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Characterization of Invertase Entrapped into Calcium Alginate Beads

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Abstract

A solution of 10 g/L of sodium alginate (Satialgine® types used [Sanofi trademark]: SG800® and S1100® with manuronic/guluronic ratio of 0.5 and 1.2, respectively) containing invertase (0.08 g of protein/L) was dropped into 0.1 *M* $CaCl_2$ solution buffered at pH 4.0, 7.0, or 8.0. The beads were left to harden in $CaCl_2$ solution for 24 h. The high immobilization yield of 60% occurred with SG800 at pH 8.0. The activity of soluble and insoluble invertase was measured against pH (2.8–8.0), sucrose concentration (4.5–45 m*M*), and temperature (30–60°C). Both forms presented an optimum pH of 4.6. However, the soluble invertase was stable at the overall pH interval studied, whereas insoluble invertase lost 30% of its original activity at pH > 5.0. At temperatures above 40°C, the insoluble form was more stable than the soluble one. The kinetic constants and activation energies (E_a) for free invertase were K_M = 41.2 m*M*, V_{max} = 0.10 mg of TRS/(min · mL), and E_a 28 kJ/mol for entrapped *invertase they were* $(K_M)_{ap}$ = 7.2 m*M*, $(V_{max})_{ap}$ = 0.060 mg of TRS/(min · mL), and $(E_a)_{ap}$ = 24 kJ/mol.

Index Entries: Invertase; entrapment in calcium alginate.

Introduction

In industry the bulk sucrose hydrolysis for obtaining the inverted sugar syrup is carried out at 75°C in the presence of HCl (pH ≅ 2.0). However, the product attained has several types of by-products (derived from glucose and fructose cyclization) that must be removed; otherwise, it is unsuitable either as a sweetener in several types of goods or as a raw material for producing more valuable commodities, such as fructose and gluconic acid *(1)*. Indeed, the low cost of HCl is the only remarkable point to be considered in acid hydrolysis, which is overrun by the drawback enhanced.

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The use of invertase (EC 3.2.1.26), an enzyme obtained from *Saccharomyces cerevisiae*, constitutes an alternative to acid hydrolysis, because the enzyme acts under mild pH (4.6–5.0) and temperature (35–40°C), leading to an inverted sugar syrup far less contaminated *(2)*.

To become a more economically attractive enzymatic process, it must be operated in a continuous mode with reutilization of the catalyst *(3)*. Thus, the immobilization of invertase in an inert matrix is a fundamental approach. However, the immobilization procedure must not be hazardous to the enzyme and the support preferably a food-grade product *(4)*. In spite of the fact that invertase has been immobilized in a great variety of supports by different immobilization methods *(5)*, only a few fulfill the requirements appointed. Among these methods, hydrogel entrapment is cheap, nontoxic, safe to the enzyme, and easy to manufacture and handle *(6)*.

One of the most common hydrogels is sodium alginate, largely used in food and pharmaceutical industries, which complexes with Ca^{2+}, forming a membrane of calcium alginate, whose porosity is adequate to retain cells and high molecular weight enzymes, such as invertase (270 kDa) *(7)*. In addition, the entrapment of this enzyme into calcium alginate beads is favored by the high aggregation capability of its molecules *(8)*.

This work deals with the catalytic performance of entrapped invertase against pH (2.8–8.0), temperature (30–60°C), and sucrose concentration (4.5–45 m*M*). The sodium alginates employed had a manuronic/guluronic ratio (M/G) between 0.5 and 1.2, and the gelification was carried out in a 0.1 *M* $CaCl_2$ solution, whose pH was set at 4.0, 7.0, or 8.0 through appropriate buffering.

Materials and Methods

Materials

Invertase from *S. cerevisiae* (EC 3.2.1.26), having a protein concentration of 1.1 mg/mL, was purchased from Biocon do Brasil Industrial (Rio De Janeiro). The sodium alginates having M/G ratios of 0.5 (Satialgine SG800) and 1.2 (Satialgine S1100) were purchased from Sanofi do Brasil (São Paulo). The physicochemical properties for SG800 were η1%, 400–490 cP; granules ≤200 μ; humidity ≤15%; and pH of the aqueous solution, 6.0–8.5. For S1100, they were η1%, 550–750 cP; granules ≤160 μ; humidity ≤15%; and pH of the aqueous solution, 6.0–8.5. In all experiments, the concentration of sodium alginate solution employed was 10 g/L. All the other reagents were commercially available products of analytical grade.

Enzyme Immobilization

The invertase was entrapped in calcium alginate beads by dropping 30 mL of aqueous solution of sodium alginate (SG800 or S1100) containing invertase (2.2 mg of total protein) into 100 mL of 0.1 *M* $CaCl_2$ solution buffered at pH 4.0, 7.0, or 8.0. The pellets formed (mean diameter of 5 mm)

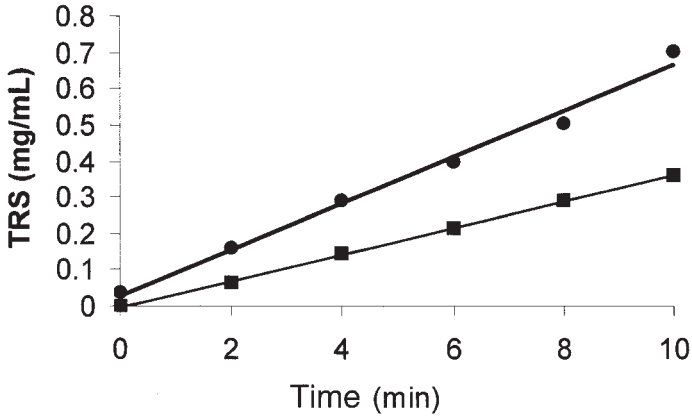

Fig. 1. Formation of reducing sugars from hydrolysis of sucrose by free invertase (●) and invertase entrapped in calcium alginate beads (■).

were left to harden in the calcium solution for 24 h. Then the beads were separated through a sieve, and the residual $CaCl_2$ solution was collected. After immobilization, the beads retained on the sieve, the recipient containing the sodium alginate–invertase solution, and the dropping device, whose geometry was previously described by Vitolo and Carreira *(9)*, were washed with deionized water. The residual calcium chloride solution and the washing waters were mixed, and the total soluble protein amount (TSPA) was determined (milligrams). The lost protein index (LPI) was calculated by the following relation: [(TSPA) × 100/2.2 mg of total protein]. Thus, the protein immobilization coefficient (PIC) was expressed as (100 – LPI). The invertase entrapment yield (IEY) was calculated by the ratio of the V_{max} of the immobilized invertase and the free enzyme.

Determination of Invertase Activity

Free Invertase

A standard test for free invertase consisted of mixing 1.0 mL of invertase (previously diluted 400 times) with 99 mL of sucrose solution (100 g/L in 0.01 *M* acetate buffer, pH 4.6). Hydrolysis was carried out for 10 min at 35°C under constant stirring (100 rpm). For monitoring hydrolysis, 0.1- to 0.3-mL aliquots were taken every 2 min, and transferred to a Folin-Wu testtube (Quimica Moderna, São Paulo) containing 1.0 mL of cupric sulfate alkaline solution *(10)*, which was quickly immersed in a boiling water bath for 10 min.

Thereafter, the procedure was followed as described by Vitolo and Borzani *(11)*. The initial invertase activity (v) was calculated (always in quintuplicate) from the slopes of the total reducing sugars (TRS) vs time of reaction plots (Fig. 1 is an example of such a plot). One invertase unit (U) was defined as the quantity of TRS (milligrams) formed per minute under the conditions of the test. The free invertase had an activity equal to 0.064 U/mL of reaction medium. The standard deviation (SD) at a 95% confidence level was ±0.0131.

Immobilized Invertase

A standard test for entrapped invertase consisted of resuspending 500 calcium alginate–invertase beads in 100 mL of buffered sucrose solution, and the reaction was carried out as already described. The immobilized invertase had an activity equal to 0.036 U/mL of reaction medium (*see* Fig. 1). The SD for this procedure at a 95% confidence level was ±0.0195.

Measurement of Soluble Protein

The measurement of soluble protein was accomplished through the conventional Bradford's method *(12)*, using bovine serum albumin (Sigma [St. Louis, MO], fraction V powder) as the standard protein.

Effect of pH, Sucrose Concentration, and Temperature on the Free and Immobilized Invertase

By changing individually the conditions of the standard test (pH from 2.8 to 8.0, temperature from 30 to 60°C, and sucrose concentration from 4.5 to 45 m*M*), the following parameters were determined: K_M and V_{max} through the Lineweaver-Burk linear plot method; the effect of pH and temperature on the invertase activity; the activation energies by the Arrhenius method; the stability of both forms of invertase against pH after 60 min of invertase-buffer contact, with the mixture maintained at 35°C; and the stability of both forms of invertase against temperature. In addition, it was verified that the nonenzymic decomposition of sucrose at different pHs and temperatures was negligible and that the total volume of samples withdrawn for testing never exceeded 5% of the total reaction volume. The thermodynamic parameters (ΔH, ΔG, ΔS, and E_a) were calculated in accordance with the conventional thermodynamic equations *(13)*.

Results and Discussion

Invertase Entrapment in Calcium-Alginate Beads

From Table 1 we observe that LPI depended on the type of alginate used and the pH of the 0.1 *M* $CaCl_2$ solution. However, an LPI of 34% at pH 8.0 was attained with both alginates in spite of the different M/G ratios of the polymers. This could be owing to the full ionization of the free carboxyl groups belonging to the manuronic and guluronic units of the alginates *(14)*, leading to the same Ca^{2+} chelating capability at this pH. As a consequence, the formation of the calcium alginate membrane at pH 8.0 would occur more efficiently than at other pHs, so an improvement on the retention of protein inside the beads is achieved (PIC = 66% in this case).

Effect of Sucrose Concentration

The kinetic constants for both forms of invertase are calculated from the straight lines of the Lineweaver-Burk plot (Fig. 2), and the values are given in Table 2.

Table 1
Variation of LPI Against the pH
of the 0.1 *M* $CaCl_2$ Solution Using SG800
and S1100 Alginates as Supports
for the Entrapment

pH	SG800 (%)	S1100 (%)
4.0	50	47
7.0	48	45
8.0	34	34

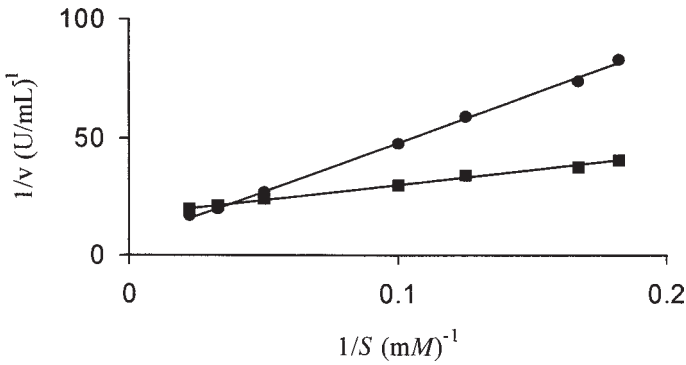

Fig. 2. Lineweaver-Burk plot for free (●) and entrapped invertase (■).

Table 2
Kinetic Constants for Free and Entrapped Invertase
Calculated Through the Lineweaver-Burk Plot

Invertase	Equation	K_M (m*M*)	V_{max} (mg of TRS/[min · mL])
Free	$(1/v) = 10 + 412 \cdot (1/S)$ ($r = 0.998$)	41.2	0.100
Immobilized	$(1/v) = 17.3 + 124 \cdot (1/S)$ ($r = 0.998$)	7.2	0.060

It is interesting to observe that V_{max} and K_M for free invertase are, respectively, 2.6 and 5.7 times higher than those of the immobilized form. For immobilized enzymes in general, a decrease in V_{max} is a common result. But a sharp diminution of K_M is seldom observed. In the case of immobilized invertase, there is no information about this point, even though this enzyme has been immobilized in a large variety of supports *(5,15–19)*.

From Fig. 3 we can observe that the immobilized invertase activity is at least 12% higher than that of the soluble form for sucrose concentration below 27.5 m*M*. Above this concentration, the activity of immobilized invertase reaches a maximum around 0.060 mg of TRS/(min · mL), whereas

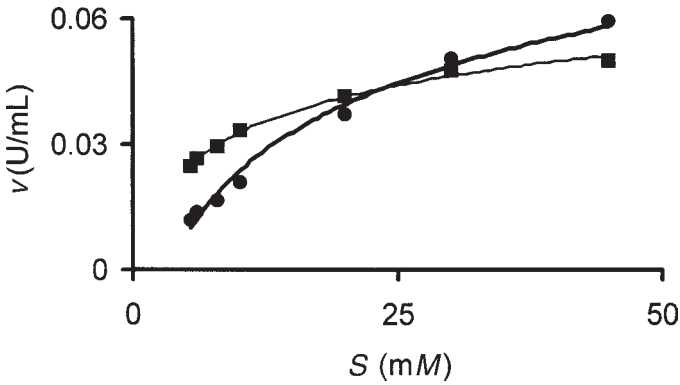

Fig. 3. Variation of free (●) and entrapped (■) invertase activity against sucrose concentration.

the activity of soluble invertase continues to grow up to 0.100 mg of TRS/(min · mL), as determined through the Lineweaver-Burk plot (Fig. 2). The behavior of immobilized invertase could be related to the simultaneous occurrence of diffusion and aggregation of enzyme molecules *(8)*, both affected by the small internal volume of the bead (about 65 mm^3). Accordingly, below 27.5 m*M* sucrose the entrapped invertase is more active than the free one, because the enhanced activity of the aggregated invertase molecules would prevail over the diffusion. Above 27.5 m*M* sucrose, at which the viscosity of the solution inside the bead increases *(20)*, the reaction rate of entrapped invertase is controlled by diffusion. At the end, the saturation condition is reached at a low sucrose concentration as compared with the free enzyme. Since less sucrose is needed to achieve $v = V_{max}/2$, then $(K_M)_{ap} < K_M$.

Taking into account the V_{max} of both forms of invertase (Table 2), the IEY was equal to 60%. This IEY is one of the best compared with those found in the literature *(15)*. The similar PIC and IEY values could be considered as evidence, even though indirect, that almost all invertase molecules in the beads are available for the substrate. This evidence, coupled with the peculiar characteristics of the alginates, such as low cost, safety for human use, high availability, ease of handling, and high *in situ* complexation capability with Ca^{2+}, undoubtedly show that this type of invertase immobilization presents good perspectives for industrial application.

Effect of Temperature on the Activity of Free and Immobilized Invertase

Activity vs Temperature

From Fig. 4 we can see that between 30 and 60°C the activities of free and entrapped invertase varied linearly with temperature, according to the Eqs. 1 and 2, respectively:

$$v = 0.0030T - 0.040 \quad (r = 0.998) \tag{1}$$

$$v = 0.0014T - 0.012 \quad (r = 0.995) \tag{2}$$

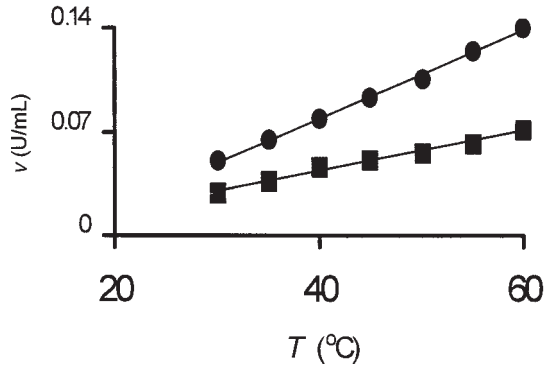

Fig. 4. Activity of free (●) and entrapped (■) invertase against temperature.

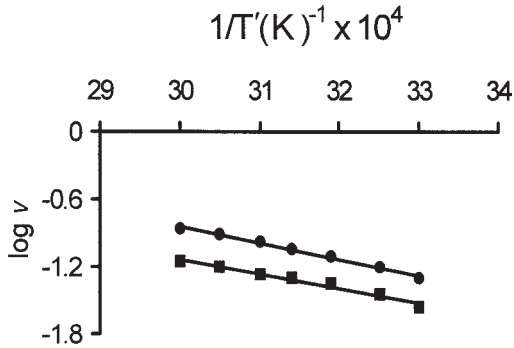

Fig. 5. Arrhenius plot for the determination of activation energy of free (●) and (■) entrapped invertase.

By comparing the angular coefficients of both equations, we observe that the free enzyme is more dependent on temperature than the immobilized one, provided that for each 1°C increase in temperature a twofold increase in the activity of soluble invertase occurs.

The activation energies (E_a) for free and immobilized invertase were calculated from Eqs. 3 and 4, respectively, established through the conventional Arrhenius plot (Fig. 5):

$$v = 3.53 - 1456 \cdot (1/T') \quad (r = -0.997) \tag{3}$$

$$v = 2.71 - 1280 \cdot (1/T') \quad (r = -0.990) \tag{4}$$

Then, the E_a for free and immobilized enzyme are 28 and 24 kJ/mol, respectively.

As can be seen, the E_a for immobilized invertase is 12% lower than that of free form. Barros and Vitolo *(5)* verified an increase of E_a when invertase was covalently immobilized on krill chitin using glutaraldehyde as a crosslinking agent. However, Oshima et al. *(21)* did not observe any variation on E_a of invertase adsorbed on ion-exchange resin. Thus, the E_a for

Table 3
Thermodynamic Parameters Related
to Free and Immobilized Invertase Calculated at 308 K

Invertase form	E_a (kJ/mol)	ΔG (kJ/mol)	ΔH (kJ/mol)	ΔS (kJ/[mol K])
Soluble	28.0	–13.6	25.2	0.13
Immobilized	24.0	–13.9	22.0	0.12

immobilized invertase depends on both the type of support and the procedure of immobilization. It is possible that in the case of calcium alginate entrapment, because the invertase molecules had been confined in a small volume (bead volume = 65 mm^3) their aggregation capability was enhanced, favoring catalysis at last. This hypothesis is in accordance with Reddy et al. *(8)* and Reddy and Maley *(22)*, who clearly showed that invertase molecules, which are dimers, associate spontaneously, forming more active catalytic structures (hexamers and octamers).

Table 3 shows the thermodynamic parameters related to both forms of invertase. As can be seen, ΔG was the same for both forms of invertase, an indication that the type of immobilization employed did not affect the global energy variation of the sucrose hydrolysis. Indeed, the enzyme continues in the solubilized form, in spite of the confinement inside the bead. This fact is corroborated through the small variation on entropy of both forms. Moreover, the 8% reduction on ΔS of immobilized invertase should reflect a little more organization of the entrapped system, represented by the supramolecular aggregates of invertase, as compared with the complete solubilized form. This fact could indirectly explain why PIC (66%) and IEY (60%) were similar. Finally, the low enthalpy for sucrose hydrolysis with immobilized invertase is in accordance with the 12% difference appointed to E_a for both forms of invertase. Thus, this result should add more interest in scaling up the sucrose conversion with entrapped invertase in calcium alginate.

Stability Against Temperature

From Fig. 6 we can observe that the free invertase was stable between 30 and 40°C. Afterward the stability diminished linearly as the temperature increased up to 55°C, as follows:

$$v = 0.24 - 0.0044T \quad (r = -0.993) \tag{5}$$

The stability of immobilized invertase decreased linearly with the temperature in the entire interval studied, as follows:

$$v = 0.14 - 0.0023T \quad (r = -0.996) \tag{6}$$

Above 40°C the thermostability of immobilized invertase was higher than that of the soluble one, as can be seen by comparison of the angular coefficients of Eqs. 5 and 6.

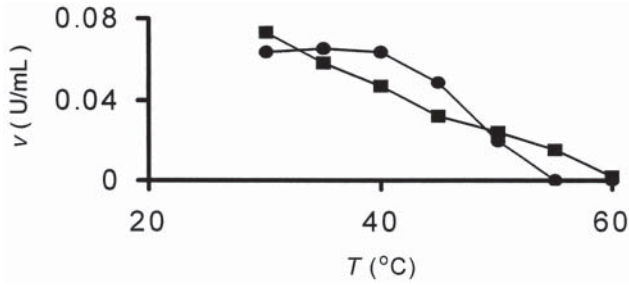

Fig. 6. Stability of free (●) and entrapped (■) invertase against temperature.

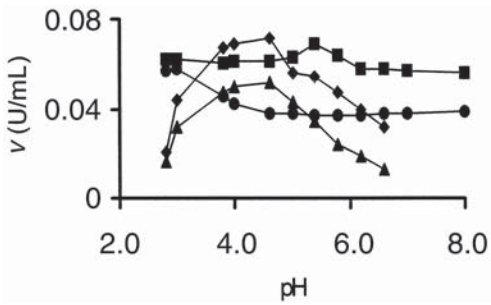

Fig. 7. Activity and stability of both forms of invertase against pH. Free invertase: activity (◆) and stability (■); entrapped invertase: activity (▲) and stability (●).

To understand this behavior, we must consider what was established by Reddy and Maley *(22)* about the enhanced catalytic performance of the hexameric and octameric forms of invertase over the dimers (its natural quaternary structure), and the adaptation of this idea for immobilized systems by De Queiroz et al. *(23)*. Accordingly, the behavior of the enzyme stability against temperature would depend on whether there was more or less protection conferred by the immobilization to the supramolecular forms of the enzyme. To the enzyme inside the beads, the octameric forms should be desegregated more slowly than the same enzyme in solution, because of the barrier imposed by the calcium alginate membrane to heat diffusion. When almost all invertase molecules are in dimer form, the tertiary structure begins to unfold, leading quickly to the complete denaturation of the protein molecule. In this work, such a turning point occurred at 50°C, because after this temperature more than 50% of the initial invertase activity is lost (Fig. 6).

Effect of pH on the Activity of Free and Immobilized Invertase

The high invertase activity occurred at pH 4.6 for both forms of the enzyme (Fig. 7). However, the enzyme activity decreased with the immobilization procedure, from 0.071 to 0.052 U/mL for free and immobilized forms.

The maintenance of optimum pH for the free and immobilized invertase could be attributed to the fact that the concentration of charged species (e.g., hydrogen ions) in the domain of the immobilized enzyme is similar to that in the bulk solution *(5)*.

From Fig. 7 we can also see that the free invertase was stable in the entire interval of pH studied, whereas the residual activity of the immobilized form diminished about 34% as the pH varied from 3.0 to 4.6. But at pH > 4.6 the residual activity remained unchanged at 0.038 U/mL. At pH 8.0 the residual activity of entrapped invertase was stable for at least 30 h, which allowed us to leave the beads to harden inside the 0.1 *M* $CaCl_2$ solution (buffered at pH 8.0) for 24 h with no additional loss in activity.

As observed, the optima pH for the activity and stability of immobilized invertase are different. Consequently, carrying out the sucrose hydrolysis with the beads containing invertase at pH 4.6 would lead to a loss in activity of about 34%. But, if the reaction were carried out at pH 3.0 (under which the stability is high), the activity would be 70% lower than that of pH 4.6. Thus, the operational pH of 4.6 must be chosen.

Conclusion

The data presented lead to the conclusion that entrapment must be carried out by dropping the 10 g/L of sodium alginate aqueous solution containing invertase (2.2 mg of total protein) into a 0.1 *M* $CaCl_2$ solution buffered at pH 8.0. The beads formed are left to harden for 24 h. The sucrose hydrolysis with the invertase beads is carried out at pH 4.6, 30°C, and a sucrose concentration of 30 m*M*. Working with such a diluted substrate solution has the advantage of avoiding any viscosity effect on the performance of the catalyst *(20)* and permits the use of a low temperature (30°C), which, in turn, reflects on energy economy of the process. These aspects are important from an industrial point of view, because they do not introduce any significant costs to the process. Indeed, they make the enzymatic sucrose hydrolysis process more attractive than the acid hydrolysis process.

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