Immobilization of Invertase onto Dimer Acid-*co*-alkyl Polyamine

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ABSTRACT: Invertase was immobilized onto the dimer acid-*co*-alkyl polyamine after activation with 1,2-diamine ethane and 1,3-diamine propane. The effects of pH, temperature, substrate concentration, and storage stability on free and immobilized invertase were investigated. Kinetic parameters were calculated as 18.2 m*M* for K_m and 6.43×10^{-5} mol dm^{-3} min^{-1} for V_{max} of free enzyme and in the range of 23.8–35.3 m*M* for K_m and 7.97–11.71 $\times$ 10^{-5} mol dm^{-3} min^{-1} for V_{max} of immobilized enzyme. After storage at 4°C for 1 month, the enzyme activities were 21.0 and 60.0–70.0% of the initial activity for free and immobilized enzyme, respectively. The optimum pH values for free and immobilized enzymes were determined as 4.5. The optimum temperatures for free and immobilized enzymes were 45 and 50°C, respectively. After using immobilized enzyme in 3 days for 43 times, it showed 76–80% of its original activity. As a result of immobilization, thermal and storage stabilities were increased. The aim of this study was to increase the storage stability and reuse number of the immobilized enzyme and also to compare this immobilization method with others with respect to storage stability and reuse number.
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Key words: invertase; dimer acid-*co*-alkyl polyamine; enzymes; kinetics (polym.); thermal properties

INTRODUCTION

The most commonly used techniques for enzyme immobilization are designed to adsorb or covalently attach a polymeric carrier to, or interwine about, an enzyme. There are numerous methods of preparing water-insoluble enzymes.[1–4] The advantages of this method are reproducibility of the carrier substance to be synthesized or isolated, reproducible bonding of the proteins to the carrier substances, no elution of the homopolar-bonded protein with both low and high molecular weight substrates, and universal use of the carrier-bounded proteins in batch, screen, column, and other reactor systems.[5] The most frequently used carriers have been based on cellulose, glass, copolymers of polyamino acids, poly(*p*-chloromethylstyrene) beads, starch, agarose, crosslinked dextran, and ethylene–maleic anhydride copolymers.[6–8] Different polypyrrole/polytetrahyrofuran/invertase electrodes were constructed by the entrapment of invertase in conducting polymer matrices by electropolymerization.[9,10] A very large number of reactions have been used for the covalent coupling of the enzymes.[11–15]

The kinetic behavior and activities of immobilized enzyme usually differ appreciably from those of free enzyme. The nature of interaction between enzyme and polymer carrier is dependent on the three-dimensional structure of the enzyme. Some changes may also be attributable to the chemical modification resulting from the coupling.[16,17]

In recent years, hydrogels have been used for the immobilization of enzymes, proteins, antibodies, and antigens because of their versatile applications in biomedicine and biotechnology.[18–20] Advantages of using hydrogel for immobilization of enzymes have been widely described in the literature.[21–24] General operational advantages of immobilized enzymes are reusability, possibility of batch continuous operational modes, rapid termination of reactions, controlled product formation, easy separation of the product, great variety of engineering design for continuous processes, and possible greater efficiency in consecutive multistep reactions. As long as the enzyme can be stabilized by modification or immobilization reuse of the enzyme may be worthwhile.[25]

The invertase (EC 3.2.1.26) enzyme that catalyzes the hydrolysis of sucrose to glucose and fructose is largely used in the food industry to prevent the crystallization of sucrose in sugar mixtures. A number of studies have been reported in the literature on the immobilization and adsorption of invertase on various polymeric agents and gels.[15,21–24,26] The optimum coupling conditions such as enzyme concentrations, time, and pH have been reported elsewhere.[12,27]

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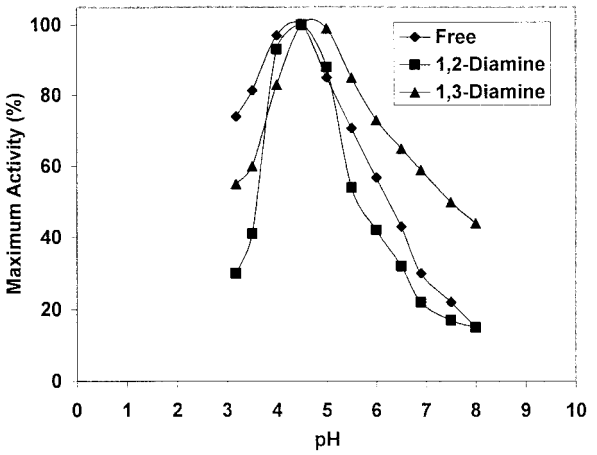

Figure 1 Effect of pH on free and immobilized invertase activity.

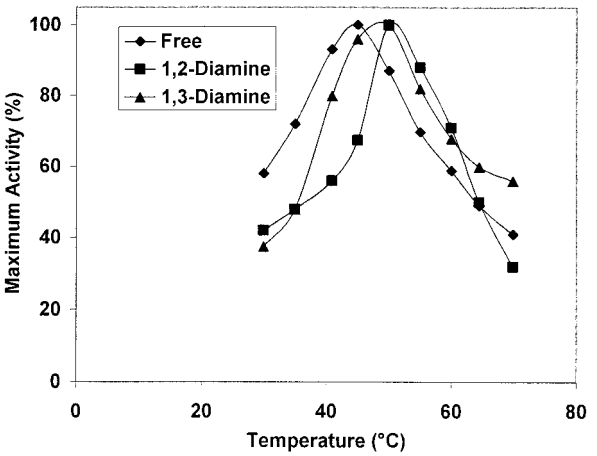

Figure 2 Effect of temperature on free and immobilized invertase activity.

In this study, dimer acid-*co*-alkyl polyamine copolymers were used. The enzymatic performance of the dimer acid-*co*-alkyl polyamine at various temperatures and pH and a comparison with the free enzyme are also reported here.

EXPERIMENTAL

Materials

The invertase (Fructofuranosidase, EC 3.2.1.26), used for the immobilization studies, was purchased from Fluka (Buchs, Switzerland). Sucrose was a product of Sigma (St. Louis, MO) and used as the substrate. Biochemical-grade glucose was provided by Fluka. Dimer acid-*co*-alkyl polyamine was supplied by Aldrich (Milwaukee, WI). 1,2-Diamine ethane and 1,3-diamine propane were supplied by Merck (Darmstadt, Germany) and used without further purification. All the chemicals used in the preparation of buffers were supplied by Merck and used without further purification.

Activation with diamine

Dimer acid-*co*-alkyl polyamine (0.1 g) was placed in 5 mL diamine solution. The activation was carried out at 30°C in a shaking water bath for 4 h and left at that temperature overnight. The activated polymer was separated and washed with phosphate buffer (pH: 4.3, 0.2*M*, 15 mL).

Enzyme immobilization

The activated dimer acid-*co*-alkyl polyamine was placed in a 20 mL solution of 40 mg dL^{-1} invertase and the immobilization reaction was carried out at 30°C in a shaking water bath for 4 h. Polymers were separated and free enzyme was removed by washing with phosphate buffer (pH: 4.3, 0.2*M*, 15 mL). The immobilized enzymes were freshly used and stored at 4°C.

Enzyme assay

The enzymatic activity of invertase was determined by an enzyme reaction using sucrose as substrate. The activities of immobilized and free invertase were determined by the Folin–Wu method. Assay solutions containing free or immobilized enzyme in phosphate buffer (0.2 mg enzyme or 0.1 g polymer in 1.0 mL phosphate buffer) were placed in a test tube. A substrate solution (1% sucrose) was added to the tubes and the incubation was continued for exactly 15 min. At the end of 15 min, the tubes were removed from the water bath (30°C); to terminate the reaction, 1 mL of alkaline copper sulfate solution was added to the tube.

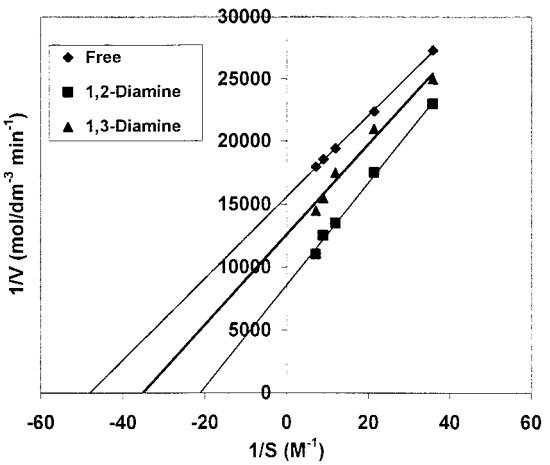

Figure 3 Lineweaver–Burk plots for free and immobilized invertase.

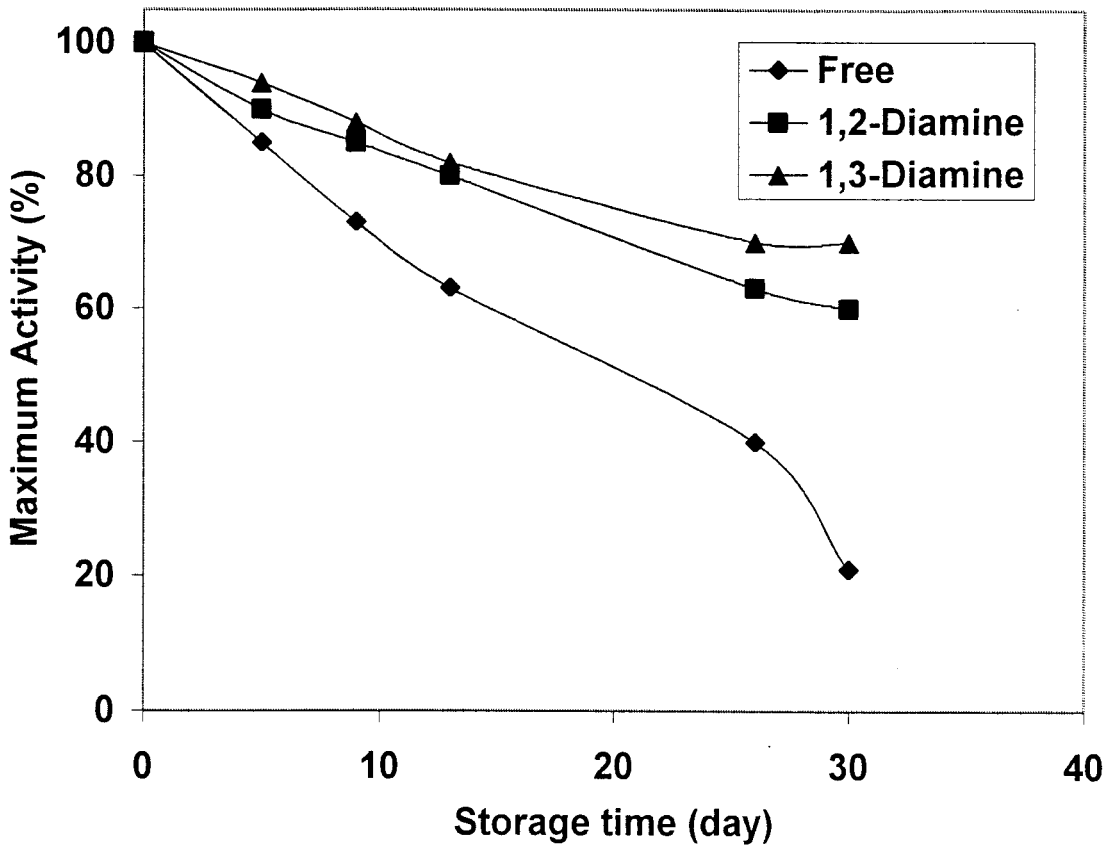

Figure 4 Storage stability of free and immobilized invertase.

The tube was allowed to remain in the boiling water bath until a brown color was observed, after which the tube was cooled in water at room temperature. Later, 1 mL of phosphofomolibdic acid reagent was added to tube and mixed thoroughly by vortex. Finally, 10.0 cm^3 of phosphate buffer was added to the tube. The absorbance was read at 640 nm versus a blank solution. The blank solution was prepared in the same manner in the absence of the enzyme. The amount of glucose was obtained from the calibration curve and used in the calculation of enzyme activity. The activity of invertase (moles of glucose + fructose formed/mg protein min^{-1}) was calculated (1 U of enzyme activity is defined as that amount of enzyme that hydrolyzes 1 μmol sucrose/min under the present assay conditions).

RESULTS AND DISCUSSION

Effect of pH and temperature on enzyme activity

The influence of pH on the enzyme activity was investigated in the pH range of 3.0–8.0 at 30°C. The pH activity profiles of free and immobilized invertase on the polymer were similar in both situations (Fig. 1). The optimum pH values of free and immobilized invertase were reported between 4.0 and 5.4 in the literature.[8,12,23,28,29] It can be seen from Figure 1 that maximum activity is observed at pH 4.5 for free and immobilized invertase. Polyionic matrices that cause partitioning of protons between the bulk phase and enzyme microenvironment are well known.[30] The pH maximum activity for immobilized invertase shifted to the more alkaline side compared to native invertase.

The effect of temperature on the activity of free and immobilized invertase is shown in Figure 2. Maximum activity is observed at 45 and 50°C for free and immobilized invertase, respectively. The optimum temperature for the immobilized invertase was 5°C higher than that of free enzyme, attributed to the creation of conformational limitations on the enzyme movement as a result of formation of lower interaction between the enzyme and the matrix. Similar observations were previously reported in the literature.[11,12,27,31] Immobilization improved both the pH stability and temperature stability of enzymes. Thermal stability was found to increase with immobilization and at 70°C.[32]

The maximum activity of free and immobilized invertase is only slightly affected by pH.[21–23,33] Immobilized invertase is more active at higher temperatures but alginate gel undergoes thermal degradation at such temperatures.[23]

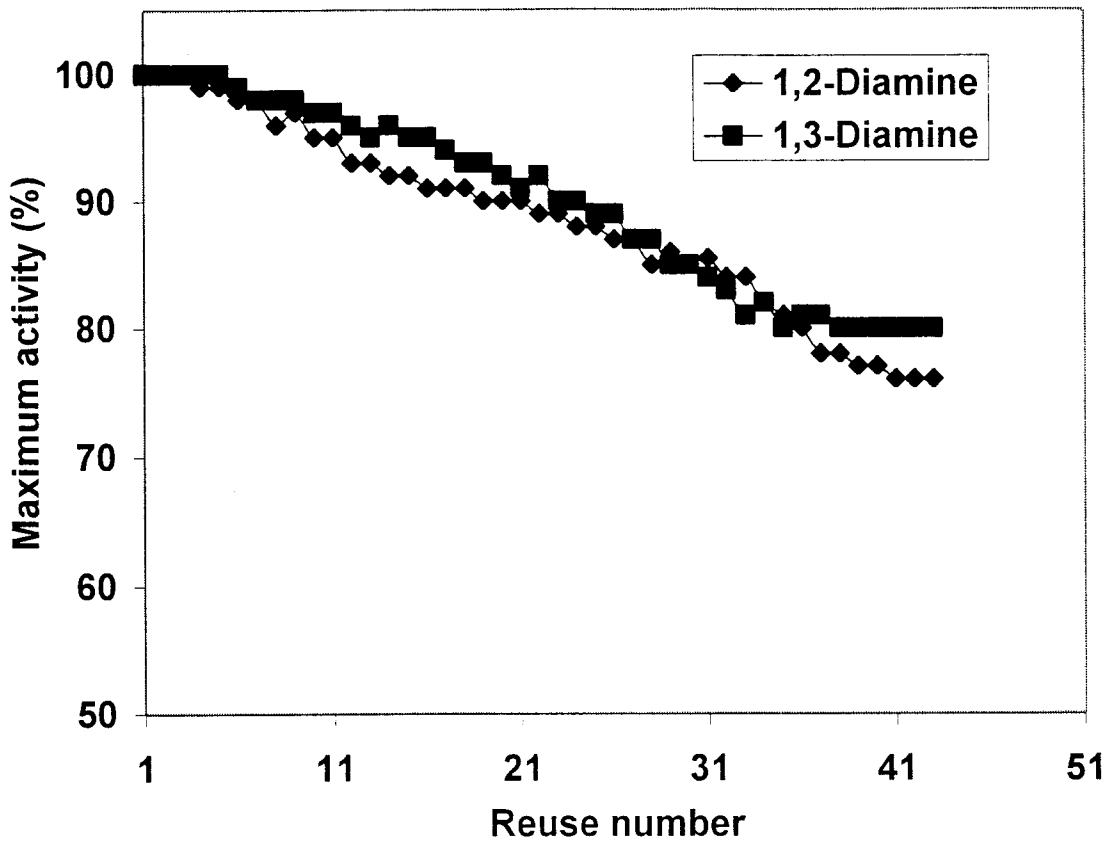

Figure 5 Effect of repeated use on the activity of immobilized invertase.

Kinetic parameters

The effect of substrate concentration on the hydrolysis of sucrose by free and immobilized invertase was also investigated. The results are presented in Figure 3. Values of the Michaelis–Menten constant K_m and V_{max} of free invertase were calculated as 18.2 m*M* and 6.43 $\times 10^{-5}$ mol dm^{-3} min^{-1}, respectively. These parameters were also calculated for immobilized 1,2-diamine ethane and 1,3-diamine propane as 35.3–23.8 m*M* (K_m values) and 11.71–7.97 $\times 10^{-5}$ mol dm^{-3} min^{-1} (V_{max} values), respectively. The K_m value of immobilized invertase (55 m*M* sucrose) was higher than that of the free enzyme (24 m*M* sucrose), whereas V_{max} values were smaller for the immobilized invertase.[12] Values of K_m of invertase were significantly larger ($\sim$ 2.5 times) upon immobilization, indicating decreased affinity by the enzyme for its substrate, whereas V_{max} was smaller for the immobilized invertase.[32] This indicates that the formation of the enzyme–substrate complex is more difficult than the immobilized invertase.[27] This increase may be a consequence of either structural changes in the enzyme, introduced by the applied immobilized procedure, or lower accessibility of the substrate to the active site of the immobilized enzyme. Kinetics parameters were calculated from the Eadie–Hofstee plot for immobilized invertase. The K_m of immobilized invertase was larger than that of native invertase, whereas the opposite tendency was observed for the V_{max}.[23] The K_m values for free and immobilized enzyme were 46 and 50 m*M*, respectively. V_{max} values for free and immobilized invertase were calculated as 45 and 38 mg fructose/mg enzyme min^{-1}, respectively.[8]

Storage stability

Enzymes are not stable in solutions and their activities decrease with time during the storage. The invertase solution was stored for 1 month at 4°C, and its activities were found to be 21 and 60–70% of the initial activity values for free, immobilized 1,2-diamine ethane, and 1,3-diamine propane, respectively (Fig. 4). The stability of immobilized invertase with 1,3-diamine propane was higher than that of 1,2-diamine ethane, which can be attributed to the increase of specific interactions between positively charged enzyme molecules and ionized polymer.

Reuse

The immobilized sample was used repeatedly 43 times within 3 days and the measured activities are pre-

sented in Figure 5. It has been observed that, after the 43rd use, immobilized enzyme retained about 76–80% of its original activity. The bound enzyme showed excellent stability to repeated use and retained about 90% of its initial activity after eight cycles of use.[34]

CONCLUSIONS

Immobilized invertase, compared with free invertase, was more stable at high pH and temperature. The storage stability of immobilized enzyme is also higher than that of the free enzyme. These immobilization properties are of great importance in biotechnological applications.

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