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Characterization and Performance of Immobilized Amylase and Cellulase

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Abstract

The performance of cellulase and amylase immobilized on siliceous supports was investigated. Enzyme uptake onto the support depended on the enzyme source and immobilization conditions. For amylase, the uptake ranged between 20 and 60%, and for cellulase, 7–10%. Immobilized amylase performance was assessed by batch kinetics in 100–300 g/L of corn flour at 65°C. Depending on the substrate and enzyme loading, between 40 and 60% starch conversion was obtained. Immobilized amylase was more stable than soluble amylase. Enzyme samples were preincubated in a water bath at various temperatures, then tested for activity. At 105°C, soluble amylase lost ~55% of its activity, compared with ~30% loss for immobilized amylase. The performance of immobilized cellulase was evaluated from batch kinetics in 10 g/L of substrate (shredded wastepaper) at 55°C. Significant hydrolysis of the wastepaper was also observed, indicating that immobilization does not preclude access to and hydrolysis of insoluble cellulose.

Index Entries: Amylase; cellulase; immobilization; inactivation; wastepaper.

Introduction

Enzymes such as "cellulases" and α-amylases are currently used by several industries to hydrolyze cellulose or starch to products such as dextrins, syrups, and sugars. Such reactions represent the key first step toward the production of a variety of useful chemicals and sweeteners and are also useful in the pulp and paper industry for fiber modification and de-inking.

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α-Amylases hydrolyze starch by cleaving the internal α-1,4-glucosidic bonds, producing polysaccharides that may be further converted by glucoamylases to produce sugars that can be utilized during fermentations. The conventional enzymatic starch hydrolysis process consists of two steps—liquefaction and saccharification—both of which are carried out at high temperatures. However, soluble α-amylases are easily inactivated at higher temperatures and longer reaction durations. Furthermore, these enzymes are inhibited by products generated during hydrolysis.

Enzyme immobilization has been reported to improve the thermal stability of enzymes *(1,2)* and may also affect binding of substrates and inhibitors to the enzyme, thereby affecting the Michaelis constant and enzyme inhibition. Several previous studies have considered the advantages of immobilized enzymes with soluble substrates, and a few studies have also investigated the properties of immobilized enzymes with insoluble substrates. The main objective of the present work was to establish the effect of immobilization on the thermal stability of these enzymes, so that they may be used at elevated temperatures without significant activity loss. The immobilization conditions were varied, and their effect on the performance of the immobilized enzymes was analyzed with reference to their physiochemical and structural properties.

Materials and Methods

Immobilization

Enzymes were immobilized onto silica gel by covalent crosslinking with glutaraldehyde in a procedure similar to that of Kondo et al. (3). Briefly, 1–4 g of silica gel was incubated in 1–13 wt% glutaraldehyde and in 100–1000 mL of enzyme solution for up to 48 h. The immobilized enzyme was recovered by filtration and washed to remove loosely bound enzyme. Samples of the soluble enzyme were collected before and after immobilization and assayed for activity, to provide an estimate of enzyme uptake onto the support. The α-amylases studied were Spezyme Fred® (Genencor), Allyzme® (Alltech), and Liquozyme® (Novozymes). The cellulases studied were Spezyme CP® and Spezyme CE® (Genencor).

Enzyme Uptake

For enzyme uptake studies, 0.1 g of substrate (corn flour or carboxymethylcellulose) and 24 mL of buffer were added to a 40-mL jacketed reactor. The temperature was maintained at 52 ± 2°C using a water bath. A 1.5-mL "blank" sample was collected from the reaction mixture and added to 3 mL of the dinitrosalicylic acid (DNS) reagent. Then 500 µL of the soluble enzyme was added to the reactor to initiate the reaction. Samples were collected every 3 min, and the DNS assay *(4)* was performed for all the samples. One unit of enzyme releases 1 µmol of reducing groups/min, the absorbance of which can be measured at 540 nm. Thus, the amount of activity transferred to the support can be determined from the sugar pro-

duction rates observed with enzyme solutions collected before (t = 0 h) and after the immobilization step. The fraction of residual enzyme (R) is defined as follows:

$$R = \frac{\text{µmol of glucosidic linkages hydrolyzed/min from sample after immobilization}}{\text{µmol of glucosidic linkages hydrolyzed/min from sample after 0 h}}$$

Therefore, the immobilization yield, or fraction of enzyme activity that was transferred to the support, Y_i, would equal $(1 - R)$. For these experiments, the uptake was determined via a point-by-point comparison of absorbance values from the "before" and "after" samples.

Amylase Assay

Kinetics studies were conducted at 65 ± 1°C in a jacketed batch reactor. Five hundred milliliters or 1 L of buffer was added to the reactor and heated to the assay temperature. The buffer pH was chosen according to the optima specified by the enzyme manufacturers. Corn flour (100–300 g/L) was then added to the reactor, along with a specified quantity of either soluble or immobilized amylase to initiate hydrolysis. Samples were collected at regular intervals over 30–60 min, and centrifuged to separate solids. The supernatant was analyzed for sugar content by measuring the %Brix with an optical refractometer.

Cellulase Assay

Kinetics studies were conducted at 55°C in a jacketed batch reactor. Shredded wastepaper (10 g/L) was added to 500 mL or 1 L of citrate buffer, pH 4.8, and heated to the assay temperature. A specified quantity of either soluble or immobilized cellulase was added to the reactor to initiate hydrolysis. Samples were collected at regular intervals over 30–60 min, and centrifuged to separate solids. The DNS assay (4) was used to detect sugars formed during hydrolysis experiments. The supernatant from the centrifuge tube and the DNS solution were mixed and cooked for exactly 5 min in boiling water. Finally, the sample was transferred to a methacrylate cuvet, and its absorbance was measured at 540 nm.

Thermal Stability of Amylase

Enzyme samples were added to test tubes and heated at a controlled temperature before conducting a standard activity assay. Samples were incubated either in boiling water for 15 min, or in a constant-temperature bath at 65, 85, or 105°C for 15 min. Samples were then cooled to room temperature and tested for activity at 65°C using the standard assay outlined above. To determine whether the substrate offered protection against thermoinactivation, another set of trials was conducted in which 0.5 g of corn was added to the enzyme during the preincubation period. The enzyme was then cooled and assayed for activity at 65°C, as outlined above. In these experiments, samples were incubated in a silicone oil bath at either 85, 105, or 125°C.

Modeling of Amylase Kinetics

%Brix values were converted to product concentrations (g/L) using maltose as a standard. Although it is extremely likely that we had a distribution of mono-, di-, and polysaccharides, we simply used a total sugars concentration for kinetics modeling at this point. Various mathematical forms were tested in an effort to represent the inhibitory effect of product on reaction rate; however, the form recommended by Lim et al. (2) (Eq. 1) provided the best overall fit to the data.

Product concentrations at each time point during the kinetics experiment were estimated by numerical integration of the following equations:

$$dP/dt = \left[V_{\max} S/\left(S + K_m\right)\right]\left[e^{\left(-K_I \cdot P\right)}\right] \quad (1)$$

$$S = S_0 - P \cdot n \quad (2)$$

in which S_0 is the initial substrate concentration (g/L), P is the product concentration (g/L), $V_{\max}$ is the maximum reaction velocity (g/[L·min]), K_m is the Michaelis constant (g/L), K_I is a constant that accounts for product-mediated inhibition (L/g), and n is the fraction of starch in the corn flour. Values of the unknown parameters ($V_{\max}$, K_m, and K_I) were estimated by nonlinear regression of the predicted product concentrations vs experimental data, using E-Z Solve software. Because of cross-correlation between parameters, and the fact that experiments were not conducted over a wide range of substrate concentrations, the model fit was relatively insensitive to K_m values.

Results and Discussion

The enzyme uptake varied significantly according to the enzyme source, type, loading, and incubation time. The typical uptake for amylase ranged between 20 and 60%, whereas for cellulase, only 7–10% uptake was obtained. Since these uptake measurements simply reflect losses in soluble enzyme activity from the immobilization solution, they set a maximum on the activity that may be expressed by the immobilized enzyme. The actual activity is expected to be less, once losses resulting from shear, conformational changes, and nonproductive binding are taken into account. Direct measurements of immobilized enzyme activity therefore provide the best measure of the effectiveness of a particular immobilization technique.

A typical profile for starch hydrolysis using soluble amylase is shown in Fig. 1. The profile predicted by the kinetics model is also shown. Clearly, the model describes the experimental concentration profiles very well. The model curves also show the insensitivity of the model fit to K_m; there is very little difference in the quality of the model predictions with K_m = 5 g/L and K_m = 50 g/L. Similarly, a typical profile for starch hydrolysis using immobilized amylase is shown in Fig. 2. The model also predicts these data very well, with little sensitivity to the K_m value.

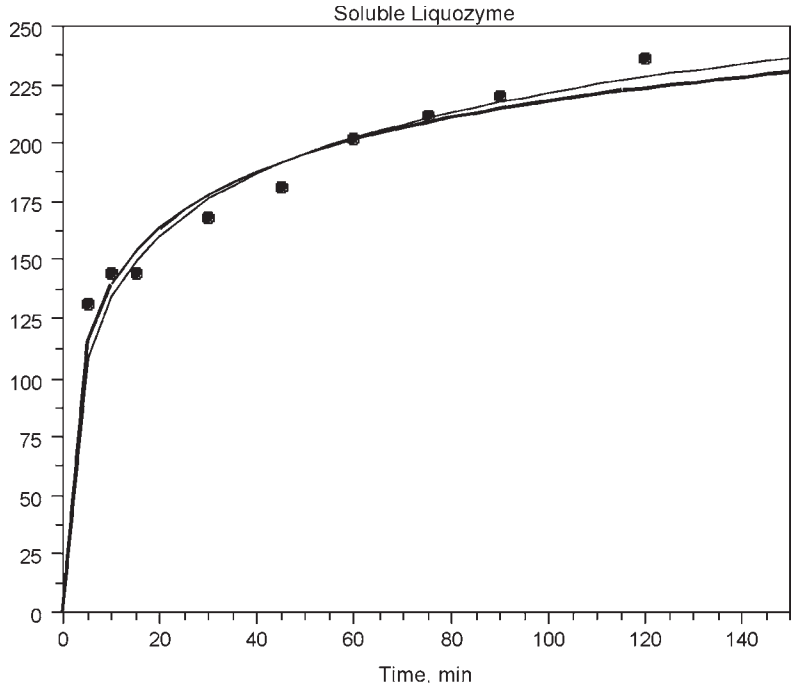

Fig. 1. Kinetics of starch hydrolysis with soluble liquozyme. V_{max} = 122 g/(L·min); K_I = 0.026 L/g; (—) K_m = 50; (– – –) K_m = 5; (●) experimental data.

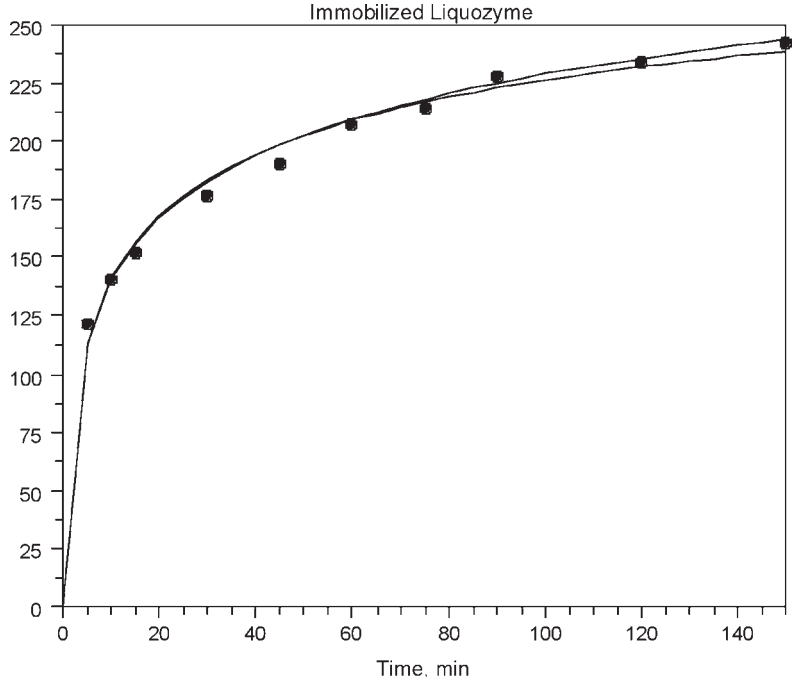

Fig. 2. Kinetics of starch hydrolysis with immobilized liquozyme. V_{max} = 144 g/(L·min); K_I = 0.023 L/g; (—) K_m = 50; (– – –) K_m = 5; (●) experimental data.

Table 1
Kinetics Parameters for Soluble and Immobilized Amylases

Enzyme	Quantity	pH	T (°C)	V_m	K_m	K_I
Soluble Allzyme	334.0 µL	5.5	65	93.0	50	0.026
Immobilized Allzyme	6.1 g	5.5	65	72.0	50	0.021
Soluble Spezyme Fred	334.0 µL	6.8	65	279.0	50	0.043
Immobilized Spezyme Fred	6.0 g	6.8	65	46.8	50	0.031
Immobilized Spezyme Fred	6.0 g	6.8	67	47.1	50	0.030
Immobilized Spezyme Fred	8.0 g	6.9	66	53.1	50	0.032
Soluble Liquozyme	334.0 µL	5.5	66	168.0	50	0.027
Immobilized Liquozyme	4.5 g	5.5	66	86.1	50	0.023
Immobilized Liquozyme	3.0 g	5.5	65	60.6	50	0.024
Immobilized Liquozyme	6.0 g	5.6	66	118.0	50	0.024

Table 1 summarizes the parameters obtained from the kinetics modeling. The modeling results indicate that soluble Spezyme Fred is more active than soluble Allzyme or Liquozyme, but Allzyme and Liquozyme are less sensitive to product inhibition. The V_{max} values for immobilized Liquozyme are greater than those for immobilized Fred or Allzyme, indicating that Liquozyme is more amenable to immobilization and provides better performance. Furthermore, the immobilized forms of enzyme, particularly Spezyme Fred, are less subject to product inhibition than the soluble forms, as demonstrated by a reduction in K_I. It is also useful to note that for immobilized Liquozyme, values of V_{max} are approximately proportional to the enzyme loading. Such scaling was not observed with immobilized Spezyme Fred, possibly indicating limitations in substrate access to this form of the enzyme.

The relative activities of the soluble and immobilized amylases were compared based on the values of V_{max}, accounting for the enzyme uptake and loading onto the support. Depending on the immobilization conditions and source of amylase, the immobilized enzyme possessed from 1 to 4% of the activity of its soluble counterpart.

It is difficult to compare directly the immobilized amylase activities (i.e., V_{max} and K_m) observed in these studies with values obtained by other researchers, owing to the fact that we used industrial corn flour as the substrate, whereas others used an activity assay based on soluble starch *(5–9)*. Moreover, the substrate concentrations used in our studies (100–300 g/L) are high enough to facilitate gelatinization, and the reaction takes place with the substrate present as a slurry. By contrast, the starch assays are typically based on a substrate concentration of ~10–20 g/L *(5–7,9)*, low enough to completely dissolve the substrate.

Aksoy et al. *(9)* did observe a 12-fold increase in K_m on immobilization to polymethacrylic acid microspheres and noted that the immobilized

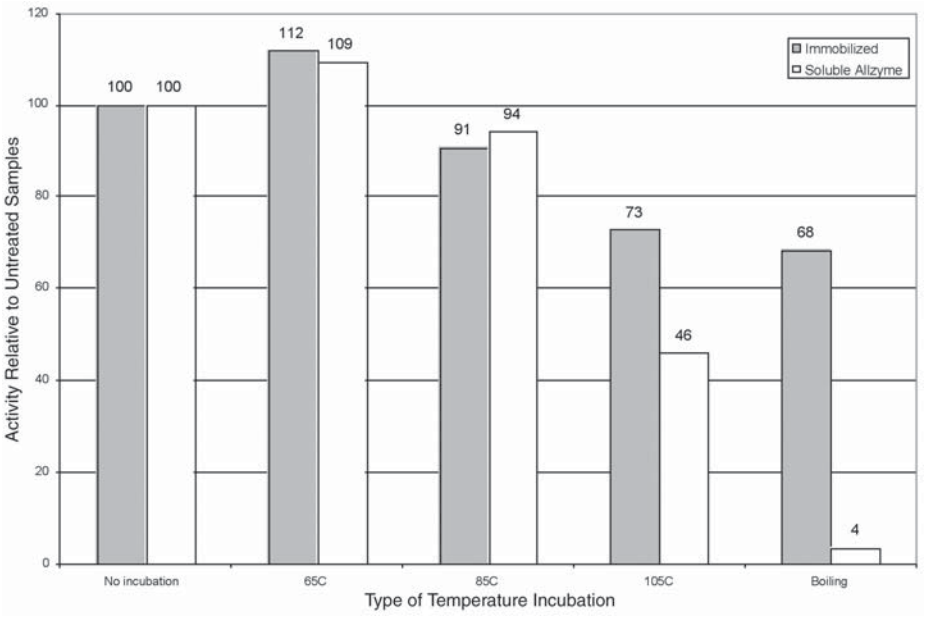

Fig. 3. Effect of temperature on activity of soluble and immobilized Allzyme®.

forms possessed between 68 and 80% of the activity of the soluble enzyme, depending on the coupling method used. Ju et al. *(7)* observed a 5% activity yield for α-amylase immobilized onto cellulose-based hollow fibers.

Figure 3 illustrates that immobilized amylase is more thermostable than its soluble counterpart. There was little difference between soluble and immobilized Allzyme following preincubation at either 65 or 85°C. However, after incubation for 15 min at 105°C, immobilized Allzyme lost ~30% of its activity, compared with ~55% activity loss for soluble Allzyme. Furthermore, after boiling for 15 min., immobilized Allzyme retained about 70% of its activity, whereas the soluble form was almost completely deactivated.

For comparison, Arasaratnam and Balasubramanian *(8)* showed that coupling praline with α-amylase during immobilization to Sepharose 4-B could increase the thermal stability of the enzyme without adversely affecting the activity of amylase (based on comparison of amylase activity following immobilization without proline). Sadhukhan et al. *(6)* observed that immobilization of α-amylase onto Sepharose, alginate beads, and polyacrylamide gels could increase the optimum reaction temperature to about 70°C, compared with about 60°C for the soluble form. In thermal stability studies analogous to ours, they observed improved thermal stability on immobilization but still observed about a 40% loss of activity following incubation of the immobilized enzyme for 15 min at 80°C. By contrast, in our studies, only a 5–10% activity loss was observed following incubation at 85°C (Fig. 3).

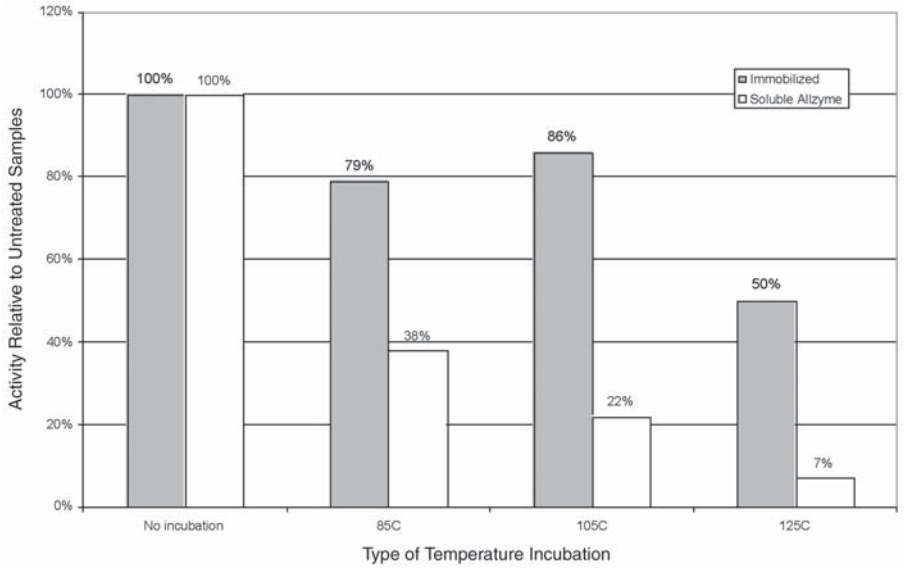

Fig. 4. Effect of substrate preincubation on activity of soluble and immobilized Allzyme®.

Figure 4 illustrates the impact of the substrate on thermal stability. Typically, the substrate is expected to have a protective effect on the enzyme, because the active site is occupied. However, Fig. 4 shows that the substrate can have both positive and negative effects. At all temperatures, incubation of the soluble enzyme with substrate decreased the retained activity of the soluble enzyme. For example, at 85°C, 94% of the original enzyme activity was retained following incubation in buffer (Fig. 3), but only 38% of the original activity was retained when the enzyme was incubated in the presence of substrate (Figure 4). This observation is in contrast to that of Ju et al. *(7)*, who suggested that starch has a stabilizing effect on α-amylase. Perhaps this is owing to differences in the source of the enzyme.

The substrate had little effect on the immobilized enzyme, perhaps a slight deleterious effect at 85°C and a slight protective effect at 105°C. Regardless, the immobilized enzyme was more stable than the soluble form, with or without substrate. This protective effect of immobilization is not surprising, since immobilization can help to distribute the thermal energy imposed on the protein at higher temperatures.

Figure 5 illustrates the performance of immobilized cellulase in comparison with that of soluble cellulase. The small difference between the "crude" and "used" Spezyme CP profiles illustrates the very low uptake of soluble cellulase onto the support. Furthermore, soluble cellulase is much more active; the dose of soluble enzyme is approx one-twentieth that of immobilized cellulase, once enzyme uptake is taken into account. Nonetheless, it is important to note that the immobilized enzyme is able to hydrolyze this insoluble substrate, albeit at a much lower rate.

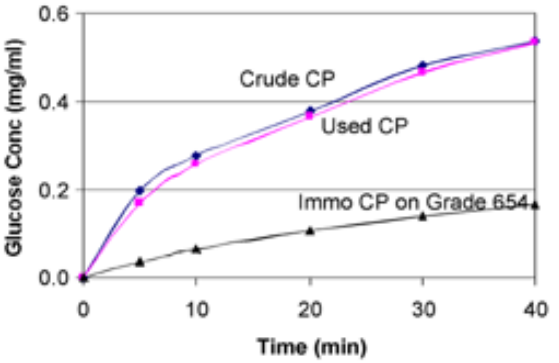

Fig. 5. Comparison of cellulose hydrolysis with soluble and immobilized cellulase. "Crude" and "Used" designations refer to the soluble enzyme collected before and after immobilization, respectively. CP, Spezyme CP.

Conclusions

Immobilized cellulase and amylase are able to hydrolyze cellulose and starch. However, the immobilized enzymes possess only about 1–6% of the activity of the soluble forms. In addition, immobilization clearly enhanced the thermal stability of amylase. Immobilized amylase retained more than half of its activity, even after incubation at 125°C. By comparison, soluble amylase was almost completely inactivated under these conditions. Furthermore, kinetics modeling indicates that the susceptibility to product inhibition is dependent on the amylase source. Finally, immobilization can reduce the susceptibility to product inhibition; K_I was less for each of the immobilized forms, compared with their soluble counterparts.

Acknowledgments

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