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Recombinant peroxidase production in species of lepidoptera frequently found in Argentina

Research Paper

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Horseradish peroxidase isozyme C (HRPC) is an important commercial biocatalyst. In this study, a screening of different lepidopteran species frequently found in Argentina to produce this protein was carried out. Two recombinant viruses were constructed: AcMNPV HRPC polyhedrin-minus (occ−), an intrahemocelical infective virus; and AcMNPV HRPC polyhedrin-plus (occ+), to achieve an oral infective baculovirus. Each lepidopteran species was infected either with AcMNPV HRPC occ− or AcMNPV HRPC occ+ and the harvesting days post-infection (dpi) were optimized. All species were susceptible to AcMNPV HRPC occ− infection, giving *Spodoptera frugiperda* the best yield: 41 μg per larva. *Rachiplusia nu* was highly susceptible to oral infection, reaching 22 μg per larva at 4 dpi. HRPC was purified by IMAC from *S. frugiperda* extracts with a yield of 86% and a purification factor of 29.

Introduction

The order Lepidoptera (butterflies and moths) is a large group of insects containing more than 100,000 described species. Many of them are considered destructive plagues during their larval stage affecting economically important crops such as soy and corn. *Spodoptera frugiperda*, *Helicoverpa zea*, *Heliothis virescens* and *Rachiplusia nu* are some of the most abundant and widely distributed lepidopteran species in Argentina. Accordingly, there is a long experience of rearing them to investigate pest control where baculovirus offers a natural alternative to chemical pesticides. By contrast, baculovirus-insect expression system is widely accepted as an excellent choice for heterologous gene expression when a eukaryotic environment is required, usually proteins with biotechnological or pharmaceutical purposes [1]. The main disadvantage of recombinant protein production in insect cell culture at industrial scale is its high cost [2]. For this reason, the production of recombinant proteins in larvae as 'bio-factories' becomes an economically advantageous strategy, mainly because the need of tissue-culture specialized facilities is minimal [3,4]. It was reported the expression of an antigen hepatitis E virus in *Spodoptera litura* larvae infected with recombinant AcMNPV that resulted three times cheaper than that expressed in *Trichoplusia ni* cells [5].

Autographa californica multiple *nucleopolyhedrovirus* – AcMNPV – (family *Baculoviridae*, genus *Nucleopolyhedrovirus*) has a broad host range and can mortally infect larvae of at least 32 lepidopteran species [6]. AcMNPV produces two structurally and functionally different phenotypes involved in the natural infection cycle of baculoviruses: budded (BV) and occlusion-derived viruses (ODV). ODV are packages of nucleocapsids within an envelope and they are embedded within a crystalline matrix of polyhedrin protein forming an occlusion body or polyhedron. ODV establish the first round of infection of feeding larva after its release from polyhedra upon exposure to the alkaline pH of the midgut lumen, and BV, produced by ODV-infected midgut cells, consist of a single enveloped nucleocapsid and it spreads infection to the whole insect until the host dies, liquefies and releases millions of polyhedra to the environment.

Almost every host species demonstrate some degree of developmental resistance, that is increasing resistance with age [7] but it is observed only when virus is administered orally [7,8]. Some species, such as *S. frugiperda* and *H. zea* are resistant to oral infection but they

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exhibit no systemic resistance to AcMNPV when budded virus is injected intrahemocelically (IH) [9]. However, *H. virescens* is known as a fully permissive host and little information exists about *R. nu* susceptibility. Resistance mechanisms result in various degrees of larval susceptibility to infection and this is decisive for choosing the infection route to be used. All mentioned species can be reared under laboratory conditions.

Horseradish peroxidase isozyme C from *Armoracia rusticana* roots (HRPC) is an important commercial biocatalyst with several chemical and biochemical applications, including diagnostics, biocatalysts, biosensors and it has been applied to the bioremediation of polluted soil and water [10,11].

In this study, *S. frugiperda*, *H. zea*, *H. virescens* and *R. nu* were evaluated as biological factories to produce recombinant HRPC. We compared their potential as expression hosts by infection with recombinant baculovirus, either by oral administration of polyhedra or by injection of BV. Then, HRPC purification by IMAC was directly performed from the larval crude extract.

Materials and methods

Materials

Horseradish peroxidase (HRP) from *A. rusticana* roots (type 8375) and 3,3′-diaminobenzidine (DAB reagent) were from Sigma–Aldrich (Saint Louis, MO, USA). *S. frugiperda* IPBL-Sf9 (Sf9) cell line was from ABAC (Buenos Aires, Argentina). Sf900 II insect tissue-culture media, Sf9 serum-free medium adapted cell line and penicillin/streptomycin (ATB/ATM) were from Invitrogen (Carisbad, CA, USA). Fetal calf serum (FCS) was from Internegocios S.A. (Buenos Aires, Argentina). AcMNPV, pAcGP67 vector and Agarplaque Plus were from BD Biosciences Pharmingen (San Diego, CA, USA). Disposable materials were from Nunc International (Naperville, IL, USA). HisTrap FF column and AKTA Purifier 10 equipment were from GE HealthCare (Piscataway, NJ, USA).

Insect cells, virus strains and larvae

In all experiments, Sf9 cell line was cultured in Sf900 II medium supplemented with 1% FCS. Virus propagation and viral titration were carried out according to the standard protocol described by O'Reilly *et al.* [12].

The synthetic HRPC gene was generously provided by Dr. PE Ortiz de Montellano (University of California). It was cloned into the pAcGP67 vector (BD Biosciences Pharmingen) containing a sequence for the glycoprotein 67 (GP67) leader peptide, which targets the recombinant protein for secretion (pAcGPHRPC). The recombinant AcMNPV HRPC polyhedrin-minus (occ−) and AcMNPV HRPC polyhedrin-plus (occ+) were previously constructed and maintained in our lab. Briefly, AcMNPV HRPC occ− was obtained by cloning the HRPC gene into the locus polyhedrin, thus giving an intrahemocelically infective virus. The polyhedrin-plus virus (AcMNPV HRPC occ+) was identical except for the polyhedrin gene presence under p10 promoter to achieve an orally infective baculovirus. The commercial AcMNPV strain was used as the wild type virus.

AcMNPV HRPC occ+ polyhedra were collected and counted at 5 dpi from a cell culture previously infected as described by O'Reilly *et al.* [12].

S. frugiperda, *H. zea*, *H. virescens* and *R. nu* larvae were obtained from a laboratory colony and maintained on an artificial high-wheat germ diet [13]. Larvae were individually reared in six-well plates at 23–25°C in a 70% humidified chamber, with a 16:8 photoperiod (L:D). A phenol-free diet was used in all oral infection experiments.

Larvae infection

Larvae of *S. frugiperda* (approximately 200 mg), *H. zea*, *H. virescens* and *R. nu* (approximately 100 mg) were injected subcutaneously into the hemocele with 50 μl recombinant baculovirus (1×10^7 pfu ml^{-1}). Control larvae were either uninfected or infected with wild type AcMNPV.

Larvae of *R. nu* and *H. virescens* (approximately 50 mg), *S. frugiperda* and *H. zea* (approximately 100 mg), were starved for 24 hours and then fed a 100 mg diet containing 1×10^6 AcMNPV HRPC occ+ polyhedra in independent experiments.

After the allotted time for each larval species, larvae were harvested and their weights determined before individual homogenization in the presence of 1 ml per larva of lysis buffer (50 mM sodium phosphate buffer, pH 6.0, 5 mM EDTA, 0.2 mg ml^{-1} PMSF, 150 mM KCl with glutathione crystals) to obtain a total extract. Each extract was centrifuged at 14,000*g* for 10 min at 4°C for HRPC activity measurement.

HRP activity measurement

A reaction mixture containing 30 μM guaiacol and 25 mM hydrogen peroxide in 100 mM potassium phosphate buffer, pH 7.0, was prepared. Guaiacol oxidation was initiated by adding a 50 μl sample to 1 ml reaction mixture. The reaction was monitored by measuring its absorbance at 470 nm within 1.5 min and activity was calculated as described by Tjissen [14]. The results are expressed as units (U) per ml.

HRP mass measurement

HRP from *A. rusticana* roots was used as the standard to construct a calibration curve to correlate HRP activity and mass protein measured using the Bradford assay [15].

HRPC purification from S. frugiperda larvae by IMAC

S. frugiperda larvae were homogenized in lysis buffer (0.3 g larva per ml) to obtain the total extract. Then, it was centrifuged at 14,000*g* for 10 min at 4°C and filtered using a Whatmann filter paper grade 1. The sample was conditioned in the equilibration buffer (5 mM sodium phosphate buffer, pH 8.0, 300 mM NaCl) by passing it through a PD-10 column. A HisTrap column (1.0-ml bed volume) equilibrated with the same buffer was loaded with the sample. Following a washing step with the equilibration buffer containing 20 mM imidazole, step elution was performed by increasing the imidazole concentration to 250 mM. Linear flow rate was 0.4 cm min^{-1} and 1.5 ml fractions were collected. Protein separation was monitored by both absorbance at 280 nm and HRP activity.

Catalase activity measurement

Catalase activity was measured as described by Aebi [16]: a reaction mixture was prepared containing 770 μl 50 mM potassium phosphate buffer, pH 7.0, and 15 μl 2.6 M hydrogen peroxide. Reaction was initiated by addition of 15 μl sample and the absorbance at 240 nm was recorded.

TABLE 1

HRPC mass expressed by different lepidopteran pest species, following infection with recombinant AcMNPV either by oral inoculation with polyhedra (1×10^6 polyhedra per larva) or by inoculating BV directly into the insect hemocele (50 μl of 1×10^7 ufp ml^{-1} per larva)

Lepidopteran species	Media weight larva (g)	Harvesting time (days post-infection)	Intrahemocelic infection (50 μl of 1×10^7 ufp ml^{-1})		Oral infection (1×10^6 polyhedra)	
			HRPC mass (mg kg^{-1})	μg $larva^{-1}$	HRPC mass (mg kg^{-1})	μg $larva^{-1}$
S. frugiperda	0.3	6	137.0 ± 17.0	41	0	0
H. virescens	0.06	6	218.1 ± 30.1	14	215.0 ± 21.0	13
H. zea	0.2	6	37.9 ± 0.6	8	0	0
R. nu	0.2	4	100.0 ± 14.0	20	110.0 ± 10.1	22

Both inocula used in the study were optimized to achieve the maximum HRPC yield by oral and intrahemocelic routes.

Electrophoretic analyses

Protein samples were separated by SDS-PAGE on 12.5% polyacrylamide gels and stained with Coomassie Blue. For western blot analysis, gels were transferred onto a nitrocellulose membrane and detection was performed using a mouse polyclonal anti-HRP (Sigma–Aldrich) or mouse polyclonal anti-GP67 as primary antibodies and HRP-conjugated anti-mouse IgG (DAKO, Copenhagen, Denmark) as the secondary antibody. For DAB staining, the gel was immersed in a 9 mg ml^{-1} DAB aqueous solution with 10 μl 2.6 M hydrogen peroxide for 2 min.

Results and discussion

To determine the capability of HRPC expression of different lepidopteran pests frequently found in Argentina, individual extracts of infected larvae were made by homogenization in lysis buffer to control the melanization process [17]. After centrifugation, HRPC activity was measured in the supernatants and enzyme mass was calculated. All species were successfully infected by AcMNPV HRPC occ−. Kinetic expression curve (not shown) for *S. frugiperda* showed a maximum of 137.0 ± 17.0 mg HRPC per kg larva, at 6 dpi. Similar HRPC yield was obtained with *R. nu* (100.0 ± 14.0 mg HRPC per kg larva) but the kinetic curve showed the maximum at 4 dpi. *H. zea* and *H. virescens* showed the maximum enzyme activity at 6 dpi but with different expression levels, being 37.9 ± 0.6 and 218.1 ± 30.1 mg HRPC per kg larva, respectively. The former had the worst capability to produce the recombinant protein probably due to its poor susceptibility to baculovirus infection [18]. By contrast, *H. virescens* showed the highest HRPC level per kg larva. However, it is important to point out that three times higher number of larvae is needed to express the same amount of HRPC in comparison with *S. frugiperda*. As a consequence, the total volume of viral inoculum has to be greater.

Table 1 shows a summary of the results to compare the HRPC expression level for each larval species. The oral infection of insect

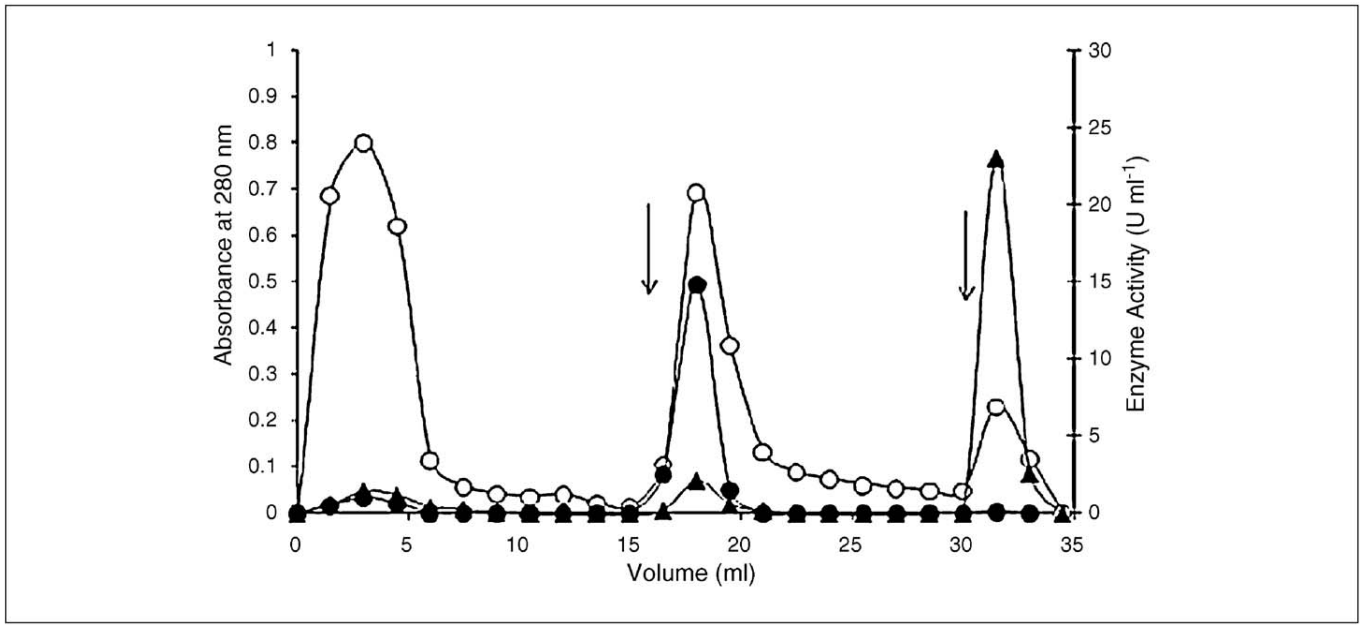

FIGURE 1

HRPC purification by IMAC. Conditioned larval extract (3 ml) was loaded onto a HisTrap (Ni) column previously equilibrated with 5 mM sodium phosphate buffer, pH 8.0, 300 mM NaCl. A washing step with 10 ml of the same buffer containing 20 mM imidazole was carried out and an elution step was performed by increasing the imidazole concentration to 250 mM. 1.5 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.

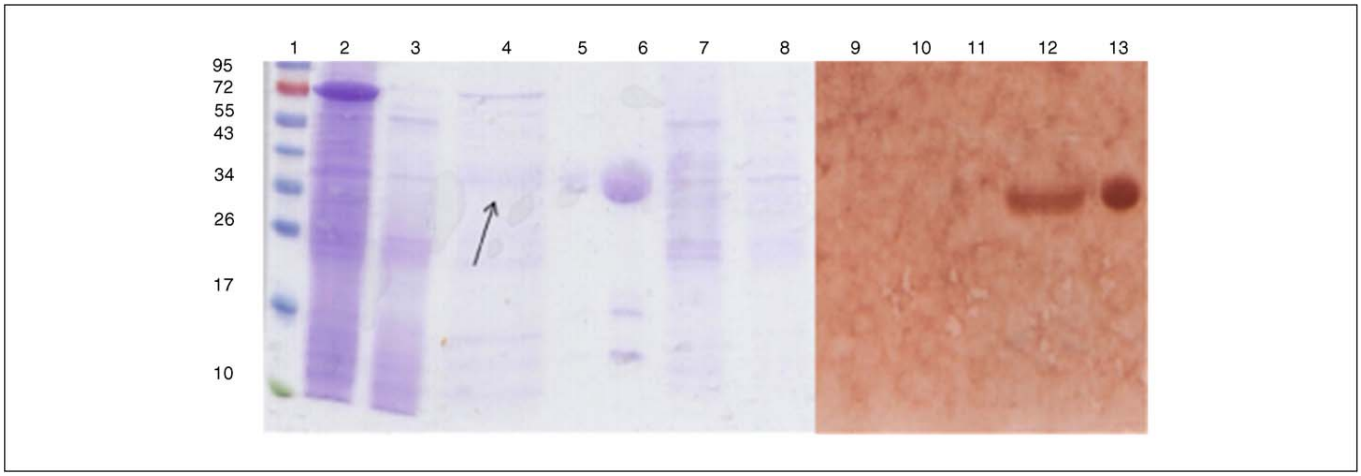

FIGURE 2

SDS-PAGE and western blot. Lane 1, molecular weight markers; lane 2, larval extract day 6 post-infection (starting material); lanes 3 and 9, IMAC pass-through; lanes 4 and 12, IMAC fraction eluted by 250 mM imidazole in equilibration buffer (final product); lanes 5 (0.3 mg ml^{-1}), 6 (1.7 mg ml^{-1}) and 13 (1.7 mg ml^{-1}) standard HRP from *A. rusticana* roots; lanes 7 and 10, IMAC washing step with equilibration buffer; lanes 8 and 11, IMAC washing step with 20 mM imidazole. Lanes 1–8 were stained with Coomassie Blue, lanes 9–13 were revealed with DAB after western blot. Western blot was developed with a specific polyclonal antiserum raised against HRP. The arrow indicates peroxidase bands.

larvae was also studied but only *R. nu* showed a high biotechnological yield, reaching 110.0 ± 10.1 mg HRPC per kg larva at 4 dpi, similar level to that obtained by via intrahemocelical. Orally infected *R. nu* larvae expressed 22 μg HRPC per larva and *H. virescens* only 13 μg per larva due to its lower weight as it was mentioned above. So, *R. nu* is the more convenient host to produce HRPC by oral inoculation.

Comparatively, *S. frugiperda* doubles the HRPC expression level obtained in *R. nu* (41 and 20 μg per larva, respectively). Moreover, its oviposition averages about 1500 eggs per *S. frugiperda* female is three times greater than *R. nu*. These characteristics indicate that *S. frugiperda* is the best host for economical HRPC production when AcMNPV occ− is used as the inoculum because the same amount of HRPC is achieved with a smaller insect number, and therefore smaller inoculum amount in comparison with other species.

Figure 1 shows the chromatographic profile of HRPC purified by IMAC from *S. frugiperda* crude extract at 6 dpi. The main contaminant was a hydrogen peroxide-consuming protein which interferes in HRP measurement, given an apparent low specific activity product. Although this contaminant binds to the affinity ligand, it was totally eluted with 20 mM imidazole. We have previously observed this pattern for catalase [17]. This enzyme is part of the antioxidant defense system against prooxidant compounds from plants [19] existing in the larval midgut of Lepidoptera. As it was also present in hemolymph of *R. nu*, it might play an important role in oxidative stress caused by radiation or pathogens [20,21]. Thus, the observed hydrogen peroxide-consuming activity was considered to be catalase.

We have previously described the HRPC purification from a larval extract of *R. nu* by IMAC with a yield of 88.8% and a purification factor of 18.9 [17]. In this study, the yield of HRPC from *S. frugiperda* extract was 86% with a purification factor of 29. The same performance was observed for this purification method when it was applied to both larval species. Figure 2 shows the corresponding SDS-polyacrylamide gel and western blot patterns of the final product compared to the starting material and the standard HRP. As it is shown in Figure 3, the recombinant baculovirus was present in the pass-through and no viral particles were detected in the elution fraction. This is an important feature because the viral inactivation reagent of the final product, such as 0.1N HCl, is not necessary. This extra step could affect the recombinant product.

These results evidence that *S. frugiperda* larvae infected with recombinant baculovirus is a highly efficient host for HRPC production.

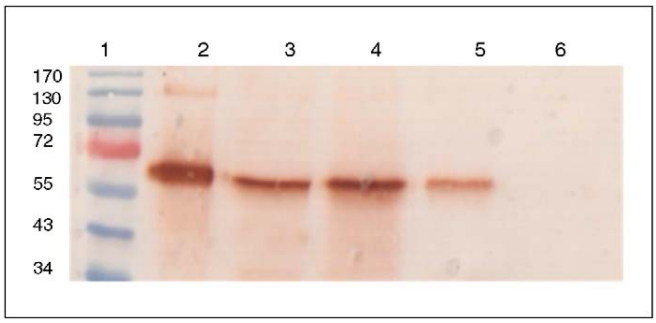

FIGURE 3

Virus localization. Western blot against GP64 capsid virus protein. Lane 1, molecular weight markers; lane 2, positive control (purified virus); lane 3, larval extract day 6 post-infection (starting material); lane 4, conditioned larval extract (PD-10); lane 5, IMAC pass-through; lane 6, IMAC fraction eluted with 250 mM imidazole in equilibration buffer (final product). Lanes 1–6 were stained with DAB after western blot. Western blot was developed with a specific monoclonal antiserum raised against GP64.

Conclusion

S. frugiperda is the best choice to HRPC expression among the lepidopteran species evaluated in this study. HRPC was purified by IMAC from the larval extract with high yield and purity, free of catalase and virus particles.

R. nu is the best option to produce HRPC by oral infection.

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