An optical biosensor employing phenylalanine ammonia lyase-immobilised films for phenylketonuria detection

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An optical biosensor has been designed to quantify the amount of phenylalanine in new born babies. Rapid detection and quantification of phenylalanine in the serum has been achieved by immobilizing phenylalanine ammonia lyase in polyvinyl alcohol-jellose biocomposite films using entrapment method. The trans-cinnamic acid formed by the enzymatic reaction is measured using optical fibre spectrophotometry. The results obtained by this method are confirmed using HPLC and microassay methods. The optimum measurement of trans-cinnamic acid formation from phenylalanine is achieved in 150 mM Tris HCl buffer pH 8.5 at 30°C and the lowest detectable concentration under these conditions is found to be 1.0 µmol/L and response time obtained is 30 min. The biosensor developed is stable for atleast 1 month when stored in buffer at 4°C and less than 10% drop in original activity is observed. The sensor developed requires minimum chemicals and the enzyme source used is also very cheap. Thus, the sensor has the credentials of commercial importance.

Keywords: Biosensor, Biofilms, Phenylketonuria, Phenylalanine, Polyvinyl alcohol-jellose

Phenylketonuria (PKU) is an autosomal recessive genetic disorder which is the first characterized metabolic inborn disease1. The main molecular cause of this disease is deficiency of enzyme phenylalanine hydroxylase (PAH) (EC1.14.16.1). PAH is necessary to metabolize amino acid phenylalanine to tyrosine. The deficiency of PAH leads to the accumulation of phenylalanine which is subsequently converted into phenylpyruvate due to oxidation of NADH to NAD+ (also known as phenylketone), and is excreted through urine2. Left untreated the condition can cause problems with brain development, leading to progressive mental retardation and seizures3. This is one of the inborn errors of metabolism which can be cured by diet control. Damage once done is irreversible and hence early detection is crucial.

Phenylalanine is a large, neutral amino acid (LNAA). LNAAAs compete for transport across the blood brain barrier (BBB) via the large neutral amino acid transporter (LNAAT). Excessive phenylalanine in the blood saturates the transporter. Thus, excessive levels of phenylalanine significantly decrease the levels of other LNAAAs in the brain. But since these amino acids are required for protein and neurotransmitter synthesis phenylalanine accumulation disrupts brain development in children, leading to mental retardation4. Incidence of PKU is 1 in 10,000 births in USA5,6 and can vary up to 1 in 4,500 births especially in the southern parts of India7.

The present techniques for detection of phenylketonuria include high pressure liquid chromatography (HPLC)8; Genetic engineering methods like screening Variable number of tandem repeats (VNTR), Short tandem repeats (STR), etc; and Guthrie test, which is a microbiological method. These techniques are either tedious or expensive which require skilled technicians9,10. Thus, in developing countries there is a lack of screening for metabolic disorders such as phenylketonuria. With the increase in the incidence of this disorder there is an urgent need to develop a cost effective screening method.

Several dehydrogenase based biosensors are available for phenylketonuria measurements. These sensors typically are dependent on the enzyme cofactor, NADH, which is detected amperometrically at the electrode surface. However, these sensors require an electron mediator11,12. Weiss et al.13 has reported a reagent-less dehydrogenase based biosensor for detection of phenylalanine in urine. While Wang et al.14 have immobilized phenylalanine ammonia lyase (PAL) on an ammonia electrode to measure blood phenylalanine for PKU prevention.

In the present study, an optical biosensor has been designed to quantify the amount of phenylalanine in...
new born babies. The enzyme phenylalanine ammonia lyase (EC 4.3.1.5) isolated and purified from phaseolus seeds is immobilized in polyvinyl alcohol (PVA) and biocomposite polymer jellose. PAL is responsible for the conversion of phenylalanine to trans-cinnamic acid (Scheme 1). The amount of trans-cinnamic acid is measured using optical spectrophotometer. Since the sensitivity of this measuring device is greater the response can be easily correlated. The sensor developed requires minimum chemicals and the enzyme source is also very cheap. The results obtained have been verified with the other available techniques such as HPLC and microassay and show good compliance. Thus, the sensor has the credentials of commercial importance.

Experimental Procedure

Chemicals
L-phenylalanine was purchased from Sigma Chemical Company (St. Louis, MO). Other chemicals were of analytical reagent grade and purchased from SRL, SD Fine Chemicals, and used without purification. Jasco V-530 UV-VIS spectrophotometer was used for the enzyme assay.

Instrumentation
Ocean Optics Spectra Suite 2000-Optical fibre spectrophotometer was used for sample quantification with a sensitivity of 1000 counts/msec integration time and wavelength range of 200-1100 nm. Spectra Physics dual pump HPLC was used to quantify the phenylalanine in the serum. Acetonitrile : water : potassium sulphate (70:20:10) mixture was used as the mobile phase on a C18 column.

Isolation and purification of enzyme
Phenyl ammonia lyase was isolated from phaseolous seeds and purified according to the method described by Saunders et al.15 with some modifications. All purification steps were carried out at 4°C as far as possible, and all buffers contained freshly added β-mercaptoethanol (5 mM). Phenylmethylsulfonyl fluoride (PMSF) (dissolved in acetone and diluted to 1.0 mm final concentration) was added to the extraction buffer. Phaseolus seeds (up to 500 g) was taken in ice-cold extraction buffer (200 mM Tris-HCl, pH 7.5) containing buffer-soaked PVP (1%) and incubated on ice for 20 min with occasional stirring. The homogenate was squeezed through four layers of buffer washed cheese cloth, brought to 0.1% (w/v) protamine sulfate, and stirred for 20 min. After centrifugation (10,000 g, 10 min, 4°C), the clear supernatant was equilibrated with extraction buffer. The filtrate was brought to 60% saturation with solid ammonium sulfate. Insoluble proteins were collected by centrifugation (10,000 g, 20 min, 4°C), redissolved in a minimum volume of start buffer (20 mM Tris-HCl, pH 7.5). The supernatant was dialyzed prior to loading on to a Sephadex G-50 column (10 mL bed volume; 50 mM Tris-HCl, pH 7.5 ) at a flow rate of 1 mL min⁻¹.

The supernatant was passed through Sephadex G-50 column. 1.5 mL of fractions was collected and absorbance was measured at 280 nm. The purified enzyme extract was further subjected for SDS-PAGE (12% gel) to determine molecular weight and purity of the eluted protein. The protein was estimated using the Bradford method with bovine serum albumin as standard.

Enzyme activity
The activity of the purified enzyme was measured using the method described by Zucker et al.16 with some modifications. To assay the PAL activity, 5 µM phenylalanine solution was added to 0.5 mL of the purified enzyme and the volume was made upto 3.0 mL with 0.05 M Tris buffer at pH 8.8. The mixture was incubated at 37°C for 60 min. At the end of incubation period, the reaction was stopped with addition of 1.0 mL of 1 M trichloroacetic acid. The absorbance of the solution was measured at 290 nm. The amount of trans-cinnamic acid formed was measured spectrophotometrically and quantified against standard curve of trans-cinnamic acid.

Assays were performed in triplicate. Under the experimental conditions, the increase in absorbance at 290 nm was linear for up to 30 min. The molar extinction coefficient of authentic trans-cinnamic acid in assay buffer was determined to be 17,400. A unit of enzyme activity (kat) was defined as the amount of enzyme required for the formation of 1 µmole of product in 1 min under the assay conditions.
Immobilization of enzyme

Immobilization was carried out using entrapment method in 3% of PVA and 1.5% of Jellose. Jellose was separated according to protocol by Kulkarni et al.\textsuperscript{17}. 50 μL of enzyme extract containing 0.0459 unit/mg of protein was added to the polymer mixture. The solution was then applied as a layer on grease free glass slides and allowed to dry overnight. The dried films were preserved at 4°C until further testing was done.

Effect of pH and temperature

The enzymatic deamination reaction was monitored at various pH. For the assay, about 20 units of PAL were incubated under standard conditions with 3.0 mL phosphate buffer (0.02M) and tris buffer (0.02 M) ranging from pH 5.5 - 9.0. The optimal pH obtained was used to measure the temperature stability between 20°C and 60°C. The temperature stability was determined based on the enzymatic deamination reaction product formed.

Kinetic analysis and micro assay

A partially purified PAL preparation (after Sephadex G-50 column purification) was used to determine the Km for phenylalanine. For Km determinations, 100 μL aliquots of enzyme preparation were incubated with 1 µM to 25 mM L-phenylalanine (approximately 10^5 dpm/assay) for 30 min.

\textit{Bacillus subtilis}, a Gram positive facultative anaerobic bacterium producing phenylalanine deaminase was taken and assayed according to Vallian et al.\textsuperscript{18,19}.

Optical fibre measurements

The enzyme (0.0459 units/mg of protein) immobilized in PVA, jellose biofilms was used for the optical fibre measurements. The substrate phenylalanine (1mL) was added to the reaction mixture. The spectral response was monitored for 30 min.

Results and Discussion

Purification of enzyme

The elution profile of phenylalanine ammonia lyase using Sephadex G-50 column is shown in Fig. 1a. Phenylalanine ammonia-lyase was purified more than 1000-fold, from phaseolus seeds. The first 10 volumes belong to non-specific proteins and are eluted out along with the buffer. Fractions 20-30 containing the purified protein are pooled. The purified protein moves as a single band and the molecular weight is found to be 33 KDa by SDS PAGE analysis (Fig. 1b)\textsuperscript{20}. Standard curve for trans-cinnamic acid was plotted with concentrations varying from 5 μM to 160 μM. The amount of trans-cinnamic acid liberated from the PAL reaction was extrapolated to calculate the enzyme activity in free and immobilized enzyme. A BSA standard curve was obtained using bovine serum albumin and the protein was estimated using Bradford method\textsuperscript{21}. The purified enzyme shows an activity of 0.0459 units /mg of protein.

Biosensor fabrication and characterization

The biosensor used in this work is schematically shown in Fig. 2. The films formed are flexible.
and could be attached to the tip of the optical fibre. It is essential to determine the minimum amount of enzyme required for immobilization. The volume of the enzyme was varied and the enzyme activity was measured. A range of concentrations 2.5-100 µM of the purified enzyme was assayed using spectrophotometric methods. 25 µM of the enzyme is required to immobilize to obtain a good data (data not shown). Thus, for a 1 cm × 1 cm film 25 µM of enzyme is immobilized in the biocomposite matrix to cast polymer films. To increase the sensitivity of the measuring device the optical fibre spectrophotometer was selected.

Effect of pH and temperature

Figure 3a depicts the pH profile wherein the absorbance is measured at different pH. The enzyme exhibits maximum activity at pH 9, for free as well as immobilized enzyme. The temperature profile for the free and immobilized enzyme shows a gaussian curve with maximum activity at around 40°C [Fig. 3b]. The initial velocity versus substrate double reciprocal Line weaver-Burk plot (Fig. 4) shows an apparent $K_m$ of 1.87 mM while the $k_{cat}$ is found to be 0.52 (ref. 20). Thus, the enzyme does not exhibit significant loss of activity or change in properties on immobilization.

Optical fibre measurements

The enzyme immobilized strips were subjected to optical fibre measurement and the spectra were monitored for 30 min, after which saturation was reached. The peak obtained at 290 nm was plotted. As the time progressed there is an increase in the absorbance at 290 nm (Fig. 5). A linear response is obtained at 290 nm with respect to time with $R^2 = 0.989$ (data not shown). The graph obeys Beers Lamberts law and thus the optical spectrometric method for measurement of phenylalanine concentration proves to be an excellent methodology. The minimum detection limit of Phenylalanine using optical fibre spectrophotometer is reduced to 1 µmol/L as compared to 30 µmol/L in the spectrophotometric detection.

Analysis of serum samples of different individuals

In the present study, 3 blood samples from patients positive for PKU were used. Blood samples treated with EDTA was collected in hemocrit microtubes.
The serum was separated and diluted 1:10 before the analysis. Appropriate working standard solution of phenylalanine was made and the results obtained are shown in Fig. 6. The micro assay, HPLC and optical methods were applied to measure the level of phenylalanine in samples. As shown in Table 1 all the three methods produce comparable results. The coefficient of variation amongst the three different readings is within 3–6%. Normal level of serum Phenylalanine is 60-240 µmol/L. Increased level of serum phenylalanine gives rise to disease conditions viz. mild phenylketonuria (PKU) [600-720 µmol/L] and moderate PKU [720-1200 µmol/L] while higher levels [1200-1800 µmol/L] are stated as classic PKU conditions. The samples collected from the three patients were examples of classic PKU and are undergoing treatment.

**Reproducibility and recycling of PAL immobilized films:**

The responses of 10 different films prepared separately were measured. The coefficient of variation is found to be ($r^2=0.9443$), showing that the film response is reproducible. The recycling experiments were carried out on the reagent films by exposing them to aqueous solutions of 0.1 M phosphate buffer pH 7.5 for 10 min after each use. The films can be used for at least five recycles before there is any deviation in the optical signal.

**Conclusion**

Instead of the enzyme phenylalanine hydroxylase, a cheaper alternative enzyme phenylalanine ammonia lyase, has been used for phenylketonuria (PKU) detection. The detection limit for this sensor is found to be 1.0 µmol/L. The response time is 30 min which is acceptable as compared to other methods. The sensor shows linear response over increasing concentrations. The sensor device is simple, fast (as compared to HPLC, Guthrie test, micro assay techniques) cost effective (much less than HPLC and commercial kits). Therefore, this assay is suitable for detection of PKU disease, especially in developing countries.
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Reference