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Biosynthesis of glycyrrhetinic acid 3-O-mono-β-D-glucuronide by free and immobilized *Aspergillus terreus* β-D-glucuronidase

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A B S T R A C T

Enzymatic conversion of glycyrrhizinic acid (GL) into glycyrrhetinic acid 3-O-mono-β-D-glucuronide (GAMG) was achieved using β-D-glucuronidase from *Aspergillus terreus* I. The partially purified β-D-glucuronidase was immobilized by entrapment in Ca-alginate beads and the immobilization yield was about 83% at 2% alginate concentration. The pH-activity profile was widened upon immobilization. The optimum temperature was shifted from 40 to 45 °C and the apparent activation energy (E_a) was increased from 7.3 to 17.3 kcal/mol by immobilization. The immobilized enzyme exhibited higher thermal stability compared to the free form. The half-life values of the immobilized and free enzyme at 60 °C were 124.9 and 33.8 min, respectively. Also, immobilization eliminates the inhibitory effect of Cu^{2+} on GAMG production. The value of Michaelis-constant K_m of the immobilized enzyme (1.4 mg/ml) was greater than that of the free form (0.88 mg/ml), whereas, V_{max} was smaller for the immobilized enzyme. The durability of the immobilized β-D-glucuronidases in repeated use was studied. The immobilized enzyme retained about 40% of its original activity after 4 cycles.

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1. Introduction

Licorice extracts and its principle component, glycyrrhizin (GL), have extensively used in foods, tobacco (as a sweetener and a flavoring ingredient) and in both traditional and herbal medicine [1]. GL is composed of one molecule of glycyrrhetinic acid (GA) as aglycone and two molecules of glucuronic acid [2]. With cleavage of one terminal glucuronic acid by hydrolysis, GL can be transformed into glycyrrhetinic acid monoglucuronide (GAMG) [3]. The flavor improving effect of GAMG and its sweetness are 20- and 5-fold greater than those of GL, respectively [4]. Moreover, it was revealed that GAMG exhibits the similar (or stronger) anti-allergic, anti-inflammatory, anti-cancer and antivirus effects to (or than) GL [5–7]. Acute toxicity of GAMG is 5000 mg/kg while that of GL was 805 mg/kg and it shows no mutagenicity by umu-test [6,8]. High doses of licorice (more than 20 g/day) may cause serious side effects such as hypertension and symptoms associated with electrolyte disturbances [9]. Biochemical studies indicate that glycyrrhizinates inhibit 11β-hydroxysteroid dehydrogenase, the enzyme responsible for inactivating cortisol. As a result, the continuous, high level exposure to glycyrrhizin compounds can produce hypermineralocorticoid-like effects in both animals and humans [1]. Based on the in vivo and clinical evidence, the same authors proposed that an acceptable daily intake of 0.015–0.229 mg glycyrrhizin/kg body weight/day [1]. It should be noteworthy that GL is hardly soluble in water, and generally used as a form of water soluble salt, while GAMG itself is readily soluble in water. Thus, it seems that GAMG is promising as a potent sweetener, flavoring agent, as well as a biologically active compound. Considering the great importance and the expanding market of GAMG, the efficient methodologies are required for the large scale production of GAMG in industry.

β-D-Glucuronidases are members of the glycosidase family 2 of enzymes that hydrolyse the glycosidic bond between two or more carbohydrate or between a carbohydrate and non-carbohydrate moiety. β-D-Glucuronidase (PGUS, EC 3.2.1.31) is type of glucuronidases that catalyzes hydrolysis of β-D-glucuronic acid residues from the non-reducing end of mucopolysaccharides [10]. PGUS was known to be present in different groups of organism such as archaebacteria, eubacteria, fungi, invertebrates and vertebrates [11]. β-Glucuronidase from both *Escherichia coli* and bovine liver cleaved the prodrugs efficiently to release O^6-benzylguanine and O^6-benzyl-2′-deoxyguanosine, respectively. These prodrugs may be useful for prodrug monotherapy of necrotic tumors that liberate β-glucuronidase or for antibody-directed enzyme prodrug therapy with antibodies that can deliver β-glucuronidase to target tumor cells [12]. Zhang et al. [13] reported that Baicalein was produced through an enzymatic hydrolysis of Baicalin by PGUS encapsulated in biomimetic alginate/protamine/silica (APSi) capsules.

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Biosynthesis of GAMG has several advantages over chemical methods due to its high specificity, efficiency, and also an eco-friendly status. GAMG can be produced from GL by lysosomal PGUS of animal livers [14]; many PGUS derived from human intestinal bacteria [15]; and *Penicillium* sp. Li-3 PGUS [8]. Enzyme immobilization is a useful process which not only ensures reusability of enzymes and permits easy biocatalyst–product separation, but also improves thermal and chemical stability of enzymes [16]. Amongst the various techniques of enzyme immobilization, entrapment into alginate gel shows unique advantages due to its non-toxicity, mechanical stability, high porosity for substrate and product diffusion and above all the simple procedural requirements for immobilization [17]. However, there have been few studies on PGUS immobilization conditions and no reports on bioconversion of GL into GAMG by immobilized PGUS.

In our continuing studies on applications of biological transformation, we screened several fungal cultures for their ability to biotransform GL into GAMG [18]. *Aspergillus terreus* I was selected as the biotransforming target strain. In the present investigation, *A. terreus* PGUS was immobilized in Ca-alginate beads and reused for the hydrolysis of GL to GAMG. The properties of the free and immobilized PGUS were also compared, such as the pH and temperature profiles, pH and thermal stabilities and the kinetic behavior.

2. Materials and methods

2.1. Microorganism

A. terreus I was kindly obtained from the Center of Cultures of Chemistry of Natural and Microbial Products Department, National Research Center, Cairo, Egypt.

2.2. Production of PGUS

A. terreus I was cultivated in a medium containing 2.5% soybean flour, 3.5% corn steep liquor, 0.5% glucose and 0.5% $CaCO_3$ for 72 h at 27 °C (pH 6). After cultivation, the mycelia were harvested by filtration and disrupted by grounding with approximately twice its weight of washed cold sand in a cold mortar. The cell contents were extracted with cold 1 M acetate buffer pH 6. Thereafter, the obtained cell homogenate was centrifuged at 5500 rpm for 15 min and the resulting supernatant "cell free extract" was lyophilized. The lyophilized material was used as the starting crude endocellular enzyme [19].

2.3. Partial purification of PGUS

The lyophilized crude enzyme (120 g) was dissolved in 500 ml of 1 M Tris–maleate buffer (pH 6). It was fractionated with ammonium sulfate of 30–60% saturation. The precipitate was collected by centrifugation at 12,000 rpm and dialyzed against the same buffer. The protein and enzyme activity was determined in the dialyzed enzymatic fraction.

2.4. PGUS hydrolytic activity assay

The assay mixture for hydrolysis, contained 1 ml of 0.2% GL and 1 ml of enzyme solution, was incubated at 40 °C for 1 h in shaking water bath. Then, the reaction was stopped with 0.1 ml HCl (1 N) and the mixture was extracted twice with 2 ml of ethyl acetate. The amounts of GL, GAMG and GA in the ethyl acetate layer were determined by TLC scanner (Shimadzu CS-9000 dual wave-length flying spot, thin layer chromato-scanner, Japan). One unit (U) enzyme activity of PGUS was defined as the amount of enzyme that capable of converting GL to 1 μg GAMG or GA per hour under certain condition.

$$\text{Enzyme activity (U/ml)} = \mu\text{g GAMG/ml/h or } \mu\text{g GA/ml/h}$$

$$\text{Specific enzyme activity (U/mg)} = \text{activity of sample/protein content}$$

$$\text{Residual activity (\%)} = (\text{activity of sample/activity of control}) \times 100$$

2.5. Determination of GAMG and GA

The ethyl acetate solution (containing the transformation products) was concentrated to a small volume and mounted on silica gel plates (Fluka, silica gel 60F-254, layer thickness 0.2 mm). The plate was first chromatographed for GA with solvent system of chloroform–petroleum ether–acetic acid (6:6:1, v/v/v) and secondly for GL and GAMG with solvent system of acetic acid–n-butanol–1,2-dichloroethane–water (4:1:4:1, v/v/v/v). GA and GAMG were detected on TLC plates under ultraviolet (UV) light. These compounds were quantitatively analyzed with TLC-scanner (λ_s = 250 nm, λ_r = 400 nm) by using calibration lines obtained with authentic samples [15].

2.6. Immobilization of partially purified PGUS

The partially purified enzyme solution was immobilized by entrapment in calcium alginate gel beads according to Fraser and

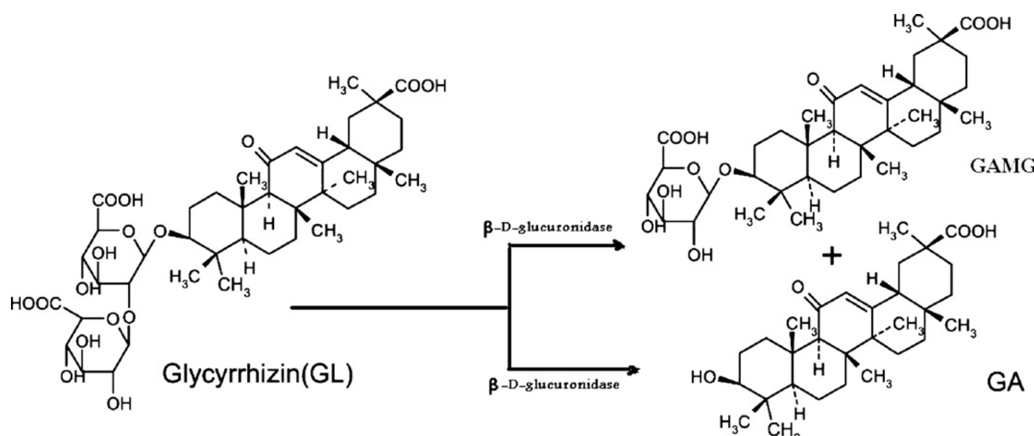

Fig. 1. Enzymatic hydrolysis of GL by *Aspergillus terreus*.

Bickerstaff [20]. Unless otherwise stated, 1 ml of the partially purified enzyme solution (containing 3 mg protein and 271.9 U) was mixed with 1 ml of 4% alginate to give a final concentration of 2% alginate (w/v). Beads were performed by dropping this mixture into constantly stirred 2% $CaCl_2$ solution (w/v) as cross-linking agent. The calcium alginate beads were cured by stirring in $CaCl_2$ for a further 20–30 min. Then, the beads were collected, and washed with Tris–maleate buffer (pH 6) three times. β-Glucuronidase activities in the initial enzyme solution, immobilized preparation and in the washing buffer (unbound enzyme) were determined as described before.

The immobilization yield (IY, %) was calculated as follows:

$$IY(\%) = \frac{I}{A-B}$$

where I is the activity units of the immobilized enzyme, A is the initial activity units offered for immobilization, and B is the activity units of the unbound enzyme.

2.7. Determination of kinetic parameters, optimum temperature and pH

K_m, V_{max}, optimum temperature and pH were determined by changing individually the conditions of the PGUS activity assay (GL concentration from 0.5 to 4 mg/ml; temperature from 25 to 55 °C; and pH from 4 to 7). K_m and V_{max} were calculated from Lineweaver–Burk plots.

2.8. pH and thermal stability

The pH stability of the free and immobilized enzymes was examined after pre-incubating the enzyme samples at room temperature for 2 h at various pH values ranging from 4.0 to 9.0. The stabilization effect was evaluated as the residual activity, which is the percentage of incubated enzyme activity relative to untreated enzyme activity.

Thermal stability of both free and immobilized PGUS was evaluated by measuring the residual activity of the enzyme samples exposed to various temperatures (from 25 to 60 °C) in Tris–maleate buffer (1 M, pH 6), with different time intervals (15, 30 and 60 min) for each temperature and the residual activity was then measured.

2.9. Activation energy (E_a)

E_a is defined as "the minimum energy required to start a chemical reaction and given in units of kcal/moles". The temperature dependence on the rate constant, for values below the temperature of inactivation, can be described by the Arrhenius equation:

$$K = A \times e^{-Ea/RT}$$

where K is the rate constant, A is the preexponential factor, E_a is the activation energy, R is the gas constant (R = 1.976 cal mol^{-1} K^{-1} or 8.314 J mol^{-1} K^{-1}), T is the absolute temperature. The apparent activation energy of free and immobilized enzymes was determined from the slope of logarithmic of the activity versus the reciprocal of Kelvin temperature (slope = $-E_a/2.303R$) [21].

2.10. Half-life and the deactivation constant rate

The half-life of an enzyme is the time it takes for the activity to reduce to a half of the original activity. It was determined by plotting the log of residual activity against time, at temperatures causing inactivation (50, 55, and 60 °C), according to the following equation [22]:

$$\text{Half life} = 0.693/\text{slope}$$
$$\text{Deactivation energy} = \text{slope of the straight line.}$$

2.11. Test for PGUS leakage

Enzyme leakage measurement was carried out by placing capsules in a test tube filled with tris buffer (pH 6) for 2 h. Then the capsules were removed, cut in half and put in phosphate buffer (pH 7.4) solution. The protein concentration was measured according to Lowry's assay and the leakage percentage was calculated from the differences between encapsulated protein at the beginning of time interval and the value found according to the above procedure.

2.12. Operational stability of immobilized PGUS

The immobilized enzyme was incubated with the substrate in Tris–maleate buffer (1 M at pH 6) at 40 °C for 1 h at shaking conditions. Then, the immobilized enzyme was collected by filtration, washed with buffer and resuspended in freshly prepared substrate to start a new run. The enzyme activity of immobilized enzyme was assayed in every run by the standard assay method.

3. Results and discussion

In our previous work [18], conversion of GL by *A. terreus* I PGUS resulted in two kinds of productions, GAMG and GA in the hydrolyzing reaction mixture (Fig. 1). An optimization for the culture production conditions as well as the reaction conditions was undergone for directing the hydrolyzing activity towards the production of GAMG and minimizing GA production. Under these optimal conditions, GAMG yield (51.5%) became about 2 times higher than GA yield (26.8%) and the cells bioconversion efficiency increased from 24.6 to 78.3% [18].

3.1. Effect of different concentrations of sodium alginate

The partially purified enzyme was entrapped with 2, 3, 4, 5% calcium alginate gel beads. The results in Table 1 show that the highest immobilization yield of PGUS (83%) was obtained using alginate concentration of 2% (w/v). Also, alginate concentration had a significant effect on enzyme leakage percentage. The maximum leakage of enzyme occurred at 1% (w/v) sodium alginate concentration was owing to the larger pores of the less tightly crosslinked gel. This means that the enzyme used in immobilization was not completely included in the gel matrix. Some PGUS molecules distributed on the

Table 1
Effect of different concentrations of sodium alginate.

Concentration of sodium alginate (g%)	Enzyme leakage (%)	Unbound enzyme (U) B	Immobilized enzyme (U) I	Immobilization yield (%) $I/(A-B) \times 100$
1	22.2 ± 2.1	7.1 ± 0.21	61.6 ± 2.16	23.3 ± 0.82
2	10.7 ± 1.5	4.2 ± 0.17	222.2 ± 5.56	83.0 ± 2.08
3	5.3 ± 1.3	5.8 ± 0.19	138.6 ± 4.16	52.1 ± 1.56
4	2.1 ± 0.5	6.6 ± 0.27	82.0 ± 2.62	30.9 ± 0.99

A = 271.9 U/reaction (added enzyme to each alginate concentration, corresponding to 3 mg protein).

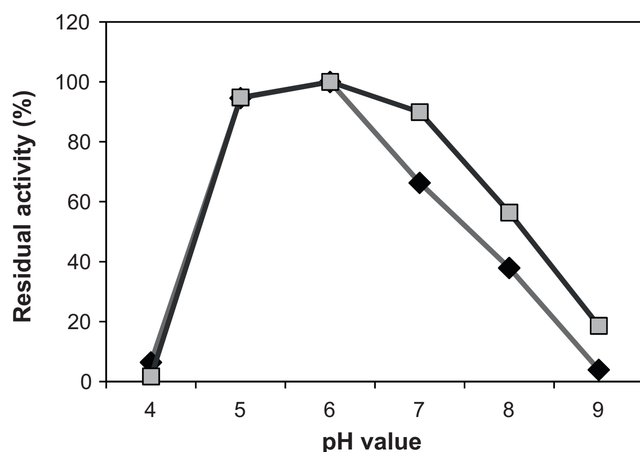

Fig. 2. pH stability of free and immobilized *A. terreus* PGUS. The 100% activity of the free (♦) and immobilized (■) PGUS was 271.9 and 222.2 U/ml, respectively. The enzyme samples were pre-incubated at room temperature for 2 h at various pH values ranging from 4.0 to 9.0. The stabilization effect was evaluated as the residual activity, which is the percentage of incubated enzyme activity relative to untreated enzyme activity. The experiments were performed in triplicate and the standard derivations were lower than ±4.5%.

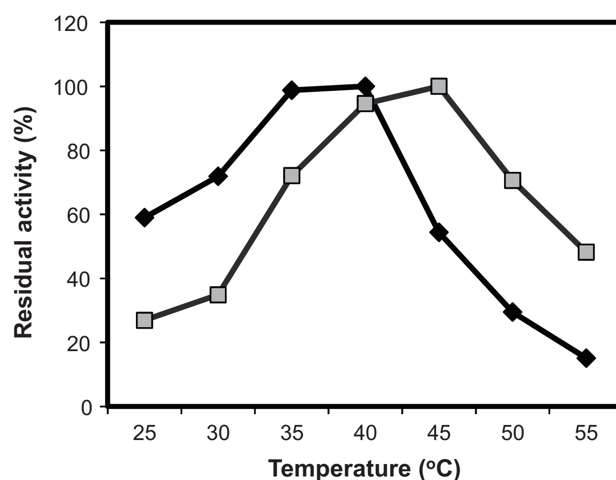

Fig. 3. Effect of temperature on activity of free and immobilized *A. terreus* PGUS. The 100% activity of the free (♦) and immobilized (■) PGUS was 271.9 and 222.2 U/ml, respectively. The reaction mixture was incubated for 1 h at different temperatures in shaking water bath. The experiments were performed in triplicate and the standard derivations were lower than ±3.2%.

surface of alginate beads diffused into the $CaCl_2$ solution during the gel beads formation. This observation was in accordance with earlier reported studies, where the yield of enzyme immobilization in alginate gel varied from 50 to 85% [17,23,24]. The decrease in yield with the increase in carrier concentration might be due to the decrease in porosity of the gel matrix which caused diffusional resistance of the substrate [25].

3.2. Effect of pH on activity and stability

The pH profiles of free and immobilized enzymes were similar at the pH range of 4–7 (data not shown); the maximal activity for both was achieved at pH 6 (see Fig. 2). The optimal pH range (5–7) at which the immobilized enzyme maintained high activity (>70%) was slightly widened compared to that of the free one (5–6), probably this change is produced by the unequal ionic distribution. This behavior might be the result of interior microenvironment of alginate capsule that is slightly cationic and separated from bulk with a semi-permeable membrane which is anionic in nature [26]. Similar behavior has been described for immobilization of neutrase on alginate glutaraldhyde beads [21]. The procedure of enzyme immobilization on insoluble carriers has a variety of effects on the protein conformation as well as on the state of ionization and dissociation of the enzyme and its environment. It is known that in some cases, when the enzyme is coupled with a polyanionic carrier the pH optimum usually shifts in the alkaline direction whereas if the carrier is polycationic the shift is in the acid direction [27]. Additionally, a very significant activity change for free enzyme at pH 8–9 was observed. Similar behavior but in lower extent was observed for immobilized enzyme, probably because immobilization could provide the enzyme some protection against buffer composition [21]. These observations suggest that the binding of the PGUS onto the support results in significant alterations of enzyme microenvironment, improving the retention of activity at alkaline pHs [27].

3.3. Effect of temperature on activity and stability

The optimal temperature of the free and immobilized PGUS was 40 and 45 °C, respectively (see Fig. 3). Similar displacement of optimum temperature for immobilized enzyme was observed; for example, a displacement of optimum temperature from 50 to 60 °C was observed for neutrase from *Bacillus subtilis* immobilized on alginate glutaraldhyde beads [21] and from 40 to 50 °C for alkaline protease from *Conidiobolus macrosporus* immobilized on polyamide using glutaraldhyde as bifunctional agent [28]. Furthermore, the immobilized enzyme was more stable than the free enzyme above 40 °C. These results would be due to the stabilization of the enzyme by immobilization to the support gel, and even at a higher temperature the immobilized PGUS could retain its active structure compared to free form. However, at temperatures lower than 40 °C, the enzyme activity of the free form was higher than that of the immobilized form. This is mainly due to the mass transport resistance to the substrate onto the porous carrier [29] and thus lower effective concentration of substrate. This explains why the calculated value of the activation energy for the free PGUS was lower than that of the immobilized enzyme form (7.3 and 17.3 kcal/mol, respectively).

Moreover, the immobilized enzyme retained 100% of its activity at 45 °C during all the incubation periods and was inactivated at much slower rate than the free form (Fig. 4). Compared to the free enzyme at 60 °C, the immobilized enzyme exhibited higher thermal stability, which was represented as more than 3.5-fold stabilization factor (expressed as the ratio of half-lives of immobilized and free enzymes). The calculated half life values of the free and immobilized enzymes at different temperatures are represented in Table 2. Also, the immobilized enzyme retained 34.5% of the original activity compared to 9.5% retained activity of the free form after 60 min incubation at the same temperature (Fig. 4). The thermal stability of immobilized PGUS increased considerably as a result of immobilization in alginate gel. Enzyme stabilization by immobilization may also be caused by the existence of a local environment for the immobilized enzyme which is less damaging than bulk solution conditions [30]. Dramatic stability enhancements have been reported based on this strategy in which gel entrapment was applied to attempt to form a local support microstructure complementary to enzyme surface [17].

3.4. Influence of various metal ions

In examining the effect of metals on the activity of the free and immobilized PGUS (Fig. 5), results show that GAMG production by the free enzyme was inhibited by Hg^{2+} and Cu^{2+} and slightly activated by Mn^{2+} and Ba^{2+}. Other tested ions, Ca^{2+}, Al^{3+} and Mg^{2+} did not show an appreciable effect on GAMG production. On the

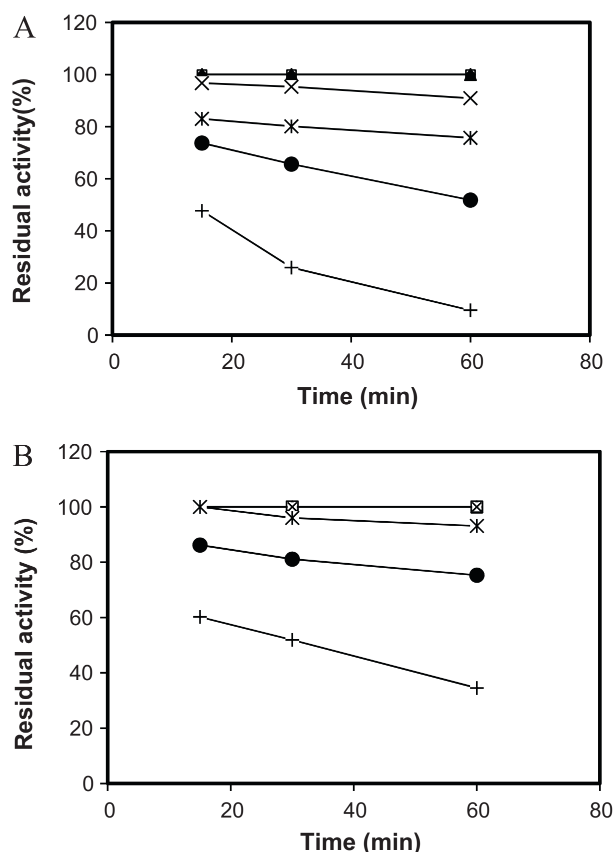

Fig. 4. Effect of temperature on the stability of the free (A) and immobilized (B) *A. terreus* PGUS. The 100% activity of the free (A) and immobilized (B) PGUS was 271.9 and 222.2 U/ml, respectively. Enzyme samples were preincubated at 30 °C (♦), 35 °C (□), 40 °C (▲), 45 °C (×), 50 °C (*), 55 °C (●), 60 °C (+) for 15, 30, 60 min. The experiments were performed in triplicate and the standard derivations were lower than ±5%. 100% residual activities were obtained by preincubating the free enzyme at 30–40 °C and the immobilized enzyme at 30–45 °C.

other hand, immobilization eliminates the inhibitory effect of Cu^{2+} on GAMG production. This can be explained as: the immobilization matrix acts as a protector for the enzyme molecule against the toxic effect of Cu^{2+} ion. The immobilization may permit a reduction of the inhibition problems by different mechanisms: (i) exclusion of the inhibitor from the enzyme environment and (ii) decrease of the affinity of the recognition places of the enzyme by the inhibitor

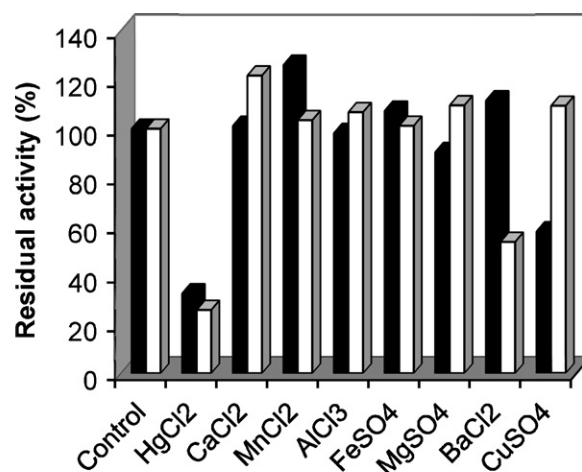

Fig. 5. Effect of metal ions on the activity of the free and immobilized enzyme. The 100% activity of the free (■) and immobilized (□) PGUS was 271.9 and 222.2 U/ml, respectively. The residual activity was presented as the percentage of the control activity without metal ion. Control = 100% activity. The experiments were performed in triplicate and the standard derivations were lower than ±3.1%.

[31]. Although, Ba^{2+} act as activator for GAMG production by the free enzyme (11.6% increase of activity compared with control), it acts as inhibitor to their production by the immobilized enzyme (46.4% decrease in activity compared with control). The inactivation for PGUS activity by the addition of salts of heavy metals, especially mercury and copper may be due to modification of enzyme thiol groups. Since many enzymes contain thiol (–SH), alcohol, or acid groups as part of their active sites, any chemical which can react with them acts as an irreversible inhibitor which cause covalent modification of enzyme structure.

3.5. Kinetic properties

The kinetic analysis was done using different concentrations of GL as a substrate (Fig. 6). The activity of the free enzyme for production of GAMG increased with the increase of the substrate concentration up to 2 mg GL/ml and decreased with the increase of the substrate concentration over the later concentration. This indicates that the free enzyme was inhibited by the substrate at levels above 2 mg/ml, whereas the immobilized enzyme fol-

Table 2
Activation energy, half-life values and deactivation rate constants of the free and immobilized PGUS.

Kinetic parameter	PGUS	
	Free	Immobilized
[a]K_m (mg/ml)	0.88	1.4
[a]V_{max} (U/mg protein)	142.9	133.3
[b]E_a (kcal/mol)	7.3	17.3
[c]Half-life (min) at		
50 °C	624.3	1248.6
55 °C	312.2	624.0
60 °C	33.8	124.9
[c]Deactivation rate constant (min^{-1}) at		
50 °C	1.1×10^{-3}	5.6×10^{-4}
55 °C	2.2×10^{-3}	1.1×10^{-3}
60 °C	2.1×10^{-2}	5.6×10^{-3}

[a] The apparent K_m and V_{max} values were determined by Lineweaver–Burk plot.
[b] The apparent E_a values were calculated by Arrhenius plot.
[c] The half-life and deactivation rate constant values were determined by plotting log residual activity versus time.

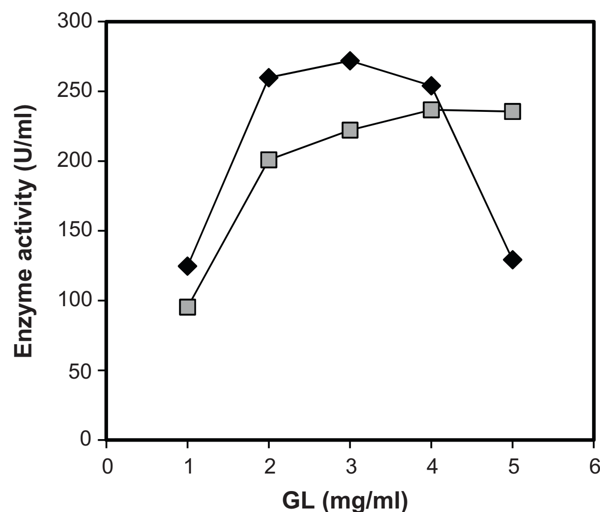

Fig. 6. Michaelis–Menten kinetics for the free (♦) and immobilized (■) *A. terreus* PGUS.

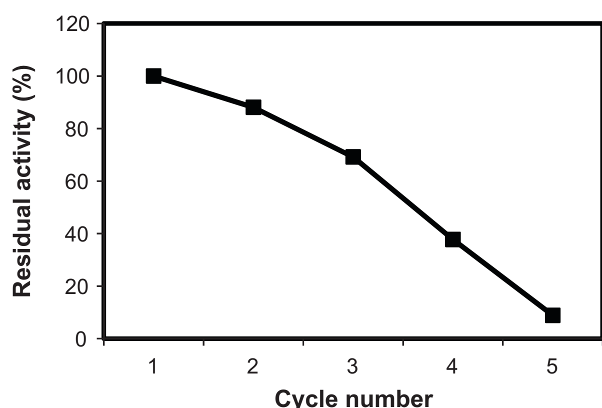

Fig. 7. Operational stability of the immobilized *A. terreus* I PGUS. The 100% residual activity corresponds to 222.2 U/ml. The immobilized enzyme was incubated at 45 °C, pH 6, 0.2% GL, and 1 h for each batch. The experiments were performed in triplicate and the standard derivations were lower than ±4.2%.

lowed Michaelis–Menten kinetics. It seems that immobilization eliminate the inhibitory effect of substrate on the PGUS activity. This effect could be attributed to limitation of the substrate diffusion rate by the alginate network. The values of kinetic prameters of the Michaelis–Menten equation were determined from Lineweaver–Burk plots. The K_m was increased from 0.8 to 1.4 mg/ml when PGUS was entrapped with calcium alginate gel, suggesting that the affinity of the enzyme for its substrate was lowered after immobilization. However, the calculated maximal reaction rate V_{max} was decreased from 142.9 to 133.3 U/mg protein after immobilization. An increase in the K_m and decrease in the V_{max} after immobilization have been already reported by many authors [13,17]. The changes in K_m and V_{max} after immobilization may result from the strict hindrance of the active site by the support or the loss of enzyme flexibility necessary for substrate binding [32]. Also, the terminal uronic acid residues from alginate matrix may compete with substrate binding. This reaction between support and the enzyme causes chemical modifications within the enzyme. These are called conformational effects [33].

3.6. Operational stability

The main advantage of immobilizing an enzyme is that it allows repeated use, which is important in the case of expensive enzyme. The operational stability of immobilized PGUS is shown in Fig. 7. The results show the durability of the immobilized enzyme in repeated use for 4 cycles. The immobilized enzyme retained about 40% of its original activity after 4 cycles. However, it lost almost all of its activity after the 4th cycle. The activity decay during recycling may have been related to inactivation of the enzyme caused by denaturation of the protein, or the adsorption and accumulation of reaction products in the biocatalyst.

Briefly, this work reports for the first time that the biotransformation of GL to GAMG is catalyzed by immobilized β-D-glucuronidase. The stability of β-D-glucuronidase against thermal and pH-induced inactivation was improved via entrapment in alginate gel. In particular, calcium alginate gel entrapment is a more suitable method than other entrapment methods involve some factors contributing to the inactivation such as high gelation temperatures, the heat of polymerization and radicals. Such preparation of immobilized β-D-glucuronidase can be used repeatedly, which determines its economical and eco-friendly status for future industrial applications.

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