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High-level expression and purification of recombinant horseradish peroxidase isozyme C in SF-9 insect cell culture

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Abstract

A method to obtain high-expression levels of recombinant horseradish peroxidase isozyme C (HRP C) in *Spodoptera frugiperda* Sf-9 cell culture and a strategy for its purification are described. HRP C was secreted into the culture medium where it accumulated to 25.6 mg/l. Addition of hemin to the insect cell culture increased the level of active enzyme expression up to 41.3 mg/l. A selective staining procedure using 3,3′-diaminobenzidine allowed visualisation of HRP C in the infected insect cells and provided an alternative staining strategy for titration of recombinant baculovirus carrying the HRP gene. Immobilised metal ion affinity chromatography using a Ni–NTA matrix with elution in the gradient-step mode yielded a 68% HRP C recovery with a RZ of 2.8. When the displacement elution mode was utilised, the yield was essentially the same and the product was electrophoretically pure, having a RZ of 3.2.

Keywords: Peroxidase isozyme C; Insect cell; *Spodoptera frugiperda* Sf-9; Expression; IMAC; Purification

1. Introduction

Peroxidases are a widely distributed class of enzymes that catalyse the oxidation of various substrates at the expense of hydrogen peroxide. Peroxidase appears as multiple isozymes in *Armoracia rusticana* roots and it is specially concentrated in the root legument. Horseradish peroxidase isozyme C (HRP C) (EC 1.11.1.7) is the most characterised peroxidase. It consists of 308 amino acid residues, an iron protoporphyrin type IX prosthetic group and two calcium ions per molecule, adding up to a molecular weight of 34520 Da. HRP C contains four highly conserved disulphide bonds and it is glycosylated at eight asparagine-linked glycosylation sites [1–4].

HRP C has long been utilised as the reporter enzyme in multiple medical diagnosis kits, in histochemical staining and in biosensors [5]. It catalyses a number of potentially interesting oxidative reactions including dehydrogenation and polymerisation of aromatic compounds [6] and is also employed as an indicator in food processing [7] and for removal of phenols and aromatic amines from wastewater [8]. Although peroxidases are not yet widely utilised in biotransformations, there is a growing interest in exploiting them as industrial biocatalysts.

The presence of a high-glycosylation level and four disulphide bonds impairs heterologous expression of HRP C in prokaryotic systems, where high levels of the polypeptide chain were accumulated in inclusion bodies but only a small amount of active enzyme (0.1 mg/l) was recovered after refolding. Heterologous expression of HRP C in *Saccharomyces cerevisiae* yielded 0.6 mg/l of active enzyme [2,9,10].

The baculovirus-insect cell expression system is widely used today to produce recombinant proteins mainly because it offers the possibility of achieving high levels of expression of eukaryotic genes in an active conformation. Heterologous genes are usually placed under the control of the strong polyhedrin promoter of *Autographa californica* nuclear polyhedrosis virus (AcNPV). *Spodoptera frugiperda* (Sf-9) cell line (ATCC CRL 1711), originally established from ovarian tissue *of Spodoptera frugiperda* larvae, is one of the most frequently used insect cell lines. In most cases,

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recombinant proteins are correctly folded; they present disulphide bonds and other important post-translational modifications. As a consequence, they display the expected biological properties. The aim of this work is to study the expression and purification strategies of recombinant HRP C using the baculovirus-Sf-9 expression system.

2. Materials and methods

Horseradish peroxidase (P-8375), hemin and 3,3′-diaminobenzidine (DAB reagent) were from Sigma (St. Louis, MO, USA). Grace's insect tissue culture media and penicillin/streptomycin (ATB/ATM) were from Gibco-BRL (Gaithersburg, MD, USA). Fetal calf serum (FCS) was from Nutrientes Naturales S.A. (Buenos Aires, Argentina). *Escherichia coli* strain DH5α was from the Facultad de Farmacia y Bioquímica (Buenos Aires, Argentina). Ni(II)-NTA agarose was from Qiagen (Valencia, CA, USA). All other reagents were AR grade.

2.1. Cell culture

S. frugiperda Sf-9 cells were obtained from ABAC (Buenos Aires, Argentina). Cultures were maintained as monolayers in T-flasks at 27 °C in Grace's medium containing 10% heat-inactivated FCS and were routinely subcultured every 2 or 3 days. Cells were counted with a hemocytometer and cell viability was assessed by Trypan blue staining.

2.2. Transfer vector

Plasmid pAcGP67HRP—containing the HRP C gene synthesised by British Biotechnologies Ltd.—was kindly donated by Dr. P.E. Ortiz de Montellano from the University of California. The sequence of the commercial gene is based on the amino acid sequence reported by Welinder [1]. The synthetic gene does not include the endogenous 5′ leader sequence found in the plant gene. The HRP C gene was oriented so that it could be expressed under the control of the baculovirus polyhedrin promoter. The vector (pAcGP67B, Pharmingen, San Diego, CA, USA) encodes a sequence for the gp67 viral envelope glycoprotein leader peptide at the 5′ end of the multiple cloning site which targets the translated HRP C gene product for secretion. Additionally, a histidine 6× tag was introduced at the 5′ extreme of the HRP C gene. pAcGP67HRP was transformed into *E. coli* DH5α, purified by means of the Wizard DNA purification kit (Promega, Madison, WI, USA) and sequenced by the fmol DNA sequencing system (Promega).

2.3. Production of recombinant virus

Sf-9 cells were plated at 1×10^6 cells per well in a six-well dish, allowed to adhere and cotransfected with 2 μg pAcGP67HRP and 0.5 μg wild linearised BaculoGold™ DNA (Pharmingen) in the presence of calcium phosphate, according to Pharmingen [11]. After a 5-day incubation at 27 °C, the cell culture supernatant was collected and centrifuged during 5 min at $2500 \times g$. Cotransfection efficiency was determined by the end-point dilution assay as per the supplier's instructions (Pharmingen). The recombinant baculovirus in the transfected cell culture supernatant was subjected to plaque purification and amplified to attain a high-titre virus stock. Viral amplification was carried out by infecting 1×10^7 Sf-9 cells in 75 cm^2 T-flasks at a multiplicity of infection (MOI) of 0.1 plaque-forming units (pfu) per cell). After two amplification rounds, a high-titre virus stock was obtained and employed to infect cultures for HRP C production.

2.4. Plaque assay using HRP activity detection

The plaque assay was used to purify recombinant baculovirus expressing high levels of HRP C and to determine viral stock titres. In this assay, 3.1×10^6 Sf-9 cells per well were infected with a low MOI (0.1 pfu per cell) so that only isolated cells got infected. A first agarose overlay was added and, after 5 days at 28 °C, a second agarose overlay containing a peroxidase substrate (DAB) and hydrogen peroxide facilitated the visualisation of the plaques and allowed identification of infected cells expressing high levels of active HRP C with the naked eye [12].

2.5. Optimisation of gene expression

HRP C expression by infected cells at various MOIs was evaluated. 2.2×10^6 Sf-9 cells were plated on six-well dishes and allowed to attach to the bottom of the plate. After a 30-min incubation at room temperature, cells were infected at MOIs of 2, 5 and 10 pfu per cell. Hemin was added at the same time at a final concentration of 2.4 μM. A negative control plate with uninfected cells was included in the analysis. Plates were incubated in the dark at 27 °C for 2 days and HRP enzymatic activity in harvested clarified supernatants was determined from days 1 up to 8 post-infection.

2.6. Recombinant HRP C production

The recombinant protein was produced in suspension cultures of 20 ml in shake-flasks or 100 ml in a spinner-flask. Cells were growing exponentially at the time of infection at cell densities of 2×10^6 cells per ml with 98% viability. The culture medium was supplemented with hemin at a concentration of 2.4 μM. Sf-9 cells were infected at a MOI of 2 pfu per cell using a high-titre virus stock. Infected cultures were incubated during 6 days in humidified incubators at 27 °C while agitated at 60 rpm.

2.7. Culture supernatant conditioning

The harvested supernatant was clarified by centrifugation (2000 × *g*, 10 min, 4 °C) to remove cells and cell debris, and concentrated using a Centricon YM-30 membrane (Millipore Corp., Bedford, MA, USA). For larger volumes (100 ml), the supernatant was concentrated by tangential flow ultrafiltration using a Sartocon Micro 10,000 MW cut-off membrane (Sartorius AG, Göttingen, Germany) with a Minitan equipment (Millipore Corp.) and diafiltered against adsorption buffer (25 mM sodium phosphate buffer, 300 mM NaCl, pH 8.0). Alternatively, samples were dialysed overnight against milliQ water with a 6000 MW cut-off dialysis membrane. Dialysed samples were then lyophilised and suspended in adsorption buffer.

2.8. Affinity chromatography

The concentrated supernatant (1 ml) was loaded on to a Ni(II)–NTA agarose column (bed volume 0.8 ml) equilibrated with 25 mM sodium phosphate buffer, 300 mM NaCl, pH 8.0. After a washing step with 25 mM sodium phosphate buffer, 300 mM NaCl, pH 6.0, HRP elution was accomplished by adding imidazole to the washing buffer either in the isocratic (100 mM) or in the gradient-step mode (20, 50 and 100 mM). Eluted fractions (1 ml) were collected for analysis at a linear flow rate of 0.4 cm/min. For column overload, 3 ml concentrated supernatant was loaded and elution was performed by addition of 100 mM imidazole to the washing buffer.

2.9. Total protein determination

Protein concentration was determined by the Bradford assay using bovine serum albumin as the standard [13]. Protein elution from chromatographic columns was monitored by absorbance at 280 nm. Reinéis Zahl (RZ) values are calculated as A_{403}/A_{275}. The RZ value measures the heme content by using the aromatic amino acid content as a reference and is a measure of the purity of HRP preparations [3].

2.10. Electrophoretic analysis

SDS-PAGE and isoelectric focusing analyses were carried out with a Phast System equipment (Amersham Biosciences, Uppsala, Sweden). Gel staining was accomplished using the silver staining method or employing a selective staining with DAB reagent to detect active HRP.

2.11. Measurement of enzyme activity

A reaction mixture containing 105 μM guaiacol and 250 μM hydrogen peroxide in 100 mM potassium phosphate buffer, pH 7.0, was prepared. Guaiacol oxidation was initiated by the addition of 10 μl sample to 1 ml reaction mixture. The reaction was monitored by measuring its absorbance at 470 nm within 1 min. Activity calculations were performed as per Tjissen [14]. For enzyme mass quantitation, a specific activity of pure enzyme of 592.3 U/mg was assumed.

3. Results and discussion

3.1. Production of recombinant virus

The linearised AcMNPV DNA (BaculoGold™, Pharmingen) is a defective baculoviral DNA incorporating a lethal deletion. The homologous recombination event between baculovirus DNA and the complementary transfer vector restores virus infectivity and, as a consequence, only the recombinant baculovirus is infective. Peroxidase activity was evidenced in harvested transfected supernatants. Transfected supernatants had a viral titre of 1.1×10^7 pfu/ml. Amplified virus stocks used for HRP production typically contained $1.0–1.2 \times 10^8$ pfu/ml.

3.2. Production of recombinant HRP C

The recombinant HRP C, expressed in Sf-9 insect cells infected with recombinant baculovirus, was catalytically active. SDS-PAGE stained with DAB revealed a single discrete band showing peroxidase activity that co-migrated with the native HRP standard. The recombinant product had a molecular mass comparable to that of the native enzyme (44 kDa) as judged by SDS-PAGE and, on the basis of this result, it can be assumed that the glycosylation degree should be similar to that of the native HRP (21.8%). The isoelectric point (pI) of the recombinant protein was 7.4.

The kinetics of HRP C expression by cells infected at various MOIs is shown in Fig. 1. Enzyme activity in harvested supernatant gradually increased up to 7 days after infection for all three MOIs tested. After day 6 post-infection, extensive cell lysis occurred and many contaminating proteins appeared. Therefore, day 6 post-infection was chosen as the best day of harvest to ensure a maximum yield of recombinant protein without significant intracellular protein contamination. Although the expression levels were higher at higher MOI at the beginning of the infection, at day 6 post-infection enzyme activity was similar for all MOI tested. Based on these results, a MOI of 2 pfu per cell was selected for further experimentation since it resulted in an optimum expression level and required less virus stock.

A selective peroxidase-specific assay has been developed in this work. The use of DAB facilitated detection of active recombinant HRP C during cloning, plaque-purification, plaque assay, isoelectric focusing and SDS-PAGE techniques. DAB was extremely useful in identifying low and high-level expressing recombinant viruses. The catalytic reaction using the DAB substrate gives a distinct dark brown product (oxidised DAB) that allows visualisation of the HRP C protein localisation in the cells by microscopy or

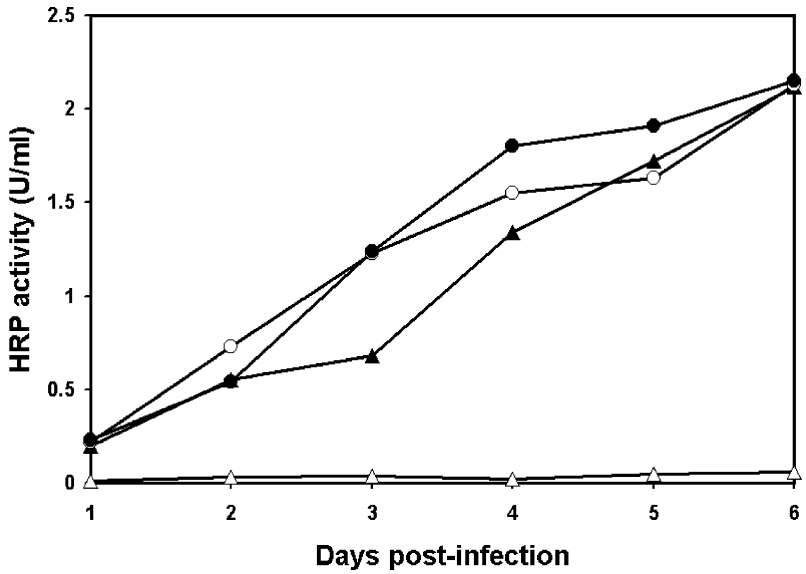

Fig. 1. Kinetics of peroxidase expression in Sf-9 cells infected with recombinant baculovirus at different multiplicities of infection (MOIs). HRP activity was measured in the culture supernatant daily after infection with 2 (▲), 5 (○) and 10 (●) pfu per cell. For comparison, Sf-9 cells not infected were also cultivated (△).

Table 1
HRP expression level under different culture conditions (mg/ml)

	Monolayer culture (10% FCS)	Suspension culture (10% FCS)	Suspension culture (5% FCS)
Negative control (uninfected cells)	0.3 ± 0.04	0.2 ± 0.04	0.3 ± 0.06
Absence of hemin	17.6 ± 1.59	23.7 ± 2.09	25.6 ± 2.03
Hemin (2.4 μM)	31.1 ± 2.52	35.7 ± 1.56	41.3 ± 2.63

Results are the mean of five independent assays ± standard deviation.

with the naked eye. The coloured reaction of the HRP C or any other peroxidase with DAB makes this system an easy, useful, inexpensive alternative to other widely used marker proteins such as GFP, beta-galactosidase or luciferase. Due to the coloured nature of the product, no expensive equipment (e.g. fluorometer, luminometer) are required to detect the enzyme but could be employed in the event that a different peroxidase substrate is utilised.

The maximum level of active HRP C produced in FCS-supplemented Grace's medium was 25.6 mg/l. HRP C

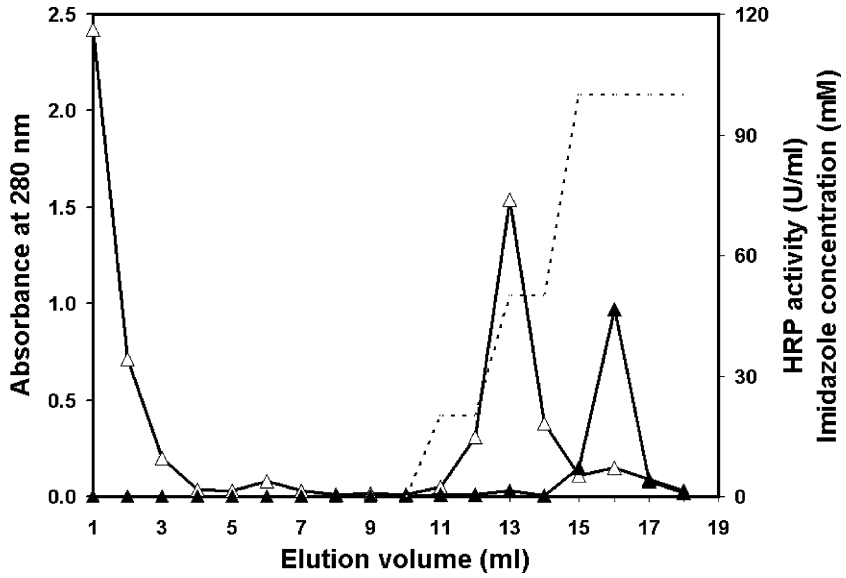

Fig. 2. Step-gradient elution pattern of HRP C on Ni–NTA agarose. A Ni–NTA column (0.5 cm × 4 cm) was loaded with 1 ml of culture supernatant conditioned by ultrafiltration/diafiltration as described in Section 2. After a washing step with 25 mM sodium phosphate buffer, 300 mM NaCl, pH 6.0, until baseline was reached, elution was performed using a step gradient (- - -) with 20, 50 and 100 mM imidazole in the washing buffer. One millilitre fractions were collected at a linear flow rate of 0.4 cm/min, and absorbance at 280 nm (△) and enzyme activity (▲) was monitored.

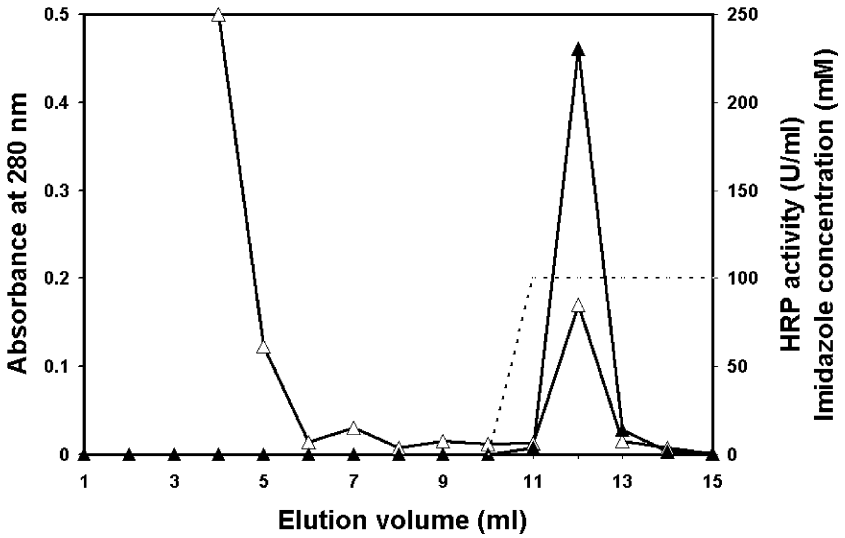

Fig. 3. Elution pattern of HRP C on Ni–NTA agarose at large sample loads. A Ni–NTA column (0.5 cm × 4 cm) was loaded with 3 ml of culture supernatant conditioned by ultrafiltration/diafiltration as described in Section 2. After a washing step with 25 mM sodium phosphate buffer, 300 mM NaCl, pH 6.0, until baseline was reached, elution was performed by adding 100 mM imidazole (- - -) to the washing buffer. One millilitre fractions were collected at a linear flow rate of 0.4 cm/min, and absorbance at 280 nm (△) and enzyme activity (▲) was monitored.

production improved (41.3 mg/l) when hemin was added to the culture after virus addition. Table 1 shows the concentration of HRP C produced under different culture conditions. In all cases, HRP C yield increased significantly (51–76%) in the presence of 2.4 μM hemin. These results confirm the limited capacity of heterologous expression systems to synthesise catalytically active hemoproteins [10]. To the best of our knowledge, this is the highest level of active recombinant peroxidase expression achieved so far.

3.3. Purification of recombinant HRP C

Different strategies were applied to purify HRP C from crude culture supernatant. Two alternative methods were assessed for HRP C concentration and conditioning from culture supernatant: ultrafiltration/diafiltration or dialysis/lyophilisation. While HRP C yield was higher with ultrafiltration/diafiltration (98% versus 72%), dialysis/lyophilisation allowed a higher concentration factor (10× versus 4×). Supernatant conditioning by dialysis or diafiltration is necessary to remove amino acids present at high concentrations in insect cell culture media as they compete with the 6× His-tagged HRP C for binding sites on a Ni–NTA matrix. On the other hand, insect culture media is acidic (pH 6.0–6.5) and must be adjusted to pH 8.0 to favour HRP C binding to the Ni–NTA chromatographic matrix.

After concentration, HRP C was purified by IMAC. Major contaminating proteins such as bovine transferrin and seroalbumin come from serum supplementation to culture media. Bovine serum albumin was not retained in IMAC with Ni as the metal ligand. In contrast, bovine transferrin, a 76 kDa glycoprotein, is concentrated by this matrix and co-elutes with HRP C when elution is performed using 0.1 M imidazole in the washing buffer. This indicates that bovine transferrin contains surface histidines that provide affinity to this protein for the immobilised Ni–NTA matrix.

When step elution with imidazole was applied as the elution strategy, it was possible to separate both proteins (bovine transferrin and HRP C). The product obtained had an RZ value of 2.8 and the yield was reasonable (68%). The corresponding chromatographic pattern is shown in Fig. 2. The small difference in affinity was exploited in an alternative elution strategy: bovine transferrin was removed from the column by displacement with HRP C loaded at concentrations exceeding 0.7 mg/ml of gel. By using this overloading strategy, it was possible to displace the contaminant present at a high concentration in the culture medium because its

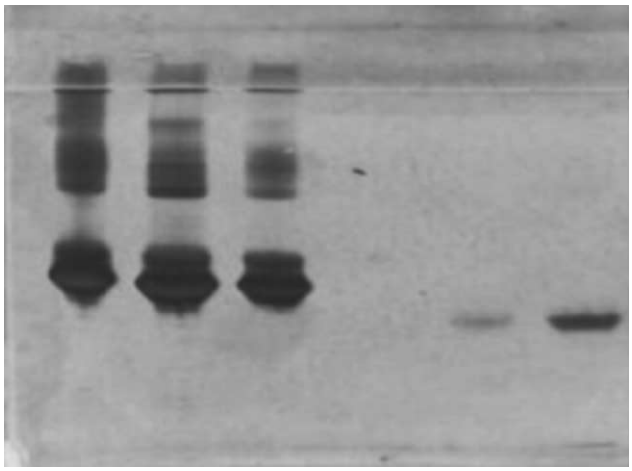

Fig. 4. SDS-PAGE of the fractions eluted from Ni–NTA agarose at large sample loads (Fig. 3). Appropriate amounts of each fraction were loaded on PhastGel 12.5. Gel was silver stained. Lane 1: starting material; lane 2: pass through (tube 1); lane 3: wash at pH 6.0 (tube 4); lane 4: wash at pH 6.0 (tube 9); lane 5: eluate (tube 12); lane 6: native HRP standard.

Table 2
Purification of recombinant HRP by column overload

Purification step	Volume (ml)	Enzyme activity (U)	Protein (mg)	Specific activity (U/mg)	Yield (%)	Purification factor
Crude						
Supernatant	100.0	2000	510.0	3.9	100.0	1.0
Ultrafiltrate	25.0	1957	312.5	6.2	97.9	1.6
IMAC	18.5	1297	2.2	584.1	66.3	149.0

affinity constant to the immobilised ion was lower than that of the HRP C (Fig. 3). In order to avoid product loss, the pass-through can be reloaded to the column. As judged by SDS-PAGE silver staining (Fig. 4) and RZ value (3.2), the latter procedure yielded a high-purity product with a purification factor of 149 and specific activity of 584.1 U/mg (Table 2) in a single chromatographic step.

Acknowledgements

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