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Calcium alginate–starch hybrid support for both surface immobilization and entrapment of bitter gourd (*Momordica charantia*) peroxidase

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ABSTRACT

Calcium alginate–starch hybrid gel was employed as an enzyme carrier both for surface immobilization and entrapment of bitter gourd peroxidase. Entrapped crosslinked concanavalin A–bitter gourd peroxidase retained 52% of the initial activity while surface immobilized and glutaraldehyde crosslinked enzyme showed 63% activity. A comparative stability of both forms of immobilized bitter gourd peroxidase was investigated against pH, temperature and chaotropic agent; like urea, heavy metals, water-miscible organic solvents, detergent and inhibitors. Entrapped peroxidase was significantly more stable as compared to surface immobilized form of enzyme. The pH and temperature-optima for both immobilized preparations were the same as for soluble bitter gourd peroxidase. Entrapped crosslinked concanavalin A–bitter gourd peroxidase showed 75% of the initial activity while the surface immobilized and crosslinked bitter gourd peroxidase retained 69% of the original activity after its seventh repeated use.

1. Introduction

Peroxidases (E.C. 1.11.1.7) are ubiquitous oxidoreductases that use hydrogen peroxide or organic hydroperoxides for oxidation of a wide range of substances [1]. Most peroxidases are glycoproteins containing N-linked oligosaccharide chains [2]. Apart from biological role, peroxidases have been found effective in analytical, clinical, biotechnological, industrial and environmental applications [3,4]. Peroxidases are being used in detoxification, decolorization and removal of various organic contaminants from polluted water [5].

However, soluble enzymes have certain inherent limitations and thus cannot be used at large-scale whereas immobilized enzymes offer several advantages and these preparations can be employed for the continuous synthesis of noble compounds and degradation of toxic pollutants in batch as well as in continuous reactors [6]. Stabilization of enzymes against the inactivation mediated by various types of denaturants has been accomplished using a multitude of immobilization strategies including covalent coupling, adsorption, microencapsulation, polymer entrapment, chemical aggregation, bioaffinity, etc. [3,7–9].

Among the various techniques employed for the immobilization of enzymes, entrapment may be a good choice owing to a relatively inert aqueous environment within the matrix and causing relatively little damage to the structure of the native enzyme [10,11]. Alginate appears to be one of the most suitable polymers for the immobilization and microencapsulation technologies because of the following advantages: hydrophilic nature, presence of carboxylic groups, natural origin, mechanical stability and stability over extreme experimental conditions [12–14].

However, it has been reported that because of the porous nature of sodium alginate, most of the entrapped material is released from the gel beads during its application. In order to optimize the encapsulation efficiency and controlled release of enzyme from the gel matrix, entrapment of crosslinked or pre-immobilized enzymes has been done [10,15]. However, the major limitation of physical entrapment is that large molecular size substrates/products cannot easily be diffused in and out of the gel [16].

In this work an effort has been made to prepare a hybrid gel of alginate and starch which could be exploited for the entrapment of enzymes as well as bioaffinity attachment of glycosylated enzymes on the surface of gel beads. These calcium alginate–starch beads were layered with concanavalin A (Con A). Con A layered calcium alginate–starch beads were used for the surface immobilization of glycosylated peroxidases from bitter gourd. Con

Abbreviations: BGP, bitter gourd peroxidases; Con A, concanavalin A; E-BGP, calcium alginate–starch entrapped crosslinked Con A-BGP; SI-BGP, BGP immobilized on the surface of concanavalin A layered calcium alginate–starch beads and crosslinked by glutaraldehyde; S-BGP, soluble BGP.

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A–bitter gourd peroxidase (BGP) complex was also entrapped in the calcium alginate–starch beads. A comparative stability study of entrapped and surface immobilized peroxidase has been carried out against various physical and chemical denaturants. Immobilized BGP preparations have also been studied for their reusability.

2. Materials and methods

2.1. Materials

Jack bean meal was procured from DIFCO, Detroit, USA. *o*-Dianisidine HCl was obtained from IGIB, New Delhi, India. Cadmium chloride ($CdCl_2$), dioxane, *n*-propanol, mercuric chloride ($HgCl_2$), starch and Tween 20 were obtained from the SRL Chem. Pvt. Ltd., Mumbai, India. Ethylene diamine tetracetic acid (EDTA) and sodium azide were purchased from the Merck Chem. Pvt. Ltd. Worli, Mumbai, India. Bovine serum albumin, glutaraldehyde and ethanolamine were procured from Sigma Chemical Co. (St. Louis, MO, USA). Bitter gourd was purchased from the local vegetable market. Other chemicals and reagents employed were of analytical grade and were used without any further purification.

2.2. Ammonium sulphate fractionation of bitter gourd proteins

Bitter gourd (100 g) was homogenized in 200 ml of 100 mM sodium acetate buffer, pH 5.0. Homogenate was filtered through four layers of cheese-cloth. The filtrate was then centrifuged at 10,000 × *g* on a Remi R-24 Cooling Centrifuge for 20 min at 4 °C. The clear supernatant was subjected to salt fractionation by adding 20–80% (w/v) ammonium sulphate. The solution was stirred overnight at 4 °C and the obtained precipitate was collected by centrifugation at 10,000 × *g* on a Remi R-24 Cooling Centrifuge for 20 min at 4 °C. The collected precipitate was redissolved in 100 mM sodium acetate buffer, pH 5.0 and dialyzed against the assay buffer Akhtar et al. [17].

2.3. Preparation of Con A–BGP complex

Jack bean extract (10%, w/v) was prepared according to the method described by Matto and Husain [11]. Insoluble residue was removed by centrifugation at 3000 × *g* for 30 min, until a clear supernatant was obtained. The collected supernatant was used as source of Con A.

BGP (1200 U) was incubated with increasing concentrations of jack bean extract (0.1–1.0 mL) containing Con A and the final volume was adjusted to 4.0 mL with 100 mM sodium phosphate buffer, pH 6.2. The mixtures were incubated at 37 °C for 12 h. The insoluble complex was collected by centrifugation at 3000 × *g* for 15 min at room temperature and the precipitates were washed thrice with sodium phosphate buffer, pH 6.2 to remove unbound protein. Finally each precipitate was suspended in assay buffer and peroxidase activity was determined.

The Con A–BGP complex (840 U) was crosslinked prior to entrapment in calcium alginate–starch beads with 0.5% glutaraldehyde for 2 h at 4 °C with constant shaking [18].

2.4. Entrapment of crosslinked Con A–BGP into calcium alginate–starch beads

Alginate is an anionic polymer composed of α-L-guluronic acid and β-D-mannuronic acid. Enzymes can be entrapped in alginate beads produced by the crosslinking between α-L-guluronic acid and divalent cations such as calcium ions [15]. The crosslinked Con A–BGP complex (792 U) was mixed with sodium alginate (2.5%, w/v) and starch (2.5%, w/v) prepared in 10.0 mL of assay buffer. The resulting mixture was slowly extruded as droplets through a 5.0 mL syringe with attached needle no. 20,200 mM calcium chloride solution and further gently stirred for 2 h. The obtained calcium alginate–starch entrapped crosslinked Con A–BGP (E-BGP) was washed with 100 mM sodium acetate buffer, pH 5.0 and stored in the assay buffer at 4 °C for its further use [11].

2.5. Immobilization of BGP on the surface of Con A layered calcium alginate–starch beads

Sodium alginate (2.5%, w/v) and starch (2.5%, w/v) beads were prepared without enzyme according to the procedure described in the above section and these beads were incubated in 10.0 mL jack bean extract, a source of Con A for 12 h at room temperature with slow stirring. After incubation period, Con A bound calcium alginate–starch beads were collected and washed with assay buffer. Con A layered calcium alginate–starch beads were then incubated with BGP (1200 U) overnight at room temperature with slow stirring. Unbound enzyme was removed by repeated washing with 100 mM sodium acetate buffer, pH 5.0. BGP immobilized on the surface of Con A layered calcium alginate–starch beads was crosslinked by 0.5% glutaraldehyde for 2 h at 4 °C [18]. Surface immobilized and glutaraldehyde crosslinked BGP (SI-BGP) was stored at 4 °C for its further use.

2.6. Effect of pH

Appropriate and equal amounts of S-BGP, SI-BGP and E-BGP were taken to determine the activity of peroxidase in the buffers of different pH. The buffers used were glycine–HCl (pH 2.0 and 3.0), sodium acetate (pH 4.0 and 5.0) and Tris–HCl (pH 6.0–10.0). The activity at pH-optimum was considered as control (100%) for the calculation of percent activity at other pH.

2.7. Effect of temperature

The activity of soluble and immobilized BGP (1.3 U) was determined at various temperatures (30–80 °C) in 100 mM sodium acetate buffer, pH 5.0. The activity at temperature-optimum was considered as control (100%) for the calculation of percent activity at other temperatures.

In another set of experiment, all three BGP preparations were incubated at 60 °C for varying time intervals in 100 mM sodium acetate buffer, pH 5.0. After each incubation period the enzyme was quickly chilled in crushed ice for 5 min. The enzyme was brought to room temperature and the peroxidase activity was measured. The activity without incubation at 60 °C was taken as control (100%) for the calculation of remaining percent activity.

2.8. Effect of urea

Soluble and immobilized BGP (1.3 U) were incubated with 4.0 M urea for varying times in 100 mM sodium acetate buffer, pH 5.0 at 37 °C. Peroxidase activity was determined at the indicated time intervals. The activity of enzyme without incubation with urea was taken as control (100%) for the calculation of remaining percent activity.

2.9. Effect of water-miscible organic solvents

Soluble and immobilized BGP (1.3 U) were incubated independently with varying concentrations of water-miscible organic solvents; dioxane and *n*-propanol (10–60%, v/v) in 100 mM sodium acetate buffer, pH 5.0 at 37 °C for 1 h. Activity of enzyme without

organic solvent was taken as control (100%) for the calculation of remaining percent activity.

2.10. Effect of sodium azide and EDTA

The inhibitory effect of sodium azide/EDTA (0.01–0.1 mM) was examined on BGP preparations (1.3 U). Soluble and immobilized BGP were pre-incubated with inhibitors in 100 mM sodium acetate buffer, pH 5.0 at 37 °C for 1 h. The activity of enzyme without exposure to sodium azide/EDTA was considered as control (100%) for the calculation of remaining percent activity.

2.11. Effect of $HgCl_2$/$CdCl_2$

Soluble and immobilized BGP (1.3 U) were incubated independently with $HgCl_2$/$CdCl_2$ (0.01–0.1 mM) in 100 mM sodium acetate buffer, pH 5.0 at 37 °C for 1 h. The activity of enzyme without exposure to heavy metal was taken as control (100%) for the calculation of remaining percent activity.

2.12. Effect of detergent and NaCl

Soluble and immobilized BGP (1.3 U) were incubated with Tween 20 (0.5–5.0%, v/v) in 100 mM sodium acetate buffer, pH 5.0 at 37 °C for 1 h. The activity of enzyme without Tween 20 was taken as control (100%) for the calculation of remaining percent activity.

In another set of experiment soluble and immobilized BGP preparations (1.3 U) were incubated with sodium chloride (0.1–1.0 M) under the identical experimental conditions as described in the above section.

2.13. Reusability of immobilized BGP

E-BGP and SI-BGP were taken in triplicates for assaying the peroxidase activity. After each assay the immobilized enzyme preparations were taken out, washed and stored in 100 mM sodium acetate buffer, pH 5.0 overnight at 4 °C. The activity was assayed for 7 successive days. The activity determined for the first time was considered as control (100%) for the calculation of remaining percent activity after each use.

2.14. Measurement of peroxidase activity

Peroxidase activity was estimated from the change in optical density (A_{460} nm) at 37 °C by measuring initial rate of oxidation of *o*-dianisidine HCl (18 mM) by H_2O_2 (6.0 mM). The assay mixture with immobilized BGP was continuously stirred for the entire duration of assay [19].

One unit (1.0 U) of peroxidase activity was defined as the amount of enzyme protein that catalyzes the oxidation of 1.0 (mol of *o*-dianisidine HCl per min at 37 °C.

2.15. Determination of protein concentration

The protein concentration was determined according to the procedure described by Lowry et al. [20]. Bovine serum albumin was used as standard.

2.16. Statistical analysis

Each value represents the mean for three independent experiments performed in duplicates, with average standard deviation, <5%. The data exposed in various studies was plotted using Sigma Plot-5.0 and expressed as mean with standard deviation of error (±). Data was analyzed by one-way ANOVA. *P*-values <0.05 were statistically significant.

3. Results

3.1. Entrapment and surface immobilization of BGP

The entrapment and surface immobilization of BGP into/on calcium alginate–starch beads is demonstrated in Fig. 1. Insoluble Con A–BGP complex retained 70% of the initial activity. In order to prevent the dissociation of Con A–BGP complex, this complex was crosslinked by 0.5% glutaraldehyde. The activity of enzyme was decreased after crosslinking and crosslinked preparation retained 66% activity. However, the entrapment of crosslinked Con A–BGP complex into calcium alginate–starch beads further resulted in a loss of 14% activity (Table 1).

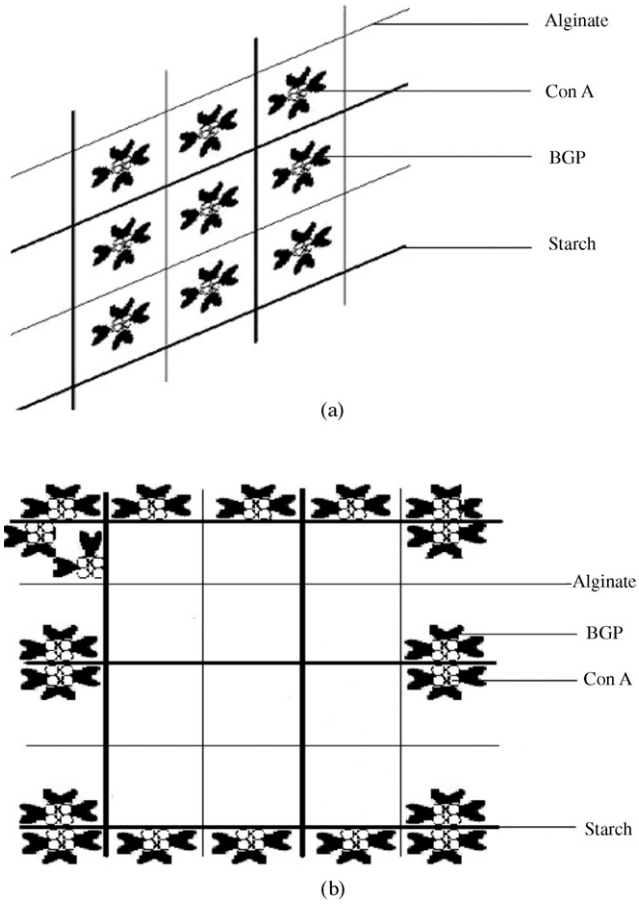

Fig. 1. Schematic diagram of (a) E-BGP and (b) SI-BGP.

Table 1
Immobilization of BGP into and on calcium alginate–starch beads

Enzyme immobilized preparations	Activity expressed (%)
Con A–BGP complex	70
Crosslinked Con A–BGP complex	66
E-BGP	52
Peroxidase immobilized on Con A layered calcium alginate–starch beads	69
SI-BGP	63

Each value shows the mean for three independent experiments performed in duplicates, with average standard deviation, <5%.

BGP immobilized on the surface of Con A layered calcium alginate–starch beads exhibited 69% of the original activity. Moreover, the surface immobilized enzyme was also crosslinked by 0.5% glutaraldehyde in order to maintain its integrity. The crosslinking of surface immobilized BGP also resulted in a marginal loss of 6% activity (Table 1).

3.2. Effect of pH

Fig. 2a demonstrates the effect of pH on the activity of soluble and immobilized BGP. Both immobilized BGP preparations showed same pH-optima as their soluble counterpart, pH 5.0. However, they exhibited a remarkable broadening in pH-activity profiles as compared to S-BGP. E-BGP retained significantly very high enzyme activity at acidic and alkaline side of pH-optima as compared to SI-BGP and S-BGP. The E-BGP showed 57% and 60% of the maximum activity at pH 3.0 and 8.0, respectively whereas soluble enzyme exhibited only 36% and 41% activity under similar incubation conditions.

3.3. Effect of temperature

Both immobilized BGP preparations exhibited same temperature-optima, at 40 °C as their free form (Fig. 2b). E-BGP retained remarkably higher fraction of catalytic activity at temperatures below and above the temperature-optima as compared to SI-BGP and S-BGP. E-BGP exhibited 50% activity at 80 °C while SI-BGP and S-BGP retained 40% and 30% activity at this temperature, respectively.

Soluble and immobilized BGP were incubated at 60 °C for various time intervals. Incubation of S-BGP at 60 °C for 2 h resulted in a loss of 53% of initial activity. However, E-BGP and SI-BGP retained 73% and 60% of the original activity under similar incubation conditions, respectively (Fig. 2c).

3.4. Effect of urea

Fig. 3 demonstrates the effect of 4.0 M urea on the activity of BGP. E-BGP and SI-BGP retained 70% and 50% of their activity after 2 h incubation in 4.0 M urea, whereas soluble enzyme lost nearly 68% activity under identical urea exposure.

3.5. Effect of organic solvents

The effect of increasing concentrations of water-miscible organic solvents; dioxane and *n*-propanol (10–60%, v/v) on the activity of soluble and immoblilized BGP is shown in Table 2. E-BGP showed more than 55% of the initial activity when exposed to 40% (v/v) dioxane/*n*-propanol for 1 h at 37 °C. However, S-BGP exhibited only 34% and 23% of the initial activity after exposure to 40% (v/v) dioxane and *n*-propanol, respectively.

3.6. Effect of sodium azide/EDTA

Table 3 demonstrates the effect of sodium azide/EDTA on the activity of soluble and immobilized BGP. E-BGP and SI-BGP retained 39% and 35% activity after 1 h exposure to 0.06 mM sodium azide. Moreover, SI-BGP and E-BGP retained 64% and 76% activity after 1 h exposure to 0.05 mM EDTA, respectively while the soluble BGP lost nearly 47% of its activity under identical EDTA exposure (Table 3).

3.7. Effect of $HgCl_2$/$CdCl_2$

The chemical contamination of water from a wide range of toxic derivatives, in particular heavy metals, is a serious environmen-

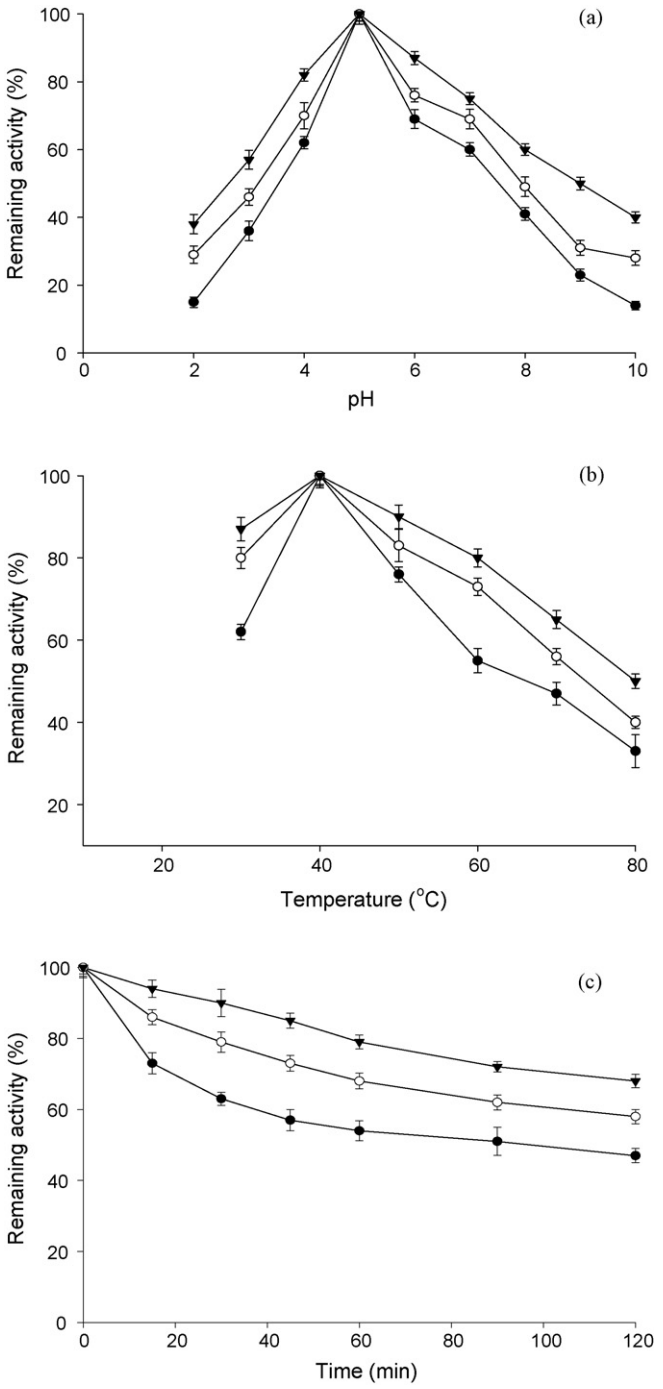

Fig. 2. Effect of pH and temperature on soluble and immobilized BGP. (a) pH-activity profile. Soluble and immobilized BGP (1.3 U) were incubated in the buffers of varying pH. The molarity of each buffer was 100 mM. The activity at pH 5.0 for all the preparations was taken as control (100%) for the calculation of remaining percent activity. The symbols show S-BGP (●), SI-BGP (○) and E-BGP (▼). (b) Temperature-activity profile. The activity of soluble and immobilized BGP (1.3 U) was measured in 100 mM sodium acetate buffer, pH 5.0 at various temperatures (30–80 °C). The activity obtained at 40 °C was taken as control (100%) for the calculation of remaining percent activity. For symbols refer to Fig. 2a legend. (c) Thermal denaturation of soluble and immobilized BGP. Soluble and immobilized BGP were incubated at 60 °C for various times in 100 mM sodium acetate buffer, pH 5.0. Aliquots of each preparation (1.3 U) were taken at indicated time intervals and chilled quickly in crushed ice for 5 min. Enzyme activity was determined as described in the text. Activity obtained without incubation at 60 °C was taken as control (100%) for the calculation of remaining percent activity. For symbols refer to (a) legend.

Table 2
Effect of organic solvents on soluble and immobilized BGP

Organic solvent (v/v, %)	Remaining activity (%)					
	Dioxane			*n*-Propanol		
	S-BGP	SI-BGP	E-BGP	S-BGP	SI-BGP	E-BGP
10	75.89 ± 1.89	85.25* ± 1.12	92.79* ± 3.97	50.97 ± 1.83	67.82# ± 1.87	89.15# ± 2.01
20	63.67 ± 2.23	72.24* ± 2.14	85.24* ± 1.34	38.24 ± 1.61	49.97# ± 2.18	80.81# ± 1.66
30	41.13 ± 1.67	57.91* ± 1.54	66.68* ± 1.25	28.69 ± 1.89	39.79# ± 1.43	77.19# ± 1.98
40	34.21 ± 2.14	39.97* ± 1.78	57.94* ± 1.78	23.23 ± 1.74	35.39# ± 1.67	55.89# ± 1.54
50	25.92 ± 1.16	30.81* ± 1.96	37.82* ± 1.97	17.91 ± 1.36	29.87# ± 1.54	42.63# ± 1.56
60	19.27 ± 2.11	23.92* ± 1.61	28.73* ± 1.91	14.40 ± 1.23	26.81# ± 1.14	35.29# ± 1.46

Soluble and immobilized BGP (1.3 U) were incubated independently with dioxane/*n*-propanol (10–60%, v/v) in 100 mM sodium acetate buffer, pH 5.0 at 37 °C for 1 h. The activity of BGP without exposure to organic solvent was taken as control (100%) for the calculation of remaining percent activity. Effect of various concentrations of organic solvents on soluble and immobilized BGP was analyzed by one-way ANOVA*. Values (#$P < 0.05$) were statistically significant when SI-BGP and E-BGP were compared with S-BGP, with respect to dioxane/*n*-propanol, respectively.

Table 3
Effect of sodium azide/EDTA on soluble and immobilized BGP

Inhibitor (mM)	Remaining activity (%)					
	sodium azide			EDTA		
	S-BGP	SI-BGP	E-BGP	S-BGP	SI-BGP	E-BGP
0.01	69.78 ± 1.07	82.17 ± 2.83	89.12 ± 1.44	82.24 ± 1.32	86.78# ± 1.41	92.15# ± 2.65
0.02	63.12 ± 2.16	71.18 ± 1.53	75.31 ± 2.17	75.79 ± 1.47	85.35# ± 1.27	89.71# ± 1.42
0.04	42.65 ± 1.23	52.33 ± 1.74	58.65 ± 1.73	59.83. ± 1.99	71.63# ± 1.68	83.22# ± 1.89
0.06	27.69 ± 1.67	34.87 ± 2.13	39.34 ± 1.19	48.61 ± 0.97	64.29# ± 0.16	76.18# ± 1.28
0.08	21.38 ± 1.27	26.84 ± 1.77	31.33 ± 1.95	41.18 ± 2.19	57.17# ± 1.67	70.17# ± 1.14
0.10	12.24 ± 1.51	19.67 ± 1.93	23.74 ± 1.49	30.71 ± 1.43	51.38# ± 1.23	61.39# ± 1.88

Soluble and immobilized BGP (1.3 U) were incubated independently with (0.01–1.0 mM) of sodium azide and EDTA in 100 mM sodium acetate buffer, pH 5.0 at 37 °C for 1 h. The activity of soluble and immobilized BGP without exposure to inhibitor was taken as control (100%) for the calculation of remaining percent activity. Effect of various concentrations of inhibitors on soluble and immobilized BGP was analyzed by one-way ANOVA*. Values (#$P < 0.05$) were statistically significant when SI-BGP and E-BGP were compared with S-BGP, with respect to sodium azide/EDTA, respectively.

tal problem owing to their potential human toxicity. In view of their presence in wastewater, it becomes important to examine the effect of some heavy metals on the activity of BGP. E-BGP and SI-BGP retained 71% and 58% activity in the presence of 0.1 mM $HgCl_2$, respectively whereas S-BGP exhibited only 53% activity under similar treatment conditions (Table 4).

Immobilized BGP preparations were more resistant to inactivation induced by $CdCl_2$. E-BGP and SI-BGP retained 69% and 59% activity after 1 h incubation with 0.1 mM $CdCl_2$, respectively. However, S-BGP lost 48% of its original activity when it was pre-incubated to 0.1 mM $CdCl_2$ (Table 4).

3.8. Effect of detergent (Tween 20) and sodium chloride

The effect of Tween 20 on the activity of soluble and immobilized BGP is shown in Table 5. E-BGP and SI-BGP retained 57% and 40% of the original activity when exposed to 5.0% (v/v) Tween 20 for 1 h at 37 °C. However, soluble enzyme was sensitive to Tween 20 exposure and it lost nearly 80% activity under similar exposure.

The effect of NaCl on the activity of BGP has been illustrated in Table 5. The activity of immobilized BGP preparations was slightly enhanced in the presence of 0.2 M NaCl. E-BGP and SI-BGP showed 104% and 114% activity after 1 h incubation with 0.2 M NaCl. However, S-BGP exhibited a marginal loss of 6% of the original enzyme activity under similar incubation conditions.

3.9. Reusability of immobilized BGP

Reusability of two immobilized preparations of BGP has been shown in Fig. 4. After 7th repeated use the E-BGP retained 75% of the original activity, whereas SI-BGP showed 69% activity.

Table 4
Effect of $HgCl_2/CdCl_2$ on soluble and immobilized BGP

$HgCl_2/CdCl_2$ (mM)	Remaining activity (%)					
	$HgCl_2$			$CdCl_2$		
	S-BGP	SI-BGP	E-BGP	S-BGP	SI-BGP	E-BGP
0.01	72.43 ± 2.41	79.87* ± 1.76	90.17* ± 2.24	77.26 ± 1.39	85.16# ± 1.96	92.15# ± 1.57
0.02	69.14 ± 1.98	72.90* ± 3.28	85.93* ± 1.54	73.41 ± 1.68	80.43# ± 1.61	88.31# ± 2.74
0.04	64.43 ± 2.19	70.36* ± 1.92	82.42* ± 2.13	69.29 ± 0.86	73.98# ± 2.49	82.43# ± 2.32
0.06	60.35 ± 1.49	63.24* ± 2.16	76.25* ± 1.78	62.14 ± 0.51	69.56# ± 1.33	75.89# ± 1.65
0.08	56.25 ± 1.75	61.28* ± 1.24	73.78* ± 1.45	56.38 ± 1.44	63.24# ± 1.13	74.15# ± 1.94
0.10	53.12 ± 1.67	57.98* ± 1.83	71.35* ± 1.61	52.34 ± 1.96	59.18# ± 1.54	69.29# ± 1.37

Soluble and immobilized BGP (1.3 U) were incubated with $HgCl_2/CdCl_2$ (0.01–1.0 mM) in 100 mM sodium acetate buffer, pH 5.0 at 37 °C for 1 h. The activity of BGP without exposure to $HgCl_2/CdCl_2$ was taken as control (100%) for the calculation of remaining percent activity. Effect of various concentrations of metals on soluble and immobilized BGP was analyzed by one-way ANOVA*. Values (#$P < 0.05$) were statistically significant when SI-BGP and E-BGP were compared with S-BGP, with respect to $HgCl_2/CdCl_2$, respectively.

Table 5
Effect of Tween 20/NaCl on soluble and immobilized BGP

Tween 20 (%, v/v)	Remaining activity (%)			NaCl (M)	Remaining activity (%)		
	S-BGP	SI-BGP	E-BGP		S-BGP	SI-BGP	E-BGP
0.5	45.67 ± 3.14	70.37* ± 2.32	86.27* ± 1.44	0.1	91.78 ± 1.91	98.32# ± 1.57	109.55# ± 2.58
1.5	41.12 ± 1.45	63.56* ± 1.84	79.35* ± 2.98	0.2	94.16 ± 2.45	103.86# ± 2.56	114.38# ± 1.72
2.5	36.82 ± 2.15	56.87* ± 2.39	76.22* ± 1.13	0.4	100.27 ± 1.73	109.71# ± 1.92	128.73# ± 1.45
3.5	31.13 ± 1.98	52.36* ± 1.12	69.51* ± 1.30	0.6	102.65 ± 1.89	111.58# ± 1.65	132.41# ± 1.78
4.5	25.21 ± 1.76	42.28* ± 1.94	61.69* ± 1.47	0.8	106.52 ± 1.44	112.33# ± 1.36	135.13# ± 1.27
5.0	21.54 ± 1.37	39.51* ± 1.67	56.24* ± 1.39	0.10	110.64 ± 2.22	119.48# ± 2.76	142.93# ± 1.87

Soluble and immobilized BGP (1.3 U) were incubated with Tween 20 (0.5–5.0%, v/v)/NaCl (0.1–1.0 M) in 100 mM sodium acetate buffer, pH 5.0 at 37 °C for 1 h. The activity of BGP without exposure to Tween 20/NaCl was taken as control (100%) for the calculation of remaining percent activity. Effect of various concentrations of detergent and salt on soluble and immobilized BGP was analyzed by one-way ANOVA*. Values (#$P < 0.05$) were statistically significant when SI-BGP and E-BGP were compared with S-BGP, with respect to Tween 20/NaCl, respectively.

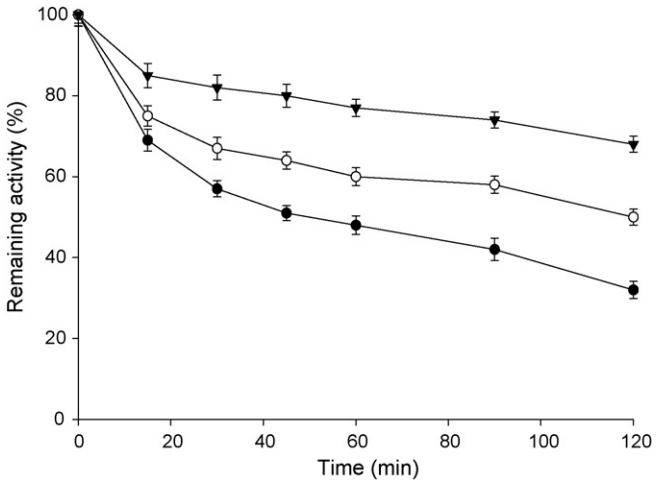

Fig. 3. Effect of 4.0 M urea on soluble and immobilized BGP. Soluble and immobilized BGP were incubated with 4.0 M urea in 100 mM sodium acetate buffer, pH 5.0 for various times. The aliquots of each preparation (1.3 U) were taken and assayed for the activity of peroxidase. Activity obtained without urea exposure was taken as control (100%) for the calculation of remaining percent activity. For symbols refer to Fig. 2a legend.

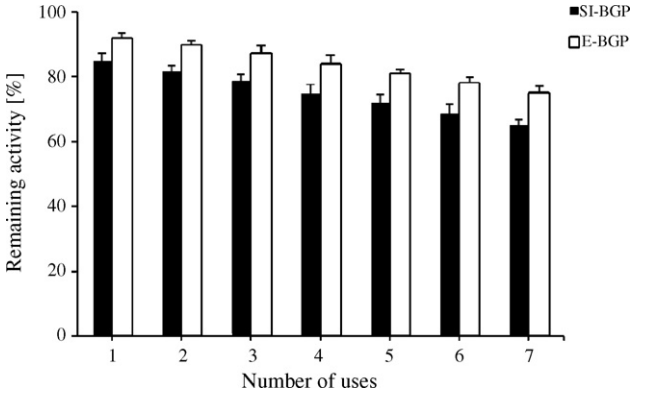

Fig. 4. Reusability of immobilized preparations of BGP. E-BGP and SI-BGP (1.3 U) were independently assayed in 100 mM sodium acetate buffer, pH 5.0. After each assay immobilized enzyme preparations were taken out and stored in assay buffer overnight till next use. This procedure was repeated for 7 consecutive days. The activity obtained on first day was considered as control (100%) for the calculation of remaining percent activity after repeated uses.

4. Discussion

Sodium alginate has been considered since a long time for the entrapment of enzymes due to its biocompatibility and processibility [13,21]. Calcium alginate entrapped enzyme preparations have one inherent limitation that the large molecular weight substrates or products cannot easily diffuse in and out of gel beads [7]. In order to circumvent this problem, the immobilization of enzymes on the surface of such beads would be a preferred choice. In this work an effort has been made to prepare a hybrid calcium alginate–starch gel beads which has successfully been employed both for entrapment of BGP and its immobilization on the surface of these beads after layering with Con A.

The immobilization of BGP on the surface of Con A layered calcium alginate–starch beads has presented a strategy to overcome the problem of diffusion limitation of the substrate/product and to increase the surface area of contact between enzyme and substrate so that such preparation could be exploited for the treatment of large volume of industrial waste. The enzyme activity expressed by SI-BGP was more than E-BGP due to the compact structure of E-BGP, which decreased the flexibility of enzyme as well as considerably limited diffusion of the substrate (Fig. 1, Table 1). It has already been reported that the enzyme immobilized on the surface of gel beads has minimum mass transfer constrain [22–24].

Immobilization of an enzyme to a support often limits its freedom to undergo drastic conformational changes and thus resulted in increased stability towards denaturants; organic solvents, thermal denaturation, inhibitors and urea (Figs. 2 and 3, Tables 2–5). However, some earlier workers have demonstrated that the stability of surface immobilized enzymes was significantly higher against pH, heat and proteolysis than the free enzyme [22]. Thus the enhanced resistance to the stability of immobilized BGP against denaturants offered a potential advantage for the application of such type of enzyme preparations in treatment of wastewater.

The pH-activity profiles of E-BGP and SI-BGP have the same pH-optima as that of S-BGP (Fig. 2a). However, entrapped and surface immobilized BGP preparations showed significant broadening in pH-activity profiles indicating a marked increased in stability in the buffers of varying pH. This predicts that the entrapment of enzyme in the gel beads provides a microenvironment for the enzyme, which might play an important role in the state of protonation of the protein molecules. However, the surface immobilized BGP provided stability due to multiple point attachment between enzyme and Con A. The crosslinking of BGP present on the Con A layered calcium alginate–starch beads by glutaraldehyde further increased stability.

Furthermore, immobilized peroxidase preparations retained their structure and remarkably high activity at elevated temperatures as compared to soluble peroxidase (Fig. 2b and c). These observations were in agreement with the findings of some earlier investigators [11,25,26]. It is well established that thermal exposure initiate unfolding of protein molecules which is followed by irreversible changes due to aggregation and formation of scrambled structures which takes place more in soluble form as compared to the immobilized enzyme [27]. However, E-BGP and SI-BGP were

superior in thermal stability as compared to S-BGP and even to the stability attained by turnip peroxidase entrapped in calcium alginate–pectin beads under similar conditions [11].

SI-BGP and E-BGP were markedly more stable to the denaturation induced by 4.0 M urea (Fig. 3). E-BGP exhibited greater stability as compared to calcium alginate–pectin entrapped turnip peroxidase under similar exposure conditions [11]. Although, the action mechanism of urea on the protein structure has not yet been completely understood some earlier workers have proposed that the presence of carbohydrate moieties in enzymes increased their resistance to inactivation induced by urea [28]

E-BGP and SI-BGP were more resistant to the inactivation mediated by water-miscible organic solvents; dioxane and *n*-propanol (Table 2). It has already been reported that the stabilization of enzyme insoluble complexes against various organic solvents which could possibly be due to low water requirement or enhanced rigidity to its structure [29]. More recently in our laboratory it has been shown that enzymes immobilized on protein supports are quite resistant to denaturation induced by various water-miscible organic solvents [36].

The immobilized peroxidase preparations showed more than 80% of their original enzyme activity in the presence of 0.1 mM sodium azide and EDTA (Table 3). A number of studies have already been performed on the inhibitory effect of such compounds on horseradish peroxidase [30]. Sodium azide has been shown to be a potent inhibitor of many hemeprotein-catalyzed reactions [31]. Peroxidase in the presence of sodium azide and H_2O_2 mediates one electron oxidation of azide ions and forming azidyl free radicals, which bind covalently to the heme moiety thus inhibiting the enzyme activity [32].

Metals induce conformational changes in enzymes, however peroxidases remain active even in the presence of a number of metal ions, as a part of their detoxifying role, BGP has exhibited more resistant to inactivation induced by heavy metals. Some recent reports have also indicated that horseradish peroxidase was remarkably inhibited by heavy metal ions [33,34]. However, in this study the strength of inhibition of immobilized BGP by heavy metal ions was quite low as compared to free enzyme (Table 4). The stability of immobilized BGP against several metal compounds showed that such preparations could be exploited to treat aromatic pollutants even in the presence of heavy metals.

Immobilized BGP was significantly more resistant to denaturation induced by Tween 20 as compared to its soluble counterpart (Table 5). These observations suggested that the presence of lower concentrations of detergents was not harmful to the enzyme's native conformation. Some workers have reported an increase in the activity of soybean peroxidase and BGP when these enzymes were exposed to low concentrations of SDS, Tween 20 and Triton X 100 [35,36].

Enzyme reuse provides a number of cost effective advantages that are often an essential prerequisite for establishing an economically viable enzyme-catalyzed process [7]. However, E-BGP and SI-BGP retained more than 60% of their original activity even after its seventh successive uses (Fig. 4). The activity loss during repeated use might be due to the inhibition of enzyme by product or by leaching of enzyme from the gel bead or due to damage of the beads [10,37].

5. Conclusions

The results presented in the present work showed that E-BGP was more stable as compared to SI-BGP against various types of denaturants. However, SI-BGP retained more activity in its immobilized form and this perpetration has no problem of substrate and product diffusion. SI-BGP can also occupy a large surface area and thus it can contact with more substrate. These beads used in a reactor would not affect the flow rate of the column. In view of these advantages offered by surface immobilized BGP preparation, we can suggest that this immobilized preparation would be most suitable for the treatment of huge volume of effluents.

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