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Extractive purification of recombinant peroxidase isozyme c from insect larvae in aqueous two-phase systems

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ABSTRACT

Aqueous two-phase systems (ATPSs) have not yet been applied to purify proteins expressed in insect larvae infected by recombinant baculovirus. This work describes the behavior of typical contaminants in the baculovirus-insect larvae expression system such as larval proteins and baculovirus particles in PEG/phosphate ATPSs, in addition to the extraction and purification of the target protein (horseradish peroxidase isozyme C, HRPC).

After assessing the influence of PEG molecular weight, system pH and added salt on the partition constants of HRPC and total protein of a clarified larvae extract, two ATPSs were selected for the first extraction step: PEG 1500/phosphate, pH 7.0 with 4.0% NaCl (System 1) and PEG/phosphate, pH 5.0 in the absence of NaCl (System 2). Both systems were found to be appropriate since a clarified enzyme-enriched top phase was obtained with a yield of 99% and 90% respectively.

The direct partition of larvae homogenized with the components of Systems 1 and 2, yielded a HRPC recovery in top phase of 71.4% and 81.1% respectively, whereas total protein recovery was 5.2% and 3.3% respectively. In both systems, the top phase was clear and particulate material remained in the interphase and the bottom phase. The bulk of immunogenic proteins of the larvae concentrated in the bottom phase of both systems. The PCR assay revealed the presence of viral DNA in both phases.

It was possible to extract the HRPC back from the PEG-rich phase by adding a fresh magnesium sulfate solution to form a new ATPS, achieving a recovery in the bottom phase of 50% and 98% in Systems 1 and 2 respectively, whereas the recovery of total protein was 69% and 24% respectively.

The HRPC global recovery of the two-step processes was 35.4% and 79.6% for Systems 1 and 2, with purification factors of 14.5 and 114.2 respectively. The final product was free of viral particles.

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1. Introduction

Aqueous two-phase partition – a technique described by Albertsson in 1986 – can be used efficiently for the separation and purification of proteins owing to its simplicity, mildness, and high yields [1]. Aqueous two-phase systems (ATPSs) are especially suited for enzyme extraction and purification from biological media, such as animal and plant tissues, since a clarification step is not required. This technique has been used as a primary purification step and – if required – it is followed by a more selective purification step such as chromatography [2].

Polymer molecular weight, polymer and salt concentration, ion strength and composition, and system pH, together with the size, charge and hydrophobicity of the biomolecule, are the main factors influencing the partition coefficient. Factors and mechanisms that causes the uneven distribution of proteins between the two phases are little understood [3–5]. As a consequence, the technique requires experimentation to find the best system for each particular application.

Nowadays, the biotechnology industry demands fast, efficient and economic downstream processes for the recovery and purification of biomolecules. Then, ATPSs are an attractive technology where clarification, concentration, and partial purification can be integrated in only one step. Moreover, this method can be easily scaled up, thus allowing wider biotechnological applications [3,6].

ATPSs have been extensively used to recover recombinant proteins from *Escherichia coli* and yeast and, recently, they have been applied to recover proteins expressed in plant tissues [7–9]. The purification of VLPs from *Spodoptera frugiperda* cells was achieved in ATPSs [4,10]; Benavides et al. [4] reported the purification of recombinant rotavirus-like particles from insect cells by ATPSs. However, there are no references on the use of ATPSs to purify recombinant proteins expressed in insect larvae.

The baculovirus-insect cell system is an excellent eukaryotic expression system for producing complex proteins for biotechnological or pharmaceutical applications [11–13]. Insect cell cultures are widely used because of their susceptibility to *Autographa cali-*

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fornica multiple nucleopolyhedrovirus (AcMNPV), the most commonly used baculovirus expression vector. However, tissue culture techniques are expensive as they need large quantities of culture medium and sophisticated instrumentation. Thus, the scaling-up of protein production using insect larvae (Lepidoptera: Noctuidae) as biofactories is becoming an attractive strategy because the method is simpler and more economical, has lower contamination risk and is less time-consuming than insect cell cultures [14].

Horseradish peroxidase isozyme C (HRPC) from *Armoracia rusticana* roots is an important commercial enzyme with several chemical and biochemical applications, including diagnostics, biocatalysis and biosensors, and has been applied to the bioremediation of polluted soil and water [15,16]. We have recently expressed and purified HRPC as a fusion protein in *S. frugiperda* and *Rachiplusia nu* larvae [17,18]. We reported that, by optimizing some parameters, the amount of HRPC recovered from a single larva of *R. nu* may be as high as 91.8 μg and we have purified the HRPC by ion exchange chromatography with a yield of 72.0% and a purification factor of 45.0 [18]. However, the use of this protein purification technique for insect larvae extracts brings about some drawbacks: the regeneration of the chromatographic matrices is tedious and their half-life decreases considerably due to the complexity of the extracts. Another important factor to control is the melanization process, which begins immediately after the tissue disruption. After the insect larvae are homogenized in a neutral buffer, the homogenate becomes black in 3–5 min due to melanization. This process involves the activation of the serine protease cascade where many proteins and lipids condense into a dark black glue mesh. This seriously impairs the recovery of the recombinant product by interfering with the chromatographic purification step, decreasing its resolution and envenoming the matrix [17,19,20]. In order to prevent the melanization process, the typical protocols include a treatment with reducing agents like glutathione in the extraction buffer [14,17,18].

We have previously studied the partition behavior of HRP from *A. rusticana* roots in different ATPSs [21–25]. Based on that knowledge, some ATPSs were selected for using in this work. Polyethylene glycol (PEG)/salt ATPSs are preferred for protein separation – in batch or continuous operational modes – because of the greater difference in density between the phases, lower viscosity and lower costs, as compared to PEG/dextran ATPSs [6]. PEG/salt systems were selected to assess the purification of recombinant HRPC from the larval extract because they provide stability and are easier to scale up [26]. The distribution of proteins in ATPSs is characterized by their partition coefficient *K*. In practice, it is useful to find systems promoting a differential partition where the difference between the *K* of the product and that of the contaminants is great, i.e. where $K_{PRODUCT} \gg 1$ and $K_{CONTAMINANTS} < 1$.

To our knowledge, ATPSs have not yet been applied to purify proteins expressed in insect larvae infected by recombinant baculovirus. Therefore, the aim of this work was not only to purify target proteins (HRPC), but also to describe the behavior of typical contaminants in the baculovirus-insect larvae expression system such as larval proteins and baculovirus particles.

2. Materials and methods

Horseradish peroxidase (type 8375) and 3,3′-diaminobenzidine (DAB reagent), PEG 600, 1000, 1540, 4000, 6000, magnesium sulfate and NaCl were from Sigma–Aldrich (St. Louis, MO, USA). Guaiacol was from Mallinkrodt Chemical Works (St. Louis, MO, USA). *R. nu* eggs and larvae were obtained from Agidea (Pergamino, Argentina). Mouse polyclonal anti-total larval protein antibodies were prepared in our laboratory. All the other reagents were AR grade.

2.1. Recombinant baculovirus

AcMNPV HRPC polyhedrin-minus (occ-) was previously constructed and maintained in our lab. Briefly, AcMNPV HRPC occ- was obtained by cloning the synthetic HRPC gene into the locus polyhedrin, thus giving an intrahemocelically infective virus. The HRPC gene was cloned downstream the sequence for the glycoprotein 67 leader peptide, which targets the recombinant protein for secretion [14].

2.2. Larval infection

R. nu larvae were obtained from a laboratory colony and reared individually in standard 6-well plates on an artificial high-wheat germ diet [27] at 23–25 °C in a 70% humidified chamber, with a 16:8 photoperiod (L:D). Fourth-instar larvae were injected subcutaneously into the hemocele with 50 μl AcMNPV HRPC occ- (5×10^6 pfu ml^{-1}).

To obtain a clarified larval extract, larvae were harvested 4 days after infection and homogenized in the presence of 2.5 ml 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) per gram of larvae. The samples were centrifuged at 14,000*g* for 10 min at 4 °C. Aliquots of the supernatants were used in trials to find optimum partition conditions.

2.3. Aqueous two-phase systems

ATPSs were prepared according to Albertsson [1]. ATPSs were prepared from concentrated stock solutions: 50% (w/w) PEG 600, 40% (w/w) PEG 1000, 40% (w/w) PEG 1500, 40% (w/w) PEG 4000, 40% (w/w) PEG 6000, 40% (w/w) phosphate buffer (as monosodium and dipotassium mixture) and 5 M NaCl solution. For partitioning experiments, the appropriate stock solutions, distilled water, and a clarified larval extract sample were added in the amount required for 1.55 g ATPS. In all experiments, the phase volume ratio was maintained at approximately 1:1 and the distance to binodial at 0.25 g water/g system. The distance to binodial was obtained by measuring the amount of water (in g) to be added to a 1-g ATPS to change it into a monophasic one and correlates closely with the tie-line length [28]. The components of the ATPS were mixed by gentle agitation and, after 20 min to ensure partition equilibrium, phase separation was accomplished by centrifugation at 1200 *g* for 10 min. Aliquots of each phase were used to measure enzyme activity and protein concentration. *K* values are the average of at least three determinations and the results are expressed as the average ± standard deviation.

The effect of the polymer molecular weight, pH, and NaCl addition on HRPC and total protein partitioning behavior was studied.

2.4. Direct HRPC extraction from larvae

R. nu larvae (1.6 g) were homogenized directly with the components of the selected ATPS in an Omni Mixer Homogenizer (Omni International, Kennesaw, GA, USA) at maximum speed in an ice bath.

2.5. HRPC activity measurement

HRPC activity was measured by assessing the 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 1 ml of 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 [29]. Results are expressed as units (U)

per ml, defined as the amount of enzyme producing a Δabsorbance of 0.302 per minute, at 25 °C. All determinations were performed in triplicate.

The partition coefficient for HRPC (K_{HRPC}) was calculated by dividing the enzyme activity in the top phase by the enzyme activity in the bottom one. All determinations were performed in triplicate.

2.6. Total protein measurement

Protein concentration was measured using the Bradford assay [30] with bovine serum albumin as the standard.

Interference from phase-forming components was avoided by using a 1/10 dilution of each phase. An identical phase system without sample was used as a blank in the spectrophotometric assay.

The partition coefficient for total protein (K_{TP}) was calculated by dividing the total protein concentration in the top phase by the protein concentration in the bottom one. All determinations were performed in triplicate.

2.7. Catalase activity measurement

Catalase activity was measured as described by Aebi [31]: a reaction mixture was prepared containing 770 μl 50 mM potassium phosphate buffer, pH 7.0 and 15 μl 2.6 M hydrogen peroxide. The reaction was initiated by addition of 15 μl sample and the absorbance at 240 nm was recorded. All determinations were performed in triplicate.

2.8. SDS–PAGE

Protein samples from each phase were analyzed by SDS–PAGE on 12.5% polyacrylamide gels stained with Coomassie Blue. For western blot analysis, gels were transferred onto a nitrocellulose membrane and HRPC, baculovirus particles and larval proteins were detected using mouse monoclonal anti-HRP and mouse polyclonal anti-total larval protein antibodies, respectively. AnHRP-conjugated anti-mouse IgG was used as the secondary antibody. For DAB staining, the gel was immersed for 2 min in a 9 mg ml^{-1} DAB aqueous solution with 10 μl of 2.6 M hydrogen peroxide.

2.9. Virus partitioning

Viral DNA was purified with the Real Genomics Viral Nucleic Acid Extraction kit (Real Biotech Corp., Banqiao, Taiwan) from samples of the top and bottom phases. Then, PCR was performed using oligonucleotides designed in our laboratory and synthesized by Operon (Huntsville, AL, USA). These primers were HRPC-forward: TGTCCAACATCGTTCGCGAC and HRPC-reverse: GCATCCTTTTCAGTGCGGAA. The PCR product had an expected length of 160 bp.

3. Results and discussion

HRPC represents 0.09% of the crude extract total protein. Other soluble proteins including enzymes like proteases and catalase, particulate material, recombinant baculovirus and lipids are typical contaminants present in insect larvae extracts. To assess the partitioning behavior of HRPC and its contaminants, different parameters such as PEG molecular weight, pH and NaCl addition were studied in a number of independent experiments.

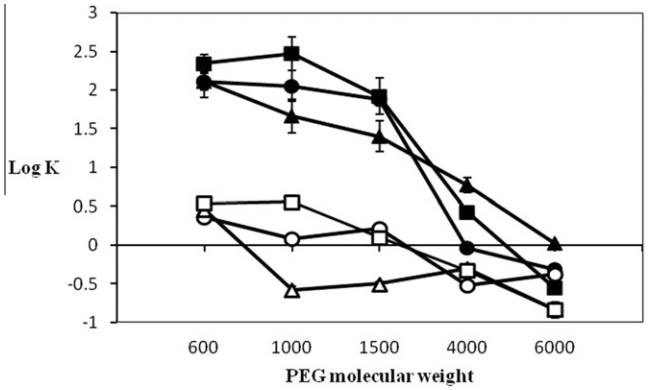

Fig. 1. Effect of PEG molecular weight on the partitioning behavior of HRPC and total protein in various PEG/phosphate systems, at pH 5.0, pH 7.0 and pH 9.0. K_{TP} at pH 5.0 (Δ), pH 7.0 (○) and pH 9.0 (□); K_{HRP} at pH 5.0 (▲), pH 7.0 (●) and pH 9.0 (■). ATPS composition, expressed as% w/w: PEG 600, 25.8 – phosphate, 19.4; PEG 1000, 21.0 – phosphate 12.9; PEG 1500, 21.0 – phosphate 12.9; PEG 4000, 21.0 – phosphate 12.9; PEG 6000, 21.0 – phosphate 12.9. Results are expressed as the average ± standard deviation (n = 3).

3.1. Effect of PEG molecular weight and pH

To evaluate the influence of PEG molecular weight and pH on the partition of HRPC and total protein, clarified larval extract samples were added to ATPS formed by PEG of molecular weights 600, 1000, 1500, 4000 and 6000 and phosphate buffer, at different pHs (5.0, 7.0 and 9.0).

Fig. 1 shows the partition coefficient of HRPC and total protein of the larvae extract in various PEG/phosphate ATPSs prepared with PEG of different molecular weights, at pH 5.0, pH 7.0 and pH 9.0. K_{HRPC} strongly depends on the molecular weight of PEG at pH 7.0. As it increases from 600 to 6000, the partition coefficient decreases from 128 to 0.5. K_{TP} also shows this trend but, in contrast to HRPC, K_{TP} for ATPSs prepared with low molecular weight PEG is not high; with PEG 600, K_{TP} is 2.3, whereas with PEG 6000, K_{TP} is 0.4. The shift of HRPC from the top to the bottom phase with the increase in PEG molecular weight may be explained by the excluded volume effect due to the presence of PEG chains in the top phase [4,32].

The effect of pH on the partition behavior of HRPC was also investigated. The pH modification causes changes in the partition of protein attributed to free-volume effect [4]. We have previously reported that pH have not a significant effect on HRP partition behavior [22]. As expected, the same results were obtained in this case. At pH 5.0, 7.0 and 9.0, the partition behavior of HRPC was similar. Moreover, the profile of contaminants was only modified for PEG of non-extreme molecular weight.

According to the results shown in Fig. 1, the best conditions to achieve differential partition between HRPC and the set of contaminant proteins are those with PEG 1000 and 1500, at pH 5.0 and 7.0. However, although all HRPC is extracted in the top phase, a significant amount of contaminants are also extracted.

In an attempt to improve the separation obtained, NaCl was added to the different ATPSs, at pH 5.0 and 7.0.

3.2. Effect of sodium chloride addition

It is known that the presence of ions has influence on the partition behavior of proteins through the "salting out" effect [1].

Fig. 2 shows the effect of increasing NaCl concentrations on the partition of HRPC and total protein in different ATPSs at pH 7.0 (2A) and 5.0 (2B).

As expected, at pH 7.0 with PEG 600, 1000 and 1500, HRPC stays in the top phase close to 100% at any salt concentration. Whereas,

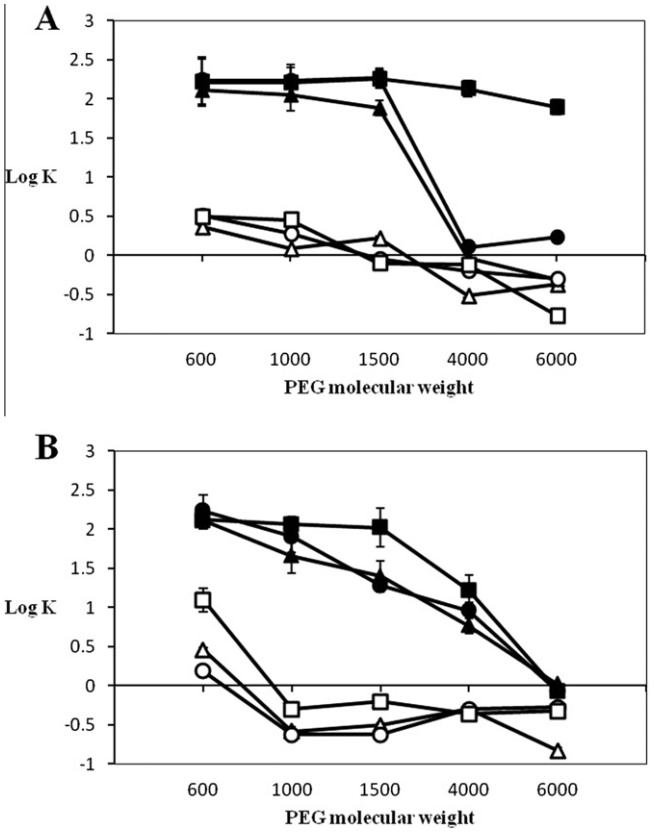

Fig. 2. Effect of NaCl on the partitioning behavior of HRPC and total protein in different PEG/phosphate systems at pH 7.0 (A) and pH 5.0 (B). Fig. 2A: K_{TP} without NaCl (Δ), 2% NaCl (○) or 4% NaCl (□). K_{HRPC} without NaCl (▲), 2% NaCl (●) and 4% NaCl (■). ATPS composition expressed as% w/w: PEG 600, 25.8 – phosphate, 19.4; PEG 1000, 21.0 – phosphate 12.9; PEG 1540, 21.0 – phosphate 12.9; PEG 4000, 21.0 – phosphate 12.9; PEG 6000, 21.0 – phosphate 12.9. Results are expressed as the average ± standard deviation (n = 3). Fig. 2B: K_{TP} without NaCl (Δ), 2% NaCl (○) or 4% NaCl (□). K_{HRPC} without NaCl (▲), 2% NaCl (●) and 4% NaCl (■). ATPS composition expressed as% w/w: PEG 600, 25.8 – phosphate, 19.4; PEG 1000, 21.0 – phosphate 12.9; PEG 1500, 21.0 – phosphate 12.9; PEG 4000, 21.0 – phosphate 12.9; PEG 6000, 21.0 – phosphate 12.9. Results are expressed as the average ± standard deviation (n = 3).

the K_{HRPC} remained high only in the presence of NaCl 4.0% due to the salt highlights the HRPC hydrophobicity. However, the influence of NaCl concentration on K_{TP} is far lower than on K_{HRPC}. With PEG 600 and 1000, proteins prefer the top phase, whereas with PEG 1500, the partition coefficient undergoes only very slight changes around 1. With PEG 4000 and 6000, total protein stays mainly in the bottom phase (Fig. 2A). According to the values of K_{HRPC} and K_{TP}, PEG 4000 or 6000 seemed to be the best option to achieve differential partition. However, despite those results, a considerable amount of HRPC precipitates at the interphase with PEG 4000 and 6000, decreasing the yield by 50%. Such behavior may be explained by the shift of HRPC to the interphase caused by the saturation of free-volume of the top phase by the high NaCl concentration and high PEG molecular weight [4]. This effect is not evident with PEGs of low molecular weight. As a consequence, only PEG 1500 with 4% NaCl was considered for further experimentation as the first step to develop a high-yield process for the direct extraction and purification of HRPC from larvae (System 1A).

At pH 5.0, the addition of salt did not result in an improvement of the selectivity between HRPC and total protein (Fig. 2B). This can be explained because the effect of the salt is opposite to the effect of pH 5.0. Salt drives the contaminants to the top phase meanwhile at pH 5.0 the contaminants are shifted to the bottom phase. Taking this fact into account, the ATPS with PEG 1500 at pH 5.0 in the absence of NaCl was considered another option for the first step in developing a HRPC purification process (System 1B).

The first step of the ATPS purification process, involving HRPC extraction, should provide a top phase with the highest HRPC yield possible. Among the systems studied, PEG 1500, 21.0% w/w-phosphate 12.9% w/w pH 7.0 containing 4% w/w NaCl (System 1) and PEG 1500, 21.0% w/w-phosphate 12.9% w/w pH 5.0 (System 2) were found to be appropriate since an enzyme-enriched top phase was obtained with a yield of 99 and 90% respectively. Although with PEG 1000 at pH 5.0 the K_{HRPC} was higher than that with PEG 1500 (Fig. 1), the recovery of HRPC in top phase with PEG 1000 was lower than with PEG 1500 due to some product precipitates in the interphase of the ATPS.

3.3. Direct HRPC purification process

In order to achieve isolation and partial purification of HRPC directly from crude extracts of insect larvae, the two systems selected for the first step (Systems 1A and 1B) were analyzed and a two-step partition procedure was developed.

The HRPC recovery in top phase by direct extraction in Systems 1A (larvae, 8.4% w/w – PEG 1500, 21.0% w/w – phosphate pH 7.0, 12.9% w/w – NaCl 4.0% w/w; $V_{top\ phase}/V_{bottom\ phase}$: 1.5) and System 1B (larvae, 8.4% w/w – PEG 1500, 21.0% w/w – phosphate pH 5.0, 12.9% w/w; $V_{top\ phase}/V_{bottom\ phase}$: 1.5) was 71.4% and 81.1% respectively, whereas total protein recovery was 5.2% and 3.3% respectively (Table 1). In both systems, the top phase was clear and particulate material remained in the interphase and the bottom phase (Fig. 3A). In some cases, the solubility of the protein in the upper phase is limited and the protein precipitates at the interphase of the system, thus decreasing the yield [33].

Purity of this preparation was assessed by SDS–PAGE. Fig. 4 shows the corresponding pattern. Lane 6 shows the proteins present in the larval crude extract. The bulk of contaminant proteins remained in the bottom phase (lanes 2 and 4) and only a low amount stayed in the top phase (lanes 3 and 5). The major contaminant in top phase is a protein of 26 kDa. On the other hand, HRPC molecular mass was comparable to that of plant enzyme – around 44 kDa – as judged by SDS–PAGE and western blot analysis.

Table 1
Performance of ATPSs direct extraction for HRPC purification from *Rachiplusia nu* larvae.

System	Phase	K_{HRPC}	K_{TP}	Specific activity	Purification factor	$Yield_{HRPC}$ (%)	$Yield_{TP}$ (%)
Larval extract	ND	ND	ND	1.4	1.0	100	100
1A	Top	10.1	0.4	26.5	18.8	71.4	5.2
2A	Bottom	0.9	0.71	21.0	14.5	50 (35.4[a])	69 (3.6[a])
1B	Top	33.3	0.12	31.8	22.7	81.1	3.3
2B	Bottom	0.1	0.71	161.2	114.2	98 (79.6[a])	24 (0.8[a])

Results are the mean of three independent experiments with an estimated error of ±5%.
System 1A: larvae, 8.4% w/w, – PEG 1500, 21.0% w/w – phosphate pH 7.0, 12.9% w/w – NaCl 4.0% w/w.
System 2A: top phase system 1A, 50% w/w – magnesium sulfate, 12.0% – phosphate pH 7, 0.6%.
System 1B: larvae, 8.4% w/w – PEG 1500, 21.0% w/w – phosphate pH 5.0, 12.9.0% w/w.
System 2B: top phase system 2A, 50% w/w – magnesium sulfate, 12.0% – phosphate pH 5.0, 0.6%.
[a] % Global yield.

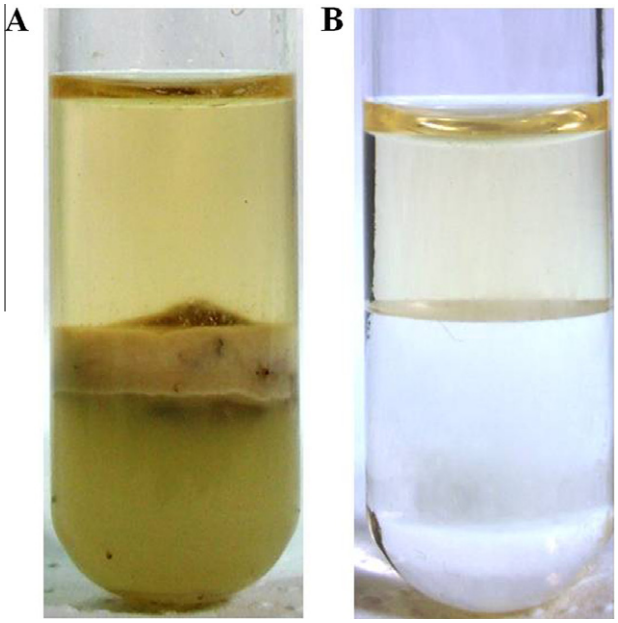

Fig. 3. Direct HRPC purification from crude larval extract. A: System 1B; B: System 2B.

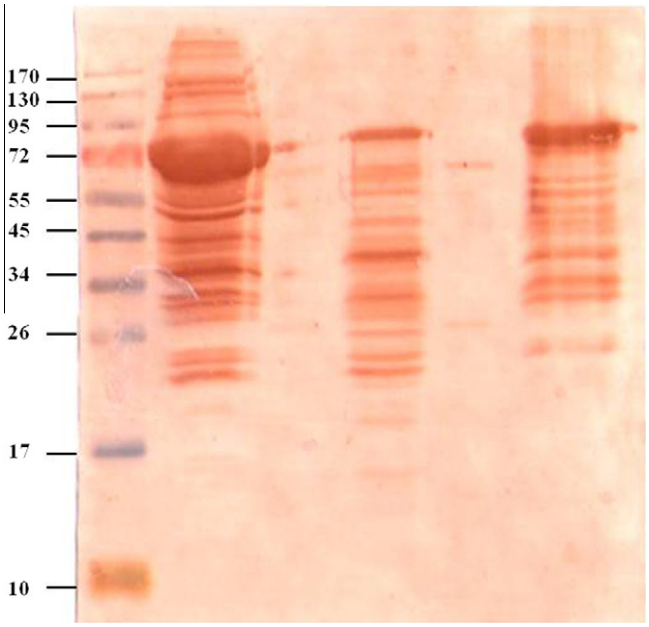

Fig. 5. Western blot analysis of larval protein after partitioning of a larval extract in the ATPS System 1. Lane 1, molecular weight marker; lane 2, larval extract; lane 3, top phase of System 1A; lane 4, bottom phase of System 1A; lane 5, top phase of System 1B; lane 6, bottom phase of System 1B. The western blot was developed with a specific polyclonal antiserum raised against total larval extract.

Catalase is an abundant enzyme in larval extracts and it is a particular contaminant because it competes with HRP for hydrogen peroxide. Catalase partition coefficient was the same for both systems ($K = 0.2$), thus indicating that this contaminant was effectively separated from HRPC in the first step.

Fig. 5 shows a western blot with serum against insect larvae proteins. The top and bottom phases in both ATPSs selected for the first step of the process (System 1A and 1B) system were shown. The bulk of immunogenic proteins of the larvae concentrated in the bottom phase of both systems (lanes 4 and 6). PCR assay revealed the presence of viral DNA in both phases (Fig. 6A). Although the first step allowed separation of the main contaminant proteins present in the larval extract, viral DNA accompanied HRPC in the top phase.

It is important to point out that – in contrast with other methods of clarification where the melanization process has to be especially controlled – in ATPSs this process is inhibited, thus preserving the HRPC integrity through the purification steps. After discarding the bottom phase of the first system, we designed a second system that involves the back-extraction of HRPC by addition of a fresh salt solution (magnesium sulfate) to form a new ATPS. Previous work on the effect of different salts on HRPC behavior in PEG/salt ATPSs evidenced the convenience of using magnesium sulfate for the back extraction of the enzyme as it allows the HRPC concentration in the bottom phase [23].

Table 1 summarizes the results obtained after applying the two-step processes developed with the selected ATPS. The recovery of HRPC in the bottom phase of Systems 2A (top phase system 1A, 50.0% w/w – magnesium sulfate, 12.0% – phosphate pH 7.0, 0.6% – $V_{top\ phase}/V_{bottom\ phase}$: 0.7) and 2B (top phase system 1B, 50.0% w/w – magnesium sulfate, 12.0% – phosphate pH 5.0, 0.6% – V_{top}

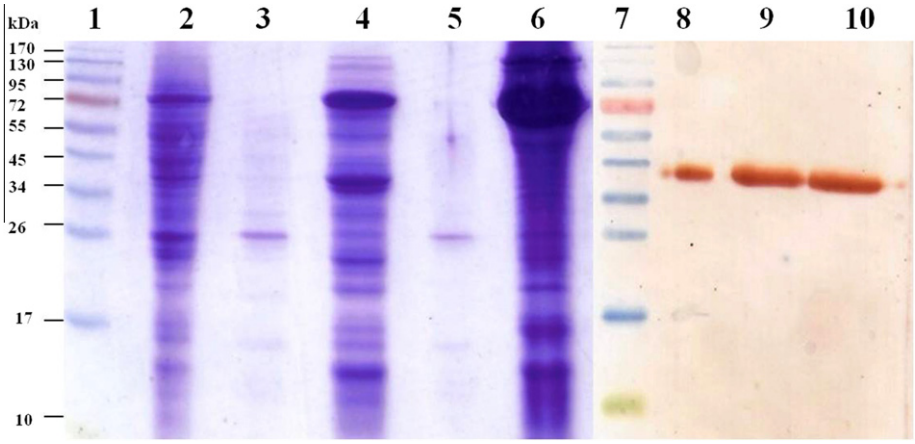

Fig. 4. SDS–PAGE analysis of HRPC and total protein distribution after partitioning of a larval extract in the ATPS System 1. Lanes 1 and 7, molecular weight marker; lane 2, bottom phase of System 1A; lanes 3 and 9, top phase of System 1A; lane 4, bottom phase of System 1B; lanes 5 and 10, top phase of System 1B; lane 6, larval extract; lane 8, standard HRP from *Armoracia rusticana* root. Lanes 2–6 were stained with Coomassie Blue. The western blot (lanes 8–10) was developed with a specific monoclonal antiserum raised against HRP.

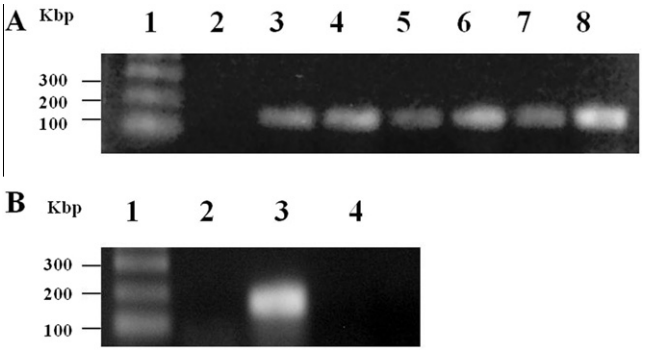

Fig. 6. Virus particle analysis by PCR with a specific primer against 160 pb HRP fragment. A: lane 1, marker 100 Kbp; lane 2, negative control; lane 3, positive control; lane 4, larval extract; lane 5, top phase of System 1B; lane 6, bottom phase of System 1B; lane 7, top phase of System 1A; lane 8, bottom phase of System 1A. B: lane 1, marker 100 Kbp; lane 2, negative control; lane 3, positive control; lane 4, bottom phase of System 2B.

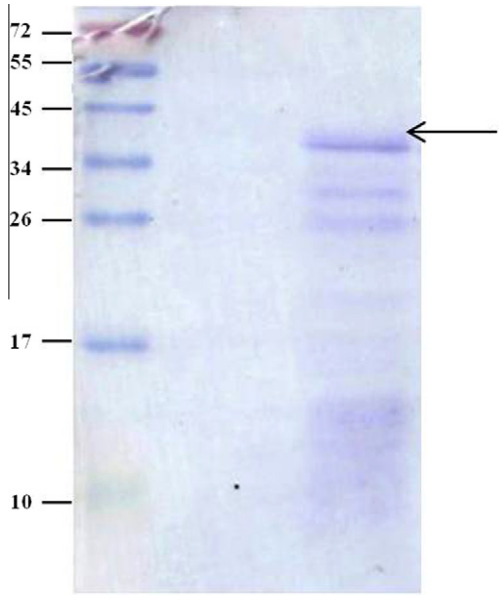

Fig. 7. SDS–PAGE analysis of the HRPC and total protein distribution after partitioning in ATPS System 2. Lane 1, molecular weight marker; lane 2, bottom phase of System 2B; lane 3, bottom phase of System 2B 10×. The arrow indicates the peroxidase band.

$_{phase}/V_{bottom\ phase}$: 0.7) was 50% and 98% respectively, whereas the recovery of total protein was 69% and 24% respectively. As expected, HRPC partitioned in the bottom phase of System 2B.

The yield of HRPC in the bottom phase of System 2A is poorer, probably as a consequence of the salting out effect due to the presence of NaCl. The effect of the magnesium sulfate is opposite to that of the NaCl. Therefore, HRPC preferred the bottom phase with magnesium sulfate alone, while in the presence of NaCl, HRPC partitioned in the top phase. A partially purified HRPC was obtained with System 2B where the purification factor was 114.2 and the global yield of the process was 79.6%. Purity of this preparation was assessed by SDS–PAGE (Fig. 7). The major contaminant present in the first system (Fig. 4), remained in the top phase in the second system. This system provides the possibility of obtaining a clear bottom phase (Fig. 3B), where the HRPC mass represented 10.3% of the total protein and it is free from viral DNA as judged by PCR (Fig. 6B). The values achieved in this paper were those expected for these systems. Rito-Palomares et al. [4] purified dlRLP from a crude extract of disrupted insect cells in ATPS, achieving a yield of 85% with a purification factor of 55. After ATPS separation, the protein represented between 6% and 11% of the total protein.

We have recently reported an efficient purification process of recombinant HRPC from insect larvae based on ion exchange chromatography rendering high yield (80%) and purity (90%). As in the numerous chromatographic schemes for HRPC purification [14,17,18], it is imperative to clarify the larval extract before chromatography. In this case, ATPSs represent an ideal previous step to avoid chromatography column damage when further HRPC purification is required.

4. Conclusions

This paper reports a simple strategy for recovery of recombinant HRPC from *R. nu* larvae. The two-step ATPS process proposed in this work allows the direct extraction and purification of recombinant HRPC directly from a larvae homogenate by mixing it with the phase components. The condition of system B was more advantageous because allowed to extracting HRPC back from the PEG-rich phase by adding a fresh magnesium sulfate solution to form a new ATPS. The second step (2B) increased the purification factor without loss of yield and it allowed separating HRPC from PEG. The recombinant product was obtained with high yield in a clear bottom phase free of virus and critical contaminants such as catalase. In addition, the melanization process is inhibited.

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