Multipoint Immobilization of Invertase on Agarose: Stability and Kinetic Properties

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Abstract

Enzyme immobilization is a specific method for restricting the enzyme freedom of movement. There are strategies for the proteins multipoint immobilization by amine-terminal residues, lysine residues and carboxylic groups. In the present work, a commercial invertase of Saccharomyces cerevisiae underwent multipoint immobilization on glyoxyl-agarose, amine-agarose and glutaraldehyde-agarose supports. Derivatives kinetic properties were determined and compared with the properties of the soluble enzyme. The copper influence on enzyme activity and its inhibition by fructose were also investigated. Amine-agarose exhibited activity closest to the soluble enzyme (93.3%). This same derivative maintained approximately 50% of initial activity when 800mM of fructose were added to the reaction medium. However, glutaraldehyde-agarose exhibited the best stability to temperature and pH and none of the derivatives lost inhibition by copper. Glyoxyl derivative exhibited the lowest Km (0.023mM) and amine derivative achieved the highest maximum velocity (1666.7 U/mg prot.).

Key words

enzyme immobilization and stabilization, invertase immobilization, multipoint immobilization

Introduction

Enzyme immobilization is a specific method for restricting the enzyme free movement (1). This method is currently among the most relevant areas of research. There are strategies that allow the multipoint immobilization of proteins through three different residue pathways: amine-terminal, and surface residues lysine and carboxylic groups (2). Invertase immobilization and its application in continuous processes is attractive, allowing the obtaining of a final product with high purity (typical of the enzymatic process) and without liberation of toxic products (3). Compared to the use of a soluble enzyme in industrial processes, immobilized invertase can reduce the process costs by reducing the amount of enzyme needed, as the immobilized form can be recovered at the end of a production cycle and reused. Recent studies have been carried out to discover new types of supports and immobilization methods. In general, there is no ideal support material or immobilization method to provide a standard for each type of immobilization (4). The supports used include polysaccharides (agarose, cellulose, starch, dextran, chitosan), proteins (collagen, gelatin, albumin), synthetic polymers (polyacrylates, polymethacrylates, polyacrylamide, polyamides and vinyl), minerals (bentonite, silica) and fabricated materials (nonporous glass, controlled-porous glass), etc.
Ahmad et al. (6) used lectin; Zhang (7) used silk fibroin; Godbole et al. (8) used strands of cotton residue; D’Souza and Godbole (9) used rice husks; Emregül et al. (10) used a system composed of carboxymethyl-cellulose-gelatin; Katchalski-Katzir & Kraemer (11), Mateo et al. (12) and Torres et al. (13) used Eupergit C and Sepabeads epoxy supports; Tanriseven & Dogan (14) used calcium alginate gel capsules; Siso et al. (15) used microcapsules of chitosan; Akgöl et al. (16) used magnetic micro-spheres of polyvinyl alcohol; Sanjay & Sugunan (17) used montmorillonite; and Basha & Palanivelu (18) used phenyl Sepharose. Invertase multipoint immobilization may generate large profits for industries that produce inverted sugar and, consequently, reduce costs for industries that use this product. The aim of the present study was to determine some characteristics of the immobilized enzyme on a glyoxyl/amine/glutaraldehyde-agarose support for possible industrial application and compare them with the soluble enzyme.

Material And Methods

Material

Saccharomyces cerevisiae invertase was acquired from Sigma. Invitrogen Ultra Pure Agarose (lot nº 1106405) was used as the enzyme immobilization support.

Reagents

Sodium borohydride and glycidol were acquired from Sigma; ethylenediamine was acquired from Merk. Other reagents were of an analytical grade.

Methods

Agarose activation

Support activation and preparation procedures were performed according with Guisán (19). The agarose was washed with distilled water, carefully homogenized and vacuum filtered. To 73g of this agarose were added 50mL of distilled water, 34 mL of 1.7N NaOH containing 0.95g of sodium borohydride and 6.7mL of glycidol (2,3-epoxi-propanol). The mixture was maintained under agitation for 18h at 200 rpm. Thus, the activated agarose presented active glyceryl groups on its structure. The support was then washed with abundant distilled water and vacuum filtered.

Preparation of glyoxyl, amine and glutaraldehyde supports

Glyoxy-agarose was prepared adding 825mL of distilled water and 175mL of 0.1M sodium periodate to the glyceryl-agarose support. The oxidation control process was performed by removing a 0.2mL sample of the supernatant and adding 1.5mL of 10% KI with 1.5mL of sodium bicarbonate saturated until absorbance at 419nm was constant. The support was then washed with abundant water and vacuum filtered.

Preparation of the amine-agarose support was performed in accordance with Guisán (20), adding 115mL of ethylenediamine pH 10.0 (adjusted with concentrated HCl) to 20g of the glyoxy-agarose support. This mixture was maintained under agitation at 100rpm for 2h in an ice bath. 1.15g of sodium borohydride was then added and maintained under agitation at 100rpm for another 2h. After reducing the derivative, it was washed with sodium acetate buffer pH 4.0, sodium borate buffer pH 9.0 and abundant distilled water.

Glutaraldehyde-agarose support was prepared adding 11mL of 0.2M sodium phosphate buffer pH 7.0 and 16.75mL of glutaraldehyde to 10g of the amine-agarose support and maintained under agitation at 150rpm for 18h at 25ºC. The support was then washed with abundant distilled water.
Obtainment of glyoxyl, amine and glutaraldehyde-agarose derivatives

**Glyoxyl derivative**: 20mL of enzyme in 0.1M sodium carbonate buffer pH 10.0 (1:1 v/v) were added to 10g of the glyoxyl-agarose support and the mixture was maintained under agitation for 24h and 8ºC. 90 mL of 0.1M sodium carbonate buffer pH 10.0 containing 0.12g of sodium borohydride were then added and maintained under agitation at 150rpm for 45min at 25ºC. The resulting derivative (glyoxyl-AIG-Sigma) was washed with abundant water, sodium acetate buffer pH 5.0 and vacuum filtered.

**Amine derivative**: 34mL of enzyme in 0.05M sodium acetate buffer pH 5.0 (1:1 v/v) were added to 10g of the amine-agarose support and the mixture was maintained under agitation for 24h at 8ºC. 140mL of 0.1M sodium carbonate buffer pH 10.0 containing 0.19g of sodium borohydride were then added and maintained under agitation at 100rpm for 30min at 25ºC. The resulting derivative (amine-AIG-Sigma) was washed with abundant water, sodium acetate buffer pH 5.0 and vacuum filtered.

**Glutaraldehyde derivative**: 20mL of 0.2M sodium phosphate buffer pH 7.0 (1:1 v/v) were added to 10g of the glutaraldehyde-agarose support and the mixture was maintained under agitation for 24h at 8ºC. 95mL of 0.1M sodium carbonate buffer pH 10.0 containing 0.13g of sodium borohydride were then added and maintained under 100rpm agitation for 40min. at 25ºC. The resulting derivative (glutaraldehyde-AIG-Sigma) was washed with abundant water and vacuum filtered.

**Determination of enzymatic activity of the derivatives and soluble enzyme**

Assays for the determination of enzymatic activity were performed at 40ºC using the reducing sugar quantification method through 3,5’-dinitrosalicylic acid (DNS) (21). For the assays with the derivatives, an agitation system was used directly in the test tube to avoid sedimentation. In the assays for the determination of Km, Vmax and influence of CuSO4 on the derivatives and inhibition by the product, a glucose-oxidase enzyme kit was used to avoid the reduction of DNS by copper, which was determined in preliminary assays.

**Determination of thermal stability of the derivatives**

0.5 mL of 0.05M sodium acetate buffer pH 5.0 were added to small quantities of the derivatives and maintained at 60ºC for periods of up to 1440min. The mixtures were then placed in an ice bath for 30s. Next, a 1% sucrose solution prepared in 0.05M sodium acetate buffer pH 5.0 was added for the determination of residual enzymatic activity. The same procedure was carried out for the soluble enzyme.

**Influence of pH on derivatives and soluble enzyme**

0.5 mL of 0.01M buffers (pH 3.0 citrate-citric acid; pH 4.0, 4.5, 5.0, 5.5 sodium acetate; pH 6.0, 6.5, 7.0, 7.5, 8.0 sodium phosphate; pH 9.0, 10.0 carbonate-sodium bicarbonate) were added to small quantities of the derivatives and maintained at 60ºC for 30min. 1% sucrose solution prepared in 0.05M sodium acetate buffer pH 5.0 was added for the determination of residual enzymatic activity. The same procedure was carried out for the soluble enzyme.

**Determination of Km, Vmax and Ki of the soluble enzyme and derivatives in the presence of CuSO4**

Different volumes of 2% sucrose solution prepared in 0.05M sodium acetate buffer pH 5.0 were diluted in the same buffer for final concentrations ranging from 0.004 to 0.058 mM.
Km and Vmax values were calculated using Lineweaver-Burk graphs for the reactions with the derivatives and soluble enzyme. CuSO_{4} concentrations ranging from 1 to 10mM for each sucrose concentration were used for Ki determination.

**Influence of fructose on invertase activity**

Soluble enzyme and derivatives were tested in the presence of sucrose, with fructose added at concentrations ranging from 50 to 800mM for the determination of possible invertase activity inhibition by the reaction product. Assays were carried out using 1% sucrose and diluted fructose for the concentrations above.

**Results and Discussion**

**Activity of AIG-Sigma derivatives and soluble enzyme**

Activity of the derivatives and soluble enzyme was determined through the formation of the reaction product, following the method described by Miller (21). Table 1 displays the results.

Amine derivative exhibited activity closest to that of the soluble enzyme (60.2%). The enzyme in this derivative is bound by carboxyl groups of the surface amino acid residues on the tertiary structure. Glutaraldehyde and glyoxyl derivatives exhibited activity of 33.6% and 6.4% in relation to the soluble enzyme, respectively. The enzyme in these derivatives is bound by the terminal-amine and amine group of lysine residues, respectively. Therefore, the different forms of binding of the enzyme to the support may account for the variation in activity values, and the best result was obtained with the amine derivative.

**Influence of immobilization on derivatives thermal stability at 60ºC**

To determine the derivatives thermal stability, 0.1g of glyoxyl derivative and 0.01g of amine and glutaraldehyde derivatives were incubated in 0.5 mL of 0.05 M sodium acetate buffer pH 5.0 for different periods of time up to 1440min; the soluble enzyme was also tested under the same conditions (Fig 1). Glutaraldehyde derivative was the most stable at the temperature of 60ºC; this derivative exhibited residual activity of approximately 40% even after 1440min (24h). Amine derivative was the second best, retaining approximately 40% of the initial activity after 480 minutes (8h). These results are interesting because they demonstrate that the derivative exhibiting the higher enzymatic activity (60.2% of the soluble enzyme activity) is not always the most stable after a particular time has elapsed at a given temperature.
derivative exhibited 30% of activity, whereas amine and glyoxyl derivatives exhibited 20% and 10%, respectively. Soluble enzyme exhibited 10% of maximum activity at both pH 3.0 and pH 10.0. The behavior of the derivatives was similar for the remaining pH values, with increased residual activity with the increase in pH, presenting maximum activity at pH 5.0 and diminishing as the pH went from neutral to alkaline. However, the soluble enzyme completely lost all activity at pH 7.0, whereas the derivatives exhibited approximately 40% (amine), 55% (glyoxyl) and 70% (glutaraldehyde) of activity at pH 5.0.

Fig 2: Influence of pH on glyoxyl, amine and glutaraldehyde derivatives at 60ºC.

Influence of different fructose concentrations on invertase activity

Derivatives amine and glutaraldehyde studied exhibited a slight increase in activity when fructose 50mM was used. However, when fructose concentrations in the reaction medium were increased, the residual activity of the derivatives gradually decreased to values of 50%, 25% and 18%, respectively (Figure 3). From these results, it was evident that amine derivative exhibits about twice greater activity than glyoxyl derivative in presence of fructose 800mM. Such a finding allows us to hypothesize that the inhibition site by fructose is in the region of the tertiary structure of the enzyme which has a high density of carboxylic groups and, as these groups form covalent bonds with the support, the access of fructose to this site is hindered in amine derivative.

Fig 3: Activity of glyoxyl/amine/glutaraldehyde derivatives in the presence of different fructose concentrations.

Kinetic constants and effect of CuSO₄ on activity of soluble invertase and derivatives

Kinetic parameters of the enzyme reaction were determined graphically using the Lineweaver-Burk direct linear method from the initial hydrolysis velocities of the sucrose in the absence and presence of CuSO₄ (Table 2, Figure 4). As Table 2 shows, glyoxyl derivative exhibited lower Km than the other derivatives and the soluble enzyme (0.023 mM). However, amine derivative exhibited the highest Vmax (1666.7 U/mg prot), even while presenting a Km only two-fold greater than glyoxyl derivative. Apparent Vmax (Vmax ap) and Vmax were obtained in the Lineweaver-Burk graphs and used to calculate Ki by the following equation:
**Table 1:** Activity of glyoxyl, amine and glutaraldehyde derivatives in comparison to the soluble enzyme.

<table>
<thead>
<tr>
<th>Form</th>
<th>mg enzyme/g support</th>
<th>U/mg prot</th>
<th>Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soluble</td>
<td>-</td>
<td>29.5</td>
<td>100.0</td>
</tr>
<tr>
<td>Glyoxyl</td>
<td>2</td>
<td>1.9</td>
<td>6.4</td>
</tr>
<tr>
<td>Amine</td>
<td>3.4</td>
<td>17.7</td>
<td>60.2</td>
</tr>
<tr>
<td>Glutaraldehyde</td>
<td>2.4</td>
<td>9.9</td>
<td>33.6</td>
</tr>
</tbody>
</table>

**Table 2:** Kinetic properties of the soluble enzyme and glyoxyl/amine/glutaraldehyde derivatives.

<table>
<thead>
<tr>
<th>Enzyme/ Derivative</th>
<th>Km (mM)</th>
<th>Vmax (U/mg prot.)</th>
<th>Ki (mM CuSO₄)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soluble</td>
<td>0.101</td>
<td>100.0</td>
<td>1.250</td>
<td>0.4</td>
</tr>
<tr>
<td>Glyoxyl</td>
<td>0.023</td>
<td>200.0</td>
<td>0.625</td>
<td>0.4</td>
</tr>
<tr>
<td>Amine</td>
<td>0.045</td>
<td>1,666.7</td>
<td>0.429</td>
<td>0.4</td>
</tr>
<tr>
<td>Glutaraldehyde</td>
<td>0.044</td>
<td>1,000.0</td>
<td>0.333</td>
<td>0.4</td>
</tr>
</tbody>
</table>

**Fig 4:** Lineweaver-Burk graphic representation for the determination of Km and Vmax in the presence and absence of the CuSO₄ inhibitor. A: soluble enzyme; B: glyoxyl derivative; C: amine derivative; D: glutaraldehyde derivative.
\[
\begin{array}{c|c|c|c}
\text{Vmax} & 1 & + & [I] \\
\text{Vmax.ap} & \text{Ki}
\end{array}
\]

where Vmax is the maximum velocity in the absence of inhibitor; Vmax.ap is the maximum velocity in the presence of the inhibitor; and [I] is the CuSO\(_4\) concentration used in the reaction. Due to the oxidation of the 3,5’-dinitrosalicylic acid (DNS) by CuSO\(_4\), overestimating activity values, a glucose-oxidase enzymatic kit was used to determine Ki. According to Kulp (22), yeast invertases are inhibited by metal ions. Ki is a dissociation constant of the enzyme-inhibitor complex to be determined. As the measurement of Ki diminishes in relation to the increased concentration of the inhibitor, inhibition percentages also increase progressively.

Since the derivatives did not lose inhibition in relation to the soluble enzyme, we may hypothesize that the metallic site is located at the region near the catalytic site. Another interesting fact was that Vmax presented by amine derivative was higher than that presented by soluble enzyme and by glyoxyl derivative. This might have happened due to changes in the tertiary structure, favoring the catalysis by this derivative, without altering the inhibition. However, glyoxyl derivative, where enzyme is immobilized by amine groups, Vmax was 8 times lower, indicating that the catalytic site is near to an area of the tertiary structure rich in asparagine, glutamine, lysine or arginine residues. But the inhibition was not also affected in this derivative.

**Conclusion**

The aim of the present work was to achieve the multipoint immobilization of invertase and determine some characteristics of the derivatives for possible industrial application. For such, the experiments on thermal stability and pH were carried out at 60°C. The temperature condition is close to that used for the sucrose production, which ranges from 65 to 75°C (23). Derivatives exhibited better efficiency over free enzyme, which retained only 15% of its initial activity. Glutaraldehyde derivative retained considerable activity even after 1440min (40%), whereas amine derivative retained approximately 25% activity at 480min. Glyoxyl derivative exhibited lower stability in relation to the other derivatives tested, retaining approximately 20% of residual activity after 480min. Multipoint covalent immobilization in a highly activated 6BCL glyoxyl-agarose support increases the rigidity and durability of the enzyme (24). Torres et al. (13) immobilized an invertase on Sepabeads and obtained residual activity of approximately 80% after 50h. D’Souza and Godbole (9) reported 69% activity retention with invertase immobilized on rice husks after 30min at 60°C. Mateo et al. (12) reported that the stability of the immobilized enzyme is highly dependent on the immobilization protocol and that the support geometry also influences derivative activity. Thus, other immobilization protocols may be used in an attempt to improve the activity of the derivatives, such as immobilization/stabilization/blocking procedures. Different enzyme immobilization procedures may exercise a significant influence over conformational alterations related to enzyme inactivation. For example, an enzyme that undergoes mild chemical alterations in the residue alone and is immobilized with a long spacer arm may undergo the same changes as the soluble enzyme (25). Another possibility for solving these problems would be the use of multifunctional epoxy supports, proposed by Mateo et al. (26) as a second generation of activated supports for covalent immobilization. According to these authors, multifunctional supports that contain epoxy groups may be used under mild
experimental conditions, such as low ionic force, neutral pH and low temperatures, altering a fraction of the epoxy groups for the adsorption of proteins.

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