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Journal of Chromatography B, 786 (2003) 229–236
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Optimisation of expression and immobilized metal ion affinity chromatographic purification of recombinant $(His)_6$-tagged cytochrome P450 hydroperoxide lyase in *Escherichia coli*

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Abstract

Fatty acid hydroperoxide lyase (HPL) is a cytochrome P450 acting on fatty acid's hydroperoxides in many plants. The optimisation of the expression of recombinant $(His)_6$-tagged HPL in *Escherichia coli* is described: the highest HPL production yield were obtained with TB medium supplemented with 2.5 m*M* δ-aminolevulinic acid and 0.5 m*M* IPTG. For the first time, the time course expression of a plant P450 in a bench-scale fermentor is detailed and the amount of recombinant HPL production is 16.3 mg/l. The UV–Visible spectrum of the recombinant $(His)_6$-tagged HPL have been recorded after a Ni^{2+}-based affinity chromatography (IMAC).

Keywords: Expression; Purification; *Escherichia coli*; Hydroperoxide lyase; Cytochrome P450; Histidine tag

1. Introduction

In higher plants, mushrooms and algae, fatty acid hydroperoxide lyase (HPL) cleaves linoleic and linolenic acid hydroperoxides into volatile compounds (short chain aldehyde, alcohol, or hydrocarbon) and oxo-acids. Two categories of HPL have been reported according to their cleavage type: homolytic HPL cleaves the bond between a saturated carbon and the one bearing the hydroperoxide function (Fig. 1) while the link between vinylic carbon and carbon bearing the hydroperoxide function is cleaved by heterolytic HPL [1]. HPL is involved in ''the lipoxygenase pathway'' in which lipoxygenase

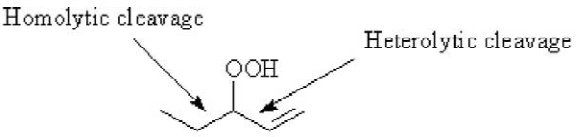

Fig. 1. Fatty acid hydroperoxides homolytic and heterolytic cleavages.

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1570-0232/02/$ – see front matter
doi:10.1016/S1570-0232(02)00815-2

(LOX) peroxydizes linoleic and linolenic acids into their 9- and 13-hydroperoxides isomers [2]. It has been demonstrated that HPL is a membrane bound heme protein [3] belonging to the cytochrome P450 class [4]. The exact mechanism of catalysis remains unclear but it has recently been demonstrated that hematin has both homolytic and heterolytic HPL activities [5]. This last result suggests that heme prosthetic group has a key function in the catalysis.

HPL has a huge industrial issue: the biosynthesis of ''green'' flavour compounds such as *Z*-3-hexenal and hexanal. Volatile C_6-aldehydes are main contributors to the characteristic odour of plant. They are widely used as food flavours to reconstitute the ''fresh green'' smell of fruits or vegetables lost during industrial processing. But plant tissues are poor in short-chain aldehydes. A biocatalytic process was needed to produce large quantities of such compounds. Plant HPL is the only enzyme source for a natural synthesis of *Z*-3-hexenal and hexanal. Another production strategy would be overexpressing HPL in *Escherichia coli* as heterologous protein. Large amount of HPL could be obtained without supplying the process with plant. Unlike plant, *E. coli* would produce only one hydroperoxide-metabolising enzyme leading to the expected product. No allene oxide synthase, peroxygenase or divinyl ether synthase synthesis that would lead to undesired by-product and loss of substrate.

Recombinant expression of HPL is a method to increase the availability of this unstable biocatalyst. Enzyme with high purity is required for studies on three-dimensional structure or catalysis mechanism. For performing a hanging drop crystallisation, large amounts of pure HPL are needed. Recombinant expression and affinity purification (IMAC) show, here again, a great interest.

This study presents the optimisation of the expression and the purification of the $(His)_6$–cytochrome P450 HPL (CYP74B) expressed in *E. coli*. The expression parameters of the recombinant enzyme and the chromatographic step (IMAC) are detailed. For the first time, the cells lysis and the enzyme extraction (and solubilisation) are performed in a single step. Amounts of $(His)_6$–HPL produced are spectrophotometrically measured. The technical feasibility of an industrial process will be evaluated.

2. Material and methods

2.1. Material

Lysozyme, IPTG and PMSF were purchased from Sigma, lipoxygenase from Sigma UK, ampicilin from Sigma Belgium and kanamycin from Sigma Japan.. His-trap affinity resin and DNAseI were purchased from Amersham Biosciences. Ni-NTA agarose and Talon resin were purchased, respectively, from Qiagen (Germany) and ClonTech (USA). *n*-Octylpolyoxypropylene was obtained from Bachem (Switzerland).

2.2. Expression of $(His)_6$-tagged CYP74B HPL

E. coli M15 expressing $(His)_6$-tagged (N-terminal form) recombinant CYP74B HPL from immature bell peppers has been furnished as described previously [4]. The cDNA encoding HPL was subcloned into the expression vector pQE-31 (Qiagen).

The His-tagged CYP74B HPL was expressed as follow. Cells from an overnight growth on a Petri dish with LB agar medium (supplemented with 200 mg/l ampicilin and 50 mg/l kanamycin) at 37 °C were harvested and resuspended in peptoned water. This suspension was added to 100 ml of TB medium supplemented with 100 mg/l ampicilin, 50 mg/l kanamycin and 2.5 m*M* δ-aminolevulinic acid in 500 ml flasks. The volume of suspension was determined in order to have an initial OD 600 nm of 0.1. M15 cells were grown at 37 °C with an orbital shaking at 150 rpm. Then, 0.1 m*M* IPTG was added when OD_{600} reached 1–1.2 and the cultures were cooled at 20 °C. The cells have been incubated at this temperature for 48 h.

2.3. A 2-l bench-scale fermentor

A 2-l fermentor Biostat (B. Braun, Biotech International, Germany) has been equipped with pH and pO_2 sensors (Ingold Messtechnik, Switzerland). The biomass production was followed at 600 nm (1 AU_{600} corresponds to 0.46 mg cells/ml, dry matter according to preliminary tests). pH was automatically regulated at 7 with 25% (v/v) H_3PO_4. The air flow was 2 l/min. HPL activity was measured each hour

using the ''cells lysis'' (1 ml medium was lysed by 1 ml lysis buffer) and ''enzyme assay'' procedures described below. TB medium supplemented with antibiotics was used.

2.4. Cell harvesting and lysis

Cells were harvested by centrifugation (10 000 *g*, 4 °C, 20 min). Supernatant was discarded. The pellet was washed (phosphate buffer pH 7) and centrifuged in the same conditions.

The composition of the lysis buffer (5 ml) follows: 50 m*M* Tris–HCl pH 8, 25 mg of lysozyme, 50 μl of 10 mg/ml PMSF, 25 μl of 1 *M* $MgCl_2$, 10 μl of DNAseI, 500 m*M* NaCl and 1% *n*-octylpolyoxypropylene (*n*-opp, detergent).

After addition of the lysis buffer, the pellet was incubated at 4 °C for 30 min with vigorous shaking (magnetic stirrer). The lysed cells were centrifuged (25 000 *g*, 4 °C, 15 min). The supernatant is the crude extract and contains heterologous HPL.

2.5. Purification of $(His)_6$-tagged recombinant HPL

The affinity resin (1 ml) was preliminary washed with 2 ml 0.1 *M* $NiSO_4$ followed by 5 ml water and equilibrated with 5 ml of buffer 1 (50 m*M* Tris–HCl pH 8, 500 m*M* NaCl and 1% *n*-opp). Prior to purification pH of the crude extract was re-adjusted to 8 with 0.1 *M* NaOH. Then, 5–7 ml of crude extract (corresponding to a 100 ml culture) were loaded on 1 ml of Ni^{2+} affinity resin (Ni-NTA Agarose, Qiagen) in a 1-cm diameter column of 10 ml. The flow-rate was 0.5 ml/min. The column was washed with 5 ml buffer 1 and 15 ml buffer 2 (50 m*M* sodium phosphate pH 6, 500 m*M* NaCl and 1% *n*-opp). $(His)_6$-tagged HPL was eluted using 10 ml buffer 3 (50 m*M* sodium acetate pH 4, 500 m*M* NaCl and 1% *n*-opp).

2.6. Enzyme assays

The substrate of HPL (13-hydroperoxy(*Z,E,Z*)-9,11,15-octadecatrienoic acid, 13-HPOTE) was synthesised, extracted and purified according to Ref. [6].

HPL activity was measured by following the decrease in absorbance at 234 nm (disruption of the conjugated diene in the substrate) using an Ultrospec 4000 UV–Visible spectrophotometer from Pharmacia.

3. Results and discussion

3.1. Expression of $(His)_6$-tagged CYP74B HPL

Preliminary tests have clearly shown that TB was better than LB. LB is not buffered and lacks specific carbon source. The consequence was a quick pH increase due to the desamination of amino acids. The high pH resulted in slow growth rate and poor HPL expression levels in our experiments.

HPL expression was only detected when the growth temperature was 20 °C. At 37 °C, 500 times lower expression was observed. As recently observed by [7], plant P450 are, at this temperature, expressed as inactive inclusion bodies in *E. coli*. An interesting method for purification of recombinant proteins expressed in inclusion bodies in denaturing conditions, followed by a refolding during the chromatographic step is proposed in Ref. [8]. Unfortunately, native HPL is very unstable and we didn't investigate in that way.

The effect of increasing inductor (IPTG) concentrations on expression rate of active HPL was investigated. The results are presented in Table 1. The best HPL expressions were obtained with 0.5 m*M* IPTG. Unexpected high HPL activity was recorded when IPTG was omitted. It seems that the repression of promoter was not optimal. This result fits with the recent publication in Ref. [9]. The

Table 1
Effect of IPTG concentration on the expression yield of recombinant $(His)_6$-tagged HPL in *E. coli*

IPTG (m*M*)	Activity (EU/ml)	Cells (dry weight, g/l)	Final pH
0	5.1	3.8	7.4
0.001	5.6	3.9	7.3
0.01	6.4	3	7.4
0.1	7	2	7.4
0.5	7.9	1.8	7.3
1	7.1	1.9	7.2
5	7.4	1.8	6.2

authors have obtained the best expression yields without IPTG. Nevertheless, the reason for this ''spontaneous expression'' remains unknown. We suppose that an undetermined ingredient of the TB medium may ''mimic'' IPTG and deregulate the repression mechanism of the *lac* repressor protein. The final biomass (the weight of cells, dry matter) decreases when IPTG concentration increases. IPTG seems to act as a growth inhibitor for *E. coli*. These results demonstrate that the optimisation of the expression of an active recombinant enzyme is not simply related to biomass production. In most of the experiments, final pH was unchanged due to the buffering capacity of TB medium.

As mentioned above, HPL is a heme enzyme. The effects of some heme precursors on HPL expression have been investigated. *E. coli* biosynthesises heme prosthetic group according to the ''C_5 route'', directly from glutamic acid via δ-aminolevulinic acid [9]. No increase of HPL activity was observed with addition (to TB medium) of 10 μ*M* hematin, 0.1 or 2.5 m*M* Fe(III) (central atom of heme) and 0.5% glutamic acid. When 2.5 m*M* δ-aminolevulinic acid was added, active HPL expression yield doubled. The beneficial effects of δ-aminolevulinic acid on recombinant plant P450s expression in *E. coli* have been noticed by some authors [7,10,11]. It is well known that cytochromes P450 need heme prosthetic group in their active forms [9]. Our results show that the ''natural'' heme synthesis enzymatic pathway of *E. coli* is insufficient to produce heme for a recombinant P450 expression. They also demonstrate that the limiting step is the bioconversion of glutamic acid to δ-aminolevulinic acid. Indeed, addition of this last compounds has increased the expression of active HPL while addition of glutamic acid did not [12] have shown that the control of δ-aminolevulinic acid synthesis in *E. coli* (and thus of the whole pathway) resides in the glutamyl-tRNA reductase (GTR). This last enzyme is one of the four steps between glutamic acid and δ-aminolevulinic acid. As we have noticed that the critical step resides between these same compounds, our observations match perfectly with the conclusion of these last authors.

Time course expression of recombinant HPL was investigated using a bench-scale 2-l fermentor. The results are presented in the Fig. 2. From 0 to 3 h, the growing temperature was maintained at 37 °C. No HPL activity was detected (expression in inclusion bodies). Glycerol was the first carbon source used by the cell and there was no pH variation. At the end of this period, the pO_2 decreased: the cells (in logarithmic growth) consumed more O_2. OD_{600} had reached 1 after 3 h. The growing temperature was decreased to 20 °C and 0.1 m*M* IPTG was added. Obviously, this temperature shift caused a break in the growing rate. The O_2 consumption decreased until pO_2 reached 100%. As soon as 20 °C was reached (around 5 h), HPL activity was detected. At this time of the production and until its end, H_3PO_4 was regularly added. Indeed, glycerol was entirely consumed. Cells started to use amino acids as a new carbon source and the desamination of this substrate caused an increase of the pH. The production was stopped after 20 h. The HPL activity maxima was not reached. Additional 500-ml flasks experiments were performed (data not presented) and demonstrated that the optimal production time was 40 h.

3.2. Cell lysis and HPL solubilisation

After M15 cells harvesting, chemical lysis (lysozyme) was performed. At 10 mg/ml lysozyme, fluorescent microscopy observations have shown that the lysis yield was nearly 100% (data not shown). No ''mechanical'' lysis (sonication or French press) was required.

To avoid a too viscous crude extract, DNAseI and its co-factor (Mg^{2+}) were added to the lysis buffer.

A PMFS/EDTA antiproteases inhibitor cocktail is generally recommended for *E. coli*. Only PMSF was used in our experiments because EDTA is not suitable with Ni^{2+}-based IMAC.

HPL is a membrane bound enzyme and thus requires a detergent for its extraction and its solubilisation. The choice of the most suitable detergent is one of the critical aspect in any study of membrane bound protein. *n*-Octylpolyoxypropylene (*n*-OPP) gave the best solubilisation after a screening including *n*-octylglucoside (1-*O*-*n*-octyl-β-D-glucopyranoside), CHAPS (3-[(cholamidopropyl)dimethyl-ammonio]-1-propanesulfonate) and Triton X-100. Although it gave nearly the same results as *n*-OPP, Triton X-100 was avoided due to its strong absorbance at 234 nm (HPL assays) and at 280 nm (protein).

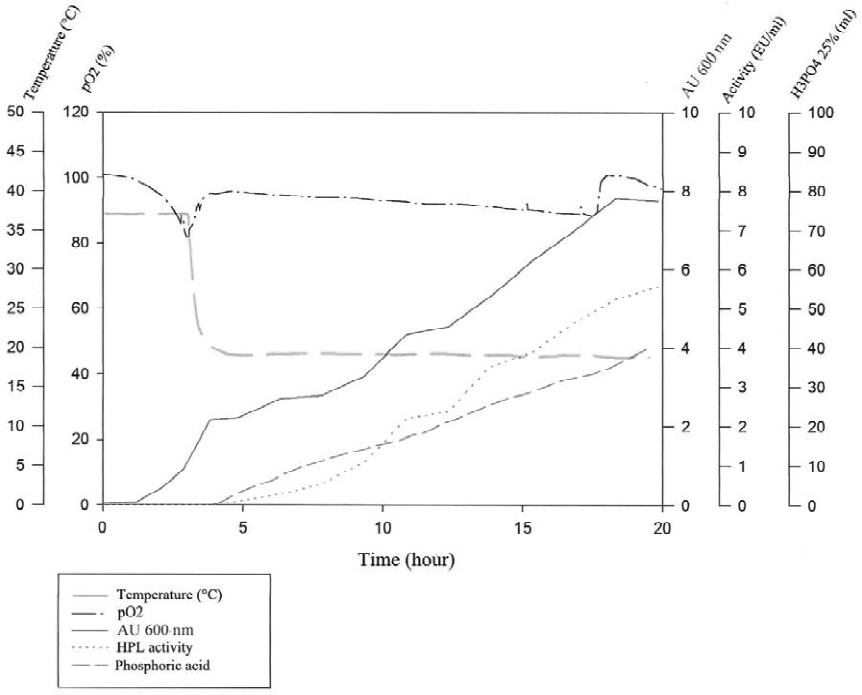

Fig. 2. Time course expression of recombinant $(His)_6$-tagged HPL in *E. coli*.

Gycerol is often used to increase solubility of P450 [13]. In our conditions, addition of 20% glycerol to crude extract was ineffective.

3.3. Purification of His-tagged HPL

3.3.1. Choice of the affinity resin

Three IMAC resins were evaluated. The metal ion was Ni^{2+} for both Ni-NTA agarose (Qiagen) and HisTrap (Pharmacia), and Co^{2+} for Talon resin (Clontech). The binding capacities of N-terminal $(His)_6$-tagged HPL were the same on the Ni^{2+}-based resins and greater than Co^{2+}-based resin. The poor N-terminal His-tagged P450 binding capacities of the Talon resin was also observed in Ref. [14]. These authors obtained better results with the C-terminal His-tagged form of their recombinant P450. The C-terminal form of $(His)_6$-tagged HPL have not been investigated because good results were obtained with Ni^{2+}-based IMAC.

3.3.2. Chromatographic parameters

Various resin bed volumes and flow-rates were investigated. Results are presented in Table 2. The best HPL recovery yields were obtained with a 5:1 ratio between crude extract and IMAC resin and a 0.5 ml/min flow-rate. The importance of the flow-rate on recovery yields was more important at a 10:1 ratio: a two times higher yield was obtained by dividing the flow-rate by 2.

Table 2
Chromatographic parameters related to IMAC purification yields of recombinant $(His)_6$-tagged HPL

Volume crude extract (ml)	Volume resin (ml)	Flow (ml/min)	η_1 (%)	η_2 (%)
5.0	0.5	1	21	25
7.2	0.5	0.5	45	55
5.0	1	1	76	–
6.4	1	0.5	80	70

η_1 and η_2 are two recovery yields (see text for details).

Two recovery yields were determined to estimate the loss of activity during the chromatographic step: η_1 and η_2 were calculated by dividing the HPL activity of the HPL-containing fractions (see Fig. 3) by, respectively, the sum of HPL activity in all fraction and the HPL activity in the crude extract. As it can be seen in Table 2, η_1 and η_2 are very closed. Thus we have considered that both the denaturation of HPL during the IMAC step and its quantities irreversibly bound on the resin were negligible.

Fig. 3 shows the chromatogram of a typical purification. The volume of the fractions were 4 ml. The collection of the HPL-containing fractions has been performed manually when a significant S/N variation was observed. V_0 was approximately 1 ml. Contaminant *E. coli* proteins were eliminated by washing the resin at pH 8 and 6. Elution of $(His)_6$-tagged HPL was performed at pH 4, without any imidazole or histidine gradient. The major 280-nm protein peak (30 ml) matches exactly with a maximum of HPL activity.

To ascertain the identification of HPL, an UV–Visible spectrum of the HPL activity containing fraction was recorded and presented as Fig. 4. The typical Soret band (393 nm) of HPL can be seen. Recombinant HPL exhibits a Soret band at the same wavelength than green pepper bells HPL [3]. Another evidence of the identification follows: (*Z*)-3-hexenal was measured (GC–MS) after the reaction of 13-HPOTE with recombinant HPL. This volatile C_6 aldehyde is obtained with heterolytic HPL from plants [2].

The SDS–PAGE electrophoregram is presented as Fig. 5. The molecular mass of HPL is around 50 kDa

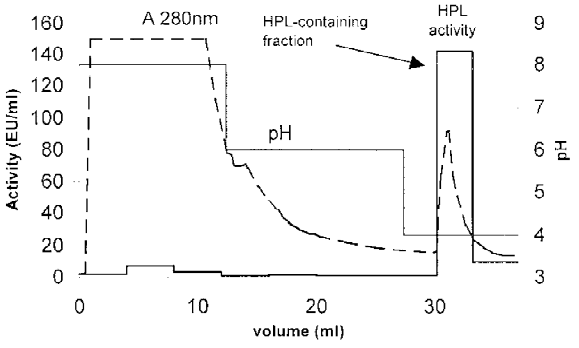

Fig. 3. IMAC purification of $(His)_6$-tagged cytochrome P450 hydroperoxide lyase.

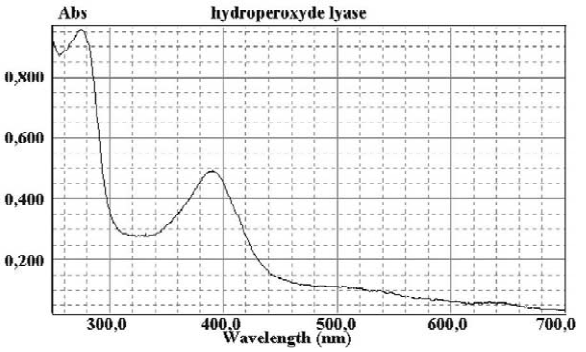

Fig. 4. UV–Visible absorption spectrum of recombinant $(His)_6$-tagged cytochrome P450 hydroperoxide lyase.

and fits the calculation from the gene (54 kDa [4]). The purity may vary between 80 and 90%.

3.3.3. Quantification of HPL

According to the molar coefficient of the Soret band [3], purified $(His)_6$–HPL amount was estimated

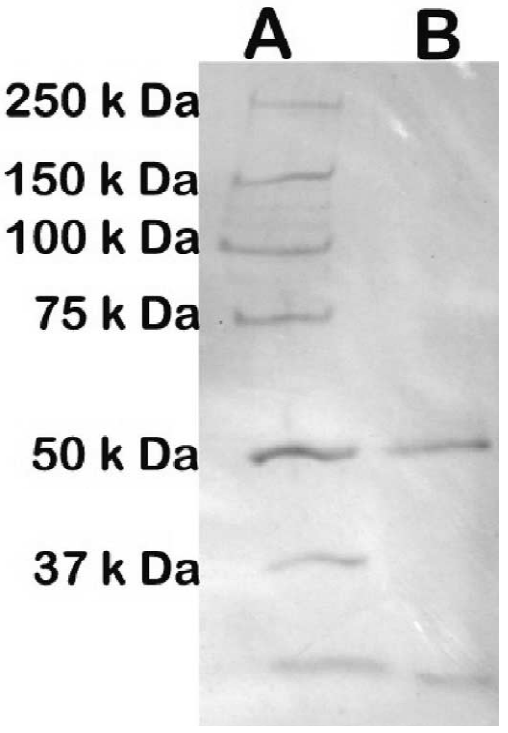

Fig. 5. SDS–polyacrylamide gel electrophoresis of purified $(His)_6$-tagged HPL (lane B). Electrophoresis was performed on a 10% polyacrylamide gel. Proteins were stained with Coomasie Blue.

to 1.3 mg. This amount corresponds to 100 ml culture and a recovery yield of 80%. Thus, enzyme expression have been estimated (for the first time) at 16.3 mg recombinant active HPL per litre.

HPL amount were also determined using a calculated A_{280} coefficient as described by [15]. The numbers of Trp, Tyr and Cys residues were deduced from the HPL gene sequence published in Ref. [4]. They are, respectively, 5, 13 and 5. HPL molecular mass is 54 055 Da [4]. According to the formula [15], the A_{280} coefficient would be 0.84 mg HPL/ml/AU. Chromophores (like heme) interferes at 280 nm. We propose the following correction: heme contribution at 280 nm=$0.45 \times A_{390}$ nm (according to pure hematin spectrum, data not shown). Thus, for HPL:

$$\text{HPL (mg/ml)} = (A_{280} - 0.45 \times A_{390}) \times 0.84$$

Using that last formula, purified HPL amount was estimated to 1.8 mg, which correspond to a 22.5-mg recombinant HPL per litre. We can see that this last estimation is very closed to the previous one that was based on the Soret band (16.3 mg/l).

4. Conclusions

From this work, we have learned that *E. coli* is a convenient expression vector for plant cytochrome P450 expression, even if this microorganism does not have any ''natural'' P450 sequence in its own gene [15]. Despite this fact, active form of P450-HPL have been detected in the recombinant strain of *E. coli* M15. This amount have been estimated to 16.3 mg enzyme per litre of TB medium. The heme prosthetic group of HPL is synthesised by the same ''enzymatic machinery'' that *E. coli* uses for its ''natural'' heme protein (like cytochrome *c*), according to the ''C_5 route''. A non negligible proportion of heme is deviated to recombinant HPL synthesis. Thus, the heme-biosynthesis capacity of *E. coli* becomes insufficient. Addition of exogenous δ-aminolevulinic acid helps the cell to produce heme. Another strategy to increase HPL synthesis could be the co-expression of glutamyl t-RNA synthase and HPL by the same cell.

E. coli culture in batch fermentor has produced between 16 and 22 mg of enzyme per litre. But glycerol was rapidly consumed by the cells that used amino acids as substrate. Another interesting way of investigation would be a batch-fed fermentor with a continuous flow of glycerol.

The efficiency of the lysis/extraction step has been considerably improved: neither mechanical lysis nor ultracentrifugation were used. Actually, some authors centrifuge the crude extract at 100 000 *g* prior to the purification of membrane bound protein. Our results have demonstrated that it is not necessary if DNAse I is added.

A total of 80–90% purity $(His)_6$-tagged HPL has been obtained from an IMAC purification. The binding capacity of the resin has been estimated to 1–2 mg of $(His)_6$-tagged protein/ml resin. The recovery yield is 80%.

Overexpressing HPL in *E. coli* is a good strategy for a ''green note'' aldehydes production: large amounts of enzyme are produced (by comparison with plant), one-step purification (thanks to 6xHis), recombinant HPL optimal pH closer to lipoxygenase optimal pH, high catalysis specificity (only hexenal is produced, data not shown). Therefore, this work has demonstrated the technical interest of the recombinant HPL.

However, before the evaluation of the profitability of the process, one more pure economical and legal information is needed: can a plant recombinant protein expression in *E. coli* be considered as ''natural'' and thus synthesised high value ''natural'' products?

Acknowledgements

Jerome Delcarte and Marie-Laure Fauconnier were research fellow and post-doctoral researcher, respectively, of the Fonds National de la Recherche Scientifique, Belgium.

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