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Genetically engineered horseradish peroxidase for facilitated purification from baculovirus cultures by cation-exchange chromatography

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Abstract

An engineered horseradish peroxidase isozyme C (HRP C) gene was constructed by the addition of a 6xArg fusion tail to 6xHis–HRP C by the PCR strategy.

The 6xHis–6xArg–HRP C cDNA was expressed in the Sf9 insect cell line from *Spodoptera frugiperda* infected with *Autographa californica* nuclear polyhedrosis virus.

The recombinant peroxidase isoelectric point was 9.5 as judged by isoelectric focusing and was purified directly from the culture medium at day-6 post-infection by cation-exchange chromatography or immobilised metal ion-affinity chromatography. While the former technique gave a yield of 98.5% with a purification factor of 130, the latter gave only a 68% yield with a purification factor of 140.

Results obtained provide evidence that the poly-Arg tag is more effective than the poly-His tag for peroxidase purification from a baculovirus expression system.

Keywords: Peroxidase; Purification; Fusion tail; Ion-exchange chromatography; Immobilised metal ion-affinity chromatography

1. Introduction

Horseradish peroxidase (HRP, EC 1.11.1.7) catalyses the oxidation of a broad variety of substrates by hydrogen peroxide. HRP isozyme C (HRP C) is the archetypal enzyme for the biochemical study of

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peroxidases and is probably, the most extensively studied member of the plant peroxidase superfamily. It consists of 308 amino acid residues, a ferric heme prosthetic group, two calcium ions per molecule and is glycosylated at eight sites of asparagine-linked glycans (Welinder, 1979; Smith et al., 1990; Dunford, 1991; Gray et al., 1998).

HRP C complexity made difficult its expression in prokaryotic systems. On the other hand, its expression in *Saccharomyces cerevisiae* and *Pichia pastoris* rendered low yields (0.6 mg l^{-1}) (Morawski et al., 2000). Baculovirus expression vectors are widely used to produce high levels of recombinant proteins during infection of insect larvae or established insect cell lines. One of the most appealing features of baculovirus–insect expression systems is the eukaryotic protein processing capability of the host. In a previous work, we expressed recombinant HRP C with a 6xHis tag (6xHis–HRP C) with high yield (41.3 mg l^{-1}) in a baculovirus–insect cell system and purified it by immobilised metal ion-affinity chromatography (IMAC) (Segura et al., 2005).

Probably, the 6xHis tail is the most commonly used tail to selectively purify recombinant proteins. However, at an industrial scale, this strategy becomes very expensive because of the matrix cost. Therefore, the growing demand for less expensive purification systems pushes the need to overcome the major drawbacks associated with this fusion tail, mainly the high purification costs and the difficulties in large-scale processes (Kweon et al., 2002). Other fusion tags specifically designed to facilitate protein purification include FLAG peptide, streptavidin, polyaspartic acid, polyarginine, glutathione-*S*-transferase, polyphenylalanine, etc. (La Vallie and McCoy, 1995).

Charged fusions are well-known by their small size and the availability of a variety of inexpensive separation methods based on charge. Brewer and Sassenfeld (1985) engineered the fusion of 6xArg tails to several proteins expressed in *Escherichia coli*, such as β-urogastrone and bacterial aspartate aminotransferase that were readily isolated from cell lysates by ion-exchange chromatography. This purification concept based on polyarginine tail was assayed by Zhang and Glatz (1999) in the canola system but, in this case, proteases degraded the fusion tail.

The aim of this work was to construct a fusion HRP C by adding a 6xArg tail to HRP C containing the 6xHis tail in order to increase its isoelectric point and therefore promote selective binding on cation exchangers directly from a baculovirus–insect cell culture medium. In addition, HRP C with both tails allowed us to compare the performance of IMAC and IEC for purification of said recombinant HRP C.

2. Materials and methods

2.1. Materials

Horseradish peroxidase (type VI), hemin and 3,3′-diaminobenzidine (DAB reagent) were from Sigma–Aldrich (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). *E. coli* strain DH5α was from Facultad de Farmacia y Bioquímica (Buenos Aires, Argentina). *Spodoptera frugiperda* Sf9 cells were obtained from ABAC (Buenos Aires, Argentina). Ni(II)–NTA HiTrap prepacked columns and SP-Sepharose FF High Trap were from Amersham Biosciences (Uppsala, Sweden).

2.2. Strategy for fusion protein construction

An engineered HRP C gene was constructed by addition of a poly-Arg fusion tail to 6xHis–HRP C gene by the PCR strategy. The template was plasmid pAcGP67HRP containing the gene synthesised by British Biotechnologies Ltd., generously provided by Dr. P.E. Ortiz de Montellano of the University of California. The synthetic gene does not include the endogenous 5′ leader sequence found in the plant. The HRP C gene including a 6xHis tag at the 5′ extreme was oriented, so that it could be expressed using the baculovirus polyhedrin promoter. The vector (pAcGP67B, Pharmingen, San Diego, CA, USA) encodes a sequence for the glycoprotein 67 leader peptide at the 5′ end of the multiple cloning site which targets the protein for secretion.

A 72 bp primer encoding the C-terminal region of HRP C was specially designed. This primer included five original codons of the native gene, a thrombin site and a tag of six arginine residues (AGG AGG AGA AGA AGG AGA). The 6xArg–HRP C amplified fragment was purified and cloned in the pGEM–T Easy

vector. JM109 competent cells were used for transformations and different clones were sequenced. The correct product and plasmid pAcGP67HRP were digested with *Sac*I and *Eco*RI restriction endonucleases. The fragments were isolated on a 1% agarose gel and then recovered by column purification and ligated to obtain pAcGPHisArgHRP.

2.3. PCR conditions

The HRP C gene was 1.0 kb double-stranded DNA. PCR products amplified from plasmid pAcGP67HRP with the primer sequences AGT ATG GAT CCA TGC AGT TAA CCC CTA CAT T (forward) and CCG AAT TCA TCG CCG ACG TCG TCT CCT TGA TCC ACG GGG AAC CAG AGA GTT GCT GTT GAC CAC TCT GCA GTT (reverse). PCR conditions (100 μl final volume): 0.8 μM each primer, 1× *Taq* buffer, 0.4 mM each dNTP and 2 U *Taq* polymerase. PCR program: 95 °C for 6 min and 40 cycles, 95 °C for 30 s, 52 °C for 30 s, 72 °C for 1 min. An additional extension step of 10 min at 72 °C was then applied. Free primers from the PCR product were removed by using the Concert™ PCR purification system (Gibco, BRL, Gaithersburg, MD, USA). DNA concentration was 0.4 μg μl^{-1}.

2.4. Recombinant baculovirus construction and amplification

Sf9 cells were co-transfected with 2 μg pAcGPHisArgHRP and 0.5 μg wild linearised BaculoGold™ DNA (Pharmingen) in the presence of calcium phosphate according to Pharmingen (Gruenwald and Heitz, 1993). After a 5-day incubation period at 27 °C, the cell culture supernatant was collected and centrifuged at 2500 × *g* for 5 min. Co-transfection efficiency was determined by the end-point dilution assay according to the supplier's instructions (Pharmingen).

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 monolayer in 15 ml Grace's medium at a multiplicity of infection (MOI) of 0.1. Following two amplification steps, virus titre was determined by a plaque assay. Typically, the titre of this stock was 4.7×10^7 to 1×10^8 pfu ml^{-1}. This amplified virus stock was used at the production step.

2.5. Cell culture

S. frugiperda Sf9 cell cultures were conserved in monolayers in T-flasks at 27 °C in a Grace's medium containing 10% heat-inactivated fetal calf serum and routinely subcultured every 2–3 days. Cells were counted with a haemocytometer and cell viability was assessed by Trypan Blue staining.

2.6. Expression of 6xHis–6xArg–HRP C in Sf9 cells

Infection was performed at a MOI 2 and, at the same time, heme was added at a 2.4 μM final concentration as was optimised as in a previous work (Segura et al., 2005). A negative control without virus was also performed. Plaques were incubated at 27 °C under light protection and, after 6 days post-infection, the culture medium was harvested, cells were separated by centrifugation (1000 × *g*, 10 min) and peroxidase activity was measured in the supernatant.

2.7. Purification of 6xHis–6xArg–HRP C by cation-exchange chromatography

The clarified supernatant brought to pH 8.5 was applied directly on a SP-Sepharose FF HiTrap column (bed volume 1.0 ml), equilibrated with 5 mM Tris–HCl buffer, pH 8.5. After a washing step with equilibration buffer, 1 ml fractions were collected at a linear flow rate of 0.4 cm min^{-1} and monitored by their absorbance at 280 nm and enzyme activity. Elution was performed with 1 M NaCl in the equilibration buffer.

2.8. Purification of 6xHis–6xArg–HRP C by IMAC

The culture supernatant was diafiltered to change the buffer to 25 mM sodium phosphate, 300 mM NaCl, pH 8.0. A Ni(II)–NTA HiTrap column (bed volume 1.0 ml) equilibrated with the same buffer was loaded with 3 ml of the conditioned sample. Following a washing step with the same buffer containing 20 mM imidazole, the elution of the enzyme was performed by raising the imidazole concentration to 500 mM. Flow rate was 0.4 cm min^{-1}, and 1 ml fractions were collected and monitored by their absorbance at 280 nm and enzyme activity.

2.9. Total protein determination

Protein concentration was determined by Bradford assay (1976) using bovine serum albumin as the standard.

2.10. Electrophoretic analysis

SDS-PAGE 12.5% and three to nine range isoelectric focusing (IEF) analyses were carried out with a Phast System Equipment (Amersham Biosciences, Uppsala, Sweden). Gel staining was accomplished using the Coomassie Blue method or employing a selective staining with the DAB reagent to detect active HRP (Segura et al., 2005).

2.11. Enzyme activity measurement

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 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 (1985). For enzyme mass quantitation, a specific activity of pure enzyme of 592.3 U mg^{-1} was assumed.

3. Results and discussion

Recombinant DNA technology was utilised to produce horseradish peroxidase with a double tail. The PCR product was cloned in an appropriate transfer vector (pAcGP67B) under the control of the strong viral polyhedrin promoter. Fig. 1 shows the construction design of the protein. The tail 6xArg is present at the C-terminus and the tail 6xHis at the N-terminus of the original template. This type of genetic construction allows comparison of the performance of purification strategies based on IMAC or ion-exchange chromatography (IEC).

As with 6xHis–HRP C (Segura et al., 2005), Sf9 cells showed a good performance in expressing recombinant 6xHis–6xArg–HRP C. At the day-6 post-infection, culture supernatant was collected and IEF analysis with DAB stain revealed a single discrete band showing peroxidase activity with p*I* 9.5 thus providing

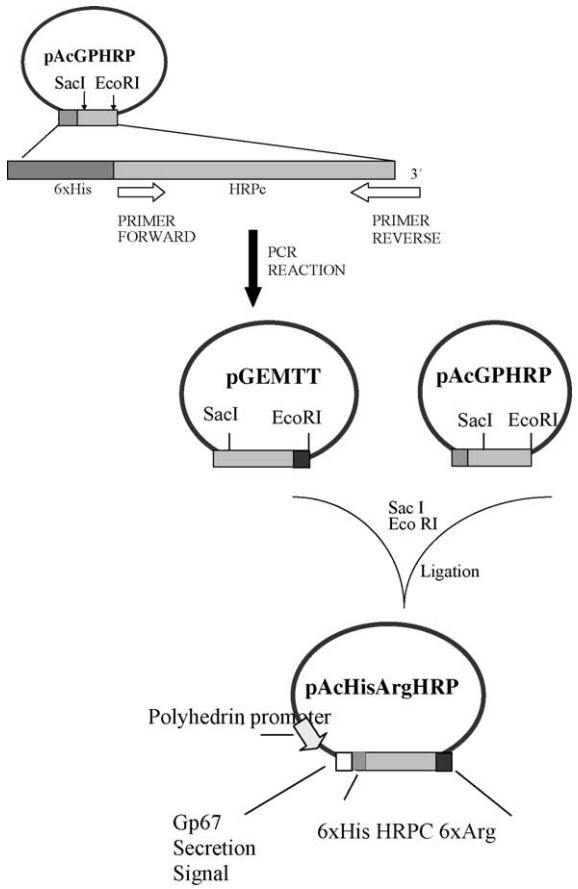

Fig. 1. Construction design of the 6xHis–6xArg–HRP C gene. The PCR product was cloned into an intermediate vector pGEMT easy. The *Sac*I–*Eco*RI restrict containing the gene of 6xArg–HRP C was then cloned into the transfer vector pAcGPHRP6xHis.

evidence on the integrity of the fusion tag as well as on the increased positive charge of the recombinant protein. Fig. 2 shows the isoelectric point increase in comparison with the 6xHis–HRP C molecule expressed in the same insect cell line. As judged by SDS-PAGE, the molecular mass of HRP C with both tags is very close to that of the native enzyme (data not shown).

The kinetics of 6xHis–6xArg–HRP C expression by Sf9 cells infected at different MOIs was very similar to that previously described for 6xHis–HRP C (Segura et al., 2005). Enzyme activity in the harvested supernatant increased continuously until day 7, but day-6 post-infection was chosen as the best

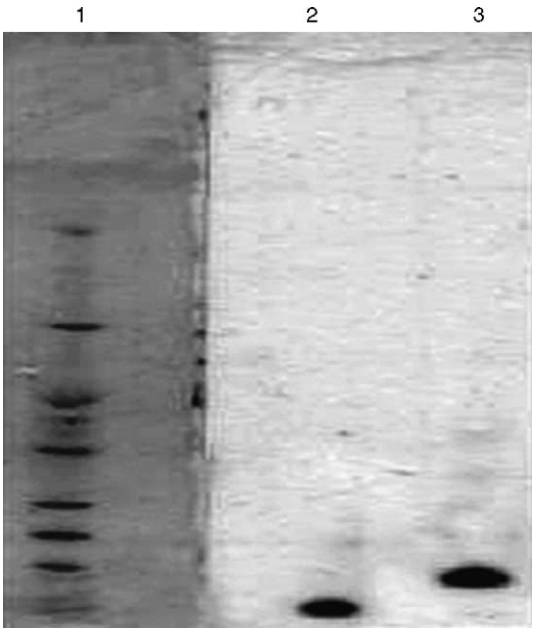

Fig. 2. Isoelectric focusing of 6xHis–6xArg–HRP C in comparison with 6xHis–HRP C. The 4 μl sample was loaded on a Phast System IEF 3–9 gel, and following separation, stained with DAB reagent as described in Section 2. Lane 1, HRP C standard; lane 2, 6xHis–6xArg–HRP C; lane 3, 6xHis–HRP C.

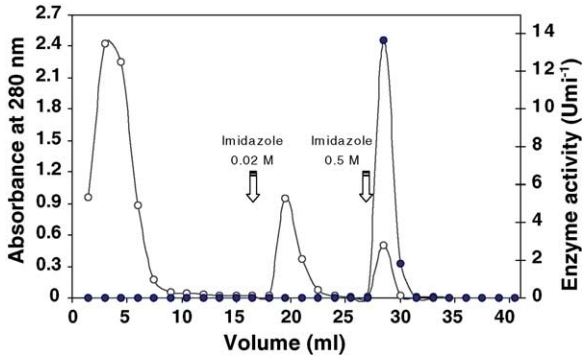

Fig. 3. Purification of 6xHis–6xArg–HRP C by immobilised metal ion-affinity chromatography. After a buffer shift to 25 mM sodium phosphate, 300 mM NaCl, pH 8.0, 3 ml of conditioned culture supernatant was loaded on a Ni(II)–NTA HiTrap column equilibrated with the same buffer. A washing step with the same buffer containing 20 mM imidazole was then performed and elution of the enzyme was carried out by raising the imidazole concentration to 500 mM; 1 ml fractions were collected at a linear flow rate of 0.4 cm min^{-1} and monitored by their absorbance at 280 nm (◯) and enzyme activity (●).

for product collection as it ensures a maximum yield of recombinant protein without significant intracellular protein contamination. The maximum level of active 6xHis–6xArg–HRP C produced in an FCS-supplemented Grace's medium containing 2.4 μM hemin was 30.0 mg l^{-1}.

In order to assess the usefulness of the tags through the performance of IMAC and IEC for enzyme purification from a culture supernatant, chromatographic runs were developed with 6xHis–6xArg–HRP C.

3.1. Purification of 6xHis–6xArg–HRP C by IMAC

The culture supernatant was conditioned by diafiltration to remove amino acids present in high concentrations in the insect cell culture medium as they compete with 6xHis–6xArg–HRP C for the binding sites on the Ni–NTA matrix. On the other hand, the insect culture medium is acidic (pH 6.0–6.5) and the pH must be raised to 8.0 and NaCl concentration up to 300 mM to promote the protein binding to the Ni–NTA matrix. Fig. 3 shows the chromatographic profile. This purification scheme yields 68% of active enzyme with a purification factor of 140.

3.2. Purification of 6xHis–6xArg–HRP C by IEC

To assess the influence of the 6xArg tail on the chromatographic behaviour of the enzyme, ion-exchange purification was performed with 6xHis–6xArg–HRP C and 6xHis–HRP C. When the culture supernatant brought to pH 8.5 was loaded to an ion-exchange column, 6xHis–HRP C was found in the pass-through. In contrast, 6xHis–6xArg–HRP C was retained and only eluted by addition of 1 M NaCl to the adsorption buffer (Fig. 4). Recovery of 6xHis–6xArg–HRP C was 98.5% and the purification factor of 130.

Both purification processes yielded electrophoretically homogeneous peroxidase.

Different codons encoding the arginine amino acid are rare in *E. coli*; therefore, those grouped in clusters should be avoided. It has been found that a long stretch of similar codons decreases the expression level in *E. coli*. Furthermore, clusters of very rare codons can create translation errors and reduce the expression level. On the other hand, polyarginine fusions have proven to be prone to degradation

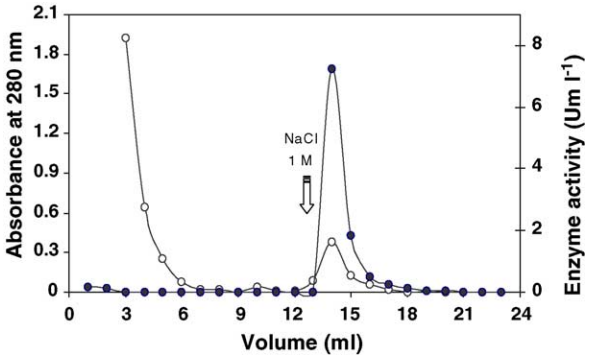

Fig. 4. Purification of 6xHis–6xArg–HRP C by ion-exchange chromatography. Clarified supernatant brought to pH 8.5 was loaded on an SP-Sepharose FF HiTrap column, equilibrated with 5 mM Tris–HCl buffer, pH 8.5. After a washing step with equilibration buffer, elution was performed with 1 M NaCl in the same buffer; 1 ml fractions were collected at a linear flow rate of 0.4 cm min^{-1} and monitored by their absorbance at 280 nm (◯) and enzyme activity (●).

by *E. coli* proteases (Skerra et al., 1991; Niederauer et al., 1994). For these reasons, the strategy of the poly-Arg fusion tag for purification was not effective in prokaryotic systems unless a special *E. coli* strain was used (BL21 (DE3) codon plus, Stratagene). Zhang and Glatz (1999) described a similar method allowing selective recovery of the recombinant T4 lysozyme from canola. Unfortunately, the authors described the presence of proteases that degraded the fusion tag.

In this work, we provide evidence that the 6xArg tag is stable in insect cells and allows us to purify recombinant peroxidase with a high yield by IEC in only one step. The enzyme purified by IMAC displays a similar purity degree but the yield is significantly lower. A culture medium supplemented with FCS was characterised by IEF and results indicate that other proteins of interest with a basic isoelectric point must be easily separated from contaminants by IEC.

4. Conclusion

The addition of a 6xArg tag to 6xHis–HRP C results in an increased p*I* of the product expressed in the baculovirus system. This gives the opportunity for facilitated direct peroxidase recovery in high yield and purity employing inexpensive cation exchangers.

Acknowledgements

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