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Purification of Recombinant N-terminal Histidine-Tagged *Arabidopsis thaliana* Phosphoglycolate Phosphatase 1, Glycolate Oxidase 1 and 2, and Hydroxypyruvate Reductase 1

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1 **Purification of recombinant N-terminal histidine-tagged *Arabidopsis thaliana***
2 **phosphoglycolate phosphatase 1, glycolate oxidase 1 and 2, and**
3 **hydroxypyruvate reductase 1**

4

5 Mathieu Jossier¹, Céline Oury, Nathalie Glab and Michael Hodges¹

6 Université Paris-Saclay, CNRS, INRAe, Université Paris Cité, Université d'Evry, Institute of
7 Plant Sciences Paris-Saclay (IPS2), Bât. 630, Avenue des Sciences, 91190 Gif-sur-Yvette,
8 France

9

10 ¹Corresponding authors: mathieu.jossier@universite-paris-saclay.fr; michael.hodges@cnrs.fr

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12

13 **Running head:** Affinity purification of His-tagged photorespiratory enzymes

14

15 **Abstract**

16 To measure the kinetic properties of photorespiratory enzymes, it is necessary to work with
17 purified proteins. Protocols to purify photorespiratory enzymes from leaves of various plant
18 species require several time-consuming steps. It is now possible to produce large quantities
19 of recombinant proteins in bacterial cells. They can be rapidly purified as histidine-tagged
20 recombinant proteins by immobilized metal affinity chromatography using Ni²⁺-NTA-agarose.
21 This chapter describes protocols to purify several *Arabidopsis thaliana* His-tagged
22 recombinant photorespiratory enzymes (phosphoglycolate phosphatase, glycolate oxidase
23 and hydroxypyruvate reductase) from *E. coli* cell cultures using two bacterial strain-plasmid
24 systems: BL21(DE3)-pET and LMG194-pBAD.

25

26 **Key words:** Photorespiration, phosphoglycolate phosphatase, glycolate oxidase,
27 hydroxypyruvate reductase, His-tagged recombinant protein, immobilized metal affinity
28 chromatography, rapid one-step protein purification, *E. coli*

29

30 **1 Introduction**

31 The photorespiratory cycle is made up of eight core enzymes located in three cell
32 compartments (chloroplasts, peroxisomes and mitochondria). To better understand their
33 kinetic properties and eventual regulatory mechanisms, it is necessary to work with purified
34 proteins. There are examples of protocols to purify photorespiratory enzymes from leaves of
35 various plant species including tobacco [1], spinach [2, 3], Arabidopsis [4, 5], corn [6] and
36 cucumber [7]. However, they are time-consuming and require multiple steps such as
37 ammonium sulphate precipitation, acetone fractionation, over-night dialysis, ion-exchange
38 chromatography, gel filtration chromatography and occasionally (depending on the enzyme)
39 affinity chromatography steps. For example, the following steps were required to purify
40 spinach leaf PGLP to 94% homogeneity: Ammonium sulphate precipitation, dialysis, DEAE-
41 SephacelTM ion-exchange chromatography, ultra-centrifugation, DEAE-cellulose 52 ion-
42 exchange chromatography and SephacrylTM S-200 gel filtration chromatography [2].

43 There are now many systems to produce and rapidly purify large quantities of recombinant
44 proteins. To produce soluble, non-toxic recombinant proteins, the *E. coli* BL21(DE3)-pET
45 expression system is often used. It is based on target genes being under the control of strong
46 bacteriophage T7 transcription. The T7 RNA polymerase gene has been introduced into the
47 *E. coli* BL21(DE3) strain under the control of an IPTG-inducible *lac* operon promoter. When
48 induced, the recombinant protein is produced under the control of a T7 promoter (which is
49 not naturally present in *E. coli*) [8]. This system often leads to high yields of recombinant
50 protein; the desired protein can comprise more than 50% of the total cell protein after only
51 a few hours of induction. To produce poorly soluble proteins, an additional maltose binding
52 protein (MBP) moiety can be added to the recombinant protein to enhance protein solubility

53 and to promote proper protein folding [9]. MBP can also be used for amylose affinity-
54 chromatography purification but since this is not very efficient, an additional histidine-tag
55 (His-tag) is often incorporated to facilitate recombinant protein purification. To produce
56 problematic toxic and/or insoluble recombinant proteins, the pBAD-LMG194 bacterial
57 expression system can be used. It is based on the araBAD operon that controls *E. coli* L-
58 arabinose metabolism. The araBAD promoter is induced by the addition of arabinose and
59 thus controls recombinant protein expression in *E.coli* strains lacking L-arabinose catabolism
60 like LMG194. Furthermore, pBAD contains the bacterial AraC gene that fixes to and inhibits
61 the araBAD promoter, in the presence of arabinose this regulatory protein changes
62 conformation and inhibition is released [10]. Together, these properties allow a tight
63 suppression of basal expression in the absence of arabinose and recombinant protein
64 expression is more controlled and highly inducible when compared to the BL21(DE3)-pET
65 system but recombinant protein production is not as high.

66 Whatever the bacterial expression system used to produce recombinant proteins, a common
67 method is to include a His-tag at either the N-terminal or C-terminal of the protein of
68 interest, produce the His-tagged recombinant protein in *E. coli* cells and then purify the
69 resulting protein using immobilized metal affinity chromatography (IMAC) [11]. This affinity
70 chromatography technique using a nickel-charged resin (Ni^{2+} -NTA-agarose) has proven to be
71 a powerful way to purify His-tagged recombinant proteins. Indeed, histidine has the
72 strongest interaction with transition metals like Ni^{2+} . Non-specific interactions can be
73 reduced by the addition of 10 mM 2-mercaptoethanol, NaCl (up to 500 mM) and 20%
74 glycerol to wash buffers. His-tagged recombinant proteins are often eluted by the addition
75 of the histidine analogue, imidazole. However, they can also be eluted by lowering the pH or

76 by the addition of EDTA (that removes the Ni²⁺ and the fixed protein from the Ni²⁺-NTA-
77 agarose column) [11].

78 In the literature, a number of photorespiratory enzymes have been successfully produced
79 and purified as recombinant proteins. The pBAD-LMG194 system has been used to purify
80 recombinant His-tagged Arabidopsis HPR (hydroxypyruvate reductase) 1 [5, 12] and HPR2
81 [13], Arabidopsis phosphoglycolate phosphatase 1 (PGLP1) [14] and Synechocystis GDC
82 (glycine decarboxylase complex) -P and GDC-H sub-units [15]. The BL21(DE3)-pET system has
83 enabled the purification of His-tagged Arabidopsis GOX (glycolate oxidase) 1 and GOX2 [16,
84 17], corn GO1 (glycolate oxidase 1) [16, 17], and Arabidopsis PGLP1 [18]. On the other hand,
85 recombinant Arabidopsis D-glycerate 3-kinase was produced using a different *E. coli* BL21
86 expression system and purified by Matrex Green A affinity chromatography [4] and although
87 *E. coli* BL21 strain and the pET28a plasmid were used to produce recombinant Arabidopsis
88 glutamate:glyoxylate aminotransferase 1, the resulting protein was purified using various
89 chromatography procedures [19].

90 In this chapter, we will describe protocols to purify His-tagged recombinant *Arabidopsis*
91 *thaliana* PGLP1, GOX1, GOX2, and HPR1 using the BL21(DE3)-pET and LMG194-pBAD
92 bacterial systems mentioned above.

93 **2 Materials**

94 **2.1 General**

95 1. Ice box with ice.

96 2. Precision balance.

97 3. Spatula.

- 98 4. pH meter.
- 99 5. Magnetic stirrer.
- 100 6. Magnetic stirrer bar.
- 101 7. Precision pipettes (0-20 μ L, 0-200 μ L, 0-1000 μ L, 0-5000 μ L).
- 102 8. Glass and plastic beakers (2 L, 1 L, 500 mL, 250 mL).
- 103 9. Glass and plastic graduated cylinders (2 L, 1 L, 500 mL 250 mL).
- 104 10. Glass bottles (500ml, 250mL).
- 105 11. Ultrapure water and bidistilled water (see **Note 1**).

106 **2.2 Bacterial culture and induction of recombinant protein expression**

- 107 1. Temperature-controlled cell culture shaker (37°C and 30°C) set to 1.5 x *g* (200 rpm).
- 108 2. Laminar flow hood.
- 109 3. Spectrophotometer with wavelength set to 600 nm.
- 110 4. 50 mL sterile glass tubes.
- 111 5. 1 L sterile conical flasks.
- 112 6. Sterile inoculation loop.
- 113 7. For *AtGOX1/2* and *AtPGLP1* recombinant proteins: Sterile bacterial Luria-Bertani (LB)
- 114 broth culture medium (see **Note 2**).

115 8. For *AtHPR1* recombinant protein: Sterile bacterial Rudic culture medium (RM) (see **Note**
116 **2**).

117 9. For *AtGOX1/2* recombinant protein: 50 g/L stock solution of kanamycin (see **Note 3**).

118 10. For *AtPGLP1* and *AtHPR1* recombinant proteins: 100 g/L stock solution of ampicillin (see
119 **Note 3**).

120 11. For *AtGOX1/2* and *AtPGLP1* recombinant proteins: 1 M stock solution of isopropyl-1-D-
121 thiogalactopyranoside (IPTG) (see **Note 4**).

122 12. For *AtHPR1* recombinant protein: 20 % (w/v) L-arabinose (see **Note 5**).

123 **2.3 Bacterial soluble protein extraction**

124 1. Refrigerated centrifuge at 4°C with swinging buckets capable of 1 400 x *g*.

125 2. Refrigerated centrifuge at 4°C capable of 35 000 x *g*.

126 3. French pressure cell press.

127 4. Pressure cell for French pressure cell press cooled to 4°C.

128 5. 250 mL centrifuge bottles cooled to 4°C.

129 6. 50 mL conical plastic centrifuge tubes.

130 7. Aluminium foil.

131 8. 30 mL centrifuge tubes rated to at least 35 000 x *g* and cooled to 4°C.

132 9. 0.5 M Tris-HCl, pH 7.5 and 0.5 M Tris-HCl, pH 8 stock solutions (see **Note 6**)

133 10. 3M NaCl stock solution (see **Note 7**)

- 134 11. 50% (v/v) glycerol stock solution (see **Note 8**)
- 135 12. 0.5 M MgCl₂ stock solution (see **Note 9**)
- 136 13. 1M imidazole stock solution (see **Note 10**)
- 137 14. 0.5M stock solutions of Na₂HPO₄ and NaH₂PO₄ for the sodium phosphate buffer (see
138 **Note 11**)
- 139 15. 10 mM stock solution of flavine mononucleotide (FMN) (see **Note 12**)
- 140 16. EDTA free Protease Inhibitor Cocktail Tablets (see **Note 13**)
- 141 17. *AtGOX1/2* extraction buffer prepared from stock solutions in ultrapure water and cooled
142 at 4°C: 50 mM Tris-HCl, pH 8, 1 M NaCl, 10% (v/v) glycerol, 10 mM imidazole, 100 μM FMN
143 (see **Note 12**), protease inhibitor cocktail (see **Note 13**).
- 144 18. *AtPGLP1* extraction buffer prepared from stock solutions in ultrapure water and cooled
145 to 4°C: 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM MgCl₂, 10 mM imidazole, protease
146 inhibitor cocktail (see **Note 13**).
- 147 19. *AtHPR1* extraction buffer prepared from stock solutions in ultrapure water and cooled to
148 4°C: 50 mM sodium phosphate buffer (pH 8), 300 mM NaCl, 5 mM imidazole, protease
149 inhibitor cocktail (see **Note 11 and Note 13**).

150 **2.4 Recombinant protein purification**

151 **2.4.1 General materials for batch and gravity-flow affinity chromatography**

- 152 2. Rolling wheel at 4 °C (see **Note 14**).

- 153 3. Immobilized metal ion affinity chromatography (IMAC) resin used for the purification of
154 His-tagged proteins (see **Note 15**).
- 155 4. 50 mL conical plastic centrifuge tubes.
- 156 5. 15 mL conical plastic centrifuge tubes.
- 157 6. Vacuum pump.
- 158 7. *AtGOX1/2* washing buffer prepared from stock solutions in ultrapure water and cooled to
159 4°C: 50 mM Tris-HCl, pH 8, 1 M NaCl, 10 % (w/v) glycerol, 20 mM imidazole, 100 µM FMN
160 (see **Note 12**).
- 161 8. *AtPGLP1* washing buffer prepared from stock solutions in ultrapure water and cooled to
162 4°C: 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM MgCl₂, either 20 mM imidazole when
163 using pDEST-His₆MBP-PGLP1 or 75 mM imidazole when using pET16b-PGLP1.
- 164 9. *AtHPR1* washing buffer prepared from stock solutions in ultrapure water and cooled to
165 4°C: 50 mM sodium phosphate buffer, pH 8, 300 mM NaCl, 20 mM imidazole (see **Note 11**).
- 166 10. *AtGOX1/2* elution buffer prepared from stock solutions in ultrapure water and cooled to
167 4°C: 50 mM Tris-HCl, pH 8, 1 M NaCl, 10 % (w/v) glycerol, 250 mM imidazole, 100 µM FMN
168 (see **Note 12**).
- 169 11. *AtPGLP1* elution buffer prepared from stock solutions in ultrapure water and cooled to
170 4°C: 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM MgCl₂, 250 mM imidazole.
- 171 12. *AtHPR1* elution buffer prepared from stock solutions in ultrapure water and cooled to
172 4°C: 50 mM sodium phosphate buffer, pH 8, 300 mM NaCl, 250 mM imidazole (see **Note 11**).

173 **2.4.2 Additional materials for gravity-flow affinity chromatography purification**

174 1. Glass wool.

175 2. 5 mL syringe.

176 3. Clamp stand.

177 **2.5 Recombinant protein desalting**

178 **2.5.1 Recombinant protein desalting by gravity flow**

179 1. PD-10 desalting columns (see **Note 16**).

180 2. Clamp stand.

181 3. AtGOX1/2 desalting buffer prepared from stock solutions in ultrapure water and cooled to
182 4°C: 50 mM Tris-HCl, pH 8, 20 % (w/v) glycerol, 100 µM FMN (see **Note 12**).

183 4. AtHPR1 desalting buffer prepared from stock solutions in ultrapure water and cooled to
184 4°C: 50 mM sodium phosphate buffer, pH 8 (see **Note 11**).

185 **2.5.2 Additional materials for recombinant protein desalting by centrifugation**

186 1. Refrigerated centrifuge cooled to 4°C with swinging bucket capable of 1 000 x *g*.

187 2. AtPGLP1 desalting buffer prepared from stock solutions in ultrapure water and cooled to
188 4°C: 50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂.

189 **2.6 Recombinant protein quantification**

190 1. Spectrophotometer with wavelength set to 595 nm.

191 2. Commercial Bradford reagent (see **Note 17**).

192 3. Bovine serum albumin (BSA) for calibration curve for protein quantification by the
193 Bradford method [20].

194 **3 Methods**

195 **3.1 Bacterial culture and recombinant protein production**

196 All following steps must be done under a laminar flow hood to maintain sterile conditions.

197 **3.1.1 His₆-AtGOX1/2 bacterial culture and recombinant protein production**

198 1. Take three colonies of freshly transformed *E. coli* BL21(DE3) by pET28a-GOX1/2 plasmid
199 [15] with a sterile inoculation loop and resuspend in a 50 mL glass tube containing 3 mL of LB
200 broth medium and add 3 µL of kanamycin stock solution (50 mg/L final). Incubate overnight
201 at 37°C in a cell culture shaker set at 200 rpm.

202 2. Transfer the 3 mL overnight culture by pouring it into a 1 L conical flask containing 200 mL
203 of LB medium (see **Note 18**) and add 200 µL of kanamycin stock solution (50 mg/L final) and
204 incubate at 37°C in a cell culture shaker until an OD_{600nm} of 0.4-0.6.

205 3. Add 200 µL of 1M IPTG stock solution (1 mM final) to induce recombinant protein
206 expression and further incubate for 20 h at 30°C in a cell culture shaker.

207 **3.1.2 His₁₀-AtPGLP1 and His₆-MBP-AtPGLP1 bacterial culture and recombinant protein 208 production**

209 1. Take three colonies of freshly transformed *E. coli* BL21(DE3) by pET16b-AtPGLP1 or pDEST-
210 His₆MBP-PGLP1 plasmid [18] with a sterile inoculation loop and resuspend in a 50 mL glass
211 tube containing 3 mL of LB broth medium and add 3 µL of ampicillin stock solution (100 mg/L
212 final). Incubate overnight at 37°C in a cell culture shaker.

213 2. Transfer the 3 mL overnight culture by pouring it into a 1 L conical flask containing 200 mL
214 of LB medium (see **Note 18**) and add 200 μ L of ampicillin stock solution (100 mg/L final) and
215 incubate at 37°C in a cell culture shaker until an OD_{600nm} of 0.8 (see **Note 19**).

216 3. Add 100 μ L of 1M IPTG stock solution (500 μ M final) to induce recombinant protein
217 expression and further incubate for 4 h at 37°C in a cell culture shaker.

218 **3.1.3 His₆-AtHPR1 bacterial culture and recombinant protein production**

219 1. Take three colonies of freshly transformed *E. coli* LMG194 by pBAD/His-AtHPR1 plasmid
220 [5, 12] with a sterile inoculation loop and resuspend in a 50 mL glass tube containing 10 mL
221 of RM medium and add 2.5 μ L of ampicillin stock solution (50 mg/L final). Incubate overnight
222 at 37°C in a cell culture shaker.

223 2. Transfer the 10 mL overnight culture by pouring it into a 1 L conical flask containing 200
224 mL of RM medium and add 100 μ L of ampicillin stock solution (50 mg/L final) and incubate at
225 37°C in a cell culture shaker until an OD_{600nm} of 0.7.

226 3. Add 200 μ L of 20 % (w/v) L-arabinose (0.2 % (w/v) final) to induce recombinant protein
227 expression and incubate for 20 h at 37°C in a cell culture shaker.

228 **3.2 Preparation of bacterial soluble protein extracts**

229 1a. Transfer the 200 mL bacterial culture to a 250 mL centrifuge bottle and pellet the cells by
230 centrifugation at 3 300 x *g* for 30 min at 4°C.

231 1b. Alternatively, transfer half of the 200 mL bacterial culture to 2x50mL centrifuge tubes
232 and centrifuge at 3 300 x *g* for 30 min at 4°C. After supernatant elimination, transfer the

233 remaining 100 mL of the culture to the same 2x50mL tubes and centrifuge at 1 400 x *g* for 30
234 min at 4°C. Discard the supernatant (see **Note 20**).

235 All of the following steps must be done on ice and protected from the light as much as
236 possible when purifying *AtGOX1/2*.

237 2. Gently resuspend each obtained bacterial cell pellet in 3 mL of the appropriate extraction
238 buffer according to the recombinant protein with a 5 mL precision pipette (see **Note 21**).
239 Transfer the resuspended pellet into a 50 mL tube (see **Note 12**).

240 3. Transfer the 3 mL of resuspended bacteria into the pressure cell using a 5 mL precision
241 pipette and place the cell onto the French press and break the bacteria by three passages
242 through the French pressure cell press at 500-to-600 Pa (see **Note 22**). During the final
243 passage, collect the broken bacterial extract in a 30 mL centrifuge tube, increase the volume
244 by adding 5 mL of the appropriate extraction buffer according to the recombinant protein
245 and gently homogenise.

246 4. Centrifuge the broken bacterial extract at 35 000 x *g* for 30 min at 4 °C. Keep it on ice prior
247 to recombinant protein purification either by gravity flow affinity chromatography (step
248 3.3.1.2) or “in batch” affinity purification (step 3.3.2.2).

249 **3.3 Recombinant protein purification**

250 **3.3.1 Recombinant protein purification by gravity flow affinity chromatography for His₆-** 251 ***AtGOX1/2* and His₆-*AtHPR1***

252 1. Centrifuge 2 mL of Ni²⁺-NTA-agarose IMAC resin (see **Note 23**) in a 50 mL centrifuge tube
253 at 5 400 x *g* for 10 min at 4 °C. Discard the supernatant by aspiration with a vacuum pump
254 and wash the resin by resuspending in 40 mL of the appropriate extraction buffer according

255 to the recombinant protein to be purified. Centrifuge again at 5 400 x *g* for 10 min at 4 °C,
256 aspirate the supernatant and resuspend the resin in 10 mL of the appropriate extraction
257 buffer (see **Note 24**) and transfer to a 15 mL centrifuge tube.

258 2. Once the centrifugation step (in step 3.2.4.) has finished, transfer the supernatant from
259 the cell extraction (see **Note 25**) to the 15 mL centrifuge tube containing the washed IMAC
260 resin. Incubate on a rolling wheel for 2 h at 4°C (see **Note 14**).

261 3. At the bottom of a 5mL plastic syringe (without a needle), place a homogeneous layer of
262 glass wool compacted with the plunger of the syringe. The glass wool layer must have a
263 thickness of about 2 mm once compacted. Fix the syringe to a clamp stand (see **Note 12**).
264 Pour the mix of resin and bacterial soluble protein extract into the syringe and let the
265 solution flow by gravity, the resin is retained by the glass wool to form the affinity
266 chromatography column.

267 4. Carefully and gradually pipette 40 mL of the appropriate washing buffer into the syringe
268 to wash the resin so as to remove proteins that do not interact with the Ni²⁺. You can discard
269 this flow through liquid.

270 5. Carefully add 500 µL of the appropriate elution buffer to the top of the resin and let it flow
271 through the resin. Discard this initial elution fraction as it does not yet contain recombinant
272 protein.

273 6. Carefully add 2.5 mL of the appropriate elution buffer to the top of the resin and collect
274 the eluate containing recombinant protein in a 15 mL tube kept on ice (see **Note 12**).

275 **3.3.2 “Batch” affinity purification of recombinant proteins for His₆-AtGOX1, His₁₀-AtPGLP1**
276 **and His₆-MBP-AtPGLP1**

277 1. Add 2.5 mL of Ni²⁺-NTA-agarose IMAC resin (see **Note 23**) to a 15 mL centrifuge tube and
278 add 10 mL of the appropriate extraction buffer and centrifuge in a swinging bucket at 5 400 x
279 g for 6 min at 4°C. Discard the supernatant by vacuum aspiration and wash the resin two
280 more times: Add 12 mL of the appropriate extraction buffer and carefully resuspend the
281 resin after each washing step (see **Note 24**). After the final wash step centrifugation,
282 carefully remove by vacuum aspiration the supernatant but keep 1 mL of extraction buffer
283 on top of the resin. Keep the tube containing the resin on ice.

284 2. Once the centrifuge step in 3.2.4. is finished, transfer the supernatant containing the
285 bacterial soluble proteins (see **Note 25**) to the 15 mL centrifuge tube containing the washed
286 resin and fill to 13 mL with the appropriate extraction buffer (see **Note 12**). Carefully mix the
287 suspension (see **Note 24**) and incubate at 4°C on a rolling wheel for 2 h (see **Note 14**).

288 4. Centrifuge the 15 mL tube containing the resin and the bacterial soluble protein extract in
289 a swinging bucket at 5 400 x g for 6 min at 4 °C and discard the supernatant by vacuum
290 aspiration.

291 5. Fill to 13 mL with the appropriate washing buffer. Carefully resuspend the resin (see **Note**
292 **24**) and after centrifugation at 5 400 x g for 6 min at 4 °C, remove the supernatant by
293 vacuum aspiration. Repeat this step twice.

294 6. To elute the recombinant protein, add 2.5 mL of the appropriate elution buffer to the
295 resin, mix by gentle shaking (see **Note 24**) and centrifuge in a swinging bucket at 5 400 x g
296 for 6 min at 4 °C. Collect the supernatant in a clean 15 mL tube and keep it on ice (see **Note**
297 **12**).

298 **3.4 Recombinant protein desalting**

299 **3.4.1 Recombinant protein desalting by gravity for recombinant protein purified by gravity**
300 **flow affinity chromatography (His₆-AtGOX1/2 and His₆-AtHPR1)**

301 1. Fix a PD-10 desalting column to a clamp stand and equilibrate the column (see **Note 12**) by
302 gradually adding 25 mL of the appropriate desalting buffer, allow it flow through the column
303 and into a plastic beaker.

304 2. Carefully add the 2.5 mL of eluted recombinant protein from step 3.3.1.6 to the top of the
305 column and let it enter by gravity flow.

306 3. Add 500 µL of the appropriate desalting buffer and let it flow through the column. The
307 flow through liquid is not kept as it does not contain recombinant protein.

308 4. Eluate the recombinant protein by carefully adding 3 mL of the appropriate desalting
309 buffer and collect the eluate in a 15 mL tube kept on ice (see **Note 26**).

310 **3.4.2 Recombinant protein desalting by centrifugation for “batch” affinity purification of**
311 **recombinant proteins (His₆-AtGOX1 and His₁₀-AtPGLP1 and His₆-MBP-AtPGLP1)**

312 1. Remove the frosted glass top filter of the PD-10 desalting column using forceps, cut the
313 sealed end and place it into a 50mL collection conical tube (see **Note 12**) using the adapter.

314 2. Equilibrate the PD-10 desalting column by filling to the top with the appropriate desalting
315 buffer until 25 mL of desalting buffer has passed through the column. Discard the flow-
316 through liquid.

317 3. Centrifuge the PD-10 desalting column (placed in its collection tube with the adapter) at 1
318 000 x *g* for 2 min at 4 °C.

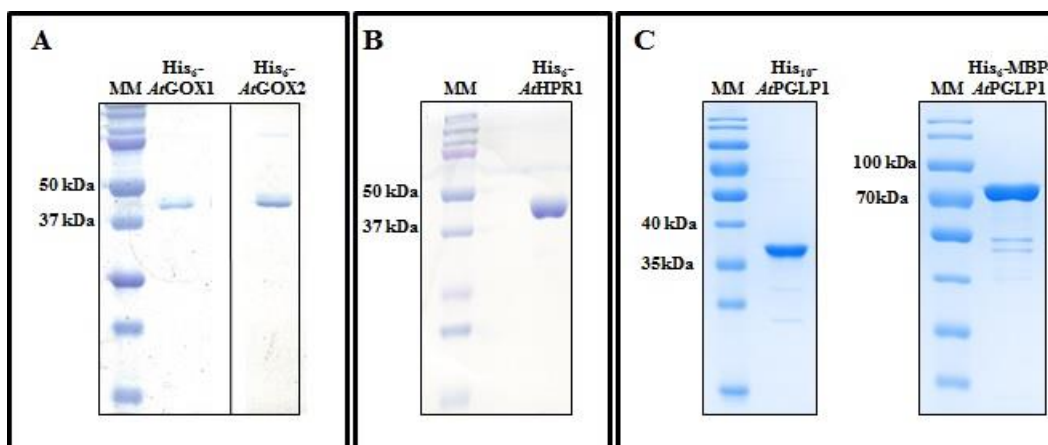
319 4. Place the equilibrated centrifuged column into a new 50 mL collection conical tube and
320 slowly add the 2.5 mL of eluted recombinant protein from step 3.3.2.6 onto the equilibrated
321 PD-10 desalting column.

322 5. Elute the desalted recombinant protein into the 50 mL tube by centrifugation at 1 000 x *g*
323 for 2 min at 4 °C (see **Note 26**).

324 **3.5 Recombinant protein quantification**

325 1. Determine recombinant protein concentration by the Bradford method [20] using a
326 commercial Bradford reagent (see **Note 17**) and by following the manufacturer's
327 instructions. This will require a calibration curve made with BSA and a spectrophotometer
328 set to 595 nm.

329 2. The purity of the recombinant protein can be checked by migrating 2 µg of desalted
330 purified recombinant protein on an SDS-PAGE gel (containing the appropriate amount of
331 acrylamide for the molecular weight of the recombinant protein) followed by staining with
332 Coomassie blue [21] (see Figure 1).



333

334

335 **4 Notes**

336 **Note 1.** In order to have ultrapure water, we use O-TEC bi-distilled apyrogenic sterile water.
337 The day before an experiment refrigerate some ultrapure water for the preparation of
338 solutions and buffers. Bidistilled water was obtained using a MilliQ apparatus.

339 **Note 2:** We use commercial LB broth (20g/L) prepared in 1 L or 2 L beakers (depending on
340 the required volume) with bidistilled water and sterilized in glass bottles at 121°C for 20 min
341 in an autoclave. Rudic culture medium (RM) is prepared as follows: 1xM9 salts, 2 % (w/v)
342 casamino acids, 0.2 % glucose, 1 mM MgCl₂ in bidistilled water and sterilized at 115°C for 20
343 min in an autoclave.

344 **Note 3:** Kanamycin stock solution (50 g/L) and ampicillin stock solution (100 g/L) are
345 prepared in bidistilled water, sterilized by filtration through a 0.25 µm filter with the help of
346 a 50 mL syringe, aliquoted and kept frozen at -20°C until use.

347 **Note 4:** 1 M IPTG stock solution is prepared in bidistilled water, sterilized by filtration
348 through a 0.25 µm filter with the help of a 50 mL syringe, aliquoted and kept frozen at -20°C
349 until use.

350 **Note 5:** 20 % (w/v) L-arabinose is prepared in bidistilled water and sterilized by filtration
351 through a 0.25 µm filter using a 50 mL syringe, aliquoted and kept frozen at 4°C until use.

352 **Note 6:** To prepare the 0.5 M Tris-HCl stock solutions at pH 7.5 and pH 8: Weigh 30.29 g of
353 Tris-base and dissolve in a beaker in 450 mL of ultrapure water, adjust the pH to 7.5 or 8
354 using concentrated HCl (12.1 N) but a lower concentrated HCl solution (1N) can be used
355 when approaching the required pH. Then adjust the volume to 500 mL in a 500 mL

356 graduated cylinder with ultrapure water, homogenize and store in 250 mL glass bottles for
357 several months at 4°C.

358 **Note 7:** To prepare the 3M NaCl stock solution: Weigh 87.7 g of NaCl, dissolve in a beaker
359 using a magnetic stirrer in 450 mL of ultrapure water and adjust the volume to 500 mL in a
360 500 mL graduated cylinder with ultrapure water, homogenize and store in 250 mL glass
361 bottles and store for several months at 4°C.

362 **Note 8:** To prepare the 50% (v/v) glycerol stock solution: Introduce directly in a graduated
363 glass cylinder 100 mL of high purity glycerol 100% anhydrous and adjust to 200 mL with
364 ultrapure water. Carefully seal the graduated cylinder with Parafilm M and handshake until
365 complete homogenisation. Store in a glass bottle at 4°C for a maximum of one month.

366 **Note 9:** To prepare the 0.5 M MgCl₂ stock solution: Weigh 20.33 g of MgCl₂.6H₂O (≥99.0%
367 purity) and dissolve in a glass beaker with 180 mL of ultrapure water before adjusting the
368 volume with ultrapure water to 200mL in a 250 mL graduated cylinder, homogenize and
369 store in a 250 mL glass bottle for several months at 4°C.

370 **Note 10:** 1M imidazole stock solution is prepared freshly and not re-used therefore you
371 should calculate the required volume before starting. Typically, to prepare 10 mL of 1M
372 imidazole solution: Weigh 680.6 mg of imidazole in a 15 mL conical tube and dissolve with
373 cold ultrapure water by vortexing. Keep on ice until use.

374 **Note 11:** To prepare 0.5 M stock solutions of Na₂HPO₄ and NaH₂PO₄ : Weigh 35.5 g Na₂HPO₄
375 (sodium phosphate dibasic) and 34.5 g NaH₂PO₄ (sodium phosphate monobasic
376 monohydrate) and dissolve them separately in beakers with 480mL ultrapure water, adjust
377 to 500 mL in a graduated cylinder, homogenize, store in a glass bottle at 4°C for one month.

378 To prepare 100 mL of 50 mM sodium phosphate buffer pH 8 for AtHPR1 purification: In a
379 100 mL graduated cylinder add 9.32 mL of 0.5 M Na₂HPO₄, 0.68 mL of 0.5 M NaH₂PO₄, and
380 adjust to 100 mL with ultrapure water. When required, for instance in the case of extraction,
381 washing and elution buffers, add required volumes of the other constituents before
382 adjusting to 100 mL.

383 **Note 12:** FMN (Riboflavin 5'-monophosphate sodium salt hydrate, store at -20°C) is unstable
384 and sensitive to light. Thus, FMN-containing buffers must be protected from light by
385 wrapping the bottle/tube in aluminium foil. Typically, to prepare 10 mL of 10mM FMN stock
386 solution: Weigh 51.4 mg of powder in a 15 mL conical tube, resuspend in 10 mL of ultrapure
387 water by vigorous vortexing. FMN is added to buffers just before the experiment and the
388 stock solution is not kept.

389 **Note 13:** We use cOmplete EDTA-free protease inhibitor from Roche (reference:
390 04693159001). One tablet must be dissolved in 50 mL of buffer just before use. To be
391 economical, a half tablet can be dissolved in 25 mL of buffer.

392 **Note 14:** The rolling wheel is set to 8 rpm for batch and to 20 rpm for gravity-flow affinity
393 chromatography.

394 **Note 15:** We use HIS-Select® Nickel Affinity Gel from Sigma-Aldrich (reference: P6611).

395 **Note 16:** We use disposable PD 10 Desalting Columns either from Cytiva (reference: 17-
396 0851-01) or GE Healthcare (reference: GE17-0851-01).

397 **Note 17:** For each new bottle of reagent, a new calibration curve is performed using BSA.
398 The baseline measurement is performed in the appropriate desalting buffer without added
399 protein.

400 **Note 18:** Conical flask and LB medium are pre-warmed to 37°C overnight to ensure fast
401 bacterial growth.

402 **Note 19:** An OD_{600nm} of 0.8 must be reached before adding the IPTG. This is important to
403 ensure a sufficient *AtPGLP1* recombinant protein production.

404 **Note 20:** For *AtPGLP1* and *AtGOX1/2* recombinant protein production, the bacterial pellet
405 can be stored at -20°C for a maximum of 3 weeks.

406 **Note 21:** The extraction buffer must be pipetted and projected onto the pellet to dissolve it
407 (avoid vortexing). Alternatively, a 1 mL precision pipette can be used.

408 **Note 22:** For the first two passages through the pressure cell, the extract can be collected in
409 the same 50 mL tube.

410 **Note 23:** Resuspend the resin by gentle shaking. Take the appropriate volume with a 5 mL
411 pipette and the appropriate tip or a 1 mL pipette with a cut tip to allow resin entry,
412 otherwise only the buffer will be taken up but not the resin.

413 **Note 24:** Resuspend the pellet of resin by gentle inversion shaking. Do not vortex.

414 **Note 25:** The supernatant contains soluble proteins, but sometimes there is floating debris.
415 Avoid taking this debris.

416 **Note 26:** PD-10 desalting columns can be re-used several times but they must be thoroughly
417 washed with at least 25 mL of ultrapure water and stored at 4°C with a top cap and stopper
418 to ensure the column does not become dry.

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428

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482

483 **Figure legend**

484 **Fig. 1. Coomassie-stained purified recombinant AtGOX1/2, AtHPR1 and AtPGLP1 His-**
485 **tagged proteins separated on SDS-PAGE gels. (A)** Two micrograms of desalted purified His₆-
486 AtGOX1 and His₆-AtGOX2 proteins purified by gravity flow affinity chromatography were
487 separated by SDS-PAGE with 10% acrylamide and stained with Coomassie Blue R-250. Each
488 AtGOX recombinant protein was estimated to be ~40 kDa. **(B)** Two micrograms of desalted
489 purified His₆-AtHPR1 protein purified by gravity flow affinity chromatography were
490 separated by SDS-PAGE with 10% acrylamide and stained with Coomassie Blue R-250.
491 AtHPR1 recombinant protein was estimated to be ~40 kDa. **(C)** Two micrograms of desalted

492 purified His₁₀-AtPGLP1 and His₆-MBP-AtPGLP1 proteins purified by “in batch” affinity
493 chromatography were separated by SDS-PAGE either with 12% or 10% acrylamide and
494 stained with Coomassie Blue R-250. His₁₀-AtPGLP1 recombinant protein was estimated to be
495 ~37 kDa and His₆-MBP-AtPGLP1 recombinant protein was estimated to be ~80 kDa.
496 Molecular mass markers (MM) and corresponding molecular masses in kDa are shown.