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Short communication

Expression and purification of horseradish peroxidase in insect larvae

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Abstract

A strategy for obtaining a high-level expression of horseradish peroxidase isozyme C (HRPC) in insect larvae is herein described. The HRPC-6xHis coding sequence was inserted into the AcNPV genome via the pAcGP67HRPC-6xHis transfer vector to originate an AcHRPC-6xHis recombinant baculovirus of phenotype occ$^-$. *Rachiplusia nu* larvae were injected with a viral stock derived from culture supernatants of Sf9 cells infected with AcHRPC-6xHis. Enzyme concentration at day 3 post-infection was 230 ± 10 mg/kg haemolymph and 100 ± 14 mg/kg larvae, an expression level never attained by other expression systems. HRPC-6xHis was purified from the crude larval extract or haemolymph by immobilised metal ion affinity chromatography with yields of 88.8% and 89.0% and purification factors of 18.9 and 14.0, respectively, in a single step. The purity of the final product was 90%.

Keywords: Horseradish peroxidase; Expression; Purification; Insect larvae; *Rachiplusia nu*; Haemolymph

1. Introduction

The baculovirus expression system – which provides a powerful promoter and post-translational modification of recombinant proteins – is an effective and versatile eukaryotic expression tool. The system utilising the *Autographa californica* nuclear polyhedrosis virus (AcNPV) and insect cell lines – such as those from *Spodoptera frugiperda* (Sf21, Sf9) and *Trichoplusia ni* (Tn-5) – is widely used for the production of many heterologous proteins [1]. In insect cell lines, the expression of heterologous proteins at the polyhedrin locus can represent up to 50% of the total protein of the infected cell [2], and the downstream processing is easier than protein derived from insect larvae, specially in serum-free medium cultures. There may also be more uniformity in the post-translational modification of the protein as only a single cell-type is supporting synthesis. However, the principal disadvantage of the scale-up of recombinant protein production in cell culture is its cost. Commercially prepared cell-culture media are expensive and costs may become too high for very large volumes. In addition, a considerable investment in highly technical equipment (e.g. bioreactors) is required [3]. Production of large quantities of recombinant protein in insect larvae is inexpensive and time saving, and eliminates the need of specialised facilities for tissue culture.

Horseradish peroxidase (HRP, EC 1.11.1.7) catalyses oxidation of a broad variety of substrates by hydrogen peroxide. HRP isozyme C (HRPC) consists of 308 amino acid residues, a ferric heme prosthetic group and two calcium ions per molecule, adding up to a molecular weight of 34,520 Da. HRPC contains four highly conserved disulfide bridges and is glycosylated at eight sites of asparagine-linked glycans [4]. Such structural complexity makes its expression difficult in prokaryotic systems and, therefore, its expression in *Saccharomyces cerevisiae* and *Pichia pastoris* rendered low yields (0.6 mg l^{-1}) [5]. Recently, HRPC with a His tail was expressed in baculovirus-infected insect cells with a yield of 41.3 mg l^{-1} [6].

The aim of this work is to design an integrated method for protein expression and purification for fast-track product delivery in *Rachiplusia nu* larvae infected by baculovirus. The HRPC with a His tail (HRPC-6xHis) was used to promote selective binding to IMAC matrices directly from the haemolymph or *R. nu* extract infected by baculovirus.

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2. Materials and methods

2.1. Materials

Horseradish peroxidase (type 8375), hemin, phenylmethylsulfonyl fluoride (PMSF) and 3,3′-diaminobenzidine (DAB reagent) were from Sigma–Aldrich (Saint Louis, MO, U.S.A.). Grace's insect tissue culture media and penicillin/streptomycin (ATB/ATM) were from Invitrogen (Carisbad, CA, U.S.A.). Fetal calf serum was from Nutrientes Naturales S.A. (Buenos Aires, Argentina). *S. frugiperda* Sf9 cells were obtained from ABAC (Buenos Aires, Argentina). Ni-HiTrap and PD-10 prepacked columns and low molecular weight markers calibration kit for SDS-PAGE electrophoresis were from GE Healthcare (Piscataway, NJ, U.S.A.). Gel runs were performed with PhastSystem equipment (Amersham Biosciences, Uppsala, Sweden). The HRPC-6xHis gene was kindly donated by Dr. Ortiz de Montellano, University of California. Mouse polyclonal anti-HRPC serum was prepared in our laboratory. Anti-mouse horseradish peroxidase-conjugated IgG was from DAKO (Copenhagen, Denmark).

2.2. Insect cells and larvae

The *S. frugiperda* cell line Sf9 was conserved in monolayers in T-flasks at 27 °C in a Grace's medium containing 10% (v/v) heat-inactivated fetal calf serum and routinely sub-cultured every 2–3 days. Cells were counted with a haemocytometer and their viability was assessed by means of Trypan Blue staining. All experiments were carried out with cells at 95–99% viability in the log phase.

R. nu larvae were obtained from a laboratory colony fed with a high-wheat germ diet. Second-instar larvae were reared in 6-well plates (one per well) at 23–25 °C in a 70% humidified chamber, with a 16:8 photoperiod (L:D).

2.3. Viral stock production

Recombinant baculovirus construction was described in a previous work [7]. After a round of plaque purification, a recombinant plaque was isolated and amplified to yield a high-titre virus stock. The purified virus was used to infect 1×10^7 Sf9 cells in a monolayer containing 15 ml Grace's medium at a multiplicity of infection (MOI) of 0.1. After two amplification steps, the virus titre was determined by a plaque assay [8]. The titre of this stock was about 3.3×10^7 pfu/ml. This amplified virus stock was used at the production step.

2.4. Larvae infection

When larvae reached the last instar (approximately 200 mg), they were chilled on ice for 5–10 min and 50 μl of the viral suspension was injected subcutaneously into the body of the larvae. After the allotted time, haemolymph was collected by cutting the larval proleg and allowing haemolymph to drop into a cold eppendorf microcentrifuge tube containing an equal volume of appropriate buffer. Haemolymph was then centrifuged at 10,000 × *g* for 10 min at 4 °C to remove haemocytes and cell debris, and the supernatant was stored at −20 °C. Due to the difficulty of totally withdrawing the haemolymph, larvae were homogenised in the presence of the buffer selected (2.5 ml buffer/g larvae) to obtain a total extract containing all their haemolymph. Taking into account that larva homogenisation would bring about cell lysis and, consequently, an increase in the protein complexity of the extract in comparison with that of the haemolymph, homogenisation with a hand blender was slight enough to preclude cell lysis. For the kinetics of HRP expression, six larvae per day were used, and results are expressed as the average ± standard error.

2.5. Purification of HRPC from larval crude extract or haemolymph by IMAC

The sample was conditioned in the equilibration buffer by passing it through a PD-10 column. A Ni(II)-HiTrap column (1.0 ml bed volume) was equilibrated with a 25 mM sodium phosphate buffer, pH 8.0, 300 mM NaCl, and loaded with the sample. After a washing step with the same buffer containing 25 mM imidazole, HRPC elution was performed with 250 mM imidazole. Linear flow rate was 0.4 cm min^{-1} and 1-ml fractions were collected and monitored for protein content and enzyme activity.

2.6. Total protein measurement

The Bradford assay [9] was used with bovine serum albumin as the standard.

2.7. Electrophoretic analyses

Fifteen percent SDS-PAGE and native PAGE 8–25% gradient were carried out with a PhastSystem equipment.

Gel staining was performed by using Coomassie Blue or by selective staining with a DAB reagent to detect active HRP. For DAB staining, the gel was immersed in a 9 mg/ml DAB aqueous solution with 10 μl 30% hydrogen peroxide for 2 min.

For Western blot, gels were electroblotted onto a nitrocellulose membrane and detection was performed using a mouse polyclonal anti-HRPC as primary antibody and HRPC-conjugated anti-mouse IgG (DAKO, Copenhagen, Denmark) as secondary antibody. Then, the membrane was stained with DAB.

For image processing, gels were scanned and then analysed with the Image Quant software (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

2.8. Measurement of HRP 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 adding a 10 μl sample to 1 ml reaction mixture. The reaction was monitored by measuring its absorbance at 470 nm within 1.5 min and activity calculations were performed as per Tjissen [10].

2.9. Measurement of catalase activity

Catalase activity was determined as described by Aebi [11]: a reaction mixture containing 770 μl 50 mM potassium phosphate buffer, pH 7.0, and 15 μl hydrogen peroxide was prepared. Reaction was initiated by adding a 15 μl sample and absorbance at 240 nm was measured within 1 min.

3. Results and discussion

The use of baculovirus-infected insect larvae as hosts for protein production was first described for the expression of α-interferon in 1985 [12]. Since that time insects – specially silkworm larvae – have been used to produce a variety of recombinant proteins [13–17]. This approach has not been widely adopted in the Western world; however, the cabbage looper moth *Trichoplusia ni* has attained some industrial interest. These larvae are prone to infection with *Autographa californica* nuclear polyhedrosis virus (AcMNPV). In Latin America, *Spodoptera frugiperda* and *R. nu* larvae are plagues of many important crops. Although the use of *R. nu* as a "biofactory" was little explored, a high-level expression of recombinant 3AB1 non-structural protein from the foot-and-mouth disease virus has recently described [18].

3.1. HRP expression

The cDNA HRPC-6xHis gene was cloned in frame with the leader peptide for signal secretion of baculovirus glycoprotein GP67 while the powerful polyhedrin promoter controlled the synthesis of the protein [6]. As the recombinant baculovirus lacked polyhedrin (occ$^-$), it did not originate occlusion bodies.

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Table 1
Influence of the addition of β-mercaptoethanol and EDTA to the buffer on the melanisation process and HRP activity of total larvae extract

β-Mercaptoethanol (mM)	EDTA (mM)	Melanisation	Enzyme activity (U/ml)
0.25	–	(−)	0.8 ± 0.3
0.50	–	(−)	0.8 ± 0.3
1.00	–	(−)	0.7 ± 0.2
5.00	–	(−)	0.4 ± 0.2
–	1.00	(+)	0.6 ± 0.3
–	5.00	(−)	1.2 ± 0.4
–	10.00	(−)	1.0 ± 0.3
–	–	(+)	1.0 ± 0.4

(+): melanisation; (−): no melanisation. Results are expressed as the average of three experiments ± S.E.

Table 2
Kinetics of HRP expression

Day post-infection	Haemolymph (mg HRP/kg)	Total extract (mg HRP/kg)
1	0	0
2	7 ± 5	1 ± 1
3	230 ± 10	100 ± 14
4	240 ± 19	100 ± 9

Larvae were homogenised with a 50 mM sodium phosphate buffer, pH 6.0, 5 mM EDTA, 0.2 mg/ml PMSF, 150 mM KCl and glutathione crystals or haemolymph was diluted with the same buffer at different post-infection days, and HRP activity was measured. Results are expressed as the average of six experiments ± S.E.

However, this virus form was infective when injected into the larval hemocoel.

Early fifth-instar *R. nu* larvae infected with the recombinant virus showed typical symptoms of AcNPV infection including discoloration of the body and loss of appetite at the second post-infection day. To measure the HRP expression, larvae were buffer-homogenised. In order to prevent the melanisation process, different homogenisation buffers were assayed. In the melanisation process, many proteins and lipids condense into a dark black gluey mess, this seriously impairing recovery of the recombinant product. The buffer selected should prevent melanisation without affecting enzyme activity. Table 1 shows the effect of β-mercaptoethanol and EDTA in the homogenisation buffer on HRP activity and melanisation. According to our results, 50 mM sodium phosphate buffer, pH 6.0, 5 mM EDTA, 0.2 mg/ml PMSF, 150 mM KCl with glutathione crystals was selected for further experiments. A few stress proteases have been described in insects [19]. In Sf9 cells, proteases produced in response to baculovirus infection have also been reported [20]. Many authors reported that most larval-expressed heterologous proteins disappeared in vivo typically 3 or 4 days after infection, presumably due to proteolysis [21,22]. In an attempt to overcome this difficulty, genetically modified baculovirus with a reduced capability for cell lysis initiation or development of a chitinase and v-cathepsin negative bacmid were described [23,24].

In order to assess the expression kinetics, HRP activity in the larval extract and haemolymph was measured at different post-infection days (Table 2). The extract of uninfected *R. nu* showed no peroxidase activity. Results indicated that production of recombinant HRP greatly increases at day 3 and remains constant thereafter. Considering that 50–70% of the larvae die at day 4, the enzyme harvest was performed at day 3 post-infection. No evidence of proteolysis was observed in recombinant HRPC according to SDS-PAGE. Similar results were observed in the case of 3AB1 expressed in *R. nu*, where the recombinant protein was harvested at day 5 post-infection without any sign of proteolysis [18].

HRPC-6xHis was expressed and accumulated in haemolymph directed by the viral GP67 signal secretion. Enzyme concentration in haemolymph was 230 ± 10 mg/kg at day 3 post-infection. When total larval extract was used, HRP concentration was only 100 ± 14 mg/kg thus evidencing HRP accumulation in haemolymph. In contrast with Sf9 cells, where the expression was improved by heme addition [6], HRP concentration did not increase significantly by addition of heme in the 40–80 mg l^{-1} range.

Catalase is a hydrogen peroxide-consuming enzyme present in larvae which interferes in HRP measurement. Taking into account that catalase molecular weight is five times that of HRP (approximately 240,000 vs. 44,000), HRP was measured after ultrafiltration of the sample through a 100,000 MWCO membrane. Catalase concentration was constant at 630 ± 57 mg/kg in haemolymph and 760 ± 54 mg/kg in total extract, from day 1 to day 4 post-infection. It was reported that catalase is able to partially prevent cell death triggered by baculovirus infection and to inhibit lipid peroxidation [25]. Therefore, the recombinant protein should be protected by catalase activity in the host.

A native PAGE with specific staining for HRP (DAB) showed the presence of a band in haemolymph and extract of infected larvae. Samples of uninfected larvae used as a control did not show any band with HRP activity (Fig. 1A).

3.2. HRP purification

Haemolymph or larval extract were conditioned to change the solvent to 25 mM sodium phosphate buffer, pH 8.0,

Table 3
Performance of IMAC for HRP6xHis purification from haemolymph and larval extract

Sample	Volume (ml)	Total protein (mg/ml)	HRP activity (U/ml)	Specific activity (U/mg)	Yield (%)	Purification factor
Haemolymph	1	4.1	50.8	12.4	100	
Peak III (not shown)	1	0.26	45.2	173.8	89.0	14.0
Larval extract	1	4.5	40.3	9.0	100	
Peak III (Fig. 2)	1	0.21	35.8	170.5	88.8	18.9

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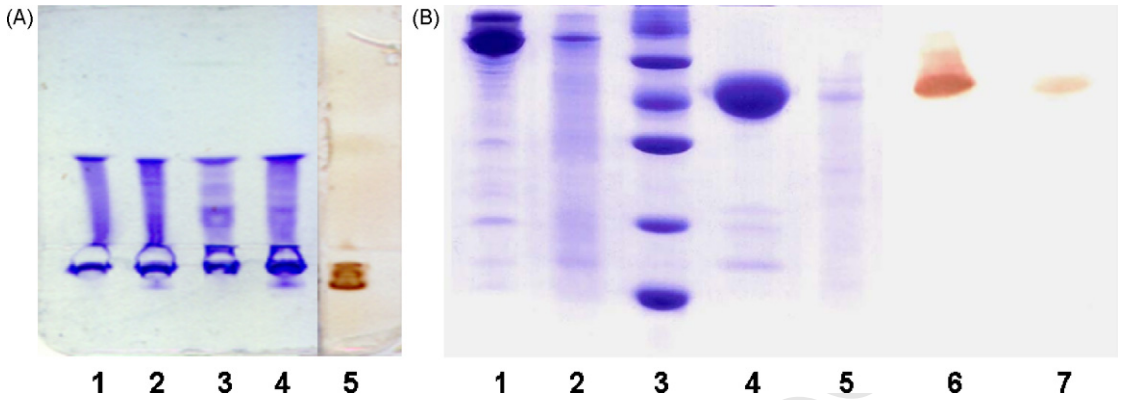

Fig. 1. Native and SDS-PAGE. A: native PAGE. Lane 1, extract from non-infected larvae; lane 2, larval extract, day 3 post-infection; lane 3, haemolymph from non-infected larvae; lane 4, haemolymph, day 3 post-infection; lane 5, larval extract, day 3 post-infection. Lanes 1 through 4 were stained with Coomassie Blue and lane 5 was stained with DAB. B: SDS-PAGE. Lane 1, haemolymph, day 3 post-infection; lane 2, larval extract, day 3 post-infection; lane 3, MW markers; lanes 4 and 7, standard HRP; lanes 5 and 6, final product from larval extract (peak III of Fig. 2). Lanes 1 through 5 were stained with Coomassie Blue and lanes 6 and 7 were revealed with DAB after Western blot. Molecular weight markers were phosphorylase b (97.0 kDa), bovine serum albumin (66.0 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (30.0 kDa), trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa).

300 mM NaCl to raise the pH to 8.0 and the NaCl concentration to 300 mM so as to promote protein binding to the Ni-NTA matrix. Fig. 2 shows the IMAC profile of larval extract. An identical elution pattern was obtained for haemolymph (not shown) displaying a pass-through (peak I), a peak eluting at 25 mM imidazole (peak II) and one containing HRPC-6xHis (peak III) eluting at 250 mM imidazole. Among other proteins, peak II contains catalase as judged by its enzyme activity. Table 3 shows the purification performance of the IMAC for larval extract and haemolymph as the starting materials. HRP yield purified from haemolymph was 89.0% while that of HRP purified from larval extract was 88.8%. Purification factors were 14.0 and 18.9, for haemolymph and larval extract respectively. When HRPC was expressed in Sf9 insect cell line, an enzyme concentration and conditioning of culture supernatant were necessary steps before IMAC purification [6]. In contrast, larval extract or haemolymph could be directly loaded onto the affinity matrix.

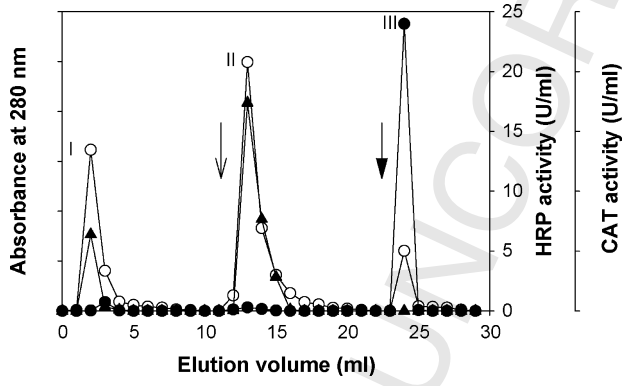

Fig. 2. HRPC-6xHis purification from larval extract by IMAC. After a buffer shift to 25 mM sodium phosphate, 300 mM NaCl, pH 8.0, 1 ml diluted larval extract was loaded onto a Ni(II)-NTA HiTrap column. A washing step with 10 ml of the same buffer containing 25 mM imidazole was carried out and a step elution was then performed by increasing the imidazole concentration to 250 mM (10 ml). One-ml fractions were collected at a linear flow rate of 0.4 cm min^{-1} and monitored at 280 nm for protein content (○) and enzyme activity; (●) HRP activity; (▲) catalase activity. Arrows indicate the buffer change.

Fig. 1B shows the SDS-PAGE pattern of the final product in comparison with that of the starting material and the standard HRPC. Western blot confirms the identity and molecular weight (around 44 kDa) of the final product. A 90% purity was estimated from the gel scan and specific activity of the final product. The final product was completely free of catalase, a probable interfering enzymatic contaminant.

Results obtained show that *R. nu* larva infected with recombinant baculovirus is highly effective for HRPC-6xHis production and can be purified directly from both the conditioned haemolymph and larval extract by IMAC, the latter being favoured due to its easy manipulation. The expression yield obtained is very high as compared with other HRP expression systems (yeast, 0.6 mg l^{-1} [5]; Sf9 cells, 41.3 mg l^{-1} [6]).

In a typical experiment, with a small lot-to-lot variation, the amount of HRPC recovered from a single larva was approximately 50 μg, this being similar to the 3AB1 expression level [18].

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