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A simple toll for *Raphanus sativus L* peroxidase purification and concentration by precipitation with polyacrylate

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ABSTRACT

We present an economical and environmentally-friendly method in which peroxidase from *Raphanus sativus L* (wild radish) is separated from a crude extract by precipitation with polyacrylate, a commercially available negatively-charged weak polyelectrolyte. Peroxidase was precipitated by adding polyacrylate at pH 4.00 to the extract. Under these conditions, the enzyme is positively charged and forms non-soluble complexes with the negatively charged polyacrylate. The purification factor of peroxidase was 1.8 with a enzyme recovery of 60%. The volume of the final product was decreased to 10% of the initial feedstock in order to concentrate the sample up to 10 times.

KEYWORDS

Raphanus sativus L peroxidase;
Polyacrylate;
Enzyme-polyelectrolyte complex;
Precipitation;
Bioseparation.

INTRODUCTION

The production of proteins by genetically engineered modified microorganisms, yeasts and animal cells became a very important technique for the preparation of pharmaceuticals and other molecules used in Biotechnology. The feedstocks from which proteins are prepared are generally complex, containing solid, soluble and dissolved biomass of various sizes and molecular mass. Enzymes have been traditionally purified from a variety of sources. Current methods of protein purification involve an extensive series of steps and processes that increase the cost of the final product. Bioseparation steps for the recovery of the final product can account for 50 – 80% of overall production costs. New techniques for large-scale protein separation are therefore of interest. One of these involves the addition of polyelectrolytes (PE), leading to selective protein phase separation. Proteins interact with PE to form soluble or non-soluble complexes according to the experimental conditions of the medium[1]. By changing these conditions, such as pH or ionic strength, the protein can be released, keeping its secondary and tertiary structure as well as its biological activity. Synthetic and natural PE have been used to purify proteins from a variety of sources such as chitosan[2], carrageenan[3], poly vinyl sulphonate[4], poly acrylic acid[5].

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. PODs are widely used as

indicator enzymes in enzyme immunoassays, enzyme electrodes, food industries, nucleic acid detection systems, in the cellulose industry to remove hydrogen peroxide excess, as a component in cleaning solutions of contact lenses, etc. Because of its extensive uses in Biotechnology, it is necessary to have a simple and economical method for POD isolation and purification.

PODs have been isolated and purified from a number of organisms including bacteria, fungi and higher plants[6, 9]. Although PODs are widely distributed, the main source of commercially available POD is horseradish roots. 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[10, 13]. It has been reported a purification factor of 9.7 with a low recovery using ammonium sulphate precipitation, dialysis, ion exchange chromatography, hydrophobic interaction chromatograpy and size exclusion chromatography[14]. Obviously these results do not offset the time and cost of the total process. Besides, the salt is difficult to recycle and cannot be discarded in the environment because has a negative impact on it. In addition, its scale-up is difficult and the final cost of the product is high.

Raphanus sativus L (wild radish) is a biennal species grown throughout the world. Its cultivation is simple, requires little time and space and it is resistant to extreme weather conditions.

In this study, we assayed the capacity of a synthetic weak anionic polyelectrolyte polyacrylate (sodium salt) (PAA) 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. Sodium polyacrylate is a non-toxic chemical used in a variety of common products, from diapers to fake snow to tampons and similar female hygiene products[15]. Some polymers derivate from acrylate acid have been registrated as trade mark, as in the case of Eudragit L100 and L50 ®. In this work we intend to use polyacrylic acid which is not protected by any patent, being its use free, on the other hand this polymer is relatively non expensive and non-toxic which adds novelty to the method that we intend to develop.

MATERIALS AND METHODS

Chemical

Pyrogallol, Poly (acrylic acid), M_w 240.000 (PAA), sodium salt, 25% w/w, solution in water, polyethylene glycol average molecular mass 4000 (PEG4000) and dextran average molecular mass 500 kDa (Dx500) were purchased from Sigma-Aldrich and used without further purification. All other reagents were also of analytical grade.

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; 10 mM phosphate-10 mM acetic acid- 0.15 M NaCl buffer; 10 mM phosphate-10 mM acetic acid- 0.3 M NaCl buffer. The pH was adjusted with NaOH or HCl in each case.

Preparation of crude extract

Radish roots (*Raphanus sativus L*) were purchased locally. Radish roots (100g) 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 -30ºC.

Measurements of the enzymatic activity of POD

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 % v/v H_2O_2 and enough enzyme concentration to produce appreciable change in the absorbance at 420 nm between 0 and 90 s. The reaction mixture without the enzyme served as 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 20ºC[16]. Changes in the absorbance of the sample were followed using a Jasco FP520 spectrophotometer with 1 cm of path length thermostatized cell. Measurements were taken every 0.1 s and the solution was kept under continuous agitation during the measurements.

Determination of total protein concentration

Total protein concentration was determined by Warburg-Christian method based in absorption measures at 280 and 260 nm[17].

POD isoelectric pH determination by partitioning in aqueous two phase system

We selected an aqueous two-phase system (ATPs) formed by PEG4000 and Dx500. The ATPs were prepared according to the binodial diagram reported by Walter *et al*[18]. Solid PEG4000 and Dx500 were weighed and dissolved in an appropriate mass of universal buffer formed by 10 mM sodium acetate – 10 mM sodium phosphate. The system was carried out at desired pH by adding small aliquots of NaOH. Several ATPs were obtained with pH ranging from 4.00 to 8.00. The same procedure was used to obtain ATPs with high ionic strength by adding solid NaCl to the buffer. The same amount of crude extract was added to each tube, mixing during 1 hs and the phases separated by low-speed centrifugation. The POD activity was determined in each phase. Also, a blank was performed to correct the possible influence of the phase medium on the enzyme activity. The results were expressed as the POD partition coefficient (Kr), calculated as the POD activity coefficient between the top and bottom phase.

POD precipitation from crude extract

All operations were carried out at 25ºC unless otherwise stated. After mixing the crude extract and the polymer, the tubes were stirred for 30 min and centrifuged 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 cases, the values reported are the mean of at least two independent determinations.

Solubility curves of POD-PAA mixtures

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

Kinetics of precipitation

In order to determine the kinetics of aggregation of POD with PAA, a fixed volume of crude extract was mixed with a fixed concentration of polymer and the tubes were stirred for different time intervals and centrifuged.

Titration of extract with PAA

Aliquots of crude extract (500 µL) at pH 4.00 were titrated with the polymer solution as the titrant (0.05% w/w). 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 different NaCl concentrations.

Complex formation was followed through a plot of activity recovered (%) vs. polymer concentration. The results were fitted with a 4-parameter sigmoidal function. 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.

Effect of temperature on the efficiency of the precipitation

Precipitation was performed at three different temperatures: 5ºC, 25ºC and 40ºC. POD activity and total protein concentration were determined in the supernatant and the re-dissolved precipitate. Yields and purification factors were obtained for each condition from these data.

RESULTS AND DISCUSSION

POD isoelectric pH value determination

Partition of proteins in aqueous two-phase systems is greatly influenced by the ionic composition[18]. Further, partition depends on the charge of

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the protein, thus partition coefficients for proteins are different in different salt media and also vary as a function of pH. The Albertsson equation[19] allows us to explain the partition behavior of a protein as a function of pH and the medium ionic strength, it can be used to determine the isoelectrical pH of a macromolecule as shown equation (2).

$$\ln K = -\ln K^0 - \frac{\mathcal{F}\,\Delta\psi\, Z_P}{RT} \qquad (2)$$

Where: K and K_o are the partition coefficients in the presence and absence of electrostatic effects, $\Delta\psi$ and Zp are the electric interfacial potential and the electrical charge of the protein and ‘ is the Faraday constant. Equation (2) shows that the electrostatic term depends on ionic strength, so its variation induced a variation in K. If the components of both phases do not have charges, as is the case of the system formed by PEG and Dx, the pH and ionic strength of the medium influence on *Zp* and “ψ values, except when Zp = 0 (at the pH value of the isoelectrical point). So the curves K vs pH, obtained at different ionic strengths, intersect at a constant pH, the isoelectric value. Figure 1 shows the partition coefficient of POD vs. pH determined at different NaCl concentrations. Walter and Sasakawa[20] reported that many proteins show a cross partition zone for the curves and not a unique cross point. This may be due to two reasons: the protein has zones with net electrical charge at the isoelectric pH or the protein presents different isoenzymes with different values of isoelectric point. Therefore, the curves cannot cross at the same point, and, as a result, a cross pH zone is obtained. By non- lineal fitting of the curves from Figure 1, it could be concluded that the cross partition pHs are in the interval between 4.61 – 5.52. It has previously reported the presence of four isoenzymes for POD, and our finding shows that the isoelectric pH of them is in this interval.

pH influence on the POD-PAA complex formation

As a starting step to carry out the POD precipitation with PAA, the pH effect on the POD precipitation was studied. Figure 2 shows the results obtained for a constant POD - PAA ratio. The formation of POD-PAA complex is dramatically influenced by the pH of the medium. When pH decreased from 5.00 to lower values, a significant increase in the POD recovered activity in the precipitate was observed, in agreement with the formation of a non-soluble POD-PAA complex. PAA is a synthetic weak anionic PE with a pKa value of 3.5, so the fraction of anion present becomes important at pH 3.50, being totally negatively charged at pH higher than 5.0. The pI of POD is in the range of 4.61-5.52. This is

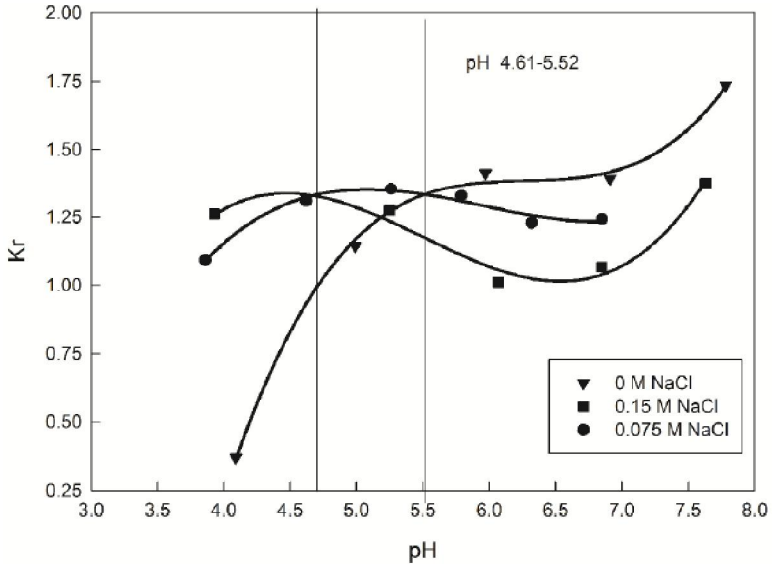

Figure 1 : Dependence of the partition coefficient (K_r) of POD in polyethylene glycol 4000- dextran system at different pH. Medium: universal 10 mM sodium acetate – 10 mM sodium phosphate buffer. Temperature 20 °C. NaCl concentrations: 0 mM (▼), 75 mM (■) and 150 mM (●).

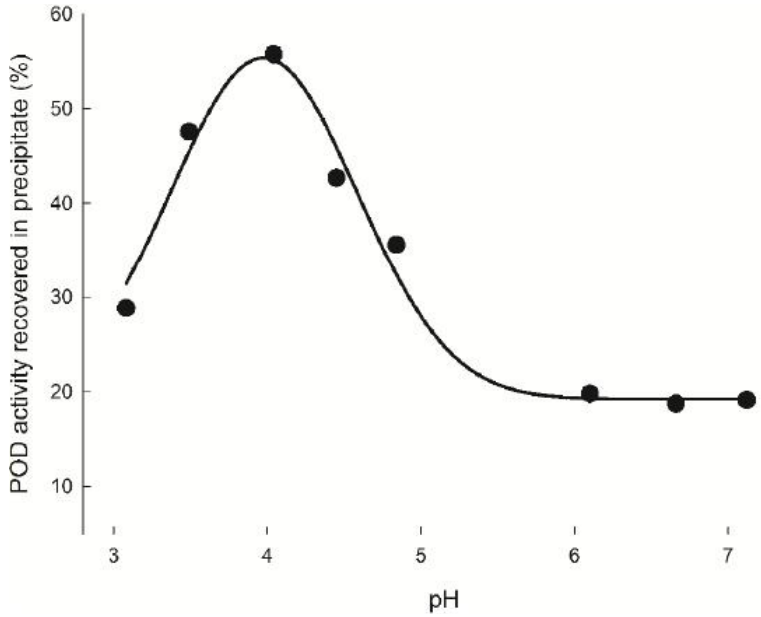

Figure 2 : Solubility phase diagrams of POD-PAA complex as pH function. Medium: 25 Mm sodium acetate-phosphate buffer, Temperature 25°C

consistent with the range where PAA-POD complex formation occurs. The increase of pH above 3.50 induced an increase in the POD recovered activity in the re-dissolved precipitate consistent with the formation de non-soluble POD-PAA complex. In this pH region, the protein and PE have opposite charges and interact between them.

Figure 2 shows that the maximal recovery of POD in the precipitate takes place at pH 4.00, suggesting that at this pH the interaction PAA –POD is maximal. At pH higher than 4.00, the recovered activity significantly decreased, due to a lesser interaction PAA- POD. Above this pH, POD lost its electrical charge because it is the pH zone of the isoelectrical pH of the enzyme. The experimental result suggests that the pH range from 3.00 to 5.00 is the best for the precipitation of the enzyme.

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 (data not shown)

Kinetic of POD–PAA complex formation

The time needed to form the complex was evaluated by measuring the time required to obtain the maximal POD recovered activity in the precipitate. To achieve this, a fixed volume of crude extract was mixed with a fixed concentration of polymer and the tubes were stirred for different time intervals and centrifuged. The experiment showed that only less than 1 min is needed for the complex formation (data not shown).

Titration curves of POD with PAA

Figure 3 shows POD activity recovered in the re-dissolved precipitate and supernatant obtained from the titration curves with PAA at pH 4.00. This pH was selected because is the pH of maximal POD activity recovery. The data were fitted with a 4-parameter sigmoidal function. From this curve, the stoichiometric of POD in extract/polymer ratio was obtained. This value is important because it allows us to know the amount of precipitant (PAA) to obtain the maximal recovery of POD. The PAA minimal concentration required to obtain the maximal enzyme activity in the precipitation process was (7.0 ± 0.6) 10^{-4} % w/w. This amount is extremely small when compared to the values obtained for other PE-protein systems[2, 3, 21] or the traditional proteins precipitants such as inorganic cations and anions[22]. Moreover, from Figure 3, it can be concluded that PAA did not affect the activity of the protein since the sum of the POD activities in the supernatant and the precipitate equaled the initial POD activity (data not shown). Also we assayed the POD activity in the presence of PAA (1. 10^{-3} w/w) at pH 6.0 where

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the complex is soluble and not modification in the enzyme activity was found (data not shown). The titration was also performed in the presence of NaCl 250 mM (Figure 3). The total POD recovered activity was not affected by the presence of NaCl; however, the PAA concentration for maximum precipitation was $(1.2 \pm 0.2)\ 10^{-3}$ % w/w. This value is almost twice that obtained for the precipitation in the absence of salt. This finding suggests a competition between the PAA anion and Cl^- for the positively charged groups in POD, so at increasing concentration of Cl^-, a higher concentration of PAA is required to displace the PAA-POD equilibrium complex formation.

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 PAA in a final concentration of $7.0\ 10^{-4}$ % w/w.

The temperature effect on the purification factor, on the POD activity recovered in PP and on the total protein yield of the process was also assayed. Three temperature values were selected because they are the most useful values used in the scaling-up production of an enzyme: 5, 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 purification factors. TABLE 1 shows the results obtained. The purification factor, POD recovered activity and protein total yield remained constant in the range of assayed temperatures.

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 PAA ($7.00\ 10^{-4}$ % w/w). 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 changed between 0.5 to 5 ml. The POD activity and total protein concentration were measured in each re-dissolved precipitate. Figure 4 shows the

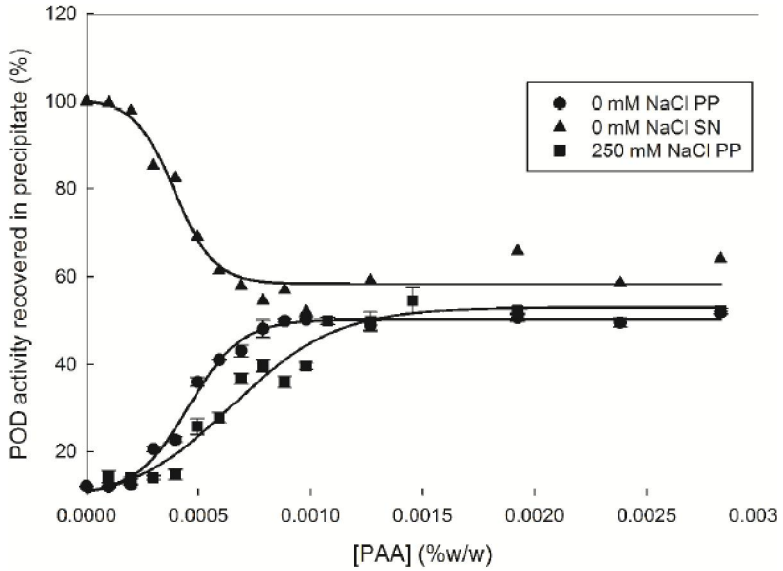

Figure 3 : Recovery of the POD activity in the precipitate (PP) and in the supernatant (SN) at different initial concentrations of precipitant agent (PAA) and different salt concentration. Crude extract with POD initial activity of 30.1 pyrogallol units/ml and total protein concentration of 1.03 mg/ml

TABLE 1 : Purification of POD from *Rhapanus sativus* extract at differents temperature

Temperature (°C)	Purification Factor	POD activity recovered in PP (%)	Total protein yield (%)
5	1.8 ± 0.1	58 ± 4	33 ± 3
25	1.8 ± 0.3	53 ± 1	30 ± 5
40	1.7 ± 0.2	51 ± 7	28 ± 4

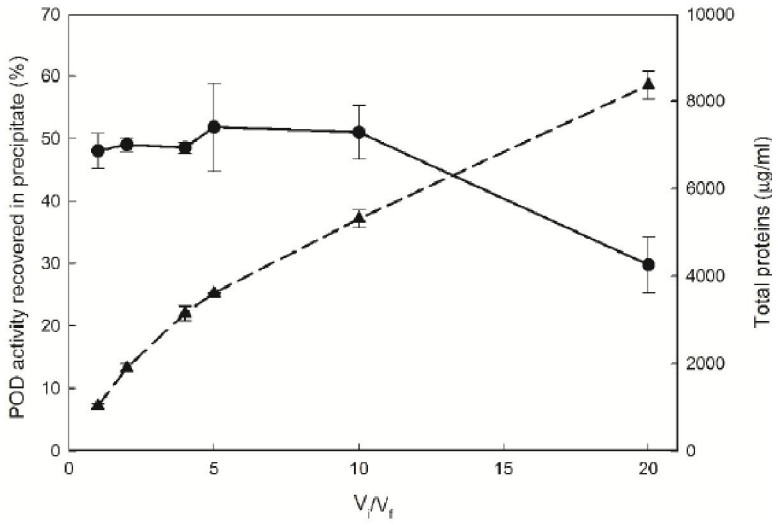

Figure 4 : POD activity and total protein recovered in the re-dissolved precipitate in function of re-dissolution volume. Medium :50 mM phosphate buffer pH 7.00. Temperature : 25°C

results obtained. POD activity recovered remains constant up to a 10-fold decrease in the volume of re-dissolution, and then it begins to decrease, indicating that not all the precipitate was re-dissolved. It is important to point out that activity measures are a standardized way to make them independent of the final volume of the sample. Figure 4 shows that total proteins increase with decreasing volumes of re-dissolution. This finding might suggest an aggregation of the protein in the sense of decreasing the free volume available.

Effect of biomass variation on the efficiency of the precipitation

Finally, the effect of the percentage of biomass present was assayed. We prepared different dilutions of extract (1, 0.75, 0.5 and 0.25) which were treated with PAA in the concentration determined above. POD recovered activity and the total protein concentration were determined in the supernatant and the re-dissolved precipitate in order to calculate the yields and purification factors. The purification factor, POD recovered activity and protein total yield remained constant and independent of biomass percentage (data not shown).

CONCLUSION

Peroxidases are versatile enzymes with a wide variety of uses in industry, medicine, research, etc. The classical methods for POD isolation are tedious and expensive due to the large quantity of reagents that consume; besides, they are not environmentally-friendly.

In a previous work[5], we isolated trypsin and chymotrypsin from fresh pancreas homogenate using precipitation with PAA. In this work, the recovery of POD from a fresh *Raphanus sativus L* homogenate was carried out by means of precipitation with polyacrylate sodium salt. This PE has proved effective in precipitating POD from fresh homogenate. Besides, the isoelectric pH of POD was determined using the POD partition in aqueous two phase systems.

The data about the isoelectric pH of POD reported previously by other authors have great dispersion of values (from 4.0 to 7.5). We think that the isoelectric pH depends on the type of POD and the source from which it was isolated. We determined the isoelectric pH of POD in order to have an idea of its true value. The interval value obtained might be due to the presence of different POD isoenzymes and this value is in agreement with the precipitation results found.

PAA has a net negative electrical charge above pH 3.5, while POD has a positive electrical charge up to pH 4.6. Therefore, in the pH interval 3.5 to 4.6 both molecules interact by appositive electrical charge through coulombic interaction. This hypothesis is in agreement with the experimental result

observed where the major interaction between PAA and POD is at pH 4.0. The interaction of PAA with other enzymes showed to be 100% coulombic, so the increase of the NaCl concentration in the medium decreased the complex formation in a significant way. However, the interaction PAA with POD was not decreased by the presence of NaCl, the only effect observed was a displacement of the titration curve (the curve that shows the affinity of PAA for the enzyme) to high concentration of PAA.

This finding suggests the presence of a highly coulombic interaction between both, which cannot be decreased by the presence of salt. In our case, a high NaCl concentration was not assayed due to a possible enzyme denaturation or precipitation by the presence of salt.

Early papers about the peroxidase purification included a first step of precipitation using ethanol and/or ammonium sulphate with a end concentration of salt around 85% of saturation[23]. Later the isolation method was modificated adding one or two step of ionic exchange and exclusion chromatography[14]. Other authors[24] have proposed a combinated methods, in a first step the homogenated is partitioned in an aqueous two- phase systems formed by polyethylen glycol and potassium phosphate. After this extraction most of the enzyme is concentrated in the bottom phase and this phase is subjected to ultrafiltration. In this case the purification factor increased until 5.9 with a recovery around 90%. However, aqueous to phase systems are not good system to isolate and purificate a protein in scaling up, because the high salt concentration that it uses.

Our finding showed that this negatively charged PE can precipitate around 58% of the POD from the fresh homogenate using only one step (ideally effective in terms of cost and processing time), with a purification factor of around 1.8. This PE did not modify the biological activity of the enzyme at the concentration used to form the complex. Also, this methodology allowed the concentration of POD activity by reduction of the final volume where the precipitate is dissolved in the order of 10 times. The non-effect of a temperature increase in the yield of the process is due to the low enthalpy change associated with the complex formation of different enzymes with polyacrylate, as previously demonstrated[25]. Previous reports[3] have shown that the PE concentration necessary to precipitate enzymes is in the order of 10^{-2} to 10^{-3} %w/w, however, the concentration of PAA necessary to obtain the maximal POD activity was about 10^{-4} %w/w, which is 100 times lower than for the other systems.

The more remarkable advantages of this protocol are that we are reducing the volume and clarifying the fresh homogenate of POD in only one step. Besides this way uses a inexpensive and non-toxic polyelectrolyte.

ABBREVIATIONS

PE; Polyelectrolyte, POD; Peroxidase, PAA; Polyacrylate, PEG; Polyethylene glycol, Dx; Dextran, ATP; Aqueous two-phase system

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