1 **Affitins as robust tailored reagents for affinity chromatography**
2 **purification of antibodies and non-immunoglobulin proteins**
3

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17 **ABSTRACT**

18 Affinity chromatography is a convenient way of purifying proteins, as a high degree of purity
19 can be reached in one step. The use of tags has greatly contributed to the popularity of this
20 technique. However, the addition of tags may not be desirable or possible for the production of
21 biopharmaceuticals. There is thus a need for tailored artificial affinity ligands. We have
22 developed the use of archaeal extremophilic proteins as scaffolds to generate affinity proteins
23 (Affitins). Here, we explored the potential of Affitins as ligand to design affinity columns.
24 Affitins specific for human immunoglobulin G (hIgG), bacterial PulD protein, and chicken egg
25 lysozyme were immobilized on an agarose matrix. The columns obtained were functional and
26 highly selective for their cognate target, even in the presence of exogenous proteins as found in
27 cell culture media, ascites and bacterial lysates, which result in a high degree of purity (~ 95 %)
28 and recovery (~100%) in a single step. Anti-hIgG Affitin columns withstand repetitive cycles
29 of purification and cleaning-in-place treatments with 0.25 M NaOH as well as Protein A does.
30 High levels of Affitin productions in *E. coli* makes it possible to produce these affinity columns
31 at low cost. Our results validate Affitins as a new class of tailored ligands for the affinity
32 chromatography purification of potentially any proteins of interest including
33 biopharmaceuticals.

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36 **Keywords:** affinity chromatography, antibody, immunoglobulin, Affitin, Sac7d, Sso7d.

1. Introduction

In the manufacture of therapeutic proteins, affinity chromatography contributes significantly to reduce the processing cost as a high degree of purity can be reached in one step. The fusion of proteins to polypeptide tags, such as the hexahistidine tag, is widely used to facilitate protein purification by affinity chromatography [1]. However, this approach is problematic when sequence modifications are neither desirable nor possible.

Thus, specific ligands for proteins of interest can be helpful for affinity purification. For instance, concanavalin A and amylose enable the purification of glycoproteins [2] and maltose binding protein [3], respectively. Among therapeutic proteins, monoclonal antibodies are of great interest, as they accounted for about half of the sales in the European Union and the USA in 2010 [4]. Several bacterial surface proteins have been identified as affinity reagents and are commonly used to purify antibodies or some of their fragments. Binding specificities of these proteins differ between source species and antibody subclasses. For instance, Protein G and Protein A from group G *Streptococci* and *Staphylococcus aureus*, respectively, are able to bind IgG mainly *via* their Fc region [5, 6], while Protein L from *Peptostreptococcus magnus* recognizes antibodies through light chain interactions [7]. Protein A strongly binds human IgG1, IgG2 and IgG4 while Protein G strongly binds all human subclasses. Proteins A and G bind rabbit IgG strongly, while Protein L binds them weakly (see [8] for a review).

Thus, depending on the application, the choice of the ligand is critical. A major drawback of these existing binders is that they may not fit specific needs. For non-antibody proteins, the problem is even more serious, as often no natural partner is known with properties suitable for use as an affinity reagent. Therefore, it is worth developing new reagents suitable for affinity chromatography by chemistry or molecular evolution, with specificity and affinity for the protein of interest.

61 The structure-based design of chemicals and peptidic mimetics have been used to obtain
62 artificial ligands for targets such as antibodies (see [9, 10] for reviews), insulin [11], and
63 plasminogen activator [12]. Although some progress has been made in recent years [13, 14],
64 obtaining a ligand with a well-defined selectivity is not easy to achieve. Another approach is to
65 develop affinity reagents from proteins, termed alternative scaffold proteins, by combinatorial
66 protein design. For example, this was used to convert an IgG-binding domain from Protein A
67 into an IgA binder [15] and to isolate Fc binders from designed ankyrin repeat protein libraries
68 [16]. Numerous alternative scaffold proteins have been proposed (see refs. [17] and [18] for
69 reviews), but few of them are really usable for demanding applications, such as affinity
70 chromatography.

71 Ideally, to be suitable for affinity purification, an affinity reagent should (i) be highly
72 specific for the target to be purified, (ii) show a cost-effective production and (iii) have a high
73 thermal and chemical stability, as this is often associated with a long column life cycle. For use
74 in single-use disposable columns, it is also important that the reagent is at least resistant to the
75 conditions used for the elution of the target. Moreover, to ensure safe reusability of columns,
76 the affinity reagent must be resistant to extreme alkaline pH used for cleaning-in-place (CIP)
77 procedures, which are part of good manufacturing practices (GMP), and for the minimization
78 of product contamination by leached ligand fragments.

79 We have previously described the use of the small (7 kDa) archaeal hyperthermophilic
80 and acidophilic Sac7d protein and its homologues as a scaffold to design tailored artificial
81 affinity proteins (Affitins) [19, 20]. Using combinatorial engineering, we have generated
82 Affitins with dissociation constants in nanomolar and subnanomolar ranges, and with
83 specificity for their targets, such as bacterial protein PulD [19, 21, 22], chicken egg lysozyme
84 [20, 23-26] and human IgGs [27, 28]. Recently, we validated the structural basis of two of their
85 modes of binding by solving the structures of three Affitins in complex with their cognate

86 targets [25]. Affitins are chemically and thermally stable (from pH = 0 up to 12, toward
87 detergents and chaotropic agents, and up to 90°C). We have shown that it is possible to further
88 increase the stability of Affitins toward alkaline conditions, up to at least pH 13, *via* a
89 grafting/mutation strategy [28]. Furthermore, we reported that Affitins are overproduced in *E.*
90 *coli* with yields from several dozen to several hundreds of milligrams per liter of culture. Thus,
91 Affitins exhibit all the desired properties for their use as reagents in affinity chromatography.

92 Here, for the first time, we present the use of Affitins, covalently immobilized in columns,
93 as reagents capable of selectively capturing three unrelated proteins from heterogeneous protein
94 mixtures: human IgGs, bacterial PulD protein and hen egg white lysozyme (HEWL).
95 Furthermore, to gain an insight into the potential of purification processes using Affitins, we
96 compare several anti-IgG Affitins and Protein A columns to assess their resistance to repetitive
97 CIP procedures using sodium hydroxide. Our results demonstrate the great potential of Affitins
98 as designed ligands for robust affinity chromatography columns.
99

100 **2. Materials and methods**

101 **2.1 Materials**

102 Affitins Sac7*6, H4, D1Sso7d and D1Sso7d-DM were expressed in the *E. coli* DH5α Iq strain
103 and purified as described previously [19, 25, 28]. IgGs used in this study were purchased from
104 Fluka: hIgG (i.e. IgG pool from human serum containing hIgG1, hIgG2, hIgG3, and hIgG4);
105 and from Sigma-Aldrich: hIgG1, hIgG2, hIgG3, hIgG4, IgG pools from mouse, rat, sheep, goat,
106 rabbit, and pig. The PulD-N protein was expressed in the BL21(DE3) *E. coli* strain transformed
107 with the plasmid pCHAP3702 and purified as described previously [29]. HEWL was purchased
108 from Sigma-Aldrich. Mouse ascites containing an hIgG1 (400 μg/ml) was obtained from the
109 "Production de Protéines recombinantes et d'anticorps" platform (Institut Pasteur, Paris).
110

111 **2.2 Immobilization of Affitins on an agarose matrix**

112 Monomeric purified Affitins were dialyzed overnight against 130 mM NaCl, 2.7 mM KCl, 10
113 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4 (PBS) and quantified spectrophotometrically at 280 nm
114 using extinction coefficients of 15220 M^{-1} cm^{-1} (anti-IgG Affitins D1Sso7d and D1Sso7d-DM),
115 3840 M^{-1} cm^{-1} (anti-PulD Affitin Sac7*6) and 13940 M^{-1} cm^{-1} (anti-HEWL Affitin H4). All
116 steps of the following coupling procedure involving columns were performed using syringes.
117 One milliliter of Affitin (10 mg/ml) was injected onto a 1 ml HiTrap N-hydroxysuccinimide
118 (NHS)-activated HP column (GE Healthcare) previously flushed with 6 ml of ice cold 1 mM
119 HCl. Immobilization occurred at room temperature for 30 min. Then, the column was washed
120 with 3 ml of PBS. This 3 ml fraction contains Affitins which were not immobilized, mixed with
121 the NHS group released during coupling reaction. NHS shows strong absorption at 280 nm at
122 pH above 6. To estimate the amount of immobilized Affitins, 1 ml of this fraction was acidified
123 with 1 ml of 2 M glycine HCl pH 2 prior quantification at 280 nm using the above mentioned
124 extinction coefficients. To deactivate any remaining active groups, the column was washed and
125 equilibrated with 6 ml of buffer A (0.5 M ethanolamine , 0.5 M NaCl, pH 8.3), then with 6 ml
126 of buffer B (0.1 M acetate, 0.5 M NaCl, pH 4), 6 ml of buffer A (for 30 min at room
127 temperature), 6 ml of buffer B, 6 ml of buffer A and 6 ml of buffer B. After a final equilibration
128 with 6 ml of PBS, the column was ready to use.

129

130 **2.3 Preparation of heterogeneous samples containing hIgGs**

131 Heterogeneous samples were prepared as follows. Dulbecco's Modified Eagle's Medium
132 (DMEM) + 10% fetal calf serum (FCS) (DMEM/FCS) was spiked with hIgGs to a final
133 concentration of 125 μg/ml. An *E. coli* crude extract prepared from DH5α Iq strain (5X
134 concentrated from the initial culture) was also spiked with hIgGs to a final concentration of 125
135 μg/ml.

136

137 **2.4 Preparation of soluble *E. coli* crude extract containing PulD-N**

138 Bacteria of a one-liter culture of BL21(DE3) *E. coli* strain that had produced PulD-N (see
139 section 2.1) were harvested by centrifugation and resuspended in 30 ml of PBS. Cells were
140 lyzed with an Avestin Emulsiflex homogenizer and cell debris were discarded in a
141 centrifugation step. This supernatant was used for affinity chromatography studies.

142

143 **2.5 Preparation of heterogeneous samples containing HEWL**

144 DMEM/FCS was spiked with HEWL to a final concentration of 125 µg/ml and used for affinity
145 chromatography studies.

146

147 **2.6 Affinity chromatography**

148 All purifications were carried out at a flow rate of 1 ml/min using either an ÄKTApurifier 10
149 system (for PulD-N) or a Bio-Rad BioLogic DuoFlow 10 System (for HEWL and IgG) and
150 affinity columns prepared as described in section 2.2 with PBS as running buffer. For
151 comparison with the Protein A purification system, a 1 ml HiTrap Protein A HP column (GE
152 Healthcare) was also used.

153 About 125 µg of each pure IgG was used to study the selectivity of anti-IgG Affitin columns.
154 The specificity of D1Sso7d and D1Sso7d-DM columns was tested by loading heterogeneous
155 samples containing 125 µg of IgG (section 2.3). One milliliter and a half of soluble *E. coli* crude
156 extract containing PulD-N (section 2.4) was injected onto the Sac7*6 immobilized column. A
157 heterogeneous sample containing 125 µg HEWL (section 2.5) was injected onto the H4
158 immobilized column.

159 Non-specific proteins were washed away with PBS and elution was carried out with an acidic
160 pH step using a 100 mM glycine buffer (pH 2.5, 150 mM NaCl). The purity of the eluted protein

161 was checked by loading fractions onto an SDS gel. Gels were stained with PageBlue protein
162 staining solution (Thermo Scientific), scanned with GelDoc EZ Imager (Bio-Rad), and
163 analyzed with Image Lab (Bio-Rad) and ImageJ softwares.
164

165 **2.7 Kinetics of deactivation of Affitins and Protein A columns with sodium hydroxide**

166 The resistance of affinity columns to a cleaning-in-place (CIP) procedure at alkaline pH was
167 evaluated with 25 cycles of affinity purification of hIgG, including elutions at pH 2.5 and 15-
168 min treatments with 0.25 M NaOH (15 min at a flow rate of 1 ml/min). For each run, 1 ml of
169 a 22 mg/ml hIgG in PBS was loaded onto the affinity column. The quantity of hIgG eluted
170 was determined for each cycle by measuring optical density at 280 nm.
171

172 **3. Results**

173 Previously, we designed and characterized several Affitins specific for human IgGs [27, 28],
174 the bacterial protein PulD-N [19, 21, 22] and chicken HEWL [20, 23, 25] (Fig. 1A). These three
175 proteins are unrelated and thus represent different systems that are interesting to test with
176 various Affitins for affinity chromatography applications.
177

178 **3.1 Affinity chromatography using anti-IgG Affitins**

179 Affitins specific for the Fc region of human hIgG1, 2 and 4 [27] were studied to investigate
180 whether they could be used for affinity chromatography. This system is essential as it enables
181 comparison with Protein A, the gold standard for purifying IgGs.
182

183 **3.1.1 Immobilization of D1Sso7d anti-IgG Affitin**

184 To evaluate whether the activity of D1Sso7d was conserved once immobilized on a column,
185 this Affitin was linked to agarose beads *via* N-hydroxy-succinimide amine coupling chemistry
186 (Fig. 1B). The reaction yield was 83% (8.3 of 10 mg were immobilized).
187 The column was then used for the capture of purified hIgG1. After washing, an elution step was
188 carried out with a glycine buffer at pH 2.5. According to the chromatogram (A_{280}), hIgG1 was
189 captured by the column and eluted as a sharp peak with acidic buffer (Fig. 2).
190

191 **3.1.2 IgG selectivity of the D1Sso7d affinity column**

192 The successful binding/recovery of pure hIgG1 using an Affitin column led us to investigate if
193 the selectivity observed in solution by ELISA [27] could be confirmed by affinity purification
194 experiments. With this aim, various purified IgGs from human (isotypes 1, 2, 3 and 4), goat,
195 rabbit, mouse, rat, sheep and pig were loaded separately onto the D1Sso7d column. The
196 chromatograms shown in Figure 2 indicated that hIgG1, hIgG2, hIgG3 and hIgG4 were fully
197 captured by the affinity column while only about 50% and 10% of IgGs from rat and sheep,
198 respectively, were trapped. By contrast, IgGs from goat, mouse, rabbit and pig were not
199 captured by the column. These results confirmed most of the previous observations by ELISA
200 [28] about the specificity of the D1Sso7d protein, except for hIgG3 and IgGs from rat and sheep
201 that were not recognized by this Affitin according to ELISA. These results suggest that
202 D1Sso7d has a weak affinity for these IgGs, which can only be detected when there is a high
203 density of ligands and a rebinding effect, as it occurs in affinity columns.
204

205 **3.1.3 IgG purification from a crude *E. coli* lysate**

206 A prerequisite for affinity chromatography applications is the specificity of the affinity reagent.
207 To test the selectivity of the D1Sso7d affinity column, the first sample was prepared with the

208 soluble fraction of an *E. coli* crude extract spiked with pure hIgG. This kind of sample is highly
209 heterogeneous, given the number of different proteins found in *E. coli*, and thus represents a
210 challenge for selectivity. Figure 3 shows the chromatogram obtained for purification of this
211 sample, with a large flow-through peak, followed by a fast return of absorbance measured at
212 280 nm to baseline level, and finally a sharp peak when glycine buffer was run through the
213 column. Two control samples, containing either the soluble fraction of an *E. coli* crude extract
214 or pure hIgG only, were loaded independently onto the column. No peak could be observed on
215 the chromatogram (data not shown) for the *E. coli* only control sample when glycine buffer was
216 run through the column, which demonstrates that the column could not capture detectable
217 amounts of *E. coli* proteins. By contrast, a peak was observed on the chromatogram (data not
218 shown) upon running glycine buffer for the control sample with only pure hIgG. An SDS-PAGE
219 analysis of the eluted fractions from the three chromatographic runs (Fig. 4A) showed two
220 bands corresponding to heavy and light chains of IgG (50 and 25 kDa, respectively), thus
221 confirming that the column was able to capture hIgG selectively from the *E. coli* protein
222 mixture.
223

224 **3.1.4 IgG purification from a mammalian cell culture medium**

225 Antibodies are often produced from hybridoma cell cultures. The medium used for these
226 cultures is supplemented with 10% FCS, which could interfere with affinity purification. To
227 explore further a possible use of our affinity columns in the purification of antibody culture
228 supernatants, pure hIgG was mixed with DMEM/FCS. Two sample controls were prepared with
229 either DMEM/FCS or hIgG only. Using reducing SDS-PAGE, the eluted fractions
230 corresponding to flow-through and elution steps (Fig. 4B) were analyzed. According to this
231 analysis, the eluted hIgGs were as pure as the hIgG used to prepare spiked samples, indicating
232 that the D1Sso7d column was able to discriminate hIgG from diverse exogenous proteins, even

233 those from FCS. For comparison, the same samples were loaded onto a Protein A column. As
234 seen from the SDS-PAGE analysis of the eluted fractions for DMEM/FCS + hIgG sample
235 purification (Fig. 4C), the degree of purity reached for hIgG with the D1Sso7d column
236 compared well with that of the Protein A purification system.
237

238 **3.1.5 IgG purification from ascites**

239 Finally, ascites containing hIgG1 were also loaded onto the D1Sso7d column and the protein
240 contents of eluted fractions were compared with those obtained from the same sample purified
241 on a Protein A column. According to the SDS-PAGE analysis (Fig. 4D), both columns
242 performed equally for recovering hIgG1 antibody at a high degree of purity over 95 %, further
243 confirming the high selectivity of D1Sso7d.
244

245 **3.1.6 Robustness of anti-IgG Affitin columns to cleaning-in-place procedures**

246 To be reusable, chromatography columns must be sanitized and regenerated with a cleaning-
247 in-place (CIP) procedure. This often consists of exposing columns to NaOH in concentrations
248 ranging from 0.1 to 1 M between purification cycles. To evaluate how the D1Sso7d column
249 withstands these harsh conditions, it was subjected to 25 cycles of hIgG purifications, including
250 elution at pH 2.5 and 15-min treatment with 0.25 M NaOH in each cycle. A commercial Protein
251 A column was used in parallel for comparison. According to the plot of the percentage of the
252 IgG binding capacity of the columns *vs.* the cycle of purification (Fig. 5), different rates of
253 degradation were observed. After a cumulative exposure to NaOH for about 6 h 15 min, the
254 activity of immobilized D1Sso7d was still high (74% of the initial capacity of the column), but
255 lower than that of the Protein A column (93%).

256 We have recently shown that D1Sso7d can be stabilized towards alkaline conditions by a double
257 mutation [28]. The corresponding mutant (D1Sso7d-DM) conserved the same specificity

258 profile for IgGs as that of D1Sso7d. In this work, we first studied the selectivity of a D1Sso7d-
259 DM column as described for D1Sso7d (see section 3.1.3) with *E. coli* crude extract samples
260 spiked with hIgGs. According to the SDS-PAGE analysis of eluted fractions (Fig. 6), similarly
261 to the D1Sso7d column, no proteins from *E. coli* were bound onto the D1Sso7d-DM column
262 (Fig. 4A). This indicated that the specificity of D1Sso7d was not altered by the double mutation,
263 even after being immobilized on a matrix.
264 Finally, the D1Sso7d-DM column was submitted to the same protocol of purification/CIP
265 cycles used for the D1Sso7d column. After a total contact time of 6 h 15 min with 0.25 M
266 NaOH, the D1Sso7d-DM column was found more resilient to the alkaline solution than the
267 D1Sso7d column (90% and 74% of the initial capacity of the column, respectively), and
268 comparable to the Protein A column (Fig. 5).
269

270 **3.2 Affinity chromatography using anti-PulD-N and anti-HEWL Affitins**

271 To investigate whether Affitins could be more generally used for affinity chromatography, two
272 non-IgG-related Affitin/protein couples were studied. The very high specificity of Sac7*6 has
273 been reported previously with western blot experiments, showing that this Affitin reacts only
274 with full length PulD protein, and PulD-N which is a soluble fragment of PulD, in crude *E. coli*
275 lysates [19]. The anti-HEWL Affitin H4 is also specific for HEWL [25]. Affinity columns were
276 prepared in the same way as that for anti-IgG Affitins. The coupling yields were 86% for H4
277 (8.6 of 10 mg were immobilized) and 85% for Sac7*6 (8.5 of 10 mg immobilized). Affinity
278 chromatography was performed for each cognate target: PulD-N in *E. coli* crude extract and
279 HEWL spiked with DMEM + 10% FCS. Chromatograms showed that Sac7*6 and H4 Affitins
280 were still able to capture PulD-N and HEWL, respectively, after immobilization (Fig. 7).
281 Indeed, after post-loading wash, a protein fraction was eluted as a sharp peak with glycine
282 buffer at pH 2.5. SDS-PAGE analysis of these fractions indicated that PulD-N and HEWL were

283 eluted with high degrees of purity, over 95 % and 92 %, respectively, according to Coomassie
284 blue staining (Fig. 7). This study also showed that the columns did not have detectable affinity
285 for background proteins from the *E. coli* crude extract or the DMEM/FCS mixture, as seen with
286 control samples.
287 .
288 **3.3 Protein binding capacities**
289 Dynamic binding capacities (DBC) of the H4-, Sac7*6- and D1Sso7d-DM Affitin-columns
290 were determined by loading a 20 ml sample containing 1 mg/ml of cognate target protein
291 (HEWL, PulD and hIgG, respectively). From the loading volumes corresponding to 10%
292 breakthrough, DBCs of cognate targets were estimated as 10.8, 16.0 and 10.0 mg per ml of
293 column packing, respectively.
294
295 **4. Discussion**
296 The high cost of protein pharmaceutical production is mainly due to the multiple downstream
297 processing steps, which include initial capture and polishing purification [30]. Affinity
298 chromatography is a widely used technique to recover these drug molecules from culture media
299 and offers a high degree of product purity in one step, thereby reducing the costs of production.
300 In this work, we report three examples of unrelated targets for which Affitins have properties
301 suitable for affinity chromatography applications.
302 Affitins were shown to be functional once immobilized *via* amine chemistry on a standard
303 matrix. The dynamic binding capacities of these columns were determined to be at least 10 mg
304 of target per milliliter of bed resin. Higher capacities can probably be reached with a careful
305 optimization of immobilization. For example, a cysteine could be added to the N- or C-terminus
306 of the Affitin to enable its oriented immobilization in order to maximize exposure of the binding
307 site to the target [30]. Furthermore, we found for the three specificities tested in this work that

308 100% of 5 mg target proteins loaded onto the columns were eluted (data not shown), indicating
309 that high recoveries can be achieved with this system.
310 We chose to challenge the specificity of Affitins with highly heterogeneous samples mimicking
311 those obtained when the protein target is produced in *E. coli*, mammalian cells or ascite fluids.
312 All Affitin columns (*i.e.* anti-PulD, anti-HEWL and anti-IgG) displayed the exact specificity
313 for their cognate target. Furthermore, we have shown in this work that the anti-IgG specificity
314 of Affitins compares well with that of Protein A, a standard ligand widely used for antibody
315 purification. Interestingly, the profile of recognition of D1 Affitins (*ie* D1Sso7d and D1Sso7d-
316 DM) is different from those of Proteins G and A (Table 1), exemplifying how artificial affinity
317 proteins can extend the panel of specificity profiles available for IgG purification, and for
318 proteins in general. Affitins are obtained from combinatorial libraries by selection against a
319 defined target [19]. To obtain D1 by selection, we used a mixture of Fc from the four IgG
320 subclasses, thus enabling a degree of freedom for the system to bind on each IgG subclass. To
321 date, most of the approved human(-ized) therapeutic antibodies are IgG1 and, to a lesser extent,
322 IgG2 and IgG4 isotypes [31], which are well recognized by D1 Affitins. Although D1 Affitins
323 have a weak affinity for hIgG3 according to ELISA, in fact they are able to capture hIgG3 upon
324 immobilization in a column. Thus D1 Affitins are suitable for the production of large quantities
325 of therapeutic antibodies of any subclasses. Thanks to versatility of the Affitin system, we
326 anticipate that by using a strict selection process, for example with only one IgG type as the
327 target or with competition with unwanted IgGs, it will be possible to develop artificial affinity
328 ligands with defined specificity for any particular IgG or its derivates.
329 Given the diversity of targets for which affinity reagents are needed and for which no natural
330 ligands are available to design affinity columns, it is important to develop tailored affinity
331 reagents. To date, we have isolated Affitins against a dozen protein targets. The
332 characterizations of some of them (anti-PulD, -IgG, -CelD, and -HEWL) have been published

333 [19, 25, 27]. Hence, we believe that the Affitin system is versatile enough to provide reagents
334 for affinity chromatography of non-antibody protein targets.
335 It is economically important that the affinity column is as resistant as possible to cleaning and
336 sanitizing protocols to ensure its reusability. This is also a prerequisite for the prevention of
337 product contamination, as the degradation fragments of affinity ligands may not be compatible
338 with the product quality analysis and/or therapeutic applications [35]. Although popular in
339 industry for antibody purification, Protein A has a high cost of production of about 6000-9000
340 €/L resin [32].. Our study showed that the anti-IgG Affitins, and particularly D1Sso7d-DM,
341 withstood the harsh CIP conditions generally applied to Protein A columns [33]. However, CIP
342 protocols have been recently optimized to further extend the lifespan of Protein A columns.
343 These days, NaOH concentrations in the 25-100 mM range are commonly advised for efficient
344 cleaning, and the removal of Protein A fragment in the following step is guaranteed [34, 35].
345 We believe the NaOH concentration of 0.25 M used to challenge our system is sufficient for
346 regeneration of Affitin columns, and such milder concentrations would provide them benefits
347 observed for the Protein A system as well. For cases which need stronger resistance to alkaline
348 CIP, given that crystal structures of Sac7d and Sso7d scaffolds are available, we anticipate that
349 stability could be further improved by performing additional mutagenesis.
350 There is an increasing trend in industry toward the use of disposable devices for the production
351 of biopharmaceuticals [36]. We have reported the high level production of several Affitins in
352 *E. coli* (up to 200 mg/L of culture in flask [19]). Thus, we believe that the low cost associated
353 with their production should also make them very attractive for the development of single-use
354 affinity columns.
355

356 **5. Conclusions**

357 With high-throughput screening approaches, an increasing number of novel biopharmaceuticals
358 have been identified. However, affinity tools available for the large-scale production of these
359 protein drug molecules are limited. We have developed the technology for the generation,
360 screening, and characterization of Affitins suitable for complementing, or even replacing
361 natural ligands in many affinity applications. Our work presented hereby establishes Affitins as
362 on demand reagents for any target of interest Future works might improve Affitin-based system
363 to get higher dynamic binding capacities with higher working flow rates, and might allow
364 milder elutions compatible with fragile targets. We believe Affitins have a wide range of
365 applications in the preparation of highly purified proteins, such as the capture of target proteins,
366 the depletion of contaminant proteins, or the enrichment of weakly represented proteins for
367 proteomic research.

368

369 **Conflict of interest**

370 F.P. is an inventor of a patent application (PCT/IB2007/004388), owned by the Institut Pasteur
371 and Centre National de la Recherche Scientifique (CNRS), which covers a process for the
372 generation of Affitins. F.P. is a co-founder of a spin-off company of the Institut
373 Pasteur/CNRS/Université de Nantes, which has a license agreement related to this patent
374 application.

375

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382

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476

477 **FIGURE CAPTIONS**

478 **Figure 1.** Sequences of the four Affitins studied and scheme for their coupling to amine-
479 reactive agarose. (A) Sac7*6, and H4 have a specificity for the bacterial PulD-N protein
480 fragment and chicken HEWL, respectively, while D1Sso7d and D1Sso7d-DM have a
481 specificity for hIgGs. Residues common to D1Sso7d and D1Sso7d-DM Affitins are indicated
482 by a dot. (B) Ten milligrams of each Affitin were coupled in PBS *via* their amines to *N*-
483 Hydroxysuccinimide-activated agarose matrix to prepare affinity columns. The Affitin in the
484 scheme is a model of the D1Sso7d structure depicted as green ribbons, with residues substituted
485 during the generation of the IgG binding site highlighted in blue [28].

486

487 **Figure 2.** Study of the ability of an affinity column prepared with D1Sso7d Affitin to capture
488 pure IgGs. One hundred and twenty-five micrograms of pure IgGs was loaded for each
489 chromatography experiment. After washings with running buffer, elution was carried out with
490 a glycine buffer at pH 2.5. The dotted line indicates the start of the elution step with glycine
491 buffer. The absorbance at 280 nm was measured to monitor the chromatography. FT: flow-
492 through fraction; EL: elution fraction.

493

494 **Figure 3.** Typical chromatogram observed for affinity purification of hIgG from a sample
495 spiked with exogenous proteins (here *E. coli* proteins). Conditions for chromatography were
496 the same as in Figure 2. FT: flow-through fraction; EL: elution fraction.

497

498 **Figure 4.** Study of the selectivity of D1Sso7d and Protein A columns. SDS-PAGE analysis
499 (under reducing conditions) of affinity purification of hIgGs from samples spiked with
500 exogenous proteins or from ascites; (A) using a D1Sso7d column: *E. coli* crude extract (1:
501 injected, 2: flow through, 3: elution), *E. coli* crude extract + hIgG (4: injected, 5: flow through,

502 6: elution), pure hIgG (7: injected, 8: flow through, 9: elution), (M) Protein markers: 250, 150,
503 100, 75, 50, 37, 25, 20 kDa from top to bottom; (B) same as in (A), but with *E. coli* crude extract
504 replaced by DMEM/FCS; (C) same as in (B), but using a Protein A column; (D) analysis of
505 collected fractions from an affinity purification of hIgG1 produced in mouse ascites using a
506 D1Sso7d and a Protein A column (1: injected, 2: flow through, 3: wash, 4: elution). "H" and
507 "L" indicate migration of bands corresponding to heavy and light chains, respectively.

508

509 **Figure 5.** Comparison of the column capacities after repeated purification/CIP cycles. Affinity
510 chromatography purifications were run as in Figure 1 using hIgG and columns prepared with
511 D1Sso7d and D1Sso7d-DM as ligands. A 15-min step with 0.25 M NaOH was used as the
512 cleaning agent between each run. A commercial Protein A was used under the same conditions
513 for comparison.

514

515 **Figure 6.** Study of the selectivity of a D1Sso7d-DM column. SDS-PAGE analysis (under
516 reducing conditions) of affinity purifications of pure hIgGs, soluble *E. coli* crude extract and
517 hIgG1 spiked with soluble *E. coli* crude extract (1, 3, 5: flow through, 2, 4, 6: elution). Lane M
518 corresponds to protein markers: 250, 150, 100, 75, 50, 37, 25, 20 kDa from top to bottom.

519

520 **Figure 7.** Study of the specificity of anti-PulD and anti-HEWL affinity columns. (A) Elution
521 profile (black line) observed for affinity purification of PulD-N from the soluble fraction of *E.*
522 *coli* lysate. After washings with running buffer, elution was carried out with a glycine buffer
523 pH 2.5 (blue line). The absorbance at 280 nm was measured to monitor the chromatography.
524 FT: flow through fraction; EL: elution fraction. In inset: SDS-PAGE analysis of the affinity
525 purification of PulD-N. Lane 1: sample injected, 10: flow through, 2 and 9: pure PulD-N as
526 controls, 3-8: elution fractions 1 to 6, which are indicated with red lines in the chromatogram.

527 (B) Similar analysis performed with an anti-HEWL column for affinity purification of HEWL
528 from DMEM/FCS medium. In inset: SDS-PAGE analysis of the affinity purifications:
529 DMEM/FCS (2: injected, 3: flow through, 4: elution); DMEM/FCS + HEWL (5: injected, 6:
530 flow through, 7: elution); HEWL (8: injected, 9: flow through, 10: elution). Lane 1: protein
531 markers (250, 150, 100, 75, 50, 37, 25, 20, 15, 10 kDa from top to bottom). Elution fractions,
532 which are indicated with red lines in the chromatogram, were pooled for SDS-PAGE analysis.
533

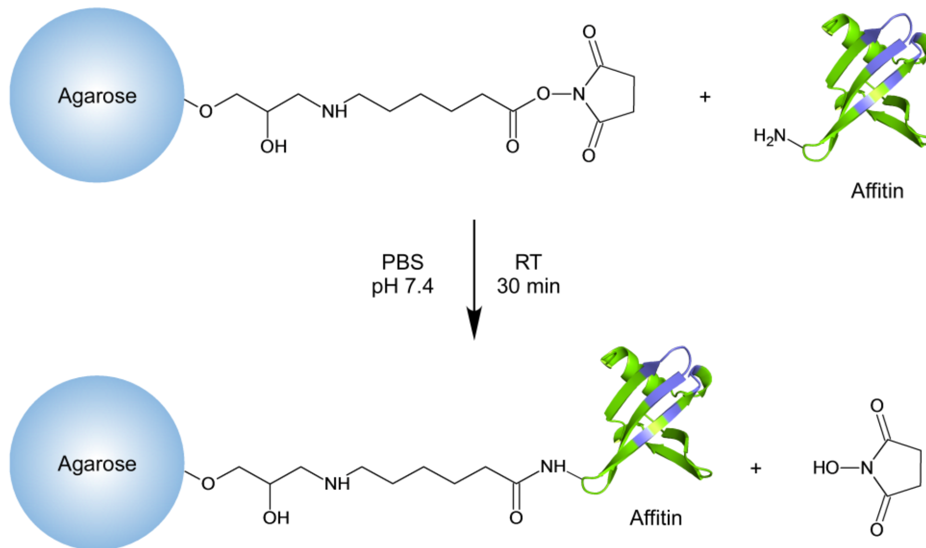
534 **Figure 1**

A

```
                             1        10        20        30        40        50        60
Sac7*6         MRGSHHHHHHGSVKVKFVLGGEEKEVDTSKIRHVYRYGKHVTFSYDDNGKLGLGLVKEKDAPKELLDMLARAEREKKLN
H4             MRGSHHHHHHGSVKVKFFWNGEEKEVDTSKIVWVKRAGKSVLFIYDDNGKNGYGDVTEKDAPKELLDMLARAEREKKLN

                             1        10        20        30        40        50        60
D1Sso7d        MRGSHHHHHHGSATVKFKYKGEEKEVDISKIKKVWRDRLAAVFTYDEGGGKTGYGWVFTKDAPKELLQMLEKQKKLN
D1Sso7d-DM     .......................L......................L.............................
```

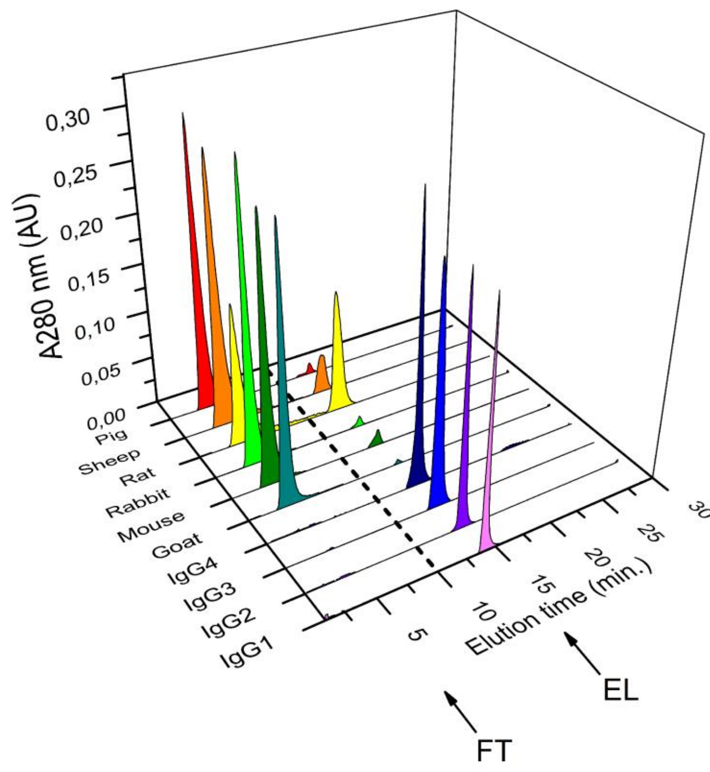
B



535

536

537 **Figure 2**



538

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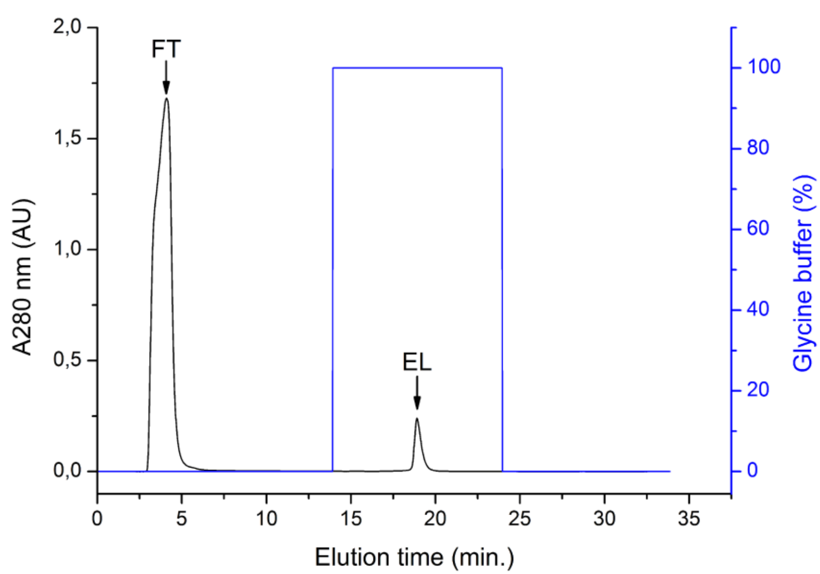
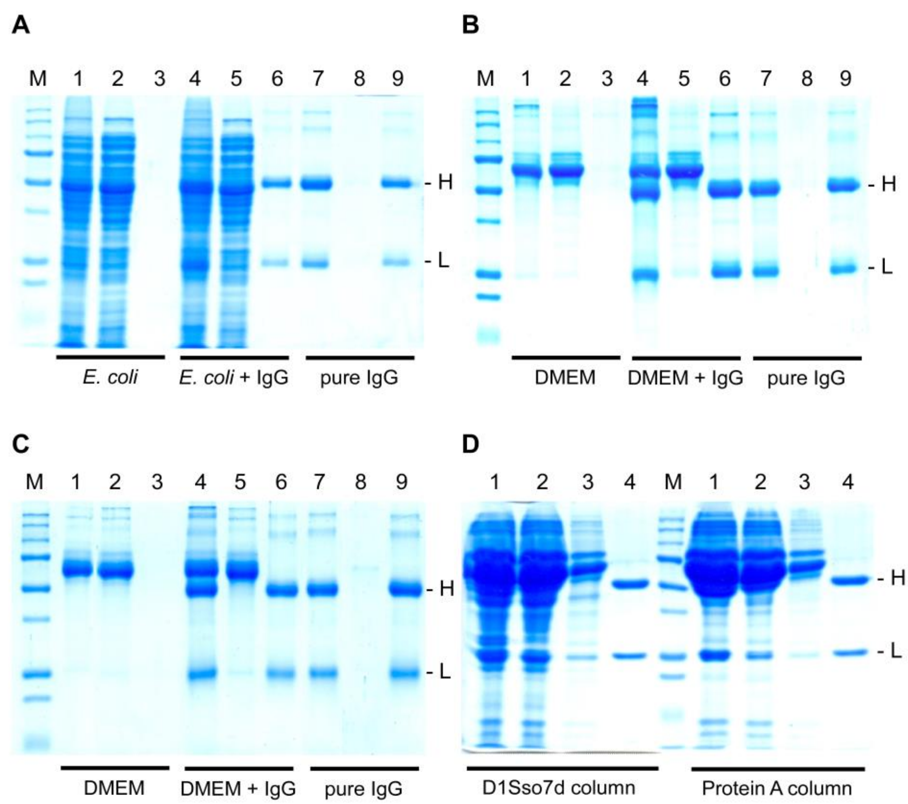
540 **Figure 3**



541

542

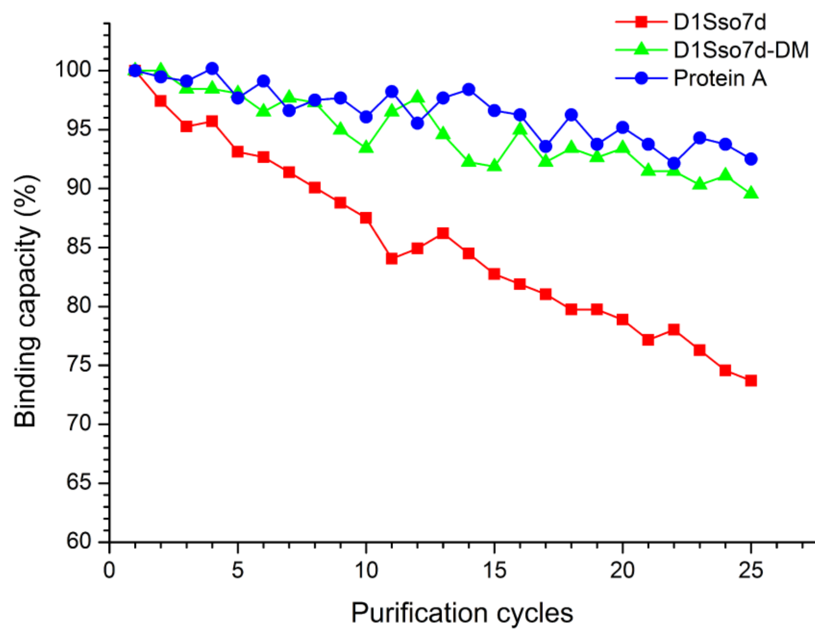
543 **Figure 4**



544

545

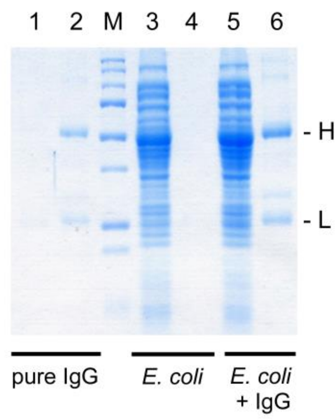
546 **Figure 5**



547

548

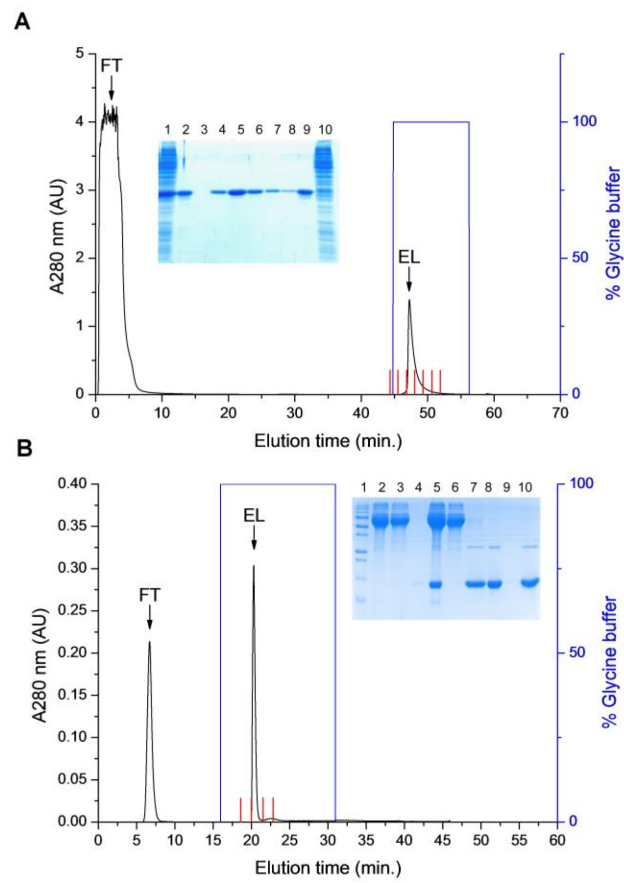
549 **Figure 6**



550

551

552 **Figure 7**



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554