Journal of Molecular Catalysis B: Enzymatic 56 (2009) 253–258

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

Biotransformation of tea catechins into theaflavins with immobilized polyphenol oxidase☆

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ARTICLE INFO

Article history:
Received 19 October 2007
Received in revised form 2 May 2008
Accepted 13 May 2008
Available online 24 May 2008

Keywords:
Biotransformation
Bioprocessing
Theaflavins
Catechins
Immobilization
Immobilized enzyme
Matrix
Substrate
Polyphenol oxidase

ABSTRACT

Theaflavins, an active antioxidant, a natural pigment and pharmacologically active molecule obtained from black tea were bioprocessed on an immobilized tea polyphenol oxidase (PPO) system by the conversion of tea catechins extracted from green tea leaves with an overall conversion efficiency of 85% about 14-fold increase over maximum achievable in normal black teas. The immobilized enzyme (IE) system consists of activated cellulose matrix on to which the freshly extracted tea leaf polyphenol oxidase was covalently linked. Cellulose as a matrix of choice was considered primarily for its non-toxic nature, natural origin, low cost and easy availability. The kinetic parameters of the IE system were; protein loading capacity 11.8 mg/g; pH optimum 5.9; temperature optimum 37.5 °C; K_m 4.76 ± 0.08 mM; V_{max} 20 ± 1.80 nmol/min; enzyme activity retention (83.58%) and number of batches per turnover without loss of activity was 14. The product from IE system was identified by HPLC and ESI-QTOF-MS spectrometry.

1. Introduction

Theaflavins, a mixture of theaflavin, theaflavin-3-gallate, theaflavin-3′-gallate and theaflavin-3,3′-digallate (Fig. 1) are bioactive molecules which account for 0.4–1.8% of the dry weight of the solids in brewed black tea and contribute greatly to the quality of tea [1–4]. They are formed during black tea manufacture from co-oxidation of selected pairs of catechins mediated by the enzyme polyphenol oxidase native to tea leaf [5]. Recently, theaflavins have attracted considerable interest because of their potential benefits to human health as natural dietary antioxidant. The antioxidant nature of these chemicals were established against lipids oxidation detected in the rabbit erythrocyte ghost system [6] and rat liver homogenates [7], and LDL oxidation in mouse macrophage cells [8]. The antimutagenic [9], anti-inflammatory [10] and cancer chemo preventive action [11] of theaflavins has also been reported.

There were some reports on the synthesis of theaflavins from tea polyphenols such as using an in vitro model fermentation system containing purified catechins and partially purified polyphenol oxidase from green tea shoots to form theaflavins and thearubigins and using the model system to assess the reaction conditions and to improve black tea quality [12–16]. These reports provided useful information on in vitro theaflavins biosynthesis albeit for different experimental purposes.

Keeping this in mind, the present work was undertaken to optimize the conditions for theaflavins bioprocessing using immobilized tea polyphenol oxidase system with a view to attain the high conversion efficiency of tea catechins into theaflavins in a cost effective manner and its easy recovery which is otherwise not achievable during black tea manufacture and also for effective utilization of biocatalyst for commercial scale production of theaflavins.

Tea polyphenol oxidase (PPO, EC 1.10.3.1) is a copper-containing enzyme which catalyses the oxidation of tea catechins into theaflavins [17] is a moderately unstable enzyme, which is susceptible to inactivation at elevated temperatures and extreme conditions of pH. It also tends to lose its catalytic activity upon storage [18], which ultimately restricts its commercial applications. Immobilization confers stability to the enzyme against denaturation by preventing conformational changes and protecting it in a confined microenvironment. Immobilized enzyme system has the advantage

Abbreviations: EC, (−)-epicatechin; ECG, (−)-epicatechin gallate; EGC, (−)-epigallocatechin; EGCG, (−)-epigallocatechingallate; PPO, polyphenol oxidase; IE, immobilized enzyme; CDI, 1,1-carbonyldiimidazole; TF, theaflavins.

☆ IHBT Publication #668.

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doi:10.1016/j.molcatb.2008.05.016

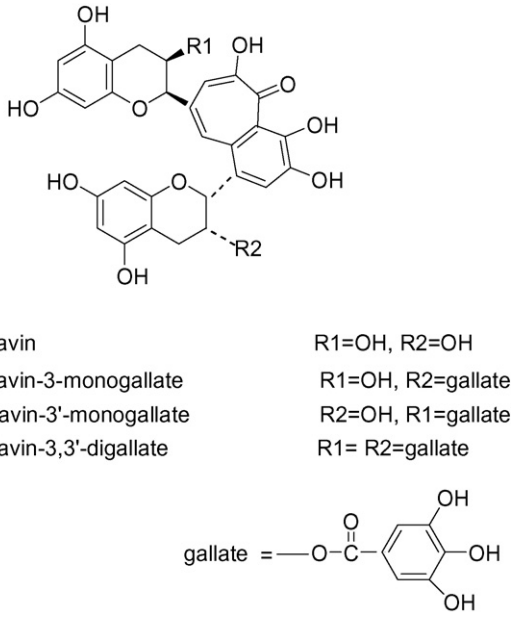

Fig. 1. Chemical structure of theaflavins.

of multiple and effective use of the enzyme. The even distribution in substrate solution, enhanced rates, higher thermal stability, easy handling and separation of reaction product besides repeated turnovers are some of the accrued benefits of the system and downstream processing and up scaling. Introduction of cellulose as matrix for immobilization of tea PPO was primarily on consideration of its low cost, over abundance, being a natural water insoluble polymer and presence of suitable groups for activation and coupling of the enzyme.

2. Experimental

2.1. Materials

Cellulose was purchased from Schleicher & Schull Gmbh (Germany). 1,1-Carbonyldiimidazole, theaflavins and BSA were from M/s Sigma–Aldrich (St. Louis, MO, USA). Solvents and all other reagents were from Merck (Darmstadt, Germany) and are of analytical grade.

2.2. Enzyme production

Tea polyphenol oxidase was extracted from young tea shoots comprising of two leaves and an apical bud according to a published method [19]. In brief, the acetone powder was made by homogenizing the tea shoots in chilled (−20 °C) acetone. The homogenate was filtered and retentate was washed free of phenolics, first with chilled acetone then with cold aqueous acetone and finally again with acetone. The acetone powder was dried, homogenized with distilled water and filtered through cotton-wool over a funnel, the filtrate was rejected and bound enzyme was eluted from residue with 0.2 M Na_2SO_4.

2.3. Substrate production and purification

The tea substrate (catechins) was prepared from young tea shoots. In brief, tea shoots were first steamed to deactivate PPO activity and then making hydro extract which then passed through column containing HP-20 resin which specifically adsorbs catechins. The catechins were then eluted with ethanol and spray dried. The extracted catechins were 90% in purity, containing 16.13% EGC, 9.17% EC, 26.26% EGCG, 11.0% ECG and less than 1% caffeine.

2.4. Activation of cellulose

The cellulose was activated for immobilization of tea PPO through carbonyldiimidazole method by activation of hydroxyl groups of cellulose with 1,1-carbonyldiimidazole [20,21]. Five-gram cellulose was added to 60 mM CDI in dichloromethane taken in a flat bottom flask, maintained at temperature 4 °C in an ice-bath. The reaction mixture was kept in an incubator shaker at 20 °C and 150 rpm for 1 h. After incubation, the reaction mixture was filtered through Buchner funnel using Whatman filter paper #14, washed with 25 ml dichloromethane and dried under vacuum. The matrix so formed was kept in a fridge at 4 °C till further use.

2.5. Immobilization of polyphenoloxidase

Ten-milliter crude tea PPO (12 mg proteins) was added to CDI activated cellulose (1.0 g) suspended in 5.0 ml 0.1 M sodium phosphate buffer solution (pH 6.2) in a plastic beaker. The immobilization reaction was carried out at 25 °C in an incubator shaker at 100 rpm for 1 h. After incubation, the immobilized PPO was washed with water and 0.1 M 6.2 pH sodium phosphate buffer solution several times to remove any unbound enzyme and filtered through funnel using Whatman filter paper #14 and stored at 4 °C until use.

2.6. Assay of PPO activity

The PPO activity was measured by monitoring the consumption of oxygen with an oxygraph using Clark type oxygen electrode having sensitivity in the range of 0.01–500 nmol/(ml min) at 20 °C (Hansatech, USA). The reaction was initiated by the addition of appropriate amount of the enzyme to the reaction mixture and initial uptake of oxygen consumption was measured. Enzyme preparation (25.0–100.0 μl) was added to the reaction mixture containing 1.5 ml of 0.1 M Na_2SO_4 and 0.9 ml of 10 mM D(+)-catechin as substrate to achieve linear rates in the range of nmol/min. One unit of enzyme activity was defined as the amount of enzyme which consumed 1 μmol of O_2 per minute at 20 °C by the use of thermostatted water bath. Overall activity displayed as oxygen consumed and expressed as μmol/(min g) protein. The total protein concentration was determined by the Lowry method [22], using bovine serum albumin as standard.

2.7. Determination of the kinetic parameters of the free and immobilized enzyme

The kinetic constants were determined using D(+)-catechin as substrate (in the concentrations range (5–30 mM) using free and immobilized PPO. The experiments were performed in triplicate and conducted under optimized assay conditions.

2.8. Optimization of reaction conditions for bioproduction of theaflavins

The reaction conditions which were optimized for bioproduction of theaflavins included pH, reaction temperature, reaction time, substrate conversion efficiency, reuse and storage stabilities of the immobilized enzyme. Reaction temperature was determined between 25 and 60 °C by performing the reaction in 0.1 M sodium phosphate buffer (pH 6.2) for 1 h at different temperatures and at 150 rpm. The effect of pH was also studied by using 0.1 M sodium phosphate buffer (pH 5–7) and reaction was performed for 1 h at

150 rpm. The influence of reaction time was determined after other conditions were optimized keeping the concentration of enzyme and substrate constant in all the cases. To evaluate the reuse stability, the immobilized PPO, after each reaction with substrate, was washed with water and reintroduced into fresh substrate to start the next batch of reaction. The process was repeated for 20 cycles. The storage stabilities of the immobilized PPO was determined by incubating the enzyme in 0.1 M sodium phosphate buffer solution (6.2 pH) at 4 and 30 °C up to 1 month and assayed for residual activity at predetermined times. At last, the substrate conversion efficiency of immobilized enzyme was determined by varying the concentration of substrate and keeping the concentration of enzyme constant under optimized reaction conditions. All the experiments were performed in triplicate.

2.9. Biotransformation of tea catechins into theaflavins

The appropriate amount of immobilized enzyme was taken in a plastic beaker and aqueous solution of catechins was added. The whole mixture was then incubated in an incubator shaker at 150 rpm for 1 h under optimized conditions of temperature and pH. After incubation, the product formed was filtered through Whatman filter paper #14 and matrix bound residual product was eluted with 30% acetone (v/v), and the immobilized enzyme matrix regenerated by washing with distilled water.

2.10. Purification of theaflavins

The bioprocessed aqueous theaflavins extract was then purified through RP-18. The aqueous solution of theaflavins was passed through RP-18 silica column which adsorbed all the theaflavins and get through unwanted polyphenols and flavonoids. The absorbed theaflavins were eluted with 40% ethanol and then lyophilized using Savant (Savant, USA) vacuum concentrator.

2.11. HPLC analysis of bioprocessed theaflavins

The HPLC analysis of bioprocessed theaflavins was performed using a Shimadzu (Shimadzu, Tokyo, Japan) liquid chromatography modular system consisting of two LC-10AD pumps, an UV Shimadzu SPD M10A UV–Vis detector, inline degasser and an LC WorkStation Class LC10 system for data processing. The samples were introduced using an injection valve fitted with a 20 μl loop (Rheodyne, California, USA). The chromatographic separation was performed on phenomenex analytical column (LUNA C_{18} (2) 250 mm × 4.6 mm I.D.) packed with 5 μm silica. The two mobile phases: (A) acetonitrile and (B) water containing 0.01% (v/v) trifluoroacetic acid were used with a linear gradient elution as follows: 0–3 min, 20% A; 3–10 min, 30% A; 10–12 min, 35% A; 12–15 min, 30% A; 15–20 min, 20% A; at a flow rate of 1.0 ml/min. The detection wavelength was set at 270 nm. The column temperature was 32 °C, and the injection volume of samples was 20 μl.

2.12. ESI-QTOF-MS spectrometry of bio-processed theaflavins

The ESI-QTOF-MS/MS spectrometry of bio-processed theaflavins was carried out with Waters Q-TOF micro using mass Lynx software. The sample was introduced into the ESI interface. The negative ion polarity mode was set for ESI ion source with the voltage on the ESI interface maintained at approximately 3 kV. The structural information of the theaflavins was obtained by tandem mass spectrometry (MS/MS) through electron spray ionization.

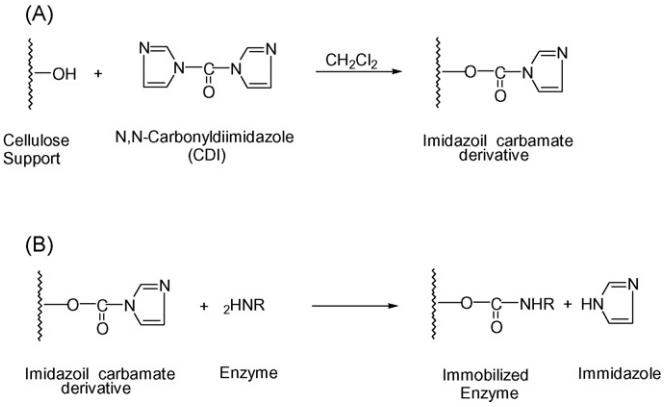

Fig. 2. (A) CDI activation of the support (cellulose) and (B) enzyme coupling.

3. Results and discussion

3.1. Enzyme immobilization

For the covalent immobilization of an enzyme on to an inert support, two discrete chemical processes are required. Initially, the activation of support with an appropriate agent and secondly, coupling of enzyme to the activated support. We preferred to use CDI for the activation step over other methods because of its ease for the activation of free hydroxyl groups on support as an active carbonylating agent and its particular suitability of the enzyme system used [23,24]. An important advantage of the CDI method compared to the standard cyanogens bromide method is the absence of the any charged groups introduced by the functional groups of the activation reagent during both the activation and ligand coupling steps as assessed over the pH range normally used. Beside this, CDI is a non-toxic reagent and reaction could be handled under mild conditions.

There are many reports in the literature that covalent binding is the best way to immobilize enzymes. However, the expensive cost of supports used in these processes has limited the general application of these protocols [25–27]. In the present work, choice of cellulose as a support was justified by its very low cost and simple activation with CDI. The activated matrix reacts smoothly with N-nucleophiles such as free amino groups in enzymes. The reaction scheme for activation and coupling involves the covalent bonding between enzyme and matrix as shown in Fig. 2. Table 1 shows the recovery of the enzyme activity. It can be noted from this table that the immobilized enzyme exhibited 83.58% retention of specific PPO activity when the ratio of protein/matrix was up to 11.8 mg/g. Fig. 3 presents the effect of the enzyme added to the matrix. It can be noted that the protein content was immobilized on 1 g matrix up to 11.8 mg. Above this quantity, as the maximum charge of the matrix was reached, a lower percentage of protein was immobilized. For instance, when 36 mg protein was used, only about 32.7% of the protein content was immobilized on matrix

Table 1
Optimization of immobilization of tea PPO on cellulose

Total activity loaded on 1.0 g of polymer, *a*	18.50
Proteins (mg)	12.00
Total specific activity of soluble PPO before immobilization, *b* (U/mg/)	1.54
Immobilized PPO activity of 1.0 g of polymer, *c*	15.20
Proteins (mg/g of polymer)	11.80
Specific activity of immobilized PPO (U/g), *d*	1.29
% PPO activity, $c/a \times 100$	82.16
% retention of specific activity, $d/b \times 100$	83.58

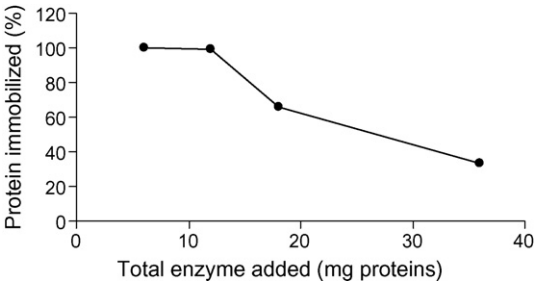

Fig. 3. Effects of enzyme loading on the CDI activated cellulose for immobilization of polyphenol oxidase. Different amounts of PPO (6, 12, 18 and 36 mg) were added to 1.0 g of CDI activated cellulose, and immobilization was performed using 0.1 M sodium phosphate buffer (6.2 pH). The immobilized enzyme was washed successively with same buffer until no proteins were released. The results presented for each experimental conditions expressed are the mean value of three replications.

3.2. Enzyme kinetics of immobilized PPO

Kinetic parameters investigated in the present study include the maximum reaction rate (V_{max}) of the enzymatic reaction and the Michaelis–Menten constant (K_m) which defines the affinity of enzyme toward its substrate. These parameters were obtained from Lineweaver–Burk Plot which is a plot of $1/V_0$ against $1/[S_0]$ for systems obeying the Michaelis–Menten equation. The K_m for free and immobilized PPO thus obtained using catechins as substrates were 3.57 and 4.76 mM, respectively (Table 4). When we compare the K_m values of free PPO with that of immobilized, very little differences in K_m values imply that the CDI activated cellulose matrix provide a more suitable microenvironment for immobilization of tea PPO and have similar kind of affinity against the catechins. The higher V_{max} of immobilized PPO as compared to free enzyme (Table 2) is an indication of more efficient turnover of the substrate towards product formation due to removal of inhibitors present in crude enzyme solution during immobilization steps and change in the microenvironment of the native enzyme.

3.3. Optimization of reaction conditions for bioproduction of theaflavins

A number of reaction parameters affecting the bioproduction of theaflavins were optimized to maximize theaflavins production. Firstly, the pH optima of the reaction mixture was determined and was found to be 5.9. The product formation below pH 5 was drastically reduced due to acidic reaction conditions and above pH 6.5 the product formation was affected due to approaching alkaline conditions and auto-oxidation of the formed product (Fig. 4). The optimum temperature for bioprocessing of theaflavins was found to be 37.5 °C as shown in Fig. 5. Tea PPO has an optimum pH of 5.5 and optimum temperature 37 °C toward (+)-catechin, and the physiological pH in the tea leaf ranges from 5.4 to 5.6 [28]. The broad stability over temperature and pH as seen in the present study could be due to modified enzyme environment in an immobilized enzyme system [17]. The influence of reaction time was also determined after conditions for pH and temperature were optimized and it was found that there was complete conversion of available catechins into theaflavins at 1 h but after 1 h the product

Table 2
Kinetic constants for free and immobilized PPO[a]

Enzyme	V_{max} (nmol/min)	K_m (mM)
Free	0.33 ± 0.02	3.57 ± 0.07
Immobilized PPO	20.00 ± 1.80	4.76 ± 0.08

[a] Data expressed as mean ($n = 3$) ± S.D.

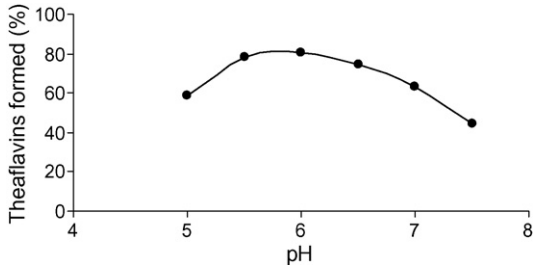

Fig. 4. pH optimum of immobilized enzyme for bioproduction of theaflavins. The reaction was performed by adding 0.1 g catechins in the reaction mixture containing 0.5 g immobilized PPO (6 mg proteins) dissolved in 2.5 ml 0.1 M sodium phosphate buffer (pH 5, 5.5, 6, 6.5 and 7) at 37 °C.

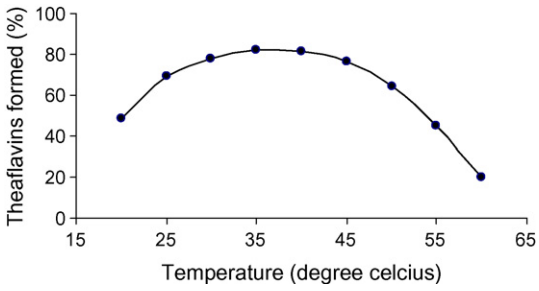

Fig. 5. Temperature optimum of immobilized enzyme for bioproduction of theaflavins. The reaction was performed by adding 0.1 g catechins in the reaction mixture containing 0.5 g immobilized PPO (6 mg proteins) dissolved in 2.5 ml 0.1 M sodium phosphate buffer (pH 5.9) at different temperatures (20–60 °C).

formation was affected mainly due to the auto-oxidation of formed product as shown in Fig. 6. The substrate conversion efficiency of immobilized enzyme was determined by keeping the concentration of immobilized enzyme (enzyme proteins) fixed at 2.0 mg and the concentration of catechins was varied and it was found that 1 mg protein equivalent of immobilized enzyme convert 25 mg of catechins into theaflavins but at higher concentration of catechins up to 1000 mg there is complete enzyme inhibition (Fig. 7). The observed continuous number of turnovers with I.E., i.e. bioconversion of catechins into theaflavins without any loss of activity was seen for first 14 numbers of batches thereafter linear loss of efficiency occurred and by the twenty second number of batch near complete loss of activity has been observed (Fig. 8). The stability of the immobilized enzyme is an important parameter for commercial production of theaflavins. The results listed in Tables 3 and 4 shows that the immobilized polyphenol oxidase was stable over a long period of storage and after many times of use for theaflavins biosynthesis. After 15 times of usage, the enzyme still had 94% of its

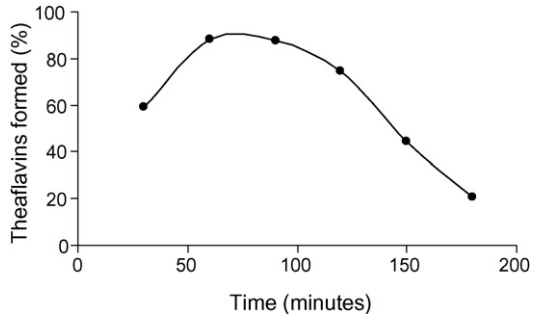

Fig. 6. Reaction time for bioproduction of theaflavins. The reaction was performed by adding 0.1 g catechins in the reaction mixture containing 0.5 g immobilized PPO (6 mg proteins) dissolved in 2.5 ml 0.1 M sodium phosphate buffer (pH 5.9) at 37.5 °C for different interval of times (1/2–2 h).

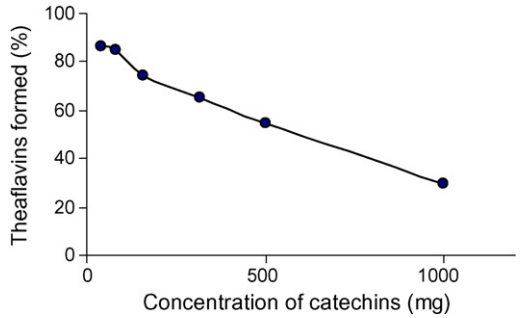

Fig. 7. Substrate conversion efficiency of immobilized enzyme for bioproduction of theaflavins.

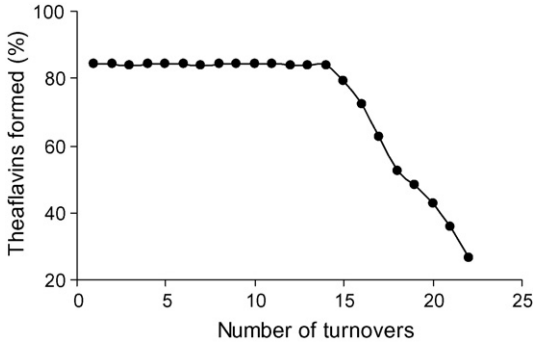

Fig. 8. Reuse stability of immobilized enzyme for bioproduction of theaflavins. The reaction was performed by adding 0.1 g catechins in the reaction mixture containing 0.5 g immobilized PPO (6 mg proteins) dissolved in 2.5 ml 0.1 M sodium phosphate buffer (pH 5.9) at 37.5 °C.

original activity. After 30 days storage in 0.1 M sodium phosphate buffer (pH 6.2) at 4 °C, the enzyme had 90% of its original activity. These results indicate that the immobilization method is effective for achieving high stability of the immobilized polyphenoloxidase system. Thus the matrix bound tea polyphenol oxidase is unique in terms of its high reactivity towards tea substrates, reusability (observed 14 number of times) without losing any activity, non-

Table 3
Yield of theaflavins in batch operations of the immobilized polyphenoloxidase

Time of usage	Amount of proteins (I.E.) (mg)	Amount of catechins added (g)	Yield of theaflavin (g)
1	125	3.2	2.76 (86.2)
5	125	3.2	2.75 (85.9)
10	125	3.2	2.73 (85.3)
15	125	3.2	2.61 (81.5)
20	125	3.2	1.36 (42.5)

Figures in parentheses represent percent conversion of catechins into theaflavins.

Table 4
Enzyme activity on different storage days of the immobilized polyphenol oxidase

Storage days	Activity (Units)	
	At 30 °C	At 4 °C
1	0.128	0.128
7	0.098	0.128
21	0.032	0.128
30	0.012	0.127
45	–	0.125
60	–	0.116

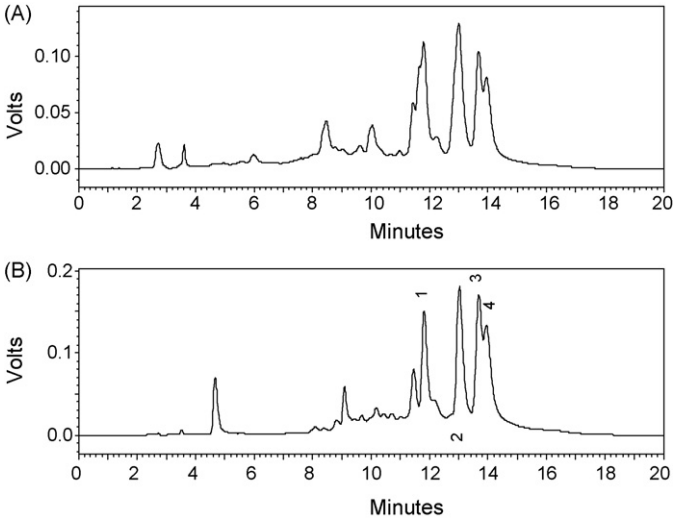

Fig. 9. (A) HPLC chromatograms of reference standard (theaflavins). (B) Bioprocessed theaflavins. Peak 1 refers to theaflavins 1 (TF 1), peak 2 to TF 2, peak 3 to TF 3, and peak 4 to TF 4.

adherence of formed product to matrix thus allaying fear of product poisoning of matrix bound enzyme system.

The bioconversion of catechins into theaflavins with polyphenoloxidase has been reported but with immobilized polyphenol oxidase very little work has been done. The immobilized PPO has been found to convert the mixed catechins into theaflavins and achieved 14 turnovers with same conversion efficiency. However, after 14 turnovers the linear loss of activity was observed till twentieth turnover when there is no appreciable product is formed with the same time. The linear loss after 14 number of turnovers may be due to number of factors such as enzyme fatigue, slow poisoning of immobilized enzyme by quinones, protein conformation, etc. Thus, the optimum reaction conditions for biotransformation of tea catechins into theaflavins were achievable at 1 mg protein equivalent immobilized enzyme, 0.04 g catechins at pH 5.9 and incubation for 1 h at 37.5 °C.

Although the green tea shoots has 15–30% of catechins but during black tea manufacture only 0.4–1.8% theaflavins are formed due to uncontrolled reaction conditions leading towards non-

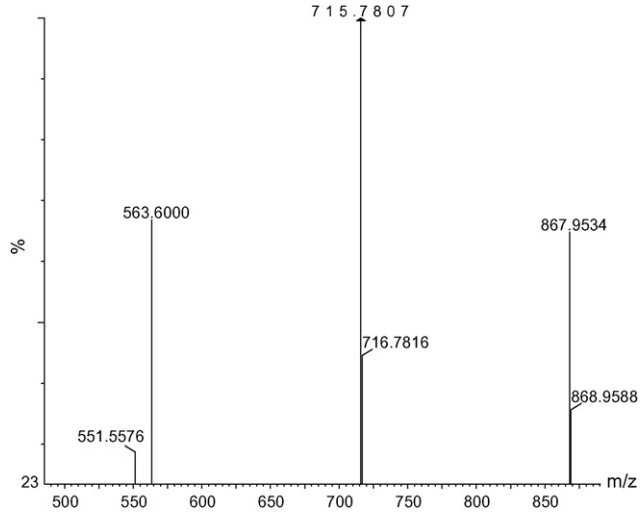

Fig. 10. ESI-QTOF-MS spectrum of bioprocessed theaflavins.

theaflavins like products but the immobilized enzyme system developed in the present study convert all the available catechins into theaflavins with 85% conversion efficiency, i.e. 85% conversion of catechins into theaflavins.

3.4. Chemical characterization of bioprocessed theaflavins

The HPLC chromatogram of theaflavins after purification with RP-18 showed the characteristic four peaks of TF1, TF2, TF3 and TF4 and was compared with reference standard, their retention times being found to match well (Fig. 9). The theaflavins were also characterized by ESI-QTOF-MS spectroscopy as they generated molecular ion peaks $(M-H)^-$ at m/z 867.9534, 715.7807 and 563.6000 corresponding to the molecular formulae of TFDG, TFMG and TF, respectively (Fig. 10).

4. Conclusions

An effective enzymatic method based on the immobilization of tea polyphenoloxidase on cellulose after suitable activation with 1,1-carbonyldiimidazole for activation of hydroxyl groups of cellulose was developed for the biotransformation of tea catechins into theaflavins, an important antioxidant that can be exploited as dietary supplement in foods and beverages or as pharmaceutical/cosmetic use.

Acknowledgements

Author (KS) is indebted to Council of Scientific and Industrial Research (CSIR), New Delhi, India, for the award of a Senior Research Fellowship. We gratefully acknowledge Director, IHBT, Palampur, for his keen interest and encouragement in this work.

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