Separation and Purification Technology 119 (2013) 1–6

Contents lists available at ScienceDirect

Separation and Purification Technology

journal homepage: www.elsevier.com/locate/seppur

A friendly method for *Raphanus sativus* L (wild radish) peroxidase purification by polyelectrolyte precipitation

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ARTICLE INFO

Article history:
Received 15 May 2013
Received in revised form 28 August 2013
Accepted 29 August 2013
Available online 7 September 2013

Keywords:
Peroxidase
Polyelectrolytes
Eudragit L 100
Eudragit S 100
Purification

ABSTRACT

The separation of radish peroxidase from a fresh *Raphanus sativus* L extract was carried out using precipitation with two commercially available negatively charged synthetic polyelectrolytes: Eudragit® L 100 and Eudragit® S 100. The enzyme was precipitated by polyelectrolyte addition at pH 4.00. The non-soluble complex formed was separated by simple centrifugation and re-dissolved by a pH change. The recovery of radish peroxidase biological activity was 50% of the initial activity in the homogenate for EuL and 45% for EuS, with 1.5-fold increase in its specific activity. The total Eudragit® concentration to precipitate the enzyme was very low: about 2×10^{-3}% w/v. The volume of the final product decreased to 10% of the feedstock, concentrating the sample up to 10 times.

1. Introduction

Peroxidases (EC 1.11.1.*x*; POD) are enzymes that catalyze the H_2O_2-dependent oxidation of a wide variety of substrates, the heme group being essential for their catalytic activity. They are ubiquitous in nature and have been implicated in a broad range of functions [1,2]. These enzymes are commonly grouped into plant and animal peroxidases superfamilies [3] and have been identified in all the higher plants studied. The super family of plant peroxidases includes related heme-containing peroxidases of diverse origins and has been further subdivided into three classes based on cellular localization and function. Class I consists of intracellular enzymes, class II comprises secretory fungal peroxidases and class III contains secretory plant peroxidases, such as those from horseradish (HRP) [3]. Two main groups of PODs have been distinguished, acidic and basic, with a pI ranging from 3.5 to 9.5. The precise role of individual isoforms of peroxidases remains unclear owing to the lack of information on the precise localization of the enzymes and the availability of their specific substrates *in vivo* [4,5]. PODs are widely used for clinical diagnosis and inmunoassays because of its high sensitivity and the large number of reactions it catalyzes [1]. Some applications of PODs have been suggested in the medicinal, chemical and food industries [6]. Other applications include synthesis of various aromatic compounds and removal of peroxide from foodstuff and industrial wastes [7], biotransformation of organic molecules, synthesis of aromatic compounds [6] and recently the combination of POD and indole-3-acetic acid has been introduced as a novel cancer therapy [8].

PODs have been isolated and purified from a number of organisms including bacteria, fungi and higher plants [2,4,9,10]. Although PODs are widely distributed, the main source of commercially available POD is horseradish roots (HPR). On the other hand, availability of PODs with different specificity would promote the development of new analytical methods and potential industrial processes [6].

The typical methods for POD purification used are the precipitation with ammonium sulphate followed by ion-exchange chromatography with salt gradient or size exclusion chromatography [1,5,6,11–13]. The latter is time-consuming and uses ammonium sulphate which cannot be disposed into the environment, being its recycling difficult and expensive. Therefore, it is necessary to develop new environmentally–friendly downstream methods. In the last years, we have been developing scaling up methods based on the formation of a complex between a polyelectrolyte and a protein [14–17]. The ability of natural and synthetic polyelectrolytes to interact with opposite charge proteins forming stable protein–polyelectrolyte complexes is well known [18–20]. This interaction will result in the formation of soluble or insoluble

Abbreviations: POD, peroxidase; HRP, horseradish peroxidase; EuL, Eudragit L 100; EuS, Eudragit S100.

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http://dx.doi.org/10.1016/j.seppur.2013.08.042

complexes depending on parameters such as pH, ionic strength, conformation, charge density and concentration of the biopolymers [21–22]. When insoluble protein–polyelectrolyte complexes are formed, the process could be used as a convenient strategy for the isolation and purification of the target protein.

Raphanus sativus L (wild radish) is a biennale species cultivated in diverse regions of the world. Its cultivation is simple and requires little time and space; it is also resistant to extreme weather conditions.

Eudragit L100 and S100 are copolymers from metacrylic and methylacrylic acids of molecular weight around 135 kD. The structures of EuS and EuL differ only in the extent of the quaternary ammonium substitutions, with EuS containing much less such substitution than EuL [23]. The ratio of the free carboxylic groups to the ester groups is approximately 1:1 in Eudragit type L, and about 1:2 in Eudragit type S. Therefore, Eudragit L polymer is more acidic than Eudragit S polymer [24]. They are soluble at neutral-alkaline pHs, non-toxic and have many applications in the pharmaceutical industry such as drug delivery [25].

In this study, we assayed the capacity of two synthetic poyelectrolytes: Eudragit 100 L and S to precipitate POD from a fresh homogenate of radish roots with the aim of applying it to the isolation of this enzyme in scaling up.

2. Materials and methods

2.1. Chemical

Peroxidase enzyme from horseradish type VI (P8375); Pyrogallol and Bicinchoninic Acid were purchased from Sigma–Aldrich and used without further purification. Eudragit® L100 and Eudragit® S100 were kindly donated by Ethilfarma (Buenos Aires- Argentina). All other reagents were also of analytical grade.

EuL100 and EuS100 were dissolved in 200 mM Tris–HCl buffer, pH 8.20. Buffers of different pH were prepared: 50 mM and 25 mM phosphate buffer, pH 7.00; 100 mM phosphate buffer, pH 6.00; 500 mM acetic acid/acetate buffer pH 4.00 and 200 mM Tris–HCl buffer, pH 8.20. The pH was adjusted with NaOH or HCl in each case.

2.2. Preparation of crude extract

Radish roots (*Raphanus sativus L*) were purchased locally. Radish roots (100 g) were processed in a blender and filtered. The volume of juice obtained was made up to 100 ml with 50 mM phosphate buffer pH 7.00. The resulting extract was divided into aliquots and frozen at −30 °C.

2.3. Peroxidase assay

POD activity was spectrophotometrically monitored by following the oxidation of pyrogallol to purpurogallin. The reaction mixture contained: 2.40 ml of 100 mM phosphate buffer pH 6.00, 300 μL of 5.3% w/v Pyrogallol solution, 200 μL of 0.6% w/w H_2O_2 and an enzyme concentration enough to produce appreciable change in the absorbance at 420 nm between 0 and 90 s. Reaction mixture without the enzyme served as a control. The activities were calculated from the slope of the initial linear portion of the absorbance vs. time curve. Activity is defined in pyrogallol units; one pyrogallol unit will form 1.0 mg of purpurogallin from pyrogallol in 20 s at pH 6.00 at 20 °C [26]. Changes in absorbance of the sample were followed using a Jasco FP520 spectrophotomether with 1 cm of path lenght thermostatized cell. Measurements were taken every 0.1 s and the solution remained under continuous agitation during the measurements.

2.4. Determination of total protein concentration

It was carried out using the bicinchoninic assay [27]. A fresh standard working reagent (SWR) was prepared mixing 100 vol of reagent *A* (Bicinchoninic acid solution purchased from Sigma–aldrich) with 2 vol of reagent *B* ($CuSO_4$ Solution 4% w/v prepared from $CuSO_4{\cdot}5H_2O$). A volume of 50 μL of protein solution (maximum concentration of 1 mg/mL) was added to 1 mL of SWR. The tubes were incubated a 37 °C for 30 min. After leaving them to cool down at room temperature, the absorbance was measured at 562 nm using a 1 cm of path length cell. The calibration curve was performed using dilutions of a standard solution of BSA 1 mg/mL.

2.5. POD purification

All operations were carried out at 25 °C unless otherwise stated. After mixing the crude extract and the polymer the tubes were maintained in stirring 30 min and centrifugated at 2000*g* for 10 min. The supernatant and the precipitate were separated and the precipitate was re-dissolved by the addition of 25 mM phosphate buffer, pH 7.00.

POD enzymatic activity in the supernatant and in the re-dissolved precipitate was measured. Data were transformed according to the following equation:

$$\text{Activity recovered } (\%) = \frac{\text{Activity POD in precipitate (or supernatant)}}{\text{Activity POD control}} \times 100\% \quad (1)$$

In all the cases, the values reported are the mean of at least two independent determinations.

2.5.1. Solubility diagram of POD–Eu mixtures

Aliquots of crude extract (500 μL) at different pHs were mixed with a fixed concentration of polymers. In each case, the crude extract at the corresponding pH served as control. The activity recovered (%) was plotted against pH. These phase diagrams show the pH range where the polymer–protein complex is soluble or insoluble.

2.5.2. Crude extract titration curves with polymer

Aliquots of crude extract (500 μL) at pH 4.00 were titrated with the polymer solution as the titrant (0.20% w/v). To avoid changes in the pH during titration, 50 mM acetic acid/acetate buffer pH 4.00 was added to the crude extract. Complex formation was studied at different ionic strengths adding NaCl, RbCl, LiCl or CsCl to the medium.

Complex formation was followed through a plot of activity recovered (%) vs. polymer concentration. The results were fitted with a four-parameters sigmoidal function or with an exponential function as appropriate. The polymer concentration required for maximum precipitation was calculated from the intersection of the tangent at the inflection point with the plateau of the plot or from the intersection of a straight line which corresponds to the prolongation of the linear zone of the curve (at low polymer concentration) with a line which gives a plateau, respectively.

2.5.3. Kinetics of precipitation

In order to determine the kinetics of aggregation of POD with Eu, a fixed volume of crude extract was mixed with a fixed concentration of polymer and the tubes were maintained in stirring for different times and centrifuged. The data were processed as activity recovered (%) vs. time and adjusted with a simple exponential function.

2.5.4. Effect of temperature on the efficiency of the precipitation

The precipitation was performed in the best conditions determined above at three different temperatures: 0 °C, 25 °C and 40 °C. POD activity and total protein concentration were determined in the supernatant and the re-dissolved precipitate. From these data yields and purification factors were obtained for each condition.

2.5.5. Native and SDS–PAGE

Native PAGE of POD was performed according to Laemmli [28] on 10% nondenaturing polyacrylamide gel, pH 6.00. A sample buffer without SDS and thiol reducing agent was used. A constant power supply of 20 mA was employed. After the run, POD bands were detected by immersing the gel in a solution of 100 mM phosphate buffer pH 6.00, 0.53% w/v Pyrogallol solution and 0.04% w/w H_2O_2. Color development occurred within 5 min.

Enzyme purity and molecular weight were analyzed by SDS–PAGE in a vertical system with 10% resolving and 8% stacking gel. Proteins were stained with coomassie brilliant blue.

3. Results

3.1. Solubility phase diagrams of POD–Eu complex as pH function

Complex formation between POD and EuL or EuS as a function of pH was studied. Fig. 1 shows the pH variation effect on the insoluble complex formation obtained for a constant extract–polymer ratio. In both cases, the formation of complex was observed to be dramatically influenced by the pH of the medium. The increase in pH above 5.00 induced a dramatic decrease in POD recovered activity in the precipitate.

Both polymers acquire negative charges around pH 4.00 where deprotonation of the carboxylic groups begins. PODs are proteins with pI from 3.5 to 9.5 and, from the native electrophoresis performed; we estimated the pI of this particular POD in the range of 5.50–8.00. This is consistent with the range where precipitation occurs: protein–polymers complexes mostly originate from electrostatic interactions between oppositely charged macromolecules [22]. At acidic conditions, proteins have a net positive electrical charge and both polymers have a net negative electrical charge which allows complex formation.

A control curve of POD activity vs. pH extract was made in the same pH range assayed and no effect of the pH in the POD activity was observed. Furthermore, after precipitation the recovered activity in the supernatant and precipitate was measured and we found that polymers did not affect the activity of the protein (data not shown).

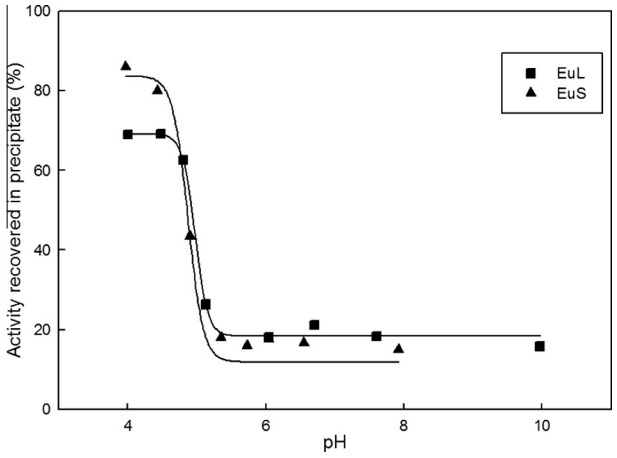

Fig. 1. Solubility phase diagrams of POD-Eu complex as pH function. Medium buffer sodium acetate–phosphate 25 mM. Temperature 25 °C.

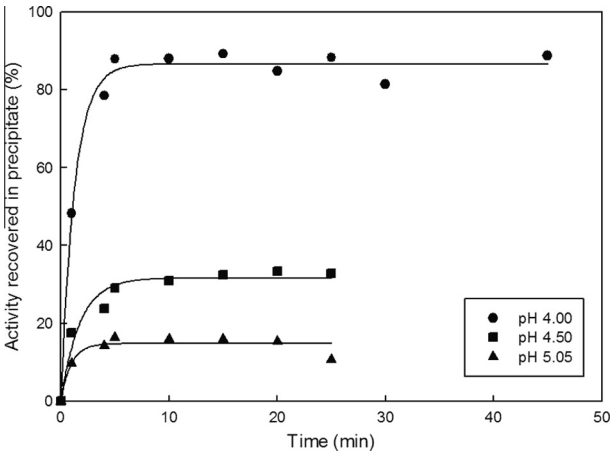

Fig. 2. Kinetics of POD precipitation with EuL (0.05% w/v) vs. time. Medium: 25 mM acetic acid/acetate buffer pHs 4.00, 4.50 and 5.05. Temperature 25 °C.

3.2. Kinetics of POD precipitation with Eudragit

It has been documented [29,30] that the PE–protein complex formation, in some cases, has kinetics of several seconds or minutes. So, the rate of the precipitate formation was assayed measuring the POD activity in the precipitate formed after PE addition. Fig. 2 shows the recovery of POD activity in the re-dissolved precipitated through time at different pH medium values after precipitation with EuL. It could be seen that the major recovery of the enzyme activity was produced (around 80% of the initial activity in the homogenate), at a time around 4–5 min at pH 4.00. At pH

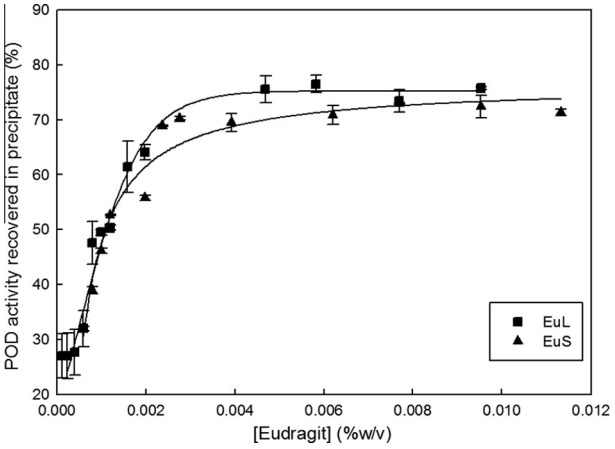

Fig. 3. Titration of crude extract with increasing concentration of EuL or EuS at pH 4.0. Medium: 25 mM acetic acid/acetate buffer, pH 4.00. Crude extract with POD initial activity of 13.84 pyrogallol units/ml and total protein concentration of 2.100 mg/ml.

Table 1
Stoichiometry of POD–Eu complex formation in the presence and absence of different salts.

Salt	Maximun POD activity recovered (%)		[Polymer] (%w/v)	
	EuS	EuL	EuS	EuL
None	72 ± 2	75 ± 2	$1.8e^{-3}$	$1.4e^{-3}$
NaCl	73 ± 2	72 ± 1	$3.1e^{-3}$	$1.5e^{-3}$
LiCl	68 ± 2	76 ± 3	$3.0e^{-3}$	$1.5e^{-3}$
RbCl	65 ± 3	75 ± 4	$3.3e^{-3}$	$1.4e^{-3}$
CsCl	60 ± 4	65 ± 4	$5.0e^{-3}$	$1.5e^{-3}$

4.50 and 5.00, the recorded activity was significantly low in agreement with the formation of low amount of complex as shown in Fig. 1. So, in all the experiments to precipitate the enzyme with EuL, the tubes were incubated 5 min at least, before separating the solid phase.

For the precipitation with EuS, the major recovery of the enzyme is produced at a time around 1 min in all the pH analyzed (data not shown). In this case, the recovery activity was also higher at pH 4.00 and decreased with increasing pH as shown in Fig. 1.

3.3. POD titration curves with polymer

Fig. 3 shows POD activity recovered in the precipitate obtained from the titration curves with EuL and EuS at pH 4.00. This pH was selected because it is the pH of best POD activity recovery. The data were fitted as described in item 2.6.2. A sigmoidal function for EuL and an exponential function for EuS were obtained for the fitting of the experimental data. From these curves, the stoichiometric of protein in extract/polymer ratio was obtained. These values are important because they allow us calculate the minimal polymer amount needed to precipitate the protein present in a extract with a given activity of POD and total protein concentration as shown in Table 1.

In both cases, the polymer concentration required to better obtain the maximal enzyme amount in the precipitation process was extremely small (in the order of 0.0015% p/v). The POD activity recovery in the precipitate was greater with EuL requiring a small quantity of polymer, which is consistent with the higher electrical charge density this polymer has.

The titration was also made in presence of NaCl (data not shown). The non-soluble complex formation was slightly affected by NaCl presence, which is consistent with a partial columbic component present in the complex formation. However, no solubilization of precipitate was observed yet in the presence of 500 mM of NaCl. This last finding is suggesting that the interaction between POD and EuL and *S* to form precipitate is partially of electrostatic nature, other forces may be taking part in the precipitate formation. A similar finding has been reported for the interaction of lipase with polyethyleneimine and chitosan [14].

To elucidate this, the titration curves were carried out in the presence of monovalent cations salts in form of chloride all at the same concentration (0.1 M) as shown in Fig. 4. The data were fitted and the stoichiometric protein in extract/polymer ratio in presence of salts was obtained from the fitting curves (Table 1).

The medium with chloride salts used have the same ionic strength. Therefore, the observed effect may be due to the structure making or breaking effect of the monovalent cations on the ordered water around the hydrophobic chain of polymer or the hydrophobic patches of the enzyme. The Hofmeister series ranks the relative influence of ions on the physical behavior of a wide variety of aqueous processes due to the structure making or breaking effect of the ions on the ordered water.

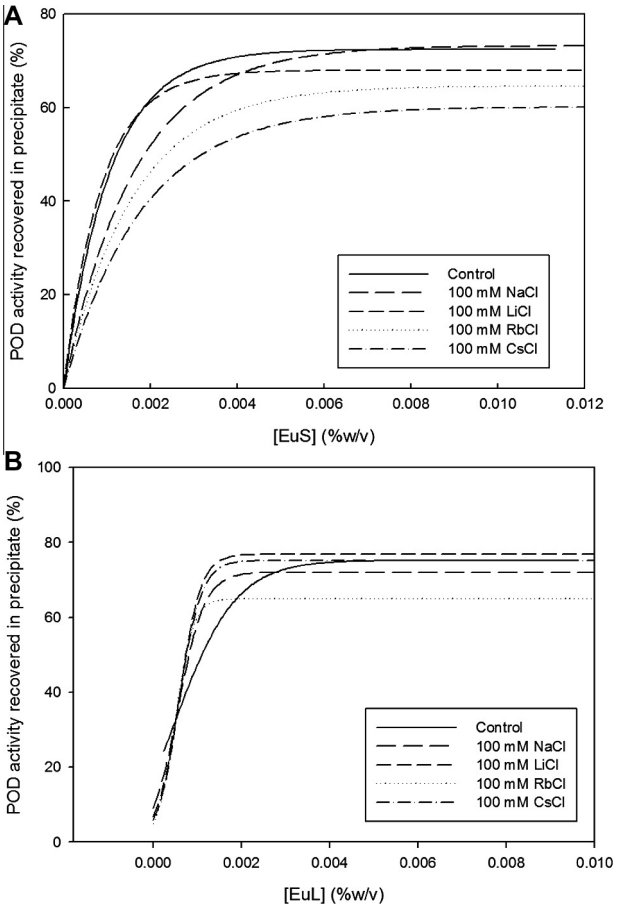

Fig. 4. Titration of crude extract at pH 4.0 in presence of different monovalent salts. All the other experimental conditions are same as Fig. 3. 4A: Titration with EuS. 4B: Titration with EuL. Medium: 25 mM acetic acid/acetate buffer, pH 4.00.

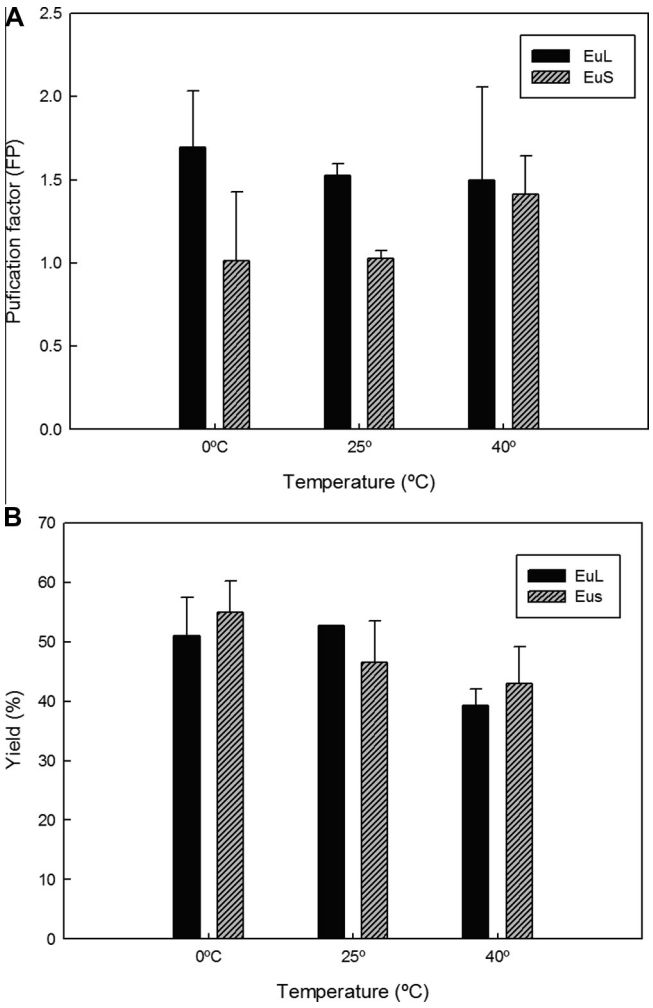

Fig. 5. Temperature effect in purification factor (5A) and yield (5B) of POD precipitation with EuL and EuS. Medium: 25 mM acetic acid/acetate buffer, pH 4.00.

These findings (Table 1 and Fig. 4) suggest that the molecular mechanisms that take part in the complex formation have a hydrophobic nature effect. EuL and EuS behave as amphipathics molecules due to the presence of carboxylic groups and the hydrocarbonate polymer chain. The molecular mechanism of PE–protein previously proposed demonstrated an interaction between the PE chains and the hydrophobic patches of the protein. When this interaction is carried out, a release of ordered water around these hydrophobic zones is produced. Since the presence of cations of the Hofmeister series induces a previous release or gain of this structured water, this has an effect on the protein PE interaction. In our case, we have found that monovalent cations influence on the complex formation, which is a proof of the presence of a hydrophobic effect as a second force.

In conclusion, the PE preferentially interact with charges on the proteins. However, at high salt concentrations there may be hydrophobic interactions playing a role.

3.4. Effect of temperature and final volume on the efficiency of the precipitation

Finally, the data were applied to the precipitation of POD from its natural source. Aliquots of extract at pH 4.00 were precipitated with EuL or EuS in the concentrations shown in Section 3.3. The temperature effect on the purification factor (FP) and on the yield (Y%) of the process was assayed. Three temperatures values were selected because they are the most useful values used in the scaling up production of an enzyme: 0, 25 and 40 °C. The POD activity recovered and the total protein concentration were determined in the supernatant and the re-dissolved precipitate in order to calculate the yields and purifications factors.

Fig. 5 shows the results obtained. When EuL was used for the precipitation, no difference in yield with respect to the two lower temperatures was observed, whereas at 40 °C a decrease in the performance was obtained. The purification factor remained practically constant in the assayed conditions. The decrease in yield may be due to a higher interaction of polyelectrolyte with other proteins since the data obtained does not show variations in the POD activity recovered when varying the temperature but there are variations in the total proteins recovered in the precipitate fractions at different temperatures (data not shown). For EuS, the purification factor was slightly higher at 40 °C. The yield, however, was not affected by temperature change. These findings are important from the biotechnological process point of view, because the precipitation method of POD using Eu is carried out at room temperature, thus avoiding the need for a monitoring system of temperature.

The purity of the enzyme was analyzed by SDS–PAGE after the precipitation. Fig. 6 shows the electrophoresis pattern of extract and re-dissolved precipitate in native and denaturalized media. Fig. 6a shows the SDS–PAGE pattern, the lane corresponding to the re-dissolved precipitates shows a predominant band with a molecular weight of 30–45 KDa, which agrees with the weights reported for POD isolates from various sources [1]. It is to be noted that the amount of protein in the re-dissolved precipitates decreased with respect to the extract. The native electrophoresis showed a single band corresponding to the POD activity (Fig. 6b).

The effect of the final volume where the non-soluble complex was dissolved was also assayed. In the experiment, a constant volume of fresh radish extract (5 ml) was treated with EuS or EuL in the concentration given in 3.3. The mixture was incubated, centrifuged and the precipitate was re-dissolved in a different final volume of 25 mM buffer, pH 7.00. The final volume was varied between 0.5 and 5 ml. The POD activity was measured in each re-dissolved precipitate. The recovered activity was inversely proportional to the final volume. The volume of the final product decreased to 10% of the feedstock, concentrating the sample up to 10 times (data not shown).

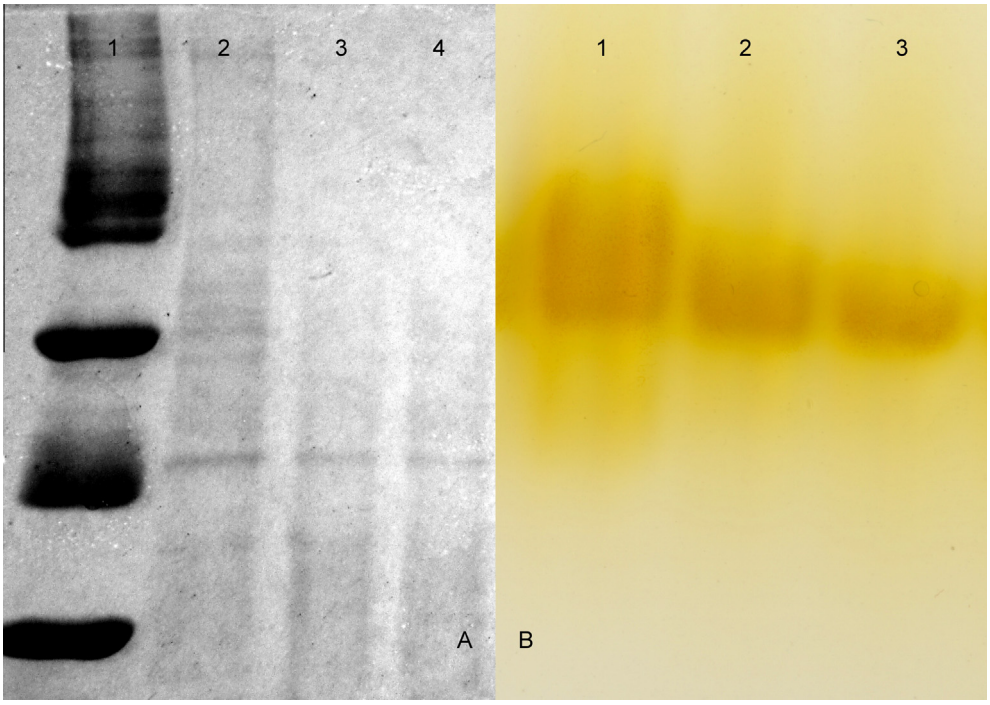

Fig. 6. (A) SDS–polyacrylamide (10%) gel electrophoresis (coomassie blue staining) of the proteins present in the crude extract (second lane); in the redissolved precipitate with EuL (third lane); in the redissolved precipitate with EuS (fourth lane) The molecular mass markers were electrophoresed in the first lane and consist of: Phophorylase b (97 KDa); Albumin (66 KDa); Ovoalbumin (45 KDa); Carbonic anhydrase (30 KDa); Trypsin inhibitor (20.1 KDa). (B) Native PAGE of POD in crude extract (first lane); redissolved precipitate with EuL (second lane) and re-dissolved precipitate with EuS (third lane).

4. Conclusions

In this work, the recovery of POD from radish roots was carried out by means of precipitation with two synthetic polyelectrolytes: Eudragit *S* and *L* 100. Both are anionic, derived from polyacrylated acid and chemically similar, but EuL has a higher electrical charge density than EuS. These polyelectrolytes are commonly used as a matrix for drug delivery. It was found to be effective in precipitating POD from fresh radish homogenate. Precipitation using these PE is more advantageous compared to other classical precipitation method for the isolation of enzymes, like ammonium sulphate addition or the use of non-electrically charged flexible chain polymers such as the polyethylene glycol family, which are not environmentally–friendly.

The results suggest that these PE can precipitate around 50% of the POD from a fresh radish homogenate in only one step (ideally effective in terms of cost and processing time), with a purification factor of around 1.5. This purification factor may be considered low, but as the initial POD concentration in the homogenate is very low this value of purification factor is appropiated. On the one hand the concentration of Eu required for the enzyme precipitation is dramatically low (in the order of 2.10^{-3} w/v) which makes this method economical and environmentally–friendly. On the other hand, these polyelectrolytes did not modify the biological activity of the enzyme at the concentration used to form the complex. EuL proved to be more effective than EuS because it has a larger number of electrically charged groups. Also, this methodology allowed the concentration of the POD activity by reduction of the final volume where the precipitate is dissolved. If necessary, this extract can be further purified by different techniques, including classical chromatography, depending on its final application. The more remarkable advantages of this protocol is that, by reducing the volume and clarifying the sample, the operation and reagent costs of the following steps in the process are reduced.

Acknowledgements

This work was supported by a grant from CONICET PIP00196. N. Woitovich Valetti thanks CONICET for their fellowship. We thank María Robson, Geraldine Raimundo, Mariana De Sanctis and Marcela Culasso for the language correction of the manuscript.

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