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Laccase mediator system for activation of agarose gel: Application for immobilization of proteins

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ABSTRACT

Cross-linked Sepharose beads were treated with laccase–TEMPO system for oxidation of the primary alcohol groups on the sugar moieties. Optimal activation conditions using *Trametes versicolor* laccase were at pH 5 and 22 °C, giving an aldehyde content of 55 μmol g^{-1} Sepharose with 28 units g^{-1} of laccase and 12.5 mM TEMPO. The activated Sepharose was used for immobilization of trypsin as model protein. Highest degree of immobilization was obtained at pH 10.5 but the activity yield was only 31% of that loaded on the gel. The yield of gel bound trypsin activity was increased to 76% (corresponding to about 43 U g^{-1} Sepharose) when the immobilization was performed in the presence of trypsin inhibitor, benzamidine. The immobilization yields were comparable to that obtained on the matrix activated using sodium periodate (containing 72 μmol aldehyde per g Sepharose). Recycling and storage of the immobilized trypsin preparations showed high stability of the enzyme bound to laccase–TEMPO activated gel.

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1. Introduction

Laccases (p-diphenol:oxygen oxidoreductase, EC 1.10.3.2) are enzymes belonging to a family of multicopper oxidases, which catalyse the oxidation of various substrates with simultaneous reduction of molecular oxygen to water. White-rot fungi constitute the major source of laccases; however these enzymes are also produced by plants, bacteria and insects [1–3]. Laccases are rather non-specific with respect to the reducing substrates and are able to oxidize a number of organic compounds including phenolic compounds, anilines, benzenethiols, etc., and some inorganic ions [1,4]. The repertoire of substrates that can be oxidized by laccases is broadened in the presence of small redox mediators – compounds that are first oxidized by laccase and the oxidized form in turn acts as an oxidant for the substrates. As the mediator can function as a diffusible electron carrier, oxidation of macromolecular structures is also enabled. The laccases and laccase mediator systems (LMS) have offered an environmentally benign means of oxidation, which has been used in a range of applications including decolorization of dyes, bioremediation, paper pulp bleaching, cosmetics, and organic syntheses [4,5].

Earlier studies have demonstrated oxidation of alcohols to carbonyl compounds by laccase in the presence of different mediators such as TEMPO (2,2,6,6-tetramethyl-1-piperidinyloxy), ABTS (2,2′-azinobis-bis (3-ethylbenzthiazoline-6-sulfonic acid)), 3-hydroxyanthranilic acid, viorulic acid, and 1-hydroxybenzotriazole [6,7]. Later, this chemo-enzymatic approach was used for regio-selective oxidation of the primary alcohols of sugar derivatives, natural glycosides, and partial oxidation of water-soluble cellulose [8,9]. Selective oxidation of the hydroxyl groups on sugar derivatives is useful for further modifications of carbohydrates to bioconjugates and biopolymers [10].

Polysaccharides are commonly used as insoluble matrices for chromatography of proteins and also for immobilization of proteins and affinity ligands. Agarose (a polymer of (1 → 3)-β-D-galactopyranose-(1 → 4)-3,6-anhydro-α-L-galactopyranose) based gels are among the most widely used matrices, having the advantages of being hydrophilic and compatible with biomolecules. Crosslinking of gels further increases their mechanical- and chemical stability. Immobilization of ligands to such matrices requires activation of the hydroxyl groups on the sugar components of the polysaccharide. Oxidation of the alcohol groups to aldehyde is one of the activation methods used and a ligand containing amino group(s) is then coupled to the oxidized groups by Schiff base reaction [11,12].

In this paper, we demonstrate the potential of LMS system for activation of a commercially available cross-linked agarose matrix, Sepharose CL-6B, for subsequent immobilization of proteins or other ligand molecules bearing amino groups. This approach has

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been compared with the conventional method for matrix activation via sodium periodate oxidation.

2. Experimental

2.1. Chemicals

Sepharose CL-6B was purchased from GE-Healthcare (Uppsala, Sweden). Laccase from *Trametes versicolor* (24 U mg^{-1}; one unit of laccase is defined as the amount of enzyme that catalyses the oxidation of 1 μmol 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid per min), trypsin (EC 3.4.21.4) from bovine pancreas, TEMPO (2,2,6,6-tetramethylpiperidine 1-oxyl), bicinchoninic acid, bovine serum albumin, N-α-benzoyl-DL-arginine-p-nitroanilide (BAPNA), benzamidine and 1,3-dinitrophenylhydrazine (DNPH) were obtained from Sigma–Aldrich (Germany). All chemicals used were of analytical quality and were obtained from standard sources.

2.2. Laccase–TEMPO activation of Sepharose CL-6B

Sepharose CL-6B was washed a few times with 25 mM sodium acetate buffer pH 5, centrifuged at 500 × *g* for 2 min and then the liquid was removed using a Pasteur pipette. One gram wet weight gel was suspended in 0.5 ml of the buffer in 15 ml tubes, supplemented with about 28 units of laccase (57 U ml^{-1}) and 12.5 mM TEMPO, and mixed on a rocking table at room temperature. Treatment with LMS was stopped at a defined time interval (up to a period of 48 h) by centrifuging the tubes and removing the liquid. The gel beads were analysed for determination of carbonyl groups.

The effect of pH (3–8) and temperature (20–45 °C) on laccase–TEMPO catalysed oxidation of Sepharose was examined. The effect of laccase (0–100 units per g of Sepharose) and mediator (0–25 mM) concentrations was evaluated by a factorial design analysis using the program statistic version 6.0.

2.3. Sodium periodate activation of Sepharose CL-6B

Oxidation of hydroxyl groups on Sepharose using sodium periodate was based on the modified method of Miron and Wilchek [13]. Sepharose CL-6B (1 g wet wt) was treated with 0.1 M sodium periodate (3 ml) solution in water for 3 h at room temperature. Subsequently, the gel was washed with water and analysed for determination of carbonyl groups.

2.4. Immobilization of trypsin to activated Sepharose CL-6B

To 0.5 g wet wt activated Sepharose CL-6B in a 15 ml tube was added 2 ml of a solution of trypsin (2.5 mg ml^{-1}, 5.7 U mg^{-1}) in 20 mM sodium phosphate buffer (pH 7), sodium bicarbonate (pH 8) and sodium carbonate buffer (pH 10.5), respectively. The tubes were placed on a rocking table, mixed for 2 h at room temperature, and then centrifuged at 500 × *g* for 2 min. Samples of the supernatant were withdrawn to quantify unbound protein concentration and trypsin activity. The gel with the bound enzyme was treated for 3 h with 2 mmol sodium borohydride dissolved in 10 ml of the respective buffers used for immobilization. Sepharose–trypsin was washed with 20 mM phosphate buffer, pH 7 and stored at 4 °C prior to analysis.

Immobilization of trypsin was also performed in the presence of 20 mM benzamidine. After immobilization, the gel was centrifuged to remove the supernatant, treated with sodium borohydride and washed with phosphate buffer, pH 7 as mentioned above. The effect of enzyme loading (2.5–10 mg ml^{-1}) and incubation time on the degree of enzyme immobilization was studied. The amount of immobilized protein (mg per g Sepharose) was estimated by the difference between the original amount used for immobilization and that left in the supernatant.

2.5. Determination of carbonyl groups on the activated gel

2.5.1. Aldehyde content

Quantitative analysis of the aldehyde content of the activated Sepharose was performed by reacting the aldehyde groups with DNPH and measuring the unreacted DNPH by HPLC (Perkin Elmer, Norwalk, CT) on a RP-18 column (4.6 × 250 mm, pore diameter 5 μm, Merck, Germany). Fifty milligram of chemically or enzymatically activated Sepharose was incubated with 50 μl 0.1 M DNPH stock solution (prepared in a solvent system composed of 1:1 2 N HCl:acetonitrile) for 20 min. The solution was extracted with 250 μl ethyl acetate and 100 μl of the extract solution was dried and dissolved in 500 μl acetonitrile for injecting into HPLC. The column was eluted by a gradient of solvent A (30% (v/v) acetonitrile in water) and B (100% acetonitrile) starting from 90 vol A:10 vol B to 10 vol A:90 vol B for 15 min, and then 10 vol A:90 vol B for 5 min at a flow rate of 1 ml min^{-1}. Elution was monitored at 356 nm ($RT_{DNPH} \approx 7$ min). The consumption of DNPH was calculated from the peak areas and compared with a standard curve of 0–0.1 M DNPH prepared and analysed under the same conditions. One mol of aldehyde corresponds to one mol of DNPH consumed [14].

2.5.2. Carboxylic group

Activated Sepharose samples were dried overnight at 60 °C and then ground in a mortar. The powder (approximately 0.1 g) was mixed with 10 ml of 0.1 N NaOH containing 0.2 M NaCl, and the reaction mixture was mixed for 2 h on a rocking table at room temperature. The insoluble fraction was separated by centrifugation (2500 × *g*, 10 min), and 5 ml of the supernatant was removed and titrated with 0.1 M HCl until the pH reached a value of 6.9. The content of carboxylic acid was calculated using the equation:

$$\text{moles per gram} = \frac{C_{HCl} \times (V_{NaOH} - V_{HCl})}{m} \quad (1)$$

where V_{HCl} (in l) is the volume of 0.1 N HCl needed for titration and V_{NaOH} represents the volume of the NaOH used, and m is the amount of the Sepharose powder.

2.6. Trypsin assay

The activity of trypsin was assayed by measuring the rate of hydrolysis of BAPNA at 22 °C [15]. The assay was performed in a cuvette in 1 ml reaction volume containing 35 mM Tris–HCl buffer pH 8, 43 mM $CaCl_2$, 0.1 mM HCl, 0.56 mM BAPNA and 0.1 ml of free enzyme. The *p*-nitroaniline released was measured spectrophotometrically at 410 nm. One unit of trypsin was defined as the amount of enzyme that catalysed the production of 1 μmol *p*-nitroaniline per min per volume of reaction. The extinction coefficient of *p*-nitroaniline at 410 nm is 8800 M^{-1} cm^{-1} [15].

Fifty milligrams of the immobilized protein was mixed with the 900 μl assay mixture in an eppendorf tube and mixed on a rocking table for 2 min, centrifuged at 3800 × *g* for 2 min and absorbance of the products accumulated in the supernatant was determined as in case of the free enzyme system.

3. Results and discussion

Oxidation of primary alcohols using TEMPO proceeds through an oxoammonium ion, which is attacked by the substrate as a nucleophile, followed by removal of α-proton to give an oxidation product and reduced-TEMPO (N–OH), which is oxidized back to TEMPO (N–O) and then to the oxoammonium ion by the catalyst [16] (Fig. 1).

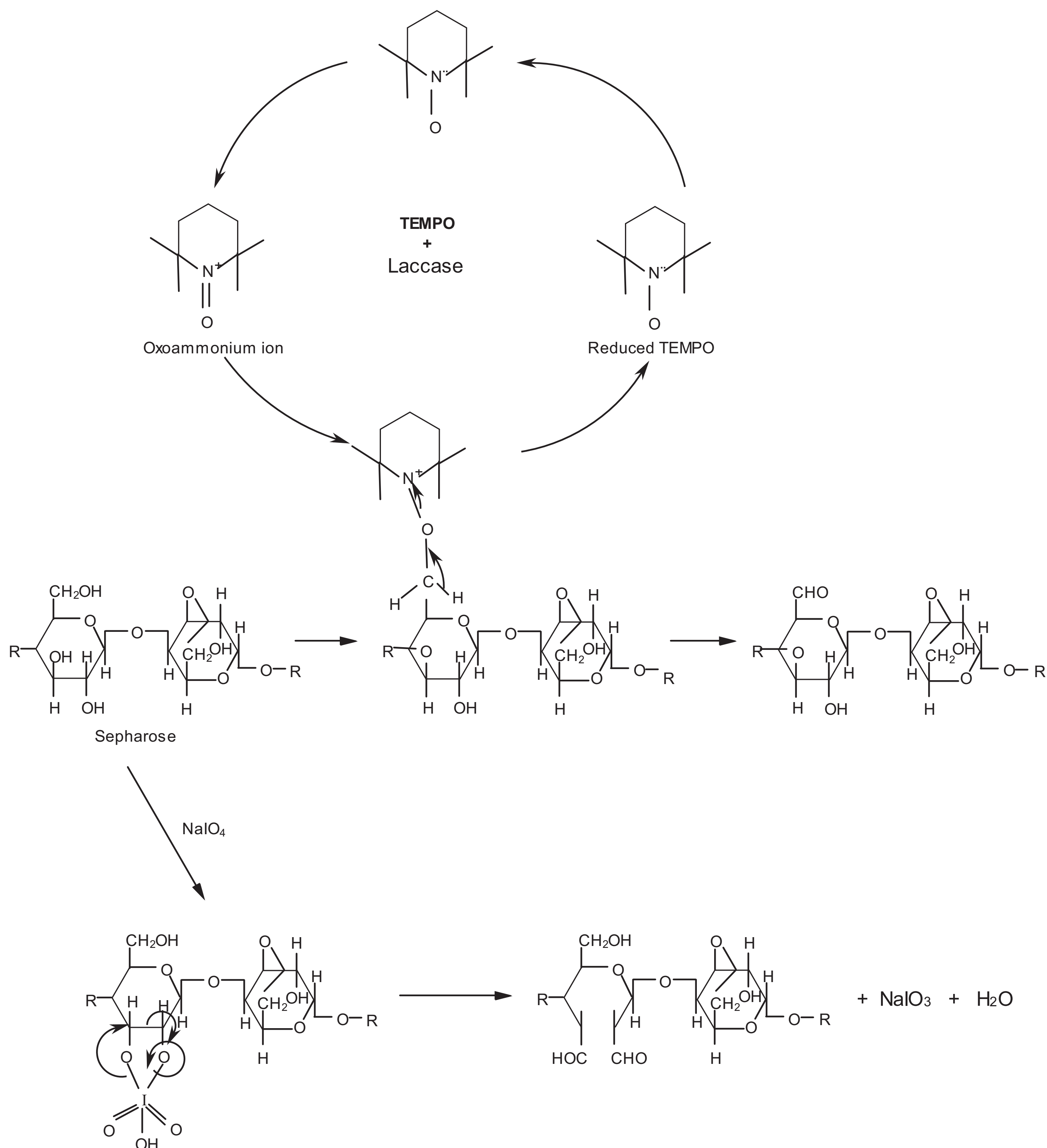

Fig. 1. Activation of agarose by oxidation using different catalysts: laccase/TEMPO and $NaIO_4$. Adapted from Galli and Gentilli [16].

Oxidation of Sepharose CL-6B using TEMPO was investigated using *T. versicolor* laccase as catalyst. Initially, Sepharose CL-6B was treated with 28 units of laccase per g (wet wt) matrix and 12.5 mM TEMPO for different periods of time and the carbonyl content of the gel was analysed. The aldehyde- and carboxyl groups generated were determined by direct measurement on the gel. Maximum aldehyde content of about 55 μmol and carboxylic acid content of 10 μmol per g (wet wt) Sepharose was obtained after 10–12 h at pH 5 and 22 °C. The degree of activation was lower when laccase treatment was performed at pH values below or above pH 5, which corresponds to the optimum pH for activity of the laccase. It has also been reported recently that carboxylic acids in the buffer systems inhibit laccase activity [17]; hence choosing an appropriate buffer (e.g. succinate or citrate buffer) could further reduce the degree of enzyme inhibition and provide the desired activation of the matrix in a shorter time or with a lower enzyme concentration.

Table 1
Immobilization of trypsin on laccase/TEMPO activated Sepharose CL-6B at different pH.

	Phosphate pH 7		Bicarbonate pH 8		Carbonate pH 10.5	
	Protein ($mg\,g^{-1}$)	Activity ($U\,g^{-1}$)	Protein ($mg\,g^{-1}$)	Activity ($U\,g^{-1}$)	Protein ($mg\,g^{-1}$)	Activity ($U\,g^{-1}$)
Buffer	3.6	3.6	5.1	12.3	4.5	17.4
Buffer + benzamidine	2.2	6.8	7.5	41.1	6.7	43.4

57 U of trypsin (specific activity of 5.7 $U\,mg^{-1}$) per g wet wt Sepharose was used for immobilization in the presence of 2 mM benzamidine.

Temperature higher than 22 °C also reduced the degree of oxidation by LMS, which is due to increasing loss of enzyme activity (data not shown). Activation of the gel was thus continued at pH 5 and 22 °C with varying laccase and TEMPO concentrations; aldehyde content of the gel was increased to 75 μmol per g (wet wt) Sepharose with 85 units of laccase and 12.5 mM TEMPO. At higher laccase (100 units per g) and mediator (25 mM) concentrations, there was a decrease in the aldehyde content. No laccase activity was detected on the Sepharose, and hence no laccase immobilization occurred during the activation period probably due to the low pH used that is not suitable for the coupling of the amino group.

The laccase/TEMPO activated Sepharose (with 55 μmol aldehyde per g wet wt gel) was then tested for immobilization of trypsin, which has 16 lysine residues besides the N-terminal residue that potentially contribute primary amino groups for the reaction with aldehyde groups on Sepharose. The coupling occurs through a Schiff base reaction which is subsequently stabilized by reduction with sodium borohydride [10]. The immobilization yield was dependent on the pH as well as the buffer used during the immobilization (Table 1). The highest yield of immobilized trypsin was obtained when carbonate buffer, pH 10.5 was used; the immobilized protein content was higher (45%) than the activity (31%) suggesting some loss of activity during the process most likely due to multipoint attachment. At pH 8, comparable yield (slightly higher than at pH 10.5) of immobilized protein was obtained, but with slightly lower activity. Blanco and Guisán [12] have earlier reported high immobilization yields for trypsin on glyoxyl agarose at pH 10, but the enzyme could also be immobilized at pH 7 due to the presence of some low pKa amino groups [18].

When the immobilization was performed in the presence of a trypsin inhibitor, benzamidine (2 mM), the activity yields of the immobilized enzyme were significantly improved to 43.4 $U\,g^{-1}$ (corresponding to 76% retention) (Table 1). Benzamidine is known to protect the active site during immobilization, preventing the amino acid residues present therein from participating in coupling to the matrix, and also protects the protease from auto-digestion [19].

The immobilization procedure using laccase activated gel was compared with the commonly used sodium periodate activation procedure for immobilization of ligands with primary amino groups [13,20,21]. Periodate oxidizes the vicinal-diols in the sugar residues to aldehydes [22] (Fig. 1). Normally, the activation by sodium periodate is measured by determining the periodate remaining in the supernatant and based on that aldehyde content on the gel is calculated [20,23]. But a significant fraction of the aldehyde groups gets further oxidized to the carboxyl groups during the processing and handling of the gel prior to immobilization. The periodate-activated gel was found to contain 72 μmol aldehyde and 20 μmol carboxylic acid per g Sepharose. Comparable yields of the immobilized trypsin (6.6 mg, 43 $U\,g^{-1}$ wet wt gel) were obtained after 2 h of incubation with this gel.

Immobilization of trypsin on laccase- and periodate-activated Sepharose was followed over a period of time. As seen in Fig. 2, the immobilized protein remained constant during incubation for 1–4 h, however the activity yield of the immobilized enzyme increased slightly with time up to 2 h and 3 h on the laccase and periodate activated Sepharose, respectively, and then decreased. Further incubation led to decrease in the yields of the immobilized trypsin activity on both kinds of activated supports probably due to the extremely high pH to which the enzyme is exposed. The amount of trypsin immobilized with respect to the initial concentration of the enzyme was also similar in both cases (Fig. 3). Although the amount of immobilized protein and -activity increased with the increase in trypsin concentration applied, there was a decrease in the percent yield.

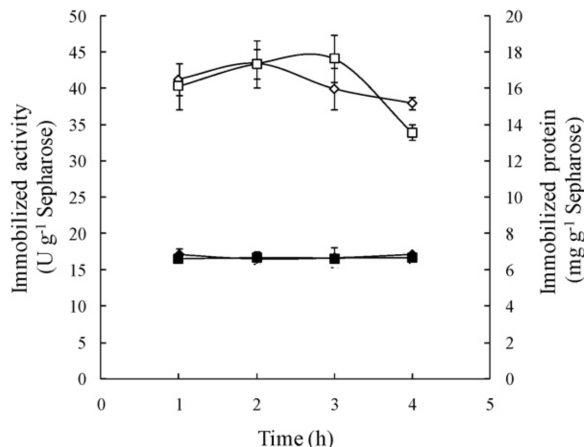

Fig. 2. Effect of reaction time on immobilization of trypsin on Sepharose CL-6B beads activated by 85 U laccase per g wet wt gel and 12.5 mM TEMPO (diamonds) and periodate (squares), respectively. Immobilized protein is denoted by filled symbols, while activity is shown by open symbols. The amount of trypsin used for immobilization was 10 mg per g gel, in a solution containing 2.5 $mg\,ml^{-1}$ enzyme in 20 mM carbonate buffer, pH 10.5 containing 2 mM benzamidine.

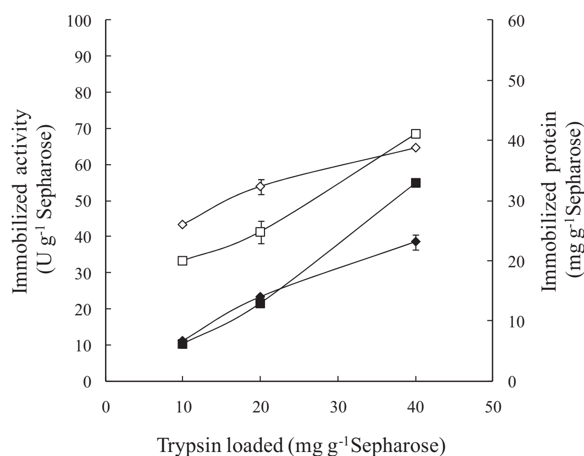

Fig. 3. Profiles of immobilized trypsin activity (open symbols) and protein (filled symbols) with respect to the initial concentration used for immobilization. The immobilization was performed for 2 h in 20 mM carbonate buffer, pH 10.5 with 2 mM benzamidine. The specific activity of soluble trypsin used was 5.7 $U\,mg^{-1}$. The symbols used for immobilization on LMS activated Sepharose are diamonds and on periodate activated gel are squares.

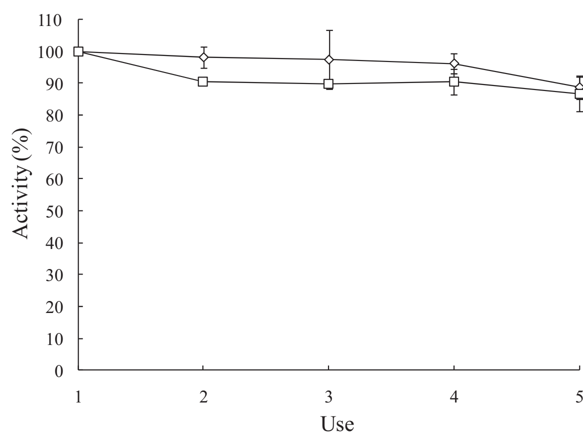

Fig. 4. Re-use of trypsin immobilized on Sepharose CL-6B beads activated using laccase/TEMPO (◊) and periodate (□).

The stability of trypsin immobilized to Sepharose, using the different activation procedures was tested during repeated use for BAPNA hydrolysis. As shown in Fig. 4, trypsin immobilized on laccase and periodate activated gel exhibited good stability, the former retained highest activity. The stability of the laccase-TEMPO immobilized trypsin was further tested during storage in 1 mM HCl containing 20 mM $CaCl_2$ at 4 °C. The enzyme retained complete activity during 4 weeks of storage, while the soluble enzyme had lost over 40% of activity during the same time period (data not shown).

4. Conclusions

This paper indicates the potential of using laccase-mediator system as an alternative method for activation of polysaccharide matrices for immobilization of proteins. The system would also be applicable for immobilization of affinity ligands bearing amino functionality. Further investigations are needed to determine the full scope of its applications. The method avoids the use of any toxic reagents and involves mild conditions, and the immobilization yields are comparable to the conventional chemical method used for the matrix activation. Treatment conditions for modification and immobilization would need to be optimized for different ligands to achieve optimal binding and specific activity. Laccase from different sources and different mediators are among the important parameters that would influence the activation of the matrix. Evaluating the potential of mediators originating from natural resources such as syringaldehyde could also be interesting from the point of view of lowering the cost and toxicity.

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