ASEAN Food Journal 15 (1): 73-78 (2008)

Biochemical Studies on the Immobilization of the Enzyme Invertase (EC.3.2.1.26) in Alginate Gel and its Kinetics

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Abstract: The biochemical properties of invertase entrapped in alginate gel were studied. The kinetic parameters were determined for immobilized and free invertase. The value of Michaelis constant K_m of the immobilized invertase (139.19 mM) was greater than that of the free invertase (93.19 mM), whereas, V_{max} was smaller for the immobilized enzyme. Immobilization impressively improved the thermal and storage stability of invertase. The half-life values of the immobilized and free enzymes at 60°C were 28 min and 8 min, respectively. In 0.1M acetate buffer (pH 4.5) at 2 - 4°C, the immobilized invertase activity was found to be quite stable after 40 days.

Keywords: Calcium alginate, enzyme immobilization, entrapment, invertase, sucrose hydrolysis

INTRODUCTION

Invertase, also known as β-fructofuranoside fructohydrolase (EC 3.2.1.26) is a catalyst for sucrose hydrolysis yielding invert sugar. Invert sugar has been used to a great extent in the food industry such as in beverage and confectionery products. Invert sugar can be produced by a chemical process (using acid as a catalyst) or biochemical process (using invertase as a catalyst). Nowadays, the biochemical process is preferred as the resulting product contains less coloured by-product and salt (Bergamasco *et al.*, 2000; Akgol *et al.*, 2001; Bayramolu *et al.*, 2003). Moreover, this process requires less energy than the chemical process.

However, the enzymatic process is more expensive than acid hydrolysis, due to the relatively high cost of invertase. To reduce the cost of the final product, the application of immobilized invertase has been considered an appropriate solution. This immobilized enzyme has many advantages because (1) it can be re-used many times and still remains active, (2) immobilization protects the activity of the enzyme from unfavourable conditions, (3) the separation and recovery of enzyme is easy and convenient, (4) it can be used in a continuous system for the production of invert syrup from sucrose solution, (5) the application of immobilized enzyme provides considerable reduction in the operating costs (Bayramolu *et al.*, 2003). Many studies were focused on the support for immobilization of invertase in different aspects namely polyvinylalcohol (Agkol *et al.*, 2001), polyacrylamide (Abdellah *et al.*, 1992; Mansour and Dawoud, 2003), chitosan (Hseih *et al.*, 2000), hen-egg white and diethylaminoethyl-cellulose (Abdellah *et al.*, 1992).

In this investigation, calcium alginate gel was chosen as a carrier for the enzyme entrapment due to its non-toxicity, high mechanical stability, high porosity for substrate and product diffusion and above all the simple procedural requirements for immobilization (Bucke, 1987). Some properties of immobilized invertase in alginate gel like kinetics, thermal and storage stability were investigated.

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MATERIALS AND METHODS

Materials

Commercial invertase (b-D-fructofuranoside fructohydrolase, E 3.2.1.26) produced from baker's yeast, *Saccharomces cerevisiae,* was obtained from Sigma Chemical Company (USA).

Alginate from *Sargassum* was procured from Biotechnology Center – Nha Trang University of Marine Products. Powder alginate contains 21.88% moisture with a viscosity (1.5% (w/v) alginate solution): 720 cp.

Saccharose was purchased from Bienhoa Sugar Company. It contained 99.8% (db) sucrose. It has 0.05% moisture and 0.03% reducing sugar. Other analytical chemicals were obtained from Merck AG (Germany) and Shantou Xilong Chemical Factory Guangdong (China).

Procedure of Invertase Immobilization in Alginate Gel

Sodium alginate solution 2.5% (w/v) and invertase solution 1.33% (w/v) were mixed in the ratio of 1:1 (v/v) to homogenization. The mixture was passed drop wise into 2% (w/v) $CaCl_2$ solution. The formed beads were retained in the stirred $CaCl_2$ solution (using a magnetic stirrer) at least for 2 hours for gel hardening. The invertase-alginate beads had 3-4 mm in diameter. Finally, the beads were separated and washed with distilled water 3 times. Before using, the beads were immersed in 0.1 M acetate buffer of pH 4.5 (Le *et al.*, 2003).

Activity Assays of Free and Immobilized Invertase

The invertase activity was assayed as follows: Ten cm^3 of immobilized invertase beads or 5 ml free invertase solution (1.005% (w/v)) was added to 100 ml sucrose solution (200 g/L in acetate buffer, pH 4.5) and incubated for 15 min at 50°C. The enzymatic reaction was stopped by crossing out immobilized invertase or boiling the solution at 100°C for 5 min. One international unit (IU) of activity was defined as the amount of enzyme that is required to hydrolyse 1.0 mmol sucrose per minute under the assay conditions. The reducing sugars produced by sucrose hydrolysis were measured by spectrophotometric method using 3,5 dinitrosalicylic acid reagent (Miller, 1959).

Determination of Invertase Immobilization Yield

The yield of invertase immobilization Y was calculated by the following equation (Abdellah *et al.*, 1992).

$Y = [(A - B)/A] \times 100$, where:

A is the total amount of enzyme (mg) added to the immobilization solution.

B is the amount of residual enzyme in the $CaCl_2$ solution and in the washing solution of the gel beads in the immobilization procedure.

Both A and B were evaluated from the amount of reducing sugars produced enzymatically in the corresponding solutions.

Determination of Kinetic Parameters

K_m and V_{max} values of the free and immobilized invertase were determined by Lineweaver-Burk method using various substrate concentrations (0.029-0.29 M) in acetate buffer (0.1 M, pH 4.5) at 55°C.

Thermal Stability of the Free and Immobilized Invertase

The thermal stability of the free and immobilized invertase were determined by measuring the residual activity of the enzyme exposed to three different temperatures (50, 55 and 60°C) in acetate buffer (0.1 M, pH 4.5) for 160 min. After every 20 minutes, a sample was taken and assayed for enzymatic activity. The inactivation rate constants, k, and the half-life, $t_{1/2}$, were calculated with the following equation (Bailey *et al.*, 1986; Bayramolu *et al.*, 2003).

$[A] = [A_o] .e^{-kt}$

k: Inactivation rate constants (min^{-1}).

A_o: The initial activity (U/mg protein enzyme).

A: The activity after a time t (U/mg protein enzyme).

Storage Stability of the Free and Immobilized Invertase

This experiment was conducted to determine the stability of the free and immobilized invertase during storage in distilled water and acetate buffer (0.1 M, pH 4.5) at 2 - 4°C for 40 days. The residual activities were measured by the assay procedure described earlier. The activity of the free and immobilized enzymes was expressed as a percentage of its residual activity compared to the initial activity. The inactivation rate constants k and the half-life $t_{1/2}$ were also calculated as described earlier.

RESULTS AND DISCUSSION

Yield of Invertase Immobilization

The results show that the yield of invertase immobilization in alginate gel was 79.34 ± 3.48%. This means that the enzymes used in immobilization was not completely included in the gel matrix. Some invertase molecules distributed on the surface of alginate beads diffused into the $CaCl_2$ solution during the gel beads formation. This observation was in accordance with earlier reported studies where the yield of enzyme immobilization in alginate gel varied from 50 to 85%. (Das *et al.*, 1998; Arruda *et al.*, 1999; Le *et al.*, 2004).

Kinetic Parameters

The kinetic parameters of the hydrolytic reaction of sucrose using the free and immobilized invertase were determined. Figure 1 presents the relation between initial rate and substrate concentration for the free and immobilized invertase. Using Lineweaver-Burk method (Figure 2), the apparent Michaelis constants K_m and V_{max} of the free invertase were 93.19 mM and 35.84 mM min^{-1}, respectively. For the immobilized invertase in alginate gel, the apparent Michaelis constants, K_m and V_{max}, were 139.19 mM and 5.97 mM min^{-1}, respectively. Therefore, K_m of the immobilized invertase was approximately 1.5-fold higher than that of the free invertase, while V_{max} was 6-fold lower. When an enzyme was immobilized in gel matrix such as alginate gel, the K_m of immobilized enzyme increased, while V_{max} decreased. This means that the affinity of the enzyme for its substrate and the velocity of enzymatic reaction decreased. This was due to the lower accessibility of the substrate to the active site of the immobilized

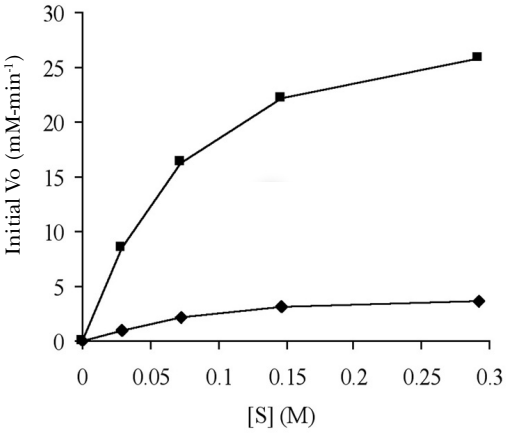

Figure 1: Michaelis-Menten plots of the free and immobilized invertase. (■) free invertase, (◆) immobilized invertase in alginate gel

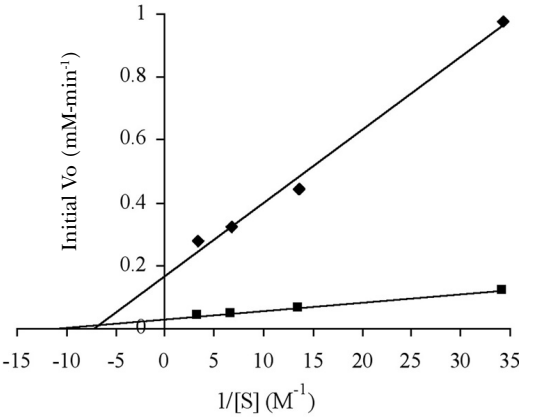

Figure 2: Lineweaver-Burk plots of the free and immobilized invertase. (■) free invertase, (◆) immobilized invertase in alginate gel

invertase and lower transporting of the substrate and products into and out the gel beads (Bailey *et al.*, 1986; Akgol *et al.*, 2001; Bayramolu *et al.*, 2003).

Thermal Stability of the Free and Immobilized Invertase

The free and immobilized enzymes were incubated in the absence of substrate at three different temperatures (50, 55 and 60°C). Figure 3 shows the heat inactivation curves of the free and immobilized invertase. At 50°C, the activity of the immobilized invertase and free enzyme retained their activities about 90 and 75%, respectively after 160 min for the same incubation period. At 55°C, the activities of the immobilized and free enzymes were retained at levels of 93 and 45%, respectively. The immobilized form was inactivated at a much slower rate than the native form. At 60°C, the free enzyme lost their initial activity after 120 min while the immobilized enzyme retained its activity about 25% after 160 min. The half-life values and thermal inactivation rate constants for the free and immobilized enzymes are presented in Table 1. The higher the temperature, the lower the half-life value, $t_{1/2,}$ and the higher the thermal inactivation rate constant k for both the immobilized and free invertase. However, at the same temperature, the half-life value, $t_{1/2,}$ of the fixed enzyme was much higher than that of the free enzyme. The thermal stability of immobilized invertase increased considerably as a result of immobilization in alginate gel. Enzyme stabilization by immobilization may also be caused by the existance of a local environment for the immobilized enzymes which is less damaging than bulk solution conditions (Bailey *et al.*, 1986).

Storage Stability of the Free and Immobilized Invertase

In general, the enzymes are not stable during storage in solution and their activities are gradually reduced or lost through time. Figure 4 indicates the activity evolution of the free and immobilized invertases in alginate gel

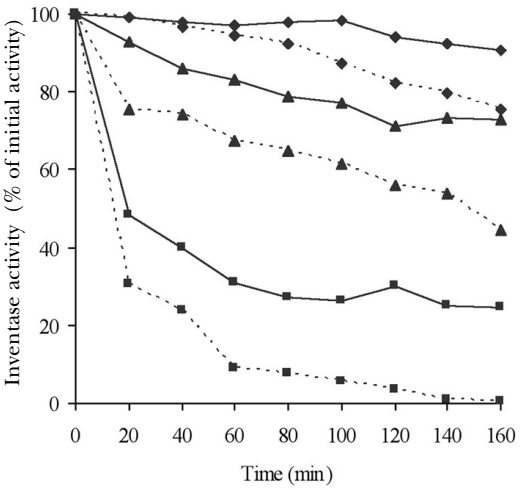

Figure 3: Influence of temperature on the stability of the free and immobilized invertase. — Immobilized invertase, ... Free invertase, (◆) 50°C, (▲) 55°C, (■) 60°C

during storage in distilled water and acetate buffer (0.1 M, pH 4.5) at 2 - 4°C. After 40 days, in acetate buffer, the immobilized enzyme preserved their activity, while, the free enzymes retained its initial activity at a level of 69%. In distilled water, the immobilized and free enzyme retained 90% and 59% of their initial activities, respectively. The half-life values and the inactivation rate constants for the free and immobilized enzymes are shown in Table 2. During storage in distilled water or in acetate buffer, the half-life values of immobilized invertase are 5 or 10-fold higher than those of the free enzyme, respectively; and the inactivation rate constants are also 5 or 10-fold lower, respectively. On the other hand, the results in Table 2 also show that the storage stability of immobilized invertase when stored in acetate buffer was 3-fold higher than when stored in distilled water. Amaya *et al.* (2006) reported similar conclusions in their investigation about invertase immobilized on nylon-6 microbeads.

In general, these results reveal that enzyme immobilization in alginate gel can reduce enzyme deactivation. First, by holding the enzyme in relatively fixed position,

Table 1
Half-lives ($t_{1/2}$) and inactivation rate constant (k) of the free and immobilized invertase in alginate gel at three different temperatures

Temperature (°C)	Immobilized invertase		Free invertase	
	$t_{1/2}$ (min)	k (min^{-1})	$t_{1/2}$ (min)	k (min^{-1})
50	1168	$0,6.10^{-3}$	403	$1,8.10^{-3}$
55	323	$2,0.10^{-3}$	143	$4,1.10^{-3}$
60	28	$7,1.10^{-3}$	8	$3,2.10^{-2}$

Table 2
Half-lives ($t_{1/2}$) and inactivation rate constant (k) of the free and immobilized invertase in alginate gel during the storaged in distilled water and acetate buffer (0.1 M, pH 4.5) at 2 - 4°C

Storage solution	Immobilized invertase		Free invertase	
	$t_{1/2}$ (days)	k (day^{-1})	$t_{1/2}$ (days)	k (day^{-1})
Distilled water	272	$2,5.10^{-3}$	59	$1,3.10^{-2}$
Acetate buffer	767	9.10^{-4}	70	$1,1.10^{-2}$

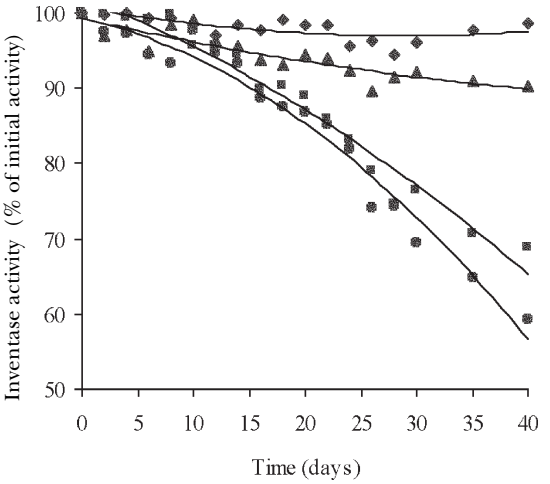

Figure 4: Storage stability of the free and the immobilized invertase. (◆) Immobilized invertase – Stored in acetate buffer, (▲) Immobilized invertase – Stored in distilled water, (■ Free invertase— Stored in acetate buffer, (●) Free invertase – Stored in distilled water

immobilization reduces interaction between enzyme molecules which contributes to deactivation by aggregation and to autolysis by proteolytic enzymes. Second, dramatic stability enhancements have been reported based on this strategy in which gel entrapment was applied to attempt to form a local support microstructure complementary to enzyme surface. Similarly, deactivation caused by dissociation of oligomeric proteins such as invertase may be reduced by immobilization which stabilised the active, multiunit structure (Bailey *et al.*, 1986; Esmon *et al.*, 1987; Bayramolu *et al.*, 2003). In summary, alginate gel provided a stable environment and prevented the loss of activity during the storage of the enzyme in solution.

CONCLUSION

In this study, the alginate was used as a support for invertase immobilization. Generally, the immobilization of invertase in alginate gel

showed a marked increase in K_m and a sharp decrease in V_{max}. However, the thermal stability of the immobilized invertase was much higher than that of the free enzyme. The rate of thermal inactivation of the immobilized enzyme decreased due to entrapment in gel matrix. In addition, the activity of the immobilized invertase was more stable in retention than that of the free enzyme during the storage in solution. Even though the activity of the immobilized enzyme was lower in comparison with the free enzyme, the enhancement of thermal and storage stability highlights the value of alginate gel as a support for enzyme immobilization. A stable immobilized system and long storage life are convenient for applications that would not be feasible with a soluble enzyme system. For instance, immobilized invertase can be used successfully in a continuous system for the production of invert syrup from sucrose solution.

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