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TITLE PAGE

**Production of *in vivo* biotinylated scFv specific to almond (*Prunus dulcis*) proteins
by recombinant *Pichia pastoris*.**

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Abstract

The methylotropic yeast *Pichia pastoris* has demonstrated its suitability for large-scale production of recombinant proteins. As a eukaryotic organism *P. pastoris* presents a series of advantages at expression and processing of heterologous proteins when compared with *E. coli*. In this work, *P. pastoris* has been used to express a scFv from a human synthetic library previously shown to bind almond proteins. In order to facilitate purification and post processing manipulations, the scFv was engineered with a C-terminal tag and biotinylated *in vivo*. After purification, biotinylated scFv were bound to avidin conjugated with HRP producing a multimeric scFv. The multimeric scFv showed to maintain their ability to recognize almond protein when assayed in ELISA, reaching a LOD of 470 mg kg⁻¹. This study describes an easy method to produce large quantities of *in vivo* biotinylated scFv in *P. pastoris*. By substituting the enzyme or fluorochromes linked to avidin, it will be possible to generate a diverse number of multimeric scFv as probes to suit different analytical platforms in the detection of almond in food products.

Keywords

Avidin; *In vivo* biotinylation; *Pichia pastoris*; *Prunus dulcis*; Multimeric scFv.

1. Introduction

Escherichia coli has been widely adopted for the production of single-chain variable fragments (scFv). However, antibody fragments produced in prokaryotic expression systems can result in unstable proteins, leading to low scFv yields (Arbabi-Ghahroudi et al., 2005; Miller et al., 2005). In this sense, *Pichia pastoris* has become an interesting alternative to *E.coli* in recombinant antibody production. It presents the advantages of single-celled organisms, such as ease of handling and cost-effectiveness, combined with the benefits of eukaryotic systems that include post-translational modifications, protein processing and a reasonably sophisticated quality control of protein folding.

Additionally, it is a more manageable and sturdy system than higher eukaryotes, such as insects and mammalian tissue culture cells systems, that besides being auxotrophic can employ cheap methanol as the sole source of carbon and energy (Cai et al., 2009; Cregg et al., 2000).

A scFv consists of the variable regions of the antibody heavy and light chains connected into a single polypeptide chain with a short flexible linker (Ahmad et al., 2012). One of the strategies aimed to enhance the avidity of antibody fragments has been to imitate the native IgG molecule by means of an engineered tetrameric complex of biotinylated recombinant antibodies fused to a core of streptavidin or avidin (Cloutier et al., 2000; Kipriyanov et al., 1995; Thie et al., 2009). Biotin molecule is typically employed as it possesses high affinity towards streptavidin ($K_d = 4 \cdot 10^{-14}$ M) (Cronan and Reed, 2000; Li and Sousa, 2012).

Although chemical or enzymatic *in vitro* biotinylation has been used for decades, the ability to target one particular amino acid residue *in vivo* has recently become very attractive (Chapman-Smith et al., 2001; Kay et al., 2008; Predonzani et al., 2008;

Scholle et al., 2006; Thie et al., 2009). In particular, the *E. coli* biotin protein ligase (BirA) has shown to catalyze the covalent attachment of a biotin molecule to a specific lysine within the biotin acceptor domain (BAD) (Beckett et al., 1999; Cull and Schatz, 2000; Li and Sousa, 2012). This reaction has the particularity of being conserved throughout evolutionary boundaries, so enzymes from different species were described to biotinylate carboxylases from different sources (Zempleni et al., 2009).

In this work we describe the *in vivo* biotinylation of a scFv employing a single clone of *P. pastoris* co-transformed with two different expression plasmids, and the subsequent multimerization of the biotinylated scFv on avidin to produce a complex antibody aimed to detect almond proteins in ELISA immunoassays.

2. Materials and methods

2.1. Materials and Reagents

E. coli XL1-Blue Chemically Competent Cells (Agilent Technologies, Santa Clara, CA, USA) were employed for the propagation of plasmids, and *P. pastoris* X-33 strain (Life Technologies, Carlsbad, CA, USA) was used for scFv and BirA enzyme expression. *P. pastoris* expression vectors pPICZ α B and pPIC6 α A were purchased from Life Technologies. Restriction enzymes *Pst*I, *Not*I, *Xba*I, *Eco*RI and *Sac*I, calf intestinal alkaline phosphatase, T4 DNA ligase, and GoTaq DNA Flexi Polymerase were purchased from Promega (Madison, WI, USA). Q5 High-Fidelity DNA Polymerase was purchased from New England Biolabs (Hitchin, UK). Synthetic oligonucleotides were purchased from Sigma-Aldrich (Gillingham, UK). Plasmid purification kit (QIAGEN Plasmid Midi Kit), PCR product purification kit (QIAquick PCR Purification Kit) and gel extraction kit (QIAquick Gel Extraction Kit) were purchased from Qiagen (Cologne,

Germany). HiTrap Protein L Column was purchased from GE Healthcare Life Sciences (München, Germany). Selection antibiotic Zeocin was purchased from Life Technologies, and blasticidin was purchased from InvivoGen (Toulouse, France). Peptone, tryptone, yeast extract, and European bacteriological agar were purchased from Pronadisa (Madrid, Spain). Methanol was purchased from Fisher Scientific (Loughborough, UK). Other chemicals were purchased from Sigma-Aldrich unless otherwise stated.

2.2. Vectors construction

The pMJA179 vector was constructed as follows: nucleotide sequence encoding the almond-specific PD1F6-scFv (GenBank accession no. **LN889750**) was amplified from the corresponding phagemid using a high fidelity DNA polymerase with primers MJA254 and MJA253 (**Table A, Supplementary material**), the former adding a new *Pst*I restriction site to the scFv sequence. After PCR reaction, purified PCR product was digested with *Pst*I and *Not*I. The resulting fragment was inserted between the *Pst*I and *Not*I sites in the pPICZαB plasmid, following a basic protocol described in Bloch and Grossmann, 1995.

The pMJA180 vector was constructed as follows: Genomic DNA from TOP10 *E.coli* strain (Life Technologies) was extracted with a phenol/chloroform/isoamyl alcohol mixture, and then precipitated with 100 % ethanol. After precipitation, DNA pellet was washed with 70 % ethanol, and resuspended in buffer TE, according to the procedure described in Moore and Dowhan, 2002. Nucleotide sequence codifying BirA enzyme (GenBank accession no. **P06709**) was PCR amplified using primers MJA255 and MJA256, the former including an *Eco*RI restriction site, the latter incorporating a *Sac*II

site. The amplified fragment was ligated between *Eco*RI and *Sac*II sites of pPIC6 α A plasmid.

The pMJA181 vector was constructed as follows: BAD sequence was obtained by enforcing hybridization between reverse-phase chromatography-purified primers MJA257 and MJA258. Primer sequences were obtained from Predonzani et al., 2008, with some modifications to include *Not*I and *Xba*I restriction sites. After resuspending primers to a final concentration of 100 μ M, 25 μ L of each primer were mixed, heated at 95 $^{\circ}$ C for 10 min, and cooled down to room temperature. The procedure was repeated 3 times. Hybridized BAD nucleotide sequence was then digested with *Not*I and *Xba*I, and inserted between the *Not*I and *Xba*I sites in the pMJA179 vector. Correct orientation of the insert (scFv + BAD) was assessed by DNA sequencing with primers pMJA254 and pMJA259 at Source BioScience (Nottingham, UK).

To direct the scFv+BAD and the BirA enzyme into the secretory pathway, the codifying sequences were inserted in frame with the methanol inducible 5'-AOX1 promoter, the α -factor secretion signal and the AOX1 transcription terminator.

2.3. Transformation of *E. coli*

Transformation of *E. coli* cells was performed according to manufacturer's protocol. Once transformed, cells were spread on pre-warmed low salt Luria-Bertani agar plates (1 % tryptone, 0.5 % yeast extract, 0.5 % NaCl, 1.5 % agar, pH 7.5) containing the selective antibiotic (25 μ g mL⁻¹ Zeocin for plasmids pMJA179 and pMJA181, and 100 μ g mL⁻¹ blasticidin for plasmid pMJA180). Plates were incubated overnight (o/n) at 37 $^{\circ}$ C.

2.4. Transformation of *P. pastoris*

The pMJA181 expression vector was linearized by *Sac*I digestion, ethanol precipitated, and transformed into *P. pastoris* X-33 with a BioRad MicroPulser electroporation apparatus, following the manufacturer's instructions (Bio-Rad, Hemel Hempsted, UK). Transformed cells were grown on Yeast Extract Peptone Dextrose Sorbitol Medium (YPDS) agar plates (1 % YE, 2 % peptone, 2 % dextrose, 1 M sorbitol, 2 % agar) with 100 µg mL⁻¹ of Zeocin for 72 h at 30 °C. Ninety-five isolated colonies were inoculated in 200 µL of Yeast Extract Peptone Dextrose Medium (YPD) with 100 µg mL⁻¹ of Zeocin, and grown o/n at 30 °C with shaking (200 rpm) to be screened for scFv production. Next day, 20 µL of each clone was inoculated in 1 mL of Buffered Glycerol-complex Medium (BMGY) (1% yeast extract, 2% peptone, 100 mM potassium phosphate pH 6.0, 1.34 % YNB, 1 % glycerol, 4·10⁻⁵ % biotin) in 24-well plates (Costar, Corning Life Sciences, NY, USA) with 100 µg/mL Zeocin. Cells were incubated o/n at 30 °C with shaking. Next day, plates were centrifuged (1800 g, 10 min, 4 °C), supernatant was removed, and cells were resuspended in Buffered Methanol-complex Medium (BMMY) (1% yeast extract, 2% peptone, 100 mM potassium phosphate pH 6.0, 1.34 % YNB, 1 % methanol, 4·10⁻⁵ % biotin) to induce scFv expression. Methanol (1 % v/v) was replenished every 12 h for 72 h. Finally, plates were centrifuged (1800 g, 10 min, 4 °C) and the supernatant was analyzed by dot-blotting analysis in search for higher expressers scFv clones.

Appropriate production of scFv by the selected clone was assessed by SDS-PAGE, using 12% resolving polyacrylamide gel, pH 8.8 but without boiling the sample buffer. Electrophoresis was run at constant voltage (150 V) using a Mini-Protean Tetra Cell (Bio-Rad).

2.5. Dot-blotting analysis

Five hundred microliters of supernatant from 95 selected *P. pastoris* clones were filtered through a dot blot microfiltration unit (Life Technologies) to coat a PVDF membrane (Immun-Blot, Bio-Rad). Membrane was then blocked with 3 % BSA (w/v) TBS (50 mM Tris-HCl, 150 mM NaCl, pH 7.5) at 37 °C for 1h. After washing the membrane 3 times for 5 minutes with TBST (0.05 % v/v Tween-20), it was incubated with anti-c-myc antibody (Sigma-Aldrich, SKU M5546) (1:100 v/v) in 1 % BSA (w/v) TBST at 37 °C for 2 h. After washing 3 times with TBST, the membrane was incubated for 2 h at 37 °C with a goat-anti-mouse IgG-ALP antibody (Sigma-Aldrich, SKU A3562) (1:20000 v/v) diluted in 1 % BSA (w/v) TBST. Then, the membrane was washed 3 times with TBST and once with distilled water. Finally, a ready-to-use solution of 5-bromo-4-chloro-3-indolyl-1-phosphate and nitroblue tetrazolium (Novex AP Chromogenic Substrate, Life Technologies) was used to detect the alkaline phosphatase enzyme activity. Reaction was stopped by rinsing the membrane with water.

2.6. *In vivo* biotinylation of scFv

One of the best scFv-expresser clones was chosen based on the intensity of the signals obtained in the blotting membrane from the different supernatants analyzed. The selected clone was transformed with the second *P. pastoris* expression vector, pMJA180, and transformed cells were grown on YPDS agar plates containing 100 µg mL⁻¹ Zeocin and 500 µg mL⁻¹ blasticidin. After 72 h, isolated colonies were picked from the selective agar plate, induced with methanol following the microscale induction described before, and supernatants were once again analyzed by dot-blotting to check for the presence of biotinylated scFv. Dot-blotting was carried out as stated before, but incubating the membrane with Avidin-Alkaline Phosphatase (Sigma-Aldrich, SKU A7294) (1:100,000

v/v) in 1% BSA, or alternatively with ExtrAvidin-Peroxidase (Sigma-Aldrich, SKU E2886) (1:5000 v/v) in 1% BSA, and developing the membrane with a chemiluminescent substrate (Clarity Western ECL, Bio-Rad).

One clone called PdBSF (*Prunus dulcis* Biotinylated Soluble Fragment, PdBSF), which showed high color intensity in the dot-blotting analysis, was used hereafter. The insertion of both plasmids was assessed by PCR with the primer pairs MJA254/MJA259 and MJA255/ MJA256. To that end, genomic DNA from PdBSF clone was isolated following the procedure described by Harju et al., 2004, with several modifications. Briefly, a single well-grown colony was resuspended into 200 μ L of lysis buffer (10 mM Tris, 1 mM EDTA, 100 mM NaCl, 1 % (w/v) SDS, 2 % (v/v) Triton X-100, pH 8.0). Then, tubes were frozen at -80 $^{\circ}$ C for 2 min, and boiled for 1 min. This procedure was repeated twice. Two hundred microliters of chloroform was added to each sample, and tubes were vortexed for 2 min and centrifuged for 3 min at high speed. The aqueous layer was transferred to a new tube, and DNA was precipitated with ethanol.

Once the insertion of both expression plasmids was confirmed, PdBSF was inoculated into 10 mL of YPD with 100 μ g mL⁻¹ Zeocin and 500 μ g mL⁻¹ blasticidin, and incubated o/n at 30 $^{\circ}$ C. Next day, 1 mL of this culture was inoculated in 600 mL Buffered Glycerol-complex Medium (BMGY) (1 % YE, 2 % peptone, 100 mM potassium phosphate, pH 6.0, 1.34 % YNB, 4 \cdot 10⁻⁵ % biotin, 1 % glycerol) containing 100 μ g mL⁻¹ Zeocin and 500 μ g mL⁻¹ blasticidin, and incubated for 18 h at 30 $^{\circ}$ C with shaking. Then, it was centrifuged at 4000 g for 15 min at 4 $^{\circ}$ C and resuspended in 600 mL Buffered Methanol-complex Medium (BMMY) (1 % YE, 2 % peptone, 100 mM potassium phosphate, pH 6.0, 1.34 % YNB, 8 \cdot 10⁻⁵ % biotin, 1 % methanol). Methanol was replenished every 12 h for 72 h. Finally, the induced culture was

centrifuged at 4000 g for 20 min at 4°C to remove yeast cells, and the biotinylated scFv purified from the supernatant as described in section 2.7.

2.7. Purification of biotinylated scFv

The supernatant containing biotinylated scFv was filtered through a 0.4 µm membrane filter (Millipore, Darmstadt, Germany) and loaded onto a 1x1 mL HiTrap protein L column (GE Healthcare Life Sciences) attached to an ÄKTA purifier FPLC system (GE Healthcare, Sweden). Chromatography was performed as described by Rouet et al., 2012 with several modifications. Briefly, 300 mL of supernatant was loaded onto the column previously equilibrated with 10 mL of PBS buffer (0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4). The column was then washed with 20 mL of PBS, and the biotinylated scFv eluted with 10 mL of 0.1 M glycine-HCl (pH 2.7). Fractions showing OD₂₈₀ above 0.05 were manually collected in 1.5 mL microcentrifuge tubes prefilled with 400 µL of 200 mM Tris-HCl (pH 8.0). Flow rate was maintained at 1 mL min⁻¹. The entire process was repeated with the remaining 300 mL of supernatant.

Recovered fractions were pooled and dialyzed against PBS buffer employing Amicon Ultra-15 Centrifugal Filter Units (Millipore) with a MWCO of 10 kDa. Protein concentration was measured in a Nanodrop (Thermo Scientific, Waltham, MA, USA), adjusted to 2 mg mL⁻¹ of total protein, and stored in 100 µL aliquots at -80 °C until further use.

2.8. Multimerization of biotinylated scFv

Multimerization was performed following the guidelines provided by the NIH Tetramer Core Facility (<http://tetramer.yerkes.emory.edu/support/protocols#10>), using as a core a

molecule of avidin conjugated with peroxidase (ExtrAvidin-HRP, Sigma-Aldrich), at a concentration of 2.5 mg/mL, with a molar ratio (ExtraAvidin: peroxidase) of ≈ 0.7 . Briefly, 0.5 μ L avidin solution was added every 10 minutes up to a total of 10 times to an aliquot of 200 μ g of biotinylated scFv. The procedure was carried out at room temperature in the dark, and with continuous but gentle rotation in a sample mixer (HulaMixer Sample Mixer, Life Technologies). After multimerization, tubes were kept in the dark at 4 °C until further use.

2.9. ScFv multimerization assessment

After multimerization, scFvs were concentrated using Amicon Ultra 50 kDa filtration unit (Merck Millipore, Darmstadt, Germany) and analysed by SDS-PAGE 12% in non-reducing conditions. Electrophoresis gel was stained with Coomassie Brilliant Blue R-250. Gel bands of interest were cut out with a scalpel and immersed in a solution 5 % (v/v) of acetic acid. Peptide mass fingerprinting was performed using a 4800 Plus MALDI TOF/TOF Analyser mass spectrometer (AB SCIEX, MA, USA), at the Unidad de Proteómica at Parque Científico de Madrid (Spain). Mascot search engine software (<http://www.matrixscience.com>) was employed to interpret mass spectra data into protein identities using the SwissProt database. Search parameters employed were: trypsin enzymatic cleavage, one possible missed cleavage allowed; peptide mass tolerance of ± 80 ppm; fragment mass tolerance of ± 0.3 Da; peptides were assumed to be monoisotopic; carbamidomethyl fixed modification; and methionine oxidation variable modification.

To further assess scFv multimerization, ultracentrifugation analyses were carried out. Sedimentation velocity experiments were carried out at 45000 rpm in an OptimaXL-A analytical ultracentrifuge (Beckman Coulter Inc., Fullerton, CA), using an AN50Ti

rotor and standard cells with double-sector epon-charcoal centrepieces. Measurements were performed in PBS at 20 °C with 400 µL of sample at protein concentrations absorbing 0.6 OD (1.2 cm optical pathway). Differential sedimentation coefficients were calculated by least-squares boundary modelling of the experimental data and corrected to $s_{20,w}$ values with the program SEDFIT (Schuck, 2000), using a partial specific volume of 0.73 mL g⁻¹. Solvent density and viscosity at 20 °C were computed using the SEDNTERP program (Laue et al., 1992). The experiments were performed at Instituto de Química-Física Rocasolano, CSIC, Madrid (Spain).

2.10. Preparation of protein extracts

Binary mixtures of wheat flour containing Marcona cultivar (100 000 to 10 mg kg⁻¹) were prepared as described elsewhere (de la Cruz et al., 2015). Protein extracts from binary mixtures and commercial food products were prepared by adding 200 mg of milled sample to 1800 µL of protein extraction buffer (0.035 M phosphate solution containing 1 M NaCl, pH 7.5.). After shaking for 10 min at room temperature to facilitate the extraction of soluble proteins, the slurry was centrifuged at 10 000 g for 10 min at 4 °C, and the supernatant was filtered through a 0.45 µm syringe filter (Sartorius, Göttingen, Germany). Bicinchoninic acid (BCA) assay (Thermo Fisher Scientific Inc., IL, USA) was used to determine protein concentration. Protein extracts were kept at -20 °C until further use.

2.11. Indirect ELISA with multimerized scFv

The ability of multimerized scFv to recognize almond protein was assessed through indirect ELISA. One hundred microliters of protein extract from binary mixtures or food samples (diluted 1:200 in PBS) was used to coat 96-multi-well polystyrene plates

(F96 MaxiSorp, Nunc immune plates, Nunc, Denmark). Plates were incubated o/n at 4 °C. Next day, plates were washed 3 times with TBST. Wells were blocked with 3 % BSA (w/v) in TBS for 1 h at 37 °C. After another washing step, 100 µL of 2 µg mL⁻¹ multimerized scFv diluted in 1 % BSA in TBST was added to each well. Plates were incubated at 37°C for 2 h. After incubation, plates were washed 3 times with TBST and once with distilled water. Then, 100 µL of tetramethylbenzidine (TMB) substrate solution was added to each well, and incubated in the dark. Color development was performed for 10 min at room temperature, and reaction was stopped with 1 M sulphuric acid. OD₄₅₀ was measured with an iEMS Reader MF (Labsystems, Helsinki, Finland). All experiments were performed in triplicate. As negative controls, different wells coated with protein extracts were also incubated with 2 µg mL⁻¹ of monomeric scFv (without ExtrAvidin) or with 0.125 µg mL⁻¹ of ExtrAvidin-HRP (without scFv) to check for non-specific reactions.

A calibration curve of different concentrations of almond in wheat flour (100 000 to 10 mg kg⁻¹) was included in each plate. Standard curve was obtained by plotting the absorbance values vs the log of almond protein concentration, and it was fitted to an exponential decay curve using Origin 8.0 software (OriginLab Crop., USA).

3. Results and discussion

3.1. Vectors construction and co-transformation into P. pastoris

P. pastoris can direct heterologous proteins either to the cytoplasm (intracellular) or to secrete them into the culture medium. For proteins to be secreted, the expressed protein must contain a signal sequence targeting the secretory pathway (Cregg et al., 2000). In this work, recombinant proteins were expressed as fusions to the secretory N-terminal

sequence of *S. cerevisiae* α -mating factor prepro-peptide. Furthermore, the vectors used induce the integration of the construct into *P. pastoris* genome, thus conferring genetic stability of the recombinant elements (Macauley-Patrick et al., 2005).

By engineering a BAD sequence into the C-terminus of the scFv (plasmid pMJA181, **Figure A, Supplementary material**) a potential biotinylation site has been created. In addition to BAD sequence, the scFv expressed by the *P. pastoris* clones contained a c-myc epitope (EQKLISEEDL) and a poly histidine tail. Thus, it was possible to assess the production of soluble scFv by dot-blotting analysis of the supernatants coated to PVDF membrane by detecting them with an anti-c-myc antibody. The screening of high expresser clones is an essential step in *Pichia* as random and multiple plasmid integration events can result in variable expression levels of heterologous proteins (Nordén et al., 2011; Zhu et al., 2009). Ninety-five *P. pastoris* clones transformed with pMJA181 were induced to produce scFv, and their supernatants checked by dot-blotting analysis to assess the production of soluble fragments. After developing the membrane with a chromogenic substrate, 55 spots (57% of clones) showed high color intensity, being indicative of a correct scFv expression. One of those 55 clones (named pMJA181-D8) was randomly selected to prepare competent cells to proceed with incorporation of the BirA codifying sequence after the second transformation with pMJA180 vector (**Figure B, Supplementary material**). It is noteworthy to mention that Zeocin resistant clones somehow resistant to usual doses of blasticidin employed in yeast were observed. Therefore, to ensure the selection of co-transformed clones, blasticidin concentration had to be increased up to 500 $\mu\text{g mL}^{-1}$. The level of antibiotic resistance reflects the recombinant gene dosage of transformed *P. pastoris* (Nordén et al., 2011), so it is usual

to find that the best *P. pastoris* expressors also show higher antibiotic resistance (Arbulu et al., 2015; Jiménez et al., 2014).

3.2. Expression and purification of biotinylated scFv

After the re-screening and selection of the best *in vivo* biotinylated clone (PdBSF) (**Figure 1**), the presence of the scFv and BirA codifying sequences were confirmed by PCR with a 780 kb band for scFv linked to BAD nucleotide sequence, and a band of about 975 kb, corresponding to BirA nucleotide sequence. Moreover, pMJA181-D8 clone only possessed pMJA181 vector, and, as expected, the non-transformed *P. pastoris* strain showed no DNA amplification with these pairs of primers.

To produce large yields of biotinylated scFv, *P. pastoris* cells were grown in buffered media (BMGY and BMMY) as pH values of 6.5-8.0 were described as optimum for scFv production (Shi et al., 2003), and the addition of peptone to the culture medium may enhance product stability through repression of protease induction caused by nitrogen limitation (Macauley-Patrick et al., 2005).

Concomitant transcription of two plasmids would theoretically allow PdBSF clone to express both the scFv and the BirA enzyme. Therefore, BirA present in supernatant would catalyze the binding of a biotin molecule to the acceptor peptide fused to the scFv, resulting in a straightforward *in vivo* biotinylated scFv. Here, *in vivo* biotinylation is presented as an alternative to *in vitro* biotinylation, which requires the previous production and purification of the enzyme, as described elsewhere (Li and Sousa, 2012). On the other hand, the presence of a BAD fused to the scFv provides a specific substrate for BirA to be selectively biotinylated, avoiding biotinylation alternatives such as amine coupling methods, that could decrease the antigen-binding activities (Kumada, 2014).

In order to characterize these products SDS-PAGE of culture supernatants (**Figure 2**) was carried out and showed the production of 30-35 kDa proteins when clones pMJA181-D8 and PdBSF were induced with methanol (lanes 2 and 3), thus demonstrating the capability of transformed clones to produce the scFv. Protein concentration of those bands greatly increased after purification of scFv (lanes 4 and 8). However, when transformed clones were not methanol induced, bands indicating the presence of the scFv were absent. Dot-blotting membranes coated with supernatants from pMJA181-D8 and PdBSF clones after methanol induction (**Figure 3, right**) and developed with anti-c-myc antibody, confirmed the ability of both transformed clones to produce scFv. On the other hand, dot-blotting analysis of the same supernatants developed with ExtrAvidin-Peroxidase (**Figure 3, left**) showed that only the scFv produced by the co-transformed *P. pastoris* clone (*i.e.* PdBSF clone) was biotinylated, thus confirming the co-expression and proper function of the BirA enzyme, and the effective production of biotinylated scFv by a single *P. pastoris* clone.

Supernatant containing biotinylated scFv was purified by affinity chromatography. The columns used (HiTrap protein L) contain an agarose matrix combined with recombinant protein L, which presents affinity towards the variable region of the kappa light chain of immunoglobulins and immunoglobulin fragments (Malpiedi et al., 2013; Muzard et al., 2009; Zheng et al., 2012). The purification process rendered 30 mg L⁻¹ of biotinylated scFv that were distributed in 100 µL aliquots of 2 mg mL⁻¹.

3.3. Multimerization assessment

To be used in ELISA, biotinylated scFv antibodies were fused to a core of ExtrAvidin-HRP to obtain multimeric scFv. SDS-PAGE in non-reducing conditions of multimeric scFv (**Figure 4**) showed a subtle band with a molecular weight of about 220 kDa, which

might coincide with the expected size of the tetramers (≈ 230 kDa). To confirm the results, the highest molecular weight band from lane 3 was excised and trypsinized to be identified by MALDI-TOF/TOF. The results obtained showed that the band contained a mixture of peroxidase from *Armoracia rusticana* and Ig from *Homo sapiens* (**Table 1**), thus being consistent with the presence of a tetramerized scFv. Moreover, when comparing MS results to scFv's amino acid sequence, coverage of 35% was found (**Figure 5**) altogether with ion scores shown in **Table 1**.

To further assess the extension of tetramerization, sedimentation velocity experiments were performed. On the basis of ultracentrifugation studies, differences between the sedimentation coefficient (S) of scFv and the scFv with ExtrAvidin-HRP were observed (**Figure 6**). Even though Extravidin-HRP is not a homogeneous reagent, its main peak showed an S value of 6.5 with an apparent Mw of 108 kDa. When Extravidin-HRP reacted with the biotinylated scFv (2.50 S, MW_{app} 27.1 kDa), a new species of 8.75 S appeared, with Mw_{app} 168 kDa, consistent with the addition of at least two biotinylated scFv molecules to a single Extravidin-peroxidase core. Nevertheless, it is possible that conjugation of peroxidase to avidin would hide biotin binding sites in the avidin molecule, hampering the production of complete tetramers. Other biotin binding proteins should be tried in further experiments.

3.4. Indirect ELISA with multimeric scFv

Due to its feasible adaptation to different applications, biotin- avidin (and its homologs) interaction continues spreading over a wide range of scientific areas. For instance, the high affinity of the moiety makes it an attractive tool for development of novel sensors (Dundas et al., 2013).

Phage display is a reliable tool to isolate antibody fragments from highly diverse antibody libraries. However, one of the drawbacks of selected antibodies is the lack of affinity maturation undergone by classical antibodies raised in animals. A typical strategy to improve antibody affinity entails the introduction of additional mutations to the specific phage binders, in a process that usually involves antibody engineering techniques such as error-prone PCR and CDR shuffling (Kobayashi and Oyama, 2011). In this sense, the multimerization of scFv on avidin or streptavidin is presented as an alternative to increase antibody's functional affinity (Cloutier et al., 2000; Kipriyanov et al., 1995).

Indirect ELISA using multimerized scFv was able to detect almond protein in the wheat flour binary mixtures assayed, with absorbance values increasing in a concentration-dependent manner, down to 500 mg kg⁻¹. The limit of detection (LOD) achieved with multimerized scFv-ELISA was of 470 mg kg⁻¹. The representative standard curve obtained is shown in **Figure 7**. Results also confirmed that despite the variation in size (due to presence of BAD peptide), scFv expressed in *P. pastoris* maintained their functionality. However, it should be noted that the monomeric scFv did not show positive signal when tested in a parallel assay employing the anti-c-myc antibody raised in mouse, and the anti-mouse antibody conjugated with alkaline phosphatase as the detection antibodies, and SigmaFast p-Nitrophenyl phosphate tablets substrate, revealing that monomeric soluble fragments lacked the ability to detect almond proteins when employed in ELISA (in these conditions, multimeric scFv were still functional). To confirm the results, monomeric scFv were also tested in dot-blotting, with the same negative results (data not shown).

To confirm whether the multimeric-scFv still possessed the same specificity to detect almond protein in foodstuffs that exhibited its phage counterpart, 10 of the 92 commercial food products previously assayed (de la Cruz et al., 2015) were selected and tested. Among the samples analyzed, 7 declared to contain almond as ingredient, two declared the possibility of containing traces of tree nuts and the last one did not declared almond as ingredient (**Table 2**). The ELISA results obtained with multimeric scFv were in accordance with the ones obtained with the phage-scFv ELISA. However, as ELISA performed with multimerized scFv is faster and requires less handling, it would be a more appropriate method to be used when a large number of samples is to be analyzed. In the recent years, the use of soluble scFv expressed in *P. pastoris* system has been proposed for detection of different biomolecules, such as Metolcarb (an insecticide), and heart failure or tumor biomarkers (Cai et al., 2014; Maeng et al., 2012; Sommaruga et al., 2011). Moreover, *P. pastoris* has been used to produce modified scFv molecules, like scFv-Fc fusion proteins aimed to detect rabies antigen (Wang et al., 2012) and T cell leukemia lymphoma CD25 marker (Wan et al., 2013), or to express anti-keratin 8 divalent scFv (sc(Fv)₂) antibodies (Jafari et al., 2011).

In vivo biotinylation of scFv was initially described by Cloutier et al., 2000, who produced “streptabodies” in an *E.coli* strain carrying the plasmid encoding the BirA enzyme after transformation with a vector encoding a scFv linked to BAD. However, due to the presence of N-terminal leader peptide (pelB), scFv expressed in *E.coli* are driven to periplasmic compartment, where they can aggregate as a result of high protein concentration (Lowe et al., 2011). In a different study, Predonzani et al., 2008 reported a bigenic plasmid that allowed the co-expression of a BAD fused scFv and the BirA enzyme in mammalian cells (HEK293 and HEK293T/17). Nevertheless, the advantage

of replacing mammalian cells with *Pichia pastoris* lies in the latter not requiring a complex growth medium or culture conditions, its easy genetic manipulation, and potential for a large scale production at high cell density (Frenzel et al., 2013).

In this work, we report for the first time the *in vivo* biotinylation of a scFv expressed in a single *P. pastoris* clone co-transfected with two different expression vectors. The system allows the production of large quantities of biotinylated scFv that once purified, are multimerized using an avidin-HRP core. The large complex maintained the ability to recognize the target almond protein in food products down to 470 ppm when used in indirect ELISA. Because the scFv multimers have demonstrated to be functional, it is possible to produce multimers on avidin molecules with suitable modifications (fluorophores, magnetic particles, etc.), so they can be used as probes in biosensors or microarrays aimed to detect proteins in complex food matrixes.

Abbreviations used

AP: alkaline phosphatase; AOX: alcohol oxidase; BAD: biotin acceptor domain; BCA: bicinchoninic acid; BirA: biotin ligase; CDR: complementary determining regions; HRP: horse radish peroxidase; LOD: limit of detection; MWCO: molecular weight cut off; ScFv: single chain variable fragment.

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Figure 1

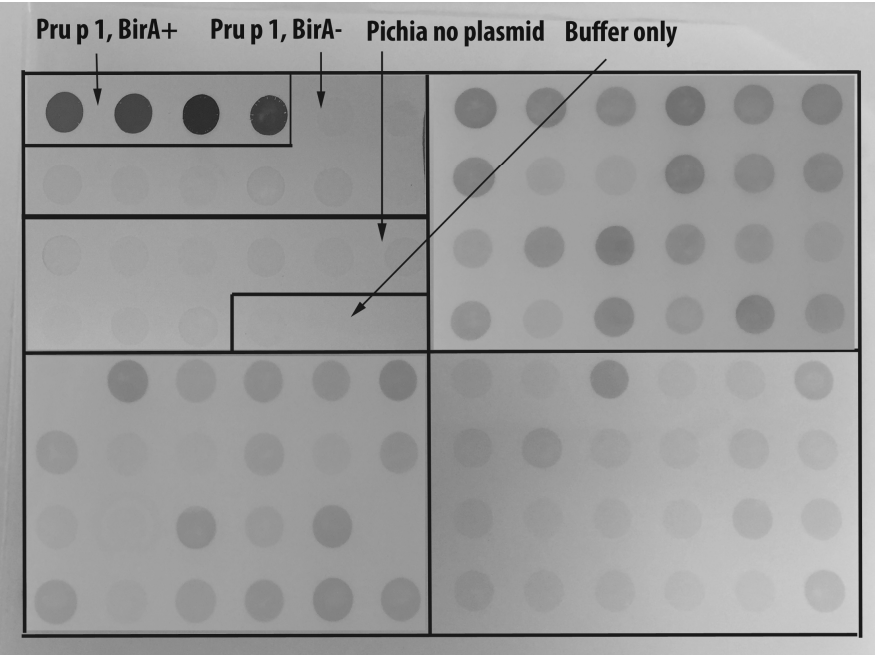


Figure 2

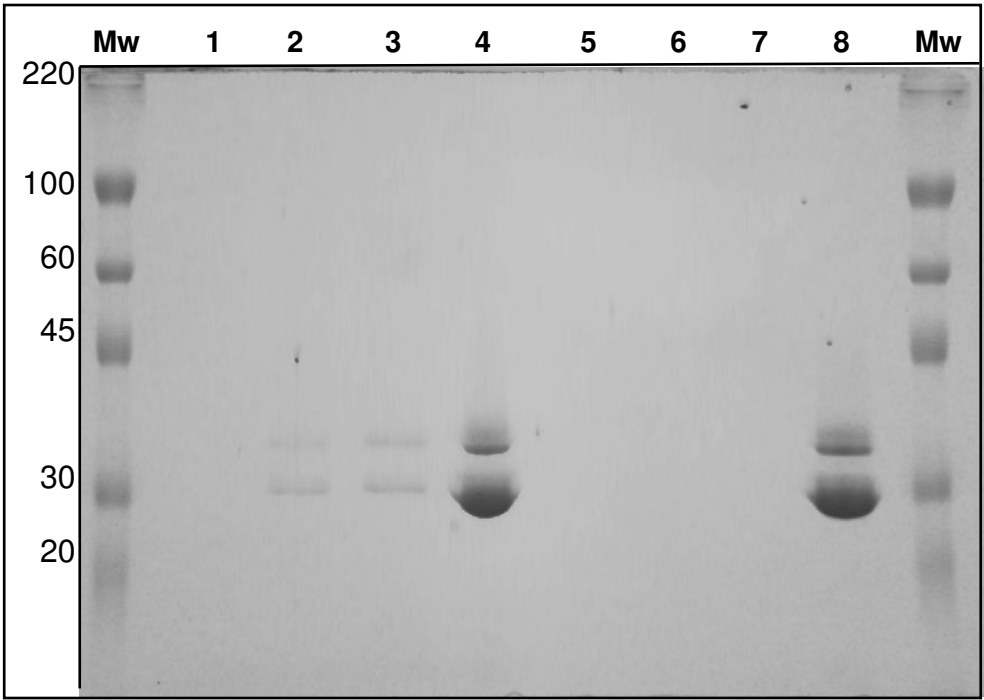


Figure 3

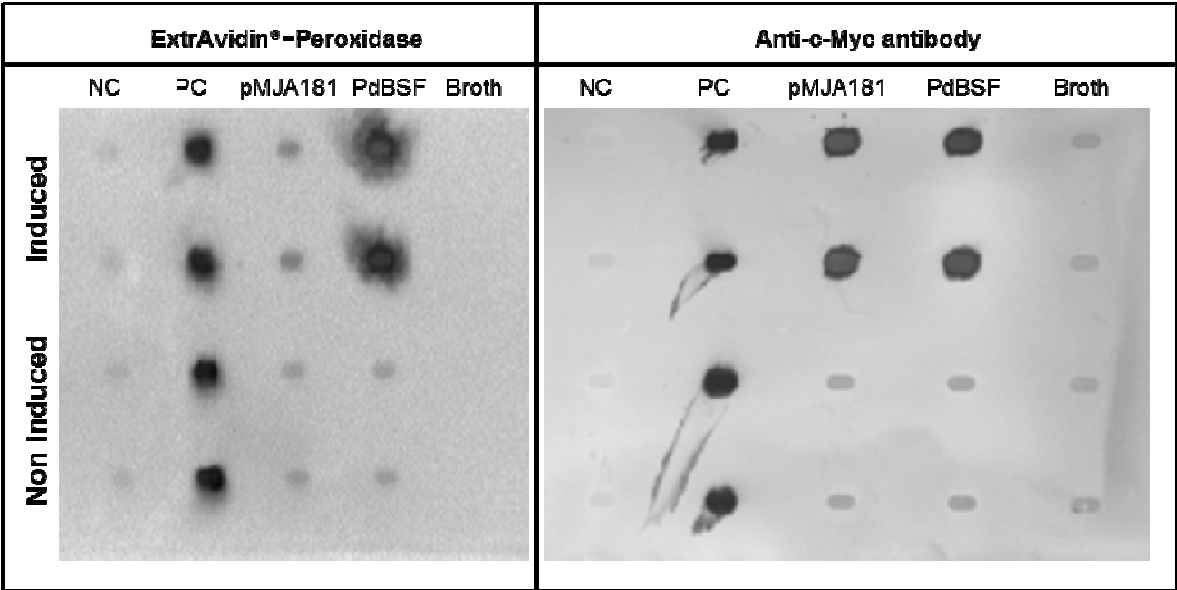


Figure 4

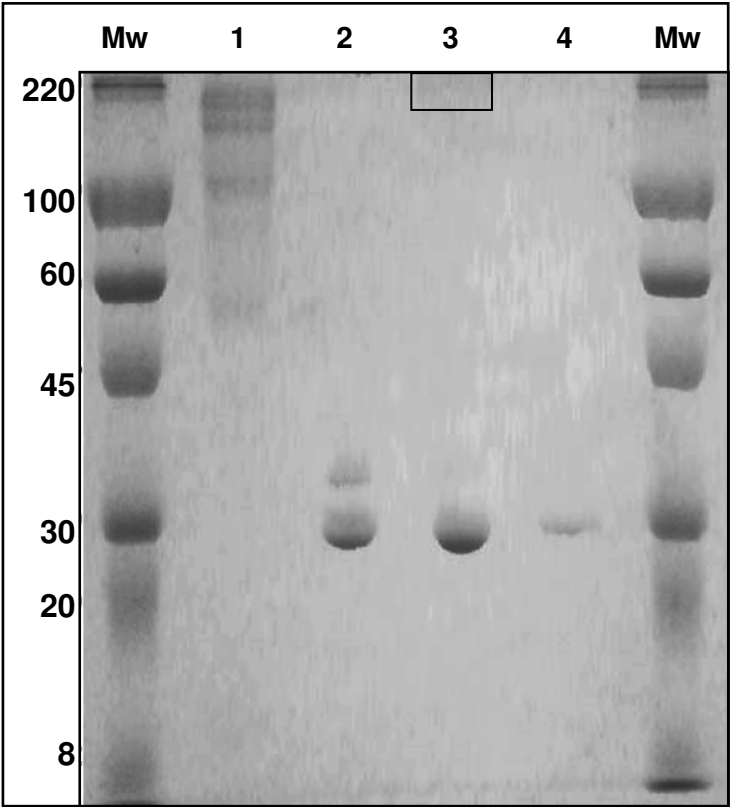
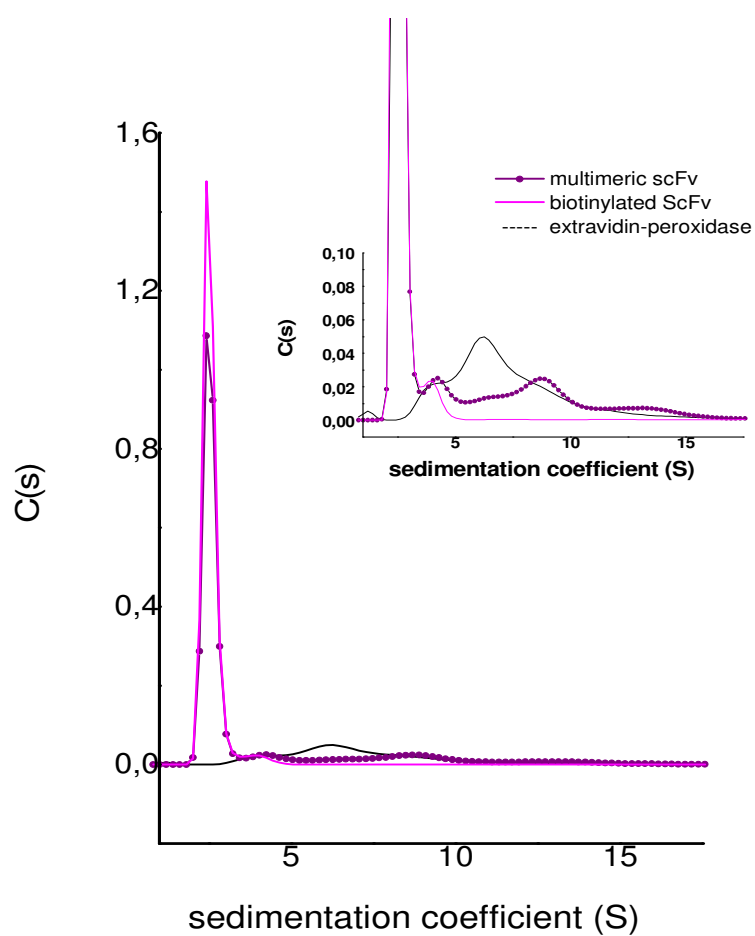


Figure 5

MRFPSIFTAV	LFAASSALAA	PVNTTTEDET	AQIPAEAVIG	YSDLEGDFDV	AVLPFSNSTN
NGLLFINTTI	ASIAAKEEGV	SLEKR <u>EAEAA</u>	<u>AEVQLLESGG</u>	<u>GLVQPGGSLR</u>	<u>LSCAASGFTF</u>
<u>SSYAMSWVRQ</u>	<u>APGKGLEWVS</u>	<u>AITSYGSDTY</u>	<u>YADSVKGR</u> ^{FT}	ISR <u>DNSKNTL</u>	<u>YLQMNSLRAE</u>
<u>DTGVYYCAKS</u>	AYDFDYWGQG	TLVTVSSGGG	GSGGGGSGGG	GSTDIQMTQS	PSSLASAVGD
RVTITCR <u>ASQ</u>	<u>SISSYLNWYQ</u>	<u>QKPGK</u> APKLL	<u>IYSASALQSG</u>	<u>VPSR</u> ^F SGSGS	GTDFTLTISS
LQPEDFATYY	CQQGASDPTT	FGQGTKVEIK	RAAAGLNDIF	EAQKIEWHEG	ALEQKLISEE
DLNSAVDHHH	HHH				

Figure 6



Sample	Peak a		Peak b		Peak 1		Peak 2		Peak3		Peak 4	
	$S_{20,W}$ (S)	Mw_{app} (kDa)	$S_{20,W}$ (S)	Mw_{app} (kDa)	$S_{20,W}$ (S)	Mw_{app} (kDa)	$S_{20,W}$ (S)	Mw_{app} (kDa)	$S_{20,W}$ (S)	Mw_{app} (kDa)	$S_{20,W}$ (S)	Mw_{app} (kDa)
extravidin-peroxidase	-	-	-	-	4.41	62.3	6.33	107	8.86	~178		
multimeric scFv	2.52	26.8	-	-	4.45	63	6.5	108	8.75	~168	13.5	~330
biotinylated scFv	2.50	27.1	3.98	54.4	-	-	-	-	-	-	-	-

Figure 7

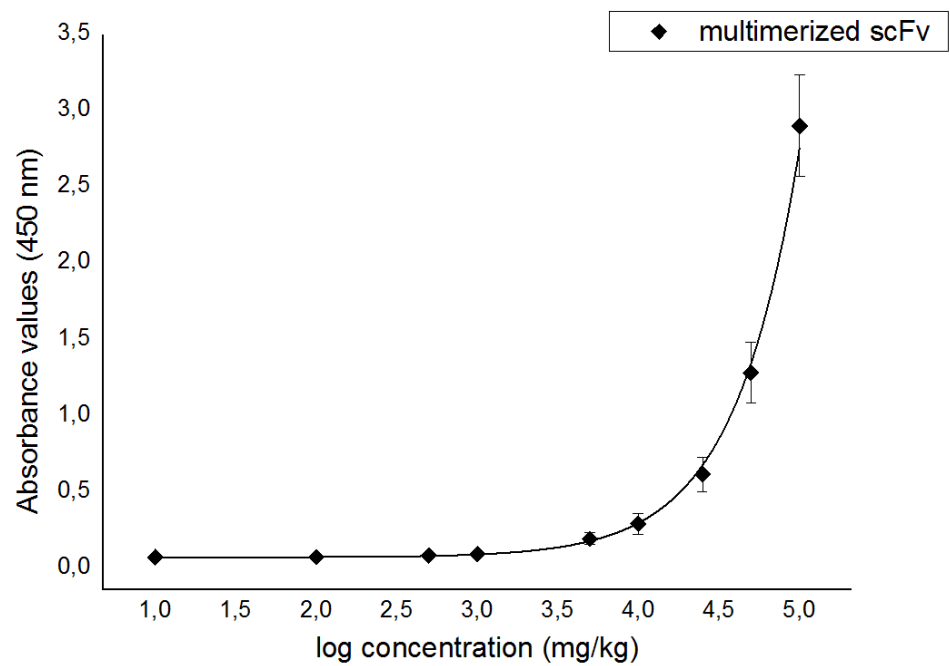


Figure captions

Figure 1: Representative screening of 24 wells expression plates after methanol induction. After induction for 72h the supernatants were dot blotted into PVDF membrane and probed with avidin-Alkaline Phosphatase. Pru p 1, a peach allergen was used as positive control. No to very low expression of Pru p 1 was detected in the absence of BirA. The other 3 panels are representative expression of different scFv clones under the BirA+ background. The selection of the higher expressers is carried out visually.

Figure 2: SDS-PAGE electrophoresis of culture supernatants from the different *P. pastoris* clones employed in this work, after methanol induction (lanes 1-3) and without induction (lanes 5-7). Lanes 1 and 5: *P. pastoris* non-transformed strain (X-33); 2 and 6: *P. pastoris* pMJA181-D8 clone; 3 and 7: *P. pastoris* PdBSF clone; 4 and 8: purified scFv. Molecular marker: ColorBurst Electrophoresis Marker (Sigma-Aldrich).

Figure 3: Dot-blotting analysis of culture supernatants produced by the different *P. pastoris* clones obtained in this work, either revealed with ExtrAvidin-peroxidase (left) or mouse monoclonal anti-c-Myc antibody (right). NC: negative control, *P. pastoris* X-33 non-transformed clone; PC: positive control, biotinylated scFv targeting walnut protein; pMJA181-D8: *P. pastoris* clone transformed with pMJA181 plasmid; PdBSF: *P. pastoris* clone co-transformed with pMJA181 and pMJA180 plasmids.

Figure 4: SDS-PAGE electrophoresis in non-reducing conditions of multimeric-scFv. Line 1: ExtrAvidin-peroxidase (Mw \approx 112 kDa); line 2: scFv (Mw \approx 30 kDa); line 3: multimeric-scFv (Mw \approx 220 kDa); line 4: flow-through recovered from Amicon Ultra-

50 Centrifugal Filter Unit. Highlighted band was excised and analyzed by MALDI-TOF/TOF.

Figure 5: ScFv amino acid sequence. Matched peptides of MS spectrum after Mascot search are highlighted to show sequence coverage obtained.

Figure 6: Distribution of the multimeric-scFv, monomeric scFv and Extravidin-Peroxidase sedimentation coefficients in PBS at 20 °C. Inset shows an amplified portion of the figure.

Figure 7: Representative standard curve of the multimerized scFv-ELISA performed with protein extracts obtained from almond/wheat flour binary mixtures. The curve shows the average value of six independent experiments and the standard deviation in each point of the curve.

Table 1: Peptides identified by MALDI-TOF/TOF Tandem Mass Spectrometry.

Protein identification	Accession number	Sequence coverage	Total score	Ion scores	Peptide sequences
Peroxidase C1A (<i>Armoracia rusticana</i>)	P00433	35%	246	49 47 61 22	R.DTIVNELR.S R.TEKDAFGNANSAR.G R.MGNITPLTGTQGQIR.L R.TVSCADLLTIAAQQSVTLAGGPSWR.V
Ig heavy chain V-III región 23 (<i>Homo sapiens</i>)	P01764	33%	84	49	K.NTLYLQMNSLR.A
pMJA181-scFv		35%	306	129 49 50	R.EAEAAAEVQLLESGGGLVQPGGSLR.L K.NTLYLQMNSLR.A K.LLIYSASALQSGVPSR.F

Table 2: Determination of the presence of almond in various commercial processed food products using multimerized-scFv ELISA and phage-ELISA.

Label statement	Product	Multimerized-scFv ELISA ^a	phage- ELISA ^b
Almond declared as ingredient	Food bar	5.29	7.1
	Breakfast cereals	2	1.8
	Chocolate	< LOD	< LOD
	Milled Flaxseed	2.5	3
	Breakfast cereals	6.56	4.5
	Nut bar	0.65	1.9
	Granola	1.82	5.4
May contain traces of tree nuts	Chocolate	< LOD	< LOD
	Biscuits	< LOD	< LOD
Almond not declared as ingredient	Chocolate	< LOD	< LOD

^a Almond concentration (expressed in w/w percentage) estimated after interpolating absorbance values obtained in ELISA in corresponding standard curves performed with binary mixtures of almond in a wheat flour matrix.

^b Results obtained following the method described in de la Cruz et al., 2015.

Supplementary material

Table A: List of primers employed in this work.

Primer	Sequence (5'→3')
MJA253	CAGATCCTCTTCTGAGATGAGTTTTTGTTTC
MJA254	AATTAACTGCAGCCGAGGTGCAGCTGTTGGAGT
MJA255	ATATTATGAATTCATGAAGGATAACACCGTGCCACTGA
MJA256	ATAATATCCGCGGTTATTTTTCTGCACTACGCAGGGATATTTC
MJA259	CACCTTCGTGCCATTCGATTTTCT
MJA257	AATTGCGGCCGCGGGTCTGAACGACATCTTCGAGGCTCAGAAAAT CGAATGGCACGAAGGTGCTCTAGAAATT
MJA258	AATTTCTAGAGCACCTTCGTGCCATTCGATTTTCTGAGCCTCGAAG ATGTCGTTTCAGACCCGCGGCCGCAATT

Figure A: pMJA181 vector containing scFv and BAD nucleotide sequences constructed in pPICZaB plasmid (Zeo^r; integrative plasmid carrying the secretion signal sequence from the *S. cerevisiae* α factor prepro-peptide and functional sites for the integration at the 5' AOX1 locus of *P. pastoris* X-33).

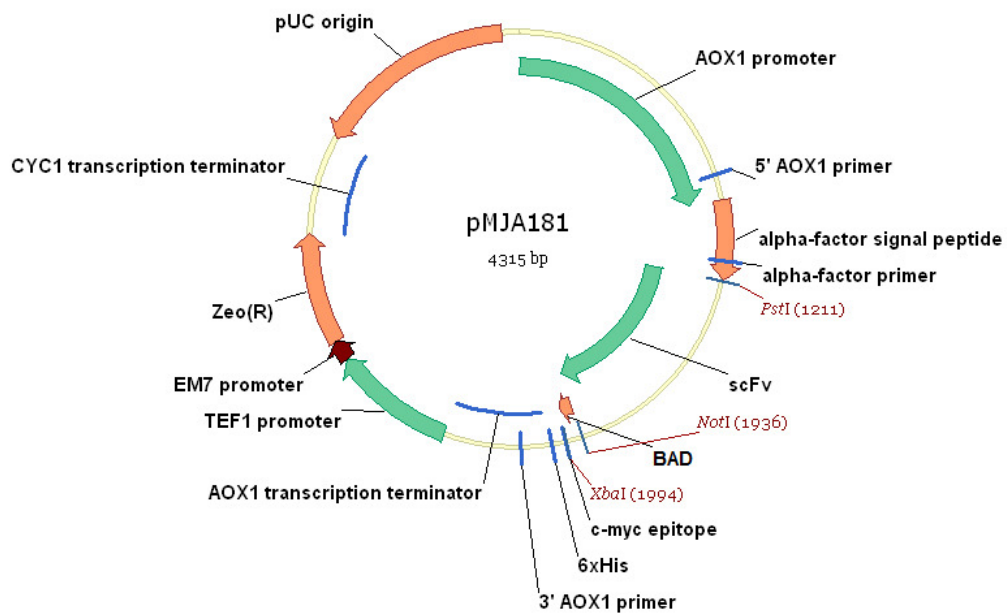


Figure B: pMJA180 vector containing BirA nucleotide sequence constructed in pPIC6 α A plasmid (Bla^r; integrative plasmid carrying the secretion signal sequence from the *S. cerevisiae* α factor prepro-peptide and functional sites for the integration at the 5' AOX1 locus of *P. pastoris* X-33).

pPIC6 α A plasmid (Bla^r; integrative plasmid carrying the secretion signal sequence from the *S. cerevisiae* α factor prepro-peptide and functional sites for the integration at the 5' AOX1 locus of *P. pastoris* X-33).

