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Fabrication of photometric dip-strip test systems for detection of β(1 → 3)-D-glucan using crude β(1 → 3)-D-glucanase from sprouts of *Vigna aconitifolia*

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ABSTRACT

Efforts have been made to fabricate enzyme dip-strip test systems for detecting β(1 → 3)-D-glucan. β(1 → 3)-D-glucanase from sprouts of *Vigna aconitifolia* (commonly known as moth bean, 8-day old) with high specific activity (244 U mg^{-1}) was co-entrapped with glucose oxidase (GOD) in different combinations of composite polymer matrices of agarose (A), gelatin (G), polyvinyl alcohol (PVA) and corn flour (CF). The enzyme immobilized membranes were checked for immobilization yield, pH and temperature optima, swelling index, thermal, operational and storage stability, and morphology by scanning electron microscopy. The 3% A–2% CF–8% G composite matrix was chosen for fabricating enzyme dip-strip systems for detection of β-glucan by spectrophotometer using DNSA method (System-I) and AAP method (System-II). Dip-strip System-I and II showed linear dynamic range for detecting glucan concentration ranged from 100 to 500 μg mL^{-1} and 10 to 50 μg mL^{-1} with contact time 10 and 5 min, respectively. The LOD of System-I and II were found to be 65 μg mL^{-1} and 10 μg mL^{-1}, respectively. Hence System-II was employed for analyzing β(1 → 3)-D-glucan contents in various pharmaceutical samples. It was found that without any sample pre-treatment the percent error of detection was less than 5.

1. Introduction

β-Glucans are natural exo-polysaccharides and polymers of D-glucose with β(1 → 3), β(1 → 4) and/or β(1 → 6) glycosidic linkages. They belong to the family of biological response modifiers (BRMs), and exist in the cell walls of some yeast, bacteria, fungi, cereal grains, bacteria and algae (Volman et al., 2008; Xu et al., 2008; Shih et al., 2008; Rhee et al., 2008). A number of studies have revealed that the positive influences of glucans on health include antibacterial, wound-healing, anti-tumor and anti-oxidative functions (Brochers et al., 1999; Kiho et al., 1992; Jaehrig et al., 2008). Consequently, functional foods and nutraceuticals containing β-glucan are gaining great attention nowadays.

The β-glucan contents of samples are traditionally determined with viscometric, enzymatic and colorimetric methods (Wood et al., 1989; McCleary and Holmes, 1985; McCleary and Shameer, 1987). Aman and Graham (1987) reported the enzymatic and fluorometric method for β-glucan detection. Wood et al. (1994) reported the use of specific chemical dyes such as Calcofluor and Congo-red for detecting β-glucans. The method of McCleary and Mugford (1997) is now recognized as AOAC International Reference Method. In addition, flow injection analysis for determination of oat and barley β-glucans was reported by Jadhav et al. (1998). These methods are time-consuming and require extensive laboratory equipment with trained personnel. Dry-dip-strip test systems in combination with sensor (bio)technology have given new analytical platform to overcome these problems. Nowadays, varieties of one-step strip test devices are widely used in various foods, clinical and environmental applications (Sithigorngul et al., 2007; Shi and Jiang, 2002; Gui et al., 2008; Schweers et al., 2008). This technology offers reliable results with rapid response, specificity and sensitivity with user friendly approach.

During sensor construction, use of crude extract/enzyme is a common practice which avoids tedious and costly efforts of enzyme purification. In addition, stability of crude enzyme is usually higher than that of purified enzyme, which may be attributed to the micro-environment which is enriched with natural proteins and other available cofactors. However, sensors with crude enzyme might suffer from low specificity due to the presence of contaminated enzymes and proteins, and long response time due to diffusion barriers (Bagal and Karve, 2006). But this issue can be obviated by selection of enzyme sources with high specific activity. Thus, high reproducibility, availability and low cost of crude enzymes from plant source may be good candidates

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for construction of biosensors (Bagal-Kestwal et al., 2007, 2008). The high expression levels of β(1 → 3)-D-glucanase in seedlings of barley (Campas et al., 2008; Morohashi and Matsushima, 2000), tomato, rye, tobacco and other dicots (Bewley and Black, 1994; Vogeli-Lange et al., 1994) have been observed. Consequently, one of the objectives of the present study was to investigate the feasibility of utilizing crude β(1 → 3)-D-glucanase from germinating seedlings to fabricate dip-strips for glucan detection using immobilization technology.

An amperometric flow injection analysis based biosensor for detection of laminarin from seaweeds and health promoting food products was reported by Miyanishi et al. (2004). However, there is lack of studies on β-glucan determination by biosensors or one-step strip tests. The objective of this study was to examine the possibility of use of crude β(1 → 3)-D-glucanase for fabrication of photometric dip-strip test systems. Since agarose (A), gelatin (G), poly vinyl alcohol (PVA) and starch (S) are often used to form bi-composite matrix for enzyme immobilization (McEvoy et al., 1985; Bagal and Karve, 2006; Yang et al., 2004; Shan et al., 2008; Bogdanovskaya et al., 2004; Gabrovska et al., 2007; Zhai et al., 2003), the combinations of these composite matrices were tested for immobilization of glucanase. The fabricated biosensor strips were compared with the standard method for β-glucan analysis in dietary supplements or drugs containing β-glucan.

2. Experimental details

2.1. Materials and chemicals

Glucose Oxidase from *Aspergillus niger* (EC. 1.1.3.4) Type X-S, lyophilized powder, 100,000–250,000 units/g solid (without added oxygen), glucose oxidase–peroxidase (GOD-POD) kit, 4-aminoantipyrine (AAP), laminarine, β(1 → 3)-D-glucan, dinitrosalicyclic acid (DNSA), D-glucose, agarose and polyvinyl alcohol (PVA) were purchased from Sigma–Aldrich, St. Louis, MO, USA. Gelatin was obtained from Hanawa Chemicals (Osaka, Japan). Corn powder (*Zea mays* L.) was purchased from a super market in Taipei, Taiwan. All other chemicals were of reagent grade and used without further purification.

2.2. Instruments

JEOL-JFC-1600E Ion Sputtering Device Fine Coat and JEOL JSM-6300F Field Emission Scanning Electron Microscope were used for SEM analysis. Hitachi F-4500 fluorospectrophotometer (Hitachi Software Engineering, Yokohama, Japan), Branson 5510 Ultrasonic instrument and Thermo Spectronic UV–visible spectrophotometer were also used during the studies.

2.3. Sprouting and β(1 → 3) glucanase extraction

2.3.1. Sprouting procedure

Different candidates including red bean (*Vigna angularis*), broad bean (*Vicia faba*), moth bean (*Vigna aconitifolia*), black eye pea (*Vigna unguiculata*) were used for germination and these sprouts were tested for β-glucanase activity. Mature seeds (25 g) of various beans were washed extensively and kept overnight in distilled water in dark. The next day, the seeds were spread on wet filter paper, and then transferred into a phytotron controlled at 25/20 °C (day/night), 80% humidity, and a photoperiod of 10–11 h. The seeds were sprinkled with water at regular time interval to maintain maximum humidity for seed sprouting. After sufficient growth (6–8 days), the sprouts were harvested and yield was estimated. The harvested sprouts were stored at 4 °C before extraction of β-glucanase.

2.3.2. Extraction of β-glucanase from sprouts

For extraction of β-glucanase, sprouts were homogenized in a mixer with PBS (phosphate buffered saline, pH 7.4) (100 mL PBS/25 g sprouts) under chilled condition. The homogenate thus obtained was stirred at 4 °C for 2 h, and then centrifuged at 9072 × *g* for 10 min. The supernatant was analyzed for protein content and β-glucanase activity and stored at −20 °C till further use. The sprout with high specific activity and enzyme yield was chosen for further study.

2.3.3. Protein estimation

The protein content of bean sprout extract was determined using bovine serum albumin as a standard by the method of Lowry et al. (1951).

2.3.4. Enzyme activity

β-Glucanase activity was determined by dinitrosalicylic acid (DNSA) method (Miller, 1959). Crude enzyme extract (400 μL) was mixed with 300 μL of phosphate buffer solution (0.2 M, pH 6.0). The β-glucan (100 μg mL^{-1}) was added at 40 °C and after 10 min reaction time; the enzyme activity was terminated by addition of 500 μL DNSA reagent. The test tubes were kept in boiling water bath (100 °C) for 10 min. After cooling, the coloured product was measured by spectrophotometer at 540 nm. One international unit is defined as the amount of enzyme which forms 1 μmol of reducing sugars equivalents (expressed as glucose) per minute using β-glucan as substrate at pH 5.5 at 40 °C.

2.4. Immobilization of β(1 → 3)-D-glucanase and GOD

2.4.1. Gel preparation

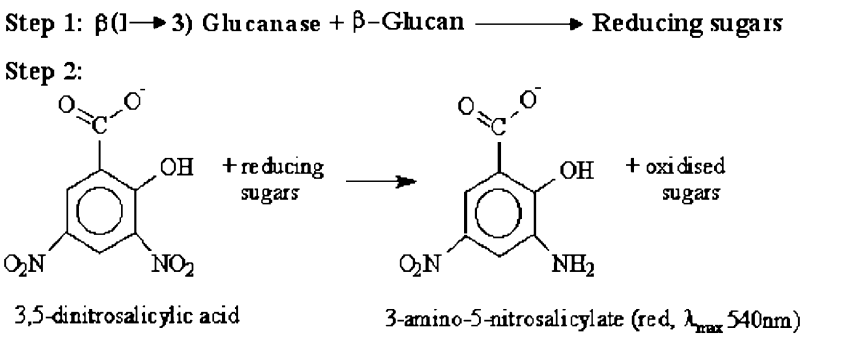
Corn flour (CF) (0.5–3%, w/v), agarose (1–3%, w/v) and gelatin (2–8%, w/v) were individually dissolved in Milli-Q water at 60 °C with constant stirring until gelation. The polyvinyl alcohol gel (0.5–3%, w/v) was prepared by mixing benzoic acid and PVA in Milli-Q water at 85 °C. The homogenized PVA gel solution was allowed to cool at room temperature and used for further studies (Kumar and D'Souza, 2008). Combinations of different gels (2:1:1 ratio) at different concentrations were tested for blank membrane (without enzyme) and the mixed gel solution was ultra-sonicated for 5 min at 60 °C for membrane casting.

2.4.2. Membrane preparation

The blank (without enzyme) and enzyme membranes were prepared for testing. The enzymes (β-glucanase: 1–12 units; GOD: 30–50 units) were added to freshly prepared gel solutions and mixed properly. Soda-lime glass coated with inert plastic material was used as support for membrane casting (Bagal and Karve, 2006). Simple drop-cast method was used for gel deposition. After overnight drying, the membranes were detached from the glass support carefully and stored at 4 °C under moisture free conditions.

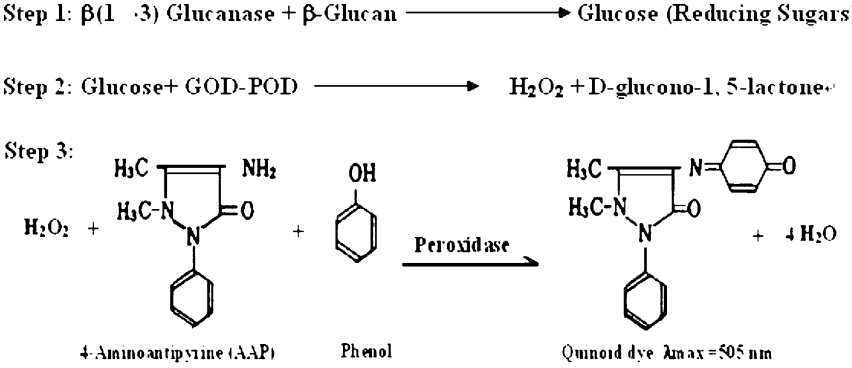
2.5. Determination of entrapped enzyme activity by DNSA method

The β-glucanase immobilized membrane (1.0 cm × 1.0 cm) was rinsed with assay buffer to remove unbound/excess enzyme and incubated with 300 μL β-glucan substrate (100 μg mL^{-1}) and 400 μL assay buffer at room temperature for 10 min. After removal of the membrane, 300 μL dinitrosalicylic acid (DNSA) was added and reducing sugars liberated in the reaction were estimated by DNSA method (Miller, 1959).



2.6. Determination of entrapped enzyme activity by 4-AAP method

A piece of co-immobilized enzyme membrane (1.0 cm × 1.0 cm) was rinsed with assay buffer and incubated with 300 μL β-glucan (300 μg mL^{-1}) and 350 μL assay buffer at room temperature for 5 min. The membrane was removed from solution to stop the enzyme-substrate reaction, and then 4-aminoantipyrine-phenol reagent (350 μL) was added to the reaction. After 5 min contact time, pink color developed was directly measured at 505 nm using UV–visible spectrophotometer (Saito et al., 1987; Kabasakalian et al., 1974).



2.7. Leaching of enzyme

A piece of membrane (1.0 cm × 1.0 cm) was rinsed with 0.2 M phosphate buffer (pH 5.5), and then incubated in 2.0 mL of fresh assay buffer for 10 min. The membrane was removed and aliquot from residual solution was assayed for β-glucanase and GOD activities by both methods as mentioned earlier.

2.8. Swelling index of the membrane

The square pieces of co-immobilized as well as blank membranes (1.0 cm × 1.0 cm) were immersed in to Milli-Q water for 20–24 h at room temperature to attain equilibrium sorption. After removal of excess water by filter paper the membranes were weighed immediately. The swelling index (S) was calculated according to the following equation (Carolina et al., 2005; Praptowidodo, 2005):

$$S(\%) = \frac{W_s - W_d}{W_d} \times 100$$

where W_s and W_d are the weights of wet membranes and dry membranes, respectively.

2.9. Fabrication of dip-strip

A supporting strip (0.5 cm × 7.0 cm) of polyethylene material was used for dip-strip fabrication. A piece of Whatman filter paper (0.3 cm × 0.3 cm, 110 mm Dia., Cat. No.1454110) was placed at the end of the strip using adhesive material. Gel solutions of agarose (3%, w/v), corn flour (CF) (2%, w/v), gelatin (8%, w/v) and enzyme(s) were mixed in 2:1:1:1 proportion and sonicated for 5 min at room temperature. The homogenized solution was overlaid on the Whatman filter paper as thin membranous layer, subjected for overnight air drying, and used as a test zone. Two sensing systems were fabricated, System-I with moth bean β-glucanase (0.5–2.5 units) and System-II comprised of moth bean β-glucanase co-immobilized with 30 units of glucose oxidase and 6 units of peroxidase (GOD-POD) reagent from kit.

2.9.1. System-I (DNSA method)

The dip-strips with immobilized moth bean soluble enzyme were washed with assay buffer (0.2 M phosphate buffer at pH 5.5) prior to use. The aqueous samples containing 200 μL substrate (200 μg mL^{-1}) and assay buffer (500 μL) were incubated with dip-strip for 10 min at 40 °C. The strip was removed and liberated reducing sugars were estimated by DNSA method.

2.9.2. System-II (4 AAP method)

The washed dip-strip was directly immersed in 300 μL of substrate (300 μg mL^{-1}), 400 μL sodium phosphate buffer for 10 min at 40 °C. The strip was removed and liberated products were estimated by 4-AAP method.

2.10. Fabrication reproducibility

The fabrication process of dip-strips was evaluated for suitability of the novel composite material used. Adhesion of the membrane to plastic support was the key point during these studies. A set of 10 experiments in different batches were carried out to check reproducibility for both Systems (I and II).

2.11. Features of the dip-strips

2.11.1. Determination of optimum temperature and pH

The response of photometric sensing strip towards β-glucan was tested at different temperatures and pH. The relationship between test strip sensitivity for analyte and temperature was studied within the range of 30–90 °C. The pH dependent studies of soluble and immobilized β-glucanase were carried out in acetate buffer (pH 3.0–5.0) and phosphate buffer (pH 5.5–7.5).

2.11.2. Operational and storage stability of the co-immobilized enzyme

The reusability of membranes was checked for its successive use for different cycles of experiment by employing same membrane. After the membrane was used, it was rinsed with phosphate buffer (pH 5.5) and then placed in a test tube, sealed with parafilm, and stored at 4 °C until next uses. The storage stability of both enzymes was checked with regular interval of time when stored at 4 °C and room temperature under dry conditions.

2.11.3. Response time of both dip-strips

The response time for both System-I and II was studied using standard solution containing 300 μg mL^{-1} of laminarin in 0.2 M phosphate buffer at pH 5.5 and 55 °C. In both cases, the contact time for dip-strip with the standard solution was checked in a range of 1–20 min.

2.12. Application of dip-strips

2.12.1. Sample preparation

The pharmaceutical samples were purchased from market and the gelatin capsules were removed. The sample powders were weighed and 1 mg mL^{-1} solutions were prepared using 0.2 M phosphate buffer solution (pH 5.5). The β-glucan in samples was extracted at 80 °C with constant stirring for 1 h. The extracts were centrifuged for 15 min at 9072 × *g* at room temperature and the supernatants were used for β(1 → 3)-D-glucan testing. Liquid β-glucan sample was directly used for testing after appropriate dilutions with assay buffer.

2.12.2. Sample testing

The fabricated strips were directly dipped into the extract of pharmaceutical samples (400 μL) for 10 min at 55 °C. Then the strips were removed and 4-AAP and phenol were added, and then measurements were obtained as mentioned for System-II.

The reliability of fabricated dip-strips was checked by standard fluorescence method using aniline blue (Kim et al., 2004; Fuchs et al., 2007; Sekiya-Kawasaki et al., 2002). Excitation wavelength, 395 nm/slit and emission wavelength 495 nm/slit with sensitivity factor 25 were used to get minimum background disturbance.

3. Results and discussion

3.1. Selection of enzyme and membrane

Various beans, including red bean, broad bean, moth bean and black eye pea were used for germination and their sprouts were tested for β-glucanase activity. The moth bean sprout of 8-day-old grown in phytotron was found to be a better source of β-glucanase. The specific activity of crude enzyme extracted from moth bean sprout was high enough (244 U mg^{-1}) to be used without further purification to fabricate the dip-strips.

Agarose (A), gelatin (G), PVA and corn flour (CF) were used to form membrane for immobilization of the plant β-glucanase and GOD to detect β-glucan. Different concentrations and combinations were tried to obtain suitable tuning of porosity and thickness of the membrane (Table 1). Membranes with corn flour gel (A, B and C) forms slightly thicker film with more transparency. The membranes B and D were self-adhesive in nature which firmly adhered to glass and plastic support during the fabrication of dip-strips. For the membranes with non-adhesive property external adhesive is needed to prepare test-zone, which may have adverse effect on enzyme entrapped in the membranes on contact with the chemical ingredients of adhesives. Further, colour of the membrane is another important characteristic as it can cause interference during photometric studies.

The swelling behavior of a material refers to the case when water molecules enter the material and combine with hydrophilic groups of material molecules. Consequently, the content of hydrophilic groups and intermolecular force are the two main factors that influence membrane's swelling property. Our study indicated that membrane B (3% A + 2% CF + 8% G) and D (8% G + 1% A + 3% PVA) had lower swelling index and were not dissolved in buffer when immersed for at least 120 min. High swelling index of membrane A and E can be attributed to the mechanical fragility as well as inferior morphological stability of membrane. The choice of composite material was made by checking membrane forming ability, physical properties of membrane and swelling index. Based on these parameters we found that membrane B and D are the suitable candidates.

3.2. Immobilization of enzymes

The immobilization of individual enzyme (β-glucanase or GOD) was carried out first and experimental conditions were optimized to achieve maximum efficiency. Table 2 compares the percent retention of both enzymes in different membranes. Leakage of the enzyme was checked for individually immobilized and co-immobilized enzyme membranes. High leaching was observed during immobilization of GOD while minimum enzyme loss was observed in case of β-glucanase. Similar findings were observed after co-immobilization. These results indicated that β-glucanase was confined by the membrane matrix more firmly than that of GOD. A decrease in the enzyme activities (from 15 ± 7% to 20 ± 8%) was observed after co-immobilization. This effect is obvious because both enzymes compete for accommodation in same

Table 1
An account of properties of novel composite matrices.

Sample (2:1:1)	Appearance	Swelling index (%)	Thickness (μm)	Mechanical properties
Membrane A (1% A + 1% CF + 2% G)	Colorless, transparent	97 ± 1.6	18–20	Self-adhesive, flexible
Membrane B (3% A + 2% CF + 8% G)	Colorless, transparent	77 ± 2.3	20–22	Self-adhesive, flexible
Membrane C (3% CF + 3% A + 8% G)	White, semi-transparent	81 ± 1.8	20–25	Non-adhesive, flexible
Membrane D (8% G + 1% A + 3% PVA)	Yellowish, semi-transparent	70 ± 2.6	15–20	Self-adhesive, rigid
Membrane E (3% PVA + 3% A + 8% G)	Whitish, semi-transparent	93 ± 1.9	13–15	Non-adhesive, semi-rigid

A: agarose; CF: corn flour; G: gelatin; PVA: polyvinyl alcohol.

Table 2
Comparison of immobilized and co-immobilized enzymes by DNSA method.

	Matrix composite	Beta glucanase		Glucose oxidase	
		% Immobilization efficiency[a]	% Leakage[a]	% Immobilization efficiency[a]	% Leakage[a]
Free enzyme		100 ± 1.2	Not applicable	100 ± 0.6	Not applicable
Immobilized system	A (1% A + 1% CF + 2% G)	91.0 ± 0.1	6.3 ± 3.1	89.0 ± 0.1	7.7 ± 2.8
	B (3% A + 2% CF + 8% G)	92.5 ± 0.5	4.1 ± 2.2	84.0 ± 0.3	2.8 ± 2.2
	C (3% CF + 3% A + 8% G)	84.5 ± 1.5	11.0 ± 1.9	70.0 ± 0.2	22.1 ± 0.6
	D (8% G + 1% A + 3% PVA)	93.7 ± 0.1	3.8 ± 3.7	87.0 ± 1.1	7.6 ± 0.9
	E (3% PVA + 3% A + 8% G)	84.0 ± 0.4	10.3 ± 0.4	81.0 ± 0.2	13.5 ± 1.1
Co-immobilized system	A (1% A + 1% CF + 2% G)	87.0 ± 1.1	9.8 ± 2.6	83.0 ± 1.3	12.3 ± 2.7
	B (3% A + 2% CF + 8% G)	91.5 ± 0.2	2.1 ± 1.6	83.8 ± 1.3	6.7 ± 2.2
	C (3% CF + 3% A + 8% G)	75.0 ± 2.2	16.0 ± 2.4	73.0 ± 1.2	19.0 ± 1.3
	D (8% G + 1% A + 3% PVA)	88.5 ± 1.0	8.3 ± 3.1	84.3 ± 1.1	8.9 ± 0.6
	E (3% PVA + 3% A + 8% G)	70.0 ± 2.1	17.0 ± 4.0	71.5 ± 0.4	19.3 ± 3.4

[a] Values are mean ± S.D.

matrix. Co-immobilization was found to be better in case of membrane A, B and D (16 ± 2% loss of activity) as compared to that of C and E (20 ± 8% loss of activity). Overall immobilization for β-glucanase was achieved in the range of 75–93%. Recent report showed that the activity retention of β(1 → 3)-D-glucanase from *Trichoderma harzianum* immobilized in calcium alginate were 51% and 34%, for pure and crude enzyme, respectively (EL-Katatny, 2008). For co-immobilization, our study showed more than 80% of GOD activity retention except in case of membrane C and E. Similar results were observed in case of co-entrapment of GOD with invertase enzyme in biopolymer with 80% immobilization efficiency in our previous study (Bagal-Kestwal et al., 2007). Thus, the matrix combinations used in this study are highly efficient for co-immobilization of β(1 → 3)-D-glucanase and GOD. Other enzymes may also be immobilized in these matrix combinations if needed, with or without modification.

3.3. Film morphology by SEM

The composite membrane B (3% A + 2% CF + 8% G) was further characterized by surface morphological study. Using a high resolution, low voltage scanning electron microscope (SEM), we examined the topology and morphology of polymer membranes with and without enzyme (Fig. 1 A_1 and B_1). Blank membrane of agarose–corn flour–gelatin appeared smooth at surface with 100× magnification. At high magnification, surface topography revealed domains like

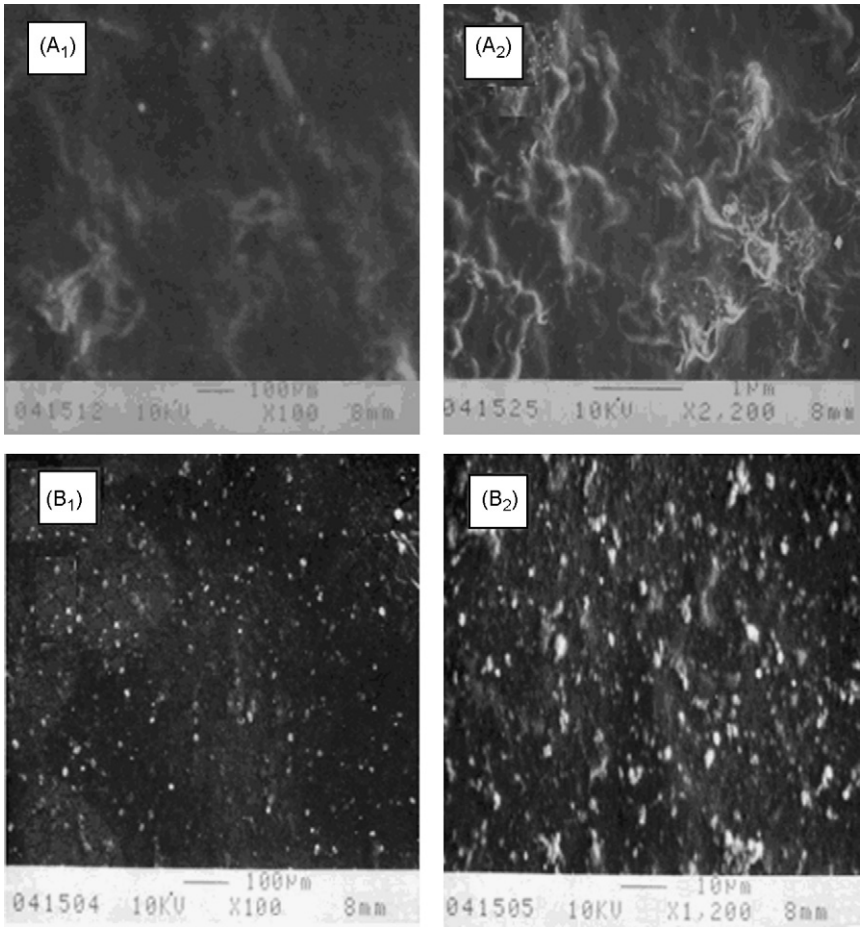


Fig. 1. SEM images of blank (A_1 and A_2) and co-immobilized enzymes (B_1 and B_2) in composite membrane of agarose–corn flour–gelatin.

structures. These structures are formed possibly due to corn starch aggregation with gelatin and agarose during sol–gel formation process. Similarly, swollen granules of maize starch were observed as 'dispersed phase' within a continuous biopolymer matrix of agarose by Mohammed and co-workers on co-gelation (Mohammed et al., 1998). These granules may form pores of different depth and diameters in which biomolecules may get entrapped firmly, regulating enzyme leaching.

As shown in Fig. 1B_1, the enzyme entrapped membrane showed uniform distribution of crude enzyme homogenate. On higher magnification the distinct particulate matter with different sizes can been seen (Fig. 1B_2). The presence of different domain like structure may be due to presence of different proteins in crude extract of β-glucanase.

3.4. Reproducibility of fabrication of dip-strip test systems

The matrix with 3% agarose, 2% corn flour, 8% gelatin (membrane-B), GOD (30 units) and β(1 → 3)-D-glucanase (2.0 units) in 2:1:1:1:0.5 was selected for fabrication of dip-strips (System-I and II) due to its superior mechanical properties and high co-immobilization efficiency. Firstly, 10 sets of experiments were carried out with enzyme extracted from different batches of sprouts to check the reproducibility of dip-strip systems. We found that the response of fabricated systems to glucan solution (0.1%, w/v) was essentially the same (see supplementary data for figure), suggesting that simple and easy physical entrapment method devoid of any chemical treatment is suitable for plant β-glucanase.

3.5. Influence of temperature and pH

A significant post-immobilization effect was observed on temperature optima of the β(1 → 3)-D-glucanase. The optimum temperature for soluble moth bean β-glucanase was around 40 °C, which increased in the range of 50–60 °C for physically entrapped glucanase in both the systems (see figure in supplementary data). In this study, the dip-strip B was found to be stable at 60 °C with retention around 70% of its initial response, while previously reported glucanase entrapped in hybrid silica-polysaccharide nano-composite was not stable above 37 °C (Shchipunov et al., 2004, 2006).

The optimum response for both enzyme dip-strips was found to be in acidic range (from pH 5.0 to 6.0) (see supplementary data). The soluble enzyme has pH optima at 5.5, while after co-immobilization a broad pH range was observed in both the systems. These findings are in line with the observations of other researchers where they have mentioned that pH optima for plant β-glucanase is in the range of 4.8–5.5 (Balance and Manners, 1978; Bewley and Black, 1994). To take advantage of maximum enzyme activity of both enzymes, pH 5.5 was used during subsequent studies.

3.6. Photometric response studies

The response time of a sensing system or sensor is a very important parameter as it determines the length of analysis. The total time for β-glucan analysis using DNSA based dip-strip (System-I) took approximately 1 h. The designed fabricated System-II allows direct measurement of reaction product formed in the solution, and total time needed for the analytical procedure is within 20 min. The optical densities increased with contact time in a linear fashion up to 10 and 5 min for System-I and II, respectively, thereafter, the photometric response reached plateau (see Fig. 2). System-I was more time consuming as compare to System-II. But, when compared with the traditional fluorescence method which required approximately 3 h for total assay, both of our fabricated enzyme dip-strips found to be faster and simpler in operation. Hence reduced number of reagents and easy method of detection was achieved by co-immobilization of β-glucanase and GOD-POD reagent in the same composite matrix.

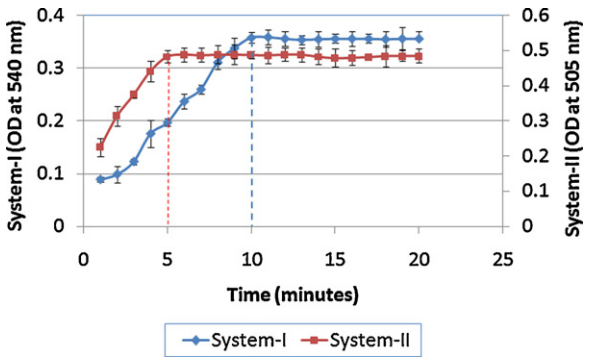


Fig. 2. The photometric response time for System-I and System-II (*assay conditions*: laminarine, 300 μL; buffer, 0.2 M sodium phosphate buffer pH 5.5; temperature, 55 °C. Measurements were taken in triplets and mean values are reported).

3.7. Operational and storage stability

The same fabricated enzyme dip-strip was used for successive experiments using 200 μg mL^{-1} β-glucan solution as substrate to check its reusability. Results suggest that strips with β-glucanase and GOD can be reused for at least 10 times with 87% of its initial sensitivity. While strip with β-glucanase and GOD-POD reagent can be used for 14 cycles with 80% response (see supplementary data section).

Both systems were preserved under moisture free conditions at 4 °C and room temperature to check the shelf life of the enzyme dip-strips. In this study, both systems showed half-life of 40 days when stored at room temperature and 65 days at 4 °C (data not shown). A short-term stability was observed in case of chemical immobilization due to chemical modification of enzyme which creates disturbance in the three-dimensional structure of the biological moiety (Munjal and Sawhne, 2002; Bora et al., 2006). The

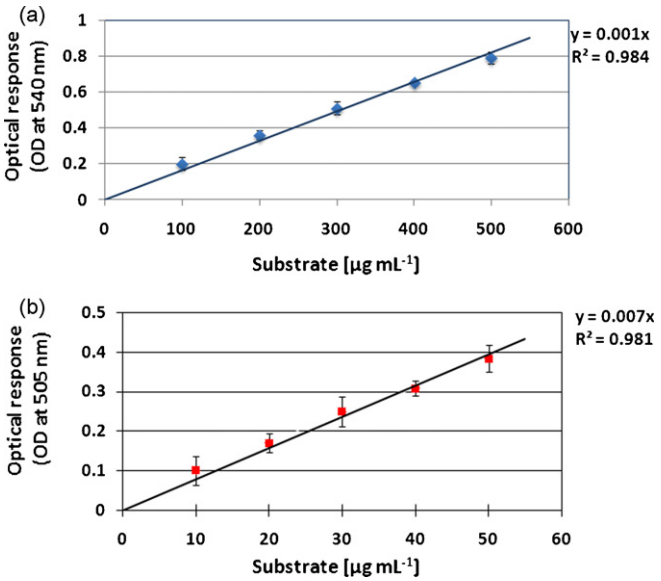


Fig. 3. Calibration curves for (a) System-I based on DNSA method and (b) System-II based on AAP method (*experimental conditions*: laminarine, 100–500 μg mL^{-1} (System-I), 10–50 μg mL^{-1} (System-II); buffer, 0.2 M sodium phosphate buffer pH 5.5; temperature, 55 °C. Measurements were taken in triplets and mean values are reported).

Table 3
Determination of β-glucan in pharmaceutical preparations using dip-strips.

Sample details	β-Glucan estimation by standard method	β-Glucan estimation by fabricated dip-strips
Sample A (1 Capsule = 500 mg) (β-glucan derived from fruit body Reishi and Hoelen extract)	389 μg/Capsule ± 6.1	373.74 μg/Capsule ± 4.0
Sample B (1 Capsule = 350 mg) (β-glucan derived from fermentation of wall-broken spores and mycelium of *Ganoderma lucidum*)	225 μg/Capsule ± 4.3	218.88 μg/Capsule ± 2.8
Sample C (1 Capsule = 300 mg) (β-glucan from mushroom extract)	270 μg/Capsule ± 5.6	257.78 μg/Capsule ± 5.0
Sample D (1 Capsule = 550 mg) (β-glucan derived from fruiting body of *Ganoderma tsugae* and *Cordyceps sinensis* mycelia)	400 μg/Capsule ± 2.1	387.03 μg/Capsul ± 3.4
Sample E (1 Bottle = 62 ml) (β-glucan isolated from Fermented product from mycelia of Cordyceps sp., Aastraglus extract, etc.)	6000 μg/Bottle ± 3.7	5818 μg/Bottle ± 3.1

Note: All the concentrations were measured in triplicate and mean values are report with percentange error.

longer half-life of enzyme dip-strips developed in this study may be attributed to hydrophilic composite membrane which may provide favorable microenvironment to the molecules imprisoned within. On further storage, slow decrease in the response was observed in both cases due to inactivation of enzymes with time.

3.8. Calibration curve for both systems

The calibration curves for β-glucan were obtained using all of the optimized parameters for both systems. The response for System-I was found to be concentration dependent in the range of 100–500 μg mL^{-1} while for System-II, it was observed to be 10–50 μg mL^{-1}. Limit of detection is considered to be the experimental lower extreme of the linear dynamic range (calibration curve). The lower detection limit for System-I and System-II were found to be 65 μg mL^{-1} and 10 μg mL^{-1}, respectively (Fig. 3a and b). Our observations suggest that both systems are quite satisfactory in terms of ease of fabrication and operation. The lower detection limit obtained by designed photometric System-II (10 μg mL^{-1}) is superior to System-I. Similar sensitivity for β-glucan has been reported by amperometric biosensor (LOD = 50 μg mL^{-1}) (Miyanishi et al., 2004). Also the linear behavior with high sensitivity towards analyte, fast response, stability presented by the System-II is much better than System-I, therefore it was chosen for analysis of different pharmaceutical samples.

3.9. Pharmaceutical sample testing

Table 3 gives the assay results of some commercial samples using standard method and the sensing strip (System-II). The results indicate that fabricated sensing strips are reliable and comparable with that of the standard fluorescence method (Kim et al., 2004; Fuchs et al., 2007; Sekiya-Kawasaki et al., 2002). The standard fluorescence method can detect all forms of soluble glucans while the enzyme based sensor specifically detect β(1 → 3)-D-glucan, this may attribute to the slightly lower values obtained by enzyme dip-strips as compared to standard method.

These results indicate that dip-strips possessed good detection precision and fabrication reproducibility. Thus, as compared to fluorometric assay method, fabricated dip-strips provide fast and stable detection with less total reaction as well as sample volume. These enzyme-strips can be used for wide concentration range of glucan with considerable reusability feature.

4. Conclusions

The enzyme β(1 → 3)-D-glucanase was isolated from a novel source, i.e. moth bean (*V. aconitifolia*) sprouts. The use of crude enzyme from plant material makes sensing system more economical as compared to use of commercial enzyme. The immobilization of moth bean β-glucanase in agarose–corn flour–gelatin composite matrices was successful, and the fabricated sensor dip-strips can detect β(1 → 3)-D-glucan specifically and rapidly. Features of the enzyme dip-strips include reproducibility, fast response time, wide optimum pH range, long shelf life and stable against thermodynamic inactivation with good operational stability. The dip-strip System-II based on co-immobilization was more sensitive and fast than System-I (single enzyme immobilization), and the detection limit was up to 10 μg mL^{-1} in the dynamic range 10–50 μg mL^{-1}. The superior dip-strip test System-II was also able to quantify β-glucan from different pharmaceutical samples available in the market with high accuracy when compared with standard fluorescence method.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2009.01.004.

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