Korean J. Chem. Eng., 40(9), 2263-2270 (2023)
DOI: 10.1007/s11814-023-1458-3

pISSN: 0256-1115
eISSN: 1975-7220

Immobilization of commercial acid phosphatases from wheat germ and potato onto ion exchangers

Frederico Alves Lima*,, Pedro Alves Martins**, Wilson Galvão de Morais Júnior***, Eloízio Júlio Ribeiro*, José Manuel Guisán**, and Miriam Maria de Resende*,†**

*Chemical Engineering Faculty, Federal University of Uberlândia, P.O. Box 593, Av. João Naves de Ávila 2121, Campus Santa Mônica, Bloco 1K, Uberlândia, MG 38408-144, Brazil
**Departamento de Biocatálisis, Instituto de Catálisis y Petroleoquímica, Consejo Superior de Investigaciones Científicas (CSIC) Campus Cantoblanco, 28049, Madrid, España
***CIETI, School of Engineering (ISEP), Polytechnic of Porto (P.Porto), R. Dr. António Bernardino de Almeida, 4249-015, Porto, Portugal
(*Received 26 October 2022 • Revised 7 March 2023 • Accepted 25 March 2023*)

Abstract−A very simple and fast immobilization technique based on ion exchange was investigated to improve the thermal stability of acid phosphatase from wheat germ and potato. Immobilization was not efficient for the DEAE-sepharose, and MANAE-agarose supports. On the other hand, Toyopearl DEAE-650s proved to be a promising support, with immobilization yield above 95% and recovery of activity above 85% for both enzymes. A second step was introduced in the immobilization protocol to improve the thermal stability of these biocatalysts. For this, oxidation and reduction of glycosidic chains of acid phosphatase were carried out, allowing the formation of aldehyde groups and subsequent interaction with the amine groups to further stabilize the different forms (free and immobilized). Both biocatalysts showed residual activity after 1 hour of inactivation at the temperature of 60 °C, a fact not observed for the free enzyme. The wheat germ acid phosphatase derivative was the most stable, with residual activity of 66.7% for the only immobilized derivative and 76.2% for the oxidized/reduced derivative. Also, the derivatives prepared by ion exchange adsorption on Toyopearl (TOYO), followed by oxidation/reduction and intramolecular crosslinking, were approximately 15 and 41 times more stable than the free enzyme from wheat germ.

Keywords: Acid Phosphatase, Immobilization, Thermal Stability, DEAE-sepharose, DEAE-Toyopearl 650s, MANAE-agarose

INTRODUCTION

Acid phosphatases (ACPase) (monoester phosphohydrolase EC 3.1.3.2) are hydrolases that promote the hydrolysis of monoester phosphate, transforming organic phosphate into a soluble inorganic form [1]. These enzymes play a specific role in mobilizing and acquiring phosphate, increasing soil fertility and plant growth [2,3]. They have been isolated from animal, plant, and microbial sources and are well characterized [2].

There are several phosphate-solubilizing microorganisms and enzyme producers with high applicability. The *Trichoderma* genus of saprophytic fungi is highlighted as being capable of colonizing root and leaf regions and promoting beneficial biochemical changes through ACPase production [4,5]. ACPase performs important functions in plants, providing inorganic phosphate since plants cannot directly use organic phosphate from the extracellular environment [6,7].

Furthermore, when used for bioremediation, acid phosphatases have been considered good pollutant indicators, acting on the degradation of hazardous pesticides present in soils and water [8,9]. In animal cells, these enzymes have been used as biomarkers in radioimmunoassay to diagnose bone metastases, chronic inflammation, and prostate cancer [10,11]. In the industrial context, ACPases are applied in feed processing for monogastric animals. These released phosphates enhance the nutritional value and reduce phosphate excreted by animals [12,13].

However, many of these plant ACPases can be highly glycosylated, playing an important role in many biological processes [14]. Glycosylated enzymes may have a glycosylic chain covering Lys residues, reducing the geometrical and chemical congruence between the enzyme and support surfaces [14]. Moreover, glycosyl chains can become interesting cross-linking polymers when modified by introduced groups capable of reacting with amino groups of Lys [14].

Enzyme immobilization poses the possibility of improving the characteristics of an enzyme not only in terms of stability and catalysis but also for process improvement, allowing the reuse of the biocatalyst for many operational cycles [15]. Many immobilization strategies focus on improving enzyme properties, like stability, recovered activity, or selectivity [16-18]. One of them is the ion exchange between ionic groups of the protein and ionic supports, which has been shown to be an essential tool in the purification

†To whom correspondence should be addressed.
E-mail: mresende@ufu.br

[19,20] and immobilization of proteins [21,22].

When used for immobilization, this method is a multipoint process; the enzyme is fixed to the support only when are established ionic bridges between the protein and the support [23-25] in a number sufficiently high to compensate for the ionic strength of the medium. During the immobilization process, some types of adsorptions can occur simultaneously. However, ionic interactions prevail because they are the majority. Considering these points of view, the ion exchange process may be a more complex and versatile immobilization tool than supposed since it may depend on the support activation and experimental conditions like pH, temperature, and concentration of the enzyme [23-25]. The main advantages of the ion exchange strategy are the reduced immobilization time and, more importantly, the support that can be reused after enzyme inactivation. The inactivated enzyme can be desorbed, leaving an unmodified support that can be used to recharge the fresh enzyme [25].

Therefore, considering the relevance of acid phosphatases in phosphate solubilization, bioremediation, and as a possible biomarker indicator, together with the importance of immobilization in the applicability of these enzymes, this work aimed to improve the stability of two plant-derivative ACPases. In this study, the effect of the immobilization of wheat germ and potato ACPases in three different ion exchange supports, Toyoperl DEAE-650s, DEAE-Sepharose, and MANAE Agarose, was analyzed.

MATERIALS AND METHODS

1. Acid Phosphatases

The acid phosphatases (ACPases) from wheat germ and potato were acquired from Sigma Aldrich Co. (St. Louis, MO, USA). Wheat germ ACPase (P3627) originated from the aleurone and scutellar tissues during germination and consists of four isoenzymes. The ACPase from potato (P3752) is a phosphomonoesterase, appearing in multiple molecular forms of similar molecular mass but with different isoelectric points.

2. Acid Phosphatase Activity Assay

Acid phosphatase activity was measured by a modified method described by Leitão et al. [26] using p-nitrophenylphosphate hexahydrate disodium salt (p-NPP, Sigma Aldrich^{TM}) as substrate. In 350 μL of 50 mM sodium acetate buffer, pH 5.0, and 100 μL of p-NPP (5 mM) as well as 50 μL of enzyme solution or suspension were added. The reaction was monitored in a spectrophotometer equipped with magnetic stirring and temperature control (Jasco, V730 Spectrophotometer) at 40 °C by measuring the increase in the absorbance at 348 nm with the time due to the p-nitrophenol (p-NP) released and taking the inclination (absorbance/min) in the linear step. One Unit (U) of acid phosphatase activity was defined as one micromole of p-NP released per min under the assayed conditions [27].

3. Supports Preparation

The commercial supports, Toyopearl 650s (TOYO), and DEAE Sepharose CL-6B (DEAE), were washed with 15 mM Tris hydrochloride (Tris) buffer pH 7.0 and vacuum filtered before use. MANAE agarose was prepared using a methodology developed by Fernandez-Lafuente et al. [28]. In a solution of 27.07 mL of 2 M ethylenediamine (EDA), distilled water was added to a volume of 165 mL and the solution pH was adjusted to 10.0 before reaching a total volume of 200 mL. Then, 35 g of glyoxyl agarose was added and kept under constant stirring [29]. After 2 h, 2.0 g of $NaBH_4$ was added to the vessel, and the new mixture was stirred again for 2 h. Finally, the obtained support was washed with approximately 1.0 L of sodium acetate buffer 100 mM at pH 4.0 and thoroughly washed with distilled water before use.

The effect of the temperature on the free ACPase stability was evaluated at 4 and 25 °C by incubating 2.0 mL of ACPase solution (5 mg/mL, in 25 mM Tris buffer pH 7.0) for 4 h. Samples were taken in one-hour intervals for ACPase activity measurement as described in section 2.2.

4. Properties and Preparation for Use

Enzyme immobilization was performed by adding 1.0 g of each support (DEAE, TOYO, or MANAE) to 10 mL of ACPase solution (5 mg/mL wheat and 10 mg/mL potato), diluted in 25 mM Tris hydrochloride (Tris) buffer, pH 7.0. The suspension was incubated for 1 hour at 25 °C under mild stirring. Finally, the immobilized derivative (ACPase-Support) was vacuum filtered and washed with three volumes of 10 mM Tris buffer pH 7.0.

The oxidation step was carried out by incubating 0.5 g of derivative in 5 mL of 25 mM Tris buffer pH 7.0 and 20 mM of sodium periodate at 25 °C for 1 hour. The derivative was then vacuum filtered and washed with three volumes of 10 mM Tris buffer pH 7.0. The derivative was resuspended in 25 mM sodium bicarbonate buffer pH 8.5 and sodium borohydride (0.1 mg/mL) at 25 °C for 30 min for the reduction. The presence or absence of aldehydes and amino groups in the derivatives was qualitatively measured using Schiff's reagent and picrylsulfocromic solution [14].

Periodically, to determine activity during the process, the immobilization yield (IY) was calculated by the ratio between the activity in the supernatant and the initial enzymatic activity (Eq. (1)). The expressed activity (EA) was calculated using the final and initial activity (Eq. (2)), according to Trobo-Maseda et al. [30].

$$IY\% = \left(1 - \frac{\text{Final supernatant activity}}{\text{Initial activity}}\right) \times 100 \quad (1)$$

$$EA\% = \left(1 - \frac{\text{Suspension activity}}{\text{Initial activity} \times IY}\right) \times 100 \quad (2)$$

5. Biochemical Characterization of Acid Phosphatase

5-1. Temperature Effect on the ACPase Activity

The temperatures evaluated for their effect on free ACPase activity were at 4 and 25 °C. The ACPase thermal sensitivity was studied by incubating 2.0 mL of soluble ACPase (5 and 10 mg protein/mL, for ACPase from wheat germ and potato, respectively) in 25 mM Tris buffer (pH 7.0) at the described temperatures for the first 4 h. The effect of the temperature on the enzymatic activity of free and immobilized ACPases was evaluated from 20 to 65 °C and pH 7.0 (Tris buffer for preparation of free enzyme solution and immobilized enzyme suspension (1 : 10 w/v). The activity at each temperature was measured according to section 2.2.

5-2. pH Effect on the ACPase Activity

To evaluate the ACPase stability in different pH conditions, assays were performed incubating the derivatives in a medium at pH val-

ues 3.0, 5.0, 7.0, 8.5, and 10.0 at 25 °C. The soluble enzyme (5.0 mg/mL) and derivatives (1 : 10 w/v) were suspended in the following buffer systems: 25 mM sodium acetate pH 3.0 and 5.0, 25 mM Tris pH 7.0, and 25 mM sodium bicarbonate pH 8.5 and 10.0. The activity was measured in the first 4 h and expressed relative to the initial activity.

6. Thermal Stability Assays of Acid Phosphatase

ACPases (free and immobilized) were incubated at 60 °C and pH 7.0. Samples of the enzyme solution were periodically withdrawn. The residual enzymatic activity was calculated as the ratio of the activity of each sample at a defined time and the initial activity before the inactivation assay.

RESULTS AND DISCUSSION

1. Stability of Free Enzymes

To identify the best conditions for immobilization, stability tests of the free enzyme were carried out at different pH values and temperatures of 4 and 25 °C, as described in 2.6. Fig. 1(a) shows that the wheat germ enzyme incubated at pH 3.0, 5.0, and 7.0 showed similar activity in one hour, inactivating 90.5% (average). Still, after 2 hours, a higher loss of activity was noted at pH 3.0, showing only 21% activity after 4 hours of the experiment. At pH 7.0, the enzyme showed higher stability when compared to other pH conditions, remaining with 87.4% activity after 4 hours. At pH 10, the enzyme suffered more than 90.0% inactivation in the first min of incubation and, after 4 hours, presented a higher inactivation. The ACPase from wheat germ was more stable at a temperature of 25 °C, maintaining its enzymatic activity after 4 hours of the experiment. At 4 °C, the enzyme retained 85.0% of its initial activity in the first hour. After 4 hours, however, it exhibited only 50.0% of the initial enzymatic activity (Fig. 1(b)).

Potato ACPase showed similar behavior to the ACPase from wheat germ. Fig. 2(a) shows that in the first hour of the experiment, the enzymes incubated at pH values of 5.0 and 7.0 maintained their activity, and at pH 3.0, the activity was 78.5%. A higher activity

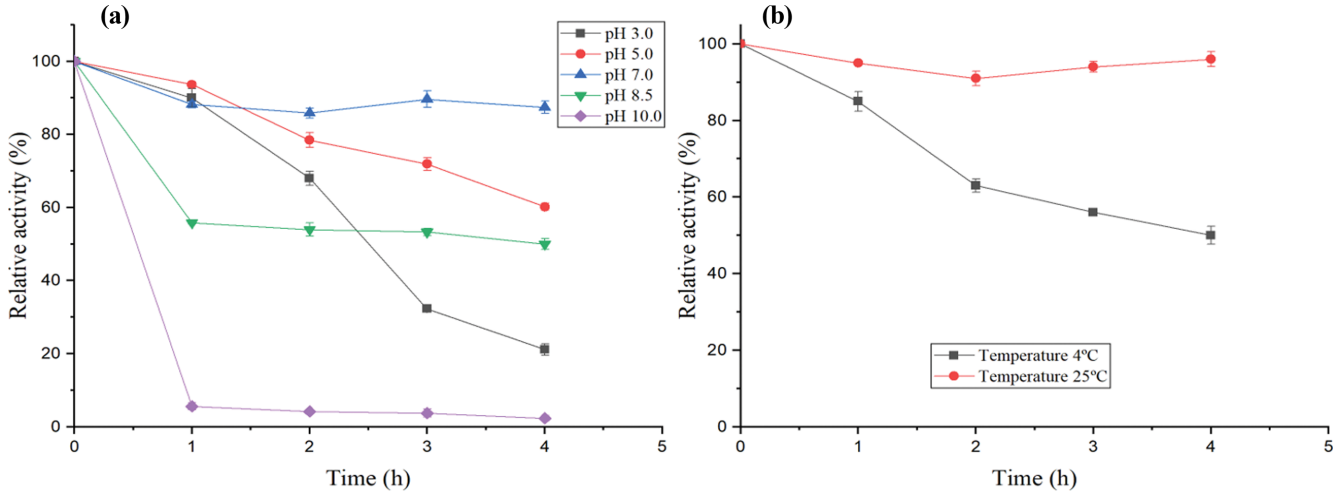

Fig. 1. Stability of free ACPase from wheat germ in different pH at room temperature (a); and temperature at pH 7.0 (b).

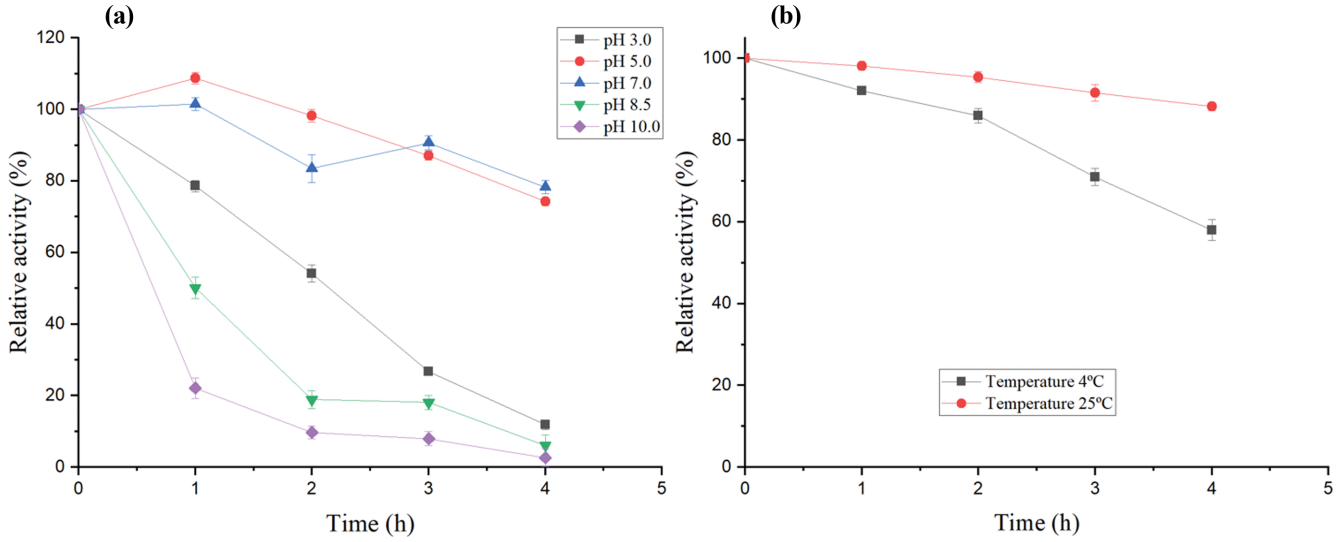

Fig. 2. Stability of free ACPase from potato in different pH at room temperature (a); and temperature at pH 7.0 (b).

loss was noted at pH 3.0, showing only 11.9% activity after 4 hours. The enzyme showed a greater residual activity (74.2 and 78.2%) at pH 5.0 and 7.0, respectively, after 4 hours of the assay. The enzyme also was inactivated by more than 80.0% at pH 10 in the first incubation hour. After 4 hours, it was almost completely inactivated. The potato ACPase was also more stable at a temperature of 25 °C, maintaining its enzymatic activity after 4 hours of experiment, while at 4 °C, the enzyme retained 92.0% of its initial activity in the first hour. After 4 hours of experimentation, only 58.0% of the initial enzymatic activity could be observed (Fig. 2(b)).

The better performance of ACPase at acid pH values (3.0-5.0) was also previously reported by Kalita and Ambasht [31] for free wheat germ ACPase (optimum pH and temperature of 5.0 and 50 °C, respectively), Souza et al. [4] for ACPase II from *Trichoderma harzianum* (optimum pH range of 3.5-4.5 and temperatures of 50 and 55 °C), and Leitão et al. [26] also for ACPase from *T. harzianum* (optimum pH range of 4.5-6.0 and 50 and 55 °C).

2. Immobilization of ACPases

Plant proteins are highly glycosylated and this can create problems for covalent immobilization on activated supports since the amino terminus and glycosylated chains can mask many lysine residues. Expressed activity of ACPases immobilized on DEAE and MANAE supports for 1 h and overnight was assessed and the results are shown in Table 1. Although the immobilization yields on DEAE and MANAE agarose were high (>98%), the structure of the support (Fig. 3(a)) allowed the enzyme immobilization inside the porous structure. The high protein load used did make it difficult to access all immobilized molecules, probably due to intraparticle diffusional delays, mainly using a substrate rapidly hydrolyzable as p-NPP. This effect likely explains the low expressed activity on these supports (<3%). This problem was not noticeable for ACPase immobilized on the Toyopearl support because its structure (Fig. 3(b)) allowed enzyme immobilization only on the external surface. In fact, for both ACPases, this support allowed high immobilization yields (>95%) and expressed activity (>85%) (Table 1).

Immobilization on the TOYO support was smoother. It occurs on the external surface where the positively charged groups are activated (Fig. 3(b)); as it is an acrylic support formed by smooth cross-linking, the resulting fibers are thin and smaller than the enzyme. The hydrophobicity of the support matrix can be enhanced by the butyl or hexyl groups that line the surface [31,33]. On the other hand, agarose is a hydrophilic support that, when activated with octyl or butyl groups (which increase the interfacial activation of the enzyme), becomes hydrophobic, providing higher enzyme-support interaction [31,33]. High immobilization yields were also previously reported by Kalita and Ambasht [31] for wheat germ acid phosphatase (Type 1) immobilized on agarose gel (75% IY) and Srivastava and Anand [33] for acid phosphatase from seeds of *Vigna aconitifolia* immobilized on chitosan granules activity with 2% glutaraldehyde (83% IY).

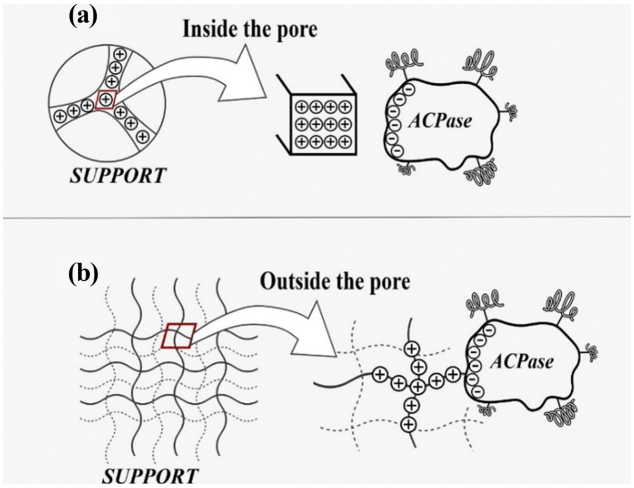

Fig. 3. Representative scheme for ionic adsorption strategy. (a) Support activated in the pore, for example DEAE Sepharose; (b) Support activated on the external surface, for example Toyopearl DEAE-650s. DEAE and MANE supports can offer diffusional delays since the enzyme is preferentially immobilized inside the pore structure. On the other hand, is not the case of Toyopearl support since the enzyme is immobilized outside the pore.

3. Effect of Oxidation/Reduction of Glycosylated ACPases Immobilized on Toyopearl on Thermal Stability

Fig. 4 shows the results of inactivation assays of ACPases from wheat germ and potato (free, adsorbed, and adsorbed followed by oxidation/reduction of glycosyl chains). The results show that immo-

Table 1. Immobilization of ACPase from wheat germ and potato in different kind of ion exchange supports

SUPPORT	Anion exchange	Condition/ Strategy	Wheat germ ACPase Derivative		Potato ACPase Derivative	
			Yield (%)	Expressed activity (%)	Yield (%)	Expressed activity (%)
Toyopearl DEAE-650s	R1-O-CH_2-CH_2-HN^+-$(C_2H_5)_2$	pH 7, 1 h (just immobilized)	97.34±0.34	96.28±3.18	95.68±2.59	85.27±2.06
Toyopearl DEAE-650s	R1-O-CH_2-CH_2-HN^+-$(C_2H_5)_2$	$NaIO_4$ and $NaBH_4$ treatment (after immobilization)	97.34±0.34	73.31±5.96	95.68±2.59	76.41±3.58
DEAE Sepharose 6BC	R2-O-CH_2-CH_2-HN^+-$(C_2H_5)_2$	pH 7, overnight	99.15±0.40	2.76±1.49	95.50±1.20	5.63±0.97
MANAE Agarose 6BC	R3-CH_2-NH_2^+-CH_2-CH_2-NH_2	pH 7, overnight	98.08±0.77	3.21±1.04	91.70±0.88	4.74±1.13

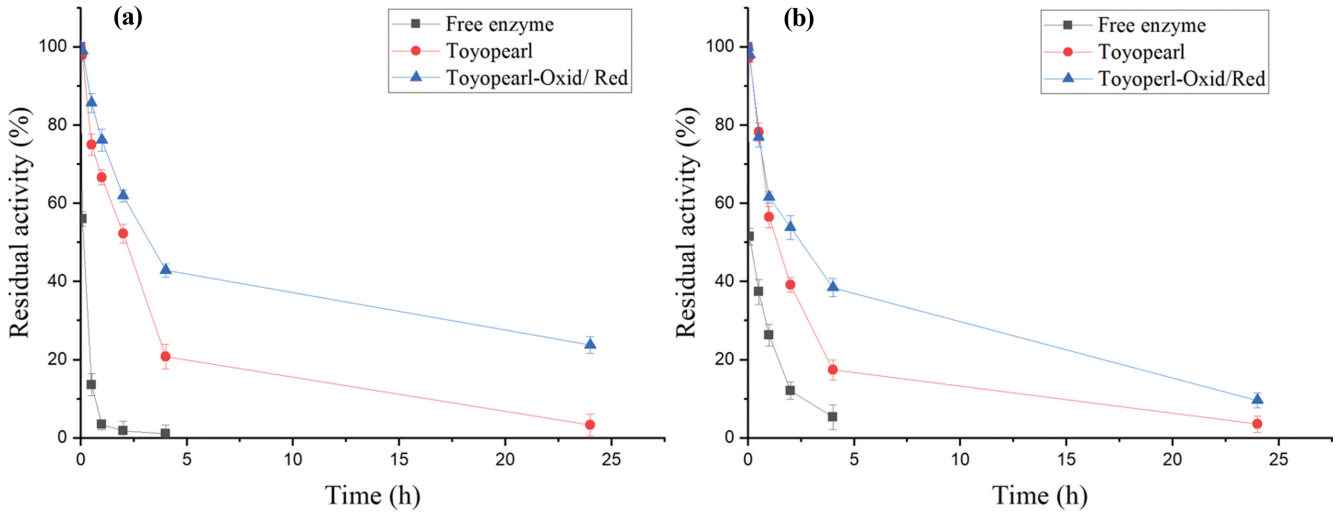

Fig. 4. Thermal inactivation profile at 60 °C and pH 7.0 of the biocatalyst: (a) ACPase from wheat germ and (b) ACPase from potato - (■) free enzyme, (●) enzyme just immobilized in Toyopearl DEAE-650s, (▲) enzyme immobilized in Toyopearl DEAE-650s and also oxidized and reduced.

bilized enzymes were more stable than the free enzymes, and the thermal stability could be improved by post-immobilization treatment (oxidation with periodate followed by reduction with sodium-borohydride).

The free ACPase from the wheat germ (Fig. 4(a)) showed a half-life of 8 min and only 13.6% of its initial activity in the first 30 min, inactivating entirely after 2 hours of incubation. Its derivatives immobilized in TOYO with different conditions showed higher stability under these conditions. In 2 h of the experiment, TOYO single step maintained 52.3% of the initial activity, exhibited a half-life of 2.05 hours, and inactivated by approximately 80% after 4 hours. Higher stability was observed for the oxidized and reduced TOYO derivative, which presented 61.9% of the initial enzymatic activity after 2 hours, a half-life of 3.45 hours, and was able to maintain 23.8% of the initial enzymatic activity after 24 hours of incubation at 60 °C. The TOYO single step and the oxidized and reduced TOYO were approximately 15 and 41 times more stable than the free enzyme from wheat germ.

The potato ACPase derivatives also showed higher stability when compared to the soluble enzyme incubated at 60 °C, pH 7.0 (Fig. 4(b)) as with the ACPase from wheat germ derivatives immobilized in TOYO. The soluble ACPase from the potato had a half-life of 5 min, was inactivated by approximately 75% in the first hour of incubation, and after 6 hours it no longer showed enzymatic activity. In the results obtained with TOYO derivatives immobilized in different conditions, both presented similar results in the first hour of the experiment, inactivated by approximately 77.8%. After 2 hours of incubation at 60 °C, the TOYO one-step derivative showed 39.2% of the initial activity with a half-life of 1.75 hours. After 4 hours, only 17.4% of the initial activity could be observed, twice as low as the oxidized and reduced TOYO activity derivative in the same incubation time. The oxidized and reduced TOYO derivative had a half-life of 2.60 hours. After 24 hours, it showed a residual activity of 9.6%, while both the soluble enzyme and the TOYO one-step derivative were completely inactivated. The TOYO single step and the oxidized and reduced TOYO were approximately 21 and 31 times more stable than the free potato enzyme.

Periodate hardly affected the enzyme activity during the oxidation stage to obtain the derivative. However, borohydride in concentrations greater than 0.1 mg/mL causes a loss of enzyme activity. Thus, considering that both soluble enzymes (wheat germ and potato) are very unstable with a half-life of less than half an hour at 60 °C, the results show that the method of oxidation and reduction of the TOYO derivative for both enzymes offers higher stability at this temperature. It is hypothesized that the crosslinking between the aldehyde groups generated in the oxidation and the amino groups of the enzymes was able to protect their active site (Fig. 5).

This is the first work to apply this technique to ACPase in the scientific literature. Still, Pinotti et al. [14] studied the immobilization of a glycosylated β-glucosidase by performing oxidation and reduction of the enzyme before immobilization on support, achieving better stability due to the conversion of the glycoside chains into similar polyhydroxy with low effect on enzymatic stability. Fig. 6 shows the results obtained in the experiments carried out to evaluate the influence of temperature on the activity of the ACPases from wheat germ and potato and their derivatives immobilized under different conditions.

The glycosylation technique with oxidation and reduction was used to stabilize the support enzyme set. Acid phosphatases from potato and wheat are a phosphomonoesterases, which can appear in multiple molecular forms of similar molecular mass but with different isoelectric point in the range of 4.7 to 7.0. The Toyopearl DEAE-650s is an anion exchanger support in the pH range of 2 to 13, maintaining stability during the pH and ionic strength changes in this range.

The enzymatic reactions were carried out under different temperature conditions. The ACPase from wheat germ derivatives immobilized in TOYO (one-step and oxidized/reduced) showed an optimum temperature around 50 °C, higher than that achieved for the soluble enzyme (45 °C) (Fig. 6(a)). In the study by Kalita and

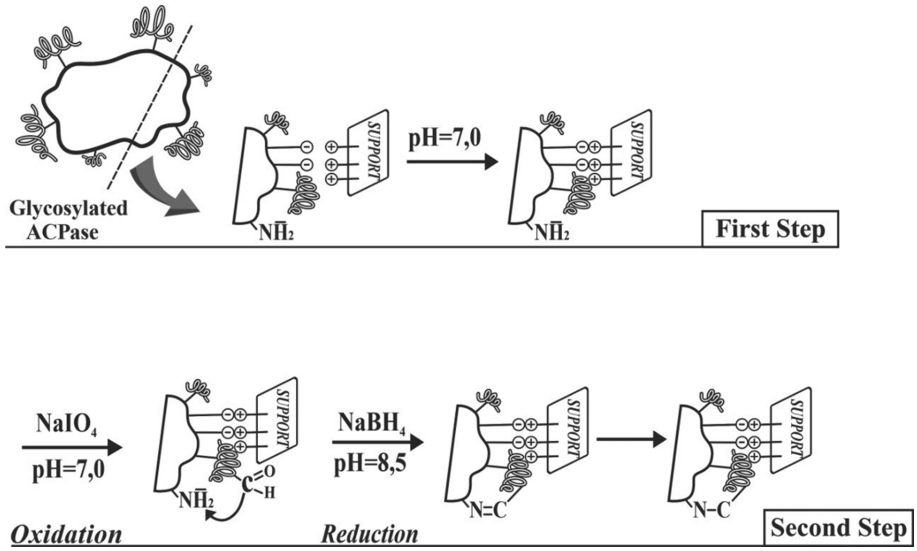

Fig. 5. Ion adsorption mechanism of ACPases in the Toyopearl DEAE-650s support. First step immobilization was performed using 25 mM, pH 7.0 of Tris hydrochloride buffer. The second stage was the oxidation and reduction of the ACPase glycoside chains in the derivative.

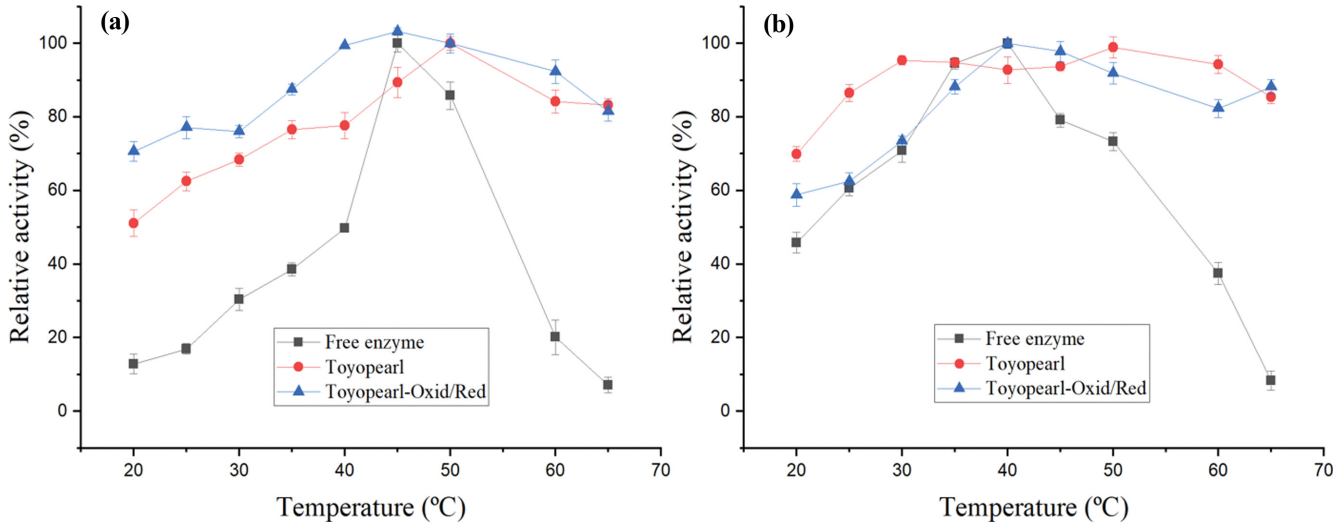

Fig. 6. Influence of temperature on the activity of: (a) ACPase from wheat germ and (b) ACPase from potato - (■) free enzyme; (●) enzyme just immobilized in Toyopearl DEAE-650s; (▲) enzyme immobilized in Toyopearl DEAE-650s and also oxidized and reduced.

Ambasht [30], the soluble wheat germ ACPase showed an optimum temperature of 50 °C while its derivative immobilized on agarose gel showed an optimum temperature of 60 °C. For the potato ACPase, the soluble enzyme and the oxidized and reduced TOYO derivative showed an optimum temperature of 40 °C. For the derivative immobilized in TOYO one-step, the optimum temperature was increased by 10 °C (Fig. 6(b)). Another result to consider was the excellent activity recovered by 70% or more at temperatures above 40 °C, indicating an increase in thermal resistance for derivatives immobilized in TOYO (one step and oxidized/reduced) in both enzymes.

The increase at an optimum temperature of immobilized ACPase has also been reported in the literature [33,34]. The reason for immobilized ACPase higher optimum temperature may be due to changes in the conformation of the enzyme after immobilization or diffusional limitation of the substrate to the enzyme's active site [35].

4. Assessment of the Presence of Aldehyde and Amine Groups in the Oxidized and Reduced TOYO Derivative

Qualitative analyses with a picrylsulfocromic solution were carried out to evaluate the presence of aldehyde and amine groups in the oxidized/reduced biocatalyst. The dyeing of the biocatalyst with orange pigmentation was indicative of free amine groups on the enzyme surface. Schiff's reagent was also used to verify the presence of aldehyde groups on the biocatalysts. The dyeing of the derivative with a purple-pink pigmentation indicated the presence of free aldehyde groups on the enzyme surface.

Once the picrylsulfocromic solution was added to the immobi-

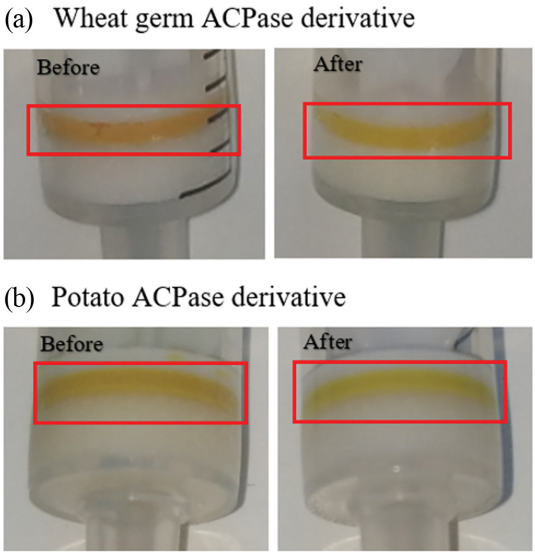

Fig. 7. ACPases immobilized on TOYO treated with pycrilsulfocromic solution. (a) ACPase from wheat germ derivative before and after oxidized and reduced; (b) ACPase from potato derivative before and after oxidized and reduced.

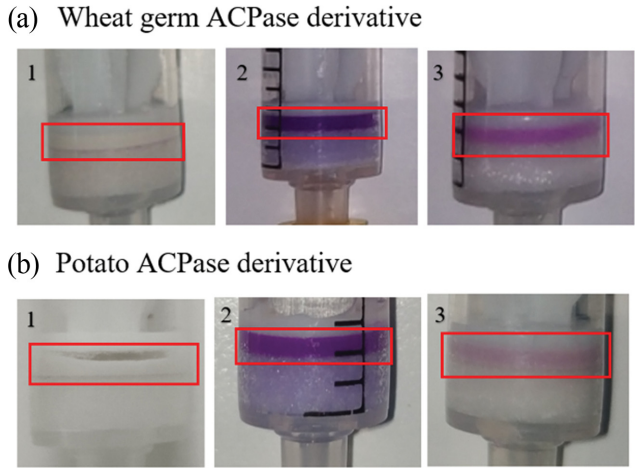

Fig. 8. ACPases immobilized on TOYO treated with Schiff`s reagent. (a) ACPase from wheat germ; (b) ACPase from potato. 1: immobilized ACPases derivatives; 2: ACPases derivatives oxidized; 3: ACPases derivatives oxidized and reduced.

lized derivatives of both ACPases, an intense orange color was observed, indicating free primary amino groups on the support surface (Fig. 7). Both products appeared yellow after the oxidation and reduction step, indicating that amine groups formed aldehyde-amine crosslinks.

After adding Schiff's reagent (Fig. 8), the immobilized derivatives showed no dyeing in pink/purple before oxidation due to the absence of aldehyde groups on the enzyme surface, for either wheat germ ACPase or potato ACPase. After the oxidation of glycosyl chains with periodate, an intense purple color was established in both cases, indicating the notable presence of aldehyde groups. After reduction with borohydride, the enzymatic derivatives were weakly dyed pink, showing that this step converted the remaining unbound aldehyde into hydroxyl groups. The results show that the oxidation and reduction steps after the immobilization of ACPases studied on TOYO contributed to the derivatives' thermal stability due to the formation of aldehyde-amine crosslinks.

CONCLUSIONS

Free soluble enzymes were unstable at low (4 °C) and high (60 °C) temperatures. They also performed poorly at some pH values (3.0, 8.5, and 10.0). On the other hand, Toyopearl DEAE-650s was a promising support for the immobilization of ACPases, with immobilization yields (%) above 95 and recovered activities (%) above 85, for both enzymes. The biocatalyst activity was further improved at high temperatures by introducing a second step in the immobilization process. Both immobilized derivatives showed residual activity after 1 hour of inactivation at a temperature of 60 °C, while these results were not observed for the free enzyme. The wheat germ ACPase derivative was the most active and stable, with residual activity of 66.7% for the only immobilized derivative and 76.2% for the oxidized/reduced biocatalyst. In addition, the TOYO single step and the oxidized and reduced TOYO were approximately 15 and 41 times more stable than the free enzyme from wheat germ. When the enzymatic reactions were carried out under different temperature conditions, both immobilized ACPases showed optimum temperatures above that obtained for the free enzyme, indicating that ACPases properties can be modulated by immobilization techniques. Excellent recovered activities of 70% or more at temperatures above 40 °C indicate increased thermal resistance for derivatives immobilized in TOYO (one-step and oxidized/reduced) in both enzymes.

ACKNOWLEDGEMENTS

The authors acknowledge the FEQUI/UFU, CNPq, FAPEMIG (Process: APQ - 00874-18) and CAPES (Brazil) for financial support and also acknowledge the ICP - CSIC institute (Spain) for allowing to this work to be carried out.

DECLARATIONS

Funding The funds are described in the acknowledgments
Conflicts of interest/Competing interests The authors declare no conflict of interest
Availability of data and material Not applicable
Code availability Not applicable
Authors' contributions - Not applicable
Ethics approval Not applicable
Consent to participate Not applicable
Consent for publication Not applicable

REFERENCES

1. A. Anand and P. K. Srivastava, *Appl. Biochem. Biotechnol.*, **167**, 8 (2012).
2. B. C. Behera, S. K. Singdevsachan, R. R. Mishra, S. K. Dutta and H. N. Thatoi, *Biocatal. Agric. Biotechnol.*, **3**, 97 (2014).

3. C. K. Tagad and S. G. Sabharwal, *J. Food Sci. Technol.*, **55** (2018).
4. A. A. Souza, V. O. Leitão, M. H. Ramada, A. Mehdad, R. C. Georg, C. J. Ulhôa and S. M. Freitas, *PLoS ONE*, **11**, 3 (2016).
5. L. Zhao, Q. Liu, Y.-Q. Zhang, Q.-Y. Cui and Y.-C. Liang, *J. Integr. Agric.*, **16**, 6 (2017).
6. S. M. G. Duff, G. Sarath and W. C. Plaxton, *Physiol Plant*, **90** (1994).
7. P. A. Granjeiro, A. D. M. Cavagis, L. C. Leite, C. V. Ferreira, J. M. Granjeiro and H. Aoyama, *Mol. Cell Biochem.*, **266** (2004).
8. L. Gianfreda and M. A. Rao, *Enzyme Microbial Tech.*, **35**, 4 (2004).
9. C. F. Hoehamer, C. S. Mazur and N. L. Wolfe, *J. Agric. Food Chem.*, **53**, 1 (2005).
10. S. Muniyan, N. K. Chaturvedi, J. G. Dwyer, C. A. Lagrange, W. G. Chaney and M. F. Lin, *Int. J. Mol. Sci.*, **14** (2013).
11. I. B. Quintero, A. M. Herrala, C. L. Araújo, A. E. Pulkka, S. Hautaniemi, K. Ovaska, E. Pryazhnikov, E. Kulesskiy, M. K. Ruuth, Y. Soini, R. T. Sormunen, L. Khirug and P. T. Vihko, *PLoS One*, **8**, 9 (2013).
12. S. Sebastian, S. P. Touchburb, E. R. Chavez and P. C. Lague, *Poult. Sci.*, **75** (1996).
13. M. R. A. Azeem, A. N. Chaudhary, R. Hayat, Q. Hussain, M. I. Tahir and M. Imran, *Arch. Agron. Soil Sci.*, **6** (2014).
14. L. M. Pinotti, P. W. Tardioli, C. S. Farinas, G. Fernández-Lorente, A. H. Orrego, J. M. Guisan and B. C. Pessela, *Appl. Biochem. Biotechnol.*, **192** (2020).
15. R. A. Sheldon and S. Van-Pelt, *Chem. Soc. Rev.*, **42** (2013).
16. D. Brady and J. Jordaan, *Biotech. Lett.*, **31** (2009).
17. A. Liese and L. Hilterhaus, *Chem. Soc. Rev.*, **42** (2013).
18. U. Guzik, K. Hupert-Kocurek and D. Wojcieszunska, *Molecules*, **19**, 17 (2014).
19. T. Kawai, K. Saito and W. Lee, *J. Chromatog. B. Anal. Technol. Biomed Life Sci.*, **790** (2003).
20. P. R. Levison, *Chromatogr. Performance Adv. Synth. Catal.*, **349** (2007).
21. R. A. Sheldon, *Synth. Catal.*, **349** (2007).
22. B. Krajewska, *Enzyme Microb. Technol.*, **35** (2004).
23. X. D. Tong, X. Y. Dong and Y. Sun, *Biochem., Eng. J.*, **12** (2002).
24. A. Lyddiatt, *Curr. Opin. Biotechnol.*, **13** (2002).
25. T. L. Albuquerque, S. Pierce, N. Rueda, A. Marzochella, L. R. B. Gonçalves, M. V. P. Rocha and R. Fernadez-Lafuente, *Process Biochem.*, **51**, 7 (2016).
26. V. O. Leitão, R. C. M. Lima, M. H. Vainstein and C. J. Ulhoa, *Biotechnol. Lett.*, **32**, 8 (2010).
27. B. N. Ames, *Meth. Enzymol.*, **8** (1966).
28. R. Fernandez-Lafuente, P. Armisén, P. Sabuquillo, G. M. Fernández-Lorente and J. M. Guisan, *Chem. Phys. Lipids*, **93** (1998).
29. J. M. Guisan, *Enzym. Microb. Technol.*, **10** (1988).
30. L. Trobo-Maseda, A. H. Orrego, J. M. Guisan and J. Rocha-Martin, *Int. J. Biol. Macromol.*, **157** (2020).
31. T. Kalita and P. K. Ambasht, *J. Protein Proteomics*, **10** (2019).
32. G. Fernandez-Lorente, Z. Cabrera, C. Godoy, R. Fernandez-Lafuente, J. M. Palomo and J. M. Guisan, *Process Biochem.*, **43** (2008).
33. P. K. Srivastava and A. Anand, *Int. J. Biol. Macromol.*, **64** (2014).
34. J. Zhu, Q. Huang, M. Pigna and A. Violante, *Colloid Surf. B*, **77** (2010).
35. K. R. C. Reddy and A. M. Kayastha, *J. Mol. Catal. B Enzym.*, **38** (2006).