JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Immobilization of Recombinant Invertase (re-INVB) from *Zymomonas mobilis* on D-Sorbitol Cinnamic Ester for Production of Invert Sugar

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The recombinant invertase (re-INVB) from *Zymomonas mobilis* was immobilized by adsorption onto the totally cinnamoylated derivative of D-sorbitol. The polymerization and cross-linking of the derivative initially obtained was achieved by irradiation in the ultraviolet region, where this prepolymer shows maximum sensitivity. Immobilization of re-INVB on this support involves a process of physical adsorption and intense hydrophobic interactions between the cinnamoyl groups of the support and related groups of the enzyme. Enzyme concentration, immobilization time, and irradiation time were important parameters affecting the immobilization efficiency. The optimum reaction pH of immobilized enzyme was 5, and the optimal reaction temperature was 40 °C. The apparent Michaelis constant and the apparent catalytic constant of re-INVB immobilized on the SOTCN derivative acting on sucrose was 78 ± 5 mM and $5 \times 10^4 \pm 3 \times 10^2$ s^{-1}, respectively, while for the free enzyme, it was 98.0 ± 4 mM and $1.2 \times 10^4 \pm 2.5 \times 10^2$ s^{-1}, respectively, suggesting a better apparent affinity of the enzyme for the substrate and a better hydrolysis rate when immobilized than when in solution. Immobilized re-INVB also showed good thermal stability and good operational stability (40% of the initial activity remaining after 45 cyles of 1 min duration and 90.6 mg of sucrose being hydrolyzed in 45 min per 2.5 mg of immobilized protein). The results showed that cinnamic carbohydrate esters of D-sorbitol are an appropriate support for re-INVB immobilization and the production of invert sugar.

KEYWORDS: INVB; *Zymomonas mobilis*; immobilization; cinnamic carbohydrate esters; enzyme kinetics; invertase INVBre stability

INTRODUCTION

Invertase enzyme catalyzes the hydrolysis of sucrose to produce invert sugar. In this study we have recombined invertase, producing recombinant invertase (re-INVB) from *Zymomonas mobilis* to obtain higher activities than with standard invertase. Invert sugar is used as fructose-rich syrup, principally in the beverage industry, and its production can be increased by immobilizing invertase in an active state (*1*). Recombinant enzymes are active in their folded and soluble forms and may be completely inactivated if produced as insoluble aggregates (*2*). It is therefore important for industrial purposes to find a method to immobilize the enzyme in an active state (*3*).

In this study, the polymeric support used for re-INVB immobilization was the totally cinnamoylated derivative of D-sorbitol (SOTCN) on glass beads, which allowed immobilization by adsorption, with little change in enzyme conformation, and provided an active immobilized re-INVB. This support was previously used to immobilize other enzymes including tyrosinase and peroxidase with good results (*4–7*). More specifically, the effect of immobilizing re-INVB on its catalytic activity and on its stability in the face of different factors was studied.

MATERIALS AND METHODS

The *invb*petK plasmid was obtained from CINVESTAV-IPN (Mexico) (*2*). Sucrose and D-sorbitol were purchased from Sigma (Spain). All other chemicals were of analytical grade and supplied by Fluka (Spain), Panreac (Spain), J. T. Baker (Holland), Sigma (Spain), and Laboratory-Scan (Ireland). Ultrapure water from Milli-Q system (Millipore Corp.) was used throughout this research.

Recombinant Protein Production. Inoculum. A fresh clone of *Escherichia coli* BL21 (*DE3*) harboring the *invb*petK plasmid, was grown in 500 mL shake flasks containing 100 mL of 2TY medium (16 g/L peptone, 10 g/L yeast extract, and 5 g/L NaCl at pH 7.5). Cultures

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10.1021/jf072646h CCC: $40.75
Published on Web 02/01/2008

were incubated at 37 °C and 250 rpm for 2 h in an orbital shaker up to an optical density of 0.8 at 600 nm (OD_{600}). For recombinant protein expression, a 25% (v/v) inoculum ratio was used to seed a 1.0 L fermenter containing 0.4 L of 2TY medium. Dissolved oxygen was manually controlled at 20–30% saturation by means of an airflow (0.2–0.25 vvm) and altering the stirring speed (400–900 rpm). Protein expression was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM.

The cells contained in 1 mL of culture broth were harvested by centrifugation (4000*g* and 4 °C for 20 min) and resuspended in Laemmli sample buffer prior to analysis of total protein extract by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) (*8*). Soluble protein was prepared from the bacterial pellet of the remaining culture broth resuspended in lysis buffer (20 mM Tris-HCl at pH 7.5) at 20:1 (w/v) ratio. Cells were lysed with lysozyme (10 mg mL^{-1}) at 4 °C for 30 min and by means of a AMINCO HA6027 Press at 16 000 psi. Cell debris was removed by centrifugation (10000*g* at 4 °C for 15 min), and the supernatant was used to analyze invertase activity and proteins by SDS−PAGE. The enzyme was purified by nickel-affinity chromatography "Ni Sepharose High Performance" (Amershan Pharmacia). Fractions (4 mL) were collected and analyzed by SDS−PAGE. Chromatography was made in a BioLogic LP (BioRad) chromatograph.

Preparation of Totally Cinnamoylated Derivative of D-Sorbitol (SOTCN). A modified version of the method proposed by Van Cleve (*9*) was used (*4*). For this, 0.02 mol of D-sorbitol was dissolved in 100 mL of pyridine by heating at 60 °C for 1 h. The resulting solution was cooled to room temperature before adding 0.15 mol of cinnamic acid chloride. The reaction was allowed to proceed at room temperature for 4 h, after which the resulting mixture was poured into vigorously stirred water at 4 °C. The precipitate obtained after decanting and filtering this mixture was dissolved in chloroform (the minimum quantity necessary to dissolve the precipitate) and purified by dropwise addition to vigorously shaken hexane. The solid obtained after redissolving and reprecipitating was dried on P_2O_5 at reduced pressure.

Re-INVB Immobilization. MICROPERL Industrial (Type A) glass beads of 0.6–1.0 mm diameter manufatured by SOVITEC IBERICA S.A. (Barcelona, Spain) were supplied by JAQUE (Murcia, Spain). Before use, the glass beads were washed and degreased (*6, 10*). A chloroform solution of SOTCN at 15 g/L was prepared, into which the glass beads were immersed. A film of prepolymer was formed on the beads, and the solvent was eliminated by evaporation and low-pressure suction. After drying, the prepolymer film was polymerized by irradiation in the ultraviolet zone for 15 min using an Osram HOL-125W mercury vapor lamp providing a power of 1.6 mW/cm^2, as determined by a Nover-Laser power/energy monitor (Ophir Optronics Ltd.).

Re-INVB was immobilized at pH 5.5, which is the optimun pH for the free enzyme. Moreover, the pI of invertase (4.9) is very near the immobilization pH chosen, with this close relation between pI and optimum immobilization pH (in the range of 4.5–5.5) having previously been observed for tyrosinase immobilized on the same support (*4, 5*). To immobilize re-INVB, a 2.5 mL solution of this enzyme in sodium acetate buffer at pH 5.5 was added to a syringe containing 3 g of glass beads covered with the immobilization support. The immobilization was allowed to proceed for 12 h at 4 °C. After immobilization, the enzyme solution was withdrawn and the immobilized enzyme was thoroughly rinsed in distilled water.

Invertase Activity Assay. Spectrophotometric measurements were made with a PerkinElmer, Lambda 35 UV/vis spectrophotometer controlled by a PC running with the software Lambda 35, adjusted to the desired wavelength. Soluble and immobilized re-INVB enzymatic activities were determined by the DNS method (*11*), and the increase in absorbance was measured at 550 nm. Equimolar mixture of glucose and fructose was used as standard. The assay medium contained 290 mM sucrose in 50 mM sodium acetate buffer at pH 5.5 for free enzyme and pH 5 for immobilized enzyme, the reaction was carried out at room temperature for 1 min. To assay the immobilized enzyme, syringes containing 3 g of glass beads with bound re-INVB were used as small packed bed continuous reactors with recirculation (6 mL) and descending flow. The substrate solution was pumped at 20 mL/min using a Minipuls 3 peristaltic pump. The enzymatic activity was expressed as UI (micromoles of sucrose hydrolyzed per minute) per milligram of immobilized protein (UI/mg). In many cases, the results were normalized, considering the highest enzymatic activity value obtained in each of the series of measurements made as 100%.

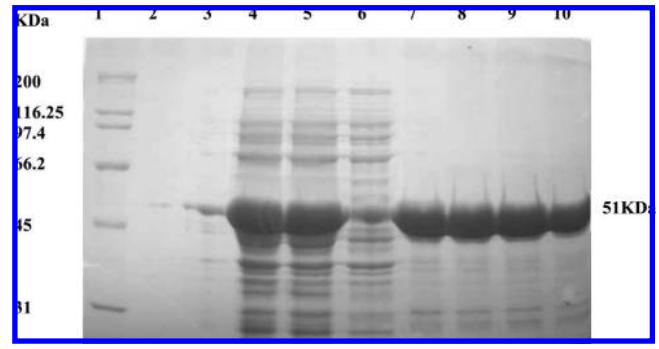

Figure 1. SDS−PAGE (10%) analysis of total protein extracts of transformed *E. coli* BL21(*DE3*). For induction and purification, 20 μL of sample containing 350 mg of protein was injected. Lane 1, MW markers; lanes 2 and 3, noninduced; lanes 4 and 5, induced with IPTG; lane 6, proteins in the washed fraction; lanes 7–10, pure re-INVB enzyme.

Protein Determination. The amount of protein in a solution was determined by the Lowry method (*12*), and the amount of immobilized protein was determined by measuring protein in the immobilization medium before and after the immobilization process.

Study of the Enzyme Concentration, Immobilization Time, and Irradiation Time. The effect of the protein concentration in the immobilization medium (0.1–4 mg/mL), immobilization time (1–70 h), and irradiation time (2.5–30 min) of the support was studied. For each measurement, a syringe containing enzyme immobilized on SOTCN derivative was used, as described in the Materials and Methods.

Dependence of Enzyme Activity on pH and Temperature. The effect of reaction pH on the enzymatic activity of the immobilized and free re-INVB was determined using a pH range of 3.5–7.5 at room temperature. For the pH range of 3.5–5.5, the sucrose solutions (290 mM) were prepared in both an aqueous solution of 50 mM sodium phosphate and 50 mM sodium acetate buffer, and for the pH range of 6–7.5, the sucrose solutions were prepared in 50 mM sodium phosphate buffer. The effect of the reaction temperature on the enzymatic activity of the samples was studied between 25 and 60 °C.

Thermal Stability. The thermal stability of immobilized re-INVB on glass bead (3 g) covered with SOTCN was studied by incubating samples at the desired temperature (25–60 °C) for different lengths of time.

Operational Stability and Hydrolyzed Sucrose Amount. Operational stability was studied by submitting a syringe containing 3 g of glass beads covered with SOTCN and immobilized re-INVB to 45 reaction cycles of 1 min each (washing with distilled water between each cycle), and the total amount of sucrose hydrolyzed was calculated.

Steady-State Kinetics and Kinetic Data Analysis. The steady-state kinetic constants, V_{max}^{app} (apparent maximum steady-state rate) and K_m^{app} (apparent Michaelis constant), of the immobilized and free re-INVB were obtained, by measuring in triplicate the initial rates (v_0) of the reaction with different sucrose concentrations and fitting the data by the Lineweaver–Burk plot (*13*) using the Sigma Plot 9.0 program for Windows. In every case k_{cat}^{app} (apparent catalytic constant) was obtained from the values obtained for V_{max}^{app}.

RESULTS AND DISCUSSION

Expression of the Recombinant INVB Protein. SDS−PAGE analysis showed the stages followed from expression to purification (**Figure 1**). The extracts from the bacteria harboring the construct *inv*BpetK showed a band of 51 kDa, which agrees with the expected theoretical molecular weight of the recombinant INVB (*2*): lanes 2 and 3 show the total protein profile from noninduced cells, while lanes 4 and 5 show the total protein

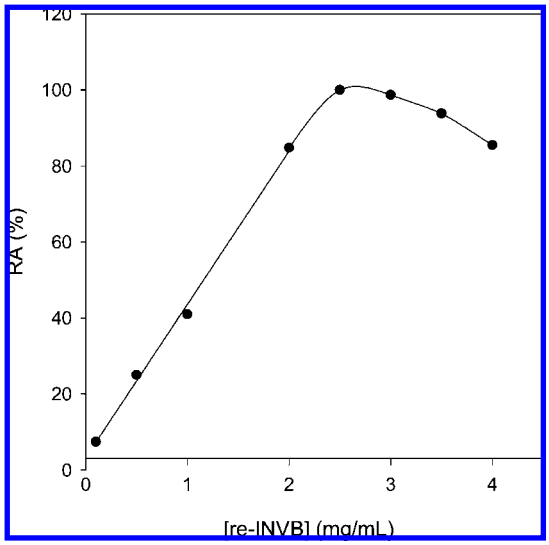

Figure 2. Effect of re-INVB concentration on enzymatic activity retained on glass beads covered with the cross-linked SOTCN. Representation of the relative activity, RA (%), versus the re-INVB concentration (mg/mL).

profile from induced cells. Fractions obtained from the nickel-affinity chromatography were also analyzed (lanes 6–10 in **Figure 1**). Lane 6 corresponds to proteins in the washed fraction, while lanes 7–10 correspond to fractions containing the purified re-INVB enzyme, after elution with 250 mM imidazol.

Cinnamic Carbohydrate Esters as Immobilization Supports. SOTCN was synthesized and used as support for re-INVB immobilization. This support was previously used to immobilize other enzymes, such as tyrosinase and peroxidase (*4–7*) with good results. One of the characteristics of this support is that all of the hydroxyl groups are esterified with cinnamoyl groups, as can be deduced from various experimental analyses. ^{1}H nuclear magnetic resonance (NMR), ^{13}C NMR, distortionless enhancement by polarization transfer (DEPT) spectra, and two-dimensional experiments (COSY and C/H ratio) of the prepared compound pointed to its complete esterification, similar to that seen for other cinnamate derivatives. The infrared spectra of the prepared compound also indicated full esterification (*4*). Differential thermal analysis showed sharp heat-absorption peaks (probably melting), which disappeared in repeat runs because of oligomerization of the sample (*4*). Therefore, the support is very heat-stable but requires cross-linking prior to use. The cross-linking of SOTCN was achieved by irradiation in the ultraviolet region, where the cinnamic carbohydrate esters showed maximum sensitivity.

The beads (i.e., the inert matrix) were covered with a film of the cinnamoyl derivative (i.e., the immobilization support) by solvent evaporation. The best results were obtained at support concentrations close to 15 g/L (*10*), when approximately 0.52 $\pm$ 0.03 mg/g of glass beads was obtained (*4*). It was assumed that the re-INVB enzyme was immobilized on the glass beads covered with the SOTCN derivative through a process of physical adsorption involving intense hydrophobic interactions between the cinnamoyl groups of the support and related groups of the enzyme.

Invertase Immobilization. Several aspects including (i) re-INVB concentration, (ii) immobilization time, and (iii) irradiation time were studied to find the best immobilization conditions. The amount of immobilized protein was also determined.

When the most suitable initial concentration of re-INVB was studied for immobilization on SOTCN (**Figure 2**), the results

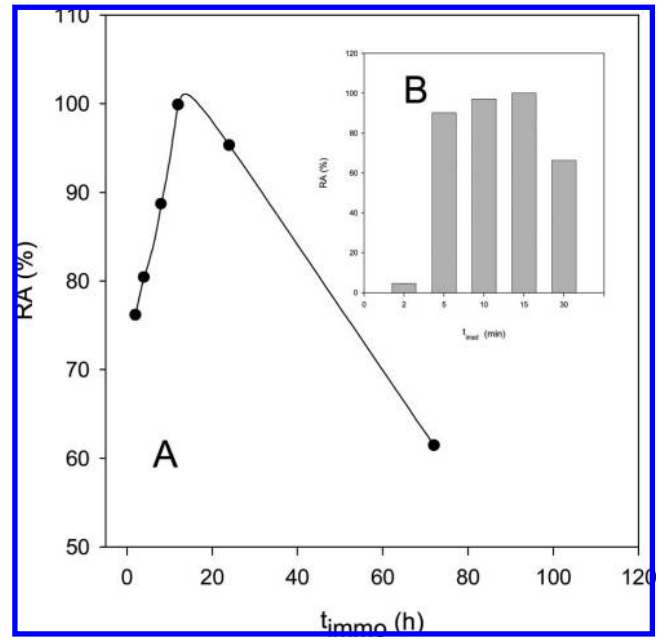

Figure 3. (**A**) Effect of immobilization time on enzymatic activity retained by re-INVB immobilized on glass beads covered with the cross-linked SOTCN. Representation of the relative activity, RA (%), versus the immobilization time, t_{immo} (h). (Inset **B**) Effect of irradiation time on enzymatic activity retained by re-INVB immobilized on glass beads covered with cross-linked SOTCN. Representation of the relative activity, RA (%), versus the irradiation time, t_{irrad} (min).

showed a lineal increase of the activity with the enzyme concentration up to 2.5 mg/mL. More concentrated enzyme solutions were not appropriate because activity did not increase further and even decreased slightly for 4 mg/mL. The increase in the activity of the immobilized enzyme with an increasing enzyme concentration in the immobilization medium was similar to that obtained for invertase immobilized on other supports (*1, 14*) and for other enzymes, such as tyrosinase, immobilized on the same support (*4*). The slight decrease in the activity at high concentrations is probably due to enzyme packaging, which would slightly inactivate the enzyme. An initial soluble re-INVB concentration of 2.5 mg/mL, which provided the greatest immobilized activity, was chosen as the optimum immobilization concentration. The values obtained for enzymatic activity were sufficiently high to be correctly measured, and good reproducibility was achieved for the different assays.

Another important feature was the effect of immobilization time on the retained enzymatic activity. In this assay, the immobilization times were varied between 1 and 70 h. The results (**Figure 3A**) indicated that maximum enzyme activity was achieved for immobilization times of 12 h, above which activity decreased. Similar behavior has been observed for invertase immobilized on other supports (*1, 3, 14*) and for other enzymes, such as tyrosinase, immobilized on the same support and for oxidases immobilized on other supports (*4, 15*).

The effect of irradiation time on the initial activity retained was studied (**Figure 3B**), irradiating the SOTCN derivative for 2.5−30 min. The activity increased sharply during the first 5 min of irradiation, after which no further effect was observed (5–20 min) probably because cross-linking was complete and the support had already reached its maximum possible molecular weight. A similar behavior was observed for tyrosinase immobilized on the same support (*4*) and for HRP immobilized

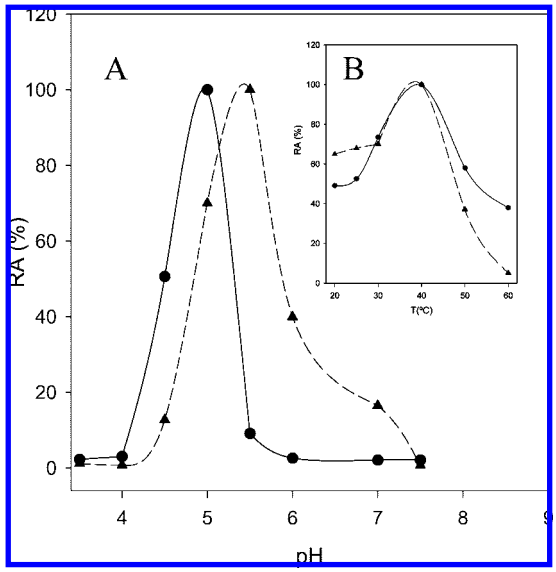

Figure 4. (**A**) Effect of reaction pH on re-INVB enzymatic activity. (●) Re-INVB immobilized on glass beads covered with cross-linked SOTCN and (▲) free re-INVB. Representation of the relative activity, RA (%), versus the reaction pH. (Inset **B**) Effect of reaction temperature on re-INVB enzymatic activity. (●) Immobilized re-INVB on glass beads covered with cross-linked SOTCN and (▲) free re-INVB. Representation of the relative activity, RA (%), versus the reaction temperature, *T* (°C).

on cinnamic carbohydrate esters (*7*). A total of 15 min was chosen as optimum irradiation time.

Finally, in the optimal immobilization conditions described above, the amount of immobilized re-INVB was 0.38 $mg_{protein}/mg_{support}$ and the specific activity was 200 UI/mg, which is much better than that described in the literature; for example, the specific activity of recombinant invertase immobilized on Avicel was 20-fold lower than that of our recombinant invertase immobilized on SOTCN (*16*), while periodate-oxidized invertase adsorbed on sepiolite showed a specific activity value of 0.18 UI/mg (lower by a factor of 10^3) (*14*); specific activity of entrapped immobilized invertase on composite gel fiber of cellulose acetate and zirconium alkoxide was 200-fold lower than in our case (*17*). This high specific activity suggest that re-INVB immobilized on SOTCN could well be used for industrial applications.

Behavior of Immobilized re-INVB in SOTCN Derivative. To study the properties of immobilized re-INVB on SOTCN and to check the applicability of this support for immobilizing this enzyme and its possible further use in industry, several aspects were investigated.

Effect of pH and Temperature on Catalytic Activity. The effect of pH on the activity of immobilized and free re-INVB was studied in the 3.5–7.5 range at room temperature (**Figure 4A**). The optimum pH for the hydrolysis of sucrose by immobilized re-INVB on SOTCN was pH 5, while for the free enzyme, it was pH 5.5. A small shift in the pH profile (*18*) to more acid pH values was observed when the enzyme was immobilized on SOTCN but not in the case of a recombinant invertase immobilized on Avicel, with optimum pH value of 5.5 in both cases (*16*). The optimum pH value obtained was similar to that mentioned for other immobilized invertases (*1, 3, 14, 19*).

The results obtained when studying the activity of immobilized and free enzyme at temperatures between 25 and 60 °C are shown in **Figure 4B**. As can be seen, the optimal reaction temperature for re-INVB immobilized on SOTCN was 40 °C, the same as for the free enzyme (*2*) and similar to that obtained

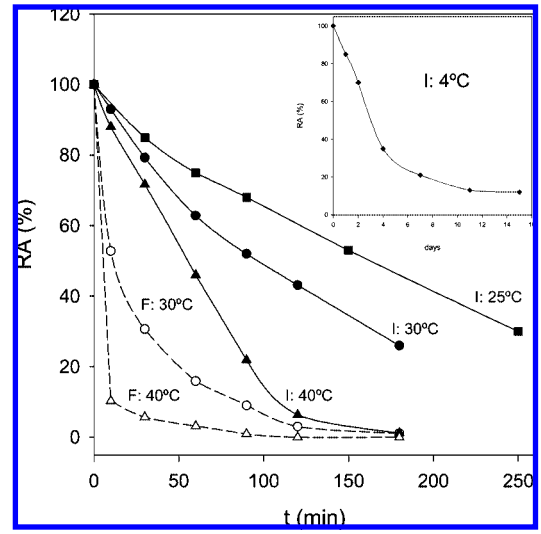

Figure 5. Thermal stability of re-INVB. Representation of residual enzymatic activity, RA (%), versus time (min). I, immobilized re-INVB; F, free re-INVB. Temperatures were I (25 °C, 30 and 40 °C) and F (30 and 40 °C). (Inset) Representation of residual enzymatic activity RA (%) versus time (min), I (4 °C).

for a recombinant invertase immobilized on Avicel (*16*) and other immobilized invertases (*16, 20–22*). At high temperatures, immobilized re-INVB retained more activity than the free enzyme, which indicates that the immobilization process stabilizes the enzyme.

Thermal Stability. The thermal stability of immobilized enzymes is one of the most important factors for possible further applications. To study the effect of immobilization on the thermal stability of re-INVB, samples of immobilized and free enzyme were incubated at 4 (inset in **Figure 5**) and 25, 30, and 40 °C in the first case and 30 and 40 °C in the second case (**Figure 5**). In both cases, free and immobilized enzyme stability decreased when the incubation temperature increased, as has been described in the literature (*1, 3, 14*). When syringes with immobilized re-INVB were stored at 4, 25, 30, and 40 °C, 50% of the initial activity remained after 3600, 250, 100, and 70 min, respectively, resulting in the same order as those obtained for other recombinant invertase immobilized on Avicel (*16*). In the case of free re-INVB, 50% of the initial activity remained after 20 min of incubation at 30 °C and after 10 min at 40 °C, meaning that free re-INVB stability is 5-fold lower at 30 °C and 7-fold lower at 40 °C than the stability of re-INVB immobilized on SOTCN. The immobilization of re-INVB by adsorption on SOTCN significantly improved its resistance to thermal inactivation, especially at high temperatures. Despite this substantial improvement in the thermal stability of immobilized re-INVB, it is not still as good as the stability of other native immobilized invertases (*1, 3, 20*) but certainly sufficient for it to be considered as of potential use in the industrial production of invert sugar, especially if we take into account the high specific activity obtained.

Operational Stability of Immobilized re-INVB and Hydrolyzed Sucrose. The operational stability of re-INVB immobilized on glass beads covered with SOTCN derivative was ascertained by reusing the immobilized enzyme for a total of 45 cycles, each of 1 min duration (**Figure 6**). There was a gradually decrease in activity after each successive use and about 42–49% of the initial activity remained after 45 cycles. These results point to the good operational stability of the immobilized enzyme compared to that described in the literature (*23, 24*) and is similar to the stability of tyrosinase immobilized on the

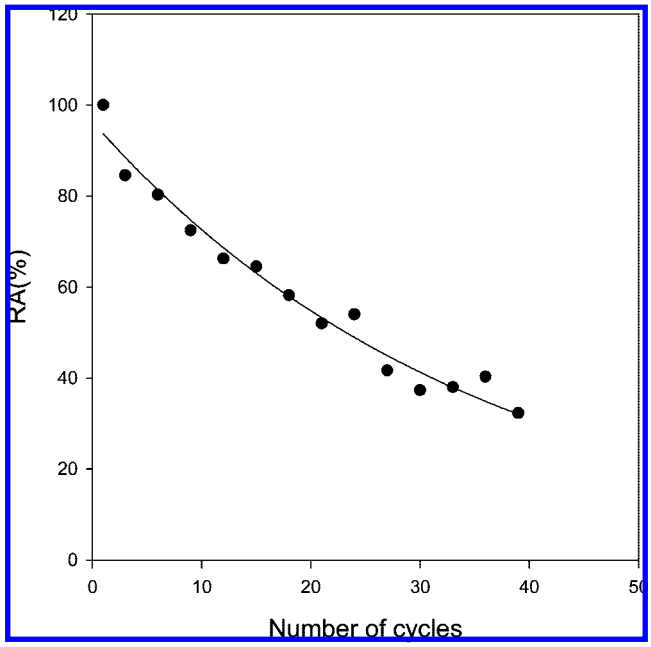

Figure 6. Operational stability of re-INVB immobilized on glass beads covered with cross-linked SOTCN. Representation of residual enzymatic activity, RA (%), versus number of cycles (*N*).

same support (*5*). The fall in the activity of immobilized re-INVB through reuse might also be due to thermal inactivation of the enzyme at the assay conditions (25 °C). The amount of sucrose hydrolyzed after each cycle was calculated, and a total amount of 90.6 mg of reducing sugars after 45 cycles was obtained. This was considered a good result and underlines the potential of the described immobilization process for obtaining reducing sugars on an industrial scale.

Kinetic Study of re-INVB Immobilized on SOTCN. The apparent maximum steady-state rate, V_{max}^{app}, the apparent Michaelis constant, K_m^{app}, and apparent catalytic constant k_{cat}^{app} values of the immobilized and free re-INVB were determined. A Michaelis–Menten type kinetic behavior was observed in both cases. After fitting the data by Lineweaver–Burk plot, we obtained values of 78 ± 5 and 98 ± 4 mM for the K_m^{app} of immobilized and free re-INVB, respectively. The k_{cat}^{app} values of immobilized and free enzyme were $5 \times 10^4 \pm 3 \times 10^2$ and $1.2 \times 10^4 \pm 2.5 \times 10^2$ s^{-1}, respectively.

The K_m^{app} was affected by the immobilization process and showed a lower value than free re-INVB, meaning that no unfavorable conformational changes of the enzyme occurred during immobilization (*24*), and the lower K_m^{app} value would be due to the higher sucrose concentrations near the enzyme because of substrate adsorption on the support (*25, 26*). Re-INVB apparent affinity for the substrate improved when it was immobilized, the same as observed for another recombinant invertase immobilized on Avicel (*16*). However, for other native invertases, the opposite effect was observed (*1, 20–22, 24*), as in the case of invertase immobilized on montmorillonite K-10 (*19*) when an increase of 14-fold in the K_m^{app} value was observed after immobilization, indicating an important loss in the affinity for the substrate.

A better (higher)k_{cat}^{app} value was obtained for immobilized re-INVB than for free re-INVB, as was observed for tyrosinase immobilized by the same method (*25, 26*). This suggests that, upon being immobilized on the hydrophobic supports, the enzyme "opens out", binding itself to the support by its hydrophobic part and enabling the substrate to reach the active center of immobilized enzyme more easily than the active center of free enzyme, explaining the higher k_{cat}^{app} A favorable conformational change occurred when re-INVB was immobilized on SOTCN, with a better hydrolisis rate being obtained than when in solution. This result is much better than that obtained for other invertases mentioned in the literature, for which a decrease about 24−60% in the activity was observed after enzyme immobilization (*17, 20, 27*), even in the case of another recombinant invertase immovilized on Avicel (*16*).

To sum up, the re-INVB enzyme immobilized on glass beads covered with cross-linked SOTCN by means of physical adsorption provided good enzymatic activity and the results obtained showed good reproducibility. The best enzymatic activity of immobilized re-INVB was reached after optimizing the initial enzyme concentration in the immobilization medium (2.5 mg/mL), the immobilization time (12 h), and the irradiation time (15 min). No great differences were observed between immobilized and free re-INVB wth regard to the optimum reaction pH and temperature. The K_m^{app} value after immobilization was lower than the corresponding value for the free enzyme, showing better apparent affinity for the substrate. The higher k_{cat}^{app} values obtained for the immobilized re-INVB than for the free enzyme showed that the rate of sucrose hydrolysis is also better after the immobilization process and better than that obtained for other immobilized invertases. Good operational stability was obtained for immobilized re-INVB, and thermal stability improved after immobilization. These results showed that the cross-linked cinnamic ester of D-sorbitol represents an appropriate support for re-INVB immobilization and the re-INVB immobilized on it could be used for the industrial production of invert sugar.

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Received for review September 6, 2007. Revised manuscript received November 26, 2007. Accepted December 5, 2007. This work was supported by a grant from the Ministerio de Educación y Cultura (Spain) Project MAT2004-01893, Consejería de Ciencia, Tecnología, Industria y Comercio (Murcia, Spain) Project 2103 SIU0043, and the Fundación Séneca (Spain) Projects 04691/bps/06. V.V.-B. gratefully acknowledges a scholarship from CONACYT, México. M.E.M.-Z. gratefully acknowledges a working contract with the University of Murcia.

JF072646H