PEG/Gelatin Composite Hydrogels as a Support of Enzyme Immobilization

Enzim İmmobilizasyonu için PEG/Jelatin Kompozit Hidrojeller

Research Article

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ABSTRACT

In the recent years composite hydrogels have gained considerable interest as a biomaterial vehicle for biomedical applications. The purpose of this study is to prepare highly biocompatible Polyethylene glycol (PEG)/Gelatin composite hydrogels for Glucose Oxidase (GOD) immobilization and evaluate the enzyme activity. PEG/Gelatin composite hydrogels were prapered by one step simultaneous technique based on UV-initiated free radical photopolymerization. PEG/Gelatin composite hydrogels were prepared by mixing Polyethylene (glycol) diacrylate PEG (30% - 50%) and Gelatin (22.000, 24.000, 40.000, 50.000, 87.500 MW) in the precense of ethylene glycol dimethacrylate (EGDMA) as crosslinker and 2,2-dimethoxy-2-phenylacetophenone (DMPA) as photo initiator and also GOD (0.1 mg/ml) for enzyme immobilization. Enzyme activity studies revealed that GOD immobilized PEG/Gelatin composite hydrogels that contain 30% PEG-DA presented a better activity, lower K_m and higher V_{max} than GOD immobilized composite hydogels that contain 50% PEG-DA. According to these results composite hydrogels are thought to be adapted for different enzyme systems with the selection of the suitable ratio.

Key words

PEG, Gelatin, Photopolymerization, Composite, Enzyme immobilization, Activity.

ÖZET

Son yıllarda kompozit hidrojeller, biyomedikal uygulamalar için biyomalzeme aracı olarak önemli bir ilgi kazanmıştır. Bu çalışmanın amacı, glikoz oksidaz (GOD) immobilizasyonu ve enzim etkinliğini değerlendirmek için oldukça biyouyumlu polietilenglikol (PEG)/jelatin kompozit hidrojeller hazırlamaktır. PEG/jelatin kompozit hidrojeller UV başlatıcılı serbest radikal fotopolimerizasyon esasına dayalı tek basamaklı simultane tekniği ile hazırlandı. PEG/jelatin kompozit hidrojeller etilen glikol dimetakrilat (EGDMA) çapraz bağlayıcı maddesi, 2,2-dimetoksi-2-fenilasetofenon (DMPA) foto başlatıcısı ve enzim immobilizasyonu için glukoz oksidaz (GOD) (0.1 mg/ml) varlığında (%30 ve 50) polietilen glikol (PEG) ve jelatin (22.000, 24.000, 40.000, 50.000, 87.500 MW) karıştırılarak hazırlanmıştır. Enzim aktivite çalışmaları, % 30 PEG içeren GOD immobilize PEG/jelatin kompozit hidrojellerin % 50 PEG içeren GOD immobilize PEG/jelatin kompozit hidrojellere göre daha iyi aktivite, düşük K_m ve daha yüksek V_{max} sergilediğini göstermiştir. Bu sonuçlar, kompozit hidrojellerin uygun oranının seçimi ile farklı enzim sistemlerine adapte edilebileceğini düşündürmüştür.

Anahtar Sözcükler

PEG, Jelatin, Fotopolimerizasyon, Kompozit, Enzim immobilizasyonu, Aktivite.

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INTRODUCTION

Immobilization of enzymes has many advantages such as increase in enzyme stability, ease of separation of the catalyst from the reaction media, continuous or repeated use of the enzyme, and low cost of the process [1]. Selection of an immobilization strategy greatly influences the properties of biocatalyst. The varying levels in activity and diffusion limitations occuring with immobilization are mainly dependent on the properties of support material and the immobilization method. Support materials play an important role in the usefulness of immobilized enzymes as it should be low cost and provide adequate large surface area together with the least diffusion limitation in the transport of substrate and product for enzymatic reactions [2]. Among all the enzyme immobilization methods reported (e.g. covalent binding, crosslinking, adsorption) the entrapment method stands out because it is mild and causes relatively little damage to the native structure of the enzymes.

In search of suitable supports for enzyme immobilization, hydrogels have been found to be among the top candidates [3-5]. Hydrogels are water swollen polymer matrices, with a huge tendency to absorb water. Their ability to swell, under physiological conditions, makes them an ideal material for biomedical applications [6]. The hydrophilicity of the network is due to the presence of chemical residues such as hydroxylic, carboxylic, amidic, primary amidic, sulphonic and others that can be found within the polymer backbone or as lateral chains. It is also possible to produce hydrogels containing a significant portion of hydrophobic polymers, by blending or copolymerizing hydrophilic and hydrophobic polymers, or by producing interpenetrating networks (IPN) or semi-interpenetrating polymer networks (s-IPN) of hydrophobic and hydrophilic polymers.

Composites are engineered materials made from 2 or more constituent materials with significantly different physical or chemical properties and these materials remain separate and distinct on a macroscopic level within the finished structure [7]. Composite hydrogels offer several benefits from a biomedical engineering perspective. The hydrogel can mask any issues regarding particle biocompatibility, as the particles will be able to "hide" within the hydrogel system. The hydrogel network provides an additional diffusive barrier for enzymes, offering potential for the generation of novel enzyme activity profiles. Composite hydrogels also offer the unique potential to independently engineer the hydrogel to optimize the enzyme activity. This will allow for tunable features to be used. The soft and hydrated environment of a swollen hydrogel can provide enzymes with near physiological conditions that minimize denaturation and help them to carry out their full biological functions.

Composite hydrogels can be prepared from natural or synthetic polymers. Several techniques have been reported for the synthesis of biomedical hydrogels [8,9]. Chemically cross-linked gels have ionic or covalent bonds between polymer chains.

Poly(ethylene glycol) (PEG) is a hydrophilic, water soluble, biocompatible polymer [10,11] that has been suggested for use in a variety of biomedical applications [12,13]. Substituting terminal hydroxyl groups with acrylates, forming poly(ethylene glycol) diacrylate (PEG), allows the polymer to be crosslinked to form a three-dimensional polymer network.

The incorporation of gelatin component presents elasticity and biodegradability to the network. Gelatin hydrogels are common for the applications in medical areas [14]. Gelatin hydrogels have often crossed-linked by chemical approach using crosslinks, such as glutaraldehyde, to improve elasticity, consistency, and stability.

In this study PEG/Gelatin composite hydrogels were prepared for the entrapment of glucose osidase by varying PEG/DA concentrations and gelatin molecular weight and the catalytic ability of immobilized glucose oxidase was investigated. For this purpose, glucose osidase was immobilized by entrapment PEG/Gelatin composite hydrogels that contain natural and synthetic polymer combination. Glucose oxidase was immobilized in these composite hydrogels by entrapment since this process takes place under mild conditions and do not cause any significant damage to the native structure of the enzyme.

MATERIALS AND METHODS

Materials

Poly(ethylene glycol)-diacrylate (PEG-DA, Mn: 700), cross-linking agent ethylene glycol dimethacrylate (EGDMA) and 2,2-dimethoxy-2-phenyl-acetophenone (DMPA) were obtained from Aldrich Chemical Company,Glucose Oxidase Type X from *Aspergillus niger* (E.C.232-601-0), Peroxidase Type II from horseradish (E.C.232-668-6), glutaraldehyde (GA) solution (25% v/v in water), D(+)- glucose was purchased from Merck. All other reagents used were of analytical grade and used without further purification.

Preparetion of GOD immobilized PEG/ Gelatin composite hydrogels

Enzyme immobilized PEG/Gelatin composite hydrogels are prepared by a process in which both component networks are polymerized concurrently, the composite hydrogels may be referred to as a simultaneous method.

Several combinations of polymers were prepared into composite hydrogels formulations to determine their potential as Enzyme immobilization system. An attempt of combining natural polymer such as gelatin and synthetic polymers such as PEG/DA composite hydrogels to improve the mechanical strength of natural polymers and to overcome the limitations of synthetic polymers. PEG/Gelatin composite hydrogels were prepared with varying, concentrations of PEG/DA mactomer and molecular weights of gelatin in the presence of the enzyme, the photoinitiator and crosslinker (Table 1).

Glucose oxidase was selected as a model enzyme. Its chemical structure is shown Figure 1. PEG/Gelatin composite hydrogels were all observed to be very homogeneous, clear and transparent. Incorporating PEG/Gelatin composite hydrogels were prepared matrices enhanced and maintained the mechanical stability [5]. The incorporation of gelatin component presented elasticity and biodegradability to the network.

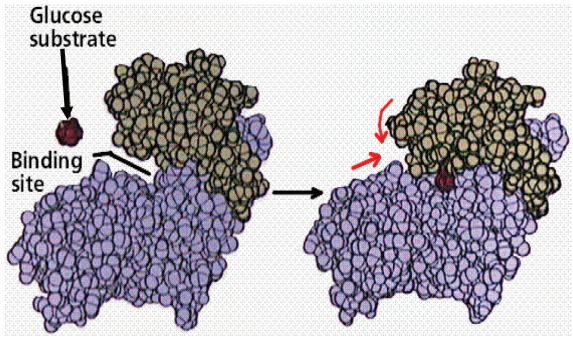

Figure 1. X-ray model Glucose oxidase enzyme.

Determination of Glucose Oxidase Activity

Activities of both free and immobilised GOD were obtained by measuring the amount of hydrogen peroxide formed from glucose conversion, spectrophotometrically. 2.5 ml of a mixture containing Peoxidase (POD) (1.5 mg) and o-dianisidine (3.3 mg) was added in 50 ml of 0.1 mM phosphate buffer (pH 7.0), and incubated for 10 min 25°C. A 100 μL sample obtained by the oxidation of D-glucose by GOD was added to the assay mixture. After 10 min, 1.5 ml of sulphuric acid solution (30%) was added to this mixture to stabilize the colour formed. The absorbance of the final solution was measured spectrophotometrically (LABOMED, Inc. Spectro UV-vis double) at 525 nm [15].

One unit of glucose oxidase activity is defined as the amount of enzyme which oxidizes 1 μM

Table 1. PEG/Gelatin composite hydrogels.

GOD immobilized PEG/Gelatin Composites	% PEG/DA	GELATIN TYPE (Bloom)
A	30	300 (M_w= 87500)
B	30	225 (M_w = 50000)
C	30	150 (M_w =40000)
D	30	75 (M_w =24000)
E	50	300 (M_w = 87500)
F	50	225 (M_w = 50000)
G	50	150 (M_w =40000)
H	50	75 (M_w =24000)

of β-D-glucose to D-gluconic acid and hydrogen peroxide per min at 25˚C and pH 7.0. Relative activity was defined as the ratio of absorbance to maximum absorbance (The relative activity of the enzyme with highest absorbance is 100% in each figure). The standard calibration curve of hydrogen peroxide was used to obtain quantitative information about the amount of product formed by the catalytic reaction of GOD.

Kinetic parameters

For enzymes, two kinetic parameters, K_m and V_{max} are important. K_m is the Michaelis constant, defines concentration of substrate at which the rate of reaction is equal to one half of the maximum rate. V_{max} defines the maximum rate obtained for an enzyme in the presence of excess substrate. K_m and V_{max} values of immobilized laccases were determined from Lineweaver–Burk graphs by measuring the initial rates of the reactions with using D(+)-glucose as substrate with a concentration range of 5.05 and 20.20 mM at room temperature.

RESULTS

Preparetion of GOD immobilized PEG/ Gelatin composite hydrogels

SEM was employed to investigate morphologies of hydrogels before and after adition of gelatin. Comparing the hydrogels as shown in Figure 2. Hydrogel that contain 30% PEG have loose network structure whereas hydrogel that contain 50% PEG have tight network structure. The SEM images show that the PEG-DA solution+1% gelatin appears to form small spaces (Figure 3). PEG/ DA gelatin composite hydrogel possessed large numbers of interconnected spaces, indicating that formation hydrogel with the hollow structure. Composite hydrogels that contain 75 bloom and 100 bloom gelatin possesses large numbers of spaces, indicating that formation of composite hydrogel would not destroy the hollow structure. In the structure of composite hydrogel the inner surface contained large numbers of the spaces connected to each other. It can be observed in the SEM image which shows structures with a great penetration of the medium into the system with spaces that form connections (channels) with the

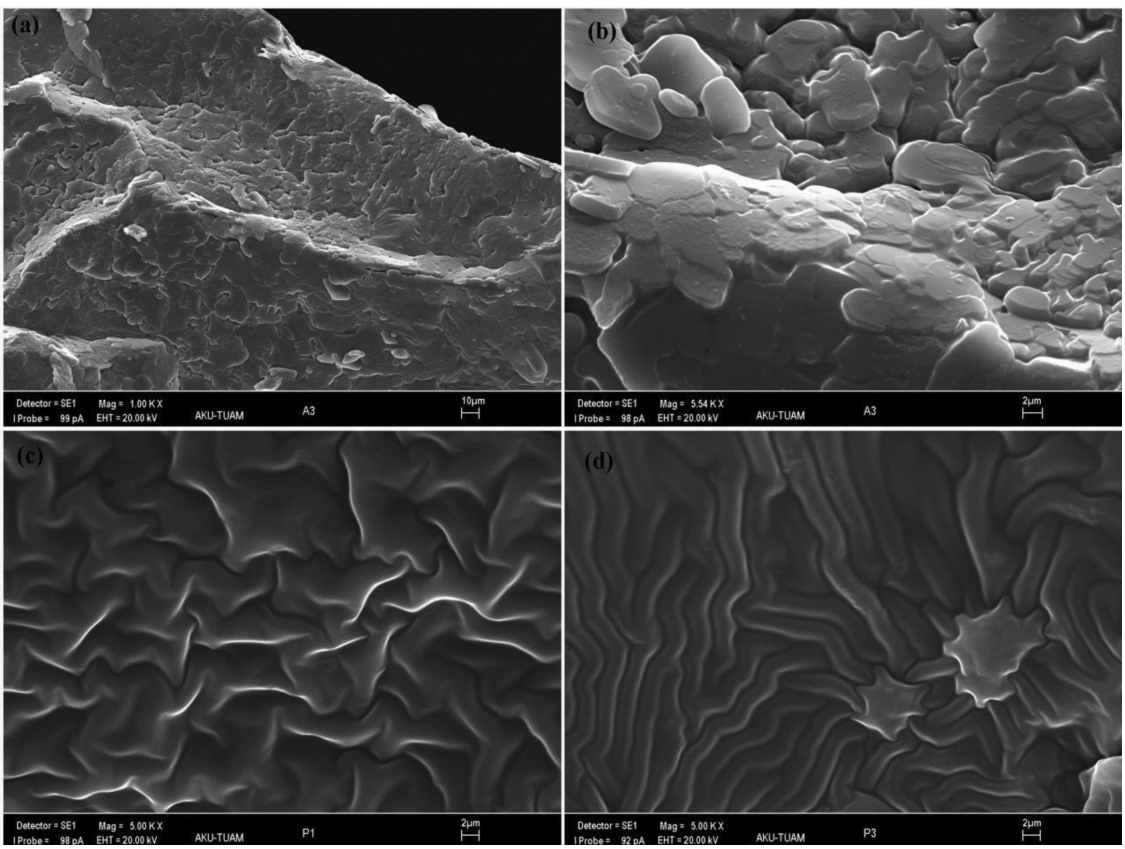

Figure 2. SEM images of PEG-DA hydrogels. (a) Surface of 30 % 1000x zoom, (b) Surface of 50 %; 5500x zoom, (c) Section of 30 % 5000x zoom, (d) Section of 50 %; 5000x zoom.

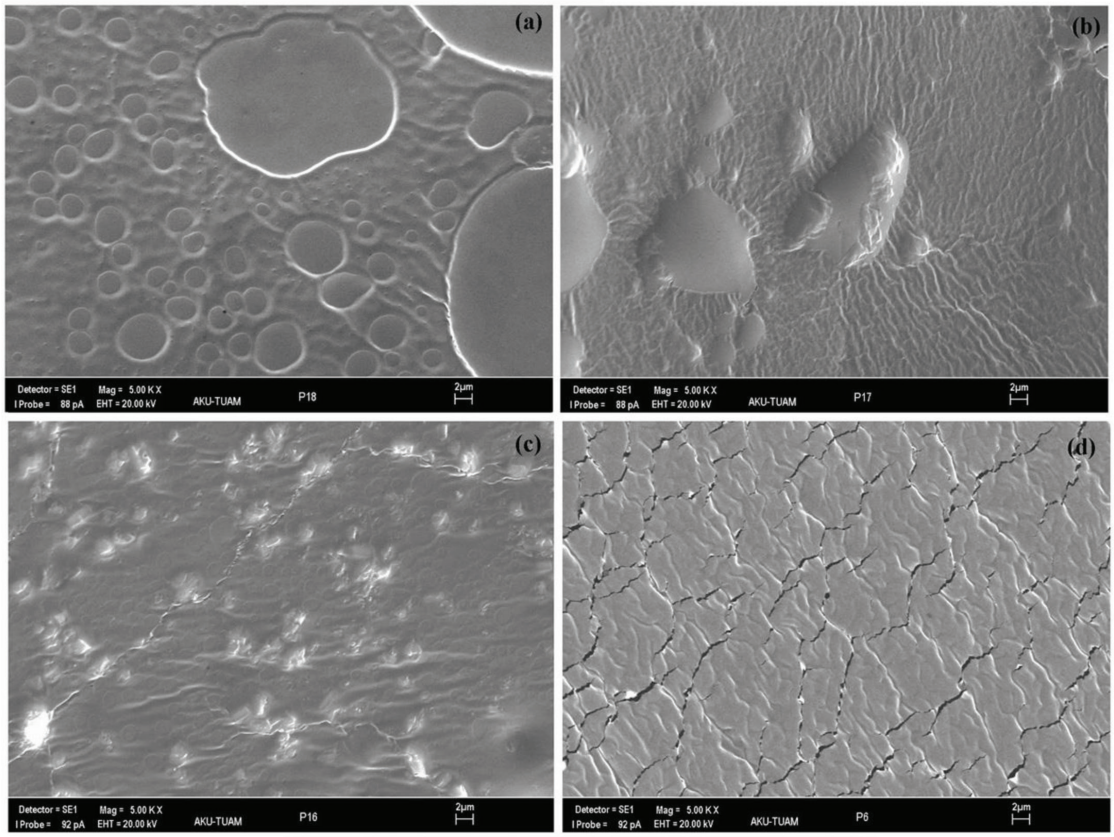

Figure 3. SEM images of PEG-DA/Gelatin composite hydrogels with 1 % gelatin (5000x zoom) (a) 75 bloom; (b) 150 bloom; (c) 225 bloom; (d) 300 bloom.

interior of the structure. The capillary channels were clearly observed from SEM image and this may enable substrate to enter into the hydrogel networks or drug molecules to diffuse out of them. Whereas composite hydrogels that contain 225 bloom and 300 bloom gelatin caused cracks in structure.

The catalytic reactions of GOD-containing PEG/Gelatin composite hydrogels based on two different concentrations of PEG/diacrylates (30%-50%) and five different moleculer weight of gelatin were monitored through a colorimetric assay, based on the oxidation of o-dianisidine through a peroxidase-coupled system.

The relative enzymatic activities and converted glucose amount of PEG/Gelatin composite hydrogels with different formulations were shown in Figure 4a-b.

As it seen in Figure 4a-b PEG/Gelatin composite hydrogels, containing 30% PEG supplemented with different MW Gelatin

Table 2. The summary of enzyme activity studies.

GOD immobilized PEG/Gelatin Composites Type	% Relative Aktivities	Glucose Amount (mmol/min)	K_m (mM)	V_m (10^{-3}mM/min)
A	47.97	0.077	0.1697	4.22
B	49.32	0.079	0.1662	4.41
C	53.63	0.086	0.1603	4.93
D	57.44	0.092	0.1558	5.10
E	25.46	0.041	0.312	2.56
F	25.95	0.042	0.307	2.40
G	26.69	0.043	0.216	2.49
H	41.08	0.066	0.173	3.72
Free Enzyme	100.00	0.16	0.1427	5.14

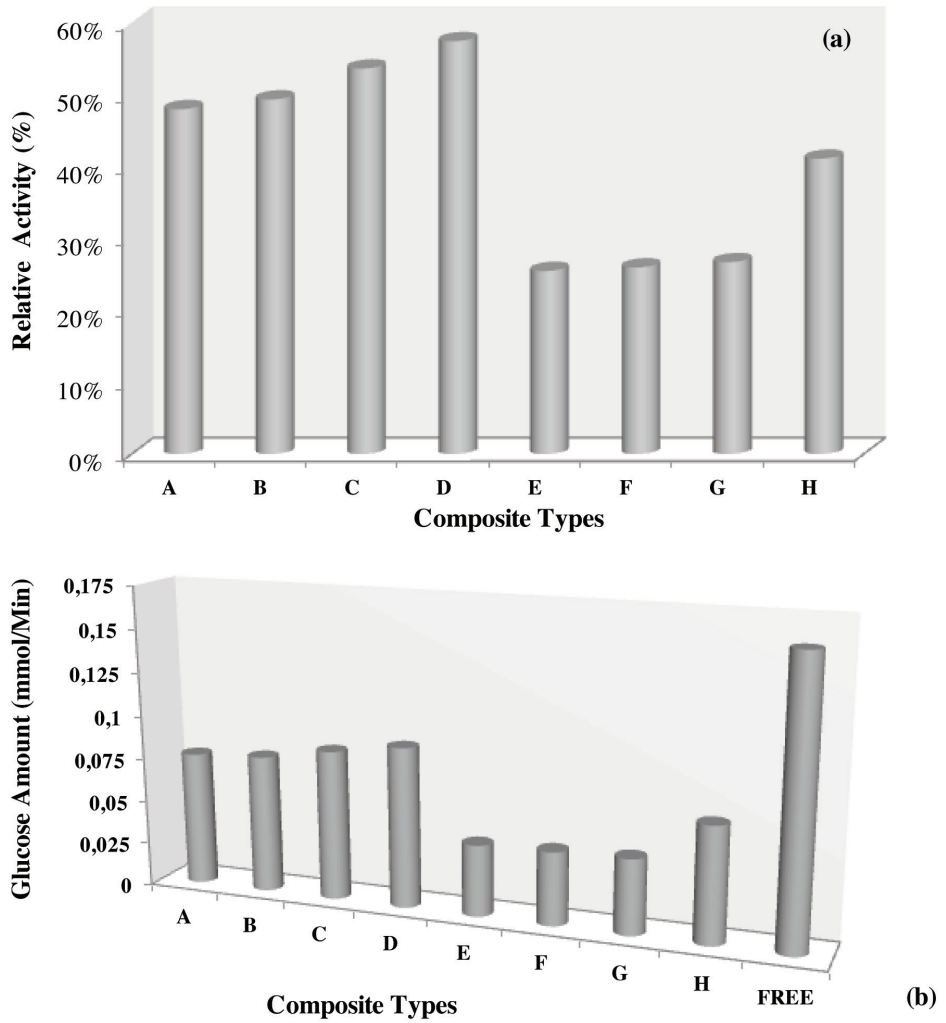

Figure 4. (a) Relative enzyme activities of composite types (b) Converted amount of GOD.

(A,B,C,D) exhibited higher activity than hydrogels, containing 50% PEG supplemented with different MW Gelatin (F,G,H,I). It means more GOD enzyme physically immobilized inside A,B,C,D, PEG/ Gelatin composite hydrogels than F,G,H,I, due to the larger mesh size of the hydrogel [16,17].

GOD-catalyzed reactions were further characterized by determination of the apparent Michaelis-Menten constant (K_m), which is calculated by relating the initial rate of the GOD-catalyzed reaction to the concentration of glucose. The change in apparent K_m values is related to the affinity of enzyme toward substrate, and a low value of apparent K_m suggests that the enzyme has a high affinity for the substrate.

As shown in Figure 5a. formulations that contain 30% PEG supplemented with different MW gelatin have lower values for K_m than the formulations that contain 50% PEG/DA supplemented with different MW gelatin. This result indicates that the hydrogel acted as a mass transport barrier, but mass transport resistance decreased with the increase in content of PEG due to the lower crosslinking density. The larger mesh size of a hydrogel prepared from higher content of PEG allowed fast diffusion of glucose into the hydrogel matrix, thus providing higher accessibility of the substrate to the active site of the immobilized enzyme, resulting in a lower apparent Km value. As shown in Fig 5b. 30% PEG supplemented with different MW gelatin composite hydrogels have higher V_{max} values, demonstrating that A,B,C,D formulations have

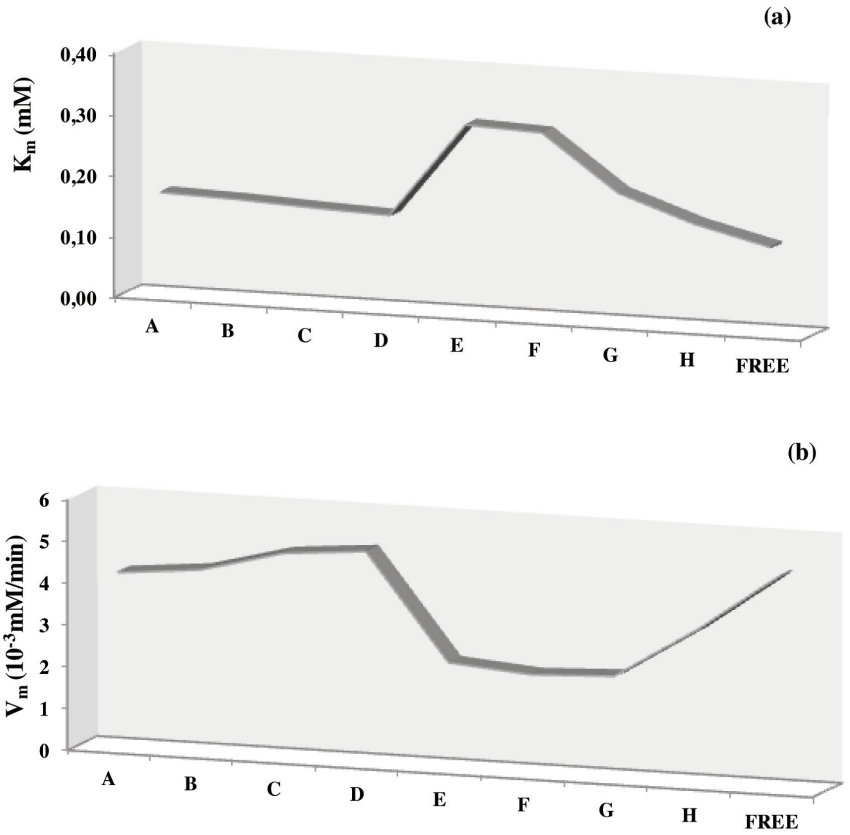

Figure 5. (a) K_m values of GOD, (b) V_m values of GOD.

better affinity towards the substrate. Therefore, it can be observed that A,B,C,D formulations are more ideal enzyme support than F,G,H,I.

Entrapment is physical enclosure of the enzyme in the microspaces formed in the matrix structure. So, the three dimensional structure of the enzymes may not be affected upon immobilization. A immobilized enzymes can be observed to be similar to that of the free enzyme, as was reported in the literature [18, 19].

Biocompatible and biodegradable polymers have been used as potential carriers for enzymes. The natural as well as synthetic polymers alone are not always able to meet the complex demands of the enzyme systems. The advantage of natural polymers are valuable in pharmaceutical industry due to their non-toxicity, low cost, biodegradability, biocompatibility and safety but some of their physical attributes are often poor while the success of synthetic polymers based on their broad range of mechanical properties.

CONCLUSIONS

PEG is one of the most widely used materials for biomedical applications and has arguably become the standard for hydrogel biomaterials. PEG is biologically inert, being both nontoxic and nonimmunogenic, and has been approved by the U.S. Food and Drug Administration for a variety of clinical applications. Numerous methods have been developed to produce PEG hydrogels through covalent crosslinking of PEG prepolymer; free radical polymerization of PEG acrylates, Michael-type addition, enzymatic reaction, and radiation [20-27].

Gelatin is an inexpensive, natural, edible, biocompatible, nontoxic material derived from acid or alkaline hydrolysis of collagen [28]. The supplementation of PEG hydrogels with gelatin has led to the development of a photocrosslinkable hydrogel that is inexpensive, easily produced with common equipment and chemicals, and biologically and mechanically tunable.

In this study PEG/Gelatin composite hydrogels were synthesized for enzyme immobilization. The study showed that both the PEG concentrations and the gelatin bloom affected the enzymatic activity. According to these results composite hydrogels are thought to be adapted for different enzyme systems with the selection of the suitable ratio.

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