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Horseradish peroxidase production from *Spodoptera frugiperda* larvae: A simple and inexpensive method

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ABSTRACT

Horseradish peroxidase is used in many biotechnological fields including diagnostics, biocatalysts and biosensors. Horseradish peroxidase isozyme C (HRPC) was extracellularly expressed in *Spodoptera frugiperda* Sf9 cell culture and in intact larvae. At day 6 post-infection, the concentration of active HRPC in suspension cultures was 3.0 ± 0.1 μg per 1×10^6 cells or 3.0 ± 0.1 mg l^{-1} with a multiplicity of infection of 1 in the presence of 7.2 μM hemin. Similar yields were obtained in monolayer cultures. In larvae, the HRPC expression level was 137 ± 17 mg HRPC kg^{-1} larvae at day 6 post-infection with a single larvae thus producing approximately 41 μg HRPC. The whole larval extract was separated by ion exchange chromatography and HRPC was purified in a single step with a yield of 75% and a purification factor of 117. The molecular weight of recombinant HRPC was 44,016 Da, and its glycosylation pattern agreed with that expected for invertebrates. The K_m and V_{max} were 12.1 ± 1.7 mM and 2673 ± 113 U mg^{-1}, respectively, similar to those of HRP purified from *Armoracia rusticana* roots. The method described in this study, based on overexpression of HRPC in *S. frugiperda* larvae, is a simple and inexpensive way to obtain high levels of active enzyme for research and other biotechnological applications.

1. Introduction

Horseradish peroxidase (HRP, EC 1.11.1.7) is a heme-containing oxidoreductase originally isolated from horseradish (*Armoracia rusticana*) roots. The HRP isozyme C (HRPC) is the most abundant and best studied enzyme among the 11 isoforms present in the plant. The HRPC mature protein is 308 amino acids in length with four highly conserved disulfide bridges and approximately 21% glycosylation [1–2]. With an average carbohydrate composition of $(GlcNAc)_2$, Man_3, Fuc, Xyl for each of its eight carbohydrate chains, the observed molecular weight of native HRPC is close to 44 kDa. HRP is useful in many biotechnological fields including diagnostics, biocatalysts and biosensors. Oxidation reactions at an industrial level require a low-cost, highly stable and catalytically efficient enzyme. To this end, site-directed mutagenesis and directed evolution techniques are now routinely employed to improve such an important biocatalyst [3]. Recombinant HRPC was expressed in *Escherichia coli* with a low yield of active enzyme after inclusion body dissolution and protein refolding [4]. Native and mutant HRPCs were active when expressed in *Pichia pastoris* but had poor specific activity [5]. Newmeyer and Ortiz de Montellano reported high expression (5–10 mg l^{-1}) of a synthetic gene of HRPC in *Trichoplusia ni* cell cultures for use in enzyme catalytic studies [6]. HRPC was recently expressed and purified as a fusion protein in *Spodoptera frugiperda* cell lines [7] and also in the larvae of *Rachiplusia nu* [8]. Both *R. nu* and *S. frugiperda* larvae are destructive plagues affecting several economically important crops such as soy and corn. These species are widely distributed in tropical and subtropical regions of the Americas.

The use of the baculovirus-based expression vector system (BEVS) in insect cell culture represents a promising option for the heterologous expression of eukaryotic proteins for biotechnological or pharmaceutical applications. *Autographa californica* nuclear polyhedrosis virus (AcMNPV) infects the clonal tissue culture line Sf9 derived from *S. frugiperda* cells. Foreign proteins are expressed at high levels by using strong viral promoters such as polyhedrin or p10 in the very late phase of the infection cycle. Proteins of eukaryotic origin expressed in insect cells are often correctly folded and glycosylated in a manner similar to that found in mammalian cells. However, in vivo production systems have not been widely exploited because of the lack of experience in rearing and maintaining larvae in laboratories involved in protein production [9]. *S. frugiperda* larvae are resistant to oral infection but are infected when budded virus is injected in the hemolymph [10]. At twice the weight of *R. nu*, *S. frugiperda* represents a better option as a recombinant protein biofactory. In this study, recombinant HPRC was overexpressed in *S. frugiperda* larvae, purified using a one-step process and characterized. The expression level of the enzyme was compared with that obtained in the Sf9 insect cell line propagated in SF900II medium containing 1% (v/v) fetal calf serum.

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2. Materials and methods

2.1. Materials

Horseradish peroxidase (type 8375) and 3,3′-diaminobenzidine (DAB reagent) were from Sigma–Aldrich (Saint Louis, MO, USA). Sf900II insect tissue culture media, *S. frugiperda* Sf9 cell line, Cellfectin and penicillin/streptomycin (ATB/ATM) were from Invitrogen Life Technologies (Gaithersburg, MD, USA). Fetal calf serum (FCS) was from Nutrientes Naturales S.A. (Buenos Aires, Argentina). Agarplaque Plus and BaculoGold Bright were from BD Biosciences Pharmingen (San Diego, CA, USA). Disposable materials were from Nunc International (Naperville, IL, USA). SP-HiTrap columns and AKTA Purifier 10 equipment were from GE HealthCare (Piscataway, NJ, USA).

2.2. Expression cassette

The synthetic HRPC gene fused to a 6xHis tag at the 5′ end – synthesized by British Biotechnologies Ltd. – was kindly provided by Dr. P.E. Ortiz de Montellano (University of California). This gene was subsequently modified to include a 6xArg tag at the 3′ end [7], and the construct was cloned into the pAcGP67 vector (BD Biosciences Pharmingen) containing a sequence for the glycoprotein 67 (GP67) leader peptide that targets the recombinant protein for secretion (pAcGPHRPC).

2.3. Virus production

One million Sf9 cells were co-transfected with 82.5 ng pAcGPHRPC and 250 ng linearized BaculGold Bright DNA (BD Biosciences Pharmingen) in the presence of Cellfectin. BaculGold Bright DNA contains the gene for green fluorescent protein (GFP). After a 4-day incubation at 27 °C, the cell culture supernatant was collected and centrifuged at 3000 × *g* for 10 min. Co-transfection efficiency was determined by assaying peroxidase activity as described below and by measuring GFP expression by fluorescence under UV light. The recombinant baculovirus polyhedrin-minus (AcMNPVHRPC) vector containing the HRPC expression cassette is shown in Fig. 1.

Following three amplification steps, the virus titer was determined by a plaque assay (1.4×10^8 pfu ml^{-1}). The amplified virus stock was used at the production step.

2.4. Insect cell infection

The Sf9 cell line was propagated in monolayers in T-flasks at 27 °C in Sf900II medium containing 1% (v/v) FCS. All experiments were carried out with cells at 95–99% viability in the log phase. HRPC expression was assessed at multiplicities of infection (MOIs) of 0.5, 1 and 5 pfu per cell on 1×10^6 Sf9 cells ml^{-1} in 6-well dishes. A negative control plate with uninfected cells was included in the analysis. Hemin was added simultaneously at final concentrations of 4.8, 7.2 and 9.6 μM. An infected plate (MOI 1) without hemin was included as a reference. The plates were incubated in the dark at 27 °C for 7 days and enzyme activity in supernatants was determined every day post-infection (dpi).

Recombinant HRPC was produced in suspension cultures of 10 ml in shake flasks. At a cell density of 1×10^6 cells ml^{-1}, the cultures were supplemented with 7.2 μM hemin and infected in duplicate at a MOI 1. A negative control with uninfected cells was also included. Infected cultures were incubated in an orbital shaker at 100 rpm. At 6 dpi, cells were removed by centrifugation (3000 × *g*, 10 min) and HRP activity was measured in the supernatant. The results are expressed as the average ± standard deviation of at least three determinations.

2.5. Larval infection

S. frugiperda larvae were obtained from a laboratory colony fed on a high-wheat germ diet [11]. Second-instar larvae were reared in 6-well dishes (one per well) at 23–25 °C in a 70% humidified chamber, with a 16:8 photoperiod (L:D). For HRP expression kinetics, 50 fifth-instar larvae (approximately 300 mg) were used per day. They were injected with 50 μl recombinant baculovirus (6×10^6 or 1.4×10^7 pfu ml^{-1}). Control larvae were either uninfected or infected with wild type AcMNPV. After the allotted time for each condition, larvae were harvested and homogenized in groups of five larvae ($n = 10$) in the presence of 2.5 ml buffer lysis (50 mM sodium phosphate buffer, pH 6.0, 5 mM EDTA, 0.2 mg ml^{-1} PMSF, 150 mM KCl with glutathione crystals) per gram of larvae to obtain larval extracts. Each extract was centrifuged at 14,000 × *g* for 10 min at 4 °C. HRP activity in the extract was measured and results are expressed as the average ± standard deviation.

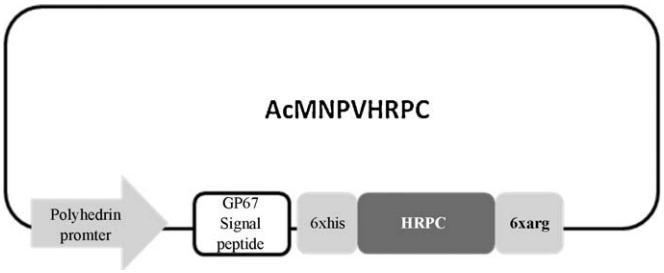

Fig. 1. Recombinant baculovirus polyhedrin-minus (AcMNPVHRPC). The synthetic HRPC gene was fused in-frame to the viral signal peptide GP67. HRPC expression is under control of the polyhedrin promoter.

2.6. HRP activity measurement

HRP activity was measured by assessing guaiacol oxidation in a reaction mixture containing 30 mM guaiacol and 25 mM hydrogen peroxide in 100 mM potassium phosphate buffer, pH 7.0. Oxidation was initiated by adding a 10 μl sample to the 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 [12]. Results are expressed as units (U) per ml.

2.7. Purification of HRPC from larval crude extract by ion exchange chromatography (IEC)

Four larvae were homogenized directly in 2.5 ml of 5 mM Tris/HCl buffer, pH 7.0 (equilibration buffer) with glutathione crystals. The crude extract was centrifuged at 14,000 × *g* for 10 min at 4 °C and then filtered using a Whatman filter paper grade 1. The ionic strength of the sample was measured with a conductivity monitor (Pharmacia Biotech, Uppsala, Sweden).

The sample was loaded on a SP-HiTrap column (1.0-ml bed volume). After a washing step with equilibration buffer, HRPC was eluted by the stepwise addition of 25, 150 and 500 mM NaCl in equilibration buffer. Protein separation was monitored by both absorbance at 280 nm and HRP activity. The linear flow rate was 0.4 cm min^{-1}.

2.8. Total protein measurement

Protein concentration was measured using the Bradford assay [13] with bovine serum albumin as the standard.

2.9. 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 HRP was detected using a mouse monoclonal anti-HRP (Sigma) as the primary antibody 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 of 2.6 M hydrogen peroxide for 2 min. For image processing, gels were scanned and then analyzed with the Image Quant software (Molecular Dynamics Sunnyvale, CA, USA).

2.10. MALDI-TOF mass spectrometry

MALDI-TOF MS spectra were recorded on a 4700 Proteomics Analyzer Instrument (Applied Biosystems, Foster City, CA, USA). Samples of IEC-purified HRPC were loaded with sinapinic acid as the matrix in 30% acetonitrile, 70% water and 0.1% trifluoroacetic acid onto a stainless steel target.

2.11. Glycosylation analysis

Glycosylation patterns of recombinant HRPC were compared with those of the standard HRP from *A. rusticana* roots (Sigma–Aldrich). A total of 80 μg of each enzyme was dissolved in 320 μl of 0.25% SDS, 0.02 M DTT and heated at 100 °C for 15 min to denature the protein. Then, 15 μg of denatured protein was incubated in 60 μl of the following buffers and glycosidases: (1) 0.05 M sodium phosphate buffer, pH 7.5, containing 1% NP-40 and 3 U N-glycosidase F; (2) 0.1 M sodium acetate buffer, pH 5.0, 0.1 mM $MgCl_2$, 0.1% Triton X-100 and 0.15 mU N-glycosidase A; (3) sodium citrate buffer, pH 5.0, 15 mU endoglycosidase H; and (4) sodium phosphate buffer, pH 7.0, 1% TritonX-100 and 1.5 mU *O*-glycosidase. As a control, 5 μg of denatured protein was incubated in 20 μl of the corresponding buffers with no added enzyme. After incubation at 37 °C for 24 h, 5 μg of each treated enzyme was mixed with sample buffer, heated at 100 °C for 5 min and analyzed by SDS-PAGE and Western blot as described above.

2.12. HRP biochemical characterization

Recombinant HRPC and standard HRP (Sigma–Aldrich) activity was spectrophotometrically assayed at 20 °C. The apparent K_m and V_{max} values were determined by fitting the data to a hyperbola by applying the Gauss–Newton algorithm [14]. The kinetic constants are the means of at least four determinations. The results are expressed as the average ± standard deviation.

The stability of recombinant HRPC was determined after a 9-h incubation period at 50 °C in 50 mM sodium acetate buffer, pH 4.5. Aliquots were withdrawn and peroxidase activity was measured at different time points.

3. Results and discussion

3.1. HRPC expression in Sf9 cell line

While a high yield of recombinant HRPC (41.3 mg l^{-1}) using Sf9 cell line in Grace's medium with 10% FCS has been previously shown [15], the often prohibitive cost of FCS makes it important to develop more economical expression systems. In this study, Sf9 insect cells in a low serum SF900II media expressed catalytically active recombinant HRPC. Its molecular mass was comparable to that of the plant enzyme (44 kDa) as judged by SDS-PAGE and Western blot analysis (Fig. 2) suggesting that the degree of glycosylation is likely similar to that of native HRPC (21.8%).

The HRPC expression kinetics at various MOIs and hemin supplementation are shown in Fig. 3. Enzyme activity gradually increased until 6 dpi for MOIs of 0.5 and 1 whereas for MOI 5 the maximum level was reached at day 5. Hemin addition significantly increased HRPC expression up to a concentration of 7.2 μM. After day 6 post-infection, extensive cell lysis occurred and many intracellular contaminants appeared (not shown). Taking these results into account, HRPC was harvested at day 6 post-infection from a cell culture infected at an MOI of 1 in the presence of 7.2 μM hemin; higher MOIs failed to increase the yield of HRPC. In monolayer culture, HRPC was expressed at a level of 3.0 ± 0.1 μg per 1×10^6 cells. The maximum HRPC level reached in suspension cultures was 3.0 ± 0.1 μg per 1×10^6 cells (or 3.0 ± 0.01 mg l^{-1}), the same as that obtained in monolayer cultures. This yield was comparable to that obtained for other proteins expressed in the same system [16].

3.2. HRPC expression in insect larvae

S. frugiperda larvae infected with the recombinant virus showed typical symptoms of AcMNPV infection such as appetite and mobility loss from 2 dpi. When larvae were homogenized, the melanization process was immediately controlled by the addition of 50 mM sodium phosphate buffer, pH 6.0, 5 mM EDTA, 0.2 mg ml^{-1} PMSF, 150 mM KCl with glutathione crystals [8]. To assess the expression kinetics, HRP activity in the larval extracts was measured at different dpi (Fig. 4). In all larvae infected with virus titers of 6×10^6 and 1.4×10^7 pfu ml^{-1}, HRPC and GFP expression were detected from 3 dpi (Fig. 5). The infection efficacy via intrahemocelical injection was 99% in all experiments. A direct correlation was observed between GFP and HRPC expression profiles, with 99% of infected larvae expressing both proteins, a fact that simplified the selection of larvae to be processed. No evidence of proteolysis of recombinant HRPC was observed by Western blot.

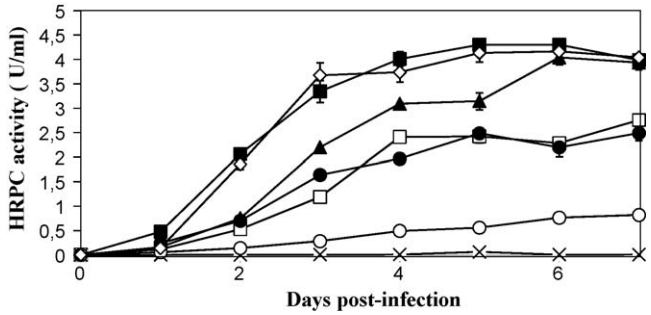

Fig. 3. Kinetics of HRPC expression in Sf9 cells infected with recombinant baculovirus at different multiplicities of infection (MOIs) and hemin supplementation. HRPC activity was measured in the culture supernatant daily after infection with MOI 0.5, 7.2 hemin (□); MOI 1 without hemin (○); MOI 1, 4.8 hemin (●); MOI 1, 7.2 hemin (▲); MOI 5, 7.2 hemin (◇) and MOI 5, 9.6 hemin (■). Uninfected Sf9 cells (×).

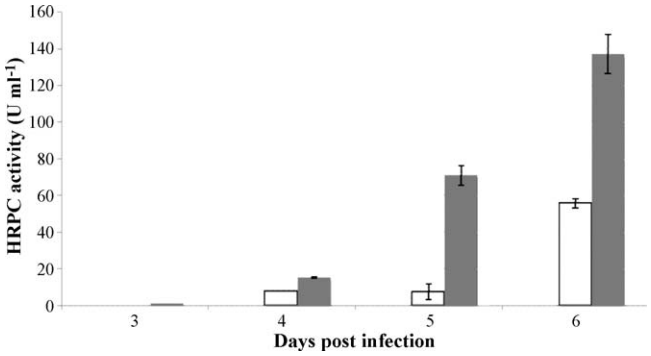

Fig. 4. HRPC activity at different times post-infection in extracts of *S. frugiperda* larvae infected with 50 μl of 6×10^6 pfu ml^{-1} (□) or 1.4×10^7 pfu ml^{-1} (■) of recombinant baculovirus. Results are expressed as the average ± standard deviation (n = 10).

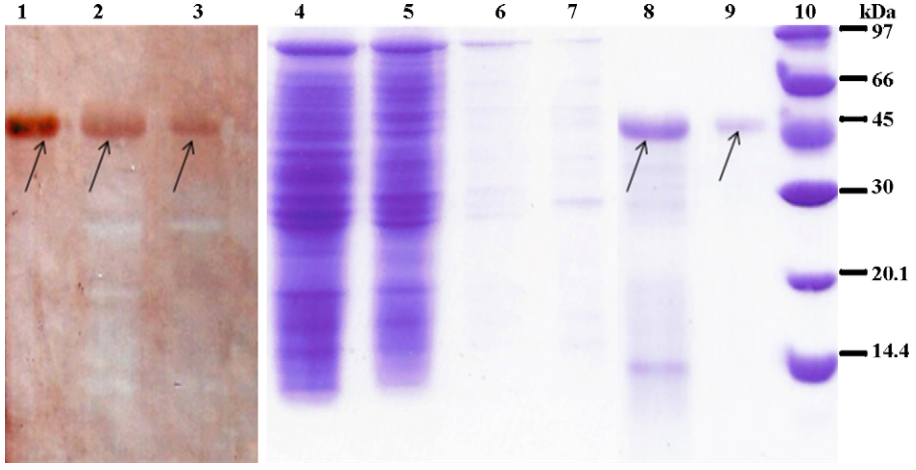

Fig. 2. SDS-PAGE. Lanes 1 and 9, standard HRP from *A. rusticana* roots; lanes 2 and 8, IEC fraction eluted by 150 mM NaCl in equilibration buffer (final product); lane 3, culture supernatant day 6 post-infection at MOI 1, 7.2 μM hemin; lane 4, larval extract day 6 post-infection (starting material); lane 5, IEC flow-through; lane 6, IEC washing step with equilibration buffer; and lane 7, IEC washing step with 25 mM NaCl in equilibration buffer; lane 10, molecular weight markers. Lanes 1–3 were developed with DAB after Western blot. Lanes 4–10 were stained with Coomassie Blue. Molecular weight markers were phosphorylase b (97.0 kDa), bovine serum albumin (66.0 kDa), ovalbumin (45 kDa), carbonic anhydrase (30.0 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa). The Western blot was developed with a specific monoclonal antiserum raised against HRP. Arrows indicate peroxidase bands.

Fig. 5. *S. frugiperda* larvae 6 days after injection with AcMNPV, expressing green fluorescent protein (GFP) throughout the body.

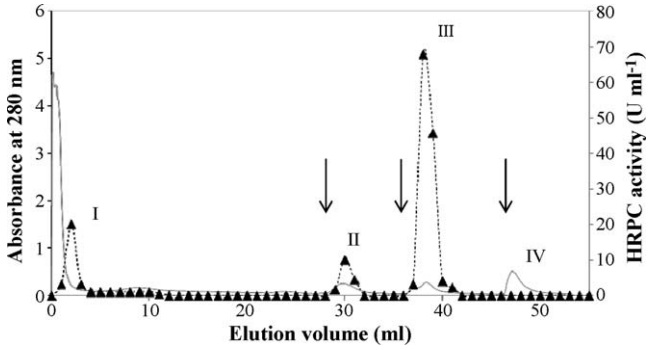

Fig. 6. HRPC purification from larval extract by IEC. 3 ml larval extract (pH 7.0) was loaded on a SP-HiTrap column. The column was washed with equilibration buffer and proteins eluted with a step gradient of increasing NaCl concentrations: 25, 150 and 500 mM. One-ml fractions were collected at a linear flow rate of 0.4 cm min^{-1} The absorbance at 280 nm (▬) and enzyme activity (▲) were monitored. Arrows indicate the buffer change.

Results indicate that recombinant HRPC expression greatly increases at 6 dpi. The expression levels were 56 ± 16 and 138 ± 17 mg HRPC kg^{-1} larvae with viral titers of 6×10^6 and 1.4×10^7 pfu ml^{-1}, respectively. Due to larval mortality at later time points (over 14% at 7 dpi), 6 dpi and a viral titer of 1.4×10^7 pfu ml^{-1} were selected as optimal conditions for further experimentation.

3.3. Crude starting material for downstream processing

In Sf9 monolayer and suspension experiments, HRPC represented 0.3% of the total protein concentration present in the supernatant using low serum media. In whole insect larvae, HRPC represented 0.6% of the total protein. A total of 1 mg of recombinant HRPC was produced in 333 ml of cell culture or in 24 larvae (18.2 ml). Tissue culture techniques are expensive as they need large quantities of culture medium and sophisticated instrumentation [17]. In addition, cell culture needs to be expanded prior to HRPC production. The whole process takes at least a month and needs 20 times more virus titer in comparison with the optimized larval method which is simpler, more economical and less time-consuming and has a lower contamination risk. After the viral stock is ready, the complete process using larvae, including expression and purification, takes only 1 week.

Despite the accumulation of HRPC in the larval hemolymph, the enzyme was purified from larval extracts due to the difficulties associated with quantitative extraction of hemolymph. Table 1 shows a comparative analysis of both processes to produce 1 g HRPC.

Table 1
Comparative analysis for expression of 1 g HRPC in *S. frugiperda* larvae and in Sf9 cell culture.

	S. frugiperda larvae	Sf9 cell culture
Starting material	24,000 fifth-instar larvae	333 l suspension culture
Harvest time after infection (days)	6	6
Viral inoculum of 1.4×10^8 pfu ml^{-1} (l)	0.12	2.40
Arbitrary cost units[a]	1	>100
Volume for downstream processing (l)	18.2	333

[a] Equipment, disposable materials, artificial diet and culture media cost were included in the analysis.

3.4. Purification of HRPC from larval crude extract by IEC

The isoelectric point of recombinant HRPC was determinate to be 9.5. The extract was prepared in the chromatographic equilibration buffer at pH 7.0 with glutathione crystals and loaded directly on the column. The chromatographic profile is shown in Fig. 6. Some HRPC activity was detected in unbound fraction but the main peak of activity eluted at 150 mM NaCl. An initial wash step with 25 mM NaCl proved essential in improving the purification factor. In the absence of this wash, the purification factor was only 40, implying that the fraction was contaminated with other proteins. However, following this wash, HRPC was eluted at 150 mM NaCl (peak III) with a purification factor of 117 and a yield of 75%. The HRPC produced by this method was estimated to be 70% purity by gel scan. The recombinant HRPC was found to have a molecular weight of 44,016 Da as judged by MALDI-TOF analysis (Fig. 7), similar to that of HRP from the roots of *A. rusticana*. An SDS-polyacrylamide gel of the final product compared to the starting material and the standard HRP is shown in Fig. 7.

3.5. Glycosylation pattern

Neither HRPC nor standard HRP purified from *A. rusticana* roots showed sensitivity to *O*-glycosidase and endoglycosidase H (not shown). HRPC was completely deglycosylated by N-glycosidase F (21% decrease in its molecular weight) and partially deglycosylated by N-glycosidase A while standard HRP showed sensitivity to N-glycosidase A but not to N-glycosidase F. The deglycosylation patterns were as expected for invertebrates and plant systems

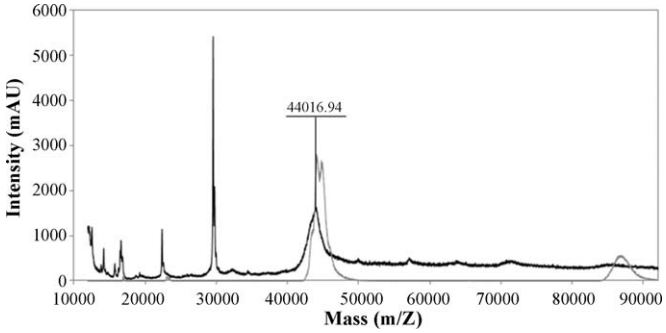

Fig. 7. MALDITOF analysis of purified recombinant HRPC (–) and HRP from *A. rusticana* roots (▬).

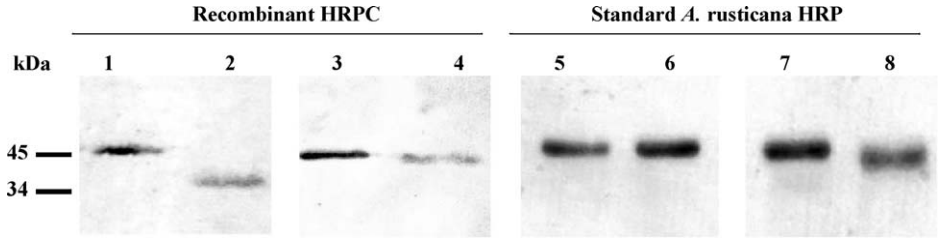

Fig. 8. Effect of glycosidase treatment on standard HRP purified from *A. rusticana* roots and on recombinant HRPC. Lane 1, HRPC incubation control; lane 2, HRPC + N-glycosidase F; lane 3, HRPC incubation control; lane 4, HRPC + N-glycosidase A; lane 5, standard *A. rusticana* HRP incubation control; lane 6, standard *A. rusticana* HRP + N-glycosidase F; lane 7, standard *A. rusticana* HRP incubation control; lane 8, standard *A. rusticana* HRP + N-glycosidase A. Lanes 1–8 were developed with DAB after Western blot.

Table 2
Kinetic constants of recombinant HRPC expressed in *Spodoptera frugiperda* larvae and standard HRP extracted from *A. rusticana* roots.

	Substrate	Co-substrate (250 μM)	Apparent K_m (mM)	V_{max} (U mg^{-1})	V_{max}/K_m (U mg^{-1} mM^{-1})
Recombinant HRPC larvae	Guaiacol	H_2O_2	12.1 ± 1.7	2673 ± 113	220 ± 40
Recombinant HRPC SF9 cell line	Guaiacol	H_2O_2	21.1 ± 2.4	2711 ± 125	129 ± 21
Standard *A. rusticana* HRP	Guaiacol	H_2O_2	18.8 ± 2.2	3102 ± 68	165 ± 23

(Fig. 8). The same pattern was determined for HRPC from the Sf9 insect cell line (data not shown).

3.6. HRP biochemical characterization

Table 2 shows the apparent K_m and V_{max} values as well as the V_{max}/K_m ratios. The K_m of both recombinant HRPC and standard HRP purified from *A. rusticana* roots were comparable. The range of K_m values is typical for peroxidase on substrates like guaiacol [18].

HRP is stable between 20 and 55° C at a pH of 5–10 [18]. Recombinant HRPC derived from larvae or the Sf9 cell line had the same thermal stability behavior as did the standard HRP. At pH 4.5, all enzymes retained 100% activity after a 9 h incubation period at 50 °C.

4. Conclusions

These results provide strong support for *S. frugiperda* larvae as an exceptional host in which to express native HRPC. The larvae have a demonstrated capability to produce active enzyme with high yields. Morawski et al. showed that *P. pastoris* expressed 0.1 mg l^{-1} HRPC with a high degree of glycosylation that negatively affected its specific activity as judged by its kinetic parameters. HRPC expressed in *P. pastoris* showed a smear in the range of 66–100 kDa in a SDS gel indicating the presence of high mannose type oligosaccharides of different grades of polymerization [5]. In contrast, recombinant HRPC from *S. frugiperda* showed a discrete band as observed in Fig. 2 at the correct size (44 kDa). In this case, the oligosaccharide structures were all mannose-terminated consisting in structures with two or three mannose residues with a core fucosylation. The slight difference observed in glycosylation patterns did not affect the enzyme activity. An HRPC expression level of 100 ± 14 mg kg^{-1} was previously reported in intrahemocelically infected *R. nu* larvae [8]. These results suggest that *S. frugiperda* larvae are a more attractive host considering that expression level was 37% higher per kilogram of larvae (137 ± 17 mg HRPC kg^{-1}) and the same amount of protein could be obtained with a smaller number of insects because *S. frugiperda* is 50% larger than *R. nu*. In a typical experiment, the amount of HRPC recovered from a single larvae was approximately 41 μg.

The enzyme was purified in only one step by ion exchange chromatography. This chromatographic mode has well-known economical advantages in comparison to other methods like metal ion affinity chromatography [8]. Recombinant HRPC was similar to the standard enzyme purified from *A. rusticana* roots with regard to thermal stability, kinetic parameters and degree of glycosylation. Therefore, this method of purification of HRPC from *S. frugiperda* larvae is an excellent way to produce a high level of active HRPC for research and other biotechnological applications at low-cost.

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