

Yeast surface display is a novel tool for the rapid immunological characterization of plant-derived food allergens

Milica Popovic · Radivoje Prodanovic ·
Raluca Ostafe · Stefan Schillberg · Rainer Fischer ·
Marija Gavrovic-Jankulovic

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Abstract High-throughput characterization of allergens relies often on phage display technique which is subject to the limitations of a prokaryotic expression system. Substituting the phage display platform with a yeast surface display could lead to fast immunological characterization of allergens with complex structures. Our objective was to evaluate the potential of yeast surface display for characterization of plant-derived food allergens. The coding sequence of mature actinidin (Act d 1) was cloned into pCTCON2 surface display vector. Flow cytometry was used to confirm localization of recombinant Act d 1 on the surface of yeast cells using rabbit polyclonal antisera IgG and IgE from sera of kiwifruit-allergic individuals. Immunological (dot blot, immunoblot ELISA and ELISA inhibition), biochemical (enzymatic activity in gel) and biological (basophil activation) characterization of Act d 1 after solubilization from the yeast cell confirmed that recombinant Act d 1 produced on the surface of yeast cell is similar to its natural counterpart isolated from green kiwifruit. Yeast surface display is a potent technique that enables fast immunochemical characterization of allergens in situ without the need for protein purification and offers

an alternative that could lead to improvement of standard immunodiagnostic and immunotherapeutic approaches.

Keywords Actinidin · Allergen characterization · Cysteine protease · Kiwifruit · Yeast surface display

Abbreviations

Act d 1	Actinidin
CRD	Component-resolved diagnosis
CRIT	Component-resolved immunotherapy
GPI	Glycosylphosphatidylinositol
PE	Phycoerythrin
YNB-CAA	Yeast nitrogen base/casamino acids
YPD	Yeast extract/peptone/dextrose
Glc	Glucose
Raf	Raffinose
Gal	Galactose
CBB	Coomassie brilliant blue
OD	Optical density
IOD	Integrated optical density
CCDs	Cross-reactive carbohydrate determinants

Introduction

Recombinant DNA technology facilitated major developments in the field of molecular allergology [1], helping to improve component-resolved diagnosis (CRD) and component-resolved immunotherapy (CRIT) [2]. Using recombinant allergens for in vitro diagnostic tests allows establishment of a patients' individual IgE reactivity profile and patient-tailored desensitization immunotherapy [3]. Cloning recombinant allergens or allergen libraries in heterologous organisms is a promising approach in allergen characterization, which is necessary for both CRD and

M. Popovic (✉) · R. Prodanovic · M. Gavrovic-Jankulovic
Department of Biochemistry, Faculty of Chemistry, University
of Belgrade, Studentski trg 16, 11000 Belgrade, Serbia
e-mail: la_bioquimica@chem.bg.ac.rs

R. Ostafe · R. Fischer
Institute for Molecular Biotechnology, RWTH Aachen
University, Aachen, Germany

S. Schillberg · R. Fischer
Department of Plant Biotechnology, Fraunhofer Institute for
Molecular Biology and Applied Ecology (IME), Aachen,
Germany

CRIT. High-throughput allergen characterization often involves phage display for the presentation of allergens and/or allergen libraries in *Escherichia coli*. This results in the presentation of peptide molecules on the surface of the filamentous phage allowing screening and selection with high affinity and specificity [4, 5]. However, many eukaryotic proteins expressed in *E. coli* do not fold properly due to the absence of suitable chaperones [6]. Therefore, the conformational IgE epitopes present in most allergens may disappear. This limits the utility of normally potent phage display technique and alternative display platforms are required in such cases. Yeast cells offer an alternative approach for the display and selection of both peptide antigens and antibodies. In some cases, yeast cells can provide a greater repertoire of correctly folded protein targets with appropriate post-translational modifications, while allowing rapid and convenient screening by fluorescence-activated cell sorting [7]. Yeast display is useful for the discovery of novel antigens and antibodies, protein evolution and engineering, immunoassay development and the screening of vaccine candidates [7–9].

Baker's yeast (*Saccharomyces cerevisiae*) has FDA “generally regarded as safe” [10] status allowing its routine use in pharmaceutical applications. There are several approaches that enable the display of proteins on the surface of yeast cells. The glycosylphosphatidylinositol (GPI) family of cell wall proteins such as α -agglutinin and flocculin are directed to the plasma membrane via GPI anchors and subsequently incorporated into the mannoprotein layer of the cell wall via a β -1, 6-glucan bridge [11]. The α -agglutinin system [12] utilizes Aga2 as the display fusion partner linked by a disulfide bridge to the GPI protein Aga1 [13] enabling co-expression of Aga1 with an Aga2 fusion protein on the cell surface (Fig. 1a) [8, 9, 11, 12].

Actinidin (also known as actinidain and Act d 1, EC 3.4.22.14) from kiwifruit (*Actinidia deliciosa*) is a cysteine proteinase from the papain superfamily synthesized a pre-protein with an endoplasmic reticulum-targeting signal peptide. The enzyme is likely to be synthesized as a zymogen and processed into an active form after secretion or sequestration in the cell. The mature protein comprises a single polypeptide chain with two domains, three disulfide bridges, a molecular weight of 23.8 kDa and a *pI* value of 3.5 [14, 15]. Despite its extensive characterization, the physiological function of the enzyme remains unknown. Act d 1 may act as a storage protein like patatin in potato [16] or may defend plant against insects and fungi [17]. Act d 1 is the most abundant IgE-reactive protein of the kiwifruit extract and is considered to be a hallmark of monosensitization toward kiwifruit [18, 19]. Furthermore, it has been designated the major kiwifruit allergen in different populations of patients [18, 20, 21]. The severity of

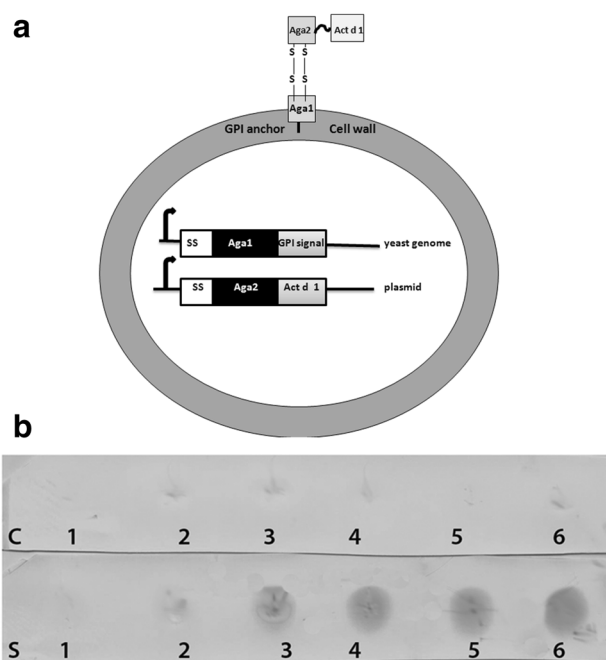


Fig. 1 **a** Principle of yeast surface display using α -agglutinin. The gene of the allergen of interest (here shown Act d 1) is cloned as a fusion with Aga2 and introduced into a yeast strain that can overexpress the Aga1 gene. The resulting proteins, Aga1 and Aga2-allergen fusion are connected by disulfide bonds and exported together to the surface of the cell where Aga1 remains attached to the cell wall by the GPI anchor. **b** Expression of rAct d 1 by yeast cells—dot blot: S1—cells expressing Aga2 protein; S2–S6—cells expressing Aga2—rAct d 1 construct; C—control of secondary antibodies

symptoms exhibited by patients was correlated with the level of IgE binding to Act d 1 [19].

We have developed a yeast surface display platform for the production and immunological characterization of functional plant allergens, allowing this potent technique to be applied in the field of molecular allergology. Act d 1, a cysteine proteinase from kiwifruit was used as a model allergen because it has a complex native structure and proteolytic activity that is easy to detect.

Materials and methods

Patient's sera

Kiwifruit-allergic patients were selected for the study according to a positive skin prick test (SPT) response to kiwifruit extract and/or the presence of kiwifruit-specific IgE. The demographic and clinical data of the patients are summarized in Table 1. Written informed consent was obtained from all patients. For in vitro IgE-binding experiments, IgG was removed from the pool of sera from seven kiwifruit-allergic individuals by protein A-Sepharose

Table 1 Subjects enrolled in the study

Patient ID	Age/Sex	Symptoms	Specific IgE ImmunoCAP (kUA/L)	SPT Kiwi extract
P1	40/F	OAS	4.31	N.D.
P2	21/M	TT	0.67	+
P3	25/M	OAS	N.D.	+
P4	75/F	OAS, TT	<0.35	+
P5	54/F	AE, TT	<0.35	+
P6	44/F	AE, TT	<0.35	+
P7	10/M	OAS, TT	N.D.	+

AE angioedema, OAS oral allergy syndrome, TT tightness of throat, N.D. not determined, SPT skin prick test

4B chromatography (GE Healthcare, Uppsala, Sweden) according to the manufacturer's instructions.

Strains and plasmids

Escherichia coli strain DH5 α (F $^-$ Φ 80*lacZ* Δ M15 Δ (*lacZYA-argF*) U169 *recA1 endA1 hsdR17* (rK $^-$, mK $^+$) *phoA supE44* λ^- *thi-1 gyrA96 relA1*) was used for cloning because it is deficient in recombinase (*recA*) and endonuclease A [22]. The *S. cerevisiae* strain was EBY 100 (*a GAL1 $^-$ AGA1::URA3 ura3-52 trp1 leu2 Δ his3 Δ 200 pep4::HIS2 prb1 Δ 1.6R can1GAL*) Trp $^-$ Leu $^-$ kindly provided by Dr. Dane Wittrup from MIT, USA. The plasmid pCTCON2 was also a gift from Dane Wittrup. For longer storage, the *E. coli* strains were stored in a LB medium with 25 % (v/v) glycerol, and yeast strains were stored in YPD with 15 % (v/v) glycerol at -80°C .

Antisera production

Natural Act d 1 (nAct d 1) has been purified from green kiwifruit (*Actinidia deliciosa*) as previously described [14]. Antibodies raised against purified natural Act d 1 (nAct d 1; *A. deliciosa*) were kindly provided by Dr. Milica Grozdanic. Antibodies against purified Act d 1 were raised in rabbits according to Harboe and Ingild [23]. Animals were injected with 0.5 mL of an emulsion (dilution 1:2, v/v) of the Act d 1 protein solution (1 mg/mL) in complete Freund's adjuvant. Bleeding was performed 50 days following the first immunization and every 2 weeks thereafter. The serum was partially purified using the ammonium sulfate fractionation (50 % saturation). Antisera specificity was confirmed in immunoblot on a kiwifruit extract (data not shown).

Cloning of kiwifruit Act d 1

The mature Act d 1 sequence (GenBank AAA32629.1) was cloned from kiwifruit cDNA and amplified using gene-specific sense and antisense primers. Total RNA was

extracted from mature kiwifruit (*Actinidia deliciosa*, Liang Ferguson) as previously described [24]. Gene-specific sense and antisense primers containing restriction sites for *NheI* and *SalI* (marked in italics) were employed for amplification, respectively: Act d 1 forward 5'-ATC *GCT AGC TTG CCG AGT TAT GTT GAT TGG AGG*-3' and reverse 5'-ATC *GTC GAC CTA AGC GCG GTA CCT CTG TCC*-3'. In the amplification reaction, 20 ng template DNA, 0.75 U Pfu DNA polymerase (Fermentas, Vilnius, Lithuania), 1 $\times$ PCR buffer, 2 mM MgCl $_2$ and 0.2 mM dNTPs were used. The reaction was performed in a MasterCycler (Eppendorf, Hamburg, Germany) programmed for 30 cycles at 94 $^\circ\text{C}$ for 1 min, 64.5 $^\circ\text{C}$ for 1 min, 72 $^\circ\text{C}$ for 1 min and final extension at 72 $^\circ\text{C}$ for 10 min. The DNA fragment of 763 bp was purified and cleaved with the restriction enzymes *NheI* and *SalI* and ligated to the expression vector pCTCON2. The construct was used for the transformation of *E. coli* DH5 α cells. Transformed *E. coli* cells harboring expression vector pCTCON2-Act d 1 were cultured in a LB agar containing ampicillin (100 $\mu\text{g/mL}$). Recombinant pCTCON2-Act d 1 constructs were isolated from *E. coli* and verified by DNA sequencing.

Transformation of EBY 100 yeast cells and Act d 1 expression

EBY 100 cells were transformed with the recombinant pCTCON2-Act d 1 vector as previously described [25]. Transformed cells were plated on an YNB-CAA agar containing 2 % glucose (Glc). Positive clones were selected among those inoculated into 10 mL YNB-CAA Glc liquid medium and incubated for 48 h at 30 $^\circ\text{C}$, 250 rpm. Act d 1 expression was induced by transferring 0.1 mL of liquid culture to 10 mL YNB-CAA medium containing 2 % galactose and raffinose (Gal/Raf) and incubating the culture under the same conditions. Expression was confirmed by dot blot using rabbit polyclonal anti-Act d 1 antisera as further described in Sect. 2.9. Expressing clones were further analyzed by flow cytometry.

Flow cytometry analysis of rAct d 1 yeast surface display

After 16 h of growth in YNB-CAA Gal/Raf liquid medium, cells expressing rAct d 1 were washed twice in phosphate-buffered saline (PBS, 20 mM sodium phosphate, 150 mM sodium chloride) pH 7.4 and diluted to approximately 10^6 cells/mL. For flow cytometry analysis, the cells were incubated with IgE from sera of kiwifruit-allergic patients (depleted for IgG as described in Sect. 2.1 and diluted 1:2) and with rabbit polyclonal antisera raised against nAct d 1 (dilution 1:50). IgE binding was visualized after incubation with a mouse antihuman IgE phycoerythrin (PE)-labeled antibody (Myltenil Biotech, Bergisch Gladbach, Germany) or after further amplification of the signal using FITC-tyramide (Perkin Elmer, Alameda, USA). FITC-tyramide staining was done after incubation with a tertiary antibody, goat anti-mouse IgG peroxidase-coupled antibody (dilution 1:500, Jackson ImmunoResearch, West Grove, USA). Color was developed using 20 μ M FITC-tyramide and 10 μ M H_2O_2 in PBS. The reaction was stopped by addition of L-ascorbic acid up to 1 mM concentration after which the cells were washed twice with PBS. Visualization of rabbit IgG binding was done after staining the cells with a goat anti-rabbit antibody labeled DyLight 680B (dilution 1:100, Thermo Scientific, Rockford, USA). Before measuring, the cells were diluted in sheath fluid (PBS, pH 7.4) and analyzed using a flow cytometer (FACS Influx v7, BD Biosciences, San Jose, CA, USA). The analysis rate was around 4,000 events/s. A blue solid state 200 mW laser at 488 nm and a 100 mW red laser at 640 nm were used for excitation. The emission was detected with 520 (FL1, FITC), 561 (FL2, PE) and 650 (FL3, DyLight 680B) nm filters. The double-positive cells were gated on the FL1-FITC, FL3-DyLight 680 plot. Cells transformed with pCTCON2 not containing an insert were used as a negative control.

Solubilization of rAct d 1

Recombinant Act d 1 displayed on the surface of yeast cells was solubilized in TRIS-buffered saline (TBS, pH 7.4) containing 1 mM dithiothreitol (DTT; Fermentas, Vilnius, Lithuania) and concentrated by ultrafiltration. After 16 h of growth in YNB-CAA Gal/Raf liquid medium, yeast cells expressing recombinant Act d 1 (rAct d 1) were harvested by centrifugation ($3,000\times g$, 5 min, 4 °C), washed twice with TBS pH 7.4, and resuspended in TBS containing 1 mM dithiothreitol (DTT, Fermentas, Vilnius, Lithuania). The suspension was incubated at 25 °C for 2 h and then centrifuged under the same conditions. The supernatant was collected and concentrated by ultrafiltration. The same method was used for buffer exchange to 50 mM TRIS pH

7.5. The concentrated supernatant was used for further analysis.

Proteolytic activity detection and MS analysis

For in-gel analysis of proteolytic activity, nAct d 1 and rAct d 1 were resolved under reducing conditions in a discontinuous buffer system in a SDS-PAGE with a 4 % (w/v) stacking gel and 12 % (w/v) resolving gel in a vertical electrophoresis slab system (Hoefer, Holliston, USA) according to Laemmli [26]. The amount of 12.5 μ g/lane of the protein was applied on the gel. To detect proteolytic activity of actinidin after SDS-PAGE, zymograms were run according to Grozdanovic et al. [14] with some modifications. Briefly, after incubation in a non-reducing sample buffer (1 h at 45 °C), protein samples were applied onto a 12 % (w/v) resolving gel co-polymerized with 0.1 % (w/v) gelatin for SDS-PAGE analysis. After electrophoresis, the gel was incubated in a 50 mM citrate, pH 6.5 with 15 mM L-cysteine, 1 mM EDTA and 5 mM DTT for 16 h, followed by staining with Coomassie blue (Serva, Heidelberg, Germany). Relative enzyme-specific activity was expressed after densitometric analysis of both SDS-PAGE and zymogram. Integrated optical density (IOD) was determined for both protein concentration and enzyme activity. Using IOD, relative specific activity was determined and expressed in arbitrary unit (AU).

To verify the primary structure of rAct d 1, mass spectrometric peptide mapping was performed. In brief, the protein sample was separated by SDS-PAGE, followed by CBB staining of the gel. Protein bands corresponding to the bands with proteolytic activity were excised from the gel, destained, and digested overnight with sequence grade trypsin from porcine pancreas (Promega, Madison, USA) as described by Shevchenko [27]. LC/MS ESI-TOF analysis was performed on an ESI-TOF spectrometer (Agilent Technologies, Santa Clara, USA). Samples were introduced into a MS detector after reverse phase chromatography using a Zorbax Eclipse C18 column (Agilent Technologies, Waldbrown, Germany) on an Agilent HPLC 1200 device (Agilent Technologies, Waldbrown, Germany). The resultant peptide masses were searched by using the Find Pept tool [28] and Protein Prospector [29].

Dot blot and immunoblot analysis

For dot blot analysis, purified nAct d 1 and rAct d 1 (1 μ g) removed from the surface of EBY 100 expressing cells, as described in Sect. 2.7, were applied onto a nitrocellulose membrane (0.45 mm, Serva, Heidelberg, Germany). For immunoblot analysis, 12.5 μ g/lane of nAct d 1 and rAct d 1 was resolved under reducing conditions as described in Sect. 2.8. After electrophoresis, the protein was transferred

to a nitrocellulose membrane (0.45 mm, Serva, Heidelberg, Germany) by semidry transfer at 0.8 mA/cm² for 45 min. The nitrocellulose membrane was cut into strips and blocked with TBST [30 mM TBS pH 7.4 with 0.5 % (v/v) Tween 20 (Serva, Heidelberg, Germany)] containing 1 % (w/v) BSA, for 1 h at RT. For visualization of rabbit IgG binding, strips were incubated for 1.5 h with polyclonal rabbit antiserum raised against purified natural Act d 1 (dilution 1:10,000) and, after the washing step, incubated in goat anti-rabbit IgG (dilution 1:30,000 Sigma-Aldrich, Steinheim, Germany). For visualization of IgE binding, strips were incubated for 12 h with a patient sera pool from kiwi allergic individuals (dilution 1:3) and, after the washing step, incubated in a mouse antihuman IgE (dilution 1:1,000 Sigma-Aldrich, Steinheim, Germany) for 6 h at 37 °C. Visualization of antibody binding was done using a BCIP/NBT solution. Densitometry of dot blot was further performed using GelPro Analyzer 3.1 (Media Cybernetics, Maryland, USA).

Direct ELISA and ELISA inhibition

Microtiter plates (96-well, MaxiSorp, NuncTM) were coated with a 0.50 µg/well of nAct d 1 and rAct d 1 in a carbonate buffer (50 mM, pH 9.6) overnight at 4 °C for direct ELISA, while for ELISA inhibition, rAct d 1 was coated on the plate (0.50 µg/well in carbonate buffer overnight at 4 °C). Blocking was performed for 1 h at 37 °C using a TBST buffer containing 5 % (w/v) skimmed milk. For direct ELISA assay, 100 µL of the patient sera pool (final dilution 1:3) was transferred to the coated wells and incubated for 1 h at 37 °C. Regarding inhibition, pre-incubation of an equal volume of the patient sera pool (final dilution 1:3) and increasing concentrations of nAct d 1 and rAct d 1 (0.01–100 µg/mL), respectively, were performed for 1 h at 37 °C. For ELISA inhibition, a volume of 100 µL of each pre-incubated sample was transferred to the coated wells for competition with coated rAct d 1 antigen, for 1 h at 37 °C. The solution obtained after solubilization of proteins from the cell surface expressing only Aga2 protein was used as a negative control (data not shown). Thereafter, for both direct and inhibition ELISA, plates were washed three times with TBS containing 0.1 % (v/v) Tween 20 (TBST). After washing, 100 µL of mouse anti-human IgE conjugated with alkaline phosphatase (dilution 1:1000, Sigma-Aldrich, Steinheim, Germany) was added to each well for 6 h of incubation at 37 °C. After thorough washing, the reaction was developed with 4-nitrophenyl phosphate (p-NPP Serva, Heidelberg, Germany) in a DEA buffer (100 mM diethanolamine, pH 9.5). The reaction was stopped by addition of 50 µL of 2 M NaOH, and the absorbance was measured at 405 nm in a plate reader (Rayto, Model RT-6100). Each analysis was carried out in

triplicate. The inhibition percentage was calculated according to the following formula:

$$\text{Inhibition (\%)} = \frac{(\text{total reactivity} - \text{remaining reactivity})}{(\text{total reactivity})} \times 100.$$

Buffer and a serum pool of three non-atopic persons were used as negative controls. Absorbance values were considered positive if they exceeded the mean A₄₀₅ nm of the negative controls by >3 SD.

Basophil activation assay

Aliquots of 100 µL heparinized whole blood of kiwifruit-allergic patients were stimulated with 10-fold dilutions of both nAct d 4 (0.01–100 µg/mL) and rAct d 1 (0.01–100 µg/mL). The solubilizate obtained from cell surface expressing only Aga2 protein was used as a negative control (data not shown). Non-stimulated cells and stimulation with antihuman IgE served as controls. Samples were incubated for 20 min at 37 °C. Afterward, 5 µL FITC-conjugated anti-CD63 and 5 µL phycoerythrin-conjugated anti-CD203c (Immunotech, Marseille, France) were added to each sample and incubated for 20 min at 4 °C. After the erythrocyte lysis and centrifugation (400×g for 5 min), the cells were resuspended in PBS (400 µL) and measurements were performed on a flow cytometer (FACS Calibur, BD Biosciences, San Jose, CA, USA).

Densitometric and statistical analyses

Densitometric analysis was performed using GelPro 3.1 Analyzer (Media Cybernetics, Maryland, USA). Relative protein abundance and relative enzyme activity were determined by analyzing Coomassie-stained gels.

Statistical analysis was carried out using GraphPad Prism v5.03 for Windows (San Diego, California, USA). A significance level of $p \leq 0.05$ was used for analysis of variance, and implemented using the Kruskal–Wallis test followed by the Dunn post-test. When appropriate, means were separated using Tukey's test ($p \leq 0.05$). For comparison of the means (dot blot densitometry, ELISA, SDS-PAGE densitometry, zymogram densitometry), Student's t test was performed at a significance level $p \leq 0.05$.

Results

Cloning and surface expression of rAct d 1

The Act d 1 coding sequence (GenBank AAA32629.1) was successfully cloned into the surface expression vector pCTCON2, which was introduced into *S. cerviciae* EBY

100. The cloning strategy included the Aga2 protein linked to the N-terminus of Act d 1. Since the Aga2-Act d 1 construct is linked to the surface of yeast cells by two disulfide bridges between Aga2 and Aga1 proteins (Fig. 1a), adding a reducing agent allowed separation of the construct from the surface of the yeast cell and solubilization of Act d 1. Several clones were tested to check whether they are expressing Act d 1, and the clone exhibiting the highest expression level according to the dot blot (determined by densitometry, Fig. 1b) was selected for further work.

Analysis of recombinant Act d 1 on the yeast cell surface by flow cytometry

After incubation in a pool of sera from patients with kiwifruit allergies (Table 1), the binding of IgE to Aga2-Act d 1 displayed on the surface of yeast cells was detected using two different approaches: Anti-IgE conjugated to phycoerythrin (PE) and a tyramide-based amplification system. The anti-IgE-PE system detected IgE binding albeit with low efficiency, since only 10.84 % of the cells expressing Aga2-Act d 1 were gated as positive (Fig. 2a2, lower right quadrant). Greater sensitivity was achieved by tyramide amplification, producing a visible shift in FL1 fluorescence intensity between the cells expressing Aga2-Act d 1 (Fig. 2b2, lower right quadrant) and negative control cells (Fig. 2b1). This approach successfully detected 40.36 % of the cells expressing Aga2-Act d 1 (Fig. 2b).

Double staining of the cells allowed the detection of two different parameters, i.e., IgE binding and IgG binding (based on rabbit polyclonal anti-Act d 1 antisera). This allowed the IgE and IgG reactivity of Act d 1 on the surface of the same cell to be measured simultaneously. IgE binding was followed by IgG binding after tyramide amplification. Double-positive cells that reacted to both IgE and IgG accounted for 24.37 % of the total cell count (Fig. 2c, upper right quadrant).

Immunological reactivity of solubilized rAct d 1

We next compared the immunological activity of recombinant and natural Act d 1. The IgE reactivity of the solubilized protein was confirmed by dot blot (Fig. 3a dot A2 and Fig. 3b) and direct ELISA (Fig. 3c) using the sera pooled from patients with kiwifruit allergies. Dot blot was further assessed by densitometry (Fig. 3b), and no significant difference was observed between IOD determined for nAct d 1 and rAct 1. In regard to direct ELISA, measured OD₄₀₅ for rAct d 1 (0.301 ± 0.010) was not significantly different than OD₄₀₅ for nAct d 1 (0.331 ± 0.033). ELISA inhibition experiments showed that the EC₅₀ of both forms of the protein were similar (Fig. 3b), i.e., 50 % ELISA

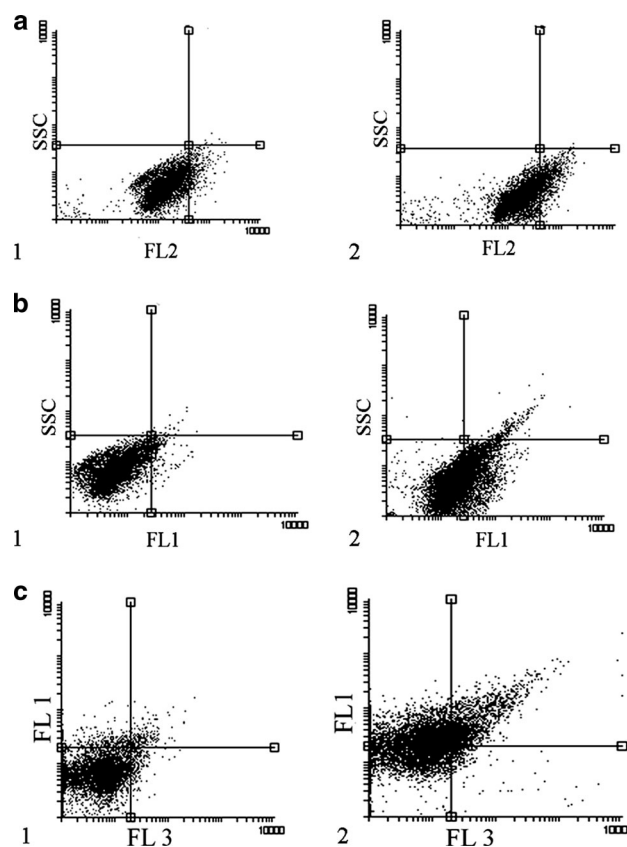


Fig. 2 Analysis of recombinant Act d 1 on the yeast cell surface by flow cytometry. **a** Detection of IgE binding using anti-IgE-PE on cells expressing only the Aga2 protein (*a1*) and the Aga2-Act d 1 construct (*a2*). **b** Detection of IgE binding using anti-tyramide amplification on cells expressing only the Aga2 protein (*b1*) or the Aga2-Act d 1 fusion protein (*b2*). **c** Simultaneous detection of IgE and IgG binding on cells expressing only the Aga2 protein (*c1*) or the Aga2-Act d 1 fusion protein (*c2*). SSC—side scattering, FL2—human IgE binding to rAct d 1 detected with an anti-IgE-PE-labeled antibody, FL1—human IgE binding to rAct d 1 detected with FITC-tyramide staining, FL3—rabbit IgG binding to rAct d 1 detected with a goat anti-rabbit antibody DyLight 680 labeled

inhibition required 1.38 ± 0.27 $\mu\text{g/mL}$ of natural Act d 1 or 1.21 ± 0.28 $\mu\text{g/mL}$ of its recombinant counterpart. Immunoblot analysis confirmed that recombinant Act d 1 was able to bind IgG from rabbit polyclonal anti-Act d 1 antisera (Fig. 4c lane 2). These data indicate that the natural and recombinant proteins share a similar surface topography.

Detection of proteolytic activity of rAct d 1 and MS analysis

The biological activity of the recombinant Act d 1 was similar to that of its natural counterpart (Fig. 4b, lanes 1 and 2). Following solubilization, the recombinant protein was able to digest gelatin (Fig. 4b, lane 2), whereas the negative control cells expressing Aga2 protein alone

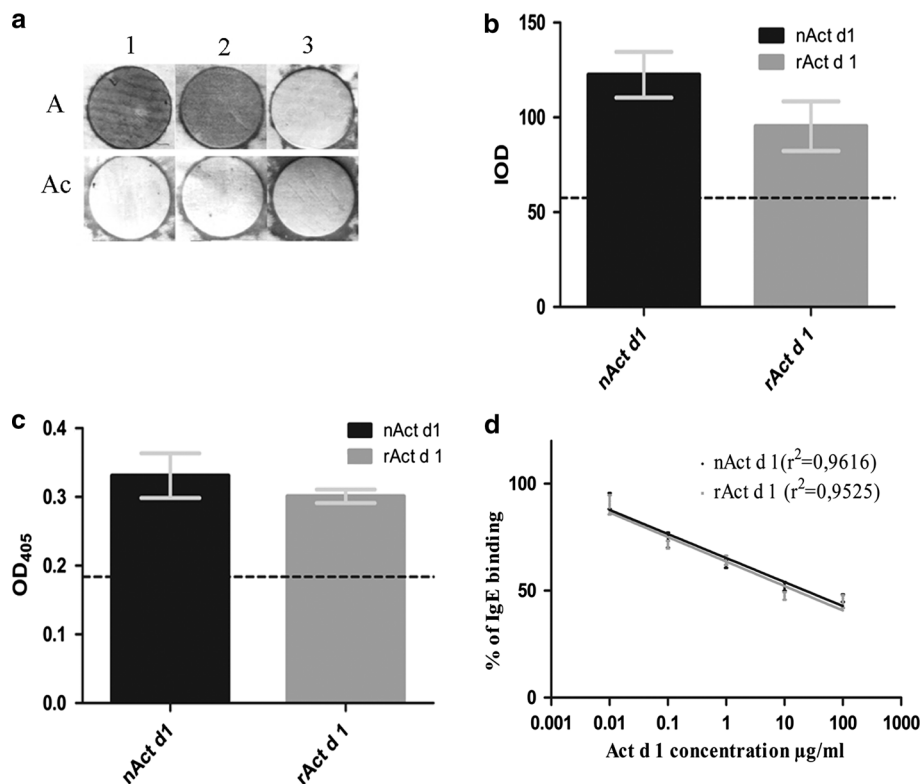


Fig. 3 IgE reactivity of rAct d 1 analyzed in **(a, b)** dot blot, **(c)** ELISA and **(d)** ELISA inhibition assays. The sera pool of seven kiwifruit-allergic individuals was used for the detection of IgE binding. **a** Dot blot A1: nAct d 1; A2: rAct d 1; A3: Aga2 protein; row Ac—control of secondary antibodies, **b** For dot blot analysis, 1 μg of purified nAct d 1 or rAct d 1 was applied onto a nitrocellulose membrane. After visualization, densitometry of dot blot was further performed and integrated optical density (IOD) was calculated. The error bars indicate standard deviations for triplicate measurements. The dashed line represents negative control IOD value + 3 SD, **c** Direct ELISA for nAct d 1 (black) and rAct d 1 (gray): The solid

phase was coated with rAct d 1 and nAct d 1 (0.05 μg/well). The binding was detected using a mouse antihuman IgE conjugated with alkaline phosphatase. The error bars indicate standard deviations for triplicate measurements. The line represents negative control OD value + 3 SD; **d** ELISA inhibition assays for nAct d 1 (black line) and rAct d 1 (gray line). The solid phase was coated with rAct d 1 (0.05 μg/well), while inhibition of IgE binding was achieved with increasing concentration (0.01–100 μg/mL) of both nAct d 1 and rAct d 1, respectively. The binding was detected using a mouse antihuman IgE conjugated with alkaline phosphatase. The error bars indicate standard deviations for triplicate measurements

showed no evidence of gelatinolytic activity when processed in the same manner (Fig. 4b, lane 3). As determined by densitometry, relative specific activity of nAct d 1 was 66.932 ± 0.016 AU, while for rAct d 1 it was 66.029 ± 0.076 AU.

Bands with proteolytic activity were excised from the gel and digested in situ with trypsin. The resulting peptides were analyzed by mass spectrometry followed by peptide mass fingerprinting, which achieved 50 % sequence coverage and confirmed the primary structure of recombinant Act d 1 (Fig. 4d). Peptides matching each of the excised bands are listed in Table 2.

Basophil activation assay

The basophil activation assay was carried out by exposing basophils from two patients with kiwifruit allergies to increasing concentrations of natural and recombinant Act d

1 (Fig. 5). In patient P1 (Fig. 5a), both proteins achieved maximal activation (35.7 and 32.1 % for the recombinant and natural forms, respectively) at a concentration of 1 μg/mL. In patient P2 (Fig. 5b), the recombinant protein achieved maximal activation (10.3 %) at a concentration of 1 μg/mL, whereas the natural protein achieved maximal activation (11.4 %) at a concentration of 10 μg/mL.

Discussion

We have produced a functional recombinant mature form of the major kiwifruit allergen Act d 1 and expressed it on the surface of yeast cells. Allergen display on the cell surface allowed the direct detection of IgE binding without initial protein purification. The high-mannose oligosaccharides that are known to be present on the surface of yeast cells did not appear to affect IgE binding, which was

Fig. 4 Characterization of rAct d 1 by **a** SDS-PAGE, **b** zymography, **c** western blot and **d** mass fingerprint: **a** SDS-PAGE: lane *a1* nAct d 1, lane *a2* rAct d 1-Aga2 expressing cell surface proteins, lane *a3* Aga2 expressing cell surface proteins; MW molecular weight markers; **b** Zymogram: lane *b1* nAct d 1, lane *b2* rAct d 1-Aga2 expressing cell surface proteins, lane *b3* Aga2 expressing cell surface proteins; **c** Western blot: lane *c1* nAct d 1, lane *c2* rAct d 1-Aga2 expressing cell surface proteins; lane *c3* Aga2 expressing cell surface proteins, lanes *b1c*–*b3c* control of secondary antibodies. **d** MS analysis: The sequence identified by MS analysis is highlighted in gray

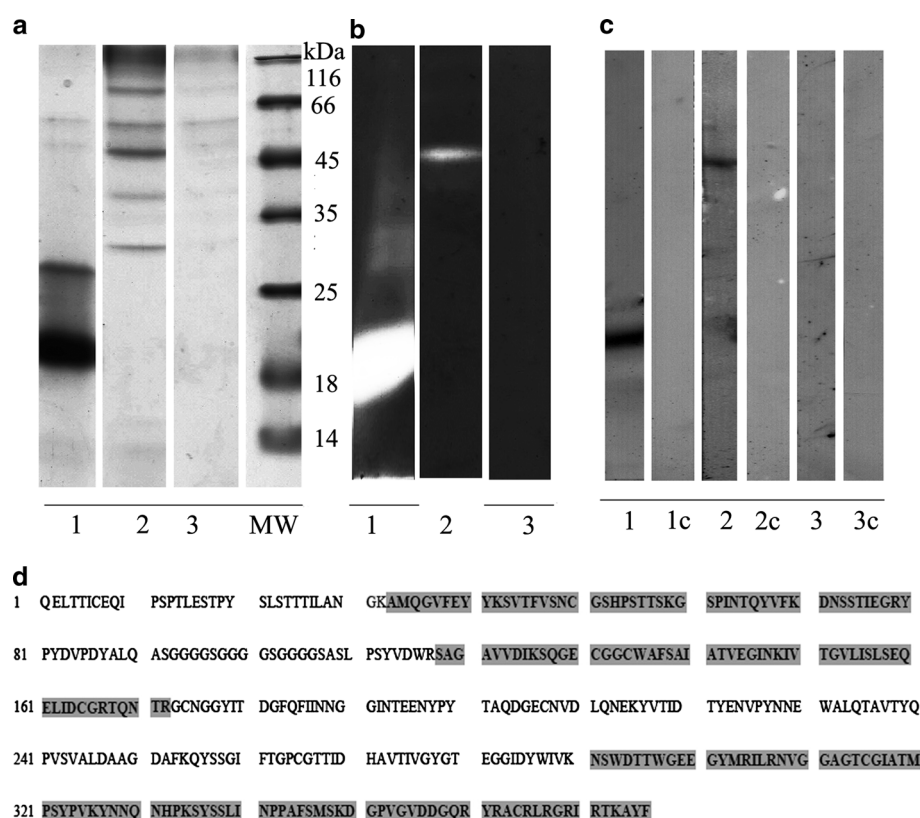


Table 2 List of the masses of predicted tryptic peptides from rAct d 1 which match the measured tryptic peptides

Gel band	Measured mass	Predicted mass	Δ mass	Matching peptides	MOWSE score	Sequence coverage
rAct d 1	1236.334	1236.427	0.093	⁵¹ AMQGVFEYYK ⁶⁰	1.59e ⁺⁴³	50 %
	1796.893	1796.949	0.056	⁶¹ SVTFVSNCGSHPTTTSK ⁷⁷		
	978.943	978.991	0.047	⁸⁹ DNSSTIEGR ⁹⁷		
	859.984	859.998	0.013	¹³⁶ SAGAVVDIK ¹⁴⁴		
	2343.672	2343.591	−0.081	¹⁴⁵ SQGECCGCWAFSAIATVEGINK ¹⁶⁶		
	2103.516	2103.438	−0.077	¹⁶⁷ IVTGVLSLSEQELIDCGR ¹⁸⁵		
	1732.772	1732.821	0.048	³⁰⁹ NSWDTTWGEEGYMR ³²²		
	401.609	401.529	−0.080	³²³ ILR ³²⁵		
	1881.031	1881.172	0.140	³²⁶ NVGGAGTCGIATMPSYPVK ³⁴⁴		
	1014.916	1015.073	0.156	³⁴⁵ YNNQNHPK ³⁵²		
	1629.845	1629.868	0.022	³⁵³ SYSSLINPPAFMSK ³⁶⁷		
	1821.969	1821.965	−0.004	³⁶⁸ DGPVGVDGQRYRACR ³⁸³		
	1769.183	1769.124	−0.059	³⁸¹ ACRLRGRIRTKAYF ³⁹⁴		

Deviations (Δ) between predicted and measured peptide masses are provided along with the location of the peptide within the full-length protein. For the peptide mass fingerprinting, proteins in excised gel slices were subjected to in-gel digestion as described in Sect. 2.8. The masses of the extracted peptides were determined by ESI-TOF MS and matched against predicted open reading frame of rAct d 1 using the search programs FindPept tool and Protein Prospector. Searches were conducted with a mass tolerance of 0.2 dalton

comparable to the binding of IgG from rabbit polyclonal anti-Act d 1 antisera.

Solubilization of the displayed allergen was attempted to allow further characterization without transferring the sequence to another expression system. We successfully

solubilized the recombinant Act d 1 protein in buffer containing DTT, and this did not appear to affect the tertiary structure because the protein retained its IgE and IgG epitopes and its biological activity. Dot blot, immunoblot and ELISA inhibition experiments clearly demonstrated

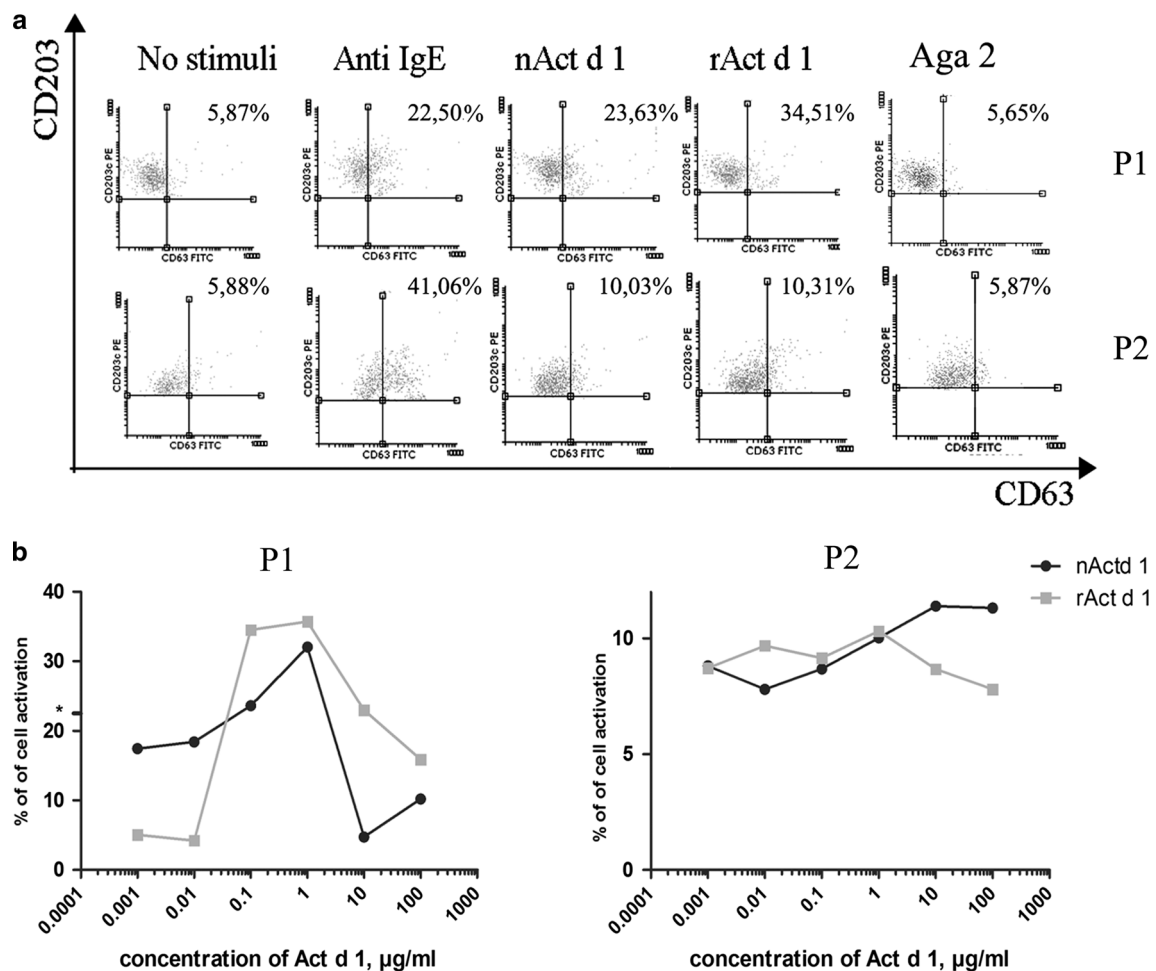


Fig. 5 Basophil degranulation in blood from two representative kiwifruit-allergic patients: **a** double-stained (CD63, CD203c) dot plot: from left to right non-stimulated cells, positive control, nAct d 1

(1 µg/mL), rAct d 1 (1 µg/mL) and Aga2 protein, respectively (1 µg/mL). **b** Percent of upregulated basophils (y-axis) induced by serial dilutions of natural and recombinant Act d 1

that these epitopes were shared with natural Act d 1, and gelatin zymography confirmed that the biological activity of the recombinant protein was almost identical to that of its natural counterpart. Finally, basophil degranulation was induced by exposing blood samples from two individuals with kiwifruit allergies to recombinant Act d 1, demonstrating that the recombinant form of the protein produced in yeast is a functional allergen.

The yeast surface display approach therefore provides a useful tool for the characterization of allergens. Detailed immunological and biochemical characterization of recombinant Act d 1 produced in yeast has shown that yeast display is suitable for the analysis of plant allergens, particularly for testing interactions with components of the immune system, without extensive protein purification. The eukaryotic nature of yeast cells allows them to fold and assemble plant proteins efficiently and form the correct disulfide bonds, which eliminates the necessity of in vitro refolding as typically required when proteins are expressed

in *E. coli* [30]. Proteins expressed in yeast are also *N*-glycosylated, which is sometimes required for protein folding, protein–protein interactions and biological activity [31]. The surface of yeast cells is abundant in high-mannose oligosaccharides which could interfere with interactions between displayed proteins and IgE molecules derived from patient sera because IgE antibodies that recognize carbohydrate epitopes (cross-reactive carbohydrate determinants, CCDs) are also present in the sera of patients that are sensitive to plant allergens. The clinical effects of CCDs are currently considered negligible, but the binding of IgE to CCDs in vitro is a diagnostic hindrance because it generates false-positive results [32, 33]. Even so, anti-CCD IgEs often recognize core α -1, 3-fucose, which is not present in yeast [34]. We found no evidence that IgE binding to Act d 1 on the surface of yeast cells was inhibited by the carbohydrates also present on the yeast surface. Allergen expression on the surface of yeast cells therefore offers new opportunities for the identification of

novel allergens in yeast display cDNA libraries, and immunotherapy based on yeast cells could help to improve standard immunodiagnostic and immunotherapeutic approaches.

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Conflicts of interest The authors do not have any conflict of interest to declare.

Ethical standard The study did not require the approval of the ethics committee.

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