Cross- Linked Glucose Oxidase Aggregates: Synthesis and Characterization

Çapraz Bağlı Glukoz Oksidaz Agregatları: Sentez ve Karakterizasyonu

Research Article / Araştırma Makalesi

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ABSTRACT

The aim of the submitted study is to realize carrier-free immobilization (as cross-linking enzyme aggregates) of *Aspergillus niger* Glucose Oxidase enzyme, to determinate the optimum immobilization conditions, and to investigate the properties of immobilized enzyme sets. Carrier-free enzyme immobilization was realised by forming cross-linked glucose oxidase enzyme aggregates (CLEA's). Different precipitants were used in different media conditions during forming CLEA's. The "immobilized enzyme activity" was measured by activity determination method with the use of glucose substrate. The optimum conditions which were determined in the light of the experiments for the cross-linked enzyme aggregates can be summarized as; the initial enzyme concentration, 0.05 mg/ml; the optimum temperature, 25°C; the precipitant type, BSA; the precipitant concentration, 5 mg/ml; the concentration of glutaraldehyde, 2% (v/v). After determining the optimum conditions, glucose solutions at different concentrations (1.0-4.0 mg/dl) were prepared for investigating the performance of the glucose oxidase aggregates. The kinetic parameters were calculated by Lineweaver-Burk plots such as; K_m=0.0115 mM; V_m=1.206 mM.min^{-1} for native enzyme and K_m= 0.025 mM; V_m=0.593 mM.min^{-1} for cross-linking enzyme aggregates.

Key Words
Glucose oxidase, carrier-freeenzyme Immobilization, cross-linked enzyme aggregates, CLEA.

ÖZET

Sunulan çalışmanın amacı *Aspergillus niger* Glukoz Oksidaz enziminin taşıyıcısız immobilizasyonunu (çapraz bağlı enzim agregatları olarak) gerçekleştirmek, en uygun immobilizasyon koşullarını bulmak ve immobilize enzim kümelerinin özelliklerini belirlemektir. Taşıyıcısız enzim immobilizasyonu çapraz bağlı glukoz oksidaz enzim agregatları (ÇBEA) oluşturarak uygulanmıştır. ÇBEA'nın oluşumu sırasında farklı çöktürücüler yine farklı ortamlarda kullanılmıştır. "İmmobilize enzim aktivitesi" aktivite tayin yöntemine göre glukoz substratı kullanılarak ölçülmüştür. Deneylerin sonunda çapraz bağlı enzim agregatları için bulunan ve en yüksek enzim aktivitesini veren en uygun koşullar: başlangıç enzim derişimi, 0.5 mg/ml; en uygun sıcaklık, 25°C; çöktürücü türü, Bovin Serum Albümin (BSA); çöktürücü derişimi, 5 mg/ml; ve glutaraldehit derişimi, % 2 (v/v). En uygun koşulların belirlenmesinden sonra glukoz oksidaz agregatlarının performanslarını belirlemek üzere farklı derişimlere sahip glukoz çözeltileri hazırlandı (1.0 - 4.0 mg/dl). Verilerin Lineweaver-Burk doğrusu olarak çizimi ile kinetik parametreler K_m= 0.0115 mM ve V_m=1.206 mM.min^{-1} serbest enzim için ve ÇBEA için K_m= 0.025 mM; V_m=0.593 mM.min^{-1} olarak hesaplanmıştır.

Anahtar Kelimeler
Glukoz oksidaz, taşıyıcısız enzim immobilizasyonu, çapraz bağlı enzim agregatları, ÇBEA

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INTRODUCTION

Researches about enzymes were devoted to the improvement of various carrier-bound immobilised enzymes with the aim of facilitating their use in continuous processes for the last 50 years, [1,2]. Enzyme immobilisation can accomplish developed enzyme performance such as activity, stability and selectivity [3,4]. Successful immobilisation is depending on selection a suitable carrier, conditions (pH, temperature, medium) and enzyme. Because of this, the design of carrier-bound immobilised enzymes relies largely on laborious and time-consuming trial and error experiments [5]. So, there is a new interest in carrier-free immobilised enzymes such as cross-linked enzyme aggregates (CLEA) [6]. CLEAs were added to the immobilised enzymes techniques more recently [6]. By altering properties that effect the proximity of soluble enzyme molecules, they can be made to form physical aggregates which after cross-linking. For example, it is possible to form aggregates by changing the hydration state of enzyme molecules or changing the electrostatic constant of the solution by adding appropriate aggregation agents [7]. Enzyme molecules were precipitated as insoluble aggregates with native enzyme conformation and these insoluble aggregates can be cross-linked by bifunctional cross-linkers [6].

CLEAs can be prepared with greater mechanical stability and tailor-made size. In principle, CLEAs are applicable to any reaction system, reactor configuration and reaction medium. Recently studies enclose the development of CLEAs of abroad range of enzymes, size control, new aggregation methods, new precipitants and new crosslinkers to construct a flexible technology platform for designing robust CLEAs for broad applications [8]. Glucose oxidase is used for production of gluconic acid in industry and in foods as a protective material. The most important usage is diagnosis for diabetes mellitus.

In this study, glucose oxidase from *Aspergillus niger* has been converted to physical aggregates by adding different precipitants. The effects of initial enzyme concentration, temperature, precipitant type and concentration, and glutaraldehyde concentration on GOD aggregates were studied.

MATERIALS AND METHODS

Materials

Glucose Oxidase Type X from *Aspergillus niger* (E.C.232-601-0), glutaraldehyde (GA) solution (25% v/v in water), bovin serum albumin (BSA, 96%, M_W: 66kDa), sodium dodecyl sulfate (SDS, M_W: 288.38) were purchased from Sigma. D(+)-glucose was purchased from Merck. All other reagents used were of analytical grade and used without further purification.

Preparation of glucose oxidase aggregates (CLEA)

Glucose oxidase aggregates were prepared as described below. Stock enzyme solutions were prepared by dissolving the known amount of enzyme in pH 5.0 acetate buffer. The precipitant was poured to a centrifuge tube, 80 μl from stock enzyme solution was added and stirred gently about 5 min until precipitant dissolves. A 20 μl of 0.1 N NaOH was added to neutralize the medium pH. Glutaraldehyde solution was added drop wise and the final volume was made up to 1 ml with distilled water. GA concentration adjustment was applied to be 2% v/v according to the final volume. The final solution was incubated at 25°C for 2 h where gentle stirring was applied for initial 15 min and the reaction mixture was left overnight at 4°C. The aggregates were collected by centrifugation at 7000 rpm for 30 min at 4°C. The residue was washed with distilled water, centrifuged for 15 min each time and the process repeated until no protein was determined in the supernatant. The existence of protein in the supernatant was measured spectrophotometrically at 219 nm [9].

The selected parameters below were investigated to determine the optimum conditions which give maximum activity. The enzyme concentration was changed between 0.8 x 10^{-3} and 8 x 10^{-3} mg/ml from the initial stock enzyme concentration of 0.100 mg/ml. The incubation temperature was selected as 4, 25 and 37°C. The two different precipitants types, BSA and SDS, were used for aggregate formation. The precipitant and GA concentrations were changed as 1, 3, 5, 7.5, 10, 15, and 20 mg/ml and 1, 2, 3, 4, and 5 % (v/v, according to the final volume), respectively. The substrate concentration, D-glucose, were kept constant as

0.5 M in activity measurements for all optimization conditions.

Measurement of native and CLEA 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 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 at 525 nm [10].

One unit of glucose oxidase activity is defined as the amount of enzyme which oxidizes 1 μM of β-D-glucose to D-gluconic acid and hydrogen peroxide per min at 25˚C and pH 7.0. The activity yield of the prepared aggregates was expressed as the percentage of the ratio of the overall activity of the aggregates to the overall activity of the initial enzyme solution (free enzyme amount that attend to aggregate formation).

The stability and reusability performance of Glucose Oxidase aggregates

CLEAs were prepared with the initial enzyme concentration depicted before. The prepared aggregates were tested for their storage stability up to 5 months. The CLEAs were stored at +4°C and the activities of the CLEAs were tested after 1, 3, and 5 months with 0.5 M D-glucose concentration. The activity measurements were carried out by the same CLEAs which was prepared with a constant initial enzyme concentration for a period of five mounts. CLEAs were washed in pH 7 phosphate buffer after each measurement and stored in 1 ml of 0.1 M NaN_2 solution until the next activity measurement. The reusability of GOD CLEAs was tested by the treatment of CLEAs with D-glucose solutions between 5.05 and 20.2 mM concentrations. The initial enzyme concentration was 4 x 10^{-3} mg/ml. All the results were given as the average of the three experimental measurements.

Determination of kinetic parameters

The kinetic parameters, K_m and V_{max}, for both free and immobilized enzyme were calculated by the Lineweaver-Burk double-reciprocal plot method of Michaelis-Menten Equation between 5.05 and 20.20 mM D(+)-glucose concentrations at room temperature. All the results were given as the average of the three experimental measurements.

Structure of CLEAs

Olympus CX-31 optic microscope and Leo 1430 vp scanning electron microscope (SEM) were used for the structural analysis of CLEAs.

RESULTS AND DISCUSSION

The cross-linked glucose oxidase aggregates were prepared by extensive cross-linking of the enzyme by glutaraldehyde. Soluble enzyme molecules can be made to form physical aggregates. By adding aggregation agents at these conditions soluble enzyme aggregates associate together to extent that they precipitate as insoluble aggregates with native enzyme conformation.

Table 1 publishes the results of the visual observations. Reaction conditions for Cross Linking Glucose Oxidase aggregates formation parameters were given in the Table 1. As it is seemed from the Table 1, at low enzyme concentrations very little insoluble aggregate formations were obtained. At 4 x 10^{-3} mg/ml enzyme concentration and at higher concentrations, same aggregate formation was seen. But enzymatic activity decreased at these (6 x 10^{-3} mg/ml and 8 x 10^{-3} mg/ml) concentrations.

This result is caused by enzyme accumulation and active sides of enzyme molecules might be restricted. At all of the temperature (4, 25, 37°C) aggregation formation was obtained. At 4 and 25˚C, the same aggregate formation was observed. Using BSA as a precipitant, aggregate formation was more successful than using SDS when both immobilization efficiency and activity values were compared.

Aggregate formation was promoted by increasing of BSA amount. At low glutaraldehyde concentrations good aggregate formation was obtained but at high concentrations, low aggregation was observed.

Table 1. Reaction conditions for cross-linked glucose oxidase aggregates.

Enzyme Concentration (mg/ml)	Temperature (°C)	Precipitant type	Precipitant Concentration (mg/ml)	GA Concentration (% v/v)	Relative Activity (%)	Immobilization Efficiency (%)	Activity (%)	Activity yield
0.8 x 10^{-3}	25	BSA	5	2	50.52	92.97	54.34	53.57
2 x1 0^{-3}	25	BSA	5	2	53.48	94.05	56.86	58.80
4 x 10^{-3}	25	BSA	5	2	94.81	88.11	107.6	106.78
6 x 10^{-3}	25	BSA	5	2	61.93	91.35	67.79	68.54
8 x 10^{-3}	25	BSA	5	2	55.40	94.59	58.57	60.34
4 x 10^{-3}	4	BSA	5	2	74.37	89.73	82.88	77.54
4 x 10^{-3}	25	BSA	5	2	94.81	88.11	107.6	106.78
4 x 10^{-3}	37	BSA	5	2	21.80	92.97	23.45	6.46
4 x 10^{-3}	25	BSA	5	2	94.81	88.11	107.6	106.78
4 x 10^{-3}	25	SDS	5	2	60.44	71.76	84.23	73.17
4 x 10^{-3}	25	BSA	1	2	49.19	94.59	52.00	41.23
4 x 10^{-3}	25	BSA	3	2	91.85	92.97	98.80	97.05
4 x 10^{-3}	25	BSA	5	2	94.81	88.11	107.6	106.78
4 x 10^{-3}	25	BSA	7.5	2	80.74	94.52	85.42	81.19
4 x 10^{-3}	25	BSA	10	2	76.45	91.89	83.20	77.99
4 x 10^{-3}	25	BSA	15	2	76.44	91.62	83.43	78.67
4 x 10^{-3}	25	BSA	20	2	72.44	92.57	78.25	72.17
4 x 10^{-3}	25	BSA	5	1	81.93	93.46	87.66	83.90
4 x 10^{-3}	25	BSA	5	2	94.81	88.11	107.6	106.78
4 x 10^{-3}	25	BSA	5	3	85.48	95.41	89.59	86.66
4 x 10^{-3}	25	BSA	5	4	63.04	93.73	67.26	59.31
4 x 10^{-3}	25	BSA	5	5	43.85	94.92	46.20	34.35

Initial Enzyme Concentration

Initial enzyme concentration is an important criterion which affects the activity of immobilised enzyme. The results of this group of experiments were submitted in Figure 1. The initial enzyme concentrations were changed between 0.8 x 10^{-3} and 8 x 10^{-3} mg/ml at 25°C with 5 mg/ml BSA precipitant concentration. GA was added to be as 2% v/v in the final solution. It was found an optimum initial enzyme concentration of 4 x 10^{-3} mg/ml which showed a 107.6% enzyme activity after cross-linking as aggregates. The immobilization efficiency decreases to 88%, while all other results were found higher than 90% as given in Figure 1.

The low initial enzyme concentrations, 0.8 x 10^{-3} and 2 x 10^{-3} mg/ml, gave efficiency more than 90% but measured activities were about 54-56% which was highly low compared to 4 x 10^{-3} mg/ml. It seems that when enzyme concentration is low, the precipitant and/or GA act as to inhibit or hinder the active site of the enzyme molecules.

The apparent enzymatic activity of CLEAs decreased at higher concentration than 4x10^{-3} mg/ml enzyme concentration probably because precipitant and cross-linker concentrations were not enough for aggregation and cross linking as the indicated values above. So, some of the enzyme molecules may not aggregate and/or enzyme molecules may interact by themselves. Also, active sites of enzyme molecules might be restricted by random bonding of molecules and this leads to decrease cross-linked enzyme activity.

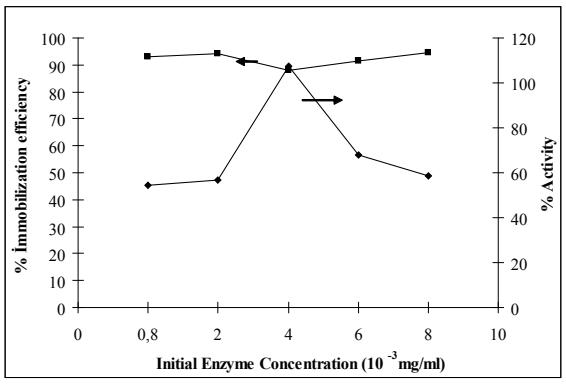


Figure 1. The change of immobilization efficiency and enzyme activity with enzyme concentration.

The initial enzyme concentration was used as 1.25 mg/ml for polyphenol oxidase, acid phosphatase, β-glucosidase, and trypsin CLEAs as reported by Tyagi et al. [11,12]. Other researchs were carried out with 30 and 50 mg enzyme/ml to obtain CLEAs of lipase PSL and commercial microbial lipases, respectively [13-15]. In our study, we reached high apparent activity values using very low initial enzyme concentration compared with the activities of the free solution forms.

Temperature

Another investigated parameter which affects the immobilization efficiency and activity was temperature. The effect of temperature has been tested at 4, 25, 37°C and the % immobilization efficiency and activity values were shown in Figure 2. The initial enzyme concentration was 4x10^{-3} mg/ml while precipitant and GA concentrations were used as indicated above. According to the Figure 2, there is a great difference in the CLEAs activity between these temperature values. The activity was measured as 82.88 and 23.45% at 4 and 37°C, respectively. Among them, 25°C seemed to be the most appropriate temperature for the enzymatic activity of glucose oxidase since it gave the biggest % activity value at defined conditions. Again immobilization efficiency at all temperature values didn't change too much.

The low and high immobilization temperature values greatly affect the three dimensional structure of the enzyme which may resulted from chemical interactions such as hydrogen bonds, London, Van der Waals, and hydrophobic forces. The CLEAs were mainly prepared at room temperature [12,13] to form CLEAs of four different enzymes and at 4°C [14,15]

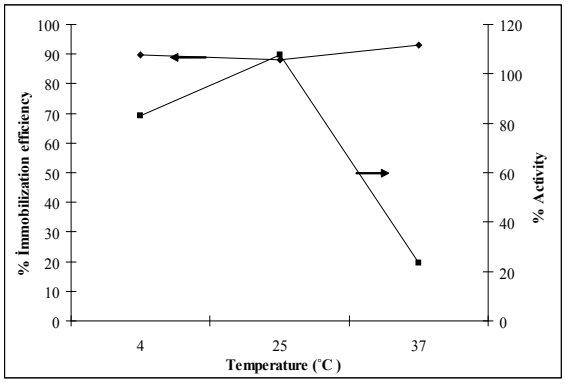


Figure 2. The change of GOD CLEAs immobilization efficiency and activity with temperature.

when seven commercial lipases and *Pseudomonas sp.* lipase were cross-linked as aggregates.

Proteic Feeder Type

For studying the effect of precipitant to the enzymatic activity, two precipitants were selected. One is a biological molecule, BSA, which is used as proteic feeder and the other is an anionic surfactant, SDS. The amounts added to the reaction medium were adjusted to be 5 mg/ml and all the preparation steps were performed at 25°C. The initial enzyme and GA concentration were 4 x 10^{-3} mg/ml and 2% v/v in the final solution, respectively. The results were shown in Table 1.

It is possible to form aggregates by changing the hydration state of enzyme molecules or by altering the electrostatic constant of the solution by adding appropriate aggregation agents. The effect of precipitant type on CLEAs forming was also investigated by Sheldon et al [6]. It was reported that the relative activity of lipase aggregates were 30%, when aggregates were prepared with SDS and ammonium sulphate together as precipitants. The relative activity of glucose oxidase aggregates using SDS was 60, 44% in our study. As it can be seen at Table 1, using BSA as a precipitant was more effective than using SDS. Cross-linking with, glutaraldehyde forms strong covalent bonds with ε-NH_2 of lysine residues. ε-NH_2 of lysine residues are found in BSA but, not found in SDS. During cross-linking, ε-NH_2 of lysine residues of BSA compete with the enzyme for GA. Since SDS hasn't any ε-NH_2 of lysine residues, this molecule didn't compete with enzyme. Consequently, GA was in excess amount in the reaction medium and caused decreasing enzymatic activity.

Proteic Feeder Concentration

Another parameter which affects the activity and immobilization efficiency was the concentration of precipitant. The results of this section were submitted in Figure 3. BSA was again used as proteic feeder to prepare glucose oxidase aggregates. The proximity of dissolved enzyme molecules was changed by adding BSA to form physical aggregates. Seven different BSA concentration values (1, 3, 5, 7.5, 10, 15, 20 mg/ml) were used for investigating the effect of precipitant concentration.

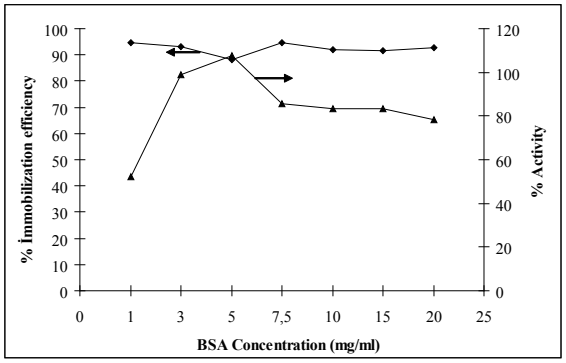


Figure 3. The Effect of BSA concentration on immobilization efficiency and enzyme activity.

As it can be seen from Figure 3, the BSA amount of 5 mg/ml was found as the optimum concentration for the formation of glucose oxidase aggregates with maximum activity.

The effect of BSA concentration on CLEAs forming was also investigated in some previous studies [11,12]. It was reported that the optimum BSA amount was found 50 mg/ml for polyphenol oxidase, acid phosphatase and β-glucosidase enzyme when the initial enzyme concentration was 1.25 mg/ml by Gupta et al [4]. As it is seen from Figure 3; at our study, 5 mg BSA amount was optimum amount for glucose oxidase when the initial enzyme concentration was 0,05 mg/ml. In fact there is an optimum range of BSA amount. At lower amount of this range, there are not enough free amino groups (contributed by BSA) to prevent excessive cross-linking. At higher than optimum range of BSA amount, the free amino groups of BSA compete with free amino groups of glucose oxidase and prevent the necessary cross-linking of glucose oxidase molecules. Within this range of employed proteic feeder concentration, there is an optimum concentration of glutaraldehyde for obtaining most active CLEA with BSA.

Glutaraldehyde Concentration

At this section, a bifunctional cross-linking agent, the glutaraldehyde, concentration effect on the activity and the immobilization efficiency were investigated and the results were shown in Figure 4. The glutaraldehyde solution concentrations were changed from 1 to 5 % v/v (26.45 mM, 52.90 mM, 79.35 mM, 105.80 mM, 132.25 mM) and it was found that the activity and immobilization of GOD CLEAs was influenced by the concentration of this agent.

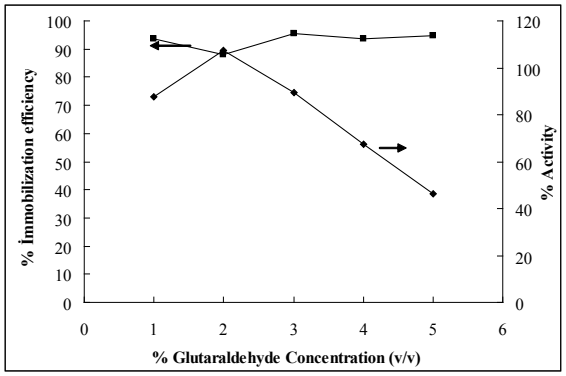


Figure 4. The Effect of glutaraldehyde concentration on immobilization efficiency and enzyme activity.

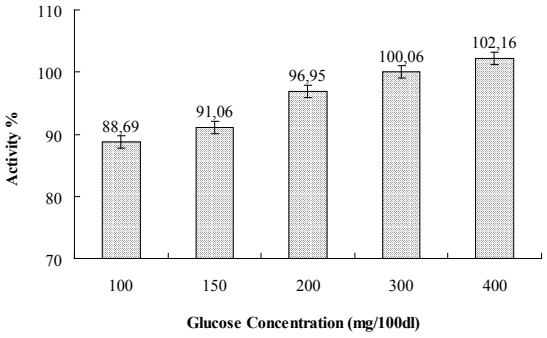


Figure 5. The activity of GOD aggregates in blood glucose concentration.

When glutaraldehyde concentration was 2 %, glucose oxidase aggregates activity has the maximum activity yield value which was 106.78. The activity values for 1 and 3% v/v GA was also high values with 84 and 87 activity yield which can also be accepted as possible cross-linking concentration values in a research. But GA concentrations above 3 % v/v resulted with a significant activity reduction. GA may act with active sides of the enzyme molecules which may lead to conformational changes and may result with activity decrease. Also, the excess GA molecules may interact to form polyglutaraldehyde and/or cross linking of GA molecules within itself may occur.

The effect of glutaraldehyde concentration on CLEAs forming was investigated in some previous studies [11,12]. It was reported that the optimum glutaraldehyde concentration was found as 0.4% (v/v) for polyphenol oxidase and acid phosphatase and 1.5 % (v/v) for β-glucosidase enzyme by Tyagi and coworkers [12]. In another study, the GA concentration was 25 mM when *Pseudomonas cepacia lipase* was aggregated and 50 mM when *Penicillin acylase* CLEAs were prepared [13].

The performance of glucose oxidase aggregates in blood glucose determination

The performance of glucose oxidase aggregates at various glucose concentrations were determined and shown in Figure 5. The enzyme activity increased with the increasing of glucose concentration.

Glucose oxidase enzyme biocatalyzes the oxidation of glucose:

$$\text{D-glucose} + O_2 + H_2O \rightarrow \text{D-gluconic acid} + H_2O_2$$

and the glucose concentration can be determined by measuring oxidation or reduction of hydrogen peroxide. The measurement of glucose is important in medical chemistry, food and other industries. There are several enzyme-based diagnostic kits for clinical glucose monitoring. Kumar et al. reported an amperometric biosensor for glucose determination. Cotton cheese cloth was used GOD immobilization as a matrix [16]. Li et al. described a glucose biosensor that was prepared the immobilization of GOD on a Prussian Blue-modified glassy carbon electrode with a silica sol gel outer layer like a sandwich. The sensor showed high selectivity and sensitivity and good performance for clinical assay of blood glucose [17]. Han et al. reported a tomato skin biosensor for measurement of blood glucose [18]. Also GOD enzyme was used for drug delivery systems. Chu et al. reported a glucose sensitive microcapsule with a porous membrane and with linear grafted polyacrylic acid chains and with bounded GOD. The glucose sensitive characteristics of the release of Vitamin B12 and sodium chloride were searched [19]. In our study, glucose oxidase aggregates are a new approach to the glucose determination for clinical assay of diabetic patient's blood glucose. The GOD aggregates exhibited good performance for different glucose concentrations, 100-400 mg/100 dl which are in the range of clinical use.

Stability of Glucose Oxidase Aggregates

Glucose Oxidase aggregates were stored at +4°C for 1, 3 and 5 months and over this period, their enzymatic activity tested. We found that the activities of glucose oxidase aggregates were

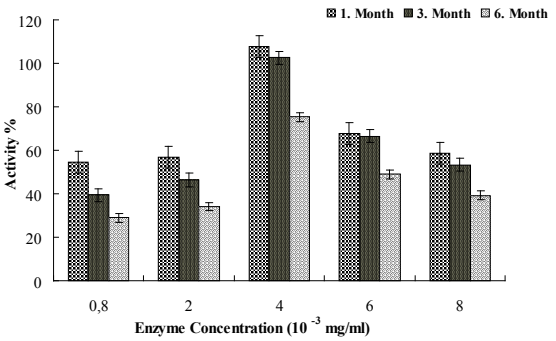


Figure 6. Shelf time of Glucose Oxidase aggregates.

retained by storage (Figure 6). According to Figure 6, the aggregates still possess enzymatic activity after five months.

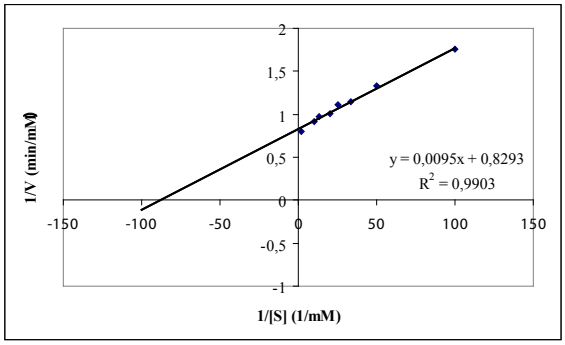
Determination of Michealis-Menten Constants

The effect of immobilization on the kinetic parameters, K_m and V_m were also calculated by Lineweaver-Burk plots. There was an appreciable difference for K_m values between free enzyme and CLEA which were calculated as 0.0115 and 0.025 mM, respectively. The possible reason of this difference could be based on the molecular disturbance and deformation which had realized in course of immobilisation procedure. Because of the immobilisation reactions affected the three dimensional structure of enzyme, the difference was a foreseeable result for such procedures. The substrate affinities of CLEA decrease as a result of increasing of K_m. But when we compare K_m value of CLEA of GOD with an earlier study about carrier-bond GOD [16], it was seen that the substrate affinity of CLEA is greater than the substrate affinity of carrier-bond immobilised GOD.

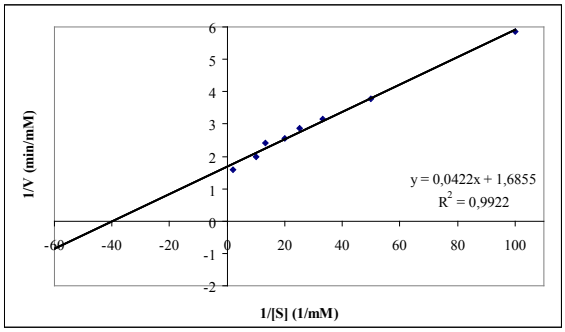
The kinetic parameters of immobilised GOD enzyme were studied in the earlier experiments [20]. It was reported that K_m values were 68.2 mM for free enzyme; 259 mM for immobilised GOD and V_m values were 435 μmol min^{-1} for free enzyme; 217 μmol min^{-1} for immobilised GOD onto magnesium silicate. In general, K_m values of immobilised

Table 2. Kinetic parameters of native and CLEAs of glucose oxidase (GOD) determined by using Lineweaver-Burk method.

	K_m (mM)	V_m (mM.min^{-1})
Native enzyme	0.0115	1.206
CLEA	0.025	0.593



(a)



(b)

Figure 7. Lineweaver-Burk plots of (a) native and (b) CLEA.

enzymes are higher and V_m values are lower than free enzymes because of steric hindrances and diffusion limitations. But, in present study, the differences of K_m (substrate affinity) between free enzyme and CLEAs are low.

Figure 8 shows the optical microscope images of GOD CLEAs prepared with BSA under the optimum conditions. The microscope images indicate a compact, amorphous, ductile and non spherical network structure. The scanning electron micrographs (SEM) of the same GOD aggregates were given in Figure 9. The GOD aggregates show spongy type structure which can be expected from optical microscope images.

Applications of GOD aggregates for clinical purposes

Several glucose biosensors have been developed by immobilizing GOD enzyme on a variety of matrices. The response to glucose was great and stability of the sensors were ranging from a few hours to a few week.

A new approach for determination of glucose is reported in this paper. No matrix was used for preparing GOD aggregates. These physical aggrega-

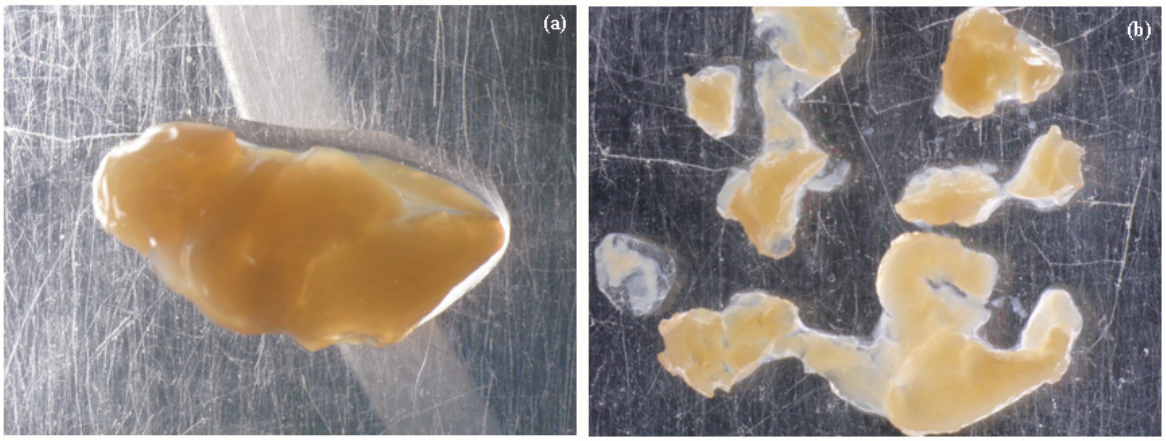


Figure 8. Optical micrographs of the GOD CLEAs. a) 20x, b) 40x magnifications.

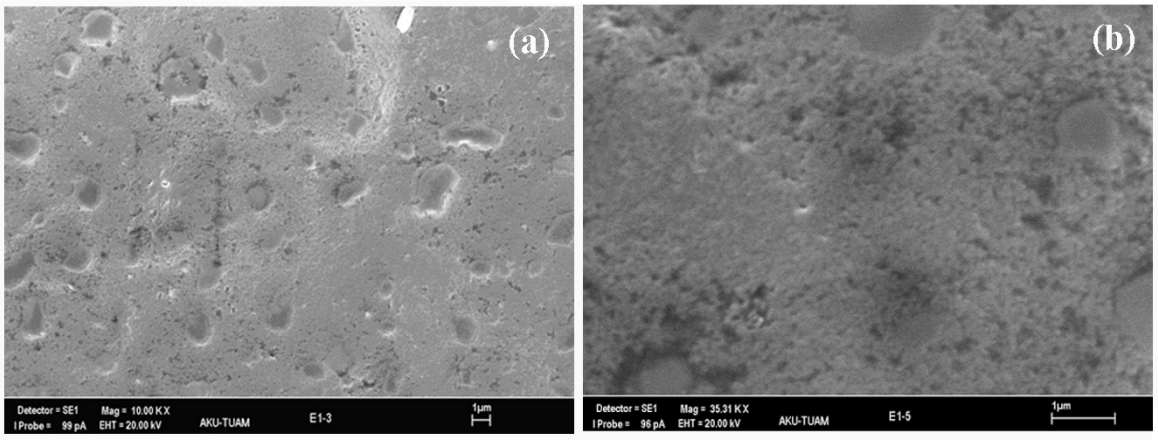


Figure 9. SEM micrographs of GOD CLEAs. a) 10000x, b) 35000x magnifications.

tes are supramolecular structures held together by noncovalent bonding. And also the stability and activity of these aggregates have been stable for five months. We offered two drafts for diagnosis and therapy applications of GOD aggregates.

Figure 10a shows the sandwich configuration in a glucose biosensor [21]. The sensor was a combination of Prussian blue-modified electrode and silica sol-gel immobilization. Similary of this sensor, figure 10b illustrates our draft model. The GOD aggregates without any matrix, held electrode surface with physical adsorption. This new model is very simple, sensitive and cost-effective.

Figure 11a shows the glucose-sensitive release principle of microcapsules with porous membrane and functional gates [22]. In Figure 11-b the microcapsule was composed of a core-shell porous membrane and GOD enzymes in pores. GOD enzyme was bounded with pore by physical adsorption. The substance to be released is dissolved in a solution inside the microcapsule interior. At neutral pH in the absence of glucose, the membrane pores are closed; when environmental glucose concentration increases because of the reduced electrostatic repulsion and then the pores open.

The aggregate enzyme activity showed better performance than immobilized enzyme activity (as biosensor or immobilized on grafted microcapsule) for clinical applications of glucoseoxidase. Also, this technique provide a new approach for drug delivery systems such as releasing of insulin.

CONCLUSION

The cross-linked enzyme aggregates of Aspergillus Niger Glucose Oxidase were investigated and the optimum conditions were determined. The enzyme aggregates showed good performance for different glucose concentrations. These aggregates can provide a new approach for drug delivery systems having drug such as insulin to

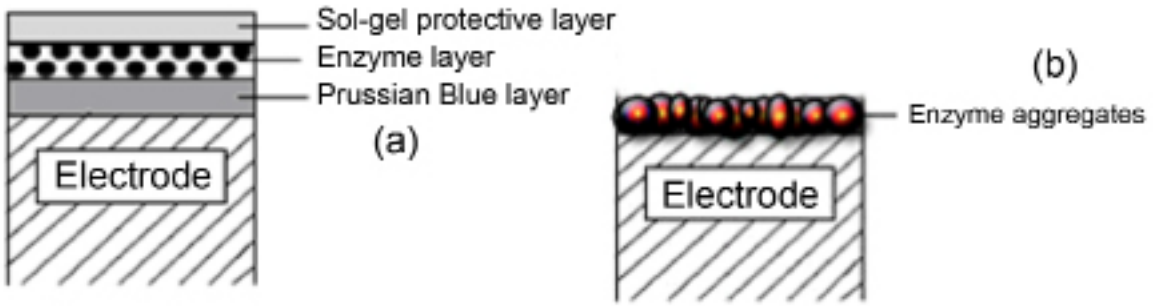


Figure 10. Application of (a) immobilised GOD, (b) GOD aggregates for diagnosis.

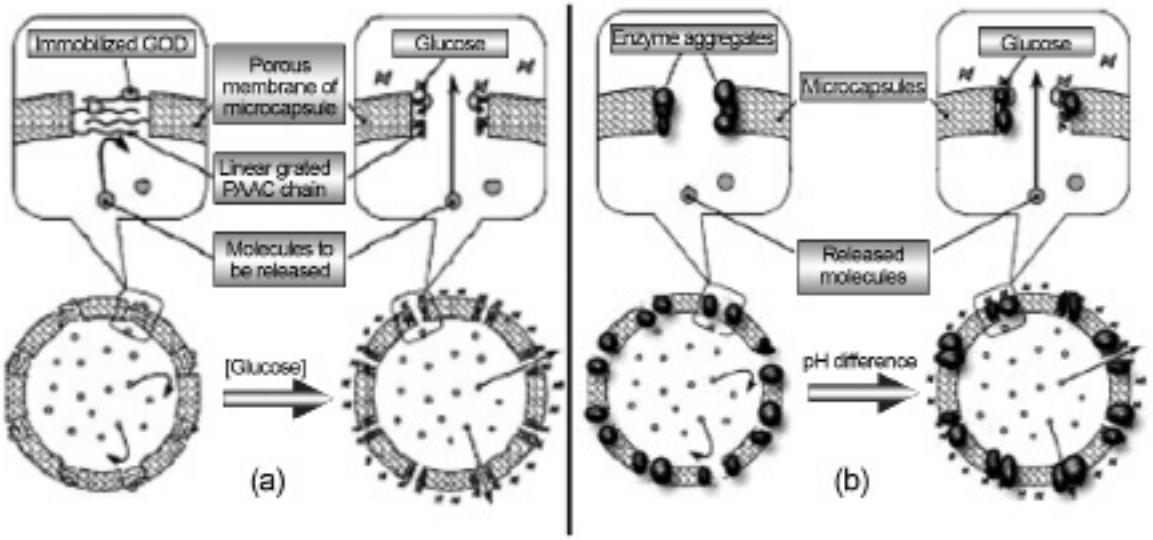


Figure 11. Application of (a) immobilised GOD, (b) GOD aggregates for therapy.

keep the blood glucose levels in normal range. It can be attractive for diabetes patients. On the other hand, further work can be continued to investigate a glucose biosensor with 'sandwiched configuration' with GOD aggregates. These sensors can be used for clinical assay of blood glucose of diabetic patients. It can be simple and cost-effective.

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