



Biotecnología



**LACTOSE-INDUCED EXPRESSION OF RECOMBINANT TURNIP PEROXIDASE IN
*Escherichia coli***

**EXPRESIÓN INDUCIDA POR LACTOSA DE PEROXIDASA DE NABO
RECOMBINANTE EN *Escherichia coli***

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Abstract

Turnip (*Brassica napus* L. var. purple top white globe) peroxidases (E.C. 1.11.1.7) may be applied free or immobilized on phenolic compounds degradation in waste water, in addition to its use in diagnostic test kits. The aim of this work was to achieve heterologous expression by lactose-induction of one peroxidase isoform (PodC) using *Escherichia coli* Rosetta 2 to produce recombinant turnip peroxidase in a fermenter. Recombinant PodC contained in inclusion bodies was refolded under oxidative conditions and purified by IMAC with a yield of 36 mg L⁻¹ of culture. The enzyme showed a specific activity of 1004 ABTS units mg⁻¹ under optimum conditions (pH 6, 45 °C). The value of K_M for ABTS was 0.38 mM and the catalytic efficiency (k_{cat}/K_M) was 5.4×10^6 s⁻¹ M⁻¹. The use of lactose as inducer and scale up of the fermentation process may effectively decrease the production cost of recombinant PodC, which in turn can be applied for biotechnological applications.

Keywords: *Brassica napus*, peroxidase, expression, lactose, fermenter.

Resumen

Las peroxidasas (E.C. 1.11.1.7) de nabo (*Brassica napus* L. var. purple top White globe) pueden aplicarse libres o inmovilizadas para la degradación de compuestos fenólicos en aguas residuales además de su uso en kits de diagnóstico. El objetivo de este trabajo fue lograr una expresión heteróloga por inducción con lactosa de una isoforma de peroxidasa (PodC) usando *Escherichia coli* Rosetta 2 para producir peroxidasa de nabo recombinante a nivel fermentador. PodC recombinante contenida en cuerpos de inclusión se replegó bajo condiciones oxidativas y se purificó por cromatografía para obtener 36 mg L⁻¹ de cultivo. La enzima tuvo una actividad específica de 1004 unidades ABTS mg⁻¹ bajo condiciones óptimas (pH 6, 45 °C). El K_M para ABTS fue 0.38 mM y la eficiencia catalítica (k_{cat}/K_M) fue 5.4×10^6 s⁻¹ M⁻¹. El uso de lactosa como inductor y el escalamiento del proceso de fermentación puede reducir el costo de producción de PodC recombinante, que a su vez puede usarse en aplicaciones biotecnológicas.

Palabras clave: *Brassica napus*, peroxidasa, expresión, lactosa, fermentador.

1 Introduction

Peroxidases (EC1.11.1.7) contain a heme iron (III) (ferriprotoporphyrin IX) as prosthetic group which catalyzes the oxidation of a variety of substrates using hydrogen peroxide (H₂O₂) as electron acceptor. These properties make peroxidases useful in many industrial applications such as removal of phenolic compounds

in contaminated water, immunoassays, biosensors and diagnostic test kits (Regalado *et al.*, 2004). Global production of peroxidase is mainly obtained from horseradish. However, some turnip (*Brassica napus* L.) isoperoxidases have better thermostability and activity characteristics than that from horseradish, which make them attractive for same applications

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(Agostini *et al.*, 2002; Quintanilla-Guerrero *et al.*, 2008). Our group has identified the cDNA encoding a turnip isoperoxidase which was labeled PodC (GenBank No. AY423440.3).

Control of recombinant peroxidase expression from *Escherichia coli* is highly regulated by the *lac* repressor and the non-metabolizable lactose analog isopropyl- β -D-thiogalactopyranoside (IPTG) that is used as inducer, which despite being efficient is costly and potentially toxic to the cell especially in large scale processes (Menzella *et al.*, 2003). Thus, commercially attractive processes need a more available inducer such as lactose, to achieve protein expression from *E. coli* resulting in cost effective, large-scale production of heterologous proteins. However, the use of lactose may affect process control and its main drawback is related to the carbon catabolite repression mechanism, in which the transport and metabolism of lactose is inhibited until glucose in the media is exhausted (Deutscher *et al.*, 2006). Thus, it seems difficult to induce foreign genes expression with the addition of lactose. A turnip recombinant peroxidase has been successfully expressed in *E. coli* using IPTG as inducer (Rodríguez-Cabrera *et al.*, 2011). Therefore, the aim of this study was to enhance the production of recombinant PodC in *E. coli* Rosetta 2 using lactose-induction.

2 Materials and methods

2.1 Materials

NaCl, Tris-HCl, imidazol, sodium phosphate, oxidized glutathione (GSSG), hemin, isopropyl- β -D-thiogalactopyranoside (IPTG), lactose, bovine serum albumin (BSA), and diammonium salt of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonate) (ABTS) were obtained from Sigma (St. Louis, MO). Electrophoresis chemicals [acrylamide, N,N'-methylenebisacrylamide, 1,2-bis(dimethylamino)ethane (TEMED), sodium dodecyl sulfate (SDS), Tris(hydroxymethyl)-aminomethane and ammonium persulfate were purchased from BioRad (Hercules, CA). A vector containing a DNA fragment coding for turnip peroxidase (PodC) was obtained as previously reported (Romero-Gómez *et al.*, 2008).

2.2 Construction of expression vector pET28-PodC

PodC was amplified by PCR using the plasmid TOPO-PodC as DNA template (Romero-Gómez *et al.*, 2008), the primers used were (Sigma): 5'-**CCATGGGCCAGTTAAACCCAACGTTT**-3' and 5'-**AAGCTTCTAGGATTGTCCATTAACCA**-3', for sense and antisense, respectively. The primers contained NcoI and HindIII restriction sites (bold) to allow directional cloning of amplified DNA. The PCR product was identified followed by ligation to the pET 28b(+) vector (Novagen, Madison, WI) previously digested with the same restriction enzymes. The construction pET28-PodC was used to transform *E. coli* Rosetta 2 (DE3) competent cells (Novagen) using heat shock (Sambrook and Russell 2001). Plasmidic DNA was isolated from transformants using the Qiagen DNA mini-prep (Hamburg, Germany) and confirmed by PCR and restriction analysis.

2.3 Small-and higher-scale expression experiments

The *E. coli* strain Rosetta 2 (DE3) transformed with pET28-PodC, was grown for small scale expression as described by Rodríguez-Cabrera *et al.* (2011). Experiments were conducted from 0.5 to 3 % (w/v) lactose as PodC inducer, followed by higher-scale expression which was performed similarly, with modifications. Transformed colonies were inoculated into 50 mL of LB-kanamycin-chloramphenicol broth. Cultures were incubated overnight at 37 °C in an orbital shaker (Lab-line, Melrose Park, IL) at 200 rpm and used to inoculate 2 L of same broth in the fermenter (Applikon, CA). pH was controlled at 7.0 and air was supplied at 30 mL min⁻¹, while agitation rate was 400 rpm, at 37 °C (Lara 2011). Bioexpert software (Applikon) was used to control process variables. Cultures were grown to an OD₆₀₀ ~0.8, measured using a Genesys 10 UV spectrophotometer (Thermoscientific, Madison, WI) and dry weight was also determined by heating at 105°C for 5 h. From small scale experiments (300 mL), it was found that recombinant PodC was best induced by adding 2% lactose (w/v), replacing IPTG. Bacterial cells in the fermenter were grown another 5 h at the same conditions and harvested by centrifugation (Eppendorf 5804R, Hamburg, Germany) at 4000 g for 10 min at 4 °C, and were either immediately used or stored at -20 °C.

2.4 Polyacrylamide gel electrophoresis (PAGE)

PodC in cell extracts and in the insoluble fraction was analyzed by SDS-PAGE using a 10% polyacrylamide gel in a Mini Protean II unit (Bio-Rad). Low molecular weight markers (GE Healthcare, Uppsala, Sweden) were used as reference and protein bands were stained using Coomassie blue G250 dye (Bio-Rad).

2.5 Solubilization of inclusion bodies, refolding and purification of PodC

Isolation and solubilization of inclusion bodies was conducted as depicted by Rodríguez- Cabrera *et al.* (2011) with modifications. Inclusion bodies were solubilized in 6 M GndCl, whereas PodC refolding was conducted before its purification using 4 M GndCl. GndCl, urea, and other denaturants are known to have ability to increase efficiency of protein refolding. However, urea may be degraded by heat or alkaline pH to cyanate, resulting in isocyanic acid, which can attack reactive groups (Lys, Arg or Cys) of recombinant protein and thus negatively affect conformational stability (Pham *et al.*, 2011). Thus, the effect of using GndCl for functionally active turnip peroxidase was tested. Refolded PodC was purified by immobilized-metal affinity chromatography (IMAC) using a 1 mL Ni-NTA column (GE Healthcare, Uppsala, Sweden) as described by Rodríguez-Cabrera *et al.* (2011). Fractions of 0.5 mL were collected and analyzed by SDS-PAGE.

2.6 Activity assay and characterization of recombinant PodC

Peroxidase activity was determined using 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid, ABTS) as the hydrogen donor. The reaction mixture consisted of 10 mM potassium phosphate buffer (pH 6.0), 2.5 mM ABTS, 50 μ L of purified enzyme and 2 mM H₂O₂, in a total volume of 1.5 mL. The change in absorbance at 414 nm ($\epsilon_{414} = 36 \text{ mM}^{-1} \text{ cm}^{-1}$) (Childs and Bardsley 1975) was followed spectrophotometrically. One unit of activity is defined as the μ moles of ABTS radical produced per min at pH 6.0 and 25 °C. Protein concentration was determined according to Bradford (1976) using bovine serum albumin (BSA) as standard.

2.7 Biochemical properties of PodC

Optimum pH and temperature experiments, and kinetic studies were conducted as described by Hernández-Martínez *et al.* (2011) and Rodríguez-Cabrera *et al.* (2011). All experiments were conducted in triplicate and results are expressed as the mean \pm standard deviation.

3 Results and discussion

3.1 Construction of the expression vector pET28-PodC

The full-length PodC comprises 922 base pairs (bp) corresponding to an open reading frame that encodes a predicted protein of 306 amino acids (Romero-Gómez *et al.*, 2008). It was successfully amplified from the pGEMT-PodC vector (Figure 1A, lane 2), and ligated to yield the expression plasmid pET28b-PodC which was verified by NcoI and HindIII digestion (Figure 1B, lane 2).

3.2 Expression of recombinant PodC

PodC contains 15 Ile (ATA), 9 Arg (AGA and CGG), 32 Leu (CTA), 16 Pro (CCC) and 25 Gly (GGA) codons which are rare for prokaryotes, but this problem was overcome by using *E. coli* Rosetta 2 (DE3) which supplies the genes of the corresponding tRNAs. Cell extracts, obtained from small-scale expression experiments, with 2% lactose (w/v) addition (Figure 2, lane 3) exhibited a differential expression band characteristic of those after induction with IPTG (Figure 2, lane 2) and showed a molecular weight of about 37 kDa. This band corresponds to the expected size of the His₆-tagged recombinant PodC, and represents about 33% of the total protein produced by *E. coli*. For subsequent higher-scale expression experiments, we used 2% lactose (w/v). However, proteins under regulation of lactose-inducible promoters, as catabolic repression, prevent lactose uptake and utilization (Tutar 2008). This suggests that the expression strain would continue to grow despite inducer addition, without target protein expression until high cells density is achieved. At this point depletion of inhibitory factors would allow lactose induction with the consequent expression of high concentrations of target protein (Studier 2005). Saturation OD₆₀₀ \sim 0.8 in bioreactor media (18 g L⁻¹) was 1.3 times higher than in flask culture (12 g L⁻¹) under similar conditions,

producing increased PodC concentration at the end of fermentation (Table 1). Process variables were controlled by Bioexpert software (Applikon) and cells density significantly increased after lactose induction (Figure 3), showing a specific growth rate of 0.0037 min^{-1} , which led to doubling time of 187.3 min. In addition, replacement of IPTG for a more available chemical such as lactose will probably result in economic benefits for turnip peroxidase production. Variation of IPTG concentration or changing induction temperature, did not improve recombinant protein solubility (Rodríguez-Cabrera *et al.*, 2011), which was similarly observed for lactose as inducer (results not shown). Recombinant PodC accumulated as cells insoluble fraction within inclusion bodies in agreement with Smith *et al.* (1990) and Hushpulia *et al.* (2003), and represented about 50% of the insoluble cellular protein. The amount of cells yield was $0.72 \text{ g cells/g LB medium}$.

3.3 Refolding of recombinant PodC

Specific activity of the refolded recombinant PodC was $1004 \pm 412 \text{ U mg}^{-1}$ under the best conditions, which is slightly lower than that shown by recombinant tobacco anionic peroxidase (1100 U mg^{-1} ; Hushpulia *et al.* 2003) and higher than that of HRP (0.58 U mg^{-1} ; Ryan and O’Fágáin 2008), both expressed in *E. coli*. Compared with other turnip peroxidases, PodC activity is slightly higher than that of BnPA (981 U mg^{-1} ; Rodríguez-Cabrera *et al.* 2011) and showed about the same activity as native partially deglycosylated turnip peroxidase (Duarte-Vázquez *et al.*, 2003), indicating an unclear effect of the carbohydrate moiety on turnip peroxidase activity.

3.4 Purification of recombinant PodC by IMAC

Refolded PodC was purified by IMAC, eluting with 0.5 M imidazole (Figure 4, lanes 5-8). It is clear that recombinant PodC was purified to homogeneity in a single step since no other protein bands were observed, with a yield of 36 mg L^{-1} of cell culture (Table 1), showing a purity of 98% according to densitometric analysis. The amount of produced PodC was $2 \text{ mg (g dry cells)}^{-1}$. BnPA purification was achieved after two chromatographic steps, IMAC followed by gel-filtration (Rodríguez-Cabrera *et al.*, 2011) with a yield of 29 mg L^{-1} , which is 1.2 times lower than that reported here for the single step purified PodC. Recombinant HRP from *E. coli* achieved yields of

16.7 mg L^{-1} (Gazaryan *et al.*, 1994), and 0.086 mg L^{-1} (Ryan and O’Fágáin 2008), which are about 2.1 and 418.6 times lower than that obtained here, respectively. Using the same system, recombinant HRP-conjugate yield was 12 mg L^{-1} (Grigorenko *et al.*, 2001), which is a third of our value. In addition, recombinant glycosylated HRP-C was expressed in insect cells culture with added hemin and the yield of active peroxidase was 28 mg L^{-1} (Segura *et al.*, 2005), which is 1.2 times lower than that obtained here. However, recombinant protein expression using tissue culture is time-consuming and may involve high cost (Ikonomou *et al.*, 2003).

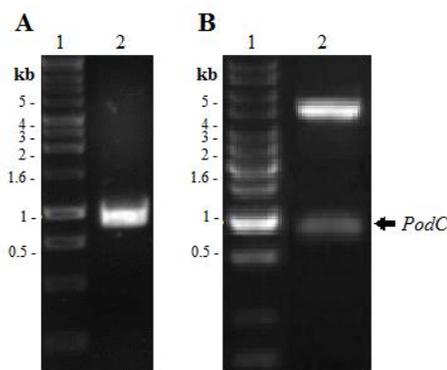


Fig. 1. Construction of recombinant vector pET28-PodC. A) Lanes: 1, 1 kb DNA ladder; 2, PCR product of PodC. B) Lanes: 1, 1 kb DNA ladder; 2, transformant clone containing the recombinant vector pET28-PodC with NcoI and HindIII.

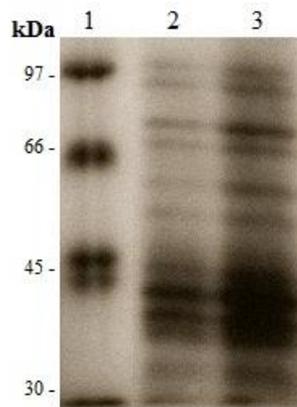


Fig. 2. SDS-PAGE analysis of PodC expression using 1 mM IPTG and 2% lactose (w/v) as inducers. Lane 1, low range molecular weight marker; lane 2, cell extract after 1 mM IPTG induction; lane 3, cell extract after 2% lactose (w/v) induction.

Table 1. Purification of recombinant PodC (mean values \pm standard deviation).

| Sample | Protein (mg·mL ⁻¹) | Total protein (mg) | Yield (%) | Total yield [mg·(Lculture) ⁻¹] |
|---------------------------------|--------------------------------|--------------------|----------------|--|
| Total cells lysate ^a | 4764 \pm 42 | 96 \pm 1 | 100 | 476 \pm 4 |
| SP ^b | 2335 \pm 21 | 44 \pm 0.7 | 49 \pm 0.6 | 233 \pm 2 |
| IMAC ^c | 643.2 \pm 5.5 | 7.2 \pm 0.1 | 13.5 \pm 0.5 | 36.0 \pm 0.3 |

^aA total of 36 g dry weight of cells from 2 L culture medium were lysed.

^bSP= solubilized protein in 6 M GndCl.

^cImmobilized metal affinity chromatography.

3.5 Recombinant PodC characterization

As expected, the effect of pH and temperature on recombinant PodC showed a profile very close to that of BnPA (Rodríguez-Cabrera *et al.*, 2011). A relative activity of 70% (i.e., ratio of actual to maximum activity) or higher was observed in a narrow pH range (5-7) with a maximum at pH 6.0, whereas maximum activity of purified recombinant PodC was observed at 45°C (results not shown). Theoretical pI was 4.6, as calculated from the bioinformatics software ExPASy (<http://www.expasy.org/>). Kinetic parameters were $K_m = 0.38 \pm 0.03$ mM, $k_{cat} = 2026 \pm 29$ s⁻¹, and catalytic efficiency ($k_{cat}K_m^{-1}$) = 5426 ± 94 s⁻¹ mM⁻¹, whereas those reported for recombinant turnip peroxidase BnPA were 0.33 ± 0.04 mM, 1722 ± 37 s⁻¹ and 5218 ± 85 s⁻¹ mM⁻¹, respectively. These values suggest that PodC was produced with similar kinetic properties as BnPA despite using lactose as inducer, and considering that in this work refolding was conducted before purification which was achieved in a single step as compared to the two steps carried out to purify BnPA (Rodríguez-Cabrera *et al.*, 2011).

Additional experiments on PodC obtained from fermentation broth involved concentration to 4 mg mL⁻¹ by means of freeze drying, followed by crystallization studies using the micro-batch method (data not shown). However, the crystals obtained did not show the quality required to perform complete X-ray diffraction studies, because higher protein concentration was required to obtain crystals suitable for structure elucidation of recombinant turnip peroxidase. More tests should be conducted on environmental applications of recombinant PodC to fully evaluate its performance as compared to native or chemically modified turnip peroxidase.

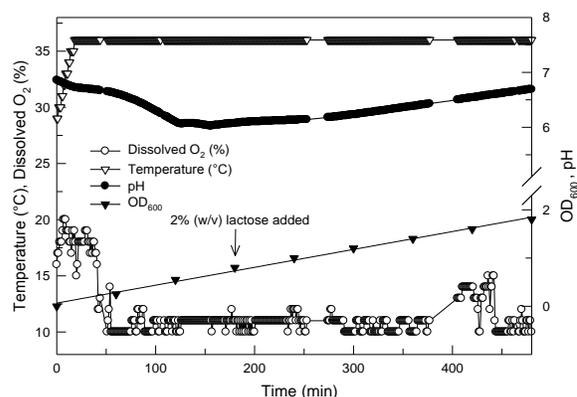


Fig. 3. Variables of the fermentation process to obtain recombinant PODC as a function of time. pH was kept at 7.0, temperature at 37°C and dissolved oxygen about 10% for the entire fermentation time.

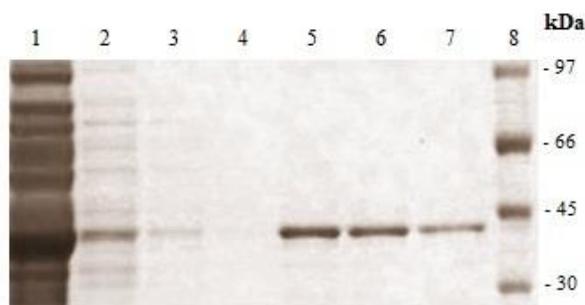


Fig. 4. SDS-PAGE analysis of purified recombinant PodC. Lane 1, cell extract after lactose induction; lane 2, insoluble fraction; lane 3, first wash from Ni-NTA column; lanes 4-7, PodC elution from Ni-NTA column; lane 8, low range molecular weight markers.

Conclusion

A methodology has been established that could be more economical for the heterologous production of active recombinant turnip peroxidase, in which lactose-induction gave an expression level of PodC comparable to conventional IPTG induction. Enhanced refolding conditions and a single step purification of recombinant PodC allowed cost effective production of active enzyme. The results presented here demonstrate that lactose may well be used as PodC inducer with the advantage of availability, and no-toxicity. Recombinant PodC production may thus be scaled-up to be used in biotechnological applications.

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