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Immobilisation of α-amylase from *Aspergillus niger* onto polyaniline

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ABSTRACT

α-Amylase from *Aspergillus niger* culture medium was immobilised on glutaraldehyde-modified polyaniline (PANIG-AMY) in a yield of 42% activity retention. Compared with the free enzyme, PANIG-AMY was less sensitive to inhibition by Zn^{2+}, Cu^{2+} and Fe^{2+}. The catalytic efficiencies of hydrolysis of starch (potato, cassava, wheat, maize and rice/maize) were similar for free and PANIG-AMY. Oligosaccharides were formed following the hydrolysis of potato starch by PANIG-AMY whereas the free enzyme produced oligosaccharides and glucose. PANIG-AMY retained 50% of its activity after repeated assay and storage at 5 °C and pH 7.0. The thermal stability of PANIG-AMY was increased by the presence of $CaCl_2$. The repeated batch-wise hydrolysis of potato starch using 5.0 mg of PANIG-AMY (0.8 U α-amylase) produced 1.7 μmol of reducing sugar per cycle, yielding a total of 25.5 μmol of reducing sugar after 15 cycles. The same yield would require 12.5 U of free α-amylase.

Keywords: α-Amylase; Immobilisation; Polyaniline; Starch hydrolysis; Oligosaccharides

1. Introduction

Amylases have attracted considerable research interest in recent years in respect of their application in the food and fermentation industries in general, and in the hydrolysis of starch and the production of oligosaccharides in particular (Kang et al., 1997). Moreover, considerable efforts have been made to produce amylases on a large-scale and at low cost for use in such industrial processes. In this context, immobilised amylases present many advantages including repeated use, application in continuous bio-processing, easy removal of the enzyme or product from the reaction medium, high thermal stability, low energy demand, and close control of product formation (Fernandes et al., 2003).

One of the most studied α-amylase is the acidic α-amylase from *Aspergillus niger*. Its amino acid sequence and three-dimensional structure have been fully elucidated (Boel et al., 1990). The enzyme exhibits some interesting pH and temperature characteristics, which could be exploited in designing novel industrial applications (Mitidieri et al., 2006). Although considerable amount of data available about acidic α-amylase from *A. niger*, there is no information relating to its immobilisation and subsequent application in the hydrolysis of starch.

In the present study a multivariate method has been employed in order to optimise a procedure for the immobilisation onto a glutaraldehyde-activated polyaniline support of α-amylase present in the culture medium of *A. niger*. The effects of immobilisation on enzymatic activity, storage, thermal stability and repeated use were also investigated.

2. Materials and methods

2.1. Chemicals

Starches derived from cassava, maize, wheat and a mixture of maize and rice were of commercial grade and obtained from the local market. Potato starch, aniline and 3,5-dinitrosalicylic acid (DNS) were purchased from Sigma–Aldrich (St. Louis, MO, USA). All other reagents were of analytical grade. Solutions were prepared using deionised distilled water.

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2.2. Production of α-amylase

Cultures of *A. niger* (strain L119) were from the collection of Fundação André Tosello, Campinas, São Paulo, Brazil. The isolation of α-amylase was performed according to a published method (Mitidieri et al., 2006). The cell-free dialysate showed a specific α-amylase activity of 0.26 U mg^{-1} protein, and was separated into aliquots that were appropriately diluted for the immobilisation experiments.

2.3. Polymer synthesis and activation

Polyaniline (PANI) was synthesised and activated with glutaraldehyde as described previously (Fernandes et al., 2003). The resulting PANIG was exhaustively washed to assure glutaraldehyde was completely removed. Then, PANIG was dried and stored at room temperature until required for immobilisation experiments.

2.4. Immobilisation of α-amylase

A multivariate method with a 2^3 factorial design was employed in order to optimise pH, immobilisation time and enzyme amounts for the efficient immobilisation of α-amylase. The responses were analysed with the aid of GNU-Octave version 2.1.50 [GNU-Octave, 2003]. Optimal immobilisation of the enzyme was carried out by mixing 5 mg of PANIG and 1.0 mL of α-amylase solution (1.92 U) prepared in 0.1 mol L^{-1} sodium acetate buffer (pH 5.0) on an orbital shaker for 30 min at room temperature. The immobilised enzyme (PANIG-AMY) was separated by centrifugation and exhaustively washed sequentially with 0.1 mol L^{-1} sodium acetate (pH 5.0), 0.1 mol L^{-1} sodium chloride, 0.1 mol L^{-1} glycine and 0.1 mol L^{-1} sodium acetate (pH 5.0) in order to remove unbound enzyme, glutaraldehyde or other reactants from fermentative extract.

2.5. Assay of amylolytic activity

The amylolytic activity of free α-amylase was determined by the formation of reducing sugars from potato starch in the presence of ADNS as described previously (Bernfeld, 1955). One unit of α-amylase was defined to be the amount of enzyme that would produce 1.0 μmol of reducing sugar per min of reaction. Immobilised α-amylase was assayed following a similar procedure except that the free enzyme was replaced by 5.0 mg of PANIG-AMY. Protein was measured following Bradford method (1976).

2.6. Characterisation of immobilised α-amylase

The enzymatic activity of PANIG-AMY was investigated in the range 40–60 °C, while the optimum pH was determined using 0.1 mol L^{-1} citrate phosphate buffer in the pH range 4.0–7.0. In each case, the enzyme reaction mixture was incubated for 15 min, a 100 μL aliquot was removed and the formation of products of starch hydrolysis estimated as described above. The effects of various ions on the activity of PANIG-AMY were evaluated by incubating the immobilised enzyme separately with 4.0 mmol L^{-1} solutions of $FeSO_4 \cdot 7H_2O$, $ZnSO_4 \cdot 7H_2O$, $CaCl_2 \cdot 7H_2O$, anhydrous $ZnCl_2$ or $ZnSO_4$ at room temperature for 10 min. After centrifugation and a thorough wash with 0.1 mol L^{-1} phosphate buffer (pH 7.0), the treated PANIG-AMY was assayed for amylolytic activity as described above.

The capacities of free and immobilised α-amylase to hydrolyse starch from different sources were studied. Solutions of starch (0.5%, w/v) derived from maize, wheat, cassava and a mixture of maize and rice were hydrolysed as described above for potato starch. The reaction products of potato starch were analysed by TLC (thin layer chromatography) on silica gel plates eluted with *n*-butanol:methanol:water (4:2:1) (Chung et al., 1995) and visualised using a dye solution containing 7.5 mL of phosphoric acid (85%, v/v), 1.0 mL of aniline, 1.0 g of diphenylamine and 50 mL of acetone. Solutions (1.0%, w/v) of glucose, maltose and maltotriose were employed as standards.

The stability of PANIG-AMY was determined following storage at 4 °C in 0.1 mol L^{-1} sodium acetate buffer (pH 4.0–5.0) or in 0.1 mol L^{-1} sodium phosphate buffer (pH 6.0–8.0). The amylolytic activity of each sample was assayed daily, following which the immobilised enzyme was washed four times with the appropriate storage buffer and returned to the original storage conditions for a further 24 h. Thermal stability was determined by incubating PANIG-AMY in 0.1 mol L^{-1} phosphate buffer (pH 7.0) at 40 °C for 2–48 h either in the presence or absence of 10 mmol L^{-1} $CaCl_2$.

2.7. Repeated hydrolysis of starch catalysed by immobilised α-amylase

The repeated batch-wise hydrolysis of starch [200 μL of 0.5% (w/v) solution per batch] by immobilised α-amylase was carried out in a reactor comprising a polypropylene flask packed with 5.0 mg of PANIG-AMY and maintained in a shaking water bath at 40 °C. Following incubation for 15 min, the products of enzymatic hydrolysis were removed and the amount of reducing sugar produced was determined. A new batch of starch was then added to the PANIG-AMY thus initiating a new 15 min reaction cycle. In a further experiment, the reaction time was increased by 3 min for each new cycle in order to compensate for losses of amylolytic activity.

3. Results and discussion

3.1. Enzyme immobilisation

The bifunctional spacer glutaraldehyde was used to bind α-amylase to PANI as part of the immobilisation strategy since this reagent promotes immobilisation through the amino groups of the enzyme and the support. Although α-amylase presents numerous carbonyl groups available for immobilisation, these groups were unused because the enzyme contains aspartic and glutamic acid residues that are essential for catalytic activity (Strol et al., 1998). Because of the architecture of the active site, such amino acids are accessible for reaction with bifunctional reagents. Additionally, the terminal carboxyl residue of α-amylase is located at the edge of the active site and immobilisation through this residue could obstruct the access of starch substrate. Since glutaraldehyde presents carbonyl groups that can react with the amino groups of PANI and of the amylase, these problems were avoided.

A multivariate method was employed for the accurate determination of the individual and interactive effects associated with the various factors influencing enzyme immobilisation. Optimisation of pH, immobilisation time and enzyme amount was attained following two consecutive factorial 2^3 analyses as outlined in Table 1. Fig. 1 displays the