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Immobilization of β-galactosidase from *Cicer arietinum* (gram chicken bean) and its catalytic actions

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Abstract

β-Galactosidase (β-D-galactosidase galactohydrolase EC 3.2.1.23) isolated and purified from *Cicer arietinum* (gram chicken bean) was immobilized on two kinds of modified resin D202 with glutaraldehyde. Both the immobilized enzymes had high protein-binding capacity and high yield of enzyme activity. Kinetics results showed that the enzyme activity attained its maximum at 57°C, pH 6 for the immobilized β-galactosidase I and 52°C, pH 6 for the immobilized β-galactosidase II, respectively. The operational pH range was increased. Kinetic constants (K_m, V_{max} and E_a) for the free and bound enzymes, with ONPG as substrate, were studied. Results showed that K_m and V_{max} of immobilized enzymes were decreased while E_a of them was increased. The effects of some compounds and organic solvents for the free and immobilized enzymes were discussed. Inhibitory constants for raffinose, lactose and D-galactose, which were all reversible inhibitors of the enzymes, were also obtained.

Keywords: β-galactosidase; Immobilization; Inhibitory

1. Introduction

Cows' milk is an extremely valuable and economic food, containing high quality protein, calcium, and phosphates. However, it also contains 5% lactose, a disaccharide which can have undesirable effects on individuals who are deficient in intestinal lactase. A simple method for removing lactose from milk is therefore desirable.

A variety of techniques based on the utilization of lactase have been proposed for hydrolyzing the lactose present in whey and milk (Dahlqvist, Mattiasson, & Mosbach, 1973; Woychik & Wondolowski, 1972). A process employing immobilized lactose has been proved to be the most desirable. This led us to develop an immobilization procedure for this enzyme. In the previous studies, β-galactosidase has been immobilized on cellulose derivatives (Kay & Crook, 1967), polyacrylamide gel (Bunting & Laider, 1972), porous glass (Wondolowski & Worchick, 1974) and polymeric resin (Rejikurnar & Deri, 1995). Each has its advantage.

The enzyme we studied comes from a plant, *Cicer arietinum*, which is an abundant product in the west of China, and is much cheaper and easier to get than enzymes from other sources, such as bacterial fungus and yeast. Enzyme activity determined showed that it had high initial activity. After being immobilized on the modified D202, it showed a higher yield of activity. We characterised this enzyme in both free and immobilized forms. Effects of some compounds and buffer solutions were also studied. The results of these investigations showed that the enzyme, β-galactosidase from gram chicken bean, would have a promising application in the utilization of milk in a convenient and cheap way and it is worth investigating further.

2. Materials and methods

2.1. Materials

DEAE-cellulose was purchased from Pharmacia. ONPG from Sigma. Bovine Serum Albumin (BSA) from Sino-American Biotechnology Company (SABC). D202, glutaraldehyde, diethanolamine, lactose, D-galactose, raffinose were all A.R. Gram chicken bean was a local product. All reagents used in the experiments were dissolved in double-distilled water.

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2.2. Enzyme purification

All the steps of extraction and purification were carried out at 4°C unless otherwise specified. Gram chicken bean seeds were soaked in the water for 24 h and then germinated in the darkroom over a period of several days at 26–30°C. The cotyledons (70–80 g) were removed, washed with distilled water and ground with high-speed tissue gravity mill, then extracted in 140–160 volumes of cold 0.01 M sodium phosphate buffer solution (PBS, pH 6.4) containing 0.1% EDTA and 1% glycerol for about 4 h. After filtering with gauze, the slurry was centrifuged for 30 min at 77,000 ***g***. The clear supernatant was collected and the sediment was discarded. After precipitating with 30% ammonium sulphate with constant stirring and leaving over a period of 1–2 h, the supernatant was centrifuged as described above. The sediment was decanted and the solubles brought to 70% saturation by the additional solid ammonium sulfate, then centrifuged under the same conditions as above. The resulting precipitate was dissolved in 0.01 M PBS (pH 6.4) and dialyzed overnight in the cold against the above buffer. The enzyme solution concentrated by PEG-20,000 (polyethylene glycol) was applied to a 2×24 cm column of DEAE-cellulose equilibrated at pH 6.8 with 0.01 M tris–HCl buffer. The column was developed at 0.4 ml min^{-1} using the above buffer, collecting 50 fractions. Protein elution was monitored with a continuous-flow uv analyzer. β-Galactosidase activity was determined with a 100 μl aliquot from each fraction according to the methods of Singh (Singh & Knor, 1985). As three different peaks of β-galactosidase appeared in the eluted fraction of DEAE-cellulose, the first peak was collected as material of immobilization.

2.3. Preparation of the carrier I

Glutaraldehyde solution and diethanolamine were mixed in a tapered flask in the proportion 1:4 (v/v) and stirred for 30 min at 20°C. D202 was added to the mixture and stirred for 2 h. After filtering and washing thoroughly with distilled water, the modified D202 was obtained. Then, it was suspended in 0.1 M HCl solution with constant stirring for 30 min. The product was obtained and washed as mentioned above and finally stored at room temperature.

2.4. Preparation of the immobilized β-galactosidase I

The carrier I was mixed with β-galactosidase and phosphate butter solution (pH 7.5) with constant stirring for one or two minutes, then left at 4°C for 3 h. After filtering and washing with distilled water, the pH of solution became constant. The immobilized β-galactosidase I was obtained and stored in the refrigerator to be used for the next step.

2.5. Preparation of the carrier II

D202 was treated with 70% CH_3CH_2OH, 1 M NaOH, 0.5 M $(NH_4)_2SO_4$, 2 M HCl and 0.01 M PBS (pH 8) for 20 min successively and finally washed with distilled water until neutral. The modified D202 obtained was reacted with 21% glutaraldehyde solution at 4°C for 12 h followed by thorough distilled water washing. Thus, the carrier II was prepared.

2.6. Preparation of immobilized β-galactosidase II

The carrier II was added into the slurry of β-galactosidase and 0.01 M PBS (pH 6.4) with constant stirring. The mixture was left at 4°C for 10 h. After filtering and washing thoroughly, the immobilized β-galactosidase II was gained and stored at 4°C to be used for the next step.

2.7. Enzyme activity assay

For the soluble enzyme activity, aliquots of it (0.1 ml) were added to the mixture containing 0.2 ml of 0.1 M citric acid buffer (pH 6) and incubated at 55°C for 20 min. Digestion of ONPG was stopped by the addition of 1.5 ml 1 M Na_2CO_3 and the amount of liberated *o*-nitrophenol was measured directly at 405 mn. For the immobilized enzyme activity, the incubation mixture consisted of 0.8 ml 0.1 M citric acid buffer (pH 6) and 0.2 ml of 5 mM ONPG. It was carried out for 20 min at 55°C and ended and analyzed as above. One unit of activity was defined as the amount of enzyme which liberated 1 μmol product/min at 55°C. Specific activities were expressed as the number of units/mg protein.

2.8. Determination of protein

This was done by the Folin–Phenol method (Lowry, Rosebrough, Farr, & Radull, 1951) with the use of crystalline bovine serum albumin as the standard.

2.9. Influence of temperature and pH

The influence of temperature on the galactosidase activity was determined using ONPG as the substrate over the range of 40–70°C. Enzyme stabilities were determined after a long duration exposure to various temperatures (40–70°C), followed by analysis at the optimum temperature.

The pH-activity curve in the range 3.5–7.5 was determined for the free enzyme and the bound enzyme at their optimum temperature using ONPG as substrate. pH stability in the range 2–14 was determined after 30 min exposure to different pH at 4°C. Activities relative to original values were determined at their optimum in pH 6 buffer for the immobilized enzyme and pH 5 buffer for the free enzyme.

2.10. Kinetics

The Michaelis constant K_m, the maximum velocity V_{max} and the inhibition constant K_i were calculated for the soluble and immobilized enzymes by assaying the enzyme in increasing ONPG concentrations ranging from 0.1 to 0.8 mM. Inhibition was examined at constant concentrations of lactose, D-galactose and raffinose, while varying the substrate concentrations. The effects of buffer solutions and some added compounds on the activity of the immobilized β-galactosidase were determined using ONPG as substrate.

For the above series of experiments, single aliquots of enzymes were used. V_{max} at different temperatures was used to calculate activation energies.

3. Results and discussion

3.1. Purification and immobilization

Purification of the galactosidase was carried out as described above and the results of a typical isolation are present in Table 1. The enzyme activity was determined by ONPG as substrate, at 55°C, pH 6 for 20 min. The protein concentration was determined by the Lowry method (Lowry et al., 1951).

β-Galactosidase was immobilized on carrier I and carrier II, respectively, as mentioned above. During the process of immobilization, no activity was detected in the washing buffer, which indicated that almost all the activity was captured. The immobilized β-galactosidases I and II obtained were dried and weighed. The enzyme activity was determined by 0.1 g immobilized enzyme at 55°C, pH 6 for 20 min, ONPG as substrate. Meanwhile, the retention of enzyme activity was found to be 78% for the immobilized I and 63% for the immobilized II, respectively. These results are shown in Table 2. Okos (Okos, Grulhe, & Syverson, 1978) reported that 6.29% retention of activity was obtained in physical adsorption method. Heng (Heng & Glatz, 1994) recently reported that approximately 50% of enzyme activity was retained for membrane reactor. Comparison above indicated that the modified D202 is successful as immobilization carrier for plant β-galactosidase.

3.2. The optimum temperature and the thermostability

As show in Fig. 1, the optimum of immobilized β-galactosidase I and II was 60 and 50°C, respectively, while the free enzyme optimum was 55°C. Enzyme activity was determined by ONPG as substrate at various temperatures (40–70°C), pH 6 for 20 min.

After all the enzyme preparations were incubated at indicated temperatures (40–70°C) for different times (20, 40, 60 min), the enzyme activity was determined in aliquots of enzyme (0.1 ml free enzyme or 0.1 g immobilized enzyme), at their optimum temperature, pH 6 for 20 min, ONPG as substrate. Fig. 2 shows that a 75% loss was observed after 20 min and complete inactivation after 60 min at 70°C for the free enzyme. Under the same conditions, the immobilized enzymes retained 76 and 72%, respectively, after 20 min and they also had 62 and 54% retentive activity separately after 60 min. Results showed that the immobilized β-galactosidase had better stability than the free enzyme.

3.3. The optimum pH and pH stability

The pH profile of the free β-galactosidase was determined and found to peak at pH 5.0, while the immobilized enzymes I and II both peaked at pH 6, representing a 1 pH unit shift from the pH optimum for the soluble enzyme. Goldstein (Goldstein, 1973) and Zaborsry (Zaborsry, 1974) reported that a carrier with ionic groups would induce the optimum pH of immobilized enzyme to change. Our experimental results in Fig. 3 indicate the same. The enzyme activity was determined by ONPG as substrate, at 55°C in various pH buffers (3.5–7.5) for 20 min.

After all the enzymes were exposed to different pH (2–14) at 4°C for 30 min, enzyme activity was determined at 55°C, pH 6 for the immobilized enzyme and pH 5 for the free enzyme for 20 min, ONPG as substrate. As shown in Fig. 4, the immobilized enzyme activity had a wider pH range than that of the free enzyme activity. The immobilized enzyme I had high activity in the range 3–7, and the activity of the

Table 1
Enzyme purification

Purification step	Total activity (ONPG units)	Total protein (mg)	Specific activity (ONPG units mg^{-1} protein)	Purification factor
Crude extract	9160	329	28.0	1.0
30–70%	6120	153	40.0	1.4
Ammonium sulfate	1750	18.3	96.0	3.4
DEAE-cellulose				

Table 2
Immobilization results of β-galactosidase on D202

Immobilized β-galactosidase	Enzyme activity (ONPG units g^{-1} dry support)	Protein content (mg g^{-1} dry support)	Activity retention (%)
E (I)	60	64	78
E (II)	48	40	63

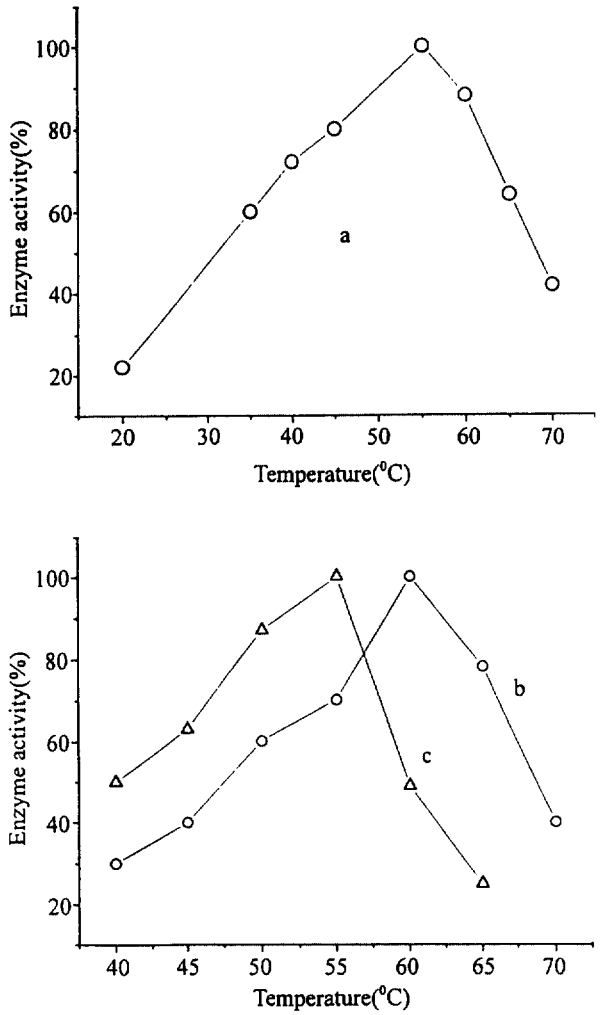

Fig. 1. Temperature dependence of β-galactosidase activity (a: soluble enzyme; b: immobilized enzyme I ; c: immobilized enzyme II).

immobilized enzyme II ranging from 6 to 10 is high. It is well known that the pH of milk is about 6.4; the wider range of immobilized enzyme offers a valuable flexibility.

3.4. Kinetic parameters

The effects of various substrate concentrations (0.1–0.8 mM) on the digestion of ONPG, by both the free enzyme and the immobilized enzymes, were investigated at the indicated temperature. The results of these experiments were contained by the Lineweaver-burk plots. The values for K_m and V_{max} were calculated from the results and are presented in Table 3. The apparent K_m of the immobilized was higher than that of the free enzyme and the values of V_{max} were lower than that of the free enzyme. The activation energies of the bound were determined from Arrehenius plots. The values of E_a obtained through the curve of $\log V_{max} - 1/T$ for two kinds of immobilized enzymes were higher than that of the free enzyme, especially the immobilized enzyme I.

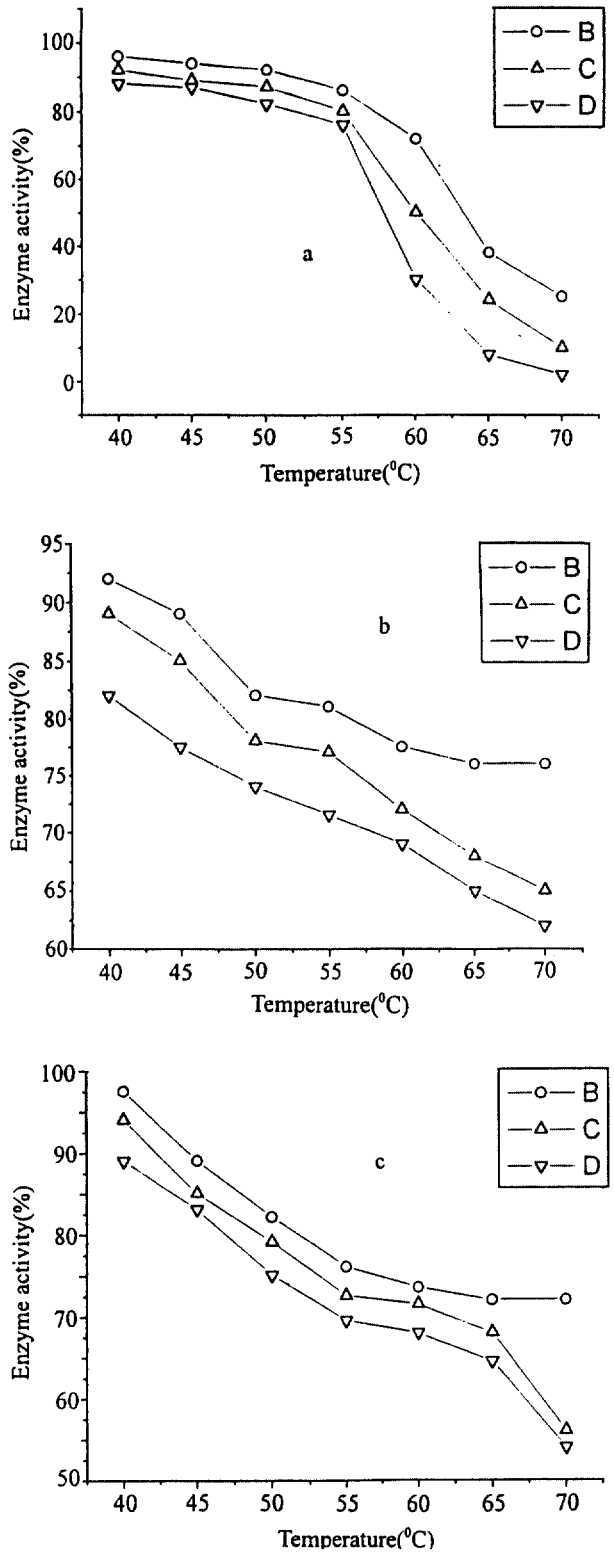

Fig. 2. Thermal stability of β-galactosidase enzyme (a: soluble enzyme; b: immobilized enzyme I; c: immobilized enzyme II; ○: 20 min; △: 40 min; ▽: 60 min).

The decreases in V_{max} and increase in K_m and E_a for bound enzymes can be attributed to steric hindrances or diffusional limitation. The results are shown in Table 3.

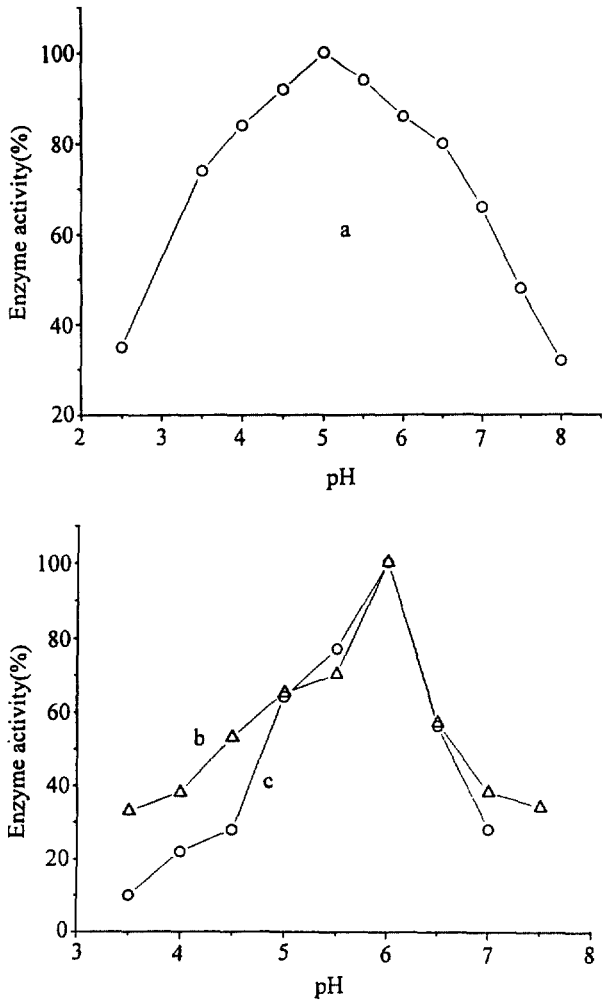

Fig. 3. pH dependence of β-galactosidase activity (a: soluble enzyme; b: immobilized enzyme I; c: immobilized enzyme II).

The inhibition by lactose, D-galactose and raffinose of the immobilized β-galactosidase and the free enzyme was investigated with ONPG as substrate. Lineweaver-Burk plots at constant inhibitor concentrations demonstrated that lactose and D-galactose were competitive inhibitors, while raffinose was a non-competitive inhibitor. Inhibition constants calculated from these data are shown in Table 4. Compared with the free enzyme, K_i of lactose and D-galactose on the immobilized enzyme was higher, i.e. inhibition by lactose and D-galactose of the immobilized enzyme was decreased.

3.5. Some compounds and buffer solutions effects

The effects or some compounds and buffer solutions on the hydrolysis of ONPG were determined after 30 min incubation of the enzyme with each of them followed by analysis with ONPG as substrate. The relative activities found are listed in Table 5. KCl and $MgCl_2$ enhanced enzymatic activity up to 0.4 M and 0.1 M

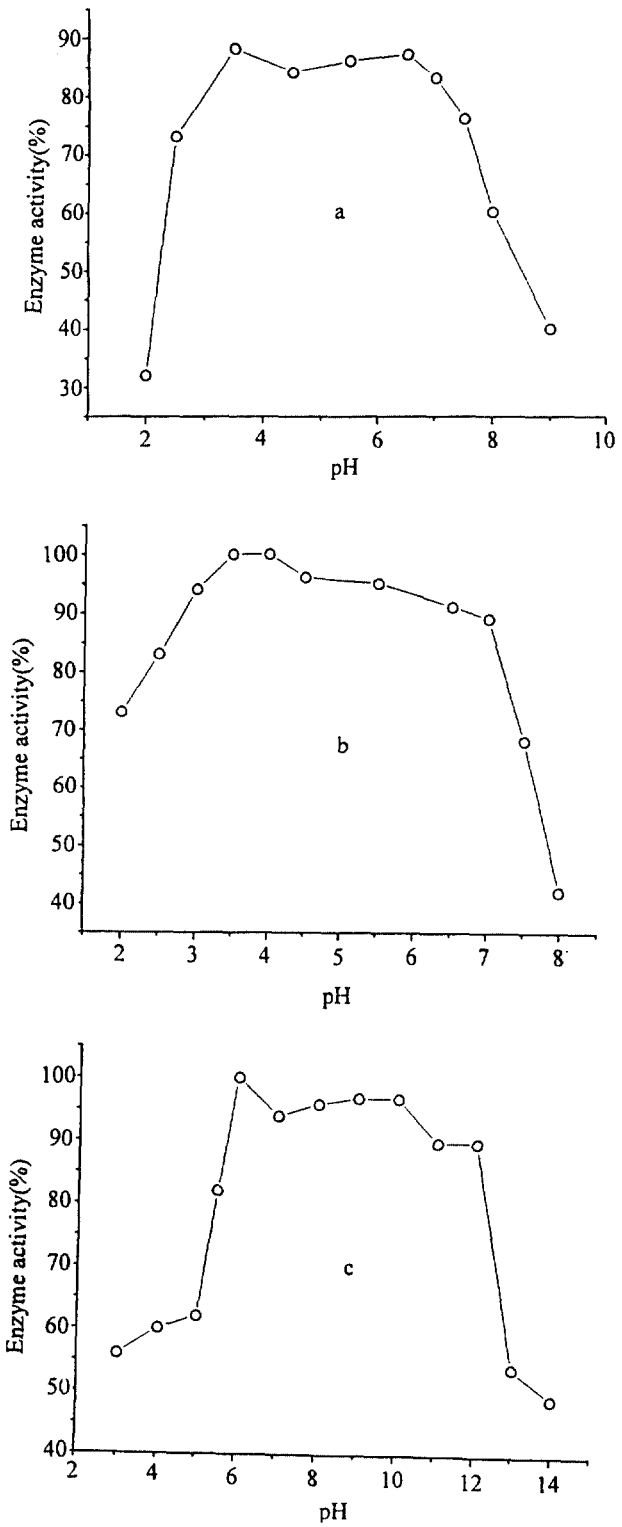

Fig. 4. pH stability of β-galactosidase activity (a: soluble enzyme; b: immobilized enzyme I; c: immobilized enzyme II).

concentrations, respectively. Some organic solvents, such as acetone and methanol, decrease the enzyme activity while trichloromethane and dichloromethane reduce the immobilized enzyme I but increase the

Table 3
K_m, V_{max} and E_a for catalytic hydrolysis of ONPG by β-galactosidase

Content	Soluble enzyme		Immobilized E (I)			Immobilized E (II)		
T (°C)	40	50	55	60	65	50	55	60
K_m (mM)	10.8	57.9	10.5	14.3	20.1	9.1	11.8	25.0
V_{max} (μM min^{-1})	20.0	33.3	2.6	6.0	13.0	2.8	4.3	6.2
E_a (kJ mol^{-1})	41.6		60.0			71.02		

Table 4
Effect of some reagents on the activity of immobilized β-galactosidase

Addition	Concentration	Enzyme relative activity		
		E (I) (%)	E (II) (%)	Free enzyme (%)
PBS	0.01 M (PH6.4)	100	100	97
Na_2HPO_4-citrate	0.01 M (PH6.5)	90	94	94
Citric acid	0.1 M	82	89	100
Acetone	10%	76	94	–
Methanol	10%	88	94	75
Formaldehyde	10%	52	89	–
Ethanol	10%	96	110	30
Trichloromethane	10%	66	142	–
Dichloromethane	10%	64	120	–
KCl	10 mM	104	116	91.6
	40 mM	121	147	98.8
$MgCl_2$	5 mM	104	100	100
	10 mM	112	137	104

Table 5
Inhibitory constants, K_i of inhibitors on β-galactosidase

Inhibitor	Soluble enzyme	Immobilized E (I)	Immobilized E (II)
Lactose	12.5	40	21
D-Galactose	6.9	24	16
Raffinose	69	59	29

immobilized II greatly. Ethanol had little effect on the immobilized enzyme. Some buffer solutions, Na_2HPO_4-citrate and citric acid, were inhibitory to the immobilized enzymes. As shown in Table 5, the inhibition by some compounds and buffer solutions of the immobilized enzymes were less than the free.

From the data presented, it is evident that, although immobilization does alter some of the properties and kinetics of β-galactosidase from gram chicken bean, the individual methods of bonding used in this study were significant in their effects. With the information generated, we are attempting to carry out an evaluation of the use of immobilized β-galactosidase for the hydrolysis of lactose. It is our hope that hydrolysis of lactose in milk by our immobilized enzyme from gram chicken bean will produce a product with a great value in a more convenient and cheaper way.

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