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Co-immobilized glucose oxidase and β-galactosidase on bovine serum albumin coated allyl glycidyl ether (AGE)–ethylene glycol dimethacrylate (EGDM) copolymer as a biosensor for lactose determination in milk

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ABSTRACT

Bovine serum albumin (BSA) was adsorbed on allyl glycidyl ether (AGE)–ethylene glycol dimethacrylate (EGDM) copolymer with 25% crosslink density (AGE-25) at pH 8.0 (16% w/w). The amino, thiol and carboxylic acid functional groups available on protein coated surface were utilized for covalent immobilization of glucose oxidase and β-galactosidase, both independently, and in a step-wise manner on the same matrix, with no more than 10% loss of enzyme activity during immobilization. Glutaraldehyde cross-linking after immobilization provided stable enzyme preparations. The pH-optima of the immobilized enzymes were similar to those for free enzyme but their thermal stability was vastly improved. The co-immobilized enzyme support was used as a biosensor for determination of lactose in milk with excellent reproducibility and reusability.

1. Introduction

During past few years, several methods, ranging from simple adsorption and entrapment to covalent binding, have been developed to prepare biosensors based on immobilized enzymes [1–6]. Since covalent immobilization provides most stable enzyme preparation, a variety of ways to fabricate a biosensor with covalently immobilized enzymes have evolved [7]. Most popular technique is to immobilize the enzyme directly on the surface of an electrode and measure the signal using an electronic devise [8]. These electrochemical biosensors need sophisticated technology and are expensive. On the other hand, colorometric methods based on colour changes due to enzymatic reaction are cheap, simple and quite reliable [9,10]. One of the important applications of a biosensor in dairy industry is assay of lactose in milk and milk products. A large population is lactose intolerant and suffers from digestive disorders due to deficiency of enzyme β-galactosidase (β-gal). For such consumers, lactose free milk products are produced after lactose hydrolysis, generally by treatment with β-gal which hydrolyzes lactose into glucose and galactose. Considering the commercial importance, a variety of immobilization protocols have been developed for immobilization of β-gal on solid supports, for example, sephadex, alginate, κ-carrageenan, chitosan, porous glass, agarose, polyvinyl alcohol, polymers, diethylaminoethyl cellulose, nylon, polyurethane foams, zeolite etc. The carrageenan–chitosan systems [11], epoxy-activated acrylic beads [12] and amino-epoxy-Sepabeads containing both amino and epoxy groups [13] are particularly interesting in production of 'lactose free' milk and processing of cheese whey.

Although highly efficient, enzymatic processing of milk does leave varying amounts of residual lactose which needs quantification. Current available methods for the determination of lactose include gravimetric method [14], mid-infrared detection [15–17], polarimetry [18,19], HPLC [20–23] and enzymatic assays [24,25]. The gravimetric method is an old method and quite tedious while infrared and polarimetric methods are not specific to lactose. Although HPLC methods are accurate, these are expensive and require sophisticated instruments. The enzymatic methods are the most preferred ones since these are highly specific and relatively

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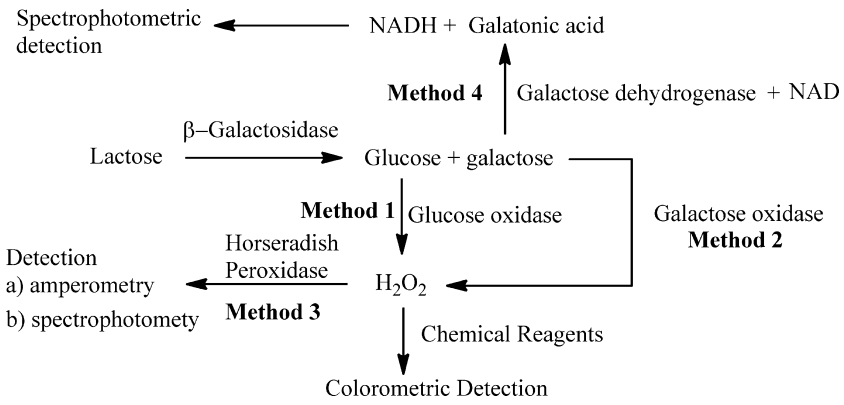

Scheme 1. Methods of lactose determination.

inexpensive. The cost of enzymes is recovered by using them in immobilized form so that they could be reused several times. The most commonly employed methodologies for lactose assay use combinations of two or three enzymes [26]. Basically, the first enzyme β-gal hydrolyses lactose to galactose and glucose. Galactose can be assayed with galactose dehydrogenase which reduces one equivalent of cofactor NAD to NADH. Alternatively, glucose produced during the reaction can be oxidized with glucose oxidase (GOx). One equivalent of hydrogen peroxide is liberated which can be assayed in different ways (Scheme 1).

Such assays can be made with all the required enzymes dissolved in same assay mixture. However, they need to be discarded after the assay. To reduce cost, the enzymes can be immobilized and reused. In this context, it is advantageous to use co-immobilized enzymes on same support rather using a mixture of different supports, each with one enzyme. Apart from the obvious advantage of using a single matrix, proximity of the co-immobilized enzymes means short diffusional distance for the product of first enzyme which acts as reactant for second enzyme. Additionally, the local concentration of the substrate for second enzyme is comparatively higher in such a microenvironment. This makes the system more sensitive towards low concentration of substrate than a corresponding system where the enzymes are separately immobilized [27,28].

There are several reports of co-immobilized enzymes for making a lactose biosensor [29–39]. Most of the methods are based on deposition or entrapment. Such enzymes are prone to leaching on repeated use. A better technique to obtain co-immobilized β-gal and GOx on a polymeric support with covalent linkages and high stability is desirable. Recently, we have developed a methodology of using bovine serum albumin (BSA) coated allyl glycidyl ether–ethylene glycol dimethacrylate copolymer for trypsin immobilization [40]. Polymer bound BSA provide naturally existing functional groups such as —SH, —NH_2 and —COOH which can be used to bind an enzyme through covalent bonds. In this communication we describe application of this methodology for immobilization of GOx from *Aspergillus niger* (E.C. 1.1.3.4; a homodimer 160 kDa) [41] and β-gal from *Aspergillus oryzae* (E.C. 3.2.1.23, monomer 105 kDa) [42], both independently and sequentially on the same matrix without going through the complicated synthesis of a specially designed functionalized polymer matrix. The co-immobilized enzyme preparation can be reliably used to analyze lactose present in raw milk using a colorometric method.

2. Materials and methods

2.1. Materials

Bovine serum albumin (fraction V, cat. no a-7030), GOx from *A. niger* (cat. no. G7141), β-gal from *A. oryzae* (cat. no. G5160), 1-ethyl-3-[3-dimethylaminopropyl]carbodimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), dithiothreitol (DTT) and 4-morpholineethanesulfonic acid sodium salt (MES) were obtained from Sigma–Aldrich, USA. *N,N′*-Disuccinimidyl carbonate (DSC) was obtained from Alfa Aesar, U.K. Xylenol orange and ferrous ammonium sulfate hexahydrate were obtained from Fluka. All other reagents and solvents were analytical grade obtained from Hi Media, India. The macroporous AGE–EGDM copolymer with relative mole ratio of the cross-linking co-monomer (ethylene glycol dimethacrylate) to epoxy functional monomer (allyl glycidyl ether) of 0.25 (AGE-25) has been described earlier [43]. The polymer beads with average particle size of 150–250 μm had specific surface area (BET) of 250 m^2/g, pore volume of 0.68 mL/g and epoxy group content of 5 mmol/g. UV–vis spectrophotometric measurements were performed on Cary 100 UV–vis spectrophotometer equipped with temperature control and Cary Win UV software. All enzyme assays were performed at 30 °C. All experiments were repeated 3 times and were reproducible within (±5%). Analysis of experimental data was performed using Graph Pad Prism 5 (www.graphpad.com).

2.2. Enzyme immobilization on BSA coated polymer

The preparation of BSA coated polymer, and enzyme immobilization experiments were performed essentially as described earlier [40]. In short, the polymer beads were treated with BSA solution (30 mg/mL) in potassium phosphate buffer (0.05 M, pH 8.0) overnight. The polymer, surface coated with BSA (160 mg/g), was washed with buffer and freeze dried. The reactive functional groups present in BSA were activated with appropriate reagents. For example, the amino groups were activated with glutaraldehyde, the thiol groups with disuccinimidyl carbonate (DSC) and the carboxylic acid groups with EDC following known protocols. The activated polymer was stirred with enzyme solution (GOx or β-gal 5 mg/mL, 5 mL) for 2 h. The polymer beads were separated, washed with phosphate buffer (5× 2 mL), freeze dried and stored in refrigerator.

2.3. Enzyme activity measurements

All enzyme assays were performed in a temperature controlled double walled vessel at 30 °C and contents were stirred at 100 rpm with a small mechanical stirrer. All experiments were repeated 3 times and were reproducible within ±5%. The specific activity of enzyme bound to the polymer (units/g) was calculated on the basis of enzyme activity observed for free enzyme (units/mg). GOx activity was assayed by measuring amount of hydrogen peroxide produced during enzyme catalyzed oxidation of glucose using Ferrous Oxidation–Xylenol orange (FOX) reagent containing 1.25 M perchloric acid [44,45]. The enzyme (free, 5 mg; or immobilized, 100 mg) were stirred with glucose solution (10 mL, 3% w/v in 0.05 M sodium acetate–0.05 M NaCl buffer, pH 5.1). Aliquots of reaction mixture (5 μL) were added to FOX reagent (3 mL) and after incubation for 15 min, absorbance was measured at 560 nm ($\Delta\varepsilon = 4.25 \times 10^4\ M^{-1}\ cm^{-1}$). For β-gal assay, the enzyme (free, 50–500 μg; or immobilized, 100 mg) was stirred with lactose solution (10 mL; 5% in sodium acetate buffer, 0.1 M; pH 4.6) for 1 h. Aliquots of samples (50–100 μL) were collected at intervals of 10 min, heated at 90° C for 10 min, cooled to 30 °C and then incubated with GOx solution (100 μL, 5 mg/mL in 0.05 M sodium acetate–0.05 M NaCl buffer, pH 5.1) for 1 h at 30 °C. An aliquot of reaction mixture (5 μL) was assayed for H_2O_2 with FOX reagent (3 mL) as described above.

2.4. Determination of lactose in milk samples

The polymer bearing co-immobilized GOx and β-Gal (25 mg) was shaken with milk sample (diluted 10 times with distilled water, 1 mL) at 30 °C for 1.5 h in an Eppendorf tube on an orbital shaker at 200 rpm. The supernatant (5–10 μL) was directly added to FOX reagent (3 mL) and assayed for H_2O_2.

Table 1
Covalent binding of GOx and β-gal on AGE-25[a].

Polymer support	Enzyme	Initial activity (units)	Specific activity of immobilized enzyme (units/g)	Activity in supernatant (units)	Loss in activity (%)
Uncoated epoxy polymer	GOx	3250	590	812	66
	β-gal	250	2.3	238	4
BSA coated AGE-25 (coupling through —NH_2 with glutaraldehyde)	GOx	3250	468	2716	9.2
	β-gal	250	17	230	1.2
BSA coated AGE-25 (coupling through —SH with DTT/DSC)	GOx	3250	1104	2430	8.2
	β-gal	250	52	178	8.0
BSA coated AGE-25 (coupling through —COOH with EDC)	GOx	3250	884	2530	8.6
	β-gal	250	170	70	4
BSA coated AGE-25 after sequential binding	GOx	3250	935	2457	10.0
	β-gal	250	207	39	2.6

[a] The activated polymer (500 mg) was treated with enzyme solution (5 mg/mL, 5 mL). All experiments were repeated 3 times and the values are mean of 3 determinations with error of ±5%

3. Results and discussion

3.1. Covalent binding of GOx and β-gal to BSA coated polymer employing different methods

In principle, the BSA coated polymer possesses free —NH_2, —COOH and —SH groups of Lys, Asp & Glu and Cys respectively, available for coupling reactions. Depending upon accessibility, they could be used after activation. Thus the amino groups of BSA were activated with glutaraldehyde, the thiols with disuccinimidyl carbonate (DSC), and the carboxylic acid groups with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC). The amino groups on the enzyme surface react with these activated functional groups and provide covalently immobilized enzyme preparations. In all the three approaches, loss of enzyme activity was no more than 10%. In comparison, GOx lost 65% activity due to adsorption induced denaturation [43,46] when native uncoated epoxy polymer was used. In case of β-gal, binding to the native polymer was negligible. The results are given in Table 1.

3.2. Sequential loading of GOx and β-gal

During the immobilization process, after using one functional group of BSA, for example —NH_2, one could expect that the other functional groups, i.e., —COOH and —SH would be still available for enzyme coupling and, in principle, it should be possible to increase enzyme loading by immobilizing the enzyme in a sequential manner. To explore this possibility, the immobilized β-gal obtained by binding through —NH_2 groups (glutaraldehyde crosslinking method, activity 17 units/g) was treated with DTT solution. The —S—S— linkages were reduced to —SH and activated with DSC. The succinimidyl activated preparation was treated with fresh enzyme solution to obtain immobilized β-Gal with activity of 63 units/g. Finally, this enzyme preparation was again coupled with β-Gal via EDC coupling. The polymer now had activity of 207 units/g. In case of GOx, the enzyme was first coupled through —SH (since it is a dimer with S-S bridges which would be destroyed when treated with DTT) to obtain immobilized enzyme with activity of 1104 units/g. Increase in enzyme loading was attempted by activating the —NH_2 and —COOH groups and coupling with fresh enzyme. In this case unfortunately, we did not see any improvement in enzyme loading, probably because the enzyme is large and cannot accommodate too many neighbouring enzyme molecules. The enzymes preparations with maximum specific activity were used for further studies.

3.3. Stability and recycle of immobilized enzymes

Stability of immobilized GOx (immobilized via —SH) and β-gal (immobilized via —COOH) were studied for 5–6 recycles. Immobilized GOx lost its activity rapidly and retained only 20% of its activity in 4 recycles while immobilized β-gal lost 30% of its activity after first use and 10% more after 2nd recycle, but retained the remaining 60% activity in further recycles. To improve the enzyme stability after binding to the polymer, the enzyme preparations were treated with glutaraldehyde to provide multi-point attachments and better stabilization.

As shown in Fig. 1, glutaraldehyde treated enzyme preparations show excellent stability. Further studies were thus made using the immobilized enzymes stabilized by glutaraldehyde treatment.

3.4. Kinetic studies

The apparent Michaelis constants, $K_{m,app}$ and $V_{max,app}$ were determined from the initial rates by using glucose and lactose as substrates for GOx and β-gal respectively (Table 2). Generally, $K_{m,app}$ values for enzymes increase after immobilization due to external as well as internal mass transfer limitations. However, the values obtained for both free and immobilized β-gal are similar, and close to reported value of 52 ± 3 mM [47]. In case of GOx, K_m for the free enzyme is close to that reported in literature [48–52] but $K_{m,app}$ for the immobilized enzyme is surprisingly lower. Similar decrease in $K_{m,app}$ for GOx has been made by several workers earlier and is attributed to a change in microenvironment after immobilization [53,54].

3.5. Effect of pH on the enzyme activity

The effect of pH on activity of GOx and β-gal was studied to understand if the basic and acidic amino acid residues of BSA cause any shift in pH optima. In these experiments, 3% glucose and 5% lactose solution were prepared in different buffers of required pH and incubated with free or immobilized enzyme for 1 h. The results in Fig. 2 show that both immobilized and free enzyme have pH-optimum near pH 5–6 for GOx and pH 4.5–5.5 for β-gal. Although immobilization on surfaces with ionic groups could be expected to alter the pH-optima as observed in case of trypsin [40], in the present case, there was no distinct change.

3.6. Effect of temperature on the free and immobilized enzymes

Temperature effect on activity of free and immobilized forms of the enzymes were studied in temperature range of 30–60 °C. The

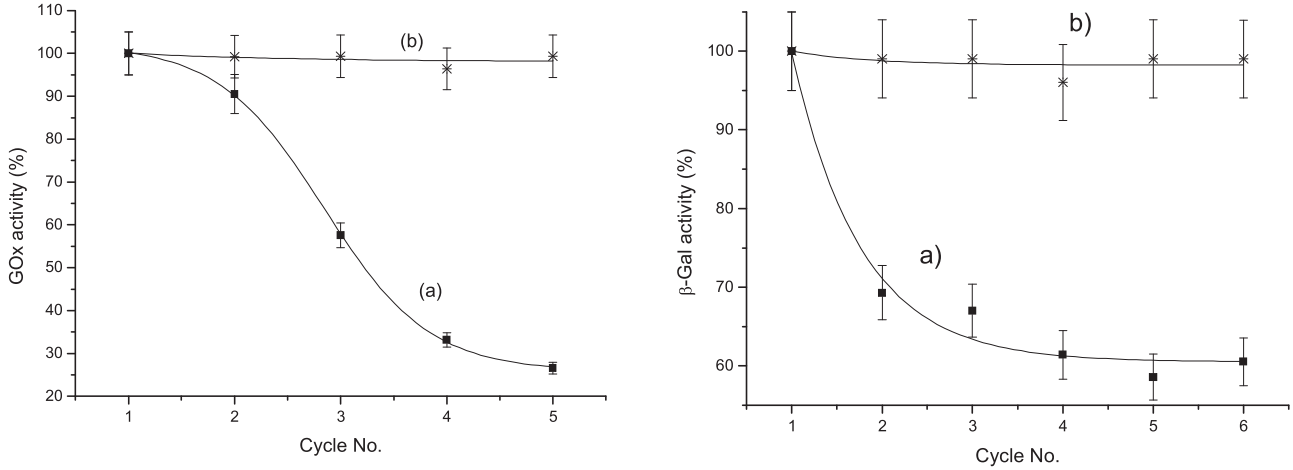

Fig. 1. Stability and recycling of GOx immobilized through —SH groups of BSA and β-gal immobilized through —COOH groups of BSA (a) without glutaraldehyde treatment, and (b) treated with glutaraldehyde after immobilization. The data points represent mean of 3 determinations reproducible within ±5%. Error bars represent the standard error of the mean.

Table 2
Kinetic parameters of free and immobilized enzymes[a].

Enzyme	K_m (mM)		V_{max}	
	Free enzyme	Immobilized	Free enzyme (units/mg)	Immobilized (units/g)
GOx	19.30 (±2)	6.1 (±0.7)	130 (±13)	1020 (±100)
β-gal	42.1 (±4)	47.40 (±4.5)	10 (±1)	208 (±20)

[a] GOx in 0.05 M acetate-NaCl buffer, pH 5.1. β-gal in 0.1 M sodium acetate buffer pH 4.6 at 30° C. Reaction volume 10 mL. Immobilized enzyme 500 mg; soluble enzyme 1 mg/mL. All assays were repeated 3 times and the calculated kinetic parameters are mean of 3 determinations.

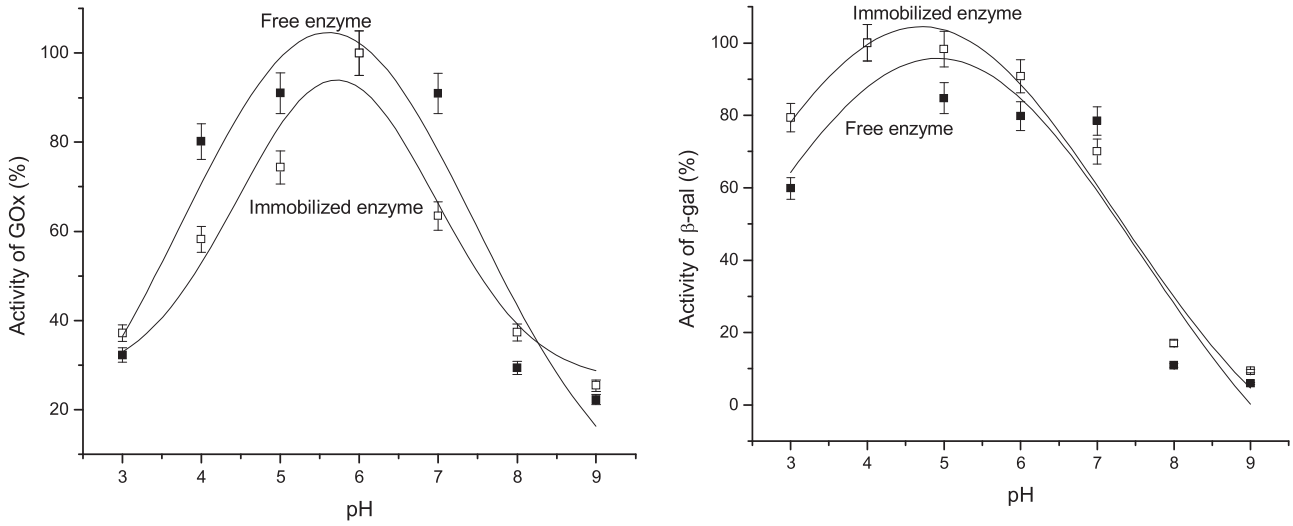

Fig. 2. Effect of pH on the activity of free and immobilized enzymes. [glucose] = 0.16 M, [lactose] = 0.1 M, [enzyme] = 5 mg/mL in buffer and [polymer] = 25 mg. Residence time 1 h at 35° C. Reaction volume 2 mL. The data points represent mean of 3 determinations reproducible within ±5%. Error bars represent the standard error of the mean.

samples were assayed after incubation at given temperature for 1 h. The optimum temperature for the activity of the GOx was 40 °C and that for β-gal was 50° C in case of soluble enzymes. Immobilized GOx is found to be thermally more stable than free enzyme and can operate over a broader range of temperatures between 30 °C and 60 °C but β-gal does not attain extra thermal stability (Fig. 3) after immobilization on BSA coated matrix.

3.7. Co-immobilization of GOx and β-gal

The success of our approach prompted us to explore if the two enzymes could be bound to the same matrix and function in a cooperative manner. This would be of great use in assay of lactose in milk and milk products. Thus immobilized GOx obtained by binding through —SH linkage using DTT-DSC protocol (activity 1104 units/g) without subjecting it to glutaraldehyde treatment was used to immobilize β-gal through —COOH functionality using EDC coupling protocol. The recovered polymer exhibited GOx activity of 487 units/g and β-Gal activity of 30 units/g. The decrease in GOx activity during β-Gal immobilization could be due to denaturation of the protein during chemical reaction of EDC or loss of loosely bound enzyme. After binding of β-Gal, the polymer beads were treated with glutaraldehyde to provide further stabilization to the immobilized enzymes. At this stage the

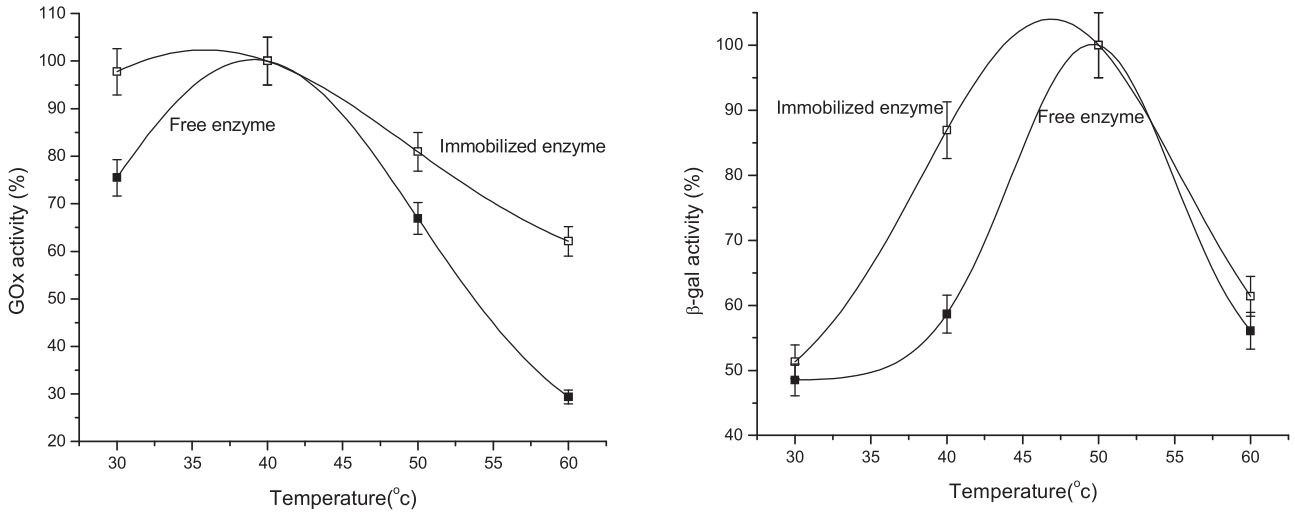

Fig. 3. Effect of temperature on the activity of free and immobilized enzymes. [glucose] = 0.16 M, [lactose] = 0.1 M, [polymer] = 25 mg and [enzyme] = 5 mg/mL in buffer. Residence time 1 h at 35° C. Reaction volume 2 mL. The data points represent mean of 3 determinations reproducible within ±5%. Error bars represent the standard error of the mean.

activities of both the enzymes were unaffected by glutaraldehyde treatment. The $K_{m,app}$ and $V_{max,app}$ were again determined for β-Gal and GOx using lactose and glucose as substrates. The values of $K_{m,app}$ were unchanged for both the enzymes. Since GOx activity (487 units/g) is sufficient to ensure complete oxidation of glucose to gluconic acid produced during hydrolysis of lactose by co-immobilized β-Gal (30 units/g), performance of this co-immobilized polymer was evaluated for assay of lactose in milk.

3.8. Choice of FOX reagent for assay of H_2O_2

Assay of hydrogen peroxide employing colorimetry can be done in a variety of ways. The most popular assay procedures employ enzyme horseradish peroxidase in combination with *p*-anisidine or *ortho*-dianisidine [55]. This procedure requires an extra enzyme and also the reaction is performed in aqueous buffer. Although we did not face any problems when using pure lactose or glucose which are completely soluble in water, raw milk, which contains fats and proteins along with lactose gave problems due to precipitation and emulsion formation. In other assay procedures, the proteins and fats are removed using clarification reagents such as Carrez reagent [56,57] and the clear filtrate is used for assay. However, this procedure is an extra step which can introduce some element of uncertainty. It was desirable to treat raw milk directly with the enzyme and measure the amount of hydrogen peroxide by injecting an aliquot into an assay reagent without needing any separation steps. FOX reagent containing 110 mM perchloric acid [44,45] was found to be most appropriate for our application. The reagent contains ferrous ammonium sulphate–xylenol orange redox system which produces a distinct change in colour from yellow to brown in the presence of hydrogen peroxide. Also, the molar extinction coefficient of 42,500 M^{-1} cm^{-1} is three times higher than that for *ortho*-dianisidine assay (11,300 M^{-1} cm^{-1}) and provides higher sensitivity. Milk sample diluted 10 times with plain distilled water was used for enzymatic reaction and very small volumes (5–50 μL) of the reaction mixture were needed for assay of hydrogen peroxide. It was possible to obtain accurate absorbance readings at 560 nm without any interference from suspended/emulsifying impurities.

3.9. Determination of lactose in milk samples

The BSA coated polymer bearing co-immobilized β-gal and GOx was shaken with milk sample for 1.5 h and the supernatant was directly assayed for H_2O_2 with FOX reagent. Measurement of absorbance as a function of time showed that an incubation period of 90 min was adequate for reliable and reproducible results. At fixed enzyme concentration, the values of Abs_{560} were accurately predicted by the Michaelis–Menten constants, $K_{m,app}$ and $V_{max,app}$ for GOx (Table 2) and the plot of Abs_{560} against [lactose] in the range 5–30 mM (1.7 mg/mL to 10 mg/mL, after taking the dilution factors into consideration) was linear as shown in Fig. 4 ($n = 6$, intercept = 0.0811 ± 0.0081; slope = 0.01112 ± 4.15734E−4; $r = 0.997$). Above this range the plot tends to curve probably due to competitive inhibition by galactose. Similarly, at fixed lactose concentration, the plot of Abs_{560nm} against [enzyme] was also linear. A standard calibration chart for absorbance vs [lactose] was prepared using lactose as substrate and several samples of milk and

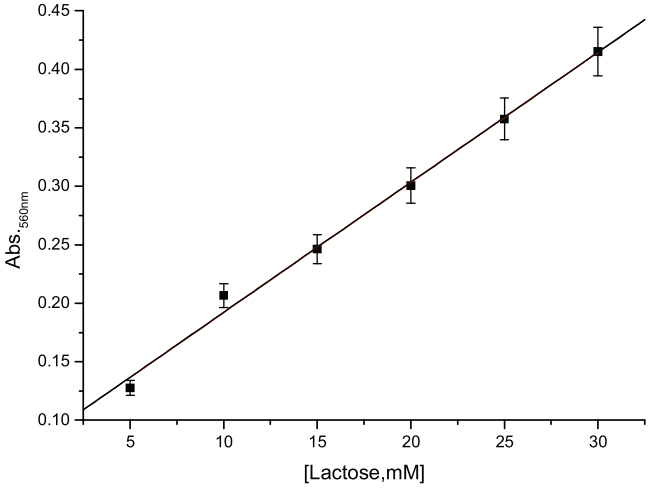

Fig. 4. Linear relationship between absorbance at 560 nm and lactose concentration after contact with co-immobilized GOx and β-gal. Wt of immobilized enzyme 25 mg, reaction volume 1 mL in distilled water. Reaction period 90 min. No. of points, $n = 6$, intercept = 0.0811 ± 0.0081; slope = 0.01112 ± 4.15734E−4; $r = 0.997$. The data points represent mean of 3 determinations reproducible within ±5%. Error bars represent the standard error of the mean.

Table 3
Concentrations of lactose in different milk samples purchased from local supermarket*.

Sample	Lactose content (%, w/v)
Full cream milk	6.3
Cow milk	5.5
Toned milk	6.2
Butter milk	2.6
Whey from full cream milk	5.8

* The values are mean of 3 determinations with error of ±5%.

milk products from local supermarket (Table 3) were assayed for lactose content.

4. Conclusion

Glucose oxidase and β-galactosidase can be easily co-immobilized on BSA coated polymers without serious loss of activity during immobilization. Glutaraldehyde crosslinking provides stable enzyme preparation which can be used for lactose assay in milk and milk products. Lactose concentrations as low as 0.17 mg ml^{-1} can be accurately detected. The method requires simple equipment and is of low cost.

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