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Biocatalytic activity of immobilized pointed gourd (*Trichosanthes dioica*) peroxidase–concanavalin A complex on calcium alginate pectin gel

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ABSTRACT

Salt fractionated pointed gourd (*Trichosanthes dioica*) peroxidase–concanavalin A (PGP–Con A) complex expressed 79% of original peroxidase activity which decreased on entrapment into calcium alginate–pectin gel. Immobilized PGP–Con A complex retained 82.7% activity even at 60 °C which was achieved at pH 4.0. Urea treatment resulted in activity loss by ~40%. With dioxane, immobilized PGP exhibited an activity of over 55% whereas an increasing concentration of dimethylformide resulted in decline. Activity of immobilized PGP–Con A was dependent on nature and concentration of detergent. Michaelis–Menten constant (K_m) and V_{max} for entrapped PGP–Con A complex was 0.08 mM and 15.7 mM/min, respectively.

This study shows the efficacy, durability and sustainability of immobilized catalytic system which could be efficiently used for the removal of synthetic dyes from industrial effluents.

1. Introduction

Peroxidases are a family of isozymes found in all plants. They are heme-containing monomeric glycoproteins that utilize either H_2O_2 or O_2 to oxidize a wide variety of molecules. These important enzymes not only have significant biological implications but find multifarious applications including use in detoxification, dye decolorization and removal of various toxic organic pollutants which contaminate water and industrial effluents [1–4]. Novel research in the area of enzyme technology has provided significant clues and strategies that facilitate using enzymes optimally at large scale by entrapping and immobilizing [5,6]. Although enzymes entrapped in porous polymeric matrices have inherent limitations of undergoing leakage and consequently are lost, however, such leakages can be minimized by controlling the pore dimensions. Alternatively, entrapping cross-linked or pre-immobilized enzyme preparations could be better and pragmatic options [7,8].

Immobilization of enzymes is a tricky and articulated approach as involvement of key amino acids must be avoided to prevent loss of enzymatic activity. In case of glycoenzymes, glycosyl moieties can safely be used in immobilizations as they do not participate in catalysis. Lectins are proteins which recognize and interact with exposed carbohydrate moieties of glycoproteins and glycoenzymes. These proteins are useful in characterizing glycoproteins and certain glycoenzymes have been immobilized on concanavalin A (Con A) affinity matrices or as Con A–glycoenzyme complexes [9–11].

The current study demonstrates a simple, inexpensive and high yield procedure for immobilization of glycosylated *Trichosanthes dioica* peroxidase. *T. dioica* popularly known as pointed gourd is widely planted in tropical areas and consumed as vegetables. Salt fractionated pointed gourd peroxidase (PGP) immobilized with lectin Con A as insoluble PGP–Con A were entrapped into calcium alginate–pectin beads. A comparative study on the biocatalytic activity of immobilized form of PGP (PGP–Con A complex and PGP–Con A–calcium alginate–pectin complex) under different experimental conditions has been presented for using such enzymes effectively in waste water treatment.

2. Materials and methods

2.1. Chemicals

Sodium alginate, bovine serum albumin, concanavalin A, *o*-dianisidine HCl was procured from Sigma Chemical Co. (St. Louis, MO, USA). Dioxane, dimethylformide (DMF) and pectin were

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obtained from SRL Chemicals, Mumbai, India. All other chemicals were of analytical grade. The pointed gourds were procured from Narendra Dev University of Agriculture and Technology, Faizabad, U.P., India. The samples were aseptically transferred into sterilized plastic bags.

2.2. Partial purification of PGP proteins by ammonium sulphate

Pointed gourd (300 g) was homogenized in 600 ml of 100 mM sodium acetate buffer, pH 5.6. The homogenate was filtered through multi-layers of cheese cloth and centrifuged at 10,000 × *g* on a Remi C-24 cooling centrifuge for 25 min at 4 °C. The supernatant was used for salt fractionation by adding 10–80% (w/v) $(NH_4)_2SO_4$. The proteins were precipitated by continuously stirring at 4 °C overnight. The precipitate was centrifuged at 10,000 × *g* on a Remi C-24 cooling centrifuge, dissolved in 100 mM sodium acetate buffer, pH 5.6 and dialyzed against the assay buffer (100 mM glycine HCl buffer, pH 4.0) [12]. This preparation of protein was aliquoted and stored for further use.

2.3. Protein estimation

Protein concentration was estimated using BSA as a standard protein and following the procedure of Lowry et al. [13].

2.4. Preparation of insoluble PGP–Con A complex

The peroxidase proteins (1100 U) were mixed with an increasing concentration of Con A (0.1–1.0 ml) in a series of tubes. Final volume of each tube was adjusted to 5 ml with 100 mM phosphate buffer (pH 5.6). The reaction mixtures were incubated overnight at 37 °C. The precipitates were collected after centrifugation at 3000 × *g* for 20 min at room temperature and washed twice with the same buffer. Finally precipitates were suspended in 2 ml assay buffer and each precipitate was analyzed for enzyme activity. The precipitate (PGP–Con A complex) exhibiting maximum activity was used for further studies.

2.5. Entrapment of PGP–Con A complex in calcium alginate–pectin beads

PGP–Con A complex (1250 U) was mixed with sodium alginate (2.5%) and pectin (2.5%) in 10 ml of 100 mM sodium acetate buffer (pH 5.6). Using a syringe the mixture was slowly extruded as droplets. Further, beads were gently stirred for 2 h in calcium chloride solution, washed and stored in 100 mM sodium acetate buffer (pH 5.6) at 4 °C for further use.

2.6. Measurement of peroxidase activity and effect of enzyme loading

Peroxidase activity was determined by measuring a change in optical density (A_{460} nm) at 37 °C of initial rate of oxidation of 6.0 mM *o*-dianisidine HCl in presence of 18.0 mM H_2O_2 in 0.1 M sodium acetate buffer (pH 5.6) for 15 min. Immobilized enzyme preparation was continuously agitated for entire duration of assay [14].

One unit (1.0 U) of enzyme activity is the amount of enzyme protein that catalyzes oxidation of 1.0 μmol of *o*-dianisidine HCl per minute at 37 °C into colored product (ε_m at 460 nm = 30,000 M/L). An increasing concentration of enzyme (110–1000 U) was mixed to calcium alginate–pectin gel in a series of tubes. Expression of loaded enzyme was monitored by assaying the peroxidase activity.

2.7. Effect of temperature and pH on soluble and immobilized PGP

Soluble PGP, PGP–Con A complex and calcium alginate–pectin entrapped PGP–Con A complex (1.20 U) were incubated at 60 °C in 100 mM sodium acetate buffer (pH 5.6) for varying time interval. Aliquots of each preparation were removed at indicated time intervals and activity was measured. The activity obtained without incubation at 60 °C was taken as control (100%). Catalytic activity of soluble, PGP–Con A complex and calcium alginate–pectin entrapped PGP–Con A complex were measured at varying temperatures (20–90 °C) in 100 mM sodium acetate buffer (pH 5.6) for 15 min.

Similarly, the activity of soluble PGP, PGP–Con A complex and calcium alginate–pectin entrapped PGP–Con A complex (1.20 U) was measured in buffers (100 mM each) of varying pHs 2–9.

2.8. Effect of urea, organic solvents and detergents on soluble and immobilized PGP

Soluble PGP, PGP–Con A complex and calcium alginate–pectin entrapped PGP–Con A preparations (1.20 U) were incubated in 4.0 M urea dissolved in 100 mM sodium acetate buffer (pH 5.6). Aliquots were removed at various time intervals and activity was determined. Similarly, soluble PGP, PGP–Con A complex and calcium alginate–pectin entrapped PGP–Con A (1.20 U) were incubated with 10–80% (v/v) of water-miscible organic solvents; dioxane and DMF prepared in 100 mM sodium acetate buffer (pH 5.6) at 37 °C for 1 h. Soluble and immobilized PGP (1.20 U) were incubated with increasing concentrations of sodium dodecyl sulphate (SDS) and non-ionic detergents; Triton X-100 and Tween-20 (0.5–6.0%, v/v) prepared in 100 mM sodium acetate buffer (pH 5.6) at 37 °C for 1 h. Catalytic activity was monitored at all the indicated detergent concentrations. Activity of enzyme without exposures to urea, organic solvents and detergent was taken as control (100%).

2.9. Effect of sodium azide and mercuric chloride on soluble and immobilized PGP

The inhibitory effect of sodium azide (0.02–0.1 mM) on PGP preparations was examined. Soluble and immobilized PGP (1.20 U) were independently preincubated for 1 h with the inhibitor in 50 mM sodium acetate buffer, pH 5.6 at 37 °C. The activity of enzyme without exposure to sodium azide was considered as control (100%) for the calculation of remaining percent activity.

Soluble and immobilized PGP (1.20 U) were incubated independently with $HgCl_2$ (0.05–6.0 mM) in 100 mM sodium acetate buffer, pH 5.6, at 37 °C for 1 h. The activity of enzyme without exposure to $HgCl_2$ was taken as control (100%) for the calculation of remaining percent activity.

2.10. Determination of K_m and V_{max} of soluble and immobilized T. dioica peroxidase with respect to o-dianisidine HCl

The initial enzymatic activity was measured at various concentrations of *o*-dianisidine HCl. The solutions having different concentrations of *o*-dianisidine HCl ranging from 0.002 to 1.2 mM were treated with PGP (1.20 U) in the presence of 18.0 mM H_2O_2 in 100 mM sodium acetate buffer, pH 5.6, at 37 °C for 15 min.

3. Results

Due to sudden increase in the level of phenolic compounds and dyes contamination in waste water emphasis on cheaper, sustainable and eco-friendly approaches of dye color removal and degradation of phenolic compounds is gathering much attention. Enzymes are environmental friendly and capable of specifically

Table 1
Activities of PGP immobilized with lectin Con A and calcium–alginate pectin gel.

Type of immobilization	Original activity	Expressed activity
PGP–Con A complex	100%	79%
PGP–Con A complex entrapped on calcium–alginate pectin gel	100%	56%

reducing hazardous wastes and therefore key to new processes. Immobilizing enzymes directly from crude homogenate is a relatively much cheaper approach [15]. Although the immobilized form of bio-molecules is commercially important, very few protocols are available for such preparations. Calcium alginate mediated entrapment is a simple and effective approach in using enzymes in crude extract for detoxification and degradation of phenolic compounds in waste water [10,16].

3.1. Purification of PGP by ammonium sulphate

The crude extract of pointed gourd exhibited an initial specific activity of 96 U/mg of protein. Peroxidase was partially purified by ammonium sulphate precipitation and specific activity of preparation was increased 3.5 fold over crude enzyme. This enzyme preparation was used for direct immobilization as enzyme–Con A complex.

3.2. Preparation of PGP–Con A complex

Peroxidases from *T. dioica* are usually glycosylated proteins. These enzymes in soluble states may leach out of beads over prolonged retention or repeated use. Thus to prevent the leakage of enzymes from porous gel beads, these enzyme molecules were complexed with lectin concanavalin A. The insoluble PGP–Con A complex was subsequently entrapped into calcium alginate–pectin gel. With 0.2 ml of Con A the PGP–Con A complex expressed an activity of 79% which on entrapment into calcium alginate–pectin gel resulted in further decrease of peroxidase activity (Table 1).

3.3. Entrapment of PGP–Con A complex in calcium alginate–pectin beads

Immobilization by means of entrapment is a simple and effective technique. Partially purified PGP precipitated with Con A was used for direct immobilization onto calcium alginate pectin beads. Entrapped PGP–Con A peroxidase complex retained only 56% of the original activity (Table 1). Further, the effect of enzyme loading on entrapped activity was evaluated by entrapping increasing concentration of enzyme. Optimum concentration (418 U/ml) was sufficient for maximum expression of peroxidase activity by entrapped preparation.

3.4. Effect of temperature and pH on soluble and immobilized PGP

Maximum activity of both the soluble and immobilized peroxidase preparations was achieved at 40 °C (Fig. 1a). Interestingly, PGP–Con A and entrapped PGP–Con A complex retained greater fraction of catalytic activity at higher temperatures as compared to its soluble counterpart.

PGP in the soluble state retained 48.4% of its initial activity after 2 h incubation at 60 °C whereas PGP–Con A complex preparation retained about 62.6% original activity under identical incubation conditions. The entrapped PGP–Con A complex expressed 82.7% activity at 60 °C in an incubation period of 2 h (Fig. 1b).

Effect of pH on activity of soluble PGP, PGP–Con A and entrapped PGP–Con A was evaluated by incubating these preparations in the buffers of varying pH values (2.0–9.0). Optimum activity was in

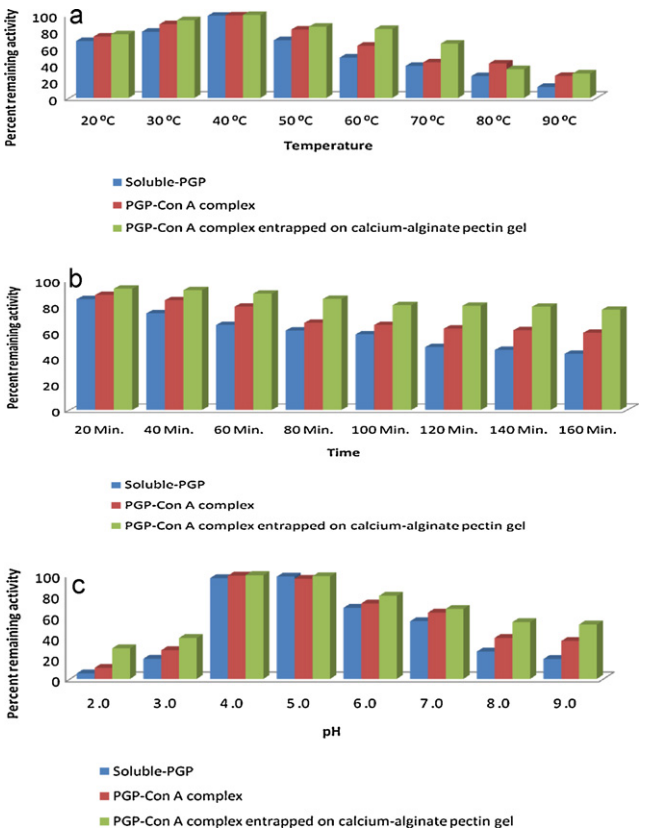

Fig. 1. Effect of (a) temperature, (b) time and (c) pH on catalytic activity of soluble and immobilized PGP (PGP–Con A complex and entrapped PGP–Con A complex).

the pH range of 4.0–5.0 (Fig. 1c). The soluble PGP fraction exhibited maximum activity (98.2%) at pH 5.0 whereas progressive loss of activity was recorded in alkaline medium. On the contrary the PGP–Con A complex and entrapped PGP–Con A performed optimally at pH 4.0 (99.1% and 99.6% respectively). These immobilized states of PGP retained profound activity in alkaline medium as compared to soluble PGP. Moreover, the entrapped PGP–Con A retained more peroxidase activity than PGP–Con A complex in alkaline range pH 7 to pH 9.

3.5. Effect of urea on soluble and immobilized PGP

Soluble and immobilized PGP preparations were incubated with 4.0 M urea for varying times. Soluble PGP lost nearly 80% of initial activity on 2 h treatment whereas activity of Con A–PGP complex diminished by ~40%. Although, with time there was progressive decline in the peroxidase activity, but the PGP–Con A complex retained much higher activity as against soluble PGP (Fig. 2a). In case of entrapped PGP–Con A complex the activity retention was profound with the progressive increase in incubation time. The loss was in the range of ~12% to ~16% in duration of 20–160 min.

3.6. Effect of organic solvents on soluble and immobilized PGP

The activity of soluble and immobilized PGP was experimented with increasing concentrations of dioxane (10–80%, v/v). At lower concentration of dioxane (10–30%, v/v) the soluble and immobilized PGP exhibited over 55% of peroxidase activity, nevertheless activities of immobilized counterparts was sufficiently high (62.9% and 75.6% for PGP–Con A complex and entrapped PGP–Con A complex, respectively) on treatment for 2 h. On exposure to dioxane (60%, v/v), soluble enzyme retained only 29.4% of its original

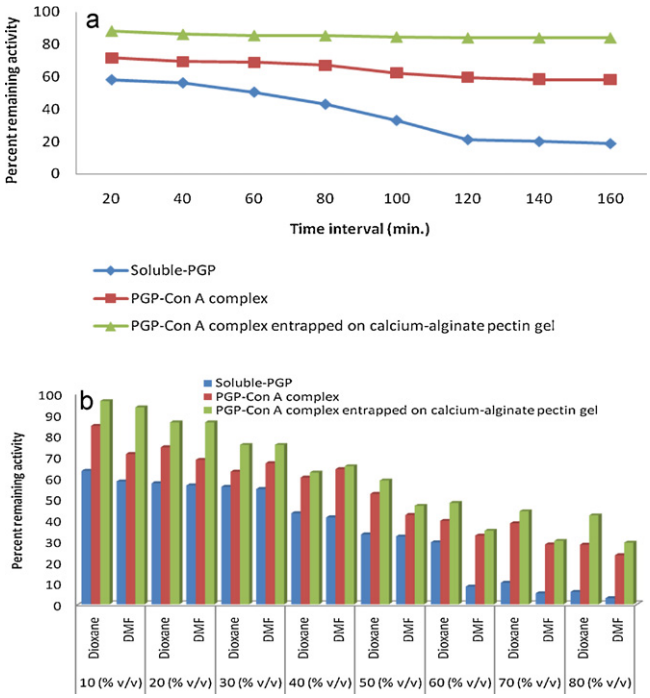

Fig. 2. Effect of (a) urea and (b) dioxane and DMF on catalytic activity of soluble and immobilized PGP (PGP–Con A complex and entrapped PGP–Con A complex).

activity while PGP–Con A complex and entrapped PGP–Con A retained nearly 39.6% and 48.1% of their initial activity, respectively. Interestingly, at 80% (v/v) dioxane, soluble PGP lost ~94% of peroxidase activity, whereas immobilized PGP exhibited 28.3% and 42.2% activity (Fig. 2b).

Incubation of soluble and immobilized PGP with increasing concentrations of DMF (10–80%, v/v) resulted in decrease of the enzyme activity. At lower concentration of DMF (10–30%) the soluble and immobilized PGP exhibited sufficiently high activity (>54%) and at 30% (v/v) concentration of DMF, activities expressed was 54.7% for soluble, 66.9% and 75.6% for PGP–Con A and entrapped PGP–Con A respectively. On exposure to DMF (60%, v/v) for 2 h, soluble PGP lost nearly 92% of its initial activity while Con A–PGP complex and entrapped Con A–PGP retained nearly 32.6% and 34.9% of original activity, respectively (Fig. 2b). On increasing the concentration of DMF further, progressive decline in activities were observed and at 80% (v/v) DMF the soluble PGP lost ~97% of activity whereas immobilized PGP exhibited 23.3% (PGP–Con A complex) and 29.2% (entrapped PGP–Con A) activities.

3.7. Effect of detergents on soluble and immobilized PGP

Several types of denaturants contaminate wastewater from various elimination sites including detergents that can strongly denature enzymes used for treatment of polluted wastewater. Soluble and immobilized PGP were treated with increasing concentrations of Triton X-100, Tween-20 and SDS for 1 h at 37 °C. There was a progressive decline in the activities of soluble and immobilized PGPs with increasing concentration of both the non-ionic and ionic detergent. The soluble PGP was adversely affected at 6.0% (v/v) of SDS as compared to Triton X-100 and Tween-20. The activity exhibited by soluble PGP was only 4.5% with 6% SDS whereas 22.9% and 25.9% activity was retained on treatment with Triton X-100 and Tween-20 respectively. Entrapped PGP–Con A complex retained 45.7%, 42.8% and 36.5% of its initial activity in the presence of 6.0% (v/v) SDS, Triton X-100 and Tween-20, respectively. However, PGP–Con A complex exhibited only 26.7%, 26.8% and 23.9% of

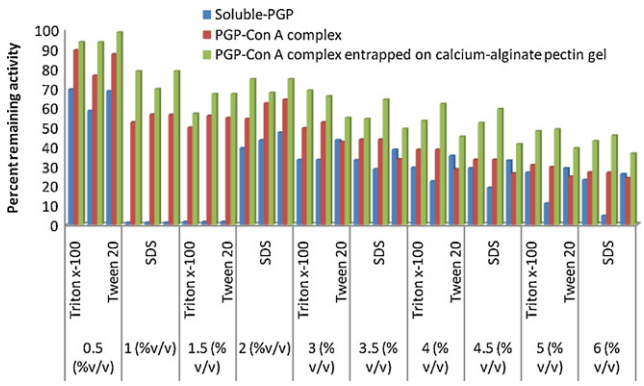

Fig. 3. Effect of detergents (SDS, Triton X-100 and Tween-20) on catalytic activity of soluble and immobilized PGP (PGP–Con A complex and entrapped PGP–Con A complex).

initial activity in presence of 6.0% SDS, Triton X-100 and Tween-20, respectively (Fig. 3).

3.8. Effect of sodium azide, EDTA and $HgCl_2$

The effect of sodium azide on the activity of soluble and immobilized PGP is shown in Fig. 4a. Soluble PGP lost 58.6% of its initial activity while entrapped PGP–Con A complex retained 88.3% of its original activity after 1 h exposure to 0.02 mM sodium azide. However, PGP–Con A complex retained an activity of 73.5% which was better than soluble PGP nevertheless lower than entrapped PGP–Con A complex. With progressive increase in concentration of sodium azide to 1.5 mM concentration there was decrease in enzymatic activities of soluble and immobilized PGP. However, the decrease in percent activity was highest in case of soluble PGP (85.7% at 1.5 mM of sodium azide) whereas the PGP–Con A complex and entrapped PGP–Con A retained 35.4% and 45.3% peroxidase activities, respectively. The effect on soluble and immobilized PGP was also studied with metal ion chelator EDTA and at 25 mM concentration and there was no remarkable effect on their activities.

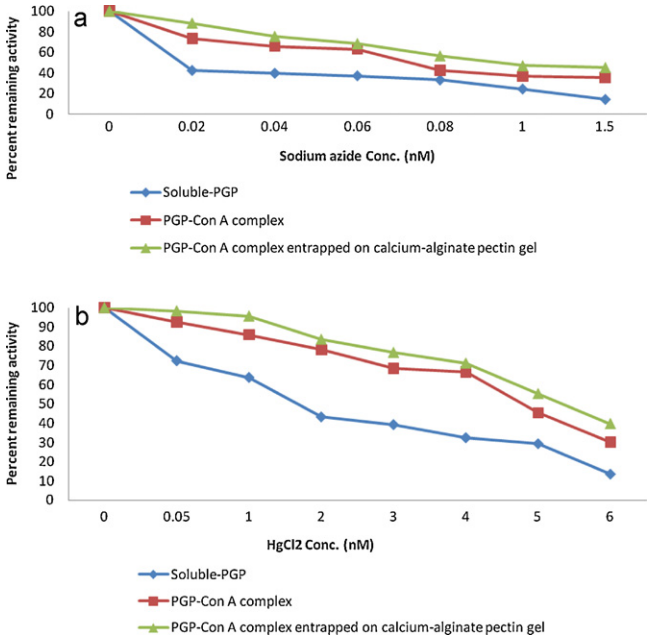

Fig. 4. Effect of (a) sodium azide and (b) $HgCl_2$ on catalytic activity of soluble and immobilized PGP (PGP–Con A complex and entrapped PGP–Con A complex).

Table 2
Properties of soluble and immobilized PGP.

Enzyme property	Enzyme preparation	
	Soluble PGP	Immobilized PGP
pH optima	4.0	5.0
Temperature optima (°C)	40	40
K_m (mM)	0.07	0.08
V_{max} (mM)	21.4	15.7

The peroxidase activity was concentration dependent in the presence of $HgCl_2$. At low concentrations of 1.0 mM immobilized PGP exhibited peroxidase activities of 95.6% (entrapped PGP–Con A complex) and 85.9% (PGP–Con A complex) which was considerably higher than soluble PGP (63.7%). Upon increasing the concentration of $HgCl_2$ further to 4.0 mM soluble PGP lost 67.6% of initial activity whereas immobilized states of PGP still retained over 65% of activities. However, at 6.0 mM $HgCl_2$ soluble PGP lost 86.7% whereas immobilized states of PGP still exhibited above 30% of peroxidase activity (Fig. 4b).

3.9. K_m and V_{max} of soluble and immobilized PGP

Experiments were performed using different concentrations of *o*-dianisidine HCl to determine the kinetic parameters of soluble and immobilized PGP. The plot of initial enzyme activity *versus* different concentrations of *o*-dianisidine HCl for both the enzyme preparations followed a hyperbolic pattern as expected according to the Michaelis–Menten kinetics (data not given). The Lineweaver–Burk plots of soluble and immobilized PGP were also found to be linear. The values of Michaelis–Menten constant, K_m, for soluble and entrapped PGP–Con A complex was 0.07 and 0.08 mM, respectively (Table 2). The V_{max} values for soluble and immobilized PGP were found to be 21.4 and 15.7 mM/min, respectively.

4. Discussion

Immobilization of enzymes by adsorption is an effective procedure for binding enzymes directly from partially purified preparations or even from crude homogenates [7,17]. Calcium alginate mediated entrapment is a simple, economical, effective and sustainable approach for using enzymes either from crude extract or directly from partially purified preparations for detoxification and degradation of phenolic compounds in waste water [10,16]. Although in this study commercial Con A has been used, but the process can be made cost effective by using simple techniques to isolate Con A from Jack Bean extract and using such preparations to improve immobilization yields of these glycosylated peroxidases.

4.1. Peroxidase activity upon immobilization of PGP

This study deals with the immobilization of pointed gourd peroxidase using concanavalin A as a lectin and entrapping on to calcium alginate gel. Major peroxidases from pointed gourd are glycosylated proteins and therefore precipitation by Con A was feasible. Generally, the soluble enzymes have inherent limitations of leaching out of beads on long standing or use [17,18]. Therefore, to prevent leaching of such enzymes out of porous gel beads, insoluble PGP–Con A complex was prepared by using commercial lectin Con A and salt fractionated PGP proteins, which was subsequently entrapped into calcium alginate–pectin gel. Using 0.2 ml of Con A maximum precipitation of peroxidase activity that could be achieved was 79% (Table 1). Thereafter, when Con A–PGP complex was entrapped into calcium alginate–pectin gel it resulted in further loss of peroxidase activity to 56%.

Cross-linked enzymes or pre-immobilized enzymes that could remain inside polymeric matrices for longer duration than soluble enzymes provide higher mechanical and operational stability to enzymes [7,17,8]. It indicated that enzymes with high molecular mass could stay for longer period inside polymeric matrix. Pre-immobilization increases molecular dimensioning of the enzyme and thus prevents its leaching from alginate beads.

4.2. Internal operational parameters of immobilized PGP

The resistance/stability of enzyme to high temperatures was greatly increased by immobilization. The enzyme expressed more than 65% peroxidase activity at reasonably high temperatures of 70 °C (Fig. 1a). Calcium alginate–pectin entrapment of PGP–Con A complex contributed strongly towards retention of its molecular structure, thus its stability and consequently high activity was observed at elevated temperatures. Such enzyme preparation could certainly be exploited where operational temperatures are relatively high. Peroxidases being glycoprotein in nature, the lectin adsorption contributed towards improvement in thermal stability of calcium alginate–pectin entrapped PGP–Con A preparation possibly due to multipoint attachment of peroxidases with Con A. This enhancement in thermal stability is possibly due to formation of several linkages between the enzyme molecules and support matrix [9,19].

The immobilized PGP preparations exhibited significant broadening in pH activity profiles indicating a marked increase in stability (Fig. 1c). This broadening in pH-activity profiles remarkably increased in case of entrapped PGP–Con A followed by PGP–Con A complex, predicting that entrapment of enzymes in gel beads provides a microenvironment for enzyme, which may play an important role in the state of protonation/deprotonation of protein molecules [17]. Formation of PGP–Con A complex promotes retention of molecular structure and consequently confers additional resistance to enzyme against extreme conditions of pH [16,20].

4.3. Effect of urea, organic solvents and detergents on immobilized PGP

Entrapped PGP–Con A preparation was remarkably resistant to urea-induced inactivation and retained nearly 84.1% of its initial activity even after 2 h incubation (Fig. 2a). On the contrary soluble PGP lost 80% of its activity under similar conditions. Urea (4.0 M) is a strong denaturant of some proteins and it irreversibly denatures soluble PGP [17,21]. Although, action mechanism of urea on protein structure is not yet completely understood, several findings have indicated that protein unfolding by direct interaction of urea molecule with a peptide backbone via non-covalent interactions contributes to maintenance of protein conformation [16,17]. Complexing of glycoenzymes with Con A has been reported in an enhancement of their resistance to denaturation mediated by urea [22]. Thus, these observations indicate that entrapment protected PGP–Con A complex from urea induced inactivation.

Enzymes exploited for the treatment of wastewaters containing aromatic pollutants would be affected by the presence of water-miscible organic solvents. Therefore, we have investigated the stability of PGP preparations against some water miscible organic solvents. It was observed that entrapped PGP–Con A complex retained remarkably high stabilization against inactivation caused by dioxane and DMF as compared to soluble PGP and PGP–Con A complex (Fig. 2b). Earlier reports also suggested that bioaffinity bound enzymes were significantly more stable perhaps due to decrease in flexibility and increased molecular rigidity against exposure to water miscible organic solvents [16].

Our study also revealed that entrapped PGP–Con A complex was markedly more stable to inactivation induced by detergents (SDS, Triton X-100 and Tween-20) (Fig. 3). Entrapped PGP–Con A could work quite efficiently in presence of contaminants like soaps and detergents. Immobilized peroxidases are reported to be significantly stabilized against denaturation induced by some commonly used detergents [16,17].

It has already been reported that immobilization of enzymes by multipoint attachment protects them from denaturation mediated by organic solvents [23]. Some other workers have also described that the stabilization of immobilized enzymes against various forms of water-miscible organic solvents could possibly be due to low water requirement or enhanced rigidity of the enzyme structure [24]. Enzymatic catalysis in organic solvents is possible if the organic solvent does not substantially disturb the active site conformation [25].

4.4. Effect of sodium azide, EDTA and heavy metal on immobilized PGP

Sodium azide, an enzyme inhibitor was found to inhibit PGP strongly (Fig. 4a). On the contrary, ethylenediamine tetra-acetic acid showed no significant effect on the activity of soluble and immobilized PGP even when its concentration was raised 30 mM (data not given). A number of studies have already been performed on the inhibitory effect of such compounds on horseradish peroxidase where sodium azide has been shown to be a potent inhibitor of many heme protein-catalyzed reactions [26,27]. Peroxidase in the presence of sodium azide and H_2O_2 mediates one electron oxidation of azide ions forming azidyl free radicals which bind covalently to the heme moiety of peroxidase, thus inhibiting the enzyme activity [28]. EDTA did not have any significant effect on the activity of PGP and such an observation on enzyme activity has already been reported [29].

The chemical contamination of water by a wide range of toxic derivatives, particularly, heavy metals are a serious environmental problem owing to their potential human toxicity. In view of their presence in wastewater, it became necessary to evaluate the effect of some heavy metals on the activity of PGP. Our results revealed that PGP exhibited more resistance to heavy metal induced inhibition; a concentration-dependent gradual inhibition of PGP activity by $HgCl_2$ was observed (Fig. 4b). Some recent reports indicated that horseradish peroxidase was remarkably inhibited by heavy metal ions [30,31]. However, the inhibition of immobilized PGP by $HgCl_2$ was quite low as compared to the soluble enzyme. Although, 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. The effect of different metal ions on the activity of different enzymes is related to their affinity to different functional groups present in the enzymatic structure [32,33]. The stability of immobilized PGP against several metal compounds showed that such enzyme preparations could be exploited for the treatment of aromatic pollutants even in the presence of heavy metals.

4.5. V_{max} and Michaelis–Menten constant (K_m) of immobilized PGP

V_{max} and Michaelis–Menten constant (K_m) for free and immobilized PGP were found from the Lineweaver–Burk plot. Although K_m values of soluble and immobilized PGP were close to each other, V_{max} of soluble enzyme was found to be more than immobilized PGP (Table 2). This observation suggested that immobilization of PGP by entrapment using calcium alginate pectin slightly altered the accessibility for the substrate whereas the conformational change in the enzyme was almost unaffected. Glutaraldehyde crosslinking enhances the structural rigidity of protein and also maintains the native structure [34]. Immobilization by entrapment serves similar functions due to which the PGP becomes more resistant to environment.

The decrease in V_{max} observed in the case of immobilized enzyme preparation indicated that since the entrapment of PGP–Con A complex is non-specific, masking of certain amino acids at or near the active site contributed towards lesser formation of enzyme–substrate complex. It is well documented that the K_m values of several immobilized enzymes were either unaltered or exhibited minor alteration as compared to those of their respective soluble counterparts [35,36]. On the basis of results obtained in the present work, it can be concluded that the stability offered by immobilized PGP against various denaturants suggested that this preparation could successfully be employed in reactors for the treatment of effluents containing phenolic and other aromatic pollutants.

5. Conclusions

The stability exhibited by calcium alginate–pectin entrapped Con A–PGP preparation was significantly higher against various physical and chemical denaturants compared to soluble PGP and Con A–PGP complex. Thus, immobilized PGP preparations could be exploited for developing bioreactors for the treatment of phenolic and other aromatic pollutants including synthetic dyes present in industrial effluents.

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