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RESEARCH ARTICLE

Simultaneous purification and immobilization of soybean hull peroxidase with a dye attached to chitosan mini-spheres

Lautaro Fidel Bracco[a,b], Gustavo Javier Levin[a,b,c], Agustín Andrés Navarro del Cañizo[a,b], Federico Javier Wolman[a,b], María Victoria Miranda[a,b] and Osvaldo Cascone[a,b]

[a]Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Buenos Aires, Argentina; [b]NANOBIOTEC, Consejo Nacional de Investigaciones Científicas y Técnicas (UBA-CONICET), Buenos Aires, Argentina; [c]Centro de Investigación y Transferencia de Entre Ríos (CITER) CONICET- UNER, Gualeguaychú, Entre Ríos, Argentina

ABSTRACT

Soybean hull peroxidase (EC 1.11.1.7, SBP) was simultaneously purified and immobilized by dye affinity chromatography with Reactive Blue 4 attached to chitosan mini-spheres. Under optimized conditions, 96% of SBP was adsorbed to the matrix. Under the most stringent condition, only 49% was desorbed, whereas 2 M NaCl failed to desorb a significant amount of SBP. This behaviour allowed proposing the dye matrix as a support to immobilize SBP from a crude extract. The pH of maximum activity shifted from 7 to 3–5. SBP gained thermostability after immobilization: after 5 h at 85 °C, the remaining activity was 54%, whereas that of the free enzyme was 31%. The optimum temperature for the immobilized SBP was 75 °C, whereas that of the free enzyme was 55 °C. After two months at 4 °C, the activity loss of the immobilized SBP was only 3%. Immobilized SBP removed 80% of 2-bromophenol from wastewater in 180 min and, after five cycles of use, the activity loss was only 12.8%.

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Soybean peroxidase; purification; immobilization; affinity chromatography; triazine dyes; bioremediation

Introduction

Peroxidases are extensively distributed in nature and can be extracted from organs and tissues from plant, animals and microorganisms. Plant peroxidases are haeme-containing enzymes that catalyse a large number of oxidative reactions in the presence of hydrogen peroxide (Dunford and Stillman 1976; Gray and Montgomery 1977), being horseradish peroxidase (HRP) the most studied one. HRP has found wide application in many fields, including health sciences and bioremediation. However, horseradish is a rather expensive raw material. In contrast, soybean hull is a by-product of low or null cost from the soybean manufacturing process, with peroxidase accounting for 3% of its total protein content. In addition, peroxidase from soybean hulls (EC 1.11.1.7, SBP) has a wide range of substrates similar to that of HRP.

SBP, either free or immobilized on different supports, has been proposed for different applications, including remediation of wastewater from different industries (Flok et al. 1999; Wright and Nicell 1999), dough bleaching (Gélinas et al. 1998) and bleaching and delignifying of wood pulp (Pokora and Johnson 1998). The advantages of SBP over HRP as the enzyme label in chemiluminescent ELISA kits have been recently demonstrated (Sakharov et al. 2010; Vdovenko et al. 2010).

SBP has been purified by means of different strategies using various chromatographic principles, such as ion exchange, affinity and hydrophobic interaction or a combination of them with or without previous precipitation with ammonium sulphate (Miranda et al. 2002; Sessa and Anderson 1981; Alyas et al. 2002; Habib et al. 2003), partitioning in aqueous two-phase systems (Miranda et al. 1998; da Silva and Franco 2000) and cooperative precipitation with ammonium sulphate and acetone (Liu et al. 2007). Besides, the purified enzyme has been immobilized on various supports, and the advantage in comparison with the free enzyme has been demonstrated. Immobilization is a technique that improves the performance of many enzymes, including peroxidase.

Dye affinity chromatography is a protein purification procedure based on the high affinity of dyes immobilized on a support for the binding sites of many proteins. It is an inexpensive and versatile method that is applicable to purification of proteins in complex raw materials (Baieli et al. 2014). Chitosan can

CONTACT Osvaldo Cascone ocascone@hotmail.com Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 956, 1113 Buenos Aires, Argentina

be used as chromatographic support since it presents several advantages, including low cost, high availability and biodegradability, biocompatibility and nontoxicity (Terbojevich and Muzzarelli 2000; Dutta et al. 2004; Prashanthand and Tharanathan 2007; Wolman et al. 2010). Chitosan mini-spheres can be used as the support for dye attachment, thus giving rise to a cheap affinity matrix. Affinity chromatography was also used to purify peroxidase from other sources, such as white and red cabbage (Somtürk et al. 2014; Erdem et al. 2015); in this case, the affinity ligand was 4-amino benzohydrazide.

The aim of this study was to use chitosan mini-spheres with triazine-attached dyes to purify and immobilize SBP from soybean seed hull aqueous extracts. The use of chitosan mini-spheres as the support of the triazine dyes is an added value that contributes to the low cost of the process.

Materials and methods

Materials

Soybean hulls were kindly donated by Entre Ríos Crushing S.A., Argentina. SBP was generously provided by Bio-Research Products, Inc. (North Liberty, IA). Guaiacol (>98%) was from Mallinkrodt Chemical Works (St. Louis, MO). Chitosan (>99%) from shrimp shells was from Sigma-Aldrich (St. Louis, MO). Epichlorohydrin (>99%) was from Fluka Analytical (Buchs SG, Switzerland). All other reagents were AR grade.

The dyes Reactive Black 5, Reactive Brown 10, Reactive Yellow 2, Reactive Yellow 86, Reactive Blue 5, Reactive Blue 4, Remazol Brilliant Violet 5R (C.I. name: Reactive Violet 5), Reactive Blue 15, Reactive Red 4, Reactive Green 19 and Cibacron Blue F3-GA were from Sigma-Aldrich (St. Louis, MO), whereas the dyes Red 7B-HE (C.I. name: Reactive Red 141), Scarlet G-A (C.I. name: Reactive Red 8), Yellow FR (C.I. name: Mordant Yellow 8), Orange R-HE (C.I. name: Reactive Orange 84), Blue R-HE (C.I. name: Reactive blue 171), Yellow HE-4R (C.I. name: Reactive Yellow 84) and Red HE-3B (C.I. name: Reactive Red 120) were from Vilmax (Buenos Aires, Argentina).

Methods

Preparation of the chromatographic matrix

A library of immobilized triazine dyes on cross-linked chitosan beads was prepared as follows: a 2% chitosan solution in 2% acetic acid with stirring overnight was prepared. The solution was centrifuged at 10,000 rpm for 10 min at 10 °C and dripped through a 15G needle on 2 M NaOH, using compressed air at 1 bar. The beads formed were left in the NaOH solution for 10 h and then washed with distilled water until pH 7.0. For the cross-linking reaction, a 500 mM epichlorohydrin solution was prepared in distilled water and the pH adjusted to 10 with 1 M NaOH. Cross linking was performed at a ratio of 25 mL epichlorohydrin solution per 15 g beads for 4 h at 60 °C. The beads were then washed with distilled water to remove residual epichlorohydrin. The dyes were immobilized on the chitosan mini-spheres by dissolving 400 mg of each dye in 24 mL distilled water per 10 g beads and incubation for 30 min at 60 °C. Then, 10 mL of 20% NaCl was added and, after 30 min at 60 °C, 4 mL of 25% Na_2CO_3 was added and the reaction mix was incubated for 3 h at 60 °C. The matrices were then washed with distilled water, methanol, 2 M NaCl, 6 M urea, 1% Tween 20, 4 h each and then with 0.5 M NaSCN and water overnight. Finally, the matrices were stored in 20% ethanol until use.

Scanning electron microscopy (SEM)

Scanning electron microscopy (SEM, Zeiss Supra 40 microscope) was used to analyse the morphology of chitosan matrices before and after dye coupling. The matrices were lyophilized and metalized with gold (gold sputtering) prior to the analysis.

Extraction of SBP from soybean hulls

SBP was extracted from soybean hulls in a 250-mL flask, with 10 g soybean hulls per 120 mL buffer. The buffer tested was 50 mM sodium phosphate at pH 5.8, 6.5 and 8.0. These three buffers were tested at three different temperatures: 4 °C, 25 °C and 40 °C overnight. Then, the extracts were centrifuged at 10,000 rpm for 30 min at 4 °C and filtered through filter paper.

Affinity screening

For the affinity screening, 10 mg of each different matrix was incubated overnight with 1 mL soybean hull extract at pH 6.5. Then, the supernatants were assayed for SBP activity. The measurements were performed in triplicate and the results expressed as the average ± standard deviation.

SBP activity assay

SBP activity was measured at 470 nm using guaiacol as the chromogen and H_2O_2 as the substrate, in sodium

acetate buffer, pH 5.0, at 25 °C. The absorbance was recorded every 20 s for 1 min and the slope was calculated. For the activity assay of immobilized SBP, 100 mg dye-chitosan mini-spheres was mixed with 10 mL of sodium phosphate buffer, pH 5.0, 35 μL guaiacol under gentle agitation, and the reaction was started by the addition of 100 μL H_2O_2. The absorbance at 470 nm was recorded every 1 min from samples of the supernatant. One unit of peroxidase activity represents the amount of enzyme that catalyses the oxidation of one μmole of guaiacol in one minute, under the conditions of the assay (Tjissen 1985).

Electrophoretic analysis

SDS-PAGE was performed in 12% disc gels, as described by Laemmli (1970). Gels were stained with Coomassie Blue R-250 under standard conditions.

Protein concentration measurement

Protein was quantified by the Bradford method (Bradford 1976), using bovine serum albumin as standard.

Thermostability of immobilized SBP

Thermostability was assessed by the measurement of the remaining enzyme activity after exposure of the dye chitosan mini-spheres at various temperatures between 25 °C and 85 °C, in 50 mM sodium phosphate buffer, pH 6.5, for 5 h. For comparison, free SBP was subjected to the same treatment. The measurements were performed in triplicate and the results expressed as the average ± standard deviation.

pH activity profile of free and immobilized SBP

The activity of free and immobilized SBP was measured at various pHs between 3.0 and 9.0: 50 mM sodium acetate buffer for pH 3.0–5.0, 50 mM sodium phosphate buffer for pH 6.0 and 7.0, and 50 mM Tris-HCl buffer for pH 8.0 and 9.0. The measurements were performed in triplicate and the results expressed as the average ± standard deviation.

Temperature activity profile of free and immobilized SBP

The activity of free and immobilized SBP was measured at 25, 35, 45, 55, 65, 75 and 85 °C. The measurements were performed in triplicate and the results expressed as the average ± standard deviation.

Storage stability of immobilized SBP

The activity of free and immobilized SBP was measured at various time periods of storage at 4 °C in 50 mM sodium phosphate buffer, pH 6.5. The measurements were performed in triplicate and the results expressed as the average ± standard deviation.

Application of immobilized SBP to a decontamination process

Water spiked with 2-bromophenol up to 2 mM was used to assess the decontamination power of the immobilized SBP. The pH of the synthetic wastewater was adjusted to 5.0 using 0.1 M HCl. To initiate peroxidase catalysis, a 1.5-mL Eppendorf tube containing 1.0 mL of wastewater was added with 100 mg of microspheres with immobilized SBP and H_2O_2 up to 2 mM final concentration. As a control, a flask containing 1.0 mL wastewater and no immobilized SBP was run simultaneously. The flasks were kept on an orbital shaker at 70 rpm at 25 °C. Aliquots of the sample (50 μL) were withdrawn after 20, 40, 60, 120 and 180 min to check the 2-bromophenol content of the supernatants with the Folin–Ciocalteu reagent (Singleton et al. 1999). After the treatment, the immobilized SBP was washed thoroughly with assay buffer. The measurements were performed in triplicate and the results expressed as the average ± standard deviation.

Reusability of immobilized SBP

The reusability of immobilized SBP was assessed by determination of the enzyme activity in successive experiments: after each experiment, the matrix was washed with 50 mM sodium phosphate buffer, pH 5.0, to remove any residual substrate and fresh buffer was added to proceed to a new activity determination. This was repeated five times.

Results and discussion

Extraction analysis

Figure 1 shows the results of the extractions performed at the different pHs and temperatures tested. At the three temperatures assayed, the extraction performance was higher at pH 6.5, and at the three pHs assayed, the extraction was higher at 25 °C. Therefore, extraction at 25 °C and pH 6.5 was chosen for further experiments.

Preparation of chitosan mini-spheres

Chitosan is a linear copolymer of residues D-GlcN (2-amino-2-deoxy-β-D-glucose) and D-GlcNAc, with free amino groups exposed, derived of chitin deacetylation. Chitosan has a structure that exhibits functional groups that promote and facilitate the adsorption and immobilization of different ligands and/or biomolecules.

The dyes were covalently attached to chitosan mini-spheres through its amino and hydroxyl groups by aromatic nucleophilic substitution to the carbon of the triazinic ring having the chlorine atom (Baieli et al. 2014). The average diameter of the mini-spheres was 1.87 ± 0.09 mm.

Figure 2 shows the SEM images of the chitosan mini-spheres with and without dye attached, where no evident changes in porosity can be observed.

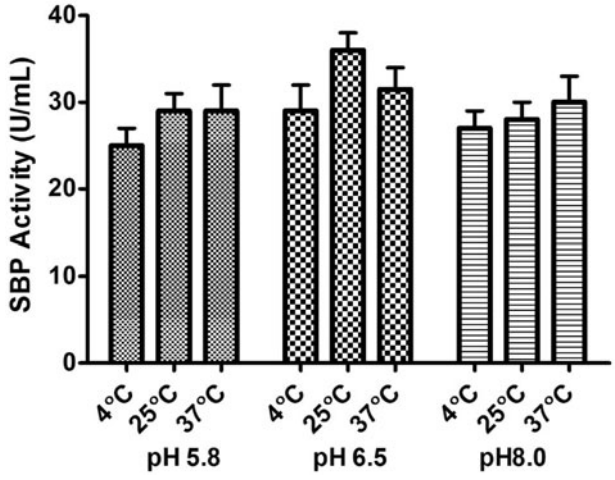

Figure 1. SBP activity (U/mL) from extracts performed at different pH and temperatures. The values are the average of three independent measurements ± standard deviation.

Affinity screening

Nineteen dyes, immobilized on chitosan mini-spheres, were assayed as possible affinity ligands for SBP. Under the conditions described in the Methods section, only three dyes showed SBP adsorption degrees over 60%: Reactive Black 5 (60.7%), Reactive Blue 4 (79.6%) and Reactive Blue 15 (68.9%) (Figure 3). Typical dyes used in affinity chromatography such as Cibacron Blue or Red HE-3B failed to adsorb significant amounts of SBP.

To find the optimal adsorption conditions of SBP on Reactive Blue 4-chitosan mini-spheres, different

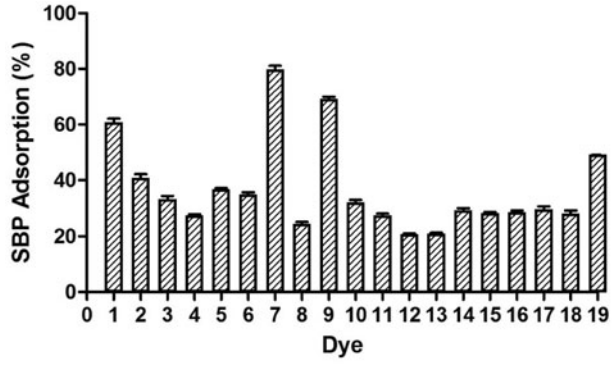

Figure 3. Percentage of SBP binding on covalently anchored triazine dyes on chitosan mini-spheres, at pH 6.5. (1, Reactive Black 5; 2, Reactive Brown 10; 3,Vilmafix Red 7B-HE 150; 4, Reactive Yellow 2; 5, Reactive Yellow 86; 6, Reactive Blue 5; 7, Reactive Blue 4; 8, Remazol Brilliant Violet 5R; 9, Reactive Blue 15; 10, Vilmafix Scarlet GA; 11, Red F5B; 12, Yellow FR; 13, Reactive Red 4; 14, Orange R-HE; 15, Blue R-HE; 16, Yellow 4R-HE; 17, Reactive Green 19; 18, Red HE-3B; 19, Cibacron Blue FG-3A). Results are expressed as a percentage of the initial SBP activity in the extract and are the average of three independent measurements ± standard deviation.

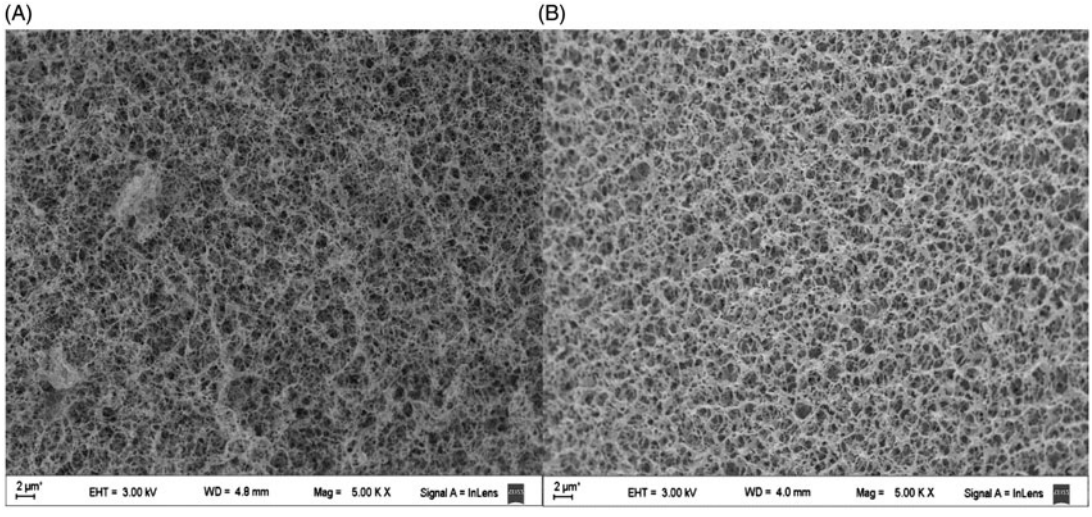

Figure 2. SEM images of the chitosan mini-spheres with (A) and without (B) dye attached, where no evident changes in porosity can be observed.

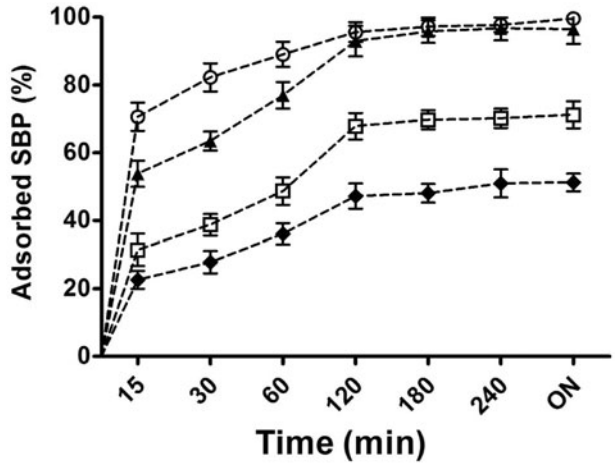

Figure 4. SBP adsorption percentage on Reactive Blue 4-chitosan mini-spheres with different matrix/extract volume ratios at different times. (○), 50 mg/mL; (▲), 25 mg/mL; (□), 10 mg/mL; (◆), 5 mg/mL. Results are expressed as a percentage of the initial SBP activity in the extract and are the average of three independent measurements ± standard deviation.

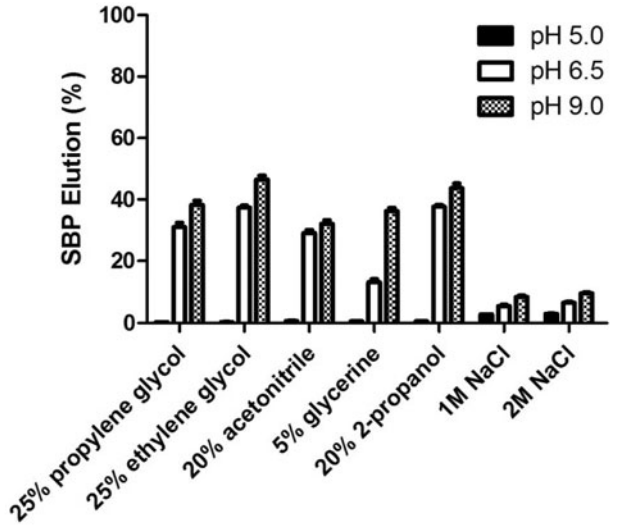

Figure 5. Percentage of SBP elution with different solvent mixtures at pHs 5.0, 6.5 and 9.0. The values are expressed as a percentage of total binding SBP activity and are the average of three independent measurements ± standard deviation.

matrix-extract volume ratios were tested at different times (Figure 4). An adsorption time of 120 min with a 25 mg/mL matrix/extract volume ratio was enough to adsorb 96% of the SBP contained in the extract (Figure 4).

Elution screening

The three dye matrices with high level of SBP binding were saturated with SBP from the extract and then different solvent mixtures were assayed for SBP elution. The solvent mixtures assayed were: 25% ethylene glycol, 25% propylene glycol, 20% acetonitrile, 5% glycerin, 20% 2-propanol, 1 M NaCl and 2 M NaCl, all of them in 50 mM sodium phosphate buffer, pH 6.5 (Figure 5). The three dye-matrices showed similar elution values, and Reactive Blue 4 matrix was thus used for the following SBP purification experiments taking into account its higher adsorption degree.

SBP purification experiments

The solvent mixtures with the highest levels of SBP elution from the Reactive Blue 4-chitosan matrix were tested at pHs 5.0, 6.5 and 9.0. Figure 5 shows the SBP elution percentage, expressed as the released SBP activity respect to the total adsorbed activity. The highest elution values for all the solvent mixtures assayed were achieved at pH 9.0 (Figure 5). According to the results obtained, 25% ethylene glycol in 50 mM sodium phosphate buffer, pH 9.0, was selected as the eluent. The addition of 1 M NaCl to the elution solution brought about a decrease in the elution percentage (47% vs. 25%), thus evidencing that the interaction between SBP and the dye is mainly hydrophobic. This is in accordance with the elution behaviour of 1 M and 2 M NaCl in the elution screening, where NaCl at these concentrations promoted only a minimal elution of SBP from the matrix.

Table 1. Performance of the purification method developed in this work.

Sample	Protein (μg/mL)	Activity (U)	Specific activity (U/mg)	Purification factor	Yield (%)
Extract	546.5	32.0	58.6	1.0	100
Eluted SBP	38.6	15.7	406.7	6.9	49.1

Table 1 shows the purification performance of the process developed under the conditions selected in this work and Figure 6 shows the SDS-PAGE pattern of the eluted enzyme and that of the supernatant and the crude extract, where it can be seen that the enzyme was specifically adsorbed to the matrix and then eluted in pure form.

Other authors have purified SBP by means of different strategies. Liu et al. (2007) reported a precipitation purification scheme that yields SBP of low purity. Gillikin and Graham (1991) prepared homogeneous SBP by means of a method based on ion-exchange, affinity and size-exclusion chromatography. Liu et al. (1998) reported a process involving precipitation, ion-exchange chromatography, affinity chromatography and size-exclusion chromatography that allowed them to purify SBP up to a homogeneous state but did not state the yield. Miranda et al. (1998), da Silva and

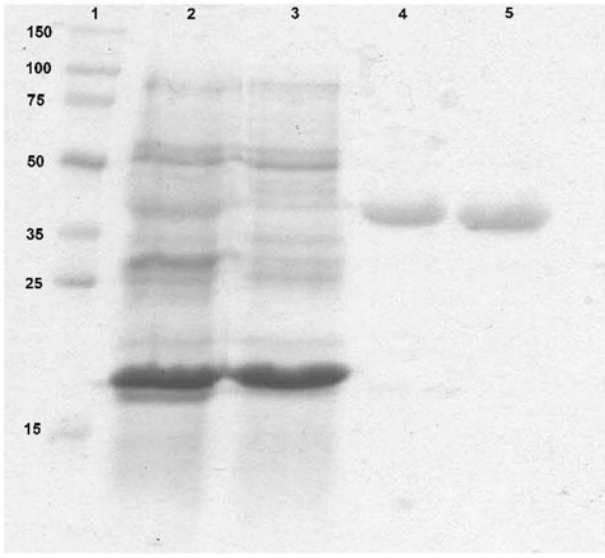

Figure 6. SDS-PAGE of the purified SBP and the crude extract. Lane1, molecular weight marker; lane 2, crude extract; lane 3, supernatant after SBP adsorption; lane 4, eluted SBP; lane 5, standard SBP.

Franco (2000), Sessa and Anderson (1981), Habib et al. (2003) and Paradkar and Dordick (1993) reported yields of 28%, 64%, 4%, 52% and 30%, respectively, but did not report the purity of their SBP final product.

As shown in Table 1, the performance of the process tested in this study was not high enough and does not represent a significant improvement respect to the reported SBP purification processes; however, taking into account the poor SBP elution from the chitosan mini-spheres with the usual eluents, especially with saline buffers, the behaviour of the matrix in immobilization processes was further assayed.

Immobilized SBP characterization

Due to the high SBP adsorption on the Reactive Blue 4-chitosan mini-spheres and very poor SBP elution with saline buffers, this matrix was assayed in immobilization reactions. After SBP adsorption in 50 mM sodium phosphate buffer, pH 6.5, and washing with the same buffer, the behaviour of the immobilized enzyme was compared with that of the free SBP.

Thermostability of immobilized SBP

Figure 7 shows the profile of temperature activity of immobilized SBP compared with that of free SBP. It is evident that SBP gained thermostability after immobilization: after 5 h at 85 °C, the activity of free SBP was 43% lower than that of the immobilized SBP. Taking into account that SBP has an intrinsic thermostability,

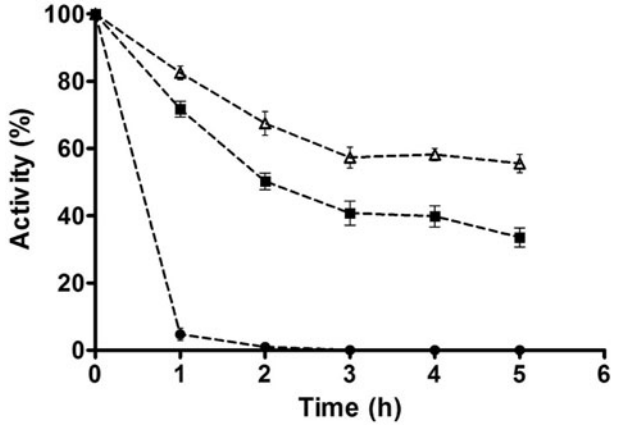

Figure 7. Effect of temperature on the activity of free SBP (■), immobilized SBP (Δ) and free HRP (●) after incubation at 85° C for 5 h. The values are the average of three independent measurements ± standard deviation.

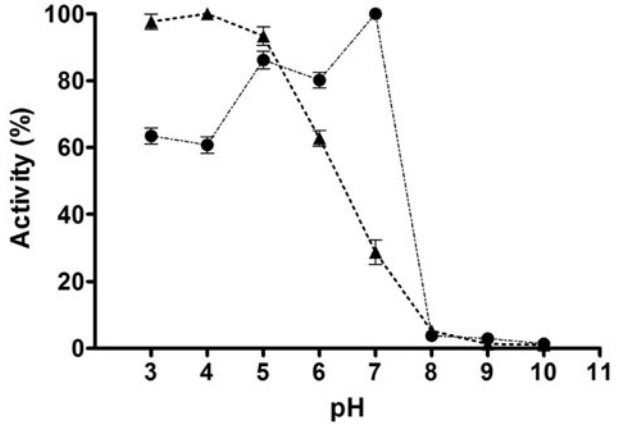

Figure 8. pH-activity profile of free SBP (●) and immobilized SBP (▲), in the pH range of 3–11. The values are the average of three independent measurements ± standard deviation.

the comparison of immobilized SBP with free HRP was still more favourable: after 5 h at 85 °C, the remaining activity of HRP was 0% while that of the immobilized SBP was 54%.

Optimum pH of immobilized SBP

Figure 8 shows the profile of pH activity of immobilized SBP compared with that of the free SBP. The pH of maximum activity shifted from 7 to 3–5, thus allowing the use of the immobilized enzyme in processes carried out at lower pHs.

Optimum temperature of immobilized SBP

Figure 9 shows the profile of temperature-activity of immobilized SBP compared with that of the free SBP. The temperature of maximum activity shifted from

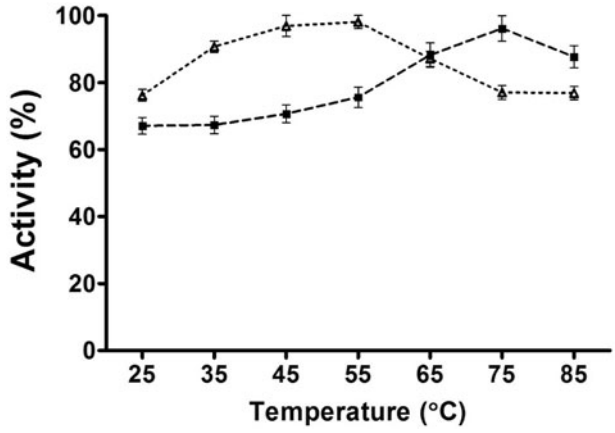

Figure 9. Temperature–activity profile of free SBP (Δ) and immobilized SBP (■), in the range of 25–85 °C. The values are the average of three independent measurements ± standard deviation.

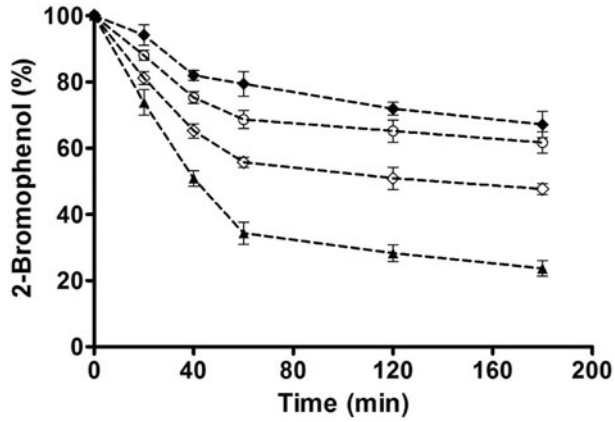

Figure 10. Removal of 2-bromophenol from synthetic wastewater at pHs 4.0 and 7.0. Immobilized SBP at pH 4.0 (▲), free SBP at pH 4.0 (◆), Immobilized SBP at pH 7.0 (◇), free SBP at pH 7.0 (○). The values are the average of three independent measurements ± standard deviation.

45–55 °C to 75 °C, thus allowing the use of the immobilized enzyme in processes carried out at higher temperatures.

Storage stability of immobilized SBP

After two months at 4 °C, the activity of the immobilized SBP decreased only 3%, while that of the free SBP decreased 21%.

Application of immobilized SBP to decontamination of 2-bromophenol in water

Figure 10 shows the performance of free and immobilized SBP in 2-bromophenol decontamination of synthetic wastewater, at pHs 4.0 and 7.0. After 180-min treatment at pH 4.0, the reduction of 2-bromophenol concentration was 80% with immobilized SBP and only 27% with free SBP. At pH 7.0, the reduction of 2-bromophenol concentration was 52% and 32%, respectively.

Reusability of immobilized SBP

SBP activity decreased only 12.8% after five cycles of utilization, thus ensuring an adequate operational stability of the matrix.

SBP has been previously immobilized on different supports: glass with gold nanoparticles (Aina et al. 2011), glycidyl methacrylate (Prokopijevic et al. 2014), aminopropyl glass (Marchis et al. 2012), aldehyde glass (Bódalo et al. 2008), activated carbon (Zheng and Jiang 2014), polyaniline (Magri et al. 2005), soybean hulls (Magri et al. 2007) and sol-gel (Wang et al. 1999; Trivedi et al. 2006). In general, all the reports indicate that SBP immobilization brings about a higher thermostability, a shift of optimum pH and optimum temperature and the possibility of reutilization for various cycles. In all reports, both the thermostability and the possibility of reutilization of SBP increase regardless of the support used, whereas the temperature and pH of optimum activity differ according to the support used for SBP immobilization. Magri et al. (2005) reported no shift in optimum activity pH after immobilization of SBP on PANIG and a shift from 65 °C to 55 °C in the optimum activity temperature. Prokopijevic et al. (2014) also reported no shift in optimum activity pH by SBP immobilization on macroporous glycidyl methacrylates with different surface characteristics. Magri et al. (2007) reported a shift in pH from 4 to 6 after immobilization of SBP on soybean hulls, whereas Marchis et al. (2012) reported a small shift from 5.8 to 6.5 as a consequence of SBP immobilization in aminopropyl glass. Probably, the orientation of the molecule of SBP must be different depending on the immobilization pathway, thus bringing about some differences in the properties of the immobilized enzyme.

Chitosan has been used as the support for immobilization of various enzymes, including HRP (Bindhu and Abraham 2003). A pioneer work of Cetinus et al. (2007) reported the possibility of immobilizing the enzyme catalase on chitosan beads with the triazine dye Cibacron Blue F3GA, with excellent results. However, although Cibacron Blue F3GA is a very used dye in affinity chromatography, its selectivity is not high. As in all previous reports, Cetinus et al. (2007) had to purify catalase prior to immobilization. In our case – thanks to the specificity of Reactive Blue 4 for SBP – purification and immobilization of the enzyme were simultaneously achieved in a single step, and

immobilized peroxidases (HRP and SBP) were used for the removal of pollutants in wastewater (Gómez et al. 2009).

Under the derivatization conditions, no leaching of dye was detected after three washings or when the matrix was incubated with soybean hulls extract. Therefore, we concluded the absence of environmental issues.

Conclusions

Derivatization of chitosan mini-spheres provides a novel matrix for the purification and immobilization of soybean hull peroxidase in only one step. Even though the yield of the SBP purification was only 50% mainly due to the elution step with stringent eluents, the chitosan-Reactive Blue 4 showed an excellent possibility to immobilize an enzyme to a matrix directly from a crude extract of soybean hulls, where the triazine dye can selectively bind to that enzyme. In most immobilization reports, the enzyme had to be purified before immobilization. Our results also suggest that the orientation of the molecule of SBP must be different depending on the immobilization pathway, thus bringing about some differences in the properties of the immobilized enzyme.

Disclosure statement

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GJI, FJW, MVM and OC are career researchers of the CONICET. LFB is a fellow of the CONICET.

No potential conflict of interest was reported by the authors.

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