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Short communication

Optimization of the immobilization of sweet potato amylase using glutaraldehyde-agarose support. Characterization of the immobilized enzyme

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ABSTRACT

A simplified procedure for the preparation of immobilized beta-amylase using non-purified extract from fresh sweet potato tubers is established in this paper, using differently activated agarose supports. Beta-amylase glutaraldehyde derivative was the preparation with best features, presenting improved temperature and pH stability and activity. The possibility of reusing the amylase was also shown, when this immobilized enzyme was fully active for five cycles of use. However, immobilization decreased enzyme activity to around 15%. This seems to be mainly due to diffusion limitations of the starch inside the pores of the biocatalyst particles. A fifteen-fold increase in the Km was noticed, while the decrease of Vmax was only 30% (10.1 U mg^{-1} protein and 7.03 U mg^{-1} protein for free and immobilized preparations, respectively).

1. Introduction

Beta-amylases are enzymes that attack the alpha-1,4-glucan bonds from the non-reducing ends of starch, and convert it and other carbohydrate polymers to maltose units. Maltose, a two glucose disaccharide, has many applications in food and pharmaceutical industries [1–4]. Amylases are widely present in microorganisms, plants and animals, and have found applications in numerous industries, mainly starch liquefaction [5–7]. Maltose, produced via beta-amylolysis, confers to cooked roots the sweetness characteristic of the traditional sweet potato [8]. Although starch liquefaction can be accomplished via chemical processes, enzymatic hydrolysis can be performed under mild conditions, and could avoid the extreme conditions required by the chemical treatments. Furthermore, the enzymatic process did not produce water pollution, which is very common in chemical processes [9].

The use of immobilized enzymes in industrial processes, in comparison with the use of soluble enzymes, could reduce process costs by reducing the quantity of enzyme required, since the immobilized derivative can be recovered at the end of a hydrolysis cycle and reused, as long as the enzyme remains active for several reaction cycles. Thus, enzyme immobilization and stabilization should be related terms and, in fact, a proper immobilization may improve enzyme stability via multipoint or multisubunit immobilization [10,11]. Enzyme immobilization and its application in continuous processes are desirable, as long as an end product of high purity is obtained, as is typical of enzymatic processes [12,13]. Different supports can be used to immobilize an enzyme via different physical or chemical phenomena, perhaps involving different regions of the protein and yielding different orientations [14]. As has been previously described, reversible immobilization and covalent immobilization may be more or less adequate depending on the requirements [15]. The ionic exchange of a very stable enzyme on an anion exchanger (e.g., aminated supports) may provide a way of reusing the enzyme several cycles, and recover the support and reuse it again after inactivation [15]. However, if stabilization is pursued, an intense multipoint covalent attachment between the enzyme and the support should be the objective of the immobilization [10]. This strategy can produce a very high stabilization of the enzyme by reducing the mobility of the enzyme structure, although may be difficult to optimize.

The choice of an appropriate support and suitable immobilization conditions can favor reaching high stabilization factors and good activity values of immobilized amylases, simplifying the use of this enzyme in different industrial applications. Glyoxyl [16] and glutaraldehyde [17] activated supports have been described as ade-

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quate to produce stabilization via multipoint covalent attachment. Glyoxyl-agarose support immobilizes the enzyme by the richest in Lys residues areas [18,19], and glutaraldehyde-agarose can immobilize by the most reactive amino group (likely the terminal one) or by the most negatively charged areas, or even by hydrophobic interactions depending on the conditions of immobilization [17,20].

In amylase immobilization, one problem to be considered is the large size of the substrate, the starch [6,21]. In these cases, enzyme activity may decrease by different causes. As it occurs for any enzyme, the activity may decrease if the enzyme areas relevant for its activity become distorted [22]. But in this case, the enzyme activity will also be hindered by two diffusion problems. First, the large size of the substrate may produce diffusion limitations to the entry of this large molecule to the pores of the biocatalyst [15]. Second, if the active center is oriented towards the support surface, it will be unavailable for the substrate [14]. Moreover, sweet potato beta amylase presents an additional problem for immobilization. Crystallographic studies demonstrate that the enzyme is a tetramers [23,24]. This means that immobilization of all enzyme subunits will be convenient to prevent subunits dissociation, stabilized also each monomer via multipoint covalent attachment, and in any case, reducing the risks of final product contamination by the enzyme [11].

In this study, amylase extracted from sweet potato was immobilized on agarose beads activated following different protocols and the best derivative was further characterized.

2. Material and methods

2.1. Materials

Agarose 6B beads were from Amersham Biosciences. Soluble starch potato, dinitrosalicylic acid, sodium periodate, sodium bohohydride were from Sigma-Aldrich. Ethylendiamine was from Fluka, and glutaraldehyde 25% was from Vetec. The sweet potatoes used were obtained at the local market.

2.2. Methods

Experiments were performed by triplicate and the values are the mean of at least 3 independent experiments. Standard deviations were always under 10%.

2.2.1. Amylase extraction

The sweet potatoes used were obtained at the local market. The fresh tuberous roots were washed, peeled and diced. Fresh sweet potato tuber just after collection were used, to avoid high alpha amylase contamination, considering that alpha amylase tended to increase during storage [8,25]. For the beta-amylase extraction, 50 g of the potato pieces were added to 100 mL of cold distilled water and pulverized. After centrifugation (7000 × *g*/20 min/4 °C) the supernatant (amylase extract) was used for all assays. The enzyme extract presented about 4.7 mg protein mL^{-1} and a specific activity of 17.05 U mg^{-1} protein. No further purification methods were used.

2.2.2. Protein determination

Protein concentration was determined by Bradford method [26], using bovine serum albumin (BSA) as standard.

2.2.3. Enzymatic activity

Enzyme activity was determined using starch as substrate (1% w/v in 100 mM citrate-phosphate buffer at pH 6.0). Starch hydrolysis (50 °C) was monitored by determination of reducing sugar using dinitrosalicylic acid method at 540 nm [27]. A standard curve was prepared with maltose. One beta-amylase activity unit (U) was defined as the amount of enzyme capable of producing 1 μMol of maltose per minute under assay conditions.

2.2.4. Supports preparation

Agarose 6B was from Amersham Biosciences. Glyoxyl-agarose was prepared with the maximal activation degree, as previously described [28]. The consumption of periodate (directly related to the aldehyde residue generated in the support) was checked after oxidation process, adding 0.2 mL of the supernatant of the oxidizing suspension to a mixture of 1.5 mL of 10% (w/v) KI and 1.5 mL of saturated sodium bicarbonate. The absorbance was read at 419 nm, considering the initial sodium periodate solution as 100% (0% aldehyde production). Monoaminoethyl-N-aminoethyl (MANAE)-agarose, was prepared as described elsewhere [29] and the glutaraldehyde-agarose support was prepared as previously described [17].

2.2.5. Enzyme Immobilization

The different immobilization supports were suspended in an enzyme solution (10 g agarose beads:100 mL enzyme solutions in immobilization buffers). The immobilization buffers differ for each immobilization protocol: 100 mM sodium bicarbonate buffer pH 10.2 when immobilization on a glyoxyl support was performed [18]; 5 mM sodium phosphate buffer pH 7.0 for immobilization on MANAE support; or 200 mM sodium phosphate buffer pH 7.0 for immobilization on glutaraldehyde support [17,20]. The immobilization suspensions were gently stirred at room temperature. Different enzyme concentrations were tested. For all immobilization experiments, samples of the suspensions and the supernatants were periodically withdrawn and enzyme activity of the samples was determined as described above. A reference enzyme suspension identical to each immobilization suspension was prepared, using inert agarose, as a reference.

The biocatalysts prepared using glyoxyl agarose were reduced by adding sodium borohydride to reach 1 mg mL^{-1} [16] and maintained under agitation for 30 min. When using glutaraldehyde supports, the immobilized enzyme was recovered by filtration and resuspended in 10 volumes of 100 mM bicarbonate buffer at pH 10.2 containing 1 mg mL^{-1} sodium borohydride for 30 min under mild stirring. This treatment reduces the remaining aldehyde groups and the imino bonds. The resulting derivatives were washed with abundant distilled water and, before use, with activity buffer.

2.2.6. Effect of temperature and pH on the free enzyme and derivatives

Enzyme activity was determined as described above, using different temperatures and different pH values (citrate-phosphate buffer for pH 3.0–8.0 range and glycine –NaOH buffer for pH 9.0 and 10.0).

2.2.7. Thermal and pH stability

Aliquots of the immobilized and free enzyme samples were incubated at different pH values for 30 min, at 25 °C, and the remaining activity was assayed at pH 6.0, for pH stability determination. When thermal stability was assayed, aliquots of the immobilized and free enzyme were incubated at pH 6.0, at 60 °C. Periodically, samples of these suspensions or solutions were withdrawn, placed in ice baths for 30 s and the remaining activities assayed. For both studies the initial activity is regarded as 100%, and residual activity was expressed as a percentage of initial activity.

2.2.8. Km and Vmax determination

In order to calculate the Km and Vmax values, starch was used in increasing concentrations, as described by Chang and Juang [30] (using 1.0–16.0 mg mL^{-1}), prepared in pH 6.0, to free enzyme, and at pH 6.5 for the enzyme immobilized form, at 50 °C. Lineweaver-Burk plots were used to determine the kinetic parameters.

2.2.9. Reuse assay

The immobilized enzyme was used as described in 2.2.3 but for several cycles. At the end of each cycle, the derivative was washed with distilled water and activity buffer and a new substrate solution was added to start a new round of reaction. The initial and remaining activities were assayed at pH 6.0, 50 °C (initial activity at these conditions is regarded as 100%).

3. Results and discussion

3.1. Selection of the immobilization support

Derivatives were prepared using high activated supports with the objective of achieving an intense multipoint covalent attachment or an intense adsorption [10,15]. Through titration of the remaining sodium periodate, an activation degree of 106 micromoles of glyoxyl groups mL^{-1} packed agarose beads was determined. Therefore, we can assume that each enzyme molecule has many reactive groups under its surface after immobilization. If the enzyme interacts with the supports through many points, enzyme stability by their three-dimensional structure “rigidification” may be obtained [10,31].

The beta-amylase immobilization was performed by using glyoxyl, MANAE and glutaraldehyde-agarose supports under the conditions described in methods. Different protein:support ratios were tested for each support (data not shown). The expressed activity gradually increased whilst 100% immobilization was maintained; with the highest possible loads being obtained using MANAE and glutaraldehyde supports (Table 1). Using the glyoxyl-agarose support, it was not possible to achieve high percentages of immobilization, even using the lowest protein loading. This low loading using glyoxyl supports could be due to the lack of a region bearing many exposed Lys residues able to react with the enzyme

Table 1
Immobilization parameters of β-amylase from sweet potato on differently activated agarose supports.

Support	Initial protein charge ($\mu g\,g^{-1}$ support)	Immobilization (%)[a]	Activity Expected[b] ($U\,g^{-1}$ derivative)	Derivative Activity ($U\,g^{-1}$ derivative)	Activity Recovered[c]
Glyoxyl	40	47.2%	0.35	0.0734	20.6%
MANAE	154.8	100%	2.6	0.1934	7.3%
Glutaraldehyde	163.9	100%	2.8	0.4643	16.6%

[a] (Initial protein concentration − final protein concentration in supernatant solution/initial protein concentration) × 100.
[b] Considering specific activity of enzyme extract equivalent to 17.05 U mg^{-1} protein.
[c] (Total activity in derivative/Total initial activity) × 100.

(glyoxyl groups are able only to fix protein to the support if multipoint covalent attachment is established [18]), or due to steric problems for the reaction between an area rich in Lys of the protein and the support due to the presence of sugar chains, as this enzyme is glycosylated [32]. Although the three supports were able to immobilize the enzyme, the expressed activities were very low, especially when the MANAE support was used (Table 1). This could be due to enzyme desorption during washings if the enzyme immobilization was quite weak. However, the activity of the suspension before washing was as low as the activity recovered after washings, suggesting that a real inactivation of the enzyme had occurred.

The glutaraldehyde-agarose derivative was the support that gives the highest immobilization yields and expressed activity among the three assayed ones (Table 1). Based on these observations, the glutaraldehyde immobilized derivative has been used for all further studies.

3.2. Kinetic parameters of beta-amylase immobilized on glutaraldehyde-agarose beads

The moderate activity recovered even in the case of the glutaraldehyde support may be due to the three reasons explained in introduction: enzyme distortions, external diffusion limitations or steric problems to the enzyme-substrate interaction generated by the support surface. Immobilization procedure raised the Km value by about 15 times, while decreasing the Vmax by only a 30% (Table 2). The apparent increase in Km and the relative good conservation of Vmax for the glutaraldehyde derivative (Table 2) suggested that the main problem is just diffusion problems for the entry of the substrate inside the biocatalyst particle. In fact, the milling (just by magnetic stirring) of the catalyst particle permitted to increase the observed activity. The negative effects of immobilization on both kinetic constants decreased the catalytic efficiency of beta-amylase (Vmax/Km values showed in Table 2) by a 20 fold factor. Chang and Juang [30] also observed a decrease in apparent affinity between beta-amylase and starch when the enzyme was immobilized on chitosan-clay composite, although the Km increase was only about 2.5 times. The Km for the free enzyme was very near to our study (2.4 mg mL^{-1}). In Roy and Edge's [33] study, when the enzyme was immobilized on polystyrene cation exchange resin equilibrated with Al^{3+} íons, its Km increased about 6 times. Differences on the particles size, pores diameter and enzyme loading may drastically influence the effect of diffusional limitations on the expressed activity of immobilized enzymes [15,22].

Table 2
Kinetic parameters for starch hydrolysis with free and immobilized β-amylase-glutaraldehyde biocatalyst.

Enzyme preparation	Km (mg mL^{-1})	Vmax (U mg^{-1} prot)	Vmax/Km
Soluble	2.17	10.1	4.7
Glutaraldehyde-agarose	34.47	7.03	0.20

3.3. Characterization of the beta amylase immobilized on glutaraladehyde agarose beads

The goal of these experiments was not to find the optimal conditions for the different enzyme preparations, but to show a first comparison between the immobilized and the free enzyme. Fig. 1A shows the effect of pH on activity of free and immobilized beta-amylase. Both preparations have better results when citrate-phosphate buffer was used, but the free enzyme presented optimal pH value at pH 6.0, while the immobilized amylase had a maximum activity at pH 6.5. Roy and Edge [33] also observed an increase in the optimum pH, from 5.0 to 5.6. Very interestingly, the immobilized amylase maintained high activity in all the range of pH values studied, even in the most extreme ones (pH 3.0, and 10.0), while the free enzyme dramatically decreased its activity by just moving

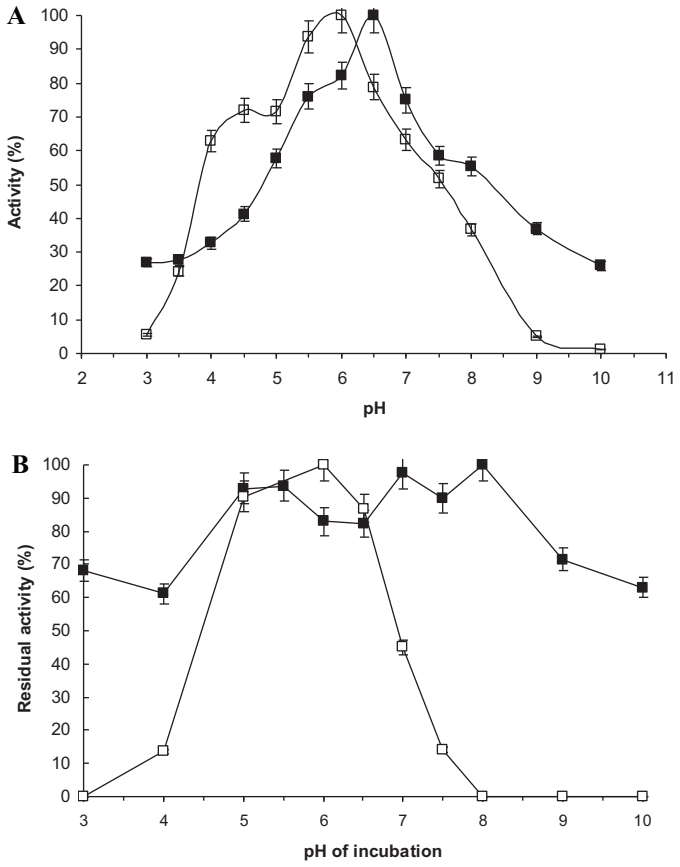

Fig. 1. Effect of pH on free (open symbol) and immobilized (closed symbol) beta-amylase activity (A) and stability (B). The effect of pH on the enzyme activity (A) was measured at 50 °C. The effect of pH on enzyme stability (B) was measured as detailed in Methods. The enzyme preparations were incubated at different pH values for 30 min at room temperature (25 °C), and the remaining activity was assayed in pH 6.0, at 50 °C (initial activity is regarded as 100%). For all measurements were used citrate-phosphate buffer (pH 3.0–8.0) and Glicin-NaOH buffer (pH 9.0–10.0).

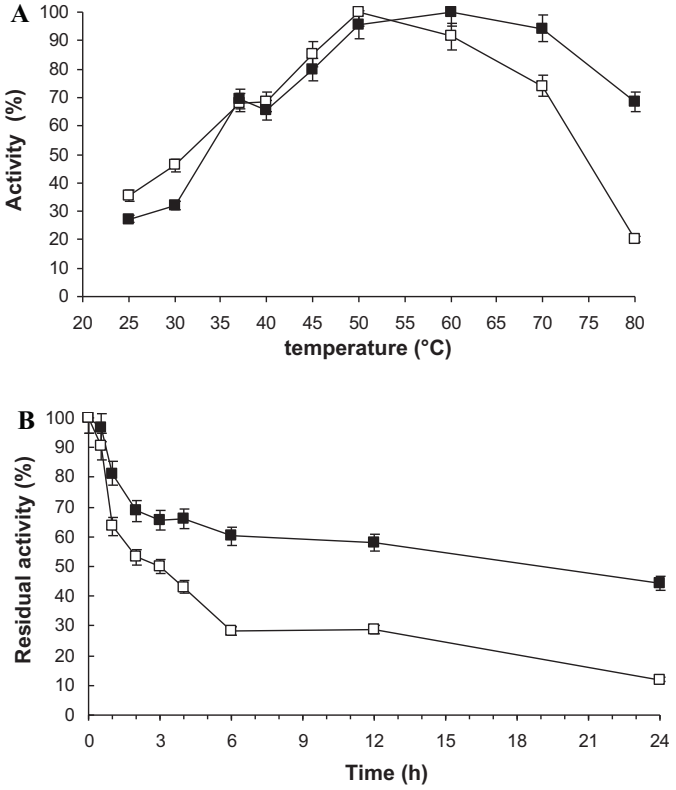

Fig. 2. Effect of temperature on activity (A) of free (open symbol) and immobilized (closed symbol) beta-amylase from sweet potato. Thermal inactivation courses of free and immobilized enzyme (B) were studied at 60 °C and pH 6.0. Remaining activity was assayed at 50 °C (The initial activity is regarded as 100%).

one pH unit far from the optimal pH value. In fact, at pHs higher than 8.0, the immobilized enzyme showed greater activity than the free form (Fig. 1A). This could be related to a higher stability of the enzyme. In fact, Fig. 1B shows that immobilization greatly enhanced the stability of the enzyme at the highest and lowest studied pH values. The free enzyme can not withstand the incubation at pH < 4.0 or pH > 7.5 for 30 min. In contrast, the immobilized enzyme presented a retained activity over 70% even at pH 3.0 or 10.0. Thus, the improved activity of the immobilized enzyme at alkaline and acid pH value can be associated to this higher stability [22], perhaps produced by prevention of enzyme dissociation [11]. Other authors have also identified an increased performance of the immobilized beta-amylase in extreme ranges of pH. For example, activity of beta-amylase was improved at pH 4.0 and pH > 7.0 when immobilized on Chitopearl BCW 3505 beads [34].

The optimum temperature for activity was also increased after enzyme immobilization (Fig. 2A). The free enzyme presented the highest activity at 50 °C; however, the immobilized enzyme presented maximum activity at 60 °C, maintaining a high activity even up to 80 °C (at this temperature the free enzyme was almost inactive). Fig. 2B shows that the enzyme was actually more stable than the free enzyme at 60 °C, and that may be the explanation for this improved features found in the immobilized enzyme. After 12 h of incubation, the immobilized derivative retained about 60% activity, whilst the free form retained just 30% activity (Fig. 2B). The time needed to reach 50% of residual activity was about 3 h for the free enzyme and about 19 h for immobilized preparation. Rigidification of the enzyme structure via multipoint attachment and reduction of dissociation problems via multi-subunit immobilization are the likeliest explanations, for these good results.

The application of this biocatalyst in industry requires the operational stability of immobilized enzymes to be high enough. Fig. 3

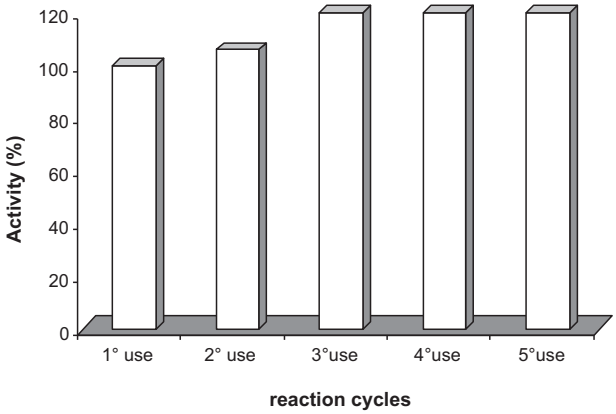

Fig. 3. Re-use of beta-amylase glutaraldehyde-agarose derivative for several reaction cycles. The initial and remaining activities were assayed at pH 6.0, 50 °C (initial activity is regarded as 100%). At the end of each cycle, the derivative was washed with distilled water and activity buffer and a new substrate solution was added to start a new round of reaction.

shows that the activity of this immobilized enzyme was fully retained for five cycles of use. This suggested that the enzyme did not suffer dissociation during use, as enzyme is submitted to several washings/dilution and the activity did not decrease.

4. Conclusions

It has been established a simplified procedure for the preparation of immobilized beta-amylase using non-purified extract from fresh sweet potato tubers. Although the procedure was very straightforward, an enzymatic derivative preparation with good catalytic properties was obtained. Beta-amylase from sweet potato is a promising element for study, in both its free or immobilized form because of its easy-to-find and cheap source, and its stability and immobilization possibility. Beta-amylase glutaraldehyde derivative has shown to be very interesting and offer advantages over their free form, very likely to the multipoint immobilization of the enzyme. It is possible to produce very stable derivatives, with both improved temperature and pH stability. The only drawback of the immobilization is the decrease in enzyme activity, that seems to be mainly due to diffusion limitations of the starch inside the pores of the enzyme, that could be modulated by using supports with higher pore diameter (e.g., agarose 4%) or by the use of lower particle size (this point may promote some difficulty to the industrial management of the biocatalyst) [22].

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