1 **TITLE PAGE**

2 **Production of *in vivo* biotinylated scFv specific to almond (*Prunus dulcis*) proteins**

3 **by recombinant *Pichia pastoris*.**

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21   **Abstract**

22   The methylotropic yeast *Pichia pastoris* has demonstrated its suitability for large-scale

23   production of recombinant proteins. As a eukaryotic organism *P. pastoris* presents a

24   series of advantages at expression and processing of heterologous proteins when

25   compared with *E. coli*. In this work, *P. pastoris* has been used to express a scFv from a

26   human synthetic library previously shown to bind almond proteins. In order to facilitate

27   purification and post processing manipulations, the scFv was engineered with a

28   C-terminal tag and biotinylated *in vivo*. After purification, biotinylated scFv were bound

29   to avidin conjugated with HRP producing a multimeric scFv. The multimeric scFv

30   showed to maintain their ability to recognize almond protein when assayed in ELISA,

31   reaching a LOD of 470 mg kg^{-1}. This study describes an easy method to produce large

32   quantities of *in vivo* biotinylated scFv in *P. pastoris*. By substituting the enzyme or

33   fluorochromes linked to avidin, it will be possible to generate a diverse number of

34   multimeric scFv as probes to suit different analytical platforms in the detection of

35   almond in food products.

36   **Keywords**

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 been 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 ($Kd = 4 \cdot 10^{-14}$ 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 pPICZαB and pPIC6αA 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 form 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,

85   Germany). HiTrap Protein L Column was purchased from GE Healthcare Life Sciences

86   (München, Germany). Selection antibiotic Zeocin was purchased from Life

87   Technologies, and blasticidin was purchased from InvivoGen (Toulouse, France).

88   Peptone, tryptone, yeast extract, and European bacteriological agar were purchased

89   from Pronadisa (Madrid, Spain). Methanol was purchased from Fisher Scientific

90   (Loughborough, UK). Other chemicals were purchased from Sigma-Aldrich unless

91   otherwise stated.

92   *2.2. Vectors construction*

93   The pMJA179 vector was constructed as follows: nucleotide sequence encoding the

94   almond-specific PD1F6-scFv (GenBank accession no. **LN889750**) was amplified from

95   the corresponding phagemid using a high fidelity DNA polymerase with primers

96   MJA254 and MJA253 (**Table A, Supplementary material**), the former adding a new

97   *Pst*I restriction site to the scFv sequence. After PCR reaction, purified PCR product was

98   digested with *Pst*I and *Not*I. The resulting fragment was inserted between the *Pst*I and

99   *Not*I sites in the pPICZαB plasmid, following a basic protocol described in Bloch and

100   Grossmann, 1995.

101   The pMJA180 vector was constructed as follows: Genomic DNA from TOP10 *E.coli*

102   strain (Life Technologies) was extracted with a phenol/chloroform/isoamyl alcohol

103   mixture, and then precipitated with 100 % ethanol. After precipitation, DNA pellet was

104   washed with 70 % ethanol, and resuspended in buffer TE, according to the procedure

105   described in Moore and Dowhan, 2002. Nucleotide sequence codifying BirA enzyme

106   (GenBank accession no. **P06709**) was PCR amplified using primers MJA255 and

107   MJA256, the former including an *Eco*RI restriction site, the latter incorporating a *Sac*II

5

108   site. The amplified fragment was ligated between *Eco*RI and *Sac*II sites of pPIC6αA

109   plasmid.

110   The pMJA181 vector was constructed as follows: BAD sequence was obtained by

111   enforcing hybridization between reverse-phase chromatography-purified primers

112   MJA257 and MJA258. Primer sequences were obtained from Predonzani et al., 2008,

113   with some modifications to include *Not*I and *Xba*I restriction sites. After resuspending

114   primers to a final concentration of 100 μM, 25 μL of each primer were mixed, heated at

115   95 ºC for 10 min, and cooled down to room temperature. The procedure was repeated 3

116   times. Hybridized BAD nucleotide sequence was then digested with *Not*I and *Xba*I, and

117   inserted between the *Not*I and *Xba*I sites in the pMJA179 vector. Correct orientation of

118   the insert (scFv + BAD) was assessed by DNA sequencing with primers pMJA254 and

119   pMJA259 at Source BioScience (Nottingham, UK).

120   To direct the scFv+BAD and the BirA enzyme into the secretory pathway, the codifying

121   sequences were inserted in frame with the methanol inducible 5'-AOX1 promoter, the

122   α-factor secretion signal and the AOX1 transcription terminator.

123   *2.3. Transformation of E. coli*

124   Transformation of *E. coli* cells was performed according to manufacturer's protocol.

125   Once transformed, cells were spread on pre-warmed low salt Luria-Bertani agar plates

126   (1 % tryptone, 0.5 % yeast extract, 0.5 % NaCl, 1.5 % agar, pH 7.5) containing the

127   selective antibiotic (25 μg mL^{-1} Zeocin for plasmids pMJA179 and pMJA181, and

128   100 μg mL^{-1} blasticidin for plasmid pMJA180). Plates were incubated overnight (o/n) at

129   37 ºC.

130   *2.4. Transformation of P. pastoris*

131   The pMJA181 expression vector was linearized by *Sac*I digestion, ethanol precipitated,

132   and transformed into *P. pastoris* X-33 with a BioRad MicroPulser electroporation

133   apparatus, following the manufacturer's instructions (Bio-Rad, Hemel Hempsted, UK).

134   Transformed cells were grown on Yeast Extract Peptone Dextrose Sorbitol Medium

135   (YPDS) agar plates (1 % YE, 2 % peptone, 2 % dextrose, 1 M sorbitol, 2 % agar) with

136   100 μg mL^{-1} of Zeocin for 72 h at 30 ℃. Ninety-five isolated colonies were inoculated

137   in 200 μL of Yeast Extract Peptone Dextrose Medium (YPD) with 100 μg mL^{-1} of

138   Zeocin, and grown o/n at 30 ℃ with shaking (200 rpm) to be screened for scFv

139   production. Next day, 20 μL of each clone was inoculated in 1 mL of Buffered

140   Glycerol-complex Medium (BMGY) (1% yeast extract, 2% peptone, 100 mM

141   potassium phosphate pH 6.0, 1.34 % YNB, 1 % glycerol, $4·10^{-5}$ % biotin) in 24-well

142   plates (Costar, Corning Life Sciences, NY, USA) with 100 μg/mL Zeocin. Cells were

143   incubated o/n at 30 ℃ with shaking. Next day, plates were centrifuged (1800 g, 10 min,

144   4 ℃), supernatant was removed, and cells were resuspended in Buffered Methanol-

145   complex Medium (BMMY) (1% yeast extract, 2% peptone, 100 mM potassium

146   phosphate pH 6.0, 1.34 % YNB, 1 % methanol, $4·10^{-5}$ % biotin) to induce scFv

147   expression. Methanol (1 % v/v) was replenished every 12 h for 72 h. Finally, plates

148   were centrifuged (1800 g, 10 min, 4 ℃) and the supernatant was analyzed by dot-

149   blotting analysis in search for higher expressers scFv clones.

150   Appropriate production of scFv by the selected clone was assessed by SDS-PAGE,

151   using 12% resolving polyacrylamide gel, pH 8.8 but without boiling the sample buffer.

152   Electrophoresis was run at constant voltage (150 V) using a Mini-Protean Tetra Cell

153   (Bio-Rad).

154   *2.5. Dot-blotting analysis*

155   Five hundred microliters of supernatant from 95 selected *P. pastoris* clones were

156   filtered through a dot blot microfiltration unit (Life Technologies) to coat a PVDF

157   membrane (Immun-Blot, Bio-Rad). Membrane was then blocked with 3 % BSA (w/v)

158   TBS (50 mM Tris-HCl, 150 mM NaCl, pH 7.5) at 37 ºC for 1h. After washing the

159   membrane 3 times for 5 minutes with TBST (0.05 % v/v Tween-20), it was incubated

160   with anti-c-myc antibody (Sigma-Aldrich, SKU M5546) (1:100 v/v) in 1 % BSA (w/v)

161   TBST at 37 ºC for 2 h. After washing 3 times with TBST, the membrane was incubated

162   for 2 h at 37 ºC with a goat-anti-mouse IgG-ALP antibody (Sigma-Aldrich, SKU

163   A3562) (1:20000 v/v) diluted in 1 % BSA (w/v) TBST. Then, the membrane was

164   washed 3 times with TBST and once with distilled water. Finally, a ready-to-use

165   solution of 5-bromo-4-chloro-3-indolyl-1-phosphate and nitroblue tetrazolium (Novex

166   AP Chromogenic Substrate, Life Technologies) was used to detect the alkaline

167   phosphatase enzyme activity. Reaction was stopped by rinsing the membrane with

168   water.

169   *2.6. In vivo biotinylation of scFv*

170   One of the best scFv-expresser clones was chosen based on the intensity of the signals

171   obtained in the blotting membrane from the different supernatants analyzed. The

172   selected clone was transformed with the second *P. pastoris* expression vector, pMJA180,

173   and transformed cells were grown on YPDS agar plates containing 100 μg mL^{-1} Zeocin

174   and 500 μg mL^{-1} blasticidin. After 72 h, isolated colonies were picked from the selective

175   agar plate, induced with methanol following the microscale induction described before,

176   and supernatants were once again analyzed by dot-blotting to check for the presence of

177   biotinylated scFv. Dot-blotting was carried out as stated before, but incubating the

178   membrane with Avidin-Alkaline Phosphatase (Sigma-Aldrich, SKU A7294) (1:100,000

179 v/v) in 1% BSA, or alternatively with ExtrAvidin-Peroxidase (Sigma-Aldrich, SKU

180 E2886) (1:5000 v/v) in 1% BSA, and developing the membrane with a

181 chemiluminescent substrate (Clarity Western ECL, Bio-Rad).

182 One clone called PdBSF (*Prunus dulcis Biotinylated Soluble Fragment,* PdBSF), which

183 showed high color intensity in the dot-blotting analysis, was used hereafter. The

184 insertion of both plasmids was assessed by PCR with the primer pairs MJA254/MJA259

185 and MJA255/ MJA256. To that end, genomic DNA from PdBSF clone was isolated

186 following the procedure described by Harju et al., 2004, with several modifications.

187 Briefly, a single well-grown colony was resuspended into 200 μL of lysis buffer (10

188 mM Tris, 1 mM EDTA, 100 mM NaCl, 1 % (w/v) SDS, 2 % (v/v) Triton X-100, pH

189 8.0). Then, tubes were frozen at -80 ℃ for 2 min, and boiled for 1 min. This procedure

190 was repeated twice. Two hundred microliters of chloroform was added to each sample,

191 and tubes were vortexed for 2 min and centrifuged for 3 min at high speed. The aqueous

192 layer was transferred to a new tube, and DNA was precipitated with ethanol.

193 Once the insertion of both expression plasmids was confirmed, PdBSF was inoculated

194 into 10 mL of YPD with 100 μg mL^{-1} Zeocin and 500 μg mL^{-1} blasticidin, and

195 incubated o/n at 30 ℃. Next day, 1 mL of this culture was inoculated in 600 mL

196 Buffered Glycerol-complex Medium (BMGY) (1 % YE, 2 % peptone, 100 mM

197 potassium phosphate, pH 6.0, 1.34 % YNB, 4·10^{-5} % biotin, 1 % glycerol) containing

198 100 μg mL^{-1} Zeocin and 500 μg mL^{-1} blasticidin, and incubated for 18 h at 30 ℃ with

199 shaking. Then, it was centrifuged at 4000 g for 15 min at 4 ℃ and resuspended in

200 600 mL Buffered Methanol-complex Medium (BMMY) (1 % YE, 2 % peptone, 100

201 mM potassium phosphate, pH 6.0, 1.34 % YNB, 8·10^{-5} % biotin, 1 % methanol).

202 Methanol was replenished every 12 h for 72 h. Finally, the induced culture was

203 centrifuged at 4000 g for 20 min at 4ºC to remove yeast cells, and the biotinylated scFv

204 purified from the supernatant as described in section *2.7*.

205 *2.7. Purification of biotinylated scFv*

206 The supernatant containing biotinylated scFv was filtered through a 0.4 µm membrane

207 filter (Millipore, Darmstadt, Germany) and loaded onto a 1x1 mL HiTrap protein L

208 column (GE Healthcare Life Sciences) attached to an ÄKTA purifier FPLC system (GE

209 Healthcare, Sweden). Chromatography was performed as described by Rouet et al.,

210 2012 with several modifications. Briefly, 300 mL of supernatant was loaded onto the

211 column previously equilibrated with 10 mL of PBS buffer (0.01 M phosphate buffer,

212 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4). The column was

213 then washed with 20 mL of PBS, and the biotinylated scFv eluted with 10 mL of 0.1 M

214 glycine-HCl (pH 2.7). Fractions showing OD_{280} above 0.05 were manually collected in

215 1.5 mL microcentrifuge tubes prefilled with 400 mL of 200 mM Tris-HCl (pH 8.0).

216 Flow rate was maintained at 1 mL min^{-1}. The entire process was repeated with the

217 remaining 300 mL of supernatant.

218 Recovered fractions were pooled and dialyzed against PBS buffer employing Amicon

219 Ultra-15 Centrifugal Filter Units (Millipore) with a MWCO of 10 kDa. Protein

220 concentration was measured in a Nanodrop (Thermo Scientific, Waltham, MA, USA),

221 adjusted to 2 mg mL^{-1} of total protein, and stored in 100 µL aliquots at -80 ºC until

222 further use.

223 *2.8. Multimerization of biotinylated scFv*

224 Multimerization was performed following the guidelines provided by the NIH Tetramer

225 Core Facility (http://tetramer.yerkes.emory.edu/support/protocols#10), using as a core a

226   molecule of avidin conjugated with peroxidase (ExtrAvidin-HRP, Sigma-Aldrich), at a

227   concentration of 2.5 mg/mL, with a molar ratio (ExtraAvidin: peroxidase) of ≈ 0.7.

228   Briefly, 0.5 µL avidin solution was added every 10 minutes up to a total of 10 times to

229   an aliquot of 200 µg of biotinylated scFv. The procedure was carried out at room

230   temperature in the dark, and with continuous but gentle rotation in a sample mixer

231   (HulaMixer Sample Mixer, Life Technologies). After multimerization, tubes were kept

232   in the dark at 4 ℃ until further use.

233   *2.9. ScFv multimerization assessment*

234   After multimerization, scFvs were concentrated using Amicon Ultra 50 kDa filtration

235   unit (Merck Millipore, Darmstadt, Germany) and analysed by SDS-PAGE 12% in non-

236   reducing conditions. Electrophoresis gel was stained with Coomassie Brilliant Blue R-

237   250. Gel bands of interest were cut out with a scalpel and immersed in a solution 5 %

238   (v/v) of acetic acid. Peptide mass fingerprinting was performed using a 4800 Plus

239   MALDI TOF/TOF Analyser mass spectrometer (AB SCIEX, MA, USA), at the Unidad

240   de Proteómica at Parque Científico de Madrid (Spain). Mascot search engine software

241   (http://www.matrixscience.com) was employed to interpret mass spectra data into

242   protein identities using the SwissProt database. Search parameters employed were:

243   trypsin enzymatic cleavage, one possible missed cleavage allowed; peptide mass

244   tolerance of ± 80 ppm; fragment mass tolerance of ±0.3 Da; peptides were assumed to

245   be monoisotopic; carbamidomethyl fixed modification; and methionine oxidation

246   variable modification.

247   To further assess scFv multimerization, ultracentrifugation analyses were carried out.

248   Sedimentation velocity experiments were carried out at 45000 rpm in an OptimaXL-A

249   analytical ultracentrifuge (Beckman Coulter Inc., Fullerton, CA), using an AN50Ti

250　rotor and standard cells with double-sector epon-charcoal centrepieces. Measurements

251　were performed in PBS at 20 ℃ with 400 μL of sample at protein concentrations

252　absorbing 0.6 OD (1.2 cm optical pathway). Differential sedimentation coefficients

253　were calculated by least-squares boundary modelling of the experimental data and

254　corrected to $s_{20,w}$ values with the program SEDFIT (Schuck, 2000), using a partial

255　specific volume of 0.73 mL g-1. Solvent density and viscosity at 20 ℃ were computed

256　using the SEDNTERP program (Laue et al., 1992). The experiments were performed at

257　Instituto de Química-Física Rocasolano, CSIC, Madrid (Spain).

258　*2.10. Preparation of protein extracts*

259　Binary mixtures of wheat flour containing Marcona cultivar (100 000 to 10 mg kg^{-1})

260　were prepared as described elsewhere (de la Cruz et al., 2015). Protein extracts from

261　binary mixtures and commercial food products were prepared by adding 200 mg of

262　milled sample to 1800 μL of protein extraction buffer (0.035 M phosphate solution

263　containing 1 M NaCl, pH 7.5.). After shaking for 10 min at room temperature to

264　facilitate the extraction of soluble proteins, the slurry was centrifuged at 10 000 g for 10

265　min at 4 ℃, and the supernatant was filtered through a 0.45 µm syringe filter (Sartorius,

266　Göttingen, Germany). Bicinchoninic acid (BCA) assay (Thermo Fisher Scientific Inc.,

267　IL, USA) was used to determine protein concentration. Protein extracts were kept at

268　-20 ℃ until further use.

269　*2.11. Indirect ELISA with multimerized scFv*

270　The ability of multimerized scFv to recognize almond protein was assessed through

271　indirect ELISA. One hundred microliters of protein extract from binary mixtures or

272　food samples (diluted 1:200 in PBS) was used to coat 96-multi-well polystyrene plates

273   (F96 MaxiSorp, Nunc immune plates, Nunc, Denmark). Plates were incubated o/n at

274   4 ºC. Next day, plates were washed 3 times with TBST. Wells were blocked with 3 %

275   BSA (w/v) in TBS for 1 h at 37 ºC. After another washing step, 100 μL of 2 μg mL^{-1}

276   multimerized scFv diluted in 1 % BSA in TBST was added to each well. Plates were

277   incubated at 37ºC for 2 h. After incubation, plates were washed 3 times with TBST and

278   once with distilled water. Then, 100 μL of tetramethylbenzidine (TMB) substrate

279   solution was added to each well, and incubated in the dark. Color development was

280   performed for 10 min at room temperature, and reaction was stopped with 1 M

281   sulphuric acid. OD_{450} was measured with an iEMS Reader MF (Labsystems, Helsinki,

282   Finland). All experiments were performed in triplicate. As negative controls, different

283   wells coated with protein extracts were also incubated with 2 μg mL^{-1} of monomeric

284   scFv (without ExtrAvidin) or with 0.125 μg mL^{-1} of ExtrAvidin-HRP (without scFv) to

285   check for non-specific reactions.

286   A calibration curve of different concentrations of almond in wheat flour (100 000 to 10

287   mg kg^{-1}) was included in each plate. Standard curve was obtained by plotting the

288   absorbance values *vs* the log of almond protein concentration, and it was fitted to an

289   exponential decay curve using Origin 8.0 software (OriginLab Crop., USA).

290   **3. Results and discussion**

291   *3.1. Vectors construction and co-transformation into P. pastoris*

292   *P. pastoris* can direct heterologous proteins either to the cytoplasm (intracellular) or to

293   secrete them into the culture medium. For proteins to be secreted, the expressed protein

294   must contain a signal sequence targeting the secretory pathway (Cregg et al., 2000). In

295   this work, recombinant proteins were expressed as fusions to the secretory N-terminal

296 sequence of *S. cerevisiae* α-mating factor prepro-peptide. Furthermore, the vectors used

297 induce the integration of the construct into *P. pastoris* genome, thus conferring genetic

298 stability of the recombinant elements (Macauley-Patrick et al., 2005).

299 By engineering a BAD sequence into the C-terminus of the scFv (plasmid pMJA181,

300 **Figure A, Supplementary material**) a potential biotinylation site has been created. In

301 addition to BAD sequence, the scFv expressed by the *P. pastoris* clones contained a *c-*

302 *myc* epitope (EQKLISEEDL) and a poly histidine tail. Thus, it was possible to assess

303 the production of soluble scFv by dot-blotting analysis of the supernatants coated to

304 PVDF membrane by detecting them with an anti-c-myc antibody. The screening of high

305 expresser clones is an essential step in *Pichia* as random and multiple plasmid

306 integration events can result in variable expression levels of heterologous proteins

307 (Nordén et al., 2011; Zhu et al., 2009). Ninety-five *P. pastoris* clones transformed with

308 pMJA181 were induced to produce scFv, and their supernatants checked by dot-blotting

309 analysis to assess the production of soluble fragments. After developing the membrane

310 with a chromogenic substrate, 55 spots (57% of clones) showed high color intensity,

311 being indicative of a correct scFv expression. One of those 55 clones (named pMJA181-

312 D8) was randomly selected to prepare competent cells to proceed with incorporation of

313 the BirA codifying sequence after the second transformation with pMJA180 vector

314 (**Figure B, Supplementary material**). It is noteworthy to mention that Zeocin resistant

315 clones somehow resistant to usual doses of blasticidin employed in yeast were observed.

316 Therefore, to ensure the selection of co-transformed clones, blasticidin concentration

317 had to be increased up to 500 μg mL^{-1}. The level of antibiotic resistance reflects the

318 recombinant gene dosage of transformed *P. pastoris* (Nordén et al., 2011), so it is usual

319   to find that the best *P. pastoris* expressors also show higher antibiotic resistance

320   (Arbulu et al., 2015; Jiménez et al., 2014).

321   *3.2. Expression and purification of biotinylated scFv*

322   After the re-screening and selection of the best in vivo biotinylated clone (PdBSF)

323   (**Figure 1**), the presence of the scFv and BirA codifying sequences were confirmed by

324   PCR with a 780 kb band for scFv linked to BAD nucleotide sequence, and a band of

325   about 975 kb, corresponding to BirA nucleotide sequence. Moreover, pMJA181-D8

326   clone only possessed pMJA181 vector, and, as expected, the non-transformed *P.*

327   *pastoris* strain showed no DNA amplification with these pairs of primers.

328   To produce large yields of biotinylated scFv, *P. pastoris* cells were grown in buffered

329   media (BMGY and BMMY) as pH values of 6.5-8.0 were described as optimum for

330   scFv production (Shi et al., 2003), and the addition of peptone to the culture medium

331   may enhance product stability through repression of protease induction caused by

332   nitrogen limitation (Macauley-Patrick et al., 2005).

333   Concomitant transcription of two plasmids would theoretically allow PdBSF clone to

334   express both the scFv and the BirA enzyme. Therefore, BirA present in supernatant

335   would catalyze the binding of a biotin molecule to the acceptor peptide fused to the

336   scFv, resulting in a straightforward *in vivo* biotinylated scFv. Here, i*n vivo* biotinylation

337   is presented as an alternative to *in vitro* biotinylation, which requires the previous

338   production and purification of the enzyme, as described elsewhere (Li and Sousa, 2012).

339   On the other hand, the presence of a BAD fused to the scFv provides a specific substrate

340   for BirA to be selectively biotinylated, avoiding biotinylation alternatives such as amine

341   coupling methods, that could decrease the antigen-binding activities (Kumada, 2014).

342   In order to characterize these products SDS-PAGE of culture supernatants (**Figure 2**)

343   was carried out and showed the production of 30-35 kDa proteins when clones

344   pMJA181-D8 and PdBSF were induced with methanol (lanes 2 and 3), thus

345   demonstrating the capability of transformed clones to produce the scFv. Protein

346   concentration of those bands greatly increased after purification of scFv (lanes 4 and 8).

347   However, when transformed clones were not methanol induced, bands indicating the

348   presence of the scFv were absent. Dot-blotting membranes coated with supernatants

349   from pMJA181-D8 and PdBSF clones after methanol induction (**Figure 3, right**) and

350   developed with anti-c-myc antibody, confirmed the ability of both transformed clones to

351   produce scFv. On the other hand, dot-blotting analysis of the same supernatants

352   developed with ExtrAvidin-Peroxidase (**Figure 3, left**) showed that only the scFv

353   produced by the co-transformed *P. pastoris* clone (*i.e.* PdBSF clone) was biotinylated,

354   thus confirming the co-expression and proper function of the BirA enzyme, and the

355   effective production of biotinylated scFv by a single *P. pastoris* clone.

356   Supernatant containing biotinylated scFv was purified by affinity chromatography. The

357   columns used (HiTrap protein L) contain an agarose matrix combined with recombinant

358   protein L, which presents affinity towards the variable region of the kappa light chain of

359   immunoglobulins and immunoglobulin fragments (Malpiedi et al., 2013; Muzard et al.,

360   2009; Zheng et al., 2012). The purification process rendered 30 mg L^{-1} of biotinylated

361   scFv that were distributed in 100 μL aliquots of 2 mg mL^{-1}.

362   *3.3. Multimerization assessment*

363   To be used in ELISA, biotinylated scFv antibodies were fused to a core of ExtrAvidin-

364   HRP to obtain mutimeric scFv. SDS-PAGE in non-reducing conditions of mutimeric

365   scFv (**Figure 4**) showed a subtle band with a molecular weight of about 220 kDa, which

16

might coincide with the expected size of the tetramers ($\approx$ 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).

389 Phage display is a reliable tool to isolate antibody fragments from highly diverse

390 antibody libraries. However, one of the drawbacks of selected antibodies is the lack of

391 affinity maturation undergone by classical antibodies raised in animals. A typical

392 strategy to improve antibody affinity entails the introduction of additional mutations to

393 the specific phage binders, in a process that usually involves antibody engineering

394 techniques such as error-prone PCR and CDR shuffling (Kobayashi and Oyama, 2011).

395 In this sense, the multimerization of scFv on avidin or streptavidin is presented as an

396 alternative to increase antibody's functional affinity (Cloutier et al., 2000; Kipriyanov et

397 al., 1995).

398 Indirect ELISA using multimerized scFv was able to detect almond protein in the wheat

399 flour binary mixtures assayed, with absorbance values increasing in a concentration-

400 dependent manner, down to 500 mg kg^{-1}. The limit of detection (LOD) achieved with

401 multimerized scFv-ELISA was of 470 mg kg^{-1}. The representative standard curve

402 obtained is shown in **Figure 7**. Results also confirmed that despite the variation in size

403 (due to presence of BAD peptide), scFv expressed in *P. pastoris* maintained their

404 functionality. However, it should be noted that the monomeric scFv did not show

405 positive signal when tested in a parallel assay employing the anti-c-myc antibody raised

406 in mouse, and the anti-mouse antibody conjugated with alkaline phosphatase as the

407 detection antibodies, and SigmaFast p-Nitrophenyl phosphate tablets substrate,

408 revealing that monomeric soluble fragments lacked the ability to detect almond proteins

409 when employed in ELISA (in these conditions, multimeric scFv were still functional).

410 To confirm the results, monomeric scFv were also tested in dot-blotting, with the same

411 negative results (data not shown).

412   To confirm whether the multimeric-scFv still possessed the same specificity to detect

413   almond protein in foodstuffs that exhibited its phage counterpart, 10 of the 92

414   commercial food products previously assayed (de la Cruz et al., 2015) were selected and

415   tested. Among the samples analyzed, 7 declared to contain almond as ingredient, two

416   declared the possibility of containing traces of tree nuts and the last one did not declared

417   almond as ingredient (**Table 2**). The ELISA results obtained with multimeric scFv were

418   in accordance with the ones obtained with the phage-scFv ELISA. However, as ELISA

419   performed with multimerized scFv is faster and requires less handling, it would be a

420   more appropriate method to be used when a large number of samples is to be analyzed.

421   In the recent years, the use of soluble scFv expressed in *P. pastoris* system has been

422   proposed for detection of different biomolecules, such as Metolcarb (an insecticide),

423   and heart failure or tumor biomarkers (Cai et al., 2014; Maeng et al., 2012; Sommaruga

424   et al., 2011). Moreover, *P. pastoris* has been used to produce modified scFv molecules,

425   like scFv-Fc fusion proteins aimed to detect rabies antigen (Wang et al., 2012) and T

426   cell leukemia lymphoma CD25 marker (Wan et al., 2013), or to express anti-keratin 8

427   divalent scFv (sc(Fv)2) antibodies (Jafari et al., 2011).

428   *In vivo* biotinylation of scFv was initially described by Cloutier et al., 2000, who

429   produced "streptabodies" in an *E.coli* strain carrying the plasmid encoding the BirA

430   enzyme after transformation with a vector encoding a scFv linked to BAD. However,

431   due to the presence of N-terminal leader peptide (pelB), scFv expressed in *E.coli* are

432   driven to periplasmic compartment, where they can aggregate as a result of high protein

433   concentration (Lowe et al., 2011). In a different study, Predonzani et al., 2008 reported a

434   bigenic plasmid that allowed the co-expression of a BAD fused scFv and the BirA

435   enzyme in mammalian cells (HEK293 and HEK293T/17). Nevertheless, the advantage

436 of replacing mammalian cells with *Pichia pastoris* lies in the latter not requiring a

437 complex growth medium or culture conditions, its easy genetic manipulation, and

438 potential for a large scale production at high cell density (Frenzel et al., 2013).

439 In this work, we report for the first time the *in vivo* biotinylation of a scFv expressed in

440 a single *P. pastoris* clone co-transfected with two different expression vectors. The

441 system allows the production of large quantities of biotinylated scFv that once purified,

442 are multimerized using an avidin-HRP core. The large complex maintained the ability to

443 recognize the target almond protein in food products down to 470 ppm when used in

444 indirect ELISA. Because the scFv multimers have demonstrated to be functional, it is

445 possible to produce multimers on avidin molecules with suitable modifications

446 (fluorophores, magnetic particles, etc.), so they can be used as probes in biosensors or

447 microarrays aimed to detect proteins in complex food matrixes.

448 **Abbreviations used**

449 AP: alkaline phosphatase; AOX: alcohol oxidase; BAD: biotin acceptor domain; BCA:

450 bicinchoninic acid; BirA: biotin ligase; CDR: complementary determining regions;

451 HRP: horse radish peroxidase; LOD: limit of detection; MWCO: molecular weight cut

452 off; ScFv: single chain variable fragment.

453 **Acknowledgements**

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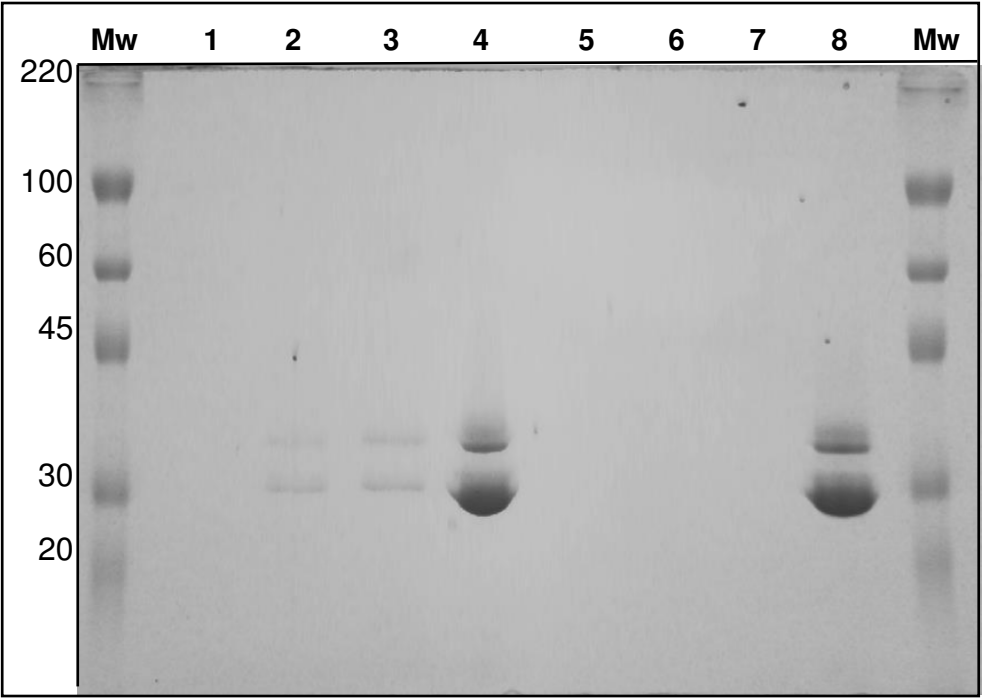618 insulin precursor in yeast *Pichia pastoris*. J. Appl. Microbiol. 107, 954–963,

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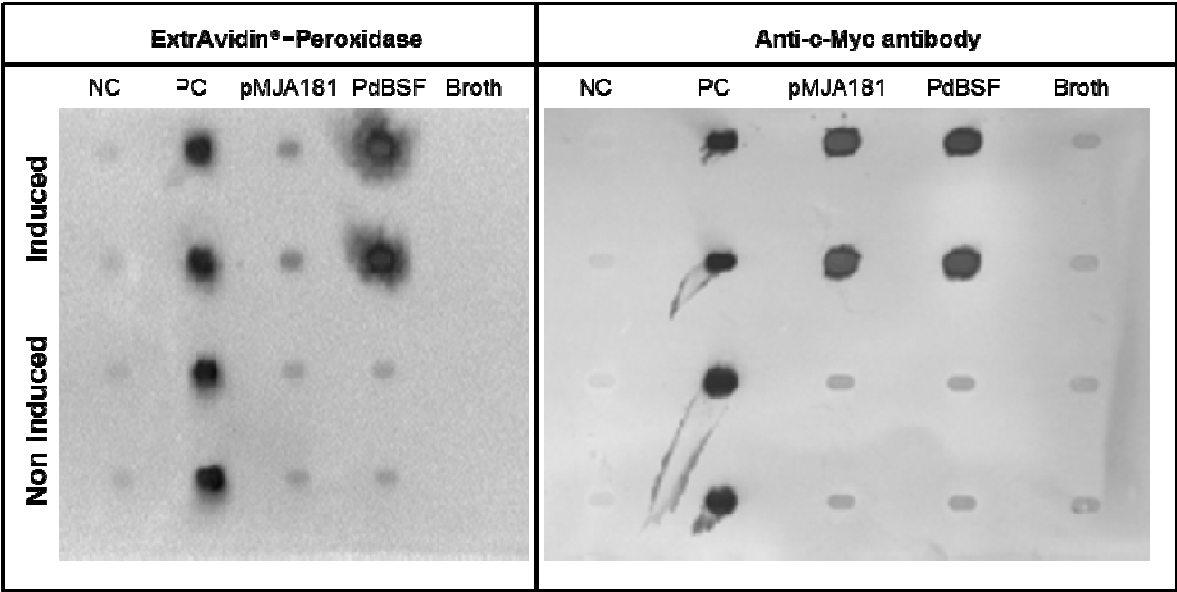620

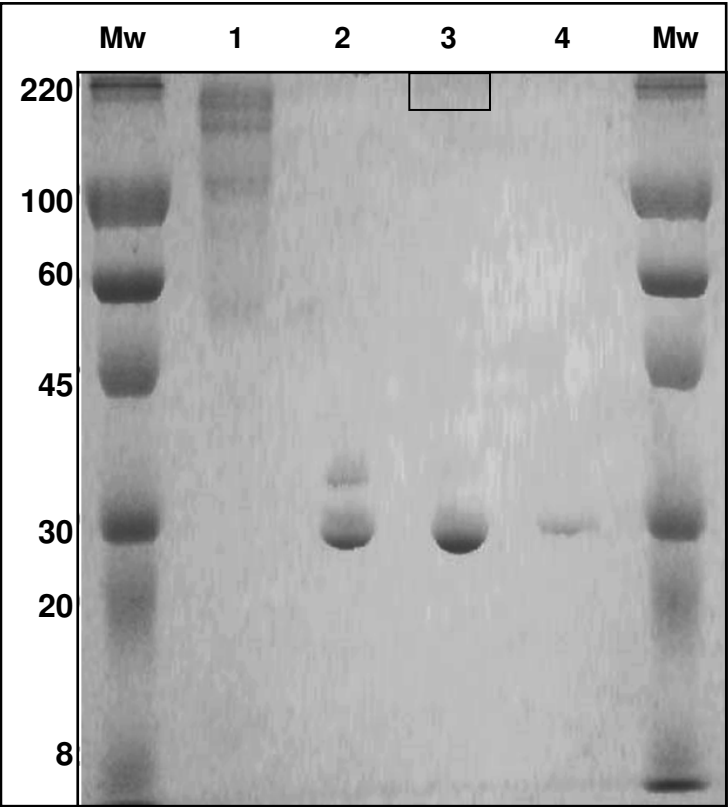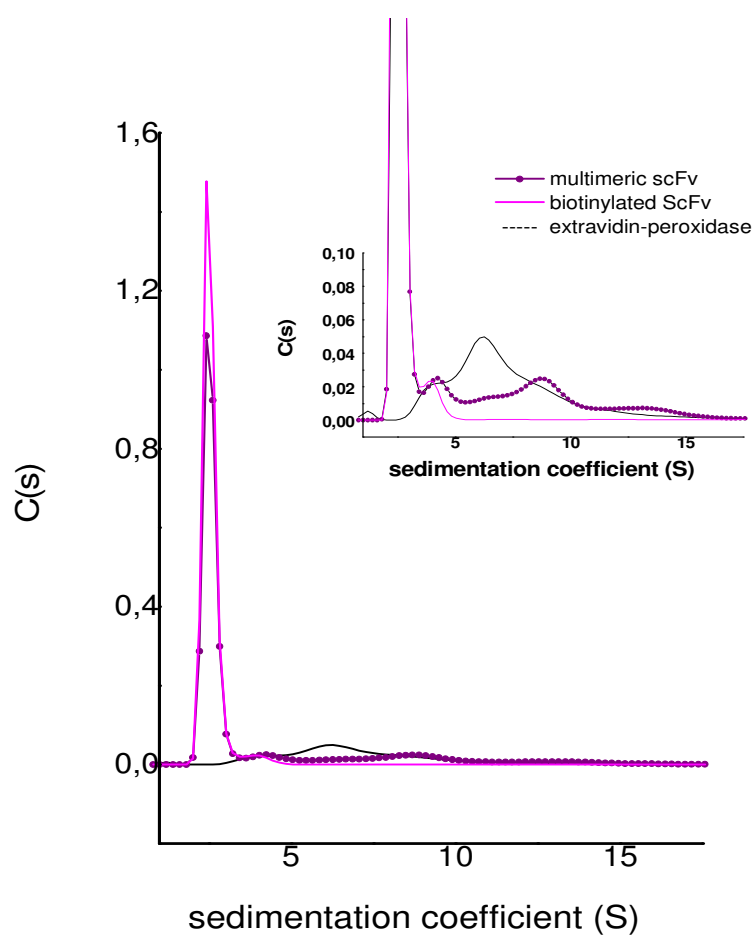**Figure 1**

Figure 2

Figure 3

Figure 4

Figure 5

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MRFPSIFTAV LFAASSALAA PVNTTTEDET AQIPAEAVIG YSDLEGDFDV AVLPFSNSTN
NGLLFINTTI ASIAAKEEGV SLEKREAEAA AEVQLLESGG GLVQPGGSLR LSCAASGFTF
SSYAMSWVRQ APGKGLEWVS AITSYGSDTY YADSVKGRFT ISRDNSKNTL YLQMNSLRAE
DTGVYYCAKS AYDFDYWGQG TLVTVSSGGG GSGGGGSGGG GSTDIQMTQS PSSLSASVGD
RVTITCRASQ SISSYLNWYQ QKPGKAPKLL IYSASALQSG VPSRFSGSGS GTDFTLTISS
LQPEDFATYY CQQGASDPTT FGQGTKVEIK RAAAGLNDIF EAQKIEWHEG ALEQKLISEE
DLNSAVDHHH HHH
```

Figure 6

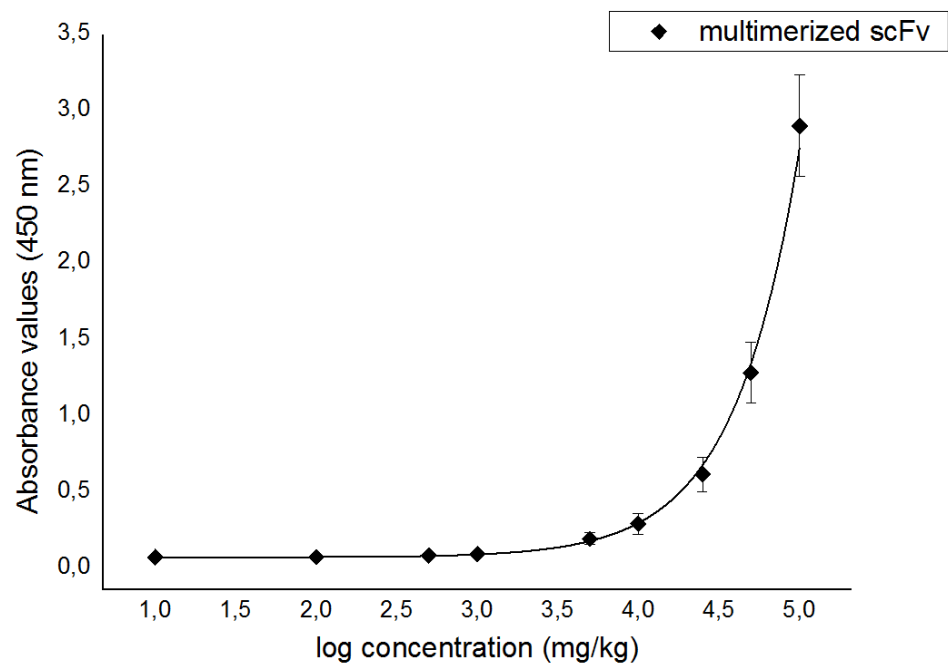|	Peak a		Peak b		Peak 1		Peak 2		Peak3		Peak 4	
Sample	$s_{20,W}$ (S)	Mw_{app} (kDa)	$s_{20,W}$ (S)	Mw_{app} (kDa)	$s_{20,W}$ (S)	Mw_{app} (kDa)	$s_{20,W}$ (S)	Mw_{app} (kDa)	$s_{20,W}$ (S)	Mw_{app} (kDa)	$s_{20,W}$ (S)	Mw_{app} (kDa)
extravidin-peroxidase	-	-	-	-	4.41	62.3	6.33	107	8.86	~178		
multimeric 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 ≈ 112 kDa); line 2: scFv (Mw ≈ 30 kDa); line 3: multimeric-scFv (Mw ≈ 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 ºC. 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 (*Armoracia rusticana*)	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 (*Homo sapiens*)	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	CAGATCCTCTTCTGAGATGAGTTTTTGTTC
MJA254	AATTAACTGCAGCCGAGGTGCAGCTGTTGGAGT
MJA255	ATATTATGAATTCATGAAGGATAACACCGTGCCACTGA
MJA256	ATAATATCCGCGGTTATTTTTCTGCACTACGCAGGGATATTTC
MJA259	CACCTTCGTGCCATTCGATTTTCT
MJA257	AATTGCGGCCGCGGGTCTGAACGACATCTTCGAGGCTCAGAAAAT CGAATGGCACGAAGGTGCTCTAGAAATT
MJA258	AATTTCTAGAGCACCTTCGTGCCATTCGATTTTCTGAGCCTCGAAG ATGTCGTTCAGACCCGCGGCCGCAATT

Figure A: pMJA181 vector containing scFv and BAD nucleotide sequences constructed in pPICZαB 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).
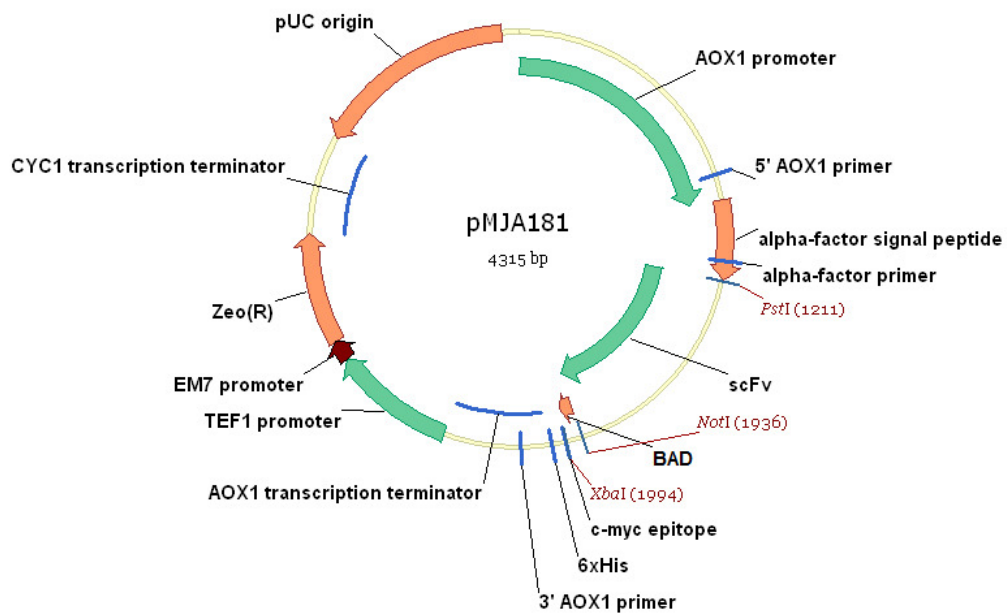
Figure B: pMJA180 vector containing BirA nucleotide sequence constructed in pPIC6αA plasmid (Bla[r]; integrative plasmid carrying the secretion signal sequence from the *S. cerevisiae* α factor prepro-peptide and functional sites for the integration at the 5'AOX1 locus of *P. pastoris* X-33).

pPIC6αA 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).