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Properties of Immobilized Phenylalanine Ammonia Lyase and Investigation of its Use for the Prediagnosis of Phenylketonuria

[İmmobilize Fenilalanin Amonyak Liyazın Özellikleri ve Fenilketonüri Öntanısı için Kullanımının Araştırılması]

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ABSTRACT

Purpose: The aim of the present study was to investigate the properties of immobilized phenylalanine ammonia lyase and its use for the determination of phenylalanine in urine.

Material and Methods: Phenylalanine ammonia lyase was immobilized into gelatin on polyester films. The enzyme activity was determined by monitoring the production of t-cinnamic acid at 290 nm. Protein concentrations were measured by Bradford's method. For the determination of phenylalanine, calibration curve was drawn from known concentrations of phenylalanine.

Results: Appearent specific activity values of free and immobilized phenylalanine ammonia lyase were $3.9x10^{-1}$ and $1.4x10^{-1}$ units.mg protein^{-1}, respectively. K_m values were 14.2 mM and 64.9 mM and V_{max} values were $9.7x10^{-6}$M.min^{-1}and $4.9x10^{-6}$M.min^{-1} for free and immobilized enzyme, respectively. Then immobilized enzyme was used to determine phenylalanine in urine for the prediagnosis of phenylketonuri. Urine samples prepared by adding phenylalanine were analyzed and the recoveries obtained were 86.2% and 94.3%.

Conclusion: Polyester film strips on which phenylalanine ammonia lyase was immobilized were stored in borate buffer (pH 8.7) at 4°C for 90 days. Although immobilized enzyme was used 17 times in 2 months, it retained its apparent activity. Immobilized enzyme retained 100% apparent activity after 30 days and as much as 75% of activity was retained after 2 months. This method is sufficiently sensitive to determine the phenylalanine concentration in phenylketonuric infants' urine.

Keywords: immobilization, phenylalanine ammonia lyase (PAL), gelatin, polyester films, phenylketonuria (PKU)

ÖZET

Amaç: Bu çalışmanın amacı immobilize fenilalanin amonyak liyazın özelliklerini ve idrarda fenilalanin tayininde kullanımını araştırmaktır.

Gereç ve Yöntemler: Fenilalanin amonyak liyaz, destek materyal olarak kullanılan poliester film (asetat) üzerine, jelatine immobilize edilmiştir. Enzim aktivitesi 290 nm'de t-sinnamik asit oluşumu izlenerek tayin edilmiştir. Protein konsantrasyonları Bradford yöntemi kullanılarak ölçülmüştür. Fenilalanin tayini için bilinen fenilalanin konsantrasyonlarından yararlanarak kalibrasyon grafiği çizilmiştir.

Bulgular: Serbest ve immobilize fenilalanin amonyak liyaz enzimin görünür spesifik aktiviteleri sırasıyla $3,9x10^{-1}$ ve $1,4x10^{-1}$ ünite.mg protein^{-1} olarak bulunmuştur. Serbest ve immobilize enzim için K_m değerleri sırasıyla 14,2 mM, 64,9 mM olarak, V_{max} değerleri de $9,7x10^{-6}$ M.dak^{-1}, $4,9x10^{-6}$ M.dak^{-1} olarak bulunmuştur. Daha sonra immobilize enzim, fenilketonüri öntanısı amacıyla idrarda fenilalanin analizi için kullanılmıştır. Fenilalanin ekleyerek hazırlanan idrar örneklerinin analizinde %86,2 ve %94,3 geri kazanım elde edilmiştir.

Sonuç: İmmobilize fenilalanin amonyak liyaz enzimi içeren poliester stripler, 4°C'da borat tamponunda (pH 8,7) 90 gün boyunca depolanmışlardır. İmmobilize enzim 2 ay boyunca 17 kez kullanılmasına rağmen görünür aktivitesini korumuştur. İmmobilize enzim 30 günde görünür aktivitesinin tamamını, 2 ay sonra ise % 75'inden fazlasını korumuştur. Yöntem fenilketonüri öntanısı için, bebeklerin idrarındaki fenilalanin konsantrasyonunu tayin edebilecek duyarlılıktadır.

Anahtar Sözcükler: İmmobilizasyon, fenilalanin amonyak liyaz (PAL), jelatin, poliester film, fenilketonüri (PKU)

Introduction

Phenylketonuria (PKU) is an inborn error of metabolism caused by a deficiency of the enzyme phenylalanine hydroxylase. This results in a deficiency in the conversion of phenylalanine to tyrosine and in an increased level of phenylalanine and other metabolites [1]. The measurement of phenylalanine (Phe) concentration in blood and urine is important in the treatment of PKU and in genetic studies. Increased excretion of Phe in urine was recognised in 1938 as one of the biochemical characteristics of phenylketonuric children [2]

Different methods have been reported for the quantitation of Phe in blood, including Guthrie's test [3], fluorometric methods [4, 5], chromatography [6, 7] and mass spectrometry techniques [8]. Some enzymatic methods [9, 10, 11, 12] have been reported for determining the quantity of Phe in blood and urine, but these methods are not related to the use of immobilized Phenylalanine ammonia lyase. Phenylalanine ammonia lyase (PAL, EC 4.3.1.5) catalyses the conversion of phenylalanine to t-cinnamic acid and ammonia [13].

Immobilized systems have the advantages of reusability, continuous operational modes, rapid termination, high efficiency in multistep reactions and product formation control [14]. Gelatin is an inexpensive, abundant and safe material, largely used as an immobilization matrix [15]. Some studies were performed on the immobilization of different enzymes into photographic gelatin on polyester film strips precoated with a thin layer of photographic gelatin by Akbulut et al. [16-18].

In the present work, PAL was immobilized for the first time into gelatin using glutaraldehyde on polyester film strips and the concentration of phenylalanine in urine was determined using immobilized PAL.

Materials and Methods

Materials

Phenylalanine ammonia lyase from *Rhodotorula glutinis* was purchased from Sigma (P1016-10UN, 7.8 mg protein/ ml) and used as received. It was stored at -20°C. Gelatin was purchased from Fluka, while polyester films were purchased from a stationer's (Turkey). The other chemicals used were of analytical grade.

Immobilization of PAL

First 1.0 g of gelatin was dissolved in 10 ml of sodium borate buffer (pH 8.7, 33 mM) at 50°C and thereafter allowed to cool to 30°C. Then 100 µl portions of gelatin solution were taken and placed onto 1x7 cm polyester film strips with a micropipette. Following this procedure 15.0 µl of enzyme was added and incubated for 2 minutes. Then cross-linker 0.1 % (v/v) glutaraldehyde solution was added followed by incubation for 3 hours at room temperature. The PAL-coated films were finally washed with distilled water and maintained in sodium borate buffer (pH 8.7) at 4°C. The filtered borate buffer solution and washings were collected for loading efficiency determination [19]. Protein concentrations were measured with Bradford Reagent using the standard method [20].

Loading efficiency (%) $= ((C_i V_i - C_f V_f) / C_i V_i) \times 100$ **Eq (1)**

where C_i is the initial protein concentration, V_i the initial volume of enzyme solution, C_f the protein concentration in the total filtrate, and V_f the total volume of filtrate.

Determination of PAL activity

Apparent free and bound enzyme activities were determined according to the method described by Havir [21]. PAL activity was determined by monitoring the production of t-cinnamic acid at 290 nm. The reaction mixture contained sodium borate (33 mM, pH 8.7), L-phenylalanine and enzyme solution in a total volume 3.0 ml and it was incubated at 30°C. Immobilized PAL-gelatin polyester film strips were immersed in the reaction mixture at 30°C and incubated for 5 minutes. After incubation, the strips were removed from the solution to stop the reaction. The amount of t-cinnamic acid formed was calculated from the increase in absorbance using a molar extinction coefficient for t-cinnamic acid of $10^4 L.cm^{-1}.mol^{-1}$ [22]. One unit of activity is defined as the amount of enzyme required for the formation of 1 µmol of t-cinnamic acid in 1 min. under the assay conditions.

Enzymatic kinetic parameters were determined by changing the substrate concentration. The concentration of substrate L-phenylalanine in sodium borate buffer varied between 5.0 and 60mM. All readings were duplicated.

Phenylalanine calibration curve

Standard phenylalanine solutions ($5.0x10^{-2}$-1.5mM) were prepared in sodium borate buffer (pH 8.7). Immobilized PAL-gelatin polyester film strips were immersed in 3 ml of L-phenylalanine solution at 30°C and incubated for 5 min. The strips were removed from the solution after incubation to stop the reaction and the absorbance was read at 290 nm. A phenylalanine calibration curve was drawn from known concentrations of phenylalanine.

2.5. Preparation of urine samples containing phenylalanine

Urine samples were prepared using a modified procedure reported by Woolf and Goodwin [23] in which 0.4 ml of saturated basic lead acetate solution was added to 3.0 ml of normal urine and centrifuged. The supernatant was filtered through Whatman 42 paper and clear filtrate was obtained. The clear filtrate was used for analytical recovery by adding known amounts of phenylalanine. For the determination of recovery, phenylalanine was added to the clear filtrate to give concentrations of 1.5 and 3.0mM. To dissolve phenylalanine, one drop of concentrated HCl was added to the filtrate.

Analysis of urine samples

A 0.5 ml of urine sample containing phenylalanine was mixed with 2.5 ml of borate buffer (pH 8.7) and used for analysis. The final concentration was 0.25 and 0.50mM. Immobilized PAL-gelatin polyester film strips were immersed in the 3 ml of urine sample at 30°C and incubated for 5 min. The absorbance was read at 290 nm. The concentration values are the average of three repeated measurements.

Results

Immobilization of PAL

Table 1 summarizes apparent specific activities for free and immobilized PAL. The specific activity is defined as units/mg protein. Loading efficiency (percent of total enzyme immobilized) calculated according to Eq (1) was 74 %. Immobilization yield was calculated as 35% as follows: specific activity of immobilized enzyme / specific activity of free enzyme.

Table 1. Appearent enzyme activities of the free and immobilized PAL

PAL	Activity (units)	Protein (mg)	Specific Activity (units/mg)	Immobilizaton Yield (%)
Free	$2.6x10^{-2}$	$6.6x10^{-2}$	$3.9x10^{-1}$	-
Immo-bilized	$6.8x10^{-3}$	$4.9x10^{-2}$	$1.4x10^{-1}$	35

Kinetic parameters

The effects of substrate concentration on the enzyme activity with free and immobilized enzyme were investigated. Figure 1 shows the Lineweaver-Burk plots based on the results obtained at pH 8.7 at 30°C. Two linear equations of the immobilized PAL and free PAL were obtained from the plots, y=1335x+20.565 with regression coefficient (R^2) of 0.9912 and y=146.28x+10.286 with R^2 of 0.9683, respectively. The Michaelis-Menten constant, $K_{m,}$ and maximum reaction rates, V_{max} were obtained from the Lineweaver-Burk plot. K_m values were 14.2 mM and 64.9mM and V_{max} values were $9.7x10^{-6}$M.min^{-1} and $4.9x10^{-6}$M.min^{-1} for free and immobilized PAL, respectively. The K_m for immobilized PAL was higher than that of free enzyme, while V_{max} for the immobilized PAL was lower than that for free enzyme. The changes in K_m and V_{max} between free and immobilized PAL enzyme were mainly caused by mass transfer limitation and conformational changes in the immobilized enzyme. Most of the reported K_m values for free PAL were in the range of $0.3x\ 10^{-1}$ to 15mM [24].

Reusability and storage stability of immobilized PAL enzyme

Reusability of immobilized PAL was analyzed by measuring apparent activities 17 times over 2 months (Figure 2). Apparent activity in sodium borate buffer at pH 8.7

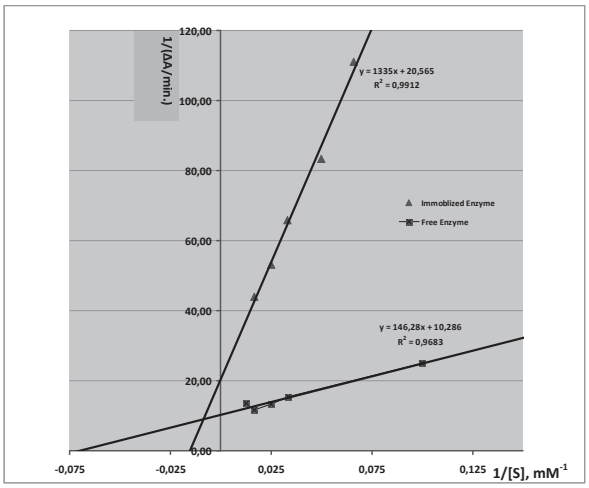

Figure 1. Lineweaver-Burk plot of free and immobilized PAL enzyme in pH 8.7 sodium borate buffer and at 30°C. Substrate concentration ranging from 5.0 to 60 mM

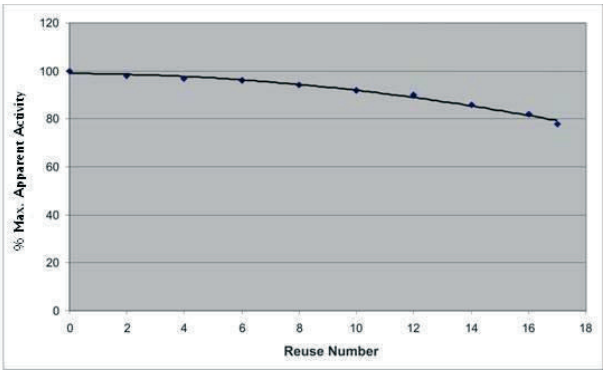

Figure 2. Reuse number of immobilized PAL enzyme. PAL activity was determined by monitoring the production of t-cinnamic acid at 290 nm (pH 8.7, 30°C)

was followed during storage at 4°C for 90 days (Figure 3). As seen in the plots, immobilized PAL enzyme retained 100 % apparent activity after 30 days and as much as 75 % of activity was retained after 2 months. Between each assay, the film strips were washed with distilled water and kept in sodium borate buffer (pH 8.7) at 4°C. The filtered borate buffer and washing solutions were used to investigate possible enzyme leakage. The enzyme (protein) in the solution was analyzed according to the Bradford's method [20]. The results showed that enzyme was immobilized tightly into gelatin by the chemical cross-linker glutaraldehyde and no significant enzyme leakage occurred during or after use.

Determination of phenylalanine concentration in urine

The phenylalanine calibration curve drawn from known concentrations of phenylalanine was linear with a regression coefficient (R^2) of 0.9948 and equation of y= 0.017x. The phenylalanine concentration in urine samples was found from the calibration curve (Figure 4).

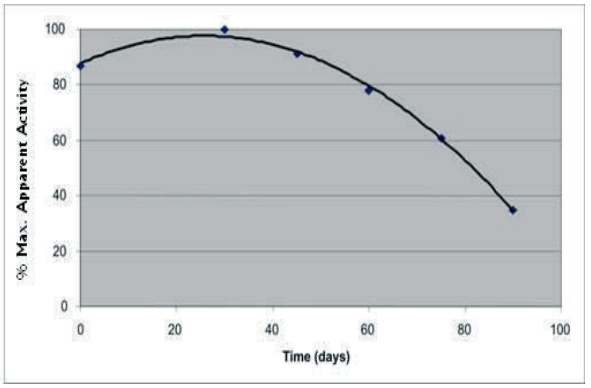

Figure 3. Storage stability of immobilized PAL. It was stored in sodium borate buffer (pH 8.7) at 4°C.

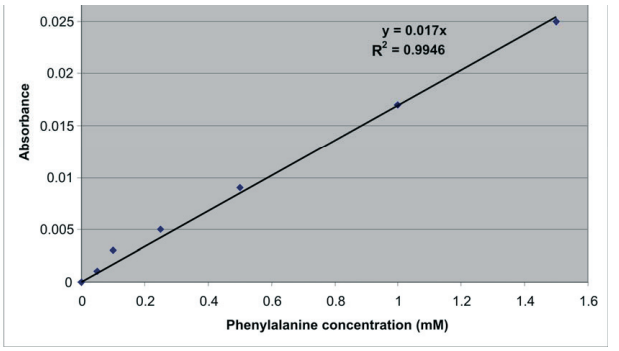

Fig. 4 Phenylalanine calibration curve in the range from $5.0x10^{-2}$ to1.5mM

Figure 4. Phenylalanine calibration curve in the range from $5.0x10^{-2}$ to 1.5 mM

Table 2 shows the recovery of phenylalanine added to urine samples. % Recovery (R) was calculated according to the following formula :

%R = (Added phenylalanine concentration / Found phenylalanine concentration) x 100

Recoveries of phenylalanine were 86.2% and 94.3%. These results show that this method can be used to determine the phenylalanine concentration in phenylketonuric infants' urine.

Table 2. The recovery of phenylalanine in urine

Sample	Phenylalanine Con. (mM)		Recovery (%)
	Added	Measured	
1	not added	unmeasurable	
2	0.25	0.29 ± 0.02	86.2
3	0.50	0.53 ± 0.01	94.3

Discussion

In the present study, PAL and uncoated polyester film were used while enzymes different from PAL and polyester films pre-coated with photographic gelatin were used in other studies in the literature. In addition, the immobilization technique was also different in this study. In previous similar studies, the gelatin, enzyme and cross-linking agents were firstly mixed and then dropped onto polyester films [16-18]. Loading efficiency for PAL immobilization using the literature technique was 50% which was very low. In our study, loading efficiency was 74% and immobilization yield was 35 %. No enzyme leakage was observed during storage or the reuse process. Therefore, the reason for this low immobilization value could have been the conformational changes in the immobilized enzyme or the limitation of substrate transfer into gelatin. In some previous studies, microencapsulated PAL was prepared for use in therapy for PKU but the encapsulated PAL had low activity i.e. only ~20 of the activity of free enzyme in buffer solution. [25-27]

PAL immobilized strips can be used 17 times without any significant difference in apparent activity. Protein analysis was performed in washing and buffer solutions. Enzyme leakage was not observed in these cases.

PAL immobilized strips were stored in borate buffer solution at 4°C for 30 days without any loss in apparent activity**.** However, after 2 months a rapid drop of activity occurred. As enzyme leakage was not observed, the reason for this decrease could be the fact that the immobilized enzyme was kept in the same buffer for 2 months. This long storage period may have led to a change in the pH of the buffer solution, which may have caused the fall in apparent activity [25].

As can be seen in Fig. 4, this method was not sensitive below the concentration of 0.05mM. Therefore PAL enzyme immobilized strips were not used in the determination of the phenylalanine concentration in normal urine. The mean phenylalanine concentration in normal urine is 0.15mM [8]. Because of dilution, this value corresponds to a mean of 0.025mM.

The mean phenylalanine concentration in phenylketonuric infants' urine is 1.6mM, ranging from 0.60 to 3.2mM. [8]. Because of dilution, this value corresponds to a mean of 0.26mM, ranging from 0.10 to 0.53mM in this study. This method can be used to determine the phenylalanine concentration sensitively in this concentration range. The assay could be used for prediagnosis of PKU. Furthermore, large-scale population studies must be carried out on the use of this method in routine clinical analysis.

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