



# A new approach for activation of the kiwifruit cysteine protease for usage in in-vitro testing

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## Abstract

Actinidin (Act d 1), a highly abundant cysteine protease from kiwifruit, is one of the major contributors to the development of kiwifruit allergy. Many studies have focused on the optimization of Act d 1 purification and its role in the development of food allergies. Testing on cell culture monolayers is a common step in the elucidation of food allergen sensitization. In the case of cysteine proteases, an additional activation step with L-cysteine is required before the testing. Hence, we aimed to evaluate whether L-cysteine already present in commonly used cell culture media would suffice for Act d 1 activation. Successfully activated Act d 1 (98.1% of proteolytic activity, as compared to L-cysteine activated Act d 1) was further tested in two commonly used 2D model systems (Caco-2 and HEK293 cells) to evaluate its role on the mRNA expression of cytokines involved in the innate immunity (*IL-1 $\beta$* , *IL-6*, *TNF $\alpha$* , *TSLP*). Furthermore, the contribution of Act d 1 in the promotion of inflammation through regulation of inducible nitric oxide synthase (*iNOS*) mRNA expression was also examined. These results demonstrate that activation of cysteine proteases can be achieved without previous enzyme incubation in L-cysteine-containing solution. Act d 1 incubated in cell culture medium was able to modulate gene expression of pro-inflammatory cytokines when tested on two model systems of the epithelial barrier.

**Keywords** Actinidin · Cysteine protease · Kiwifruit allergen · Pro-inflammatory cytokines

## Introduction

Cysteine proteases are ubiquitous enzymes that play important roles in the protection of the fruit from attack by pathogens [1]. Papain-like cysteine proteases are synthesized as preproteins and require extensive posttranslational modifications to be converted into their active form. The auto-inhibitory N-terminal prodomain, present within the catalytic site cleft, is removed upon activation of the protease [2]. Cysteine proteases, such as bromelain, papain, and actinidin require a free sulfhydryl group (Cys 151) to exert their catalytic activity [3]. When isolated under native conditions, Act d 1 preparation represent a mixture of both active and inactive forms [4]. Actinidin is expressed as an

inactive zymogen approximately 15 kDa larger than the native enzyme, with both N- and C-terminal extensions. The mechanism of activation in vivo is unknown [5]. Hence, an additional activation step is necessary to ensure the complete activity of the purified proteins [3]. Activation of cysteine proteases in vitro is usually achieved by incubation with mild reducing agents, such as cysteine. The standard activation conditions which are used, actinidin activity assays require a buffer solution containing 52 mM L-cysteine and 2 mM EDTA [6, 7].

In the case of actinidin (Act d 1), a major kiwifruit allergen, purified preparations contain a mixture of inactive and active enzyme forms [4, 8]. In this study, Act d 1 was selected as a model protein with cysteine protease activity as it seems to have a role in sensitization to kiwifruit [9, 10]. There are already well-established protocols for its purification [4, 6–8]. Act d 1 activation protocols require the addition of L-cysteine and incubation of the protein sample for one hour at 37 °C [7]. Nevertheless, most frequently used cell culture media already contain a certain amount of L-cysteine. Thus, it appeared prudent to evaluate

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the suitability of L-cysteine -containing media for activation of Act d 1 [11].

Act d 1 is a major kiwifruit allergen, out of thirteen officially identified and classified allergens by the International Union of Immunological Societies Allergen Nomenclature Subcommittee ([www.allergen.org](http://www.allergen.org)) [12]. This cysteine protease represents over 50% of the total kiwifruit protein content [5, 9, 13] and is denoted as the major allergen in monosensitized individuals suffering from kiwifruit allergy [13, 14]. When an allergen-sensitized person is exposed to the food antigen, a process of IgE-mediated degranulation of effector immune cells is initiated, leading towards the fast development of allergic symptoms. On the molecular level, the food allergen binds to the IgE molecule, which is bound to high-affinity IgE receptors present on the surface of effector cells. Afterward, the process of epitope-specific crosslinking of IgE receptors occurs, resulting in the secretion of prestored histamine and other important cytokines such as interleukin-4 (IL-4), IL-5, and IL-13 [15]. Gastro-intestinal disorders, urticaria, and anaphylaxis are clinical manifestations that accompany the ingestion of allergenic foods [16]. Among them, anaphylaxis represents a serious challenge as this allergic reaction, involving several organs that may prove to be life-threatening [17].

During food allergen exposure epithelial cells crosstalk with dendritic cells (DCs) by producing pro-inflammatory cytokines such as IL-1 $\beta$ , IL-6, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), thymic stromal lymphopoietin (TSLP), IL-25, and IL-33 [18–21]. These innate immune cytokines have a role in pro-inflammatory cascades during food allergy pathogenesis [15]. To reach their full proteolytic activity and maturation, IL-1 family members require proteolytic cleavage by caspase-1 [22].

Nitric oxide in exhaled breath is synthesized by a family of NO synthase (NOS) enzymes and serves as a useful marker of airway inflammation [23]. In addition to being produced by a wide range of immune and inflammatory cell types, NO exerts regulatory effects on these cells and at the same time promote Th2 responses, leading to enhanced production of IgE and promotion of IgE-mediated diseases such as asthma [24]. Besides neuronal and endothelial nitric oxide synthase (NOS), which are constitutively expressed, an additional inducible NOS (iNOS) is expressed in airway epithelial cells during inflammation [23, 25, 26]. Expression of iNOS can be significantly up-regulated in the presence of inflammatory agents, for example, food allergens [23, 25, 27]. Increased expression of iNOS correlates with activation of the NF- $\kappa$ B signaling pathway in the airway epithelium of patients with food allergies [27].

Previous studies have suggested that actinidin activates epithelial cells due to its protease activity, which is also

responsible for cleavage of tight junction proteins and hence impairment of epithelial barrier integrity [7, 28, 29]. Upon activation of epithelial cells by actinidin, pro-inflammatory and pro-allergenic cytokines are synthesized and thus can contribute to the sensitization process [28, 29].

This study aimed to investigate the ability of cysteine available in the cell culture medium to activate actinidin. To this end, several most commonly used cell culture media were selected and evaluated in enzymatic activity assays. Thereafter, we aimed to evaluate the effects of such activated Act d 1 on mRNA expression levels of pro-inflammatory molecules.

## Materials and methods

### Purification of Act d 1

Act d 1 was purified from fresh kiwifruit (*Actinidia deliciosa*, ‘Hayward’ cv) as previously reported [7, 8]. Briefly, kiwifruit was peeled and the pulp was homogenized in 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 $\times$ g, 30 min) and loaded onto a QAE-Sephadex A-50 column (GE Healthcare, Uppsala, Sweden) preequilibrated 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 was applied onto an SP-Sephadex C-50 ion-exchange column (GE Healthcare, Uppsala, Sweden) equilibrated in the dialysis buffer and the column was eluted by a salt step-gradient ranging from 0.1 to 1 M NaCl with 10 column volumes. A total of 100 mg of actinidin was isolated from 300 g of kiwifruit. The purity of the obtained actinidin preparation was estimated to be 97% by SDS-PAGE. The pure enzyme was lyophilized and stored at –20 °C. The determination of the proteolytic activity of the purified enzyme was achieved using casein as a substrate [30]. The LPS concentration was determined to be 0.53 EU/mg (Pierce LAL Chromogenic Endotoxin Quantitation Kit, ThermoScientific, USA).

### Proteolytic activity of purified actinidin

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. [8, 31]. In brief, a 2% casein solution was prepared in 0.1 M potassium phosphate buffer, pH 7.6. A 400  $\mu$ L aliquot of casein was added to 40  $\mu$ L of 17 mM L-cysteine, 120  $\mu$ L 1 M KH<sub>2</sub>PO<sub>4</sub> pH 7.0, 40  $\mu$ L of 17 mM EDTA, 40  $\mu$ L of 250 mM NaOH, and

80 µL of distilled water. The reaction mixture was equilibrated to 37 °C, after which 80 µL of actinidin was added and the assay mixture was incubated for 1 h at 37 °C. The enzyme reaction was stopped by the 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 A280 per minute at 37 °C under the specified conditions.

### Activation of purified Act d 1

Lyophilized Act d 1 (1 mg) was dissolved in 1 mL of the following cell culture mediums: Minimum Essential Medium (MEM, *Sigma-Aldrich*, St. Louis, USA); Dulbecco's Modified Eagle's Medium (DMEM, *Sigma-Aldrich*, St. Louis, USA); MEM Eagle with Earle's BSS (Lonza, Basel, CH); DMEM Ham's F12 (*Gibco*, Invitrogen, Paisley, UK), pre-equilibrated at 37 °C. Activation was achieved by incubation of dissolved Act d 1 in the cell culture medium for up to 1 h, during which aliquots were taken at different time points and their proteolytic activity determined in the caseinolytic assay. The L-cysteine concentration of selected mediums is listed in Table 1.

### Cell culture

Caco-2 and HEK293 cells (ATCC, Manassas, VA, USA) (passages 15–25) were cultivated in complete Minimum Essential Medium (MEM, *Sigma-Aldrich*, St. Louis,

USA), which contained 10% heat-inactivated fetal bovine serum (FBS) (*Sigma-Aldrich*, St. Louis, USA, penicillin (100 IU), streptomycin (100 µg/mL) (*Sigma-Aldrich*, St. Louis, USA) and 200 mM L-glutamine (*Sigma-Aldrich*, St. Louis, USA). Cells were grown in the incubator at 37 °C in the presence of 5% CO<sub>2</sub> until confluency.

### Treatment of the cell monolayers

The density of seeded cells was  $0.3 \times 10^6$  cells/well (12 well plate) and cultivation proceeded until 80–90% confluence was reached. Before the treatment, activation of Act d 1 was achieved by incubation of dissolved Act d 1 in cell culture medium for 1 h at 37 °C. Act d 1 was inactivated by adding the cysteine protease inhibitor E-64 (*Sigma-Aldrich*, St. Louis, USA) in an equimolar ratio (v/v). Before the treatment, the nutritive medium was aspirated and cells were incubated in the serum-free medium for 1 h. Actinidin preparations were applied to the cell monolayer and then incubated for 6 h according to the previously published protocol [28]. Monolayers treated with serum-free cell culture medium served as a negative control to allow normalization of the data and statistical analysis.

### Gene expression analysis

Upon treatment, the supernatants were discarded and cells were washed with PBS and centrifuged (800×g, 7 min) for RNA isolation. Total RNA was isolated using TRI REAGENT® BD kit (*Sigma-Aldrich*, St. Louis, USA) according to the manufacturer's instruction. The complementary cDNA and PCR were performed according to a previously published protocol [28]. Analysis of mRNA expression level was achieved using software for densitometry evaluation of agarose gels (Image Studio Lite v5.2 (LI-COR Biosciences) [28]. Gene-specific primers (Table 2) for *IL-1β*, *TNFα*, *IL-6*, *TSLP*, and *iNOS* were purchased from Invitrogen (Paisley, UK). Results were normalized to the housekeeping gene (*GAPDH*) and presented as the mean ± standard error of the mean (SEM) of the three independent experiments. mRNA levels of treated (in-medium-activated or E-64 inactivated Act d 1) and non-treated samples (taken as 1) were compared and presented using GraphPad Prism v6.07 (*Graph-Pad Software*, California, US).

### Activation of the NF-κB signaling pathway in HEK293 and Caco-2 cells

For transient transfection, HEK293 and Caco-2 adherent cells were grown in a T-75 flask (Nunc™ EasYFlask™ Cell Culture Flasks, Thermo Fisher Scientific, Waltham,

**Table 1** Amount of L-cysteine in different cell culture mediums

| Media                                                                             | L-cysteine (mM) |
|-----------------------------------------------------------------------------------|-----------------|
| Minimum essential medium (MEM, <i>Sigma-Aldrich</i> , St. Louis, USA);            | 0.84            |
| Dulbecco's modified Eagle's medium (DMEM, <i>Sigma-Aldrich</i> , St. Louis, USA); | 0.69            |
| DMEM Ham's F12 ( <i>Gibco</i> , Invitrogen, Paisley, UK)                          | 0.26            |
| MEM Eagle with Earle's BSS (Lonza, Basel, CH)                                     | 0.15            |

**Table 2** Human primer sequences used in PCR analysis

| Target gene (reference)    | Human primer sequence (5'–3')                               | AT (°C) |
|----------------------------|-------------------------------------------------------------|---------|
| <i>IL-1β</i> (NM_000576.2) | For: AACCTCTTCGAGGCACAAGG<br>Rev: GGCGAGCTCAGGTACTTCTG      | 49      |
| <i>IL-6</i> (NM_000600.3)  | For: ACCCCAATAAATATAGGACTGGA<br>Rev: GAGAAGGCAACTGGACCGAA   | 49      |
| <i>TNFα</i> (NM_000594.3)  | For: GACAAGCCTGTAGCCCATGT<br>Rev: CTCTGATGGCACCACCAACT      | 49      |
| <i>TSLP</i> (NM_033035.4)  | For: TATGAGTGGGACCAAAAGTACCG<br>Rev: GGGATTGAAGGTTAGGCTCTGG | 51      |
| <i>iNOS</i> (NM_000625.4)  | For: ACCAGTACGTTTGGCAATGG<br>Rev: TCAGCATGAAGAGCGATTCT      | 49      |
| <i>GAPDH</i> (NM_009386.2) | CGAGGCATCATCCCAAATAAGAAC<br>TCCAGAAGTCTGCCCGATCAC           | 58.7    |

AT annealing temperature

MA, USA) and cultivated in Minimum Essential Medium (MEM, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 IU), and streptomycin (100 µg/mL) (Sigma-Aldrich, St. Louis, MO, USA) and in 200 mM L-glutamine (Sigma-Aldrich, St. Louis, MO, USA) in 5% CO<sub>2</sub> at 37 °C. Before transfection, cells were passaged using 1 × trypsin/EDTA solution (Sigma-Aldrich, St. Louis, MO, USA) and seeded in complete growth medium containing serum and antibiotics on glass coverslips in a 24-well plate (8 × 10<sup>4</sup> cells/well). Cells were cultured until they reach ~80% confluence. On the day of transfection 0.4 µg/coverslip of an appropriate DNA (Signal NF-κB-GFP Pathway Reporter Assay Kit, Qiagen, Hilden, Germany) was diluted with medium without serum, antibiotics, and glutamine (Opti-MEM I Reduced Serum Media, Thermo Fisher Scientific, Waltham, MA, USA) to a total volume of 60 µL/coverslip. Then, 1.5 µL/coverslip of Attractene Transfection Reagent (Qiagen, Hilden, Germany) was added to the DNA solution mix and incubated for 20 min at RT. The medium was replaced, and the transfection complex was added drop-wise onto the cells. After 48 h of incubation, cells were treated with activated and E-64 inactivated Act d 1 (1 mg/mL). Treatment was performed for 24 h at normal growth conditions. Activation of the NF-κB-GFP was assessed by fluorescence microscopy.

## Statistics

Differences between sample means were evaluated using a two-way analysis of variance (ANOVA). To adjust *P* value for multiple comparisons, the Sidak post-test was employed. Differences were considered significant at *P* ≤ 0.05 when compared to control.

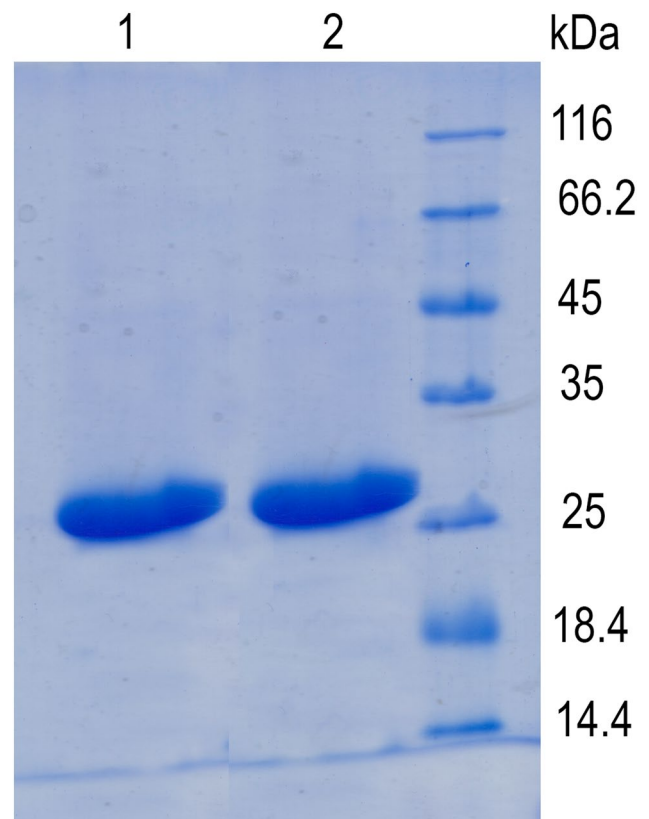


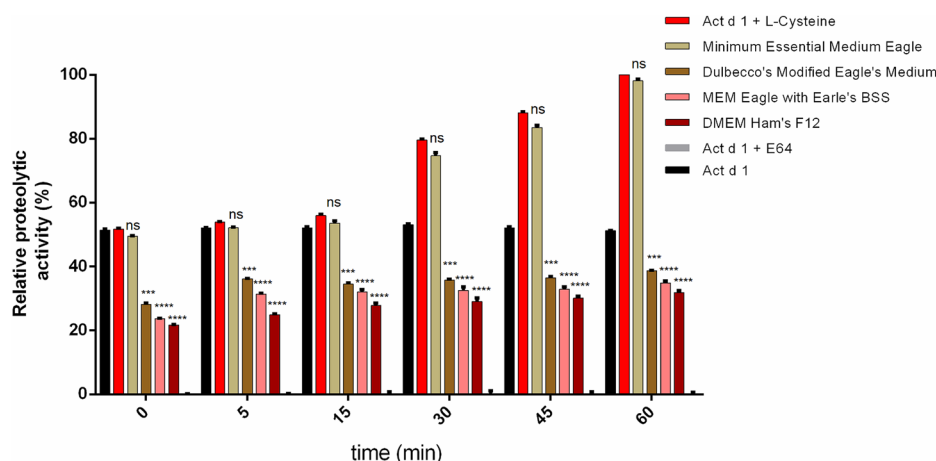
**Fig. 1** SDS-PAGE analysis of Act d 1 preparations. Lyophilized Act d 1 was dissolved in (1) cell culture medium (2) phosphate buffer pH 6.5. Proteins were stained by Coomassie Brilliant Blue (CBB)

## Results and discussion

### Activation of Act d 1 by L-cysteine present in the medium

Act d 1 was isolated from fresh kiwifruit by a combination of two ion-exchange chromatographies as previously

**Fig. 2** Activation of Act d 1 in cell culture medium. The enzyme was incubated in L-cysteine-containing nutritive medium. Results are expressed as relative proteolytic activity of the samples compared to Act d 1 activated with L-cysteine after 1 h (100%). Values are presented as mean  $\pm$  SEM. ns, not statistically significant; \*\*\* $P \leq 0.001$ , \*\*\*\* $P \leq 0.0001$  vs. L-cysteine-activated Act d 1



described [4, 8]. The purity of the isolated protein was > 97% as observed by SDS–PAGE (Fig. 1).

The activation of Act d 1 was usually performed by using protocols that involve a pre-incubation step with L-cysteine for one hour at 37 °C [7]. Therefore, we evaluated whether the activation of Act d 1 could be achieved by the amount of L-cysteine, already present in the commonly used cell-culture media.

Relative proteolytic activity of medium-activated Act d 1 was analyzed at different time points (Fig. 2). As depicted in Fig. 2, Act d 1 was activated in a time-dependent manner and reached activation (98% of relative proteolytic activity) in Minimum Essential Medium after one hour, as compared to the positive control (Act d 1 activated with 0.2 mM L-cysteine). At zero time-point, non-activated Act d 1 exerted ~50% of the proteolytic activity, compared to the control sample upon one hour of incubation (100%). For the negative control, Act d 1 was irreversibly inactivated with E-64, an inhibitor of cysteine proteases. As expected, it showed no proteolytic activity during the tested incubation period. Incubation of Act d 1 in other tested media led to a moderate increase of activity, i.e. 38.71% in Dulbecco's Modified Eagle's Medium; 34.84% in MEM Eagle with Earle's BSS, 31.87% in DMEM Ham's F12 compared to Act d 1 activated with L-cysteine enzyme after an incubation period of 1 h (100%). The reason for this is most likely due to the different concentrations of L-cysteine present in the media (0.84 mM for Minimum Essential Medium; 0.69 mM for Dulbecco's Modified Eagle's Medium; 0.26 mM DMEM Ham's F12; 0.15 mM MEM Eagle with Earle's BSS) (Table 1). This is the first protocol showing that Act d 1 activation can be achieved with the L-Cys already present in the medium.

### Effect of Act d 1 on mRNA expression of IL-1 $\beta$ , IL-6, TNF $\alpha$ and TSLP

Sensitization to food antigens may occur in the different mucosal tissues such as skin, the gastrointestinal, and the respiratory tract [22]. After ingestion, food proteins are proteolytically processed by enzymes that are present in the gastrointestinal tract [32]. Despite this acidic and proteolytic environment, Act d 1 can keep its functional structure, proteolytic activity, and immunological reactivity as long as 2 h upon simulated gastric digestion [4], and it can trigger the sensitization process [33].

The activation of different dendritic cell subsets in the gastrointestinal tract (GIT) and the expression of specific co-stimulatory molecules are important in the development of the Th2 immune response [34]. Sensitization to kiwi-fruit develops, in part, as a consequence of the proteolytic activity of Act d 1, which can disrupt the intestinal barrier integrity through proteolysis of tight junction proteins [7, 29]. Disruption of the intestinal barrier by allergens with protease activity, such as Act d 1, leads to the production of pro-inflammatory and pro-allergenic cytokines. These allergens are capable of inducing changes in the morphology of the epithelial barrier and of affecting intestinal permeability which leads to the interaction of the allergen with the cells of the immune system and the initiation of a specific immune response and cytokine synthesis [29].

The capacity of the activated Act d 1 to modulate the innate immune response was evaluated at the level of mRNA expression for IL-1 $\beta$ , IL-6, TNF $\alpha$ , TSLP, and iNOS in two in vitro 2D model systems (Caco-2 and HEK293). Epithelial cells are the main source of secreted TSLP in the lungs, gut, and skin [21]. Increased level of TSLP expression was

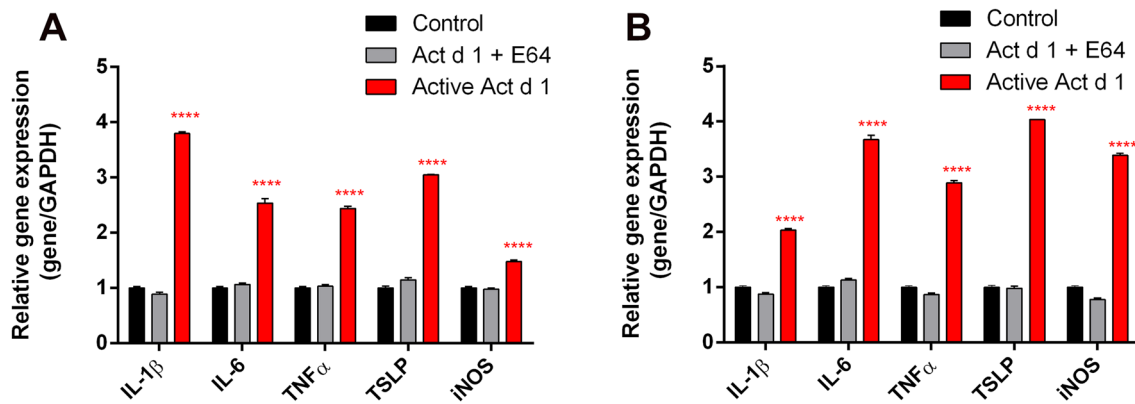


**Fig. 3** Effect of Act d 1 on the mRNA expression of cytokines normalized to GAPDH in vitro. Caco-2 (A) and HEK293 (B) cells were treated with activated or inactivated Act d 1 for 6 h. Subsequently,

RNA was isolated and gene expression was evaluated by PCR. Values are expressed as mean  $\pm$  SEM of three independent experiments. \*\*\*\* $P \leq 0.0001$  vs. control

observed in airway epithelial cells which promote the role of TSLP in the development of a Th2 type allergic inflammation [35, 36]. Our data obtained from two cell lines are consistent with the published data, showing an up-regulation of TSLP mRNA expression (Fig. 3).

Allergens with protease activity, such as Der p allergens, can modulate the expression of pro-inflammatory cytokines in sensitized subjects [37]. Cultured airway epithelial cells can release IL1- $\beta$  as a specific response to allergen exposure [37, 38]. Our results are in line with those, as Act d 1 was able to induce an up-regulation of the mRNA gene expression of these cytokines in both cell lines investigated (Fig. 3).

#### Act d 1 induces up-regulation of mRNA expression for iNOS

NO synthesized by iNOS holds a key position in the modulation of inflammatory symptoms [26]. An increased concentration of NO was detected in the exhaled air of patients with asthma [15]. Results obtained from the two employed cell lines demonstrate that Act d 1 leads to an up-regulation of the mRNA expression of iNOS and therefore could contribute to the promotion of inflammation during type I hypersensitivity reactions (Fig. 3).

#### Act d 1 Activates the NF- $\kappa$ B Signaling Pathway

Act d 1 activation of NF- $\kappa$ B signaling in transfected HEK293 and Caco-2 cells were detected as induced GFP expression by fluorescence microscopy (Fig. 4) in comparison to negative control and E-64-inactivated Act d 1. Before treatment, Act d 1 was activated by incubation in MEM. The results show that Act d 1 activated in this way leads to activation of NF- $\kappa$ B Signaling Pathway.

#### Conclusions

In the current study, a novel activation protocol for kiwi-fruit cysteine protease Act d 1 was demonstrated. Incubation at 37 °C for one hour in cell culture media without the addition of L-cysteine led to the activation of Act d 1. This, proteolytically active form of Act d 1, was tested on both HEK293 and Caco-2 cells monolayers, and up-regulation of mRNA expression of the innate immunity cytokines (IL-1 $\beta$ , IL-6, TNF $\alpha$ , TSLP) and iNOS were observed. This is of high importance as these cytokines are involved during the allergen sensitization phase, while iNOS upregulation could lead to enhanced production of NO, which in return contributes to the inflammation process.

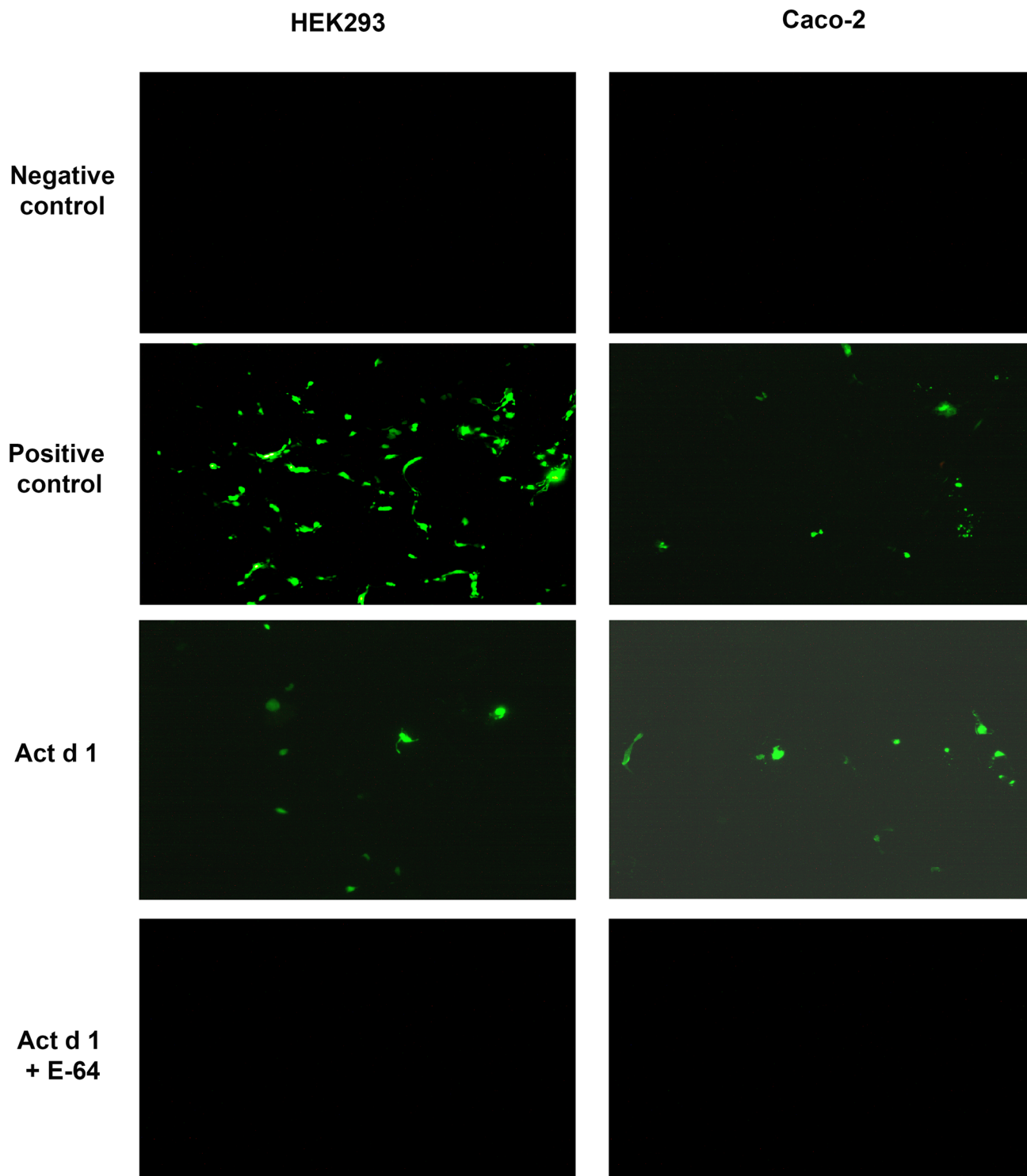


**Fig. 4** Visualization of Act d 1-induced activation of NF- $\kappa$ B signaling by detecting GFP expression with fluorescence microscopy: HEK293 and Caco-2 cells were grown on glass coverslips, trans-

fectected with NF- $\kappa$ B-GFP plasmid, and treated with Act d 1 (in MEM activated or E-64 inactivated). After fixation, cells were mounted on glass slides and examined by fluorescence microscopy

## Declarations

**Conflicts of interest** The authors declare that they have no conflict of interest.

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