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Covalent immobilization of soybean seed hull urease on chitosan mini-spheres and the impact on their properties

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ABSTRACT

Urease (EC 3.5.1.5) is found in bacteria, fungi, algae, plants and some invertebrates, and in soils. Soil ureases are partly extracellular being liberated during microbial and plant root metabolism and death. In plants, urease is present in most of Leguminosae seeds, accounting for 0.2–0.3% of the extractable soybean seed protein, being also present in the hulls, in lesser amounts. Soybean hull is a low-cost agro-industrial by-product from the soybean manufacturing process. In a way of exploiting its possible applications, urease was covalently immobilized on thiolated chitosan mini-spheres through its sulphydryl groups. Almost quantitative (98%) immobilization from a crude extract of soybean seed hull was obtained. After 4 h at 70 °C, the activity of free soybean hull urease (SBU) was 50% lower than that of the immobilized enzyme. The pH of maximum activity shifted from 7 to 5 after immobilization, thus allowing its use in processes performed at acid pH (pH 3–5). The immobilization brought about an increase in the optimum activity temperature from 65 °C to 75 °C. An 84% of the activity of the immobilized SBU was retained after 25 cycles of utilization, and the activity was maintained after 90 days at 4 °C.

1. Introduction

Urease catalyzes the hydrolysis of urea into ammonia and carbamate, which is spontaneously decomposed to form carbon dioxide and a second molecule of ammonia (Real-Guerra et al., 2013), at a rate of 1014 times faster than the non-catalyzed reaction (Jabri et al., 1995). This enzyme is found in bacteria, fungi, algae, plants and some invertebrates, and in soils. Soil ureases are partly extracellular being liberated during microbial and plant root metabolism and death (Dharmakeerthi and Thenabadu, 1996). Ureases are members of amidohydrolase and phosphotriesterases families, which have active metal (s) in their active sites: two Ni2+ ions in their active sites in the case of ureases. The number of polypeptide chains that form the monomer or functional unit is formed by different numbers of polypeptide chains, and it varies according to the source of urease. In plant and fungal the functional unit is a single polypeptide chain (α), the most common structure being a dimer of trimers (α3)2 (Kappaun et al., 2008). Although originally reported to be urea-specific, nowadays many urease substrates are known which, yet, are hydrolyzed at a much lower rate than urea, such as hydroxyurea, dihydroxyurea, semicarbazide, formamide, acetamide, thiourea, methylurea, ethylurea, ethyl carbamate, etc. (Krajewska, 2009). Additionally, many inhibitors have been reported for ureases, such as hydroxamic acids, phosphorous compound, phosphate, fluoride, polyphenols, heavy metals, etc. (Kappaun et al., 2008).

On the other hand, urease was the first example of the presence of sulfhydryl groups in a protein (Summer, 1951) and of a nickel metalloenzyme (Dixon et al., 1975). Urease allows the organisms to utilize external and internal urea as a nitrogen source. Then ammonia can be taken up and used by soil microbes and plants, which allows the utilization of urea as a fertilizer, in addition to its low cost and high nitrogen content (Qin and Cabral, 2002).

Urease is a protein present in most of Leguminosae seeds. Takeuchi et al. found in 1909 the first evidence of the presence of urease in

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higher plants, when observed that crude extracts of soybean (*Glycine max*) seeds contain high amounts of this protein. Until that moment, urease had been found only in microorganisms and in algae (Real-Guerra et al., 2013). Urease accounts for 0.2%–0.3% of the extractable soybean seed protein (Polacco et al., 1982), being also present in the hulls, in lesser amounts. In contrast to jack bean urease (JBU), soybean hull is a low-cost agro-industrial by-product from the soybean manufacturing process.

Soybeans worldwide production is forecasted up at 369.3 million tons. Since soybean seed hull represents about 5% of the whole seed weight, 18.5 millon tons of soybean seed hull will be produced in this season. Most of this by-product is used to feed cattle, taking advantage of its high assimilable fiber content. However, valuable proteins, like SBU, could be extracted from hulls prior to pelletizing for feed cattle.

Many are the possible applications of urease: analysis of urea, creatinine and arginine concentrations in serum and in wastewater, analysis of heavy metal ions (since urease is inhibited by them), urea removal for kidney failure, treatments of industrial wastewater, urea removal from alcoholic beverages by acid urease, ammonia or carbon dioxide production from urea by enzymatic hydrolysis, and others (Qin and Cabral, 2002). Urease from soybean hulls displays similar characteristics, such as molecular weight, isoelectric point and substrate specificity as JBU (Karplus et al., 1997).

There are a lot of reports on urease immobilization, in a way of exploiting its possible applications. Enzymes as biocatalysts were widely used in the field of bioprocess technology. Immobilized enzyme shows many advantages, such as amenability to continuous and batch formats, reusability capacity, improvement of stability over soluble enzyme forms, favorable alterations in pH and optimal temperature, sequestration of enzyme from product stream and the possibility of co-immobilization with other enzymes. However, immobilization processes have been associated with some disadvantages, such as the loss of enzyme activity upon immobilization, unfavorable alterations in kinetic properties and high cost of carrier, fixing agents and immobilization process, and mass transfer limitations (Sheldon and van Pelt, 2013; DiCosimo et al., 2012; Rios et al., 2016; Rodrigues et al., 2015). Carriers of different types, sizes and forms such as cellulose, alginate or chitin/chitosan polymers, etc. have been used for urease immobilization (Krajewska et al., 1988), with different degree of success. On this way, immobilization by adsorption is a simple and cheap method but sometimes reversible while covalent immobilization is irreversible and durable.

Chitin is a high molecular weight linear polymer consisting of 2-acetamido-2-deoxy-β-D-glucose (N-acetyl D-glucosamine, D-GlcNAc) and it is the second natural polymer in abundance in the world after cellulose. Chitosan is a polysaccharide derived of chitin deacetylation. Chitosan has a structure that exhibits functional groups that allow and facilitate the adsorption and immobilization of different molecules (Melo et al., 2017; dos Santos et al., 2017). This polymer has numerous industrial applications in food, pharmaceutical, biotechnology and materials fields because it has the advantage of being highly commercially available, biocompatible, biodegradable and ecologically safe (Baieli et al., 2017). Since chitosan has many functional groups that allow the enzyme adsorption, the immobilization process could occur by one-point or multipoint interaction, and then may occur that matrix and ligand continue increasing the number of the interactions (dos Santos et al., 2015).

Jack bean urease has been immobilized by covalent attachment to thiolated Sepharose via a mixed disulphide derivative of Sepharose (Carlsson et al., 1974). Additionally, in 2009, Kumar et al. reported the immobilization of pure soybean urease on chitosan beads by glutaraldehyde activation, demonstrating the superior performance of the immobilized enzyme over the free form as regards its thermal stability and reusability. Some previous reports described the urease immobilization on different supports, including chitosan. However, this is the first report on immobilization from this starting material and using this immobilization method employing a chitosan-based matrix.

The reversible covalent immobilization of enzymes via disulfide bonds has been widely studied. These methods allow the formation of a stable and reversible covalent bond, of disulfide (−S−S−) type (Batista-Viera et al., 2011). Thus, enzymes bearing exposed nonessential thiol (SH) groups can be immobilized on thiol-reactive matrices under mild conditions. A particular method to perform this covalent immobilization uses thiosulfonate gels, which involves the formation of disulfide bonds between the protein thiol-groups and the disulfide oxide structures on the supporting matrix (Ovsejevi et al., 2013). These disulfide oxide structures on the matrix supports can be achieved easily, cheaply and under mild conditions on different matrices such as agarose.

The aim of this paper was the valorization of an agro-industrial by-product, by immobilization and purification of SBU directly from soybean seed hull aqueous extracts in few steps with thiosulfonate-derivatized chitosan mini-spheres, in microscale, in batch and using the covalent chromatography principles, since this enzyme had never been reported to be immobilized by this immobilization method, and this method had never been assayed on chitosan matrix. Additionally, properties of SBU immobilized by this method were studied.

2. Materials and methods

2.1. Materials

Soybean hulls were kindly donated by Entre Ríos Crushing S.A., Argentina. Chitosan from shrimp shells was from Sigma-Aldrich (St. Louis, MO, USA). Epichlorohydrin was from Fluka Analytical (Buchs SG, Switzerland). All other reagents were AR grade.

2.2. Methods

2.2.1. Preparation of the chromatographic matrix

Cross-linked chitosan mini-spheres were prepared as per Baieli et al. (2012): 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 200 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 preparation of mercaptohydroxypropyl ether chitosan gel (thiol-chitosan) was carried out as described for thiol-agarose (Carlsson et al., 1974): the crosslinked chitosan was incubated with an excess of epichlorohydrin solution (2.55 M) with a pH adjusted to 10 with 1 M NaOH. This step was performed at a ratio of 40 mL epichlorohydrin solution per 15 g beads for 6 h at 60 °C. The beads were then washed with distilled water to remove residual epichlorohydrin, before equilibrating the beads in 0.5 M sodium phosphate buffer, pH 6.3, at room temperature. The beads were then immersed in 15 mL 0.5 M sodium phosphate buffer, pH 6.3, at ratio of 15 mL buffer per 10 g beads, and 15 mL 2.6 M sodium thiosulfate was added before incubating overnight at 22 °C. The Bunte-salt gel formed was washed with distilled water to eliminate excess of sodium thiosulfonate, before suspending it in 10 mL 0.2 M sodium bicarbonate buffer, pH 8.5, per 10 g beads. 3 g DTT or 2.75 mL β-mercaptoethanol was added to the Bunte-salt gel suspension and the incubation was carried out for 1 h, under shaking, at 22 °C. The gel was washed with 0.2 M sodium bicarbonate buffer, pH 8.5, distilled water and 0.1 M acetic acid, until absence of DTT or β-mercaptoethanol. Then, 15 g thiol chitosan was suspended in 45 mL of 0.2 M sodium acetate, pH 5.0. Aliquots of 30% hydrogen peroxide were added under continuous shaking, 1.8 mL initially and then 2.2 mL after 30, 90 and 150 min. The incubation was continued until a total reaction time of 30 h. The

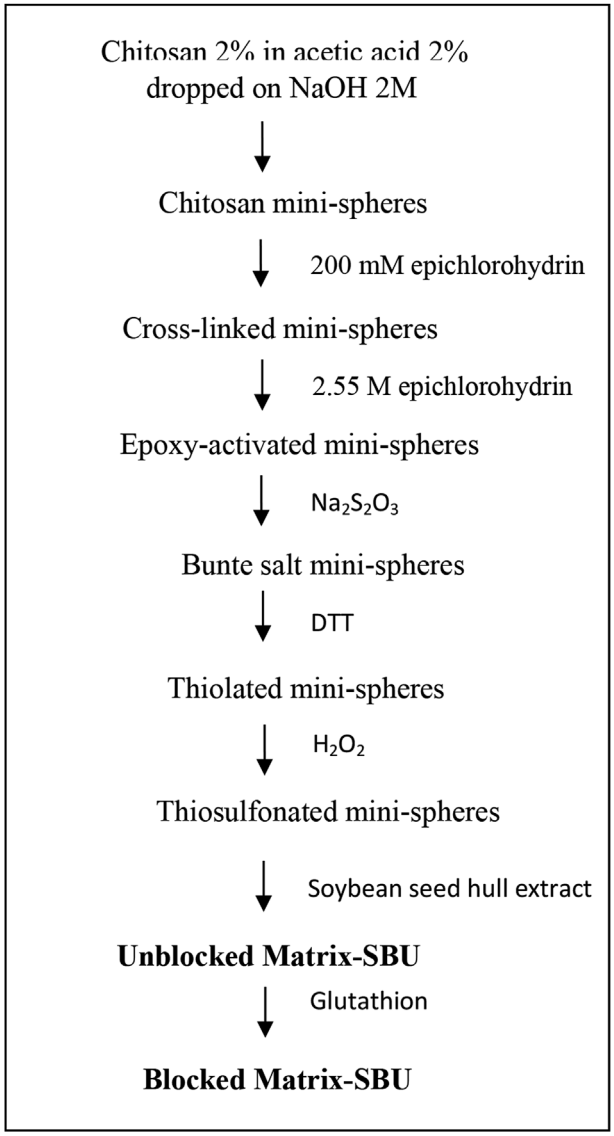

Fig. 1. Matrix preparation scheme. Mini-spheres were made by dropping a chitosan acidic solution over a NaOH solution. After a few steps, thiosulfonate matrix is achieved (see Materials and Methods). Then, thiosulfonated mini-spheres were incubated with soybean seed hull extract to immobilize SBU. After SBU immobilization process, matrix is treated with glutathione to block the thiosulfonate groups which did not react.

oxidized gel was washed with 0.1 M acetic acid until they were free of hydrogen peroxide. The activated gel was stored in 0.2 M sodium acetate, pH 5.0, at 4 °C, until use. Fig. 1 shows the scheme of the matrix preparation.

2.2.2. Extraction of SBU from soybean hulls

Soybean seed hull was used without any previous treatment. SBU was extracted from soybean hulls in a 5 L beaker, with 200 g soybean hulls per 2 L buffer, overnight at room temperature. The buffer tested was 50 mM sodium phosphate at pH 6.0 and 7.5 (Bracco et al., 2017). Then, the extract was centrifuged at 10,000 rpm for 30 min at 4 °C and the supernatant concentrated 10X by ultrafiltration. The extract was stored at 4 °C until use.

2.2.3. Immobilization of urease on thiosulfonate-chitosan beads

100 mg chitosan beads (referred henceforth as dry weight), containing 305 μmol thiosulfonate groups/g matrix, were incubated at room temperature with 1 mL SBU extracts (17 mg SBU/L) at pH 6.0 and 7.5, and at pH 7.5 with 10 mM β-mercaptoethanol or DTT. Previously, the low molecular weight material was removed from the extract by gel filtration on Sephadex G-25. The incubation was carried out overnight, at 25 °C.

After the immobilization process, to block the excess of active groups, 100 mg chitosan beads were incubated with 1.0 mL of fresh 8 mM glutathione solution, for 30 min. After incubation, chitosan beads were washed with 50 mM sodium phosphate buffer, at pH 7.0.

2.2.4. Scanning electron microscopy (SEM)

Scanning electron microscopy (SEM, Zeiss Supra 40 microscope) was used to visualize the morphology of chitosan matrices before and after urease coupling. Prior to the analysis, the matrices were lyophilized and metallized with gold (gold sputtering).

2.2.5. Enzyme and protein assay

For each experiment, 100 μL with a dilution factor which allowed a correct interpolation within the calibration curve, depending on the sample, was incubated with 0.2 M urea in 100 mM Tris-HCl buffer, pH 7.0, containing 10 mM EDTA, for 5 min at 25 °C. The ammonia concentration after the reaction was measured by adding 200 μL 1 M NaOH and then 50 μL Nessler reagent (Wu and Cao, 2013). The absorbance at 405 nm was measured spectrophotometrically. One unit of urease activity liberates 1 μmol of ammonia from urea per minute at pH 7.0, at 25 °C. Protein was quantified as per Bradford (1976), using bovine serum albumin as the standard. Specific activity was calculated as units/mg protein. Activity of the mini-spheres was calculated as units/mini-sphere (average weight).

2.2.6. Thermostability of immobilized urease

It was assessed by the measurement of the remaining enzyme activity after exposure of the SBU-thiosulfonate-chitosan mini-spheres at 70 °C and 80 °C, in 50 mM sodium phosphate buffer, pH 7.0, for 4 h. For comparison, free SBU and JBU were subjected to the same treatment, at 70 °C. The measurements were performed in triplicate and the results expressed as the average ± standard deviation.

2.2.7. pH activity profile of free and immobilized SBU

The activity of free and immobilized SBU was measured at various pH between 3.0 and 11.0. The buffers were: 50 mM sodium acetate for pH 3.0, 4.0 and 5.0, 50 mM sodium phosphate for pH 6.0 and 7.0, 50 mM Tris-HCl for pH 8.0 and 9.0, and 50 mM sodium bicarbonate for pH 11.0. The measurements were performed in triplicate and the results expressed as the average ± standard deviation.

2.2.8. Temperature-activity profile of free and immobilized SBU

One mini-sphere or 20 μL of free SBU were incubated with 100 μL of 0.1 M urea in 50 mM Tris-HCl buffer, pH 7.0, at 25, 35, 45, 55, 65, 75 and 85 °C. After 5 min reaction samples were cooled until 25 °C, then 200 μL of 1 M NaOH and 50 μL of Nessler reagent were added, and the absorbance at 405 nm was measured. The measurements were performed in triplicate and the results expressed as the average ± standard deviation.

2.2.9. Reusability of immobilized SBU

The reusability of immobilized SBU was assessed by determination of the enzyme activity in successive experiments. Three mini-spheres were incubated with 300 μL of 0.1 M urea in Tris-HCl buffer, pH 7.0, for 5 min at 37 °C and 1000 rpm to allow substrate and product diffusion through the matrix pores. After that, 200 μL of reaction mixture were mixed with 200 μL of 1 M NaOH and 50 μL of Nessler reagent and, after 5 min, the absorbance at 405 nm was measured. The residual activity was calculated by difference between the initial and final activity. After each experiment, the matrix was washed twice with 100 mM Tris-HCl buffer, pH 7.0, to remove any residual substrate and fresh buffer was added to proceed to a new activity determination. This was repeated

twenty-five times.

2.2.10. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

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

2.2.11. Storage stability of SBU

The activity of immobilized SBU was measured at different storage time periods, at 4 °C in 50 mM sodium phosphate buffer, pH 7.0, containing 1 mM EDTA. For comparison, free SBU was subjected to the same treatment. The measurements were performed in triplicate and the results expressed as the average ± standard deviation.

3. Results and discussion

3.1. Extraction of SBU from soybean hulls

The aqueous soybean seed hull extracts, performed at pH 6.0 and 7.5 and at room temperature, have protein concentrations of 2.8 and 3.0 mg/mL, of which 15.2 and 17 μg/mL, respectively, correspond to urease.

3.2. Urease immobilization yield

Fig. 2 shows the results for SBU immobilization on chitosan beads incubated with the concentrated extract. Two different pH extracts were assayed, pH 6.0 (A) and 7.5 (B), as well as pH 7.5 with the addition of 10 mM DTT or β-mercaptoethanol, with the previous removal of the low molecular weight material from the extract by gel filtration with Sephadex G-25, to unmask any buried disulfide (C). Is important to mention that when the removal of DTT or β-mercaptoethanol was not carried out, not significant interaction between SBU and thiolated chitosan was detected. An immobilization yield of 40% of the SBU contained in the extract (15.2 μg SBU/mL) was achieved at pH 6.0, whereas an immobilization yield of 70% was achieved at pH 7.5, this corresponding with reported results about the improvement of covalent immobilization via thiosulfonate groups at pH over 7.0 (Batista-Viera et al., 2011). However, the highest immobilization percentage was obtained with the DTT-pre-treatment of the soybean seed hull extract, with an immobilization percentage of 98% of the soybean seed hull extract SBU content (17 μg SBU/mL), thus suggesting the presence of buried disulfides in SBU. Fig. 3 shows the SBU immobilization and release mechanisms.

Kumar et al. (2009) obtained 77% immobilization of SBU on chitosan beads of similar size, but immobilization was performed by glutaraldehyde activation of the beads before attachment of purified urease. Krishna et al. (2011) attained 97% immobilization of purified urease from *Momordica charantia* seeds on chitosan beads after glutaraldehyde activation. In our case, the immobilization yield was over 98% by using covalent immobilization from an urease crude extract. Since SBU extract content is only 17 mg/mL, this 98% was not achieved under saturated conditions. In fact, the total capacity of the TC mini-spheres is 18.5 mg SBU/g mini-spheres.

Carlsson et al. (1974) immobilized urease from a crude extract to thiolated Sepharose, evidencing the selectivity for urease of this immobilization via. Fig. 4 shows the SDS-PAGE of the crude extract before and after the immobilization reaction, evidencing that mainly urease was attached to the chitosan mini-spheres and then released, suggesting that the enzyme is one of the major high-sulfhydryl-containing protein in the extract. In this regard, Kumar and Kayastha (2010) reported a total of 34 accessible sulfhydryl groups per SBU hexamer, with two types of accessible sulfhydryl groups (one types being more reactive than the other).

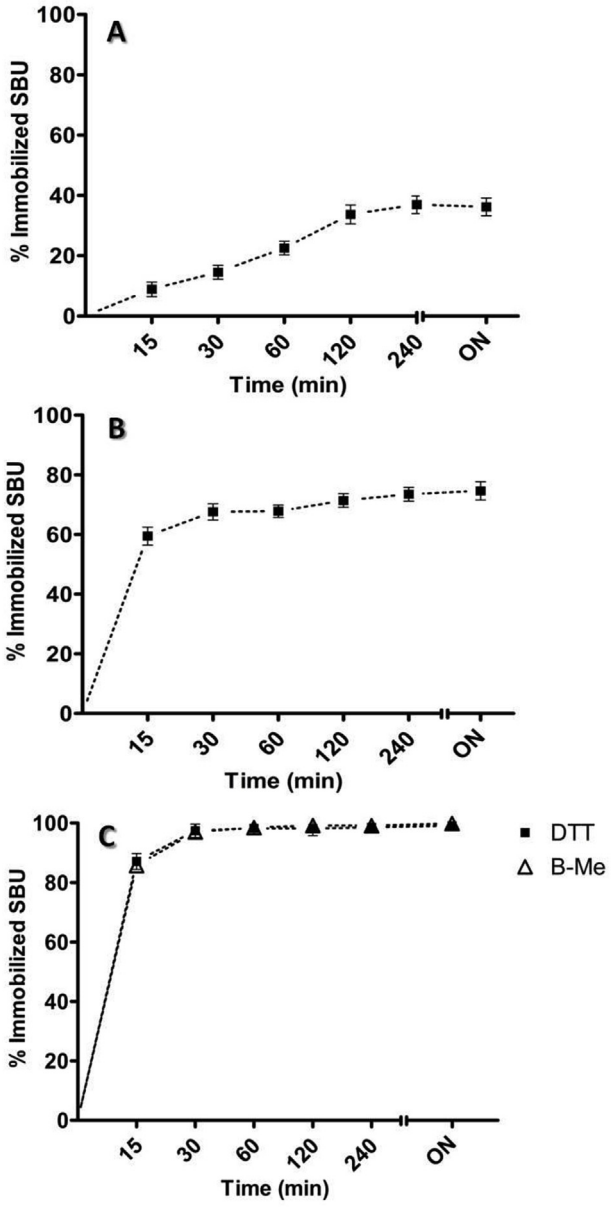

Fig. 2. Immobilization of SBU on chitosan mini-spheres. Chitosan mini-spheres incubated with SBU extracts at pH 6.0 (A), 7.5 (B), and pH 7.5 with the addition of 10 mM β-mercaptoethanol or DTT (C).

3.3. Scanning electron microscopy (SEM)

Fig. 5 shows the SEM images of chitosan matrices before and after SBU coupling. No significant differences can be observed in the morphology, thus evidencing that the attachment of urease to the matrix did not alter its pore structure. Chitosan mini-spheres had a diameter of 1.6–1.8 mm. SEM images showed a mesoporous material, containing pores between 2 and 50 nm according to IUPAC nomenclature. Mesoporous materials display a usually high surface area (over 1000 $m^2 g^{-1}$) compared with other materials, with a narrow pore size distribution, allowing the exhibition of unique catalytic, adsorption, and separation properties.

3.4. Thermostability of immobilized SBU

Fig. 6 shows the profile of thermostability of immobilized SBU as compared with that of free SBU. As expected, SBU gained stability after its immobilization on thiosulfonate-chitosan beads: after 4 h at 70 °C,

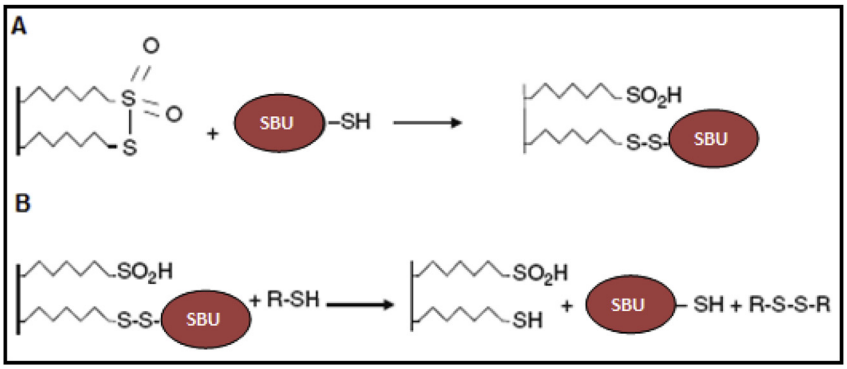

Fig. 3. SBU immobilization and release mechanism. When thiosulfonated matrix is exposed to a thiol-containing enzyme, this enzyme reacts with the more electrophilic of the two sulfur atoms (A) The sulfinic acid leaving group remain attached to the matrix. Release of the thiol-immobilized enzyme can be achieved by incubating the matrix with an excess of DTT or β-mercaptoethanol (B).

the activity of free SBU was approximately 50% lower than that of the immobilized SBU. Additionally, the comparison of immobilized SBU with free JBU was even more favorable: after 4 h at 70 °C, the remaining activity of JBU was less than 10% while that of the immobilized SBU was 60%. After 4 h at 80 °C, the activity of immobilized SBU was 45% while that of the free enzyme was 8%. As suggested by Kumar et al. (2009), the matrix could aid to the higher heat stability of the immobilized enzyme, protecting it against denaturation by absorbing a considerable amount of heat. Thermal stability of enzymes is one of the most important criteria for different applications. This stability depends on the immobilization strategy and support utilized (Manoel et al., 2015). In this sense, immobilization improves enzyme stability to several subunit enzymes, or if several matrix-enzyme bonds are promoted. This fact increases the enzyme rigidity and then it reduces the conformational changes, may be without reductions on enzyme activity (Sánchez et al., 2016). Since SBU has many potential thiosulfonate matrix attachment points (34 accessible sulfhydryl groups per SBU hexamer), the immobilization process clearly is carried out by multipoint covalent attachment. Multipoint covalent attachment allows increase the overall enzyme rigidity, but that does not occur with the same intensity in all the enzyme structure. The immobilized enzyme will have some conformational movements slower than others that result in a new more rapid conformational change which may be affecting a different area when compared to a non-stabilized enzyme. Furthermore, the multipoint attachment could produce some conformational changes, allowing the generation of new paths for further enzyme conformational changes (Sánchez et al., 2016).

3.5. pH-activity profile of free and immobilized SBP

Fig. 7 shows the profile of pH activity of immobilized SBU compared with that of the free enzyme. Surprisingly, pH of maximum activity shifted from 7 to 5, thus allowing the use of the immobilized enzyme in processes carried out at lower pHs since immobilized SBU activity was around 50% in a pH range from 3 to 4, while free SBU activity was only 10% at the same pH range. Since several applications of urease occur at acid pH, the shift after immobilization is highly useful for industrial purposes.

Kumar et al. (2009) studied the effect of pH on the activity of urease immobilized on chitosan or alginate: while immobilization by entrapment in alginate shifted the optimum pH from 7.0 to 6.2 respects to the free enzyme, immobilization on chitosan shifted the optimum pH from 7.0 to 7.9, thus evidencing the influence of the immobilization mode on the optimum pH. Our results in comparison with those of Kumar et al. also demonstrate the influence of the immobilization route on the optimum activity pH of SBU.

Additionally, other authors have reported shifts in optimal pH after the enzyme immobilization on different supports, such as graphene oxide composites (Li et al., 2015), chitosan nanoparticles (Ling et al., 2016) and SnO2 hollow nanotubes (Anwar et al., 2017). A possible explanation of the pH shift after enzyme immobilization is that the immobilization process causes both the change of the enzyme structure and the change of enzyme microenvironment. Thus, the enzyme adopts a spatial rigid structure due to the strong covalent multipoint bonds which could influence the intramolecular forces, thus changing the enzyme conformation. On the other hand, the enzyme

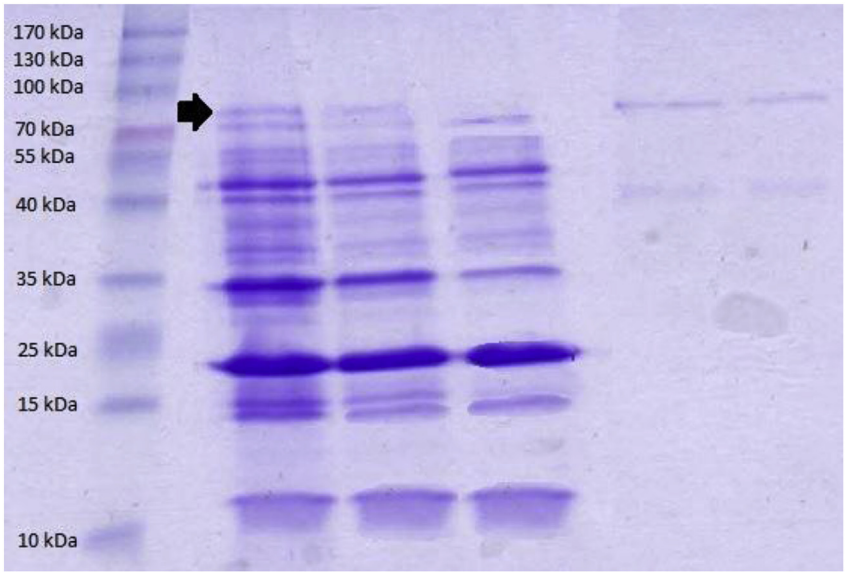

Fig. 4. SDS-PAGE analysis of the immobilization reaction. SDS-PAGE of the crude extract before and after the immobilization reaction. Lane 1, molecular weight marker; lanes 2 and 3, crude extract before the immobilization reaction; lane 4, crude extract after the immobilization reaction; lanes 5 and 6, elution of urease from the matrix with 0.5 M DTT. Coomassie Blue staining was made under the standard conditions. The arrow indicates the SBU electrophoretic position. . (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

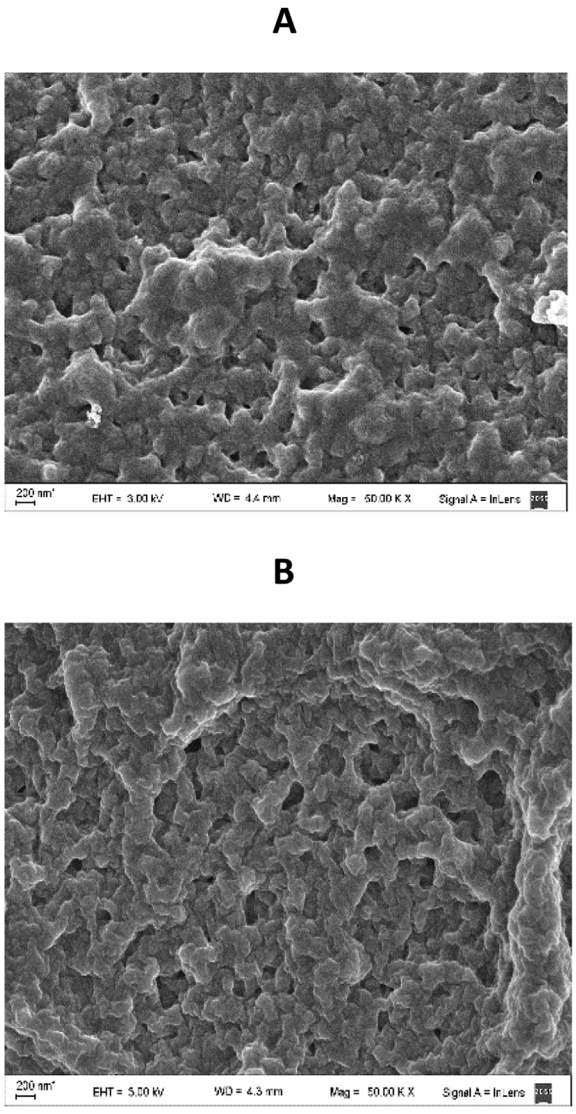

Fig. 5. Scanning electron microscopy of chitosan matrices. Morphology of chitosan matrices before (A) and after urease coupling (B). Prior to the analysis, the matrices were lyophilized and metallized with gold (gold sputtering).

microenvironment is not the same as in free enzyme, and is specially given by the chemical properties of the matrix (Li et al., 2015).

3.6. Temperature-activity profile of free and immobilized SBU

Fig. 8 shows the temperature-activity profile of free vs immobilized SBU. Immobilization of SBU brought about an increase of optimum activity temperature of 10 °C (from 65 °C to 75 °C). The same effect was observed by Kumar et al. (2009). Therefore, this effect, which allows immobilized enzyme to have industrial application at high temperature conditions, seems to be general and independent of the immobilization matrix. A possible explanation to this phenomenon is that immobilized-enzyme denaturation is not observed due to the protection of amino acid on the enzyme's surface as well as at the active site. However, when the temperature increases, substrate molecules increase kinetic energy and reach the active site more easily (Dwevedi, 2016). Additionally, immobilized enzymes by multipoint covalent attachment have shown to be more stabilized than non-multipoint covalent attached enzymes and free enzymes (Branco et al., 2015).

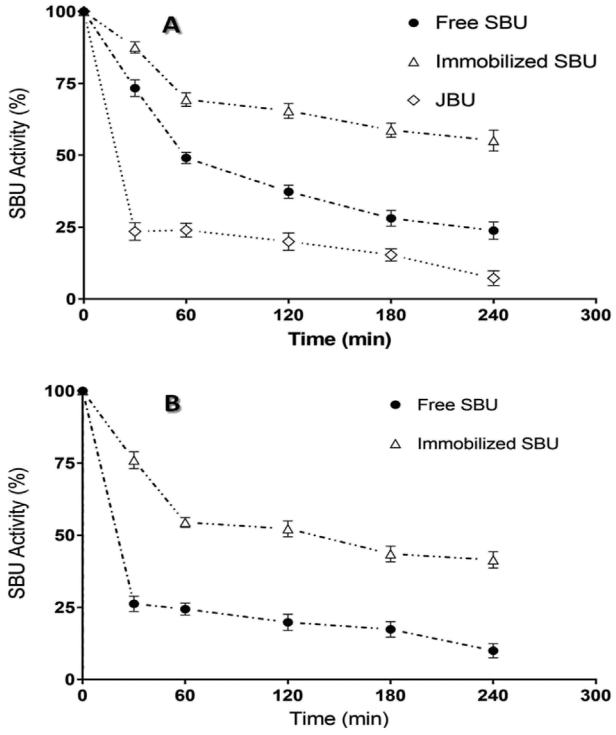

Fig. 6. Thermostability profile of immobilized SBU compared with those of free SBU and jack bean urease (JBU). Samples of immobilized SBU (Δ), free SBU (●) and free JBU (◊) were incubated to 70 °C for 4 h (A), and immobilized (Δ) and free SBU (●) at 80 °C (B) for the same time. Activity was measured at 60, 120, 180 and 240 min.

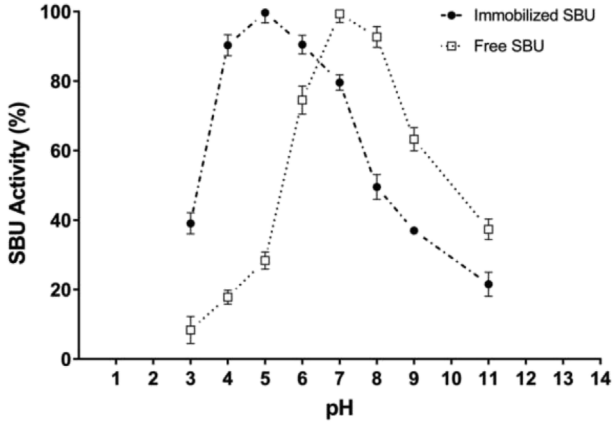

Fig. 7. pH-activity profile of immobilized SBU compared with that of the free SBU. The activity of free (□) and immobilized SBU (●) was measured at various pH between 3.0 and 11.0. The buffers were: 50 mM sodium acetate for pH 3.0, 4.0 and 5.0, 50 mM sodium phosphate for pH 6.0 and 7.0, 50 mM Tris-HCl for pH 8.0 and 9.0, and 50 mM sodium bicarbonate for pH 11.0.

3.7. Reusability of immobilized SBU

From the industrial point of view, the reusability of an immobilized enzyme is an important factor affecting the costs of a biotransformation process. Kumar et al. (2009) reported that SBU immobilized on chitosan beads retained 80% activity after 14 uses. In our case, after 25 cycles of utilization, the matrix retained 84% activity (Fig. 9), evidencing again the importance of the immobilization on the performance of the matrix. In this sense, immobilization of enzymes inside the porous structure of a matrix allows to dispose the molecules fully dispersed, thus without the interaction with any external interface. Then, immobilization stabilizes

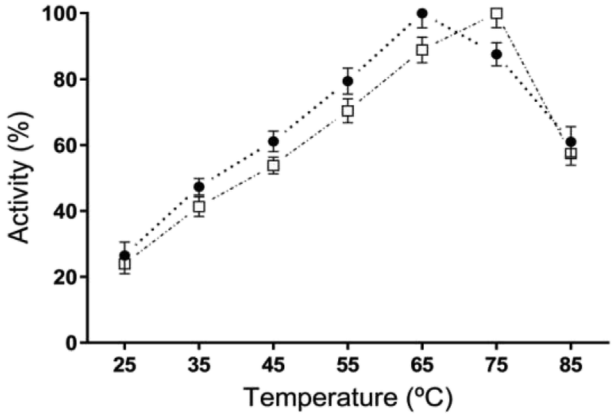

Fig. 8. Temperature-activity profile of immobilized SBU compared with that of the free SBU. One mini-sphere (□) or 20 µL of free SBU (●) were incubated with 100 µL of 100 mM urea in 50 mM Tris-HCl buffer, pH 7.0, at 25, 35, 45, 55, 65, 75 and 85 °C. After 5 min reaction, the enzyme activity was measured as described in Methods.

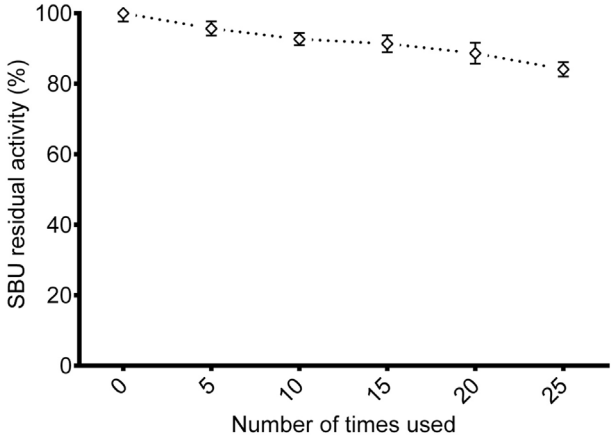

Fig. 9. Reusability of immobilized SBU. 0.05 g of mini-spheres were incubated with 300 µL of 0.1 M urea in Tris-HCl buffer, pH 7.0, at 37 °C and 1000 rpm. After 5 min reaction, the enzyme activity was measured as described in Methods. After each experiment, the matrix was washed twice with 100 mM Tris-HCl buffer, pH 7.0, to remove any residual substrate and fresh buffer was added to proceed to a new activity determination.

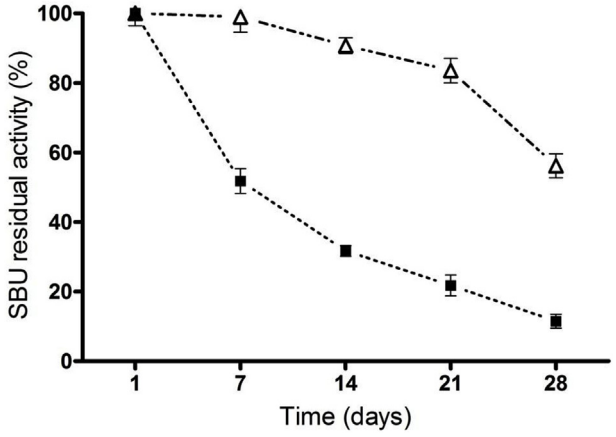

Fig. 10. Storage stability. The activity of immobilized SBU (Δ) was measured at different storage time periods, at 4 °C in 50 mM sodium phosphate buffer, pH 7.0, containing 1 mM EDTA. For comparison, free SBU was subjected to the same treatment (■). The measurements were performed in triplicate and the results expressed as the average ± standard deviation.

the enzyme against interaction with extract's contaminants, avoiding aggregation, proteolysis by extract's proteases or autolysis. Operational stability also depends on the immobilization support and the immobilization method selected (Mateo et al., 2007).

The possibility of using the obtained biocatalyst matrix in many catalyst cycles, allows its use in packed column process decontaminating urea solutions, sensing heavy metals pollution levels or sensing urea blood levels (data not shown).

3.8. Storage stability of SBU

As is shown in Fig. 10, after 1 month of storage at 4 °C, the residual activity of the immobilized SBU was 60%, while that of the free SBU was only 10%. This is congruent with many reported results, since immobilizaed enzymes tend to gain stability after the immobilization process. For instance, Kumar et al. (2009) reported a four-fold increment in storage stability after soybean urease immobilization on chitosan beads (over a period of 1 month), while Mishra (2015) reported a two-fold increment in storage stability after jackbean urease immobilization on alginate beads (over a period of 1 month). On the other hand, Selvamurugan et al. (2007) reported a retained residual activity of 60% for immobilized jackbean urease on nylon beads and gelatin films over a period of 1 month, while urease immobilized on sepharose and silica gel was stable over a period of only 10 days.

4. Conclusion

Covalent immobilization of SBU via thiosulfonate provides a matrix displaying a better performance than the free enzyme as regards thermal stability and optimum activity temperature. Besides, as judged by its reuse behavior, the immobilization via thiolation would be better than other immobilization modes.

The decrease in optimum pH due to the SBU immobilization via thiolation allows its utilization in processes operated at acid pH.

Even though the urease content of soybean seed hull is only 0.17 mg/g (much less than the whole seed content), the low cost of this agro-industrial by-product and the possibility of the purification of other proteins with many applications in different fields (Bracco et al., 2018), make this starting material economically attractive. Furthermore, this immobilization and purification method could be applied to the whole seed as the starting material, which has higher urease content than hulls.

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