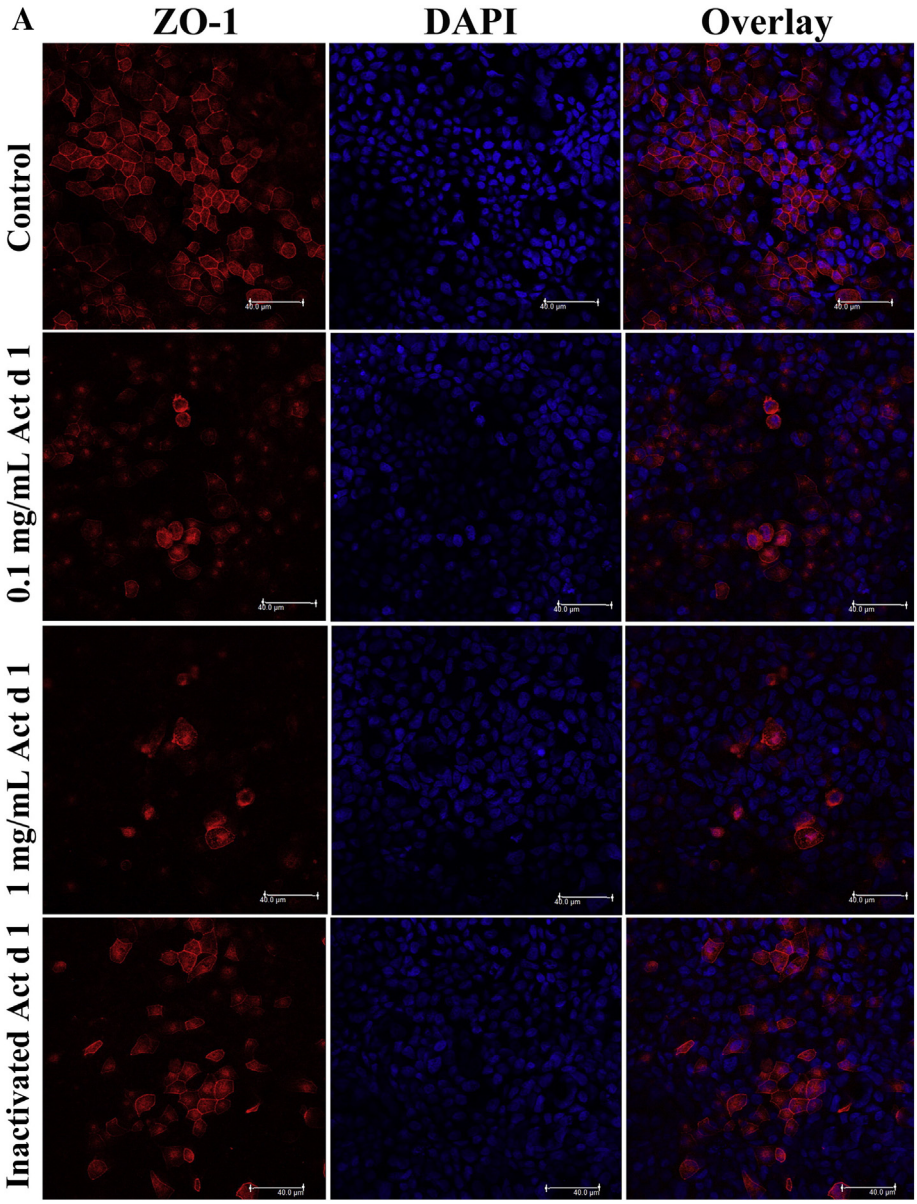


**Fig. 6.** Confocal microscopy images of ZO-1 and occludin distribution in Caco-2 epithelial cell monolayers after 4 h of treatment with actinidin: A) ZO-1 staining, B) Occludin staining. ZO-1 and occludin staining is represented in red. Nuclear signal was obtained by epifluorescence of DAPI staining and superimposed over confocal images of ZO-1 or occludin staining to create an overlaid image.

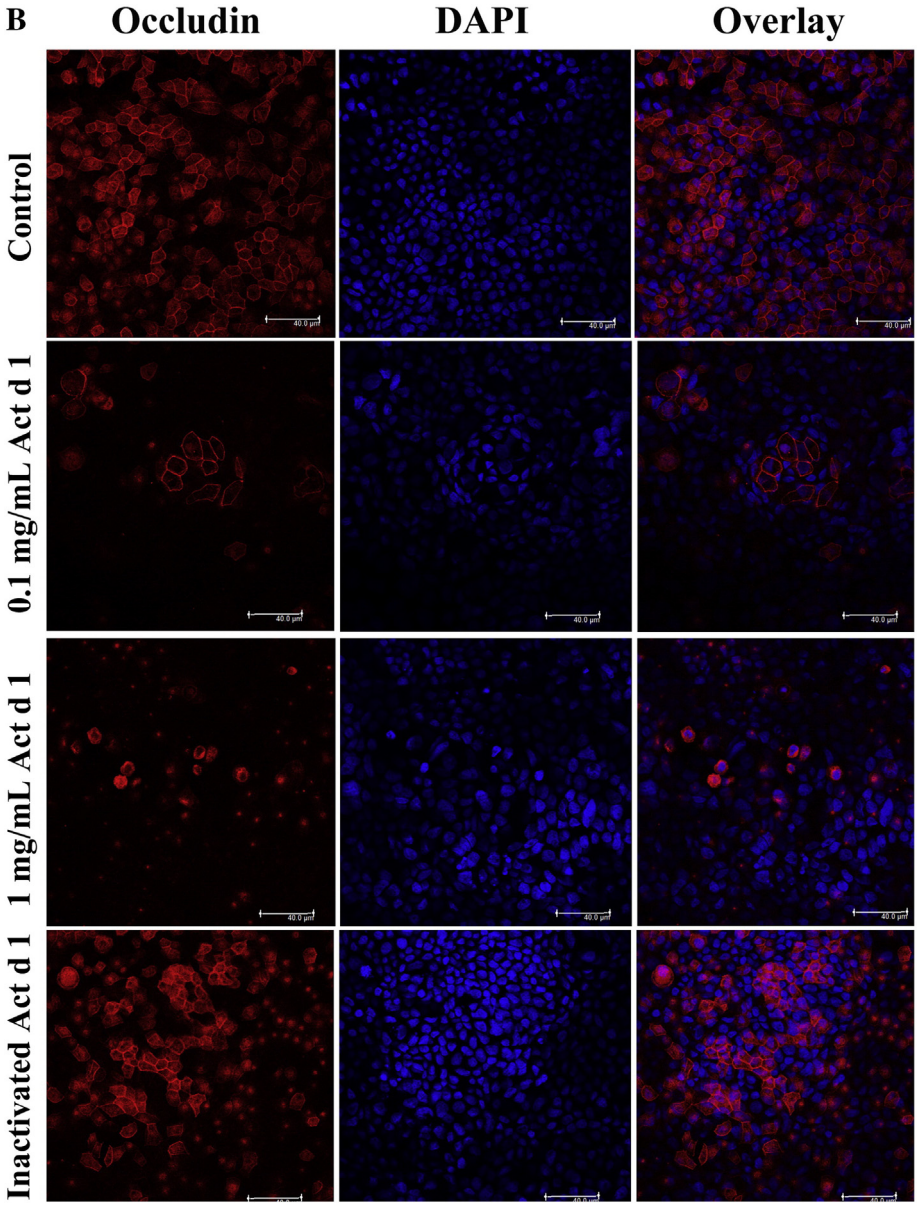


**Fig. 6** (*continued*).

was dissolved in 20 mM phosphate buffer pH 6.6 with 2 mM EDTA, and was incubated with active actinidin (20 μg) for 3 h at 37 °C. Amino acid sequences of peptides were analyzed with Ultraflex MALDI TOF/TOF (Bruker, Bremen, Germany), in reflectron positive mode for MS and LIFT mode for MS/MS analysis using α-Cyano-4-hydroxycinnamic acid

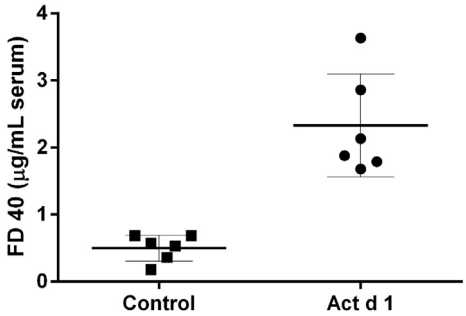


**Fig. 7.** Serum levels of 40- kDa FITC-dextran (FD-40) 4 h after administration by oral gavage in Act d 1 treated and control mice. $P < 0.05$.

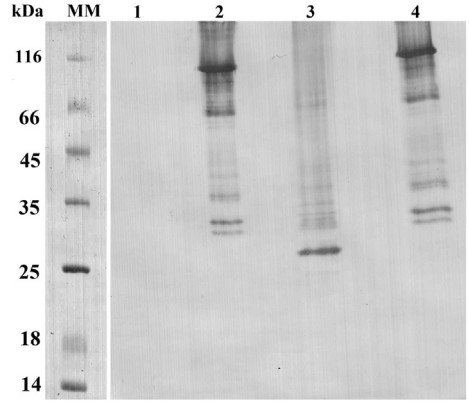


**Fig. 8.** Occludin detection in immunoblot following digestion with actinidin. 1) actinidin, 2) occludin, 3) occludin digested with active actinidin, 4) occludin digested with heat inactivated actinidin, MM) molecular markers.

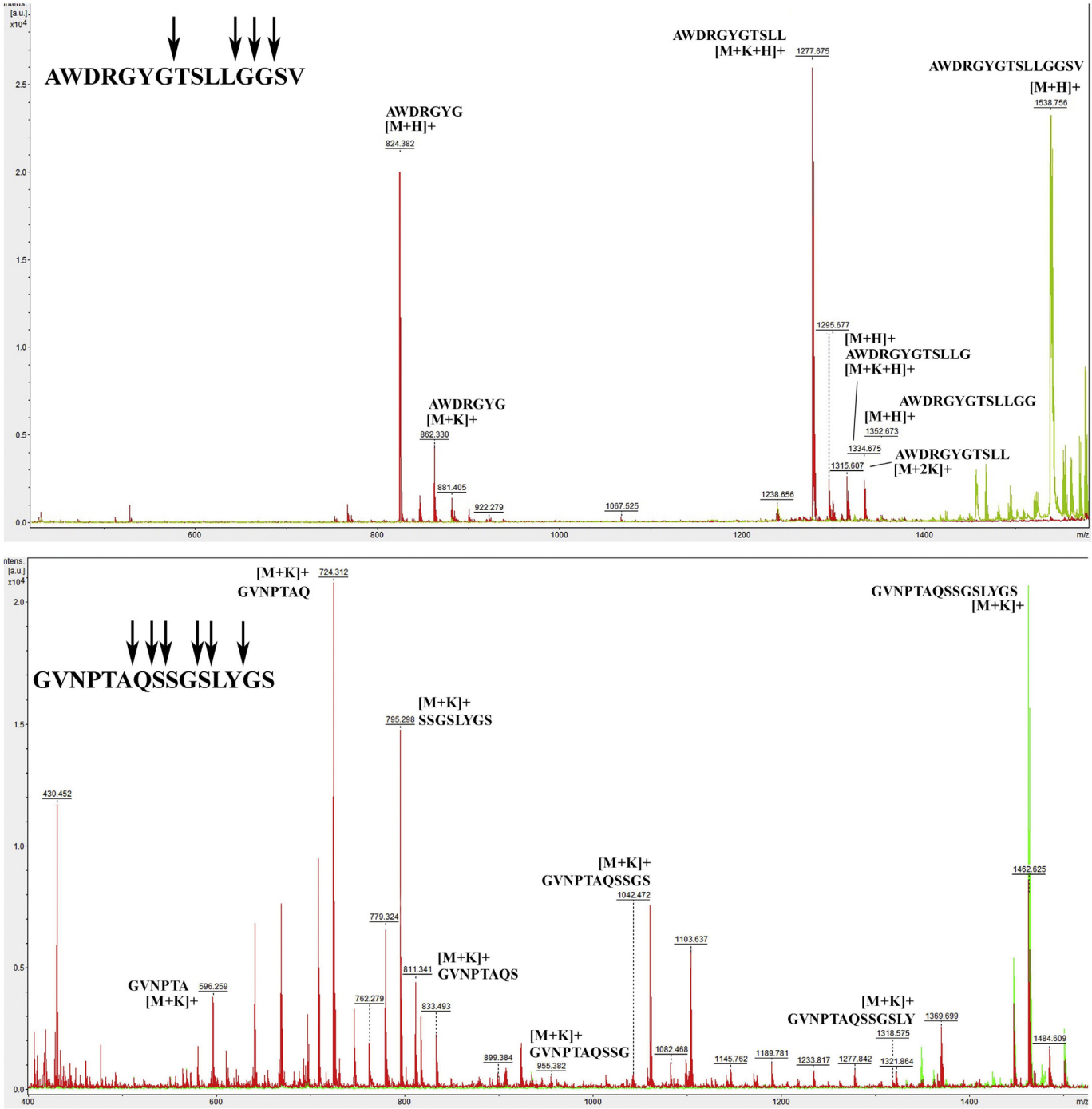


**Fig. 9.** Detection of occludin fragments by MALDI TOF/TOF after digestion with actinidin: Act d 1 digestion of peptide $^{88}$AWDRGYGTSLLGGSV$^{102}$ from the first extracellular loop of human occludin, and peptide $^{196}$GVNPTAQSSGSLYGS$^{210}$ from the second extracellular loop of human occludin;

as a matrix. Peptide calibration standard mixture (Bruker 206195) for mass calibration was used. Prior to MS analysis samples were cleaned over C18 ZipTip (Millipore, Massachusetts, USA).

### 2.12. Statistical analysis

GraphPad Prism V6.00 (GraphPad Software, San Diego, CA) was used to compare sample means in the monolayer resistance and Act d 1 translocation measurements by 2 way analysis of variance (ANOVA). When an overall statistically significant difference was seen, post-tests were performed to compare treatment pairs, using the Bonferroni method to adjust the *P*-value for multiple comparisons. Sample means of the two mice groups in the *in vivo* FITC-dextran permeability experiment were compared using the paired t- test. Differences were considered significant if $P < 0.05$. *P*-values for each analysis are indicated in the corresponding Figure legend.

## 3. Results and discussion

### 3.1. Effects of Act d 1 proteolytic activity on Caco2 cell monolayer

The LDH (Fig. 1A) and MTT (Fig. 1B) assays confirmed that actinidin purified and prepared as described in this study does not influence the viability of Caco-2 cells. Results from both assays revealed no significant

difference between the control cells, cells treated with 0.1 mg/mL or 1 mg/mL of active actinidin, or cells treated with 1 mg/mL of heat inactivated actinidin ($P > 0.05$).

In order to explore the effects of Act d 1 proteolytic activity on Caco-2 cell monolayer permeability, transepithelial electrical resistance (TEER) and translocation of Act d 1 across the monolayer were measured. Act d 1 samples (0.1 mg/mL, 1 mg/mL, and 1 mg/mL of heat inactivated Act d1) were added to the apical side of the epithelial monolayers.

TEER was measured after 1, 2, 3, and 4 h of the treatment. Control cells maintained a basal TEER over the 4 h period (Fig. 2). After 1 h of Act d 1 (1 mg/mL) treatment TEER was reduced to 81.9% of control ($P < 0.01$). This effect was shown to be more pronounced with time, and after 4 h of treatment TEER decreased to 74.4% ($P < 0.001$). The effect was also noticeable with 10 × lower Act d 1 concentrations (0.1 mg/mL), with the TEER showing a significant difference in comparison to initial values after a period of 2 h (83.1%, $P < 0.01$).The TEER of monolayers exposed to heat inactivated actinidin was not significantly changed ($P > 0.05$) in the measured time period, indicating that the observed effect was protease-dependent.

To investigate whether the observed changes in epithelial integrity induced by actinidin led to its diffusion across the Caco-2 cell monolayer, samples of the basolateral medium were analyzed by SDS-PAGE (Fig. 3A), zymography (Fig. 3B), and immunoblot with rabbit anti-actinidin antibodies (Fig. 3C). SDS-PAGE analysis showed that after 1 h a band of about 23 kDa could be detected in samples treated with 0.1 or 1 mg/mL of activated actinidin (Fig. 3A, lanes 2a and 3a), and after 4 h of the treatment (Fig. 3A, lanes 2b and 3b) the intensity of these bands was increased. The same results were confirmed by detecting proteolytically active actinidin in the basolateral compartment of treated cells after 1 and 4 h by zymography (Fig. 3B, lanes 2 and 3) and, also, by detection in immunoblot (Fig. 3C, lanes 2 and 3). As with SDS-PAGE, Act d 1 was not detected by zymography or immunoblot in untreated cells or cells treated with heat inactivated actinidin (Fig. 3A and B, lanes 1 and 4).

In order to explore whether actinidin performs proteolysis of the tight junction protein occludin, whole Caco-2 cell lysates after actinidin treatment (0.1 mg/mL, and 1 mg/mL for 1 h and 4 h, respectively) were immunoblotted and probed with anti-occludin antibodies produced against a peptide corresponding to amino acids 387–403 of human occludin. In non-treated cells occludin was detected as a band of about 65 kDa. The intensity of this 65 kDa band was reduced in all actinidin treated samples and immunoreactive degradation fragments were detected at about 53 kDa and about 45 kDa (Fig. 4). In a similar experimental set-up we failed to detect proteolytic degradation of claudin-1, another tight junction protein (not shown).

To assess the impact of Act d 1 on tight junctions Caco-2 monolayers were stained for tight junction proteins ZO-1 and occludin after 1 h and 4 h of treatment with Act d 1 samples (0.1 mg/mL, 1 mg/mL, and 1 mg/mL heat inactivated Act d 1) and compared with non-treated monolayers by confocal microscopy. Both tight junction proteins, ZO-1 (Fig. 5A) and occludin (Fig. 5B), form sharp, continuous rings at the boundaries of adherent polygonal Caco-2 cells grown as a confluent monolayer. A visible disruption in the continuity of ZO-1 (Fig. 5A) and occludin rings (Fig. 5B) was determined after 1 h of Act d 1 (1 mg/mL) treatment, along with a difference in cell shape when compared to the non-treated monolayer. After 4 h of treatment these effects were more pronounced, with extensive disruption of both ZO-1 (Fig. 6A) and occludin networks (Fig. 6B). The observed effects were now also visible in the monolayers treated with a 10 × lower concentration of Act d 1 (0.1 mg/mL). These results show that the effects of Act d 1 are time and concentration dependant.

### 3.2. Permeability in vivo in mice

The influence of Act d 1 on the permeability of the mouse intestinal barrier was determined by measuring FITC-dextran levels in mouse sera 4 h after administering FITC-dextran by oral gavage (Fig. 7). One group of mice were administered proteolytically active Act d 1 by oral gavage 30 min prior to being given FITC-dextran (40 kDa), while the other group received PBS (control group). The obtained results showed that Act d 1 treatment significantly increased the intestinal permeability, since serum FITC-dextran levels were significantly higher (mean value of 2.33 μg/mL) in mice belonging to the group that had received Act d 1 treatment in comparison to the mice in the control group (mean value of 0.5 μg/mL) ($P < 0.05$).

### 3.3. Human occludin degradation

Immunoblot detection of recombinant human occludin (theoretical MW 82.94 kDa including GST tag) upon treatment with actinidin revealed that after 4 h of digestion with active Act d 1, only an occludin fragment of app. 25 kDa could be detected. An intact 83 kDa molecule was readily detected in the control sample, as well as in the sample to which heat-inactivated Act d 1 was added (Fig. 8).

In order to distinguish the presence of Act d 1 cleavage sites within the extracellular loops of human occludin, MALDI TOF/TOF analysis was employed to detect the putative products of proteolytical cleavage by Act d 1 of two synthetic peptide segments of occludin loops. Sequence $^{88}$AWDRGYGTSLLGGSV$^{102}$ of the first extracellular loop (EL1) of human occludin underwent cleavage at positions: $^{94}$GT$^{95}$ (product AWDRGYG), $^{98}$LG$^{99}$ (product AWDRGYGTSLL), $^{99}$GG$^{100}$ (product AWDRGYGTSLLG), and $^{100}$GS$^{101}$ (product AWDRGYGTSLLGG). Sequence $^{196}$GVNPTAQSSGSLYGS$^{210}$ of the second extracellular loop (EL2) underwent cleavage at positions: $^{201}$AQ$^{202}$ (product GVNPTA), $^{202}$QS$^{203}$ (products GVNPTAQ and SSGSLYGS), $^{203}$SS$^{204}$ (product GVNPTAQS), $^{205}$GS$^{206}$ (product GVNPTAQSSG), $^{206}$SL$^{207}$ (product GVNPTAQSSGS), and $^{208}$YG$^{209}$ (product GVNPTAQSSGSLY). These results have been summarized in Fig. 9 and Table 1.

The integrity of the epithelial barrier relies on the function of numerous tight junction proteins [22]. Many of the clinically relevant inhalant allergens with serine and cysteine protease activity have been shown to promote paracellular access to intraepithelial dendritic cells (DCs) by cleaving tight junction proteins (occludin, several claudin family members, zonula occludens proteins) of the airway epithelium [23–25]. Our study focused on the still unexplored effect of allergenic food proteases on the permeability of intestinal epithelial tight junctions. Occludin was

**Table 1**
List of detected occludin peptides by MALDI TOF/TOF after digestion with actinidin. Mmu - milli-mass unit.

Amino acid sequence of Occludin (Homo sapiens)
with marked (*) sites of cleavage by Actinidin detected by mass spectrometry
analysis of peptides from both extracellular domains (underlined, bolded)

MSSRPLESPPPYRPDEFKPNHYAPSNDIYGGEMHVRPMLSQPAYSFYPEDEILHFYKWTSPPGVIRILSMLIIVMCI
AIFACVASTLAWDRGYG*TSLL*G*G*SVGYPYGGSGFGSYGSGYGYGYGYGYGYGYTDPRAAKGFMLAMAAFCFI
AALVIFVTSVIRSEMSRTRRYYLSVIIVSAILGIMVFIATIVYIMGVNPTA*Q*S*SG*S*LY*GSQIYALCNQFY
TPAATGLYVDQYLYHYCVVDPQEAIAIVLGFMIIVAFALIIFFAVKTRRKMDRYDKSNILWDKEHIYDEQPPNVEEW
VKNVSAGTQDVPSPPSDYVERVDSPMAYSSNGKVNDKRFYPESSYKSTPVPEVVQELPLTSPVDDFRQPRYSSGGNF
ETPSKRAPAKGRAGRSKRTEQDHYETDYTTGGESCDELEEDWIREYPPITSDQQRQLYKRNFDTGLQEYKSLQSELD
EINKELSRLDKELDDYREESEEYMAAADEYNRLKQVKGSADYKSKKNHCKQLKSKLSHIKKMVGDYDRQKT

| Cytoplasmic domains | Transmembrane domains | Extracellular domain 1 | Extracellular domain 2 |
|---|---|---|---|

List of peptides observed by MALDI TOF/TOF MS

| Observed ion MW (Da) | Observed ion species | Ion calculated MW (Da) | Delta (mmu) | Peptide sequence (confirmed by MS/MS) |
|---|---|---|---|---|
| 1538.756 | [M + H]$^+$ | 1538.760 | 0.004 | AWDRGYGTSLLGGSV |
| 1352.673 | [M + H]$^+$ | 1352.659 | 0.014 | AWDRGYGTSLLGG |
| 1295.677 | [M + H]$^+$ | 1295.638 | 0.039 | AWDRGYGTSLLG |
| 1334.675 | [M + K + H]$^+$ | 1334.602 | 0.073 | AWDRGYGTSLLG |
| 1315.607 | [M + 2K]$^+$ | 1315.536 | 0.071 | AWDRGYGTSLL |
| 1277.675 | [M + K + H]$^+$ | 1277.580 | 0.095 | AWDRGYGTSLL |
| 824.382 | [M + H]$^+$ | 824.368 | 0.014 | AWDRGYG |
| 862.330 | [M + K]$^+$ | 862.324 | 0.006 | AWDRGYG |
| 1462.625 | [M + K]$^+$ | 1462.621 | 0.004 | GVNPTAQSSGSLYGS |
| 1318.575 | [M + K]$^+$ | 1318.567 | 0.008 | GVNPTAQSSGSLY |
| 1042.472 | [M + K]$^+$ | 1042.42 | 0.052 | GVNPTAQSSGS |
| 955.382 | [M + K]$^+$ | 955.388 | 0.006 | GVNPTAQSSG |
| 811.341 | [M + K]$^+$ | 811.334 | 0.007 | GVNPTAQS |
| 724.312 | [M + K]$^+$ | 724.302 | 0.01 | GVNPTAQ |
| 596.259 | [M + K]$^+$ | 596.244 | 0.015 | GVNPTA |
| 795.298 | [M + K]$^+$ | 795.292 | 0.006 | SSGSLYGS |

employed as a tight junction model protein as it is of crucial importance for the regulation of paracellular permeability of epithelial monolayers. The data observed in this study are consistent with direct, protease-dependent disruption of cellular tight junctions induced by kiwifruit allergen Act d 1. Proteolytic activity of this cysteine protease decreases the transepithelial resistance of Caco-2 cell monolayers, while at the same time increasing the monolayer permeability. These findings were well correlated with occludin proteolytic degradation upon actinidin treatment as detected in immunoblot.

Confocal microscopy revealed that occludin, which is involved in regulation of size-selective diffusion [26], forms a continuous network around the boundaries of tightly connected adherent Caco-2 cells. Exposure to Act d 1 disrupts the sharp continuous boundary and markedly reduces the intensity of occludin immunostaining in the tight junctions, probably due to its degradation and/or redistribution, as it has been previously shown that Act d 1 had no effect on occludin expression at the genetic level [18]. A delocalization-inducing effect of Act d 1 was also shown on the ZO-1 protein, an important linker between occludin and the cell cytoskeleton [27]. This effect of Act d 1 is also in accordance with our previous findings on the reorganization of the actin cytoskeletal network that contributes to the morphological changes of T84 cells [17]. As tight junction proteins have complex interactions in the membrane and with membrane-associated structures, it is most probable that Act d 1 has direct and indirect effects on various proteins of the TJ network which ultimately leads to an increase in epithelial permeability [28,29]. To the best of our knowledge, Act d 1 is the first food allergen with potential to compromise intestinal permeability.

High abundance of an allergen in the food source, its relative resistance to degradation by digestive processes and compromised intestinal permeability are very important factors for the development of food allergy [30]. Actinidin constitutes about 50% of soluble protein content of kiwifruit. It has previously been shown that Act d 1 exerted proteolytic activity under simulated gastric and intestinal conditions [16], and the contribution of Act d 1 to the digestion of several food proteins was confirmed *in vivo* [31]. In our study, an increased intestinal permeability to FITC-dextran was detected in mice which had been given Act d 1 by intragastric gavage when compared to the control mice, indicating that the enzyme preserved its proteolytic activity in the mouse gastrointestinal tract. The increased passage of the protein-sized 40 kDa FITC-dextran molecules through the intestinal epithelium implies that Act d 1 action on tight junction proteins might increase the probability of other jointly ingested food proteins/peptides permeating into the sub-epithelial layers of the intestine. As a papain-like cysteine protease, Act d 1 reveals a wide pH activity range [4–10] and wide substrate specificity [32], including collagenolytic activity [33]. Apart from contributing to the disruption of tight junctions, the collagenolytic activity of Act d 1 can lead to connective tissue loosening in the lamina propria. This could increase the accessibility of other antigens to antigen-presenting cells that lie beneath the epithelium.

The relevance of tight junctions in maintaining tissue homeostasis is demonstrated in disease states such as asthma [34], inflammatory bowel disease [35], or kidney disease [36]. Tight junctions (TJs) are localized at the apical margins of the cell–cell junctions and provide the epithelial cell polarity [37]. TJ occludin is a member of the MARVEL family of proteins which display an M-shaped topology of a four-transmembrane-helix region architecture with cytoplasmic N- and C-terminal regions. While this protein was originally implicated in the formation of the TJ barrier [38], novel data suggested that occludin is a regulator of barrier properties [39,40]. In this study it was shown that occludin in the Caco-2 cell monolayer was proteolytically truncated by actinidin. Also, a full-length recombinant human occludin was digested by incubation with Act d 1. We were able to identify several putative Act d 1 cleavage sites located in two extracellular loops within the MARVEL domain of human occludin. Interestingly, among the detected cleavage sites for Act d 1 was $^{94}$GT$^{95}$ in the first extracellular loop, a cleavage site shared with cysteine protease allergen Der p 1 from house dust mite [7], which is capable of increasing paracellular permeability in the airways, contributing to the development of allergic asthma.

## 4. Conclusions

In this study, we show for the first time that a plant-derived food cysteine protease Act d 1 increases intestinal permeability in both *in vivo* (mice) and *in vitro* (Caco-2 cell culture) models. We further provide mechanistic evidence of increased permeability by demonstrating proteolytical degradation of human occludin, a key epithelial tight junction transmembrane protein. These findings indicate that food allergens with intrinsic proteolytic activity can compromise the gut epithelial barrier, contributing to the sensitization process in food allergy pathogenesis.

The results of this study warrant future investigation into the effects that the multitude of food proteases may have on the integrity of the intestinal barrier, hoping to further enhance our knowledge on the complex interplay of processes that lead to food sensitization and the development of allergenic disease.

## Transparency document

The Transparency document associated with this article can be found, in the online version.

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