

Studies on calcium alginate-pectin gel entrapped concanavalin A-bitter gourd (*Momordica charantia*) peroxidase complex

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Ammonium sulfate fractionated proteins of bitter gourd (*Momordica charantia*) were precipitated using jack bean extract as a source of concanavalin A. Concanavalin A-bitter gourd peroxidase retained nearly 86% original activity. This complex was entrapped into calcium alginate-pectin gel. Entrapped concanavalin A-peroxidase complex retained 51% original activity. Soluble and immobilized peroxidase preparations exhibited maximum activity at 40°C and pH 5.5. Concanavalin A-peroxidase complex and entrapped concanavalin A-enzyme complex retained greater fraction of catalytic activity at higher temperatures as compared to soluble form and also showed more broadening in pH-activity profiles, indicating a marked increase in stability. Concanavalin A-bitter gourd peroxidase and calcium alginate-pectin entrapped concanavalin A-bitter gourd peroxidase preparations were more stable as compared to soluble counterpart against denaturation induced by heat, pH, urea, organic solvents and detergents.

Key words: Alginate, Bitter gourd, Concanavalin A, Entrapped, Pectin, Peroxidase

Introduction

Peroxidases (E.C.1.11.1.7.) are ubiquitous heme containing oxidoreductases, which utilize hydrogen peroxide (H₂O₂) for oxidation of a wide range of substrates¹. Apart from biological role, peroxidases have been found effective in analytical, clinical, biotechnological, industrial and environmental applications^{2,3}. Peroxidases are being used in detoxification, decolorization and removal of various organic contaminants from polluted water/industrial effluents^{4,5}.

However, use of soluble enzymes is limited due to end-product inhibition, high cost, instability, non-reusability and difficult recovery⁶. Intensive research in enzyme technology has provided approaches that facilitate their practical application at large scale including biosensors⁷ and immobilized enzymes^{5,6,8}. There are reports^{9,10} about leakage of enzymes entrapped into porous polymeric matrices. A number of attempts have been made to increase the molecular dimensions of enzymes prior to their entrapment. The leakage could be avoided by entrapping cross-linked or pre-

immobilized enzyme preparations^{11,12}. Immobilization of enzymes through their amino acid side chain groups sometimes results in the loss of enzyme activity. However, an alternative strategy has been suggested for immobilization of glycoenzymes via their glycosyl moieties. Since carbohydrate parts of enzymes do not participate in catalysis, therefore, immobilization of such enzymes via glycosyl moieties is quite safe. Lectins, carbohydrate-binding proteins, interact specifically with glycoproteins/glycoenzymes. Glycoenzymes have been successfully immobilized on concanavalin A (Con A) bound supports or immobilized as Con A-glycoenzyme complexes¹³⁻¹⁵.

This study presents an inexpensive, simple and high yield procedure for immobilization of glycosylated bitter gourd (*Momordica charantia* L.) peroxidase (BGP). Insoluble Con A-BGP complex, prepared by salt fractionated bitter gourd proteins and jack bean extract, was entrapped into calcium alginate-pectin beads. In a comparative stability study of soluble BGP, Con A-BGP complex and entrapped Con A-BGP, calcium alginate-pectin entrapped Con A-BGP preparation exhibited a high yield of immobilization and stabilization against various forms of denaturation.

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Materials and Methods

Materials

Sodium alginate was the product of Koch-Light Lab (Colnbrook, UK). Bovine serum albumin and *o*-dianisidine HCl were obtained from Sigma Chemical Co. (St Louis, MO) USA. Jack bean meal was purchased from DIFCO, Detroit, USA. Dioxane, dimethyl formamide (DMF) and pectin were obtained from SRL Chemicals, Mumbai, India. Bitter gourd was purchased from local vegetable market. Other chemicals and reagents were of analytical grade and used as such.

Methods

Ammonium Sulphate Fractionation of Bitter Gourd Proteins

Bitter gourd (250 g) was homogenized in 500 ml of 0.1 M sodium acetate buffer (pH 5.5). Homogenate was filtered through four-layers of cheesecloth. Filtrate was then centrifuged at the speed of 10,000 \times *g* on a Remi C-24 cooling centrifuge for 20 min at 4°C. The clear solution thus obtained was subjected to salt fractionation by adding 0-80% (w/v) (NH₄)₂SO₄. The mixture was continuously stirred overnight at 4°C for complete precipitation of proteins. The precipitates were collected by centrifugation at 10,000 \times *g* on a Remi C-24 cooling centrifuge in 0.1 M sodium acetate buffer (pH 5.5) and dialyzed against assay buffers¹⁶. This enzyme preparation was stored for further use.

Preparation of Jack Bean Extract

Jack bean extract was prepared by adding jack bean meal (10 g) to 100 ml sodium phosphate buffer (pH 6.2). The mixture was kept overnight on a magnetic stirrer at room temperature. The clear jack bean extract obtained after centrifugation at 3000 \times *g* for 10 min was used for precipitation of peroxidases from ammonium sulphate fractionated bitter gourd proteins.

Preparation of Insoluble Con A-BGP Complex

To a series of tubes, constant amount of peroxidase (1200 U) was mixed with increasing concentration (0.1-1.0 ml) of 10% jack bean extract. Final volume of each tube was adjusted to 2.0 ml with 0.1 M sodium phosphate buffer (pH 6.2). The reaction mixtures were incubated overnight at 37°C. The precipitates were collected after centrifugation at 3000 \times *g* for 15 min at room temperature and were washed again with same buffer. Finally, precipitates were suspended in 2.0 ml assay buffer. Each precipitate was analyzed for enzyme activity. The precipitate (Con A-peroxidase complex) exhibiting maximum activity was taken for further studies.

Entrapment of Con A-BGP Complex in Calcium Alginate-Pectin Beads

Con A-peroxidase complex (1350 U) was mixed with sodium alginate (2.5%) and pectin (2.5%) in 10 ml of 0.1 M sodium acetate buffer (pH 5.5). The mixture was slowly extruded as droplets through a 5.0 ml syringe with attached needle No. 20 into 0.2 M calcium chloride solution to form alginate-pectin beads. Beads were further gently stirred for 2 h in calcium chloride solution¹⁰, washed and stored in 0.1 M sodium acetate buffer (pH 5.5) at 4°C for further use.

Peroxidase Activity— Measurement and Effect of Enzyme Loading

Peroxidase activity was determined by a change in optical density (A₄₆₀ nm) at 37°C by measuring initial rate of oxidation of 6.0 mM *o*-dianisidine HCl in presence of 18.0 mM H₂O₂ in 0.1 M sodium acetate buffer (pH 5.5) for 15 min¹⁷. Immobilized enzyme preparation was continuously agitated for entire duration of assay, which was highly reproducible with immobilized preparation. One unit (1.0 U) of enzyme activity is the amount of enzyme protein that catalyzes oxidation of 1.0 μ mole of *o*-dianisidine HCl per min at 37°C into colored product (ϵ_m P% 30, 000/M/l).

To a series of tubes, increasing concentration of enzyme (125-1000 U) was mixed to calcium alginate-pectin gel. Expression of loaded enzyme was monitored by assaying the activity.

Protein Estimation

Proteins concentration using BSA as standard was estimated by reported procedure¹⁸.

Effect of Temperature and pH on Soluble and Immobilized BGP

Soluble BGP, Con A-BGP complex and calcium alginate-pectin entrapped Con A-BGP complex (1.40 U) were incubated at 60°C in 0.1 M sodium acetate buffer (pH 5.5) for varying times. 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%). Enzyme activity of soluble, Con A-BGP complex and calcium alginate-pectin entrapped Con A-BGP were measured at varying temperatures (30-80°C) in 0.1 M sodium acetate buffer (pH 5.5) for 15 min.

Activity of soluble BGP, Con A-BGP complex and calcium alginate-pectin entrapped Con A-BGP complex (1.40 U) was measured in buffers (0.1 M each) of varying pH (3-10).

Effect of Urea, Organic Solvents and Detergents on Soluble and Immobilized BGP

Soluble BGP, Con A-BGP complex and calcium alginate-pectin entrapped Con A-BGP preparations (1.40 U) were incubated in 4.0 M urea dissolved in 0.1 M sodium acetate buffer (pH 5.5). Aliquots were removed at various time intervals and activity was determined. Soluble BGP, Con A-BGP complex and calcium alginate-pectin entrapped Con A-BGP (1.40 U) were incubated with 10-60% (v/v) of water-miscible organic solvents; dioxane and DMF prepared in 0.1 M sodium acetate buffer (pH 5.5) at 37°C for 1 h. Soluble and immobilized BGP (1.40 U) were incubated with increasing concentrations of non-ionic detergents; Triton X-100 and Tween-20 (0.5-5.0%, v/v) prepared in 0.1 M sodium acetate buffer (pH 5.5) at 37°C for 1 h.

Enzyme activity was monitored at all the indicated detergent concentrations¹⁶. Activity of enzyme without exposure to urea, organic solvents and detergent was taken as control (100%).

Statistical Analysis

Each value represents the mean for three-independent experiments performed in duplicates, with average standard deviation <5%. The data expressed in various studies was plotted using Sigma Plot-10.0 and expressed as mean with standard deviation of error (\pm). Data was analyzed by one-way ANOVA. *P*-values <0.05 were considered statistically significant.

Results and Discussion

Very few procedures meet requirements of enzyme immobilization directly from crude homogenate¹⁹. Calcium alginate mediated entrapment has attracted much attention in detoxification of phenolic compounds present in industrial effluents^{14,16}.

Partial Purification of BGP

Crude extract of bitter gourd exhibited initial specific activity, 88 U/g of the vegetable. 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 Con A-enzyme complex.

Preparation of Insoluble Con A-BGP Complex

Major peroxidases from bitter gourd are glycosylated²¹. Soluble enzymes could be leached out of beads on long standing or use^{10,20}. To prevent leaching

of enzymes out of porous gel beads, insoluble Con A-BGP complex was prepared by using jack bean extract and salt fractionated BGP proteins, which was subsequently entrapped into calcium alginate-pectin gel. Maximum precipitation of peroxidase activity using 0.4 ml of 10% jack bean extract was 86% (Table 1). Con A-BGP complex was entrapped into calcium alginate-pectin gel and it resulted in further loss of peroxidase activity.

Entrapment of Con A-BGP Complex in Calcium Alginate-Pectin Beads

Immobilization by means of entrapment is a very rapid and simple technique. 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 enzymes¹⁰⁻¹². It suggested 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 beads¹⁰. Entrapped Con A-peroxidase complex retained 51% original activity (Table 1).

Effect of enzyme loading on entrapped activity was evaluated by entrapping increasing concentration of enzyme. Optimum concentration (500 U/ml) was sufficient for maximum expression of peroxidase activity by entrapped preparation.

Effect of Temperature and pH on Soluble and Immobilized BGP

Soluble and immobilized peroxidase preparations showed maximum activity at 40°C (Fig. 1). However, Con A-BGP and entrapped Con A-BGP complex retained greater fraction of catalytic activity at higher temperatures as compared to its soluble counterpart. Soluble BGP lost nearly 60% of its initial activity after 2 h incubation at 60°C whereas entrapped Con A-BGP preparation retained about 75% original activity under identical incubation conditions (Fig. 2). These observations show that resistance of enzyme to high temperatures was greatly increased by immobilization. Calcium alginate-pectin entrapped Con A-peroxidase complex retained its structure and remarkably high activity at elevated temperatures. Therefore, such enzyme preparation could be exploited at relatively high temperatures. Improvement in thermal stability of calcium alginate-pectin entrapped Con A-BGP

Table 1—Immobilization of BGP by using Con A and calcium alginate-pectin gel

Type of immobilization	Original activity %	Expressed activity %
Con A-peroxidase complex	100	86
Calcium alginate-pectin gel entrapped Con A-peroxidase	100	51

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

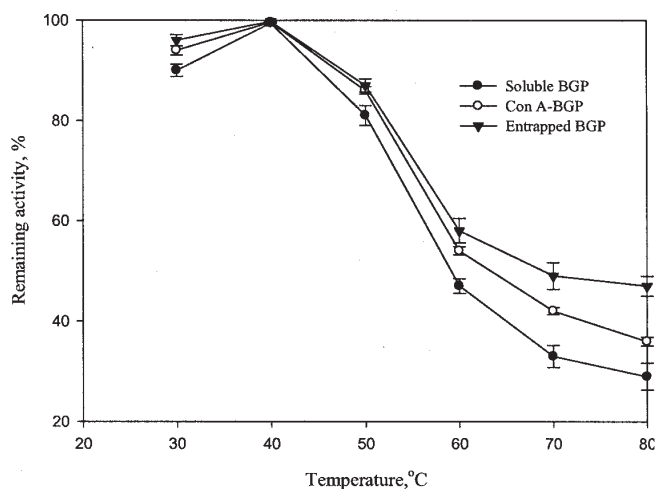


Fig. 1 — Temperature-activity profiles of soluble and immobilized BGP

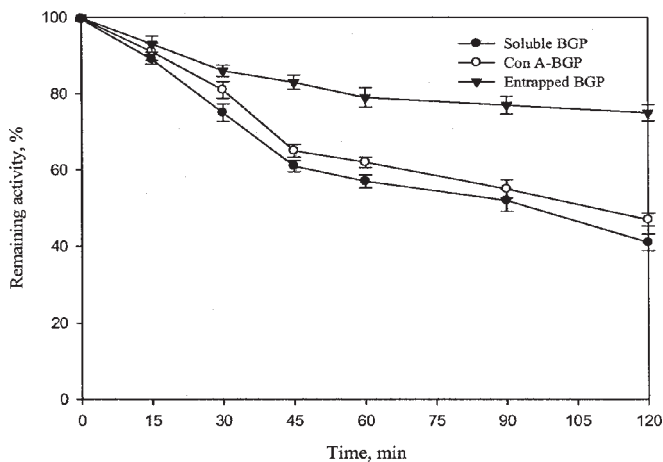


Fig. 2 — Thermal denaturation of soluble and immobilized BGP

preparation may come from multipoint attachment of peroxidases with Con A. This enhancement in thermal stability is due to formation of several linkages between enzyme and support^{13,21}.

Effect of pH on activity of soluble BGP, Con A-BGP and entrapped Con A-BGP was evaluated by incubating these preparations in the buffers of varying pH values (3.0-10.0). pH 5.5 was optimum in all enzyme preparations (Fig. 3). However, immobilized BGP preparations showed significant broadening in pH activity profiles indicating a marked increase in stability.

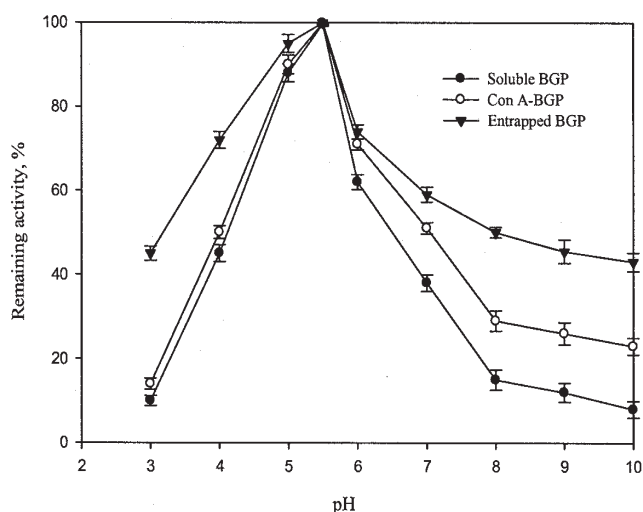


Fig. 3—pH-activity profiles of soluble and immobilized BGP preparations

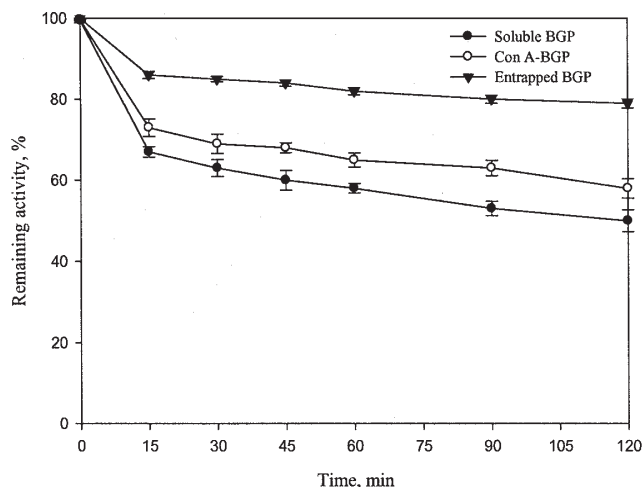


Fig. 4—Effect of urea on soluble and immobilized BGP preparations

Table 2—Effect of organic solvents on soluble and immobilized BGP

Organic solvent %, v/v	Remaining activity, %					
	Dioxane			DMF		
	Soluble BGP	Con A-BGP complex	Entrapped Con A-BGP	Soluble BGP	Con A-BGP complex	Entrapped Con A-BGP
10	58.65±1.36	81.79*±1.56	94.76*±1.21	54.67±1.43	68.82*±1.78	92.79*±0.97
20	50.12±2.23	73.67*±1.11	84.12*±1.34	52.68±2.11	66.25*±1.43	85.25*±1.12
30	48.24±1.45	63.10*±2.23	65.97*±0.97	52.22±1.68	62.79*±2.12	77.89*±0.89
40	38.67±1.67	54.23*±1.98	62.78*±1.11	44.17±1.47	57.94*±1.78	64.79*±1.32
50	33.11±2.07	43.69*±1.23	51.22*±1.23	23.92±1.23	43.24*±1.32	46.98*±1.24
60	22.98±2.66	25.92*±1.16	42.94*±1.23	6.24±1.99	28.22*±0.98	32.24*±1.76

Effect of various concentrations of organic solvents on soluble and immobilized BGP was analyzed by one-way ANOVA* and #denotes that values ($P<0.05$) were statistically significant when Con A-BGP complex and entrapped Con A-BGP were compared with soluble BGP, with respect to dioxane and DMF, respectively.

Table 3—Effect of detergents on soluble and immobilized BGP

Detergent %, v/v	Remaining activity, %					
	Triton X-100			Tween-20		
	Soluble BGP	Con A-BGP complex	Entrapped Con A-BGP	Soluble BGP	Con A-BGP complex	Entrapped Con A-BGP
0.5	64.69±1.23	87.34*±1.56	92.24*±1.32	65.23±1.24	86.78*±1.45	99.89*±1.32
1.0	44.24±1.76	67.87*±2.16	83.97*±1.26	54.98±1.67	68.24*±1.67	74.23*±2.13
1.5	40.65±2.14	61.32*±1.45	74.79*±2.43	44.75±2.56	57.89*±2.12	65.89*±1.68
2.0	36.28±1.98	52.12*±1.89	73.36*±1.96	42.24±2.18	43.79*±2.24	57.91*±1.54
2.5	32.13±1.67	49.78*±2.56	71.24*±2.11	38.96±2.11	43.34*±1.68	49.24*±2.16
3.0	30.68±2.23	42.12*±2.15	66.85*±2.23	36.32±1.23	40.21*±1.57	42.11*±1.88
3.5	30.27±2.42	39.33*±1.57	55.79*±2.16	33.22±1.97	38.24*±1.83	39.97*±1.76
4.0	28.22±2.76	36.24*±1.37	54.21*±1.86	31.25±1.23	35.79*±2.43	39.11*±1.45
4.5	26.14±1.77	35.21*±2.14	52.11*±1.76	28.69±1.65	33.68*±1.33	37.43*±2.25
5.0	23.23±0.47	30.81*±1.98	42.16*±1.11	25.87±1.45	33.12*±1.36	35.69*±2.14

Effect of various concentrations of detergents on soluble and immobilized BGP was analyzed by one-way ANOVA* and #denotes that values# ($P<0.05$) were statistically significant when Con A-BGP complex and entrapped Con A-BGP were compared with soluble BGP, with respect to Triton X-100 and Tween-20, respectively.

Broadening in pH-activity profiles remarkably increased in case of entrapped Con A-BGP followed by Con A-BGP complex, predicting that entrapment of enzymes in gel beads provide a microenvironment for enzyme, which may play an important role in the state of protonation of protein molecules¹⁰. Formation of Con A-peroxidase complex confers additional resistance to enzyme against extreme conditions of pH^{16,22}.

Effect of Urea on Soluble and Immobilized BGP

Soluble and immobilized BGP preparations were incubated with 4.0 M urea for varying times. Soluble

BGP and Con A-BGP complex lost nearly 50% and 40% of initial activity, respectively. However, entrapped Con A-BGP preparation was quite resistant to urea induced inactivation and retained nearly 80% of its initial activity even after 2 h incubation (Fig. 4). Urea (4.0 M) is a strong denaturant of some proteins and it irreversibly denatures BGP^{10,23}. However, action mechanism of urea on protein structure has not yet been completely understood, several earlier findings have suggested that protein could be unfolded by direct interaction of urea molecule with a peptide backbone via hydrogen bonding

and/or hydrophobic interaction, which contributes to maintenance of protein conformation²⁴. Complexing of glycoenzymes with Con A also reported^{10,16} in an enhancement of their resistance to denaturation mediated by urea. Thus, these observations indicate that entrapment protected Con A-peroxidase complex from urea induced inactivation.

Effect of Organic Solvents on Soluble and Immobilized BGP

Effect of increasing concentrations of dioxane (10-60%, v/v) on the activity of soluble and immobilized BGP was observed. On exposure to dioxane (60%, v/v), soluble enzyme retained only 23% of its original activity while Con A-BGP complex and entrapped Con A-BGP retained nearly 26% and 43% of their initial activity, respectively. Incubation of soluble and immobilized BGP with increasing concentrations of DMF (10-60%, v/v) resulted in decreasing the enzyme activity. On exposure to DMF (60%, v/v) for 2 h, soluble BGP lost nearly 94% of its initial activity while Con A-BGP complex and entrapped Con A-BGP retained nearly 29% and 32% of original activity, respectively (Table 2). These observations showed that entrapped Con A-BGP complex retained remarkably high stabilization against inactivation caused by dioxane and DMF as compared to soluble BGP and Con A-BGP complex. Earlier reports¹⁶ also suggested that bioaffinity bound enzymes were significantly more stable against exposure to water miscible organic solvents.

Effect of Detergents on Soluble and Immobilized BGP

Wastewater from various elimination sites also includes several types of denaturants, including detergents that can strongly denature enzymes used for treatment of polluted wastewater. Soluble and immobilized BGP were treated with increasing concentrations of Triton X-100 and Tween-20 for 1 h at 37°C. Entrapped Con A-BGP retained 42% and 36% of its initial activity in the presence of 5.0% (v/v) Triton X-100 and Tween-20, respectively. However, soluble BGP exhibited only 23% and 26% of initial activity in presence of 5.0% Triton X-100 and Tween-20, respectively (Table 3). Thus, entrapped Con A-BGP complex was markedly more stable to inactivation induced by detergents (Triton X-100 and Tween-20). Entrapped Con A-BGP could work quite efficiently in presence of contaminants like soaps and detergents. Immobilized peroxidases are reported^{10,16} significantly stabilized against denaturation induced by some household detergents.

Conclusions

Calcium alginate-pectin entrapped Con A-BGP preparation exhibits significantly higher stability against various physical and chemical denaturants compared to soluble BGP and Con A-BGP complex. Thus, immobilized BGP preparations could be exploited for developing bioreactors for the treatment of phenolic and other aromatic pollutants present in agro-industrial wastewaters.

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