Immobilization and Characterization of β-Galactosidase from the Plant Gram Chicken Bean (*Cicer arietinum*). Evolution of Its Enzymatic Actions in the Hydrolysis of Lactose

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β-Galactosidase (β-D-galactosidase galactohydrolase, EC 3.2.1.23) isolated and purified from gram chicken bean was immobilized on cross-linked polyacrylamide gel. The activity yield was high and attained up to 72%. Compared with the free enzyme, the immobilized enzyme had a wider operational pH range and better thermal stability. Lyophilized pieces exhibited good stability when stored at room temperature for 60 days and a favorable operational stability when used eight times repeatedly without loss of enzymatic activity under the same conditions. Kinetic data (K_m, V_m, and E_a) for the free and the immobilized enzymes were determined using *O*-nitrophenyl-β-D-galactoside (ONPG) and lactose as substrates. The result of time courses of hydrolysis of lactose showed that β-galactosidase from the plant gram chicken bean would have a promising application in the hydrolysis of lactose in milk.

Keywords: *β-Gactosidase; immobilization; lactose; gram chicken bean*

INTRODUCTION

Cow's milk is an extremely valuable and economic food, containing high-quality protein, calcium, phosphates, and cirtains. However, it also contains 5% lactose, a disaccharide that 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 et al., 1973; Woychik and Wondolowski, 1972; Wondolowski and Woychik, 1974). A process employing immobilized lactase had been proved to be very effective. This led us to develop an immobilization procedure for this enzyme.

In previous studies, a large number of methods had been used to immobilize β-galactosidase (Stanley and Palter, 1973; Okos et al., 1978; Dahlqvist et al., 1973; Ohmiya et al., 1975; Bunting and Laider, 1972; Oestergaard and Martiny, 1973; Beddows et al., 1980; Ariga et al., 1989, 1993; Park and Huffman, 1990; Bodalo et al., 1991; Chen et al., 1992; Berger et al., 1995, Rejikumar and Deri, 1995). Each had its advantage. Of the methods investigated for immobilization, entrapping methods for the enzyme within a cross-linked polyacrylamide gel appeared to immobilize a larger amount of enzyme than the other methods with convenient procedure.

In this paper, β-galactosidase from plant was immobilized using a polyacrylamide-entrapped method. Dahlqvist et al. (1973) immobilized a yeast lactase on cross-linked polyacrylamide in an interfacial copolymerization method. The enzyme studied comes from a plant, gram chicken bean, which is abundant in western China. The enzyme activity determined showed that it had a high initial activity. We characterized this enzyme in both free and bound forms by determining pH profiles, thermal profiles, storage stability, and operational stability, with *O*-nitrophenyl-β-D-galactoside (ONPG) as substrate. Meanwhile, kinetic parameters (K_m, V_m, and E_a) were also obtained with ONPG and lactose as substrate. Finally, we also determined the time courses of hydrolysis of lactose in the buffer system (pH 6.4). This offered a base for controlling the decomposition level of lactose in milk. The results achieved showed that β-galactosidase from gram chicken bean would be highly desirable to remove the lactose present in milk using such immobilization methods as described above.

MATERIALS AND METHODS

Materials. DEAE-cellulose was purchased from Pharmacia Co. (Uppsala, Sweden). ONPG was from Sigma Co. (St. Louis, MO). $(CH_2CHCONH)_2CH_2$ (MBA) was from Serva Co. (Germany). The kit for determination of glucose (GOD-POD methods) was purchased from the Clinical Diagnostic Reagent Institute of Lanzhou (China). Gram chicken bean was a local product from western China.

Enzyme Purification. All of the steps of extraction and purification were carried out in the same way as Li et al. (1996) described. Gram chicken bean seeds were soaked in water for 24 h and then germinated in a dark room over a period of several days at 26−30 °C. The cotyledons (70−80 g) were removed, washed with distilled water, ground with a high-speed tissue gravity mill, and then exacted 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 ∼4 h. Filtered with gauze, the slurry was centrifugated for 30 min at 77000*g*/min. The clear supernatant was collected and the sediment discarded. Precipitated with 30% ammonium sulfate with constant stirring and left over a period of 1−2 h, the supernatant was centrifugated as described above. The sediment was decanted, and the soluble was brought to 70%

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saturation by the addition of solid ammonium sulfate and 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 [poly(ethylene glycol)] was applied to 2.0 × 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, and 50 fractions were collected. 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 Sigh and 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.

All of the steps of extraction and purification were carried out under 4 °C unless otherwise specified.

Enzyme Activity Assay. For the soluble enzyme activity, aliquots of the enzyme (0.1 mL) were added into the mixture containing 0.7 mL of 0.01 M Na_2HPO_4−citric buffer (pH 5.0) and 0.2 mL of 5 mM ONPG (pH 5.0) and then incubated at 60 °C for 15 min. Digestion of ONPG was stopped by the addition of 2 mL of 1 M Na_2CO_3, and the amount of liberated *O*-nitrophenol was measured directly at 405 nm. For the immobilized enzyme activity, 0.05 g of the immobilized enzyme was soaked in 0.8 mL of 0.01 M Na_2HPO_4−citric buffer (pH 5.0) and incubated at 60 °C for 3 min. The reaction was started by adding 0.2 mL of 5 mM ONPG (pH 5.0). It was carried out for 15 min at 60 °C, ended, and analyzed as above. One unit of activity was defined as the amount of enzyme that liberated 1 μmol of product/min at 60 °C.

Determination of Glucose Concentration. Amounts of glucose liberated in our reaction solution (the total volume was 1 mL) were determined according to the glucose oxidase method using the kit for the determination of glucose. One milliliter of mixture including 0.5 mL of diluted glucose oxidase and the same volume of phenol (4-aminophenazone-coupled phenol) was added into the reaction product ended with boiled water and incubated at 35 °C. After 15 min, the reactive mixture was spectrophotometrically studied at 505 nm. The amount of liberated glucose was calculated according to the standard curve of glucose concentration−absorptivity.

Immobilization of β-Galactosidase. Two milliliters of free β-galactosidase was added into 7.2 mL of 20% CH_2-$CHCONH_2$ (Acr) solution (dissolved in 0.01 M, pH 7.0, Na_2-HPO_4−citric buffer) including 7.5% MBA of the total amount of monomer. Immediately after addition of the catalyst system [comprising 0.3 mL of $(CH_3)_2NCH_2CH_2N(CH_3)_2$ (TEMED) and 0.5 mL of 5% $(NH_4)_2S_2O_3$ dissolved in the same buffer mentioned as the Acr Sol] and being blended completely, the solution was poured into a culture dish to react. Within a few minutes, polymerization started and was complete after ∼15 min. The polyacrylamide gel was obtained and cut into little square pieces, each side being ∼1 mm. The "enzyme square pieces" acquired were washed on a glass filter with the buffer (pH 7.0) thoroughly until no protein was separated out. After being lyophilized, the little gel pieces were stored in a desiccator at 4 °C when they were not in use.

In the above experiment, all solution was kept at 0−4 °C and the temperature during the course of the polymerization was kept below 10 °C. Whether the protein existed in the washing buffer or not was determined by ninhydrin.

Operational Stability of Immobilized Enzyme. The operational stability of the immobilized enzyme was determined according to the following procedures. Two grams of the immobilized enzyme was taken and soaked in 30 mL of Na_2-HPO_4−citric buffer (pH 5.0) over a period of 30 min. After incubation at 65 °C for 5 min, the reaction was started by adding 10 mM ONPG (30 mL, pH 5.0, dissolved in the same buffer as described above) for 15 min at 65 °C, and then the reactive mixture was analyzed. Afterward, the solid was filtered and washed thoroughly with distilled water. Finally, the used immobilized enzyme was dried at under room temperature and the above experiment was repeated under the same conditions.

Table 1. Summary of Purification Procedure of β-Galactosidase

purifn step	total act. (ONPG units)	total protein (mg)	sp act. (ONPG units/mg)	purifn factor[a]
crude extract	9160	329	28.0	1
30−70% $(NH_4)_2SO_4$	6120	153	40	1.4
DEAE-cellulose	1750	18.3	96	3.4

[a] Purification factor is defined as the specific activity of enzyme solution in the process of purification divided by the specific activity of the original enzyme extraction.

Kinetic Parameters (Nicholass and Stevens, 1982). The Michaelis constant K_m and the maximum velocity V_m were calculated for the soluble and the immobilized enzyme by assaying the enzyme in increasing ONPG concentrations ranging from 1.25 to 10 mM and lactose concentration from 12 to 90 mM in Na_2HPO_4−citric buffer (0.01 M, pH 6.4). V_m at different temperature was used to calculate activation energies.

RESULTS AND DISCUSSION

Purification of Enzyme. Purification of the enzyme was carried out as described above, and the results of a typical isolation are presented in Table 1. The enzyme activity was determined by ONPG as substrate, at 60 °C for 15 min. The protein concentration was determined according to the method ofLowry et al. (1951).

Immobilization of Enzyme. The activity yield of the immobilized enzyme was defined as

$$\text{activity yield (\%)} = [\text{immobilized enzyme activity obtained (units)}/\text{free enzyme activity used in immobilization (units)}] \times 100$$

According to the immobilization method mentioned above, the activity yield of the immobilized enzyme was calculated to be 72%. Prior to the present investigation, Dahlqvist et al. (1973) and Ohmiya et al. (1975) had immobilized β-galactosidase on cross-linked polyacrylamide separately. The activity yields were 63 and 45%, respectively, both of which were lower than our result. This could be attributed to different courses used. Both Dahlqvist and Ohmiya had used interfacial copolymerization that had a great amount of organic solvent and vigorous stirring. The two kinds of effects resulted in lower enzymatic activity, whereas we used copolymerization to react in low temperature (0−10 °C). Another reason seemed to depend on the difference in enzyme source (from the plant gram chicken bean in the present work and from *Asperigillus oryaze* in Ohmiya's studies and from *Sacchromyce lactis* in the Dahlqvist's work). Finally, it is worth pointing out that the immobilized enzymes obtained had no toxicity and could be used in milk to catalyze decomposition of lactose under sanitary conditions. This could be explained by two factors: first, it is well-known that the highly cross-linked polyacrylamide gel has no toxicity; second, the "enzyme square pieces" obtained were washed thoroughly in the process of immobilization; thus, not only the unimmobilized protein but also the little poisonous molecules including unreactive monomer and initiator were washed off the carrier. The conclusion above indicates that the procedure described in this paper successfully immobilized this enzyme.

pH Optima and pH Stability. Figure 1 shows that the pH profile of the immobilized enzyme peaked at pH

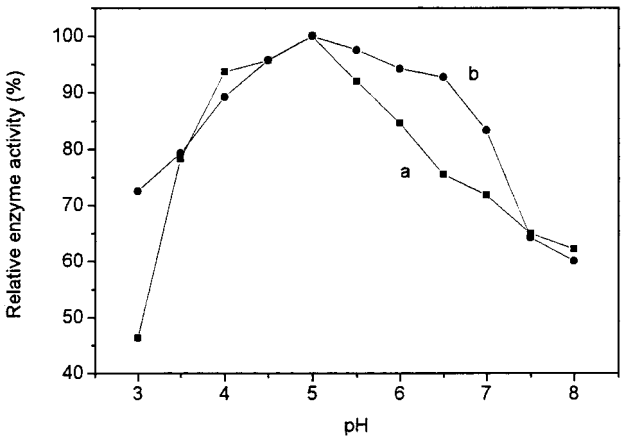

Figure 1. pH dependence of enzyme activity: (a) free enzyme; (b) immobilized enzyme. The reaction was performed at different pH values (pH 3−8, Na_2HPO_4−citric) at 60 °C for 15 min, ONPG as substrate.

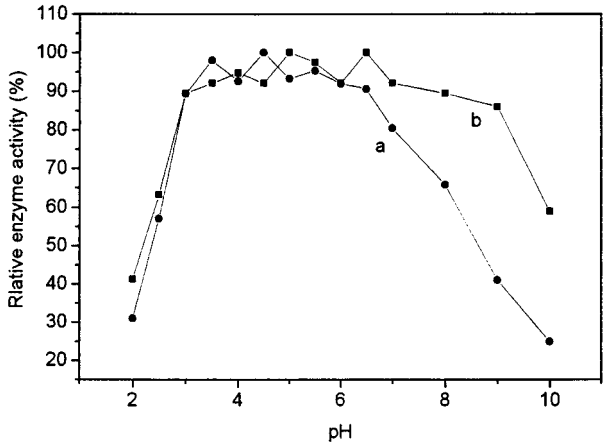

Figure 2. pH stability of enzyme activity: (a) free enzyme; (b) immobilized enzyme. The following buffer systems were used at a concentration of 0.1 M: pH 2.0−8.0 Na_2HPO_4−citric buffer; pH 9.0−10.0 Gly−NaOH.

5.0, with no shift from the pH optima for the soluble enzyme. Goldstein (1973) and Cha et al. (1988) reported that the carrier with charge would induce optimum pH of immobilized enzyme to change (big for the carrier with cationic charger and small for the carrier with anionic charge). It is well-known that polyacrylamide gel has no charge (Michael, 1980), so our experimental results in Figure 1 indicate the same results as above.

After all of the enzymes were exposed to different pH values (2.0−10.0) at room temperature for 30 min, enzyme activity was determined at pH 5.0 with ONPG as substrate. As shown in Figure 2, the immobilized enzyme activity had a wider pH range than that of the free enzyme. In the range of pH 3−9, the immobilized enzyme activity remained >86%. It is well-known that the natural pH of milk is ∼6.4; the wider range of the immobilized enzyme offers a valuable characteristic for use in milk.

Optimum Temperature and Thermostability. As seen in Figure 3, the immobilized enzyme temperature optimum was 65 °C, whereas the free enzyme's was 60 °C. Enzyme activity was determined by ONPG as substrate at various temperatures (30−80 °C) at pH 5.0 (Na_2HPO_4−citric buffer) for 15 min.

Figure 4 shows that the immobilized enzyme was more stable than the free enzyme. At 50 °C, the activities of the free enzyme and the immobilized

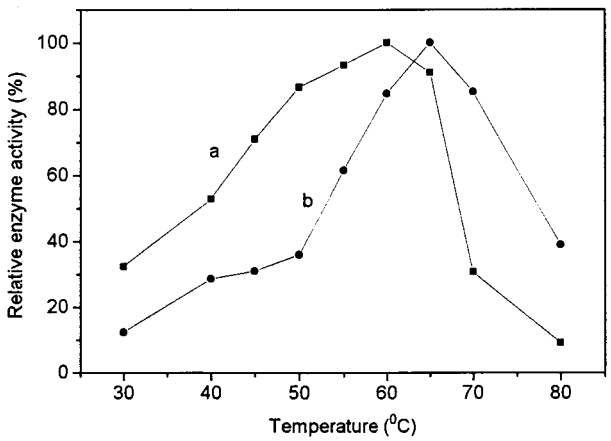

Figure 3. Temperature dependence of β-galactosidase: (a) free enzyme; (b) bound enzyme.

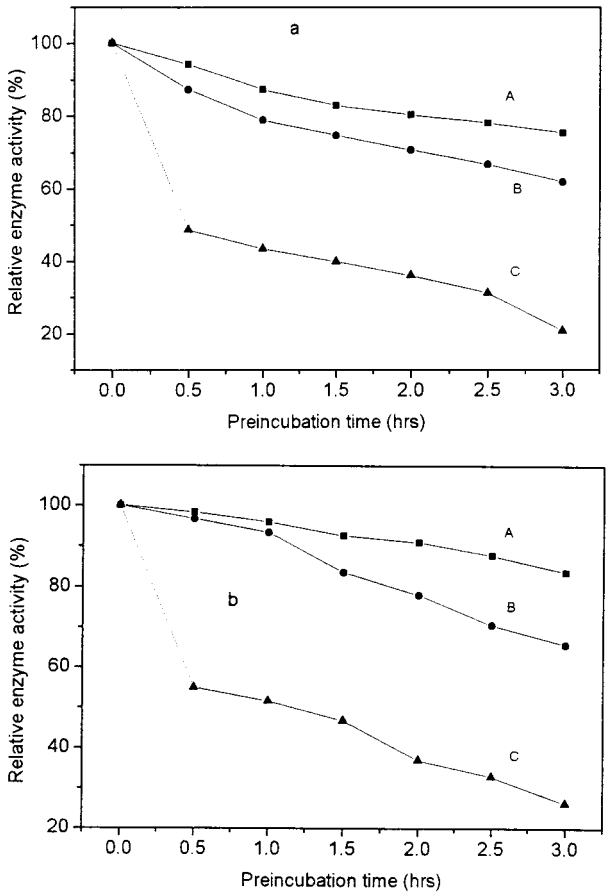

Figure 4. Thermal stability of β-galactosidase: (a) free enzyme; (b) immobilized enzyme [(A) 50 °C; (B) 60 °C; (C) 70 °C]. All of the enzyme preparations were preincubated at indicated temperature for different times. At the time indicated, aliquots (0.5 mL) were removed and assayed for activity at 60 °C, with ONPG as substrate. Initial activity corresponds to 100%.

enzyme slowly decreased; after 30 min, the remaining activity of the former was 94.3%, but the latter's was 98.4%. At 70 °C, over a period of the same time, the residual activity of the free enzyme was 48.7%, whereas that of the bound was 54.9%. Meanwhile, we could see the thermal stability of the two kinds of enzyme forms had the same decreasing trend at high temperature according to the graph. At the initial stage, the activity quickly decreased but then decreased slowly with the time. For example, a 51.3% loss was observed at 70 °C

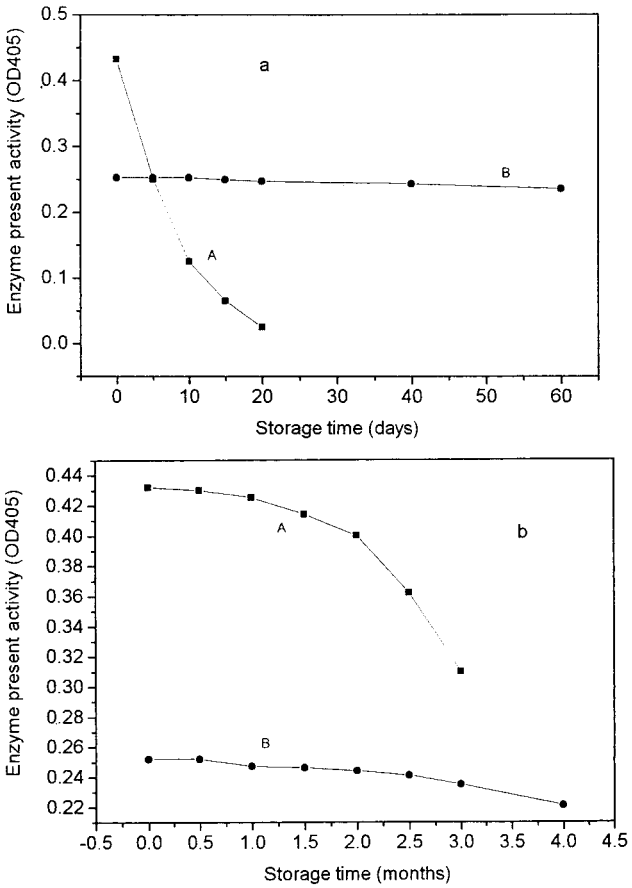

Figure 5. Storage stability of β-galactosidase: (a) enzyme was stored at room temperature (17−25 °C); (b) enzyme was stored at 0−4 °C [(A) free enzyme; (B) immobilized enzyme.

after 30 min for the free enzyme, but the activity reduced only by another 5.3% after an additional 30 min. For the immobilized enzyme, the same decreasing tendency was observed. This might be explained by the enzyme's having some heat-resisting component.

Storage Stability of Enzyme. Both the free enzyme and the immobilized enzyme were stored at 4 °C and at room temperature (17−25 °C). Almost no loss in activity of the free enzyme was observed after 2 months of storage at 4 °C, nor was any loss seen for the bound enzyme after 4 months. While at room temperature, a 42.1% loss was seen only after 5 days for the free enzyme, whereas the immobilized enzyme could retain its activity without serious loss for 60 days. The results are shown in Figure 5.

Operational Stability. The experiment was repeated eight times by using the procedures mentioned above with the same immobilized enzyme at the same initial concentration of ONPG. The results are summarized in Figure 6. It is interesting to note that the enzyme activities in all of the experiments were almost the same. Therefore, no enzyme was dissociated from the surface of the gel in the course of reaction. The operational stability of the immobilized enzyme obtained is very good.

Kinetic Parameters. The enzyme obeyed simple Michaelis−Menten kinetics toward both ONPG and lactose as demonstrated by straight lines obtained for double-reciprocal plots. Lineweaver−Burk plots for the free and immobilized enzymes using ONPG or lactose as substrate were made, and the values for K_m and V_m calculated from those graphs are shown in Tables 2 and 3. Table 2 shows that the apparent K_m of both kinds of enzyme for ONPG is irregular to change. This could be explained by the fact that K_m is affected by many sorts of complicated factors, such as the velocity constant of every step of reaction. The apparent K_m of the immobilized enzyme was higher than that of the free enzyme for both ONPG and lactose. The value of E_a obtained through the curve of log $V_m - 1/T$ for the bound enzyme was a little higher than that of the free enzyme. The increase in K_m and E_a for the bound enzyme could be attributed to steric hindrances or diffusional limitation.

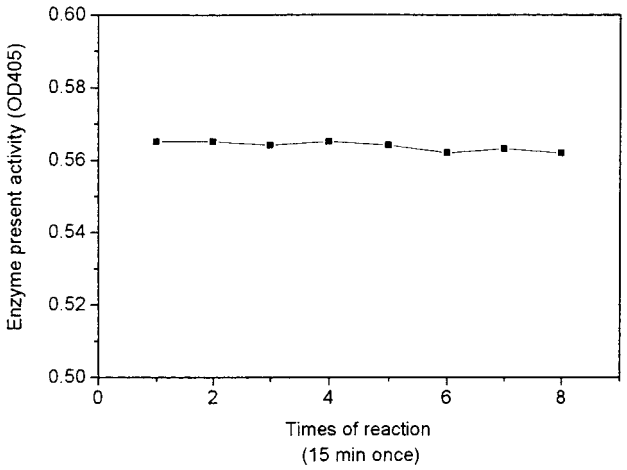

Figure 6. Operational stability of β-galactosidase, with 0.5 mL of reactive mixture each time. After 1.5 mL of 1 M Na_2CO_3 had been aded, the enzyme activity was measured at OD405.

Table 2. K_m, V_m, and E_a for Catalytic Hydrolysis of ONPG by β-Galactosidase

	soluble enzyme				immobilized enzyme			
T (°C)	35	45	50	55	35	45	50	55
K_m (mM)	3.64	2.98	3.19	3.00	6.50	5.13	6.92	4.40
V_m (μM mol^{-1})	34.38	61.67	82.50	100.8	29.00	48.75	82.50	98.75
E_a (kJ mol^{-1})			42.06				49.21	

Table 3. K_m, V_m, and E_a for the Decomposition of Lactose by β-Galactosidase

	free enzyme			immobilized enzyme		
T (°C)	30	40	50	30	40	50
K_m (mM)	44.40	38.07	24.54	53.67	43.51	28.69
V_m (μM mol^{-1})	0.59	1.23	2.47	0.42	0.90	1.84
E_a (kJ mol^{-1})		56.75			58.61	

Determination of Time Courses of Hydrolysis of Lactose. Figure 7 shows the time courses of hydrolysis of lactose in Na_2HPO_4−citric buffer (pH 6.4). It was found that 32.8 and 59.3% of 150 mM lactose were hydrolyzed during 1 and 2 h incubations separately at 50 °C, and the values were 26.2 and 43.4%, respectively, at 40 °C. As we all know, lactose can supply energy to organic bodies; it also has many kinds of functions of nutrition and health protection, which cannot be replaced by other sugars. Therefore, we cannot hydrolyze completely lactose in milk. Most sugar makers have found that a 70−80% decomposition rate of lactose was the most suitable, because the milk obtained not only has no undesirable effect on individuals deficient in intestinal lactase but also has good nutrition and nice flavor (Li et al., 1990). By using time courses of the hydrolysis of lactose, we can design the amount of enzyme and the time of reaction necessary according to the request of the decomposition level.

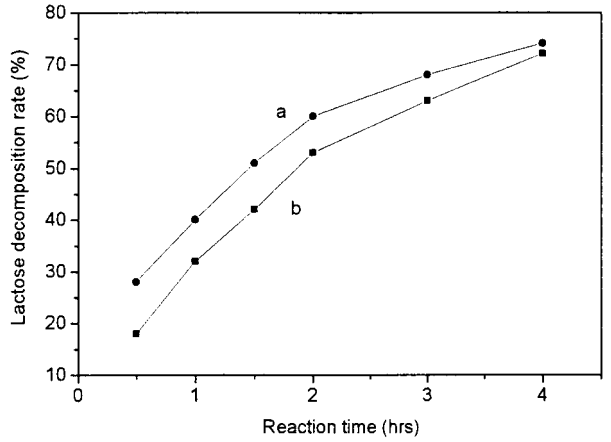

Figure 7. Time course of hydrolysis of lactose with 1 mL of reactive mixture at indicated time (if glucose concentration was high, it was diluted), according to the procedure of determination of glucose concentration to ascertain the decomposition rate of lactose: (a) 50 °C; (b) 40 °C.

From the data acquired, we could see that the immobilization of β-galactosidase from gram chicken bean, which is cheap and easy to get, had a satisfactory result in enzyme activity yield, pH stability, thermal stability, and operational stability. The results described above showed that the immobilized enzyme obtained would be highly promising to remove the lactose present in milk.

ABBREVIATIONS USED

β-Galactosidase, β-D-galatosidase galactohydrolase; ONPG, *O*-nitrophenyl-β-D-galactoside; MBA, *N*,*N*′-methylenebis(acrylamide) [$(CH_2CHCONH_2)_2CH_2$]; Acr, acrylamide ($CH_2CHCONH_2$); TEMED, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine [$(CH_3)_2NCH_2CH_2N(CH_3)_2$].

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