PREPARATION OF ASCORBATE OXIDASE ENZYME ELECTRODE AND ITS USAGE FOR L-ASCORBIC ACID DETERMINATION

Erol Akyılmaz

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Erol AKYILMAZ'ın Yüksek Lisans Tezi olarak hazırladığı "Preparation of Ascorbate Oxidase Enzyme Electrode and Its Usage for L-Ascorbic Acid Determination" başlıklı bu çalışma jürimizce Lisansüstü Yönetmeliği'nin ilgili maddeleri uyarınca değerlendirilerek oybirliği ile kabul edilmiştir.

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Üye ; Doç. Dr. E. Dinçkaya
Üye ; Yrd. Doç. Dr. F. Zihnioğlu

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ÖZET

ASKORBAT OKSİDAZ ENZİM ELEKTRODU HAZIRLANMASI VE L-ASKORBİK ASİT TAYİNİNDE KULLANILMASI

AKYILMAZ, Erol
Yüksek Lisans Tezi, Biyokimya Bölümü
Tez Yöneticisi : Doç.Dr.Erhan Dinçkaya
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Bu tezde, salatalıktan (*Cucumis sativus* L.) izole edilip kısmi olarak saflaştırılan Askorbat oksidaz [(L-Askorbat:oksijen oksidoredüktaz) (EC 1.10.3.3)] glutaraldehid yardımıyla jelatin ile çapraz bağlanarak, çözünmüş oksijen probu membranı üzerinde bir biyoaktif tabaka oluşturuldu ve L-askorbik aside duyarlı bir askorbat oksidaz enzim elektrodu geliştirildi. Ölçümler enzimatik reaksiyon uyarınca L-askorbik asit konsantrasyonu ile orantılı olarak tüketilen oksijen düzeyinin belirlenmesi yoluyla çizilen standard grafikler yardımıyla gerçekleştirildi.

Hazırlanan biyosensörün optimizasyon çalışmalarında en uygun askorbat oksidaz, jelatin ve glutaraldehid oranları sırasıyla 24,8 U/cm^2, 5,9 mgjelatin/cm^2 ve %2,5 olarak bulundu. Optimum çalışma ortamı olarak 50 mM'lik pH:7,5 olan fosfat tamponunun kullanılması ve ölçümlerin 35^{o}C de yapılması gerektiği sonucuna varıldı. Geliştirilen biyosensörün karakterizasyonu amacıyla yapılan çalışmalar sonucunda 5,0x10^{-5} - 1,2x10^{-3} M L-askorbik asit konsantrasyon aralığında doğrusal sonuçların alınabildiği tespit edildi. Tekrarlanabilirlik denemelerinde (n=11), 4,0x10^{-4} M'lık L-askorbik asit konsantrasyonu için ortalama değer ($\bar{x}$)=3,99x10^{-4} M, standard sapma (S.S):±0,001 ve varyasyon katsayısı (V.K):%0,251 olarak bulundu. Ayrıca substrat spesifikliği, depo kararlılığı ve bazı maddelerin girişim etkileri araştırıldı. Son olarak bazı meyva ekstraktları ve vitamin tabletlerinde, geliştirilen biyosensörle L-askorbik asit tayini yapıldı ve elde edilen sonuçlar ve metodun duyarlılığı 2,6 diklorofenolindofenol metodu ile kıyaslandı.

Anahtar Kelimeler : Askorbat Oksidaz, L-Askorbik Asit, Enzim Elektrodları, Biosensörler

ABSTRACT

PREPARATION OF ASCORBATE OXIDASE ENZYME ELECTRODE AND ITS USAGE FOR L-ASCORBIC ACID DETERMINATION

AKYILMAZ, Erol
MsC in Biochemistry
Supervisor : Assoc.Prof.Dr.Erhan Dinçkaya
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In this thesis, ascorbate oxidase [(L-Ascorbate:oxygen oxidoreductase) (EC1.10.3.3)], isolated and partially purified from cucumber (*Cucumis sativus* L.) was crosslinked with gelatine by using glutaraldehyde and a bioactive layer was formed on the dissolved oxygen probe, therefore, ascorbate oxidase enzyme electrode which was sensitive for L-ascorbic acid determination was developed. Measurements were carried out by standard curves which were obtained by the determination of consumed oxygen level, related to L-ascorbic acid concentration in the enzymatic reaction.

In the optimization studies of the biosensor prepared, the most suitable ascorbate oxidase, gelatine and glutaraldehyde ratios were determined as 24.8 U/cm^2, 5.9 mggelatine/cm^2 and, 2.5% respectively. The phosphate buffer (50 mM, pH:7.5) and 35^{o}C were established as providing the optimum conditions. The characterization studies proved a linearity in the ascorbic acid concentration range 5.0x10^{-5}-1.2x10^{-3} M. The reproducibility experiments (n=11) revealed that for 4x10-4 M L-ascorbic acid, the average value ($\bar{x}$) was 3.99x10-4 M,standard deviation (S.D) was ±0.001 and, variation coefficient (C.V) was 0.251%. Moreover, substrate specification, storage stability and the interference effects of some substances were investigated. Finally, L-ascorbic acid determination in some fruit extracts and vitamin tablets was carried out. Both the results obtained and the sensitivity of the method were compared with the 2,6 dichlorophenolindophenol method.

Keywords: Ascorbate Oxidase, L-Ascorbic Acid, Enzyme Electrodes, Biosensors

CONTENTS

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I would like to thank Assoc. Prof.Dr.Erhan DİNÇKAYA for allowing me to this project.

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August, 1996 Erol AKYILMAZ

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1. INTRODUCTION

L-Ascorbic acid (Vitamin-C) has been known as a very important substance for medicine, food and, drug industries, especially for preventing scurvy. A large number of investigations on this area suggested that vitamin-C played an important role in the immune system, cholesterol metabolism, cancer and cataract protection and the reactions concerning with the metabolism of some amino acids.

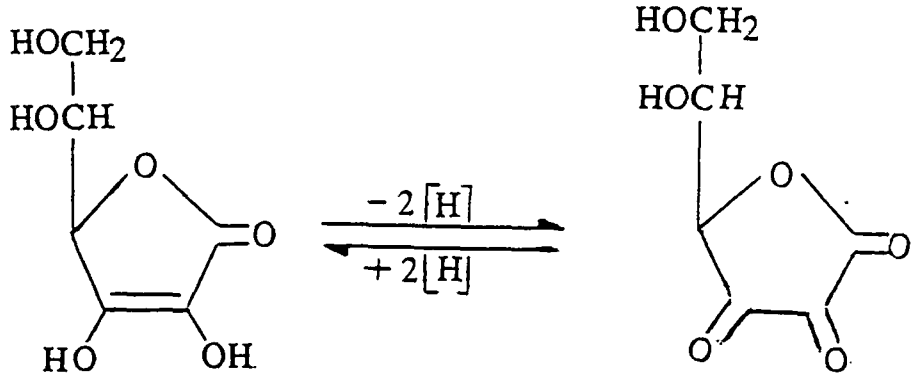
The importance of vitamin C arises from its being essential for humanbeing and behaving as an antioxidant for L-ascorbic acid determination in various natural or ready prepared food and vitamin tablets or physiological fluids. Although several chemical methods were developed for L-ascorbic acid determination in recent years,most of them were known as having no characteristics and moreover, they were prone to interferences. However, the enzymatic methods which were sensitive, convinient and special for L-ascorbic acid determination were usually preferred, as the other alternatives such as HPLC, gas chromatography and, polarographic methods were more expensive and time-consuming. Enzymes are generally immobilized before they are coupled with a suitable sensor when developing a biosensor as they are very expensive materials. Biosensors are cheap, sensitive and, simple so that, they can easily be used in all laboratories.

In this thesis dissolved oxygen probes and immobilize ascorbate oxidase were used to develop a specific biosensor for L-ascorbic acid determination.

1.1 Properties of L-Ascorbic Acid

L-Ascorbic acid was first isolated in 1928 and it had been characterized in 1933. L-Ascorbic acid is coulourless and crystall. Its melting point is 192°C and molecular weight is 176.13 g (Metzler, 1977). It contains an ene-diol group (a double bond between two adjacent

hydroxyl groups) and is a strong reducing agent, as are all ene-diols. L-Ascorbic acid dissociates the enolic hydrogen at C-3 with a pK of 4.2 and thus exist as the enolate anion at physiological pH. With the loss of hydrogen, it is converted to dehydroascorbic acid. The reaction is reversible (Karlson, 1975).



L-Ascorbic acid Dehydro-L-ascorbic acid

In some biological hydroxylations, ascorbate is a hydrogen donor (Gershoff, 1993). Ascorbate dissolves in water, methanol and ethanol but does not soluble in benzen, ether, chloroform and oil. The ox-red potential of L-ascorbic acid at 35°C and pH:4.0 is E_o=0.166 V. L-Ascorbic acid is the least stabil of all the vitamins and it is especially sensitive for the oxidation in the presence of Cu and Fe ions. It is most stable as dry crystals and fairly stable in an acid solution (Milles et al., 1949).

Humanbeing are one of a handful of animals unable to synthesize vitamin-C (Gershoff, 1993). As a result it's essential for them.

1.1.1 Biosynthesis of L-ascorbic acid

The oxidation of glucose from C-6 gives glucuronic acid. The reaction occurs enzymatically on UDP-glucose (Metzler, 1977). UDP-glucose dehydrogenase catalyzes this reaction and H-acceptore is NAD^+. Excess glucuronic acid is disintegrated in the liver and developed hemi-acetal groups (=aldehyde groups) are enzymatically reduced and as a

result, gulonic acid is formed. In the biosynthesis of L-ascorbic acid; gulonic acid is used as the starting material, at first a lacton (glunolacton) occurs and then it is aerobically dehydrogenated by L-gulonolacton oxidase (a flavoprotein) to yield ascorbic acid (Karlson, 1975). Figure 1.1 shows the biosynthesis of L-ascorbic acid.

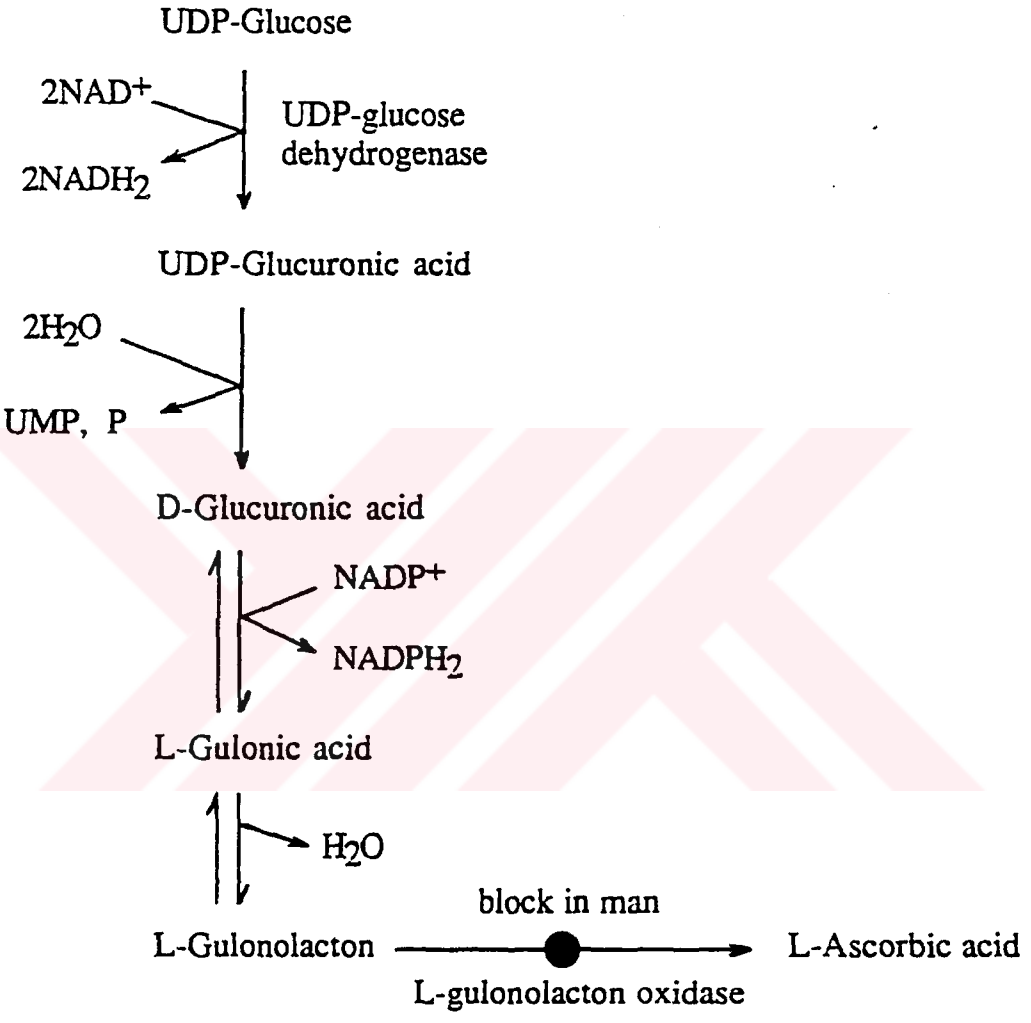


Figure 1.1 The biosynthesis of L-ascorbic acid.

1.1.2 The metabolism of L-ascorbic acid

There is an enormous amount of investigation carried out on vitamin C (L-ascorbic acid) intake and health in animals, cell cultures, and

human. Beyond its function in collagen formation, ascorbic acid is known to increase absorption of inorganic iron to have essential roles in the metabolism of folic acid, some amino acids and hormones, and to act as an antioxidant (Gershoff, 1993).

Since collagen formation requires synthesis of hydroxyproline and hydroxylysine,both dependent on the presence of L-ascorbic acid, the human diet must provide each day at least the minimum of L-ascorbic acid necessary to prevent scurvy, a condition characterized by formation of defective collagen resulting in damaged connective tissue. A large number of studies suggest that vitamin C plays a role in wound healing, the immune response (Anderson and Theron, 1990; Hemila, 1992 and Moser, 1987), and metabolism of some amino acids, hormones and folic acid.

L-Ascorbic acid is a nutrient such as (vitamin E and β-carotene) and shown to be effective in protecting against oxidative damage in tissues (Sies et al., 1992). In addition, L-ascorbic acid may suppress formation of carcinogens such as nitrosamines (Tannenbaum and Wishnok, 1987) and quinones (Terrar and Matsuskita, 1988).

L-Ascorbic acid acts as an antihistamine by reacting enzymatically with histamine (Chatterjee et al, 1975). There are many important effects of L-ascorbic acid as a cofactor and co-substrat. In the metabolism of phenylalanine, tyrosine and proline, L-ascorbic acid plays a role as the cofactor.

1.2 Properties of Ascorbate Oxidase (EC 1.10.3.3)

Enzymes involved in direct oxygen activation are oxidases and oxygenases. Oxygenases introduce either one atom of dioxygen into substrate and reduce the other atom to water (monooxygenases) or transfer two oxygen atoms into substrate (dioxygenases). Oxidases can be divided in two-electronand four-electron transferring enzymes. The first group reduces dioxygen to hydrogen peroxide and the second one

dioxygen to water. Most of the oxygenases as well as oxidases contain as prosthetic groups either flavin, iron (heme or non-heme) or copper (Messerschmidt, 1993).

Ascorbate oxidase (EC 1.10.3.3) is a blue multi-copper oxidase that catalyzes the four-electron reduction of dioxygen to water with concomitant one-electron oxidation of the reducing organic substrate (Malkin and Malmström, 1970). Copper-dependent ascorbate oxidase is found only in higher plants (Chichiricco et.al., 1989). The enzyme from Cucurbita pepo medullosa (green zucchini) is a dimer of 140.000 Mr containing eight copper ions. The immunohistochemical localization of ascorbate oxidase in green zucchini reveals that ascorbate oxidase is distributed in all specimens examined ubiquitously over vegetative and reproductive organs. At the cellular level the enzyme is linked with the cell wall and cytoplasm. (Chichiricco et al., 1989). The invivo role in plants of ascorbate and ascorbate oxidase is still under debate. As catechols and polyphenols are also substrates in vitro. Ascorbate oxidase might be involved in biological processes like fruit ripening. A role in a redox system, as an alternative to the mitochondrial chain, in growth promotion or in susceptibility to disease has also been postulated (Butt, 1980). Primary structures of ascorbate oxidase from cucumber (Ohkawa, et al., 1989) and pumpkin have recently been reported.

Ascorbate oxidase catalyzes the following reaction :

$$2\text{L-Ascorbate} + O_2 + 2H^+ \xrightarrow{\text{Ascorbate Oxidase}} 2\text{-Dehydro-L-ascorbate} + 2H_2O$$

A role of this enzyme in a redox system, alternative to the mitochondrial chain, in growth promotion or in susceptibility to diseases, has been postulated (Butt, 1980).

1.3 The Determination Methods of L-Ascorbic Acid

For the determination of L-ascorbic acid there are many different

methods such as titrimetric, spectrophotometric, polarographic, chromatographic, enzymatic. In addition recently methods based on biosensors were developed.

In this section, the above methods were compared with the methods based on biosensors.

1.3.1 Titrimetric methods

Titrimetric methods are the oldest methods to determine L-ascorbic acid. These methods lack specificity and are prone to interferences by other reducing agents present in the sample (Greenway and Ongomo, 1990).

In the titrimetric methods L-ascorbic acid was titrated with 0.1 N chloramin T (only for pharmaceutical preparations containing no other oxidizible substances). Another titrimetric method consist of a titration with 2,6-dichlorophenolindophenol solution (suitable for rough estimations for production control in the manufacture of fruit juices if great accuracy is not required) (Strohecker and henning, 1995). In addition to these methods, L-ascorbic acid was titrated with 2,6-dichlorophenolindophenol solution (Strohecker et al., 1955), or N-bromoimides [N-bromophthalimide (NBP) and N-bromosaccharin (NBSA)] (Kumar and Indrosenan, 1989).

The titrimetric methods are cheaper than other methods but they are not suitable for specific, sensitive and quantitative determination of L-ascorbic acid.

1.3.2 Spectrophotometric methods

The spectrophotometric methods are commonly used for the determination of L-ascorbic acid. By 2,4-dinitrophenyl hydrazine, the spectrophotometric determination of L-ascorbic acid has been carried out. This method is based on the formation of 2,4-dinitrophenyl hydrazone,

the product of the reaction of dehydroascorbic acid with 2,4-dinitrophenyl hydrazine. When dissolved in sulfuric acid (85%), the solution is red with an absorption maximum at 520-525 nm (Roe and Kuether, 1942). Disadvantage of this method is that glucose, fructose and glucuronic acid react to form phenyl hydrazones. The result causes difficulties for this method as it needs the separation of phenylhydrazones by thin layer chromatography.

For measuring L-ascorbic acid, enzymic oxidation of L-ascorbic acid to dehydoascorbic acid by ascorbate oxidase,samples were assayed by spectrophotometrically monitoring the kinetics of the concentration dependent absorbance changes of dehydro-L-ascorbic acid. L-Ascorbic acid was determined as the difference between dehydro-L-ascorbic acid and total L-ascorbic acid content. In this method, ascorbic acid is determined indirectly and the detection limit is <0.5 µmol/l so this method can be used only for physiological fluids (Moeslinger et al., 1995). Otherwise L-ascorbic acid was determined on the basis of the fall in UV absorption at 265 nm due to its enzymatic oxidation by ascorbate oxidase (List and Schuettler, 1978). There is a spectrophotometric method which is based on the absorbance measurement at 593 nm of the complex of ferrous ion with 2,4,6-tris (pyridly)-5-triazine, which is produced by the reduction of ferric ion by L-ascorbic acid, versus a blank sample treated with ascorbate oxidase (Skaltsa et al., 1987) but this method is time-consuming and complicated.

1.3.3 Polarographic methods

Polarographic determination of L-ascorbic acid, using either a standard curve or an internal standard, is possible (Krauze and Bozyk, 1959). The method is subject to errors introduced by other reducing agents but has the advantage of specifity and case of determination when compared with either photometric or titrimetric procedures. The best pH range for determination is between 3 and 6. Above this, L-ascorbic acid is subject to rapid oxidation and below it, the measurement of the anodic wave is difficult. A useful method for the determination of L-ascorbic

acid in a vegetable product (asparagus) by differential pulse polarographic has been set up and evaluated (Esteve et al, 1995). Detection limit of this method is 0.182 µg/ml.

Polarographic methods need different equipments and preparations so these methods are time-consuming and expensive.

1.3.4 Chromatographic methods

There are a few chromatographic methods to determine L-ascorbic acid such as ion chromatography, gas chromatography, high pressure liquid chromatography (HPLC), and etc. (Washko et al., 1992).

1.3.4.1 Ion chromatography

This method is a general one for the determination of L-ascorbic acid as it can be applied to sulfide, sulfite and thiosulfate determination as well. L-Ascorbic acid,sulfide, sulfite, and thiosulfate are completely separated from their mixtures by a single run of ion chromatography using columns of a sulfanated styrene divinylbenzene copolymeric cation-exchange resin of low crosslinking (1.0 %) (Yasuyuki et al., 1995).

Ion chromatography is not a high sensitive and a suitable method and it's also time-consuming for the determination of L-ascorbic acid, but it can be an alternative method for the chromatographic determination of ascorbic acid.

1.3.4.2 Gas chromatography

Ascorbic acid and dehydroascorbic acid could also be determined by gas chromatography. The tert-butyldimethylsilyl derivatives of ascorbic acid and dehydroascorbic acid were characterized by gas chromatorgraphy-mass spectrometry, and an isotope dilution investigations

for ascorbate and dehydroascorbate were carried out using [$^{13}C_6$] ascorbic acid, and [$^{13}C_6$] and [6,6.$^{2}H_2$] dehydroascorbate (Deutsch and Kolhouse, 1993). L-Ascorbic acid loss can easily be monitored by using this method and the resulting rise of dehydroascorbic acid in aqueous solutions and plasma. The method is time consuming and also very expensive for the determination of L-ascorbic acid.

Another gas chromatographic method is capillary gas chromatography method (Morvau and Molnar, Perl, 1992) in which the gas chromatography was used as a capillary for the simultaneous determination of L-ascorbic acid.

1.3.4.3 High pressure liquid chromatography (HPLC)

The HPLC method is often used to determine ascorbic acid in biological and food samples (Washko et al., 1992; Nisperos-Carriedo et al., 1992; and Mogele et al., 1992). The HPLC methods are very sensitive and spesific but some of them need extraction procedures (Irache et al., 1993). In this method sometimes a procelumn was used for the stationary phase such as RP-18 (Romero Rodriguez et al., 1992). L-Ascorbic acid was determined by reverse phase ion interaction HPLC (Zapata and Bufour, 1992) and isocratic reversed-phase HPLC (Vanderslice and Higgs, 1993). In addition, HPLC determination of L-ascorbic acid in soft drinks and fruit juices using tris (2,2'-bipyridine)ruthenium (II) electrochemiluminescence was carried out (Xi and Masanori, 1995).

L-Ascorbic acid determination at low concentrations is possible by HPLC methods although they need very expensive equipments and chemicals, and are time-consuming methods.

1.3.5 Enzymatic methods

The enzymatic methods are known as very sensitive spesific, simple,

and useful methods in which the immobilized forms of the enzymes were generally used. A method for determination of L-ascorbic acid via reduction of [3-(4,5-dimethylthiazolyl-2]-2 bromide by L-ascorbic acid in combination with ascorbate oxidase was developed and a new enzymatic rate method was disclosed to determine L-ascorbic acid using ascorbate oxidase (Tulley, 1995).

1.3.5.1 L-Ascorbic acid determination methods based on immobilized enzymes

The enzymes are very expensive materials so that they are immobilized. L-Ascorbic acid determination by using an immobilized ascorbate oxidase was investigated by researchers very efficiently. In this method, ascorbate oxidase was immobilized by utilizing various immobilization materials such as polyamide (List and Knechtel, 1980), CM-cellulose gel beads of polyacrylamide and polyethyleneterephthalate (also known as Dacron) (L.B. Carvalho et al.,1989).

The immobilized ascorbate oxidase was incorporated in a flow-injection system with amperometric detection at a glassy carbon electrode (Greenway and Ongomo, 1990). In this system L-ascorbic acid was converted into dehydroascorbic acid and the decrease in signal was measured. Another method needs a flow-through system equipped with a polarographic detector which monitors the oxygen depletion due to the reaction;

$$\text{L-Ascorbic acid} + 1/2O_2 \longrightarrow \text{Dehydro-L-ascorbic acid} + H_2O$$

(Stevanato et al., 1985). This method was utilized to determine ascorbate concentrations as low as 3×10^{-7} M in biological samples.

1.3.5.2 L-Ascorbic acid determination based on ascorbate oxidase electrodes

Recently biosensor technology was improved very efficiently. Since it

provides clear, suitable, specific, sensitive results for the determination applications of some substances such as L-ascorbic acid. The first bioselective electrode for ascorbic acid, consisting of a bipolar oxygen electrode and squash ascorbate oxidase cross-linked on polyamide netting with albumin and glutaraldehyde, proved efficient in analyzing different kinds of fruit (Posadka and Macholan, 1979). The enzyme electrode for the determination of L-ascorbic acid needs the immobilized ascorbate oxidase and a low-cost oxygen electrode which is applied as a transducer.

Amperometric experiments for vitamin C using an assorbate oxidase enzyme electrode was carried out (Uchiyama and Suzuki, 1993; Uchiyama and Umetsu, 1991). In addition, L-ascorbic acid membrane electrodes based on the use of biocatalysts immobilized on oxygen electrode were developed (Vincke et al., 1985). A vitamin C sensor based on the cyclic reaction between L-ascorbic acid and dehydroascorbic acid was carried out. In this method, the current response of L-ascorbic acid at an ascorbate oxidase membrane electrode has been amplified by adding dithiothreithol into the sample solution. The signal amplification is based on the cyclic reaction between L-ascorbic acid and dehydroascorbic acid (Uchiyama et al., 1993).

A plant tissue based on membrane biosensor for L-ascorbic acid was developed (Macholan and Chmelikova, 1986).

The biosensors have very advantages such as long-term storage, substrate specificity, and they are active for more than two months and after 200-250 measurements. They can be applied in all laboratories. The reproducibilty of the analysis results is very high and there are no interference effects result from different substances. The method is not time-consuming and does not need very expensive equipment or chemical material.

2. MATERIALS AND METHODS

2.1 Materials

For the isolation and purification of ascorbate oxidase (EC 1.10.3.3) from cucumber (*Cucumis sativus* L.) a Moulinex type 127.2.3 (France) was used to mince the cucumber. Then the obtained material was homogenized with SilversonModel L2R (England) homogenisator. To separate the vegetable residues and to precipitate the proteins as fractions Hettich Universal 30 RF Model (Germany) centrifuge was used and a criyostat Heto (Birkerød, Denmark) was employed for cooling.

For the measurements which was made by using developed enzyme electrodes, a dissolved oxygenmeter was used (YSI Model 54 A) (U.S.A) To develop the enzyme electrodes, dissolved oxygen probes from YSI 5700 series were used as a basic electrode.

Absorbance measurements required for the protein determination were carried out with a spectrophotometer Pharmacia LKB. Novaspec II (England). Gilson P100 and P1000 otomatic micropipettes (France) were used for pipetting.

In order to remove the salt from ascorbate oxidase solutions which was obtained at the various purification steps, dialysis pockets were used (Sigma 250-7U) (St.Louis, U.S.A).

For the incubations, a water bath (Stuart Scientific, Linear,shaker bath SBS 35) (U.K) was used. For all measurements an ultrathermostat (Colora,Germany) and a thermostat type 03 (Bresden, Germany) were used.

Ammonium sulphate,boric acid, dipotassium hydrogen phosphate, 2,6 dichlorophenolindophenol (DCIP), copper sulphate, glutaraldehyde (25%), hydrochloric acid, L-ascorbic acid, oxalic acid dihydrate, potassium dihydrogen phosphate, citric acid mono hydrate, sodium dihydrogen phosphate, sodium carbonate and trichloroacetic acid (TCA) were obtained from E.Merck (Darmstadt, Germany).

L-Aspartic acid, catechol, gelatine (Type III, 225 Bloom), glycine, hydroquinone, 8-hydroxyquinolyne, sodium dodesylsulphate and tartaric acid were obtained from Sigma (St.Louis. U.S.A)

Bovine Serum Albumin, tris-(hydroxymethyl)-aminomethan were obtained from Calbiochem (San Diego, U.S.A)

Disodium hydrogen phosphate, sucsinic acid were obtained from Riedel-De Haen AG (Hannover, Germany)

L-Glutamic acid from BDH (Poole, England),

Sodium hydroxide from Fluka (Burchs, Switzerland) and

D(-)-Fructose, D(+)-Glucose were obtained from Prolaba (Paris, France.)

The solutions used in experimental studies were given below.

- Copper sulphate (2.0%)
- 2,6Dichlorophenolindophenol (DCIP) (2.0x10-4 M)
- Phosphate buffer (pH:7.5; 25, 50 and 100 mM)
- Phosphate buffer (pH:5.5, 6.0, 6.5, 7.0, 7.5 and 8.0; 50 mM)
- Citrate buffers (pH:3.0, 3.5, 4.0, 4.5 and 5.0; 50 mM)
- Glycine buffers (pH:8.5, 9.0, 9.5 and 10.0; 50 mM)
- Tris buffer (pH:7.5; 50 mM)
- Glutaraldehyde (2.5 %, 5.0% and 10.0%)
- L-Ascorbic acid-stock solution I (3mM, in the phosphate buffer; 50 mM)
- L-Ascorbic acid-stock solution II (40 mM, in the phosphate buffer; 50 mM)
- L-Ascorbic acid-stock solution III (80 mM, in the phosphate buffer; 50 mM)
- Standard solutions of L-ascorbic acid (0.044, 0.264, 0.440, 0.881, 1.321, 1.744 mg)
- Standard solutions of L-ascorbic acid (0.05, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6 mM)
- Sodium/Potassium tartarate (2.0%)
- Protein stock solution; BSA (1mg/ml)
- Sodium dodesylsulphate (SDS) (0.5 %; in the phosphate buffer; pH:7.5 50 mM)

- Sodium hydroxide (5M)
- Sodium carbonate (2.0%; in 0.1 N NaOH)
- Folin-Ciocalteu Phenol reagent (2N)

For the substrate specifity, the stock solutions given below were used.

- L-Ascorbic acid
- L-Aspartic acid
- Glycolic acid
- D(-)-Fructose
- D(+)-Glucose
- L-Glutamic acid
- Hydroquinone
- 8-Hydroxyquinoline
- Catechol
- Oxalic acid
- Citric acid
- Sucsinic acid

(All of the stock solutions were preparaed as 0.4 and 1.0 mM in the phosphate buffer, pH:7.5; 50 mM)

2.2 Methods

2.2.1 Protein determination

The amount of protein ascorbate oxidase solutions obtained at various isolation and purification steps was determined by modification of the method suggested by Lowry et al. The method is explained below.

Reagents;

Reagent I	-	2.0% $CuSO_4.5H_2O$
Reagent II	-	2.0% K/Na tartarate
Reagent A	-	2.0% Na_2CO_3 in 0.1 M NaOH
Reagent B	-	1 ml reagent I, 1 ml reagent II up to 100 ml with reagent A.
Folin's	-	Dilute stock Folin's reagent 1:1 with destile water.

Standards;

1. Stock 100 mg BSA in 10 ml destile water (10 mg/ml)

2. Working Standards : 0.02, 0.05, 0.10, 0.15 and 0.20 mg/ml. The standards were stored in the refrigerator for 1-2 months to inhibit microbial growth.

Measurements for all samples (standards and diluted unknown protein sample) are given below in Table 1.

Table 2.1 The protein determaniton for ascorbate oxidase.

Process Number	Procedure
1	0.5 ml of standards. unknown protein and distilled water (for blank) were pipetted into tubes.
2	2.5 ml of Reagent B was transferred into each tube and vortexed, then the mixture was allowed to stand at room temperature for 10 minutes.
3	0.25 ml of Folin's reagent was added to the each tube and wortexed.
4	The tubes were allowed to stand at room temperature for 30 minutes.
5	The absorbance of the each tube was recorded at 660 nm after the reaction.

2.2.2 Activity determination

The activity determination process for ascorbate oxidase by using the modified DCIP method is as follows.

Table 2.2. The activity determination process for ascorbate oxidase

Process Number	Reagents	Blank I (ml)	Blank II (ml)	Standards (ml)						Sample (ml)
				1	2	3	4	5	6	
I	Stock L-Ascorbic Solution($3.0x10^{-3}$M)	—	2.50	0.25	0.50	1.50	2.50	3.50	5.00	2.50
	Stock Enzyme Solution (66 mg/l)	—	—	—	—	—	—	—	—	0.10
	Phosphate Buffer (pH:7.0; 0.1M)	5.00	2.50	4.75	4.50	3.50	2.50	1.50	—	2.40
II	Incubation for 10 minutes at 37°C									
III	TCA Solution (5.0%)	10	10	10	10	10	10	10	10	10
IV	Titration with DCIP solution ($2.0x10^{-4}$ M)									

After the titration, the obtained data evaluated by means of formula is given below;

Consumption DCIP(ml) (standard) - Consumption DCIP(ml) (Blank I) = A(ml)

Between real DCIP consumptions which belong to each standard and L-ascorbic acid amount of each standard (mg), a calibration graphe was plotted out and the activity of ascorbate oxidase was calculated.

Consumption DCIP(ml) (Blank II-Blank I) - Consumption DCIP(ml) (Sample) = B(ml)

The amount of L-ascorbic acid was found for B value, then the specific

activity of ascorbate oxidase was calculated by employing the formula given below.

$$C \times 10^3 / D \times E \times F = G$$

C : Amount of L-ascorbic acid belongs to B value on calibration graphe 10^3 is to converted to µg.
D : Molecular weight of L-ascorbic acid : 176.13 g/mol.
E : Incubation time of enzymatic reaction (10 minutes)
F : Protein amount of sample in reaction tube (mg) which is determined by Lowry method.
G : Specific activity of ascorbate oxidase (µmol/min.mgprotein)

(Unit/mgprotein)

In all experiments at least 3 samples were examined. Stock solution of L-ascorbic acid was prepared in phosphate buffer (pH:7.0, 0.1 M). DCIP and TCA 5.0% (w/v) were prepared in distilled water.

The reaction for activity determination is as follows,

L-Ascorbic acid + DCIP $\xrightarrow[\text{(EC 1.10.3.3)}]{\text{Ascorbate oxidase}}$ Dehydro-L-Ascorbic acid + Reduced form of DCIP

2.2.3 Isolation and partially purification of ascorbate oxidase

2.2.3.1 Homogenization and centrifugation

After the cucumber provided from the local market waspeeled. It was cut into small pieces and homogenized with a blender in phosphate buffer 250 ml (pH:7.0, 0.1 M). The homogenate was filtered through cheese-cloth to remove the cellulose residue and then the filtrate was

centrifuged at 10000 rpm for 10 minutes. The supurnatant obtained was frozen in deep-freeze for a day and then the frozen supernatant was dissolved and centrifuged at 10000 rpm for 5 minutes again to remove the precipitate.

2.2.3.2 Precipitation with ammonium sulphate

After the last centrifugation process is completed, the solid ammonium sulphate (80%) was added to supernatant. The supernatant was allowed to stand for one hour at 4°C and then the resultant precipitate which contained ascorbate oxidase, was collected by centrifugation at 10000 rpm for 10 minutes and the collected precipitate was dissolved in the phosphate buffer (pH:7.0; 0.1 M).

2.2.3.3 Dialysis

Dissolved protein solutions, obtained after ammoniumsulphate precipitated, were dialysed for 48 hours against distilled water. Dialysis step was done in cold room at 4°C, by stirring. During this operation, distilled water was frequently changed to make the dialysis more effective.

2.2.3.4 Lyophilisation

In order to remove the precipitate, which was obtained by dialysis, was centrifuged at 10000 rpm for 5 minutes. The supernatant was frozen in deep-freeze and then vaporized in lyophilisator, the solid form of ascorbate oxidase preparate was obtained. The preparate was stored in a deep-freeze.

2.2.4 The working principle of dissolved oxygen probe

Dissolved oxygen probes based on amperometric consists of Au (cathode), Ag-AgCl (anode), half-saturated KCl (electrolyte) and a teflon membrane which is selective for oxygen (Dinçkaya and Telefoncu, 1993).

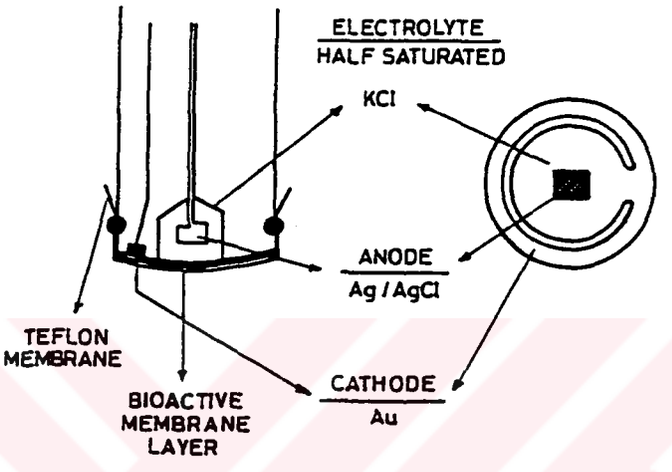


Figure 2.1. The scheme of a YSI 5739 dissolved oxygen probe.

The overall polarographic electrode reactions are as follows;

cathodic reaction : $O_2+2H_2O+2e^- \longrightarrow H_2O_2 + 2HO^-$

$H_2O_2+2e^- \longrightarrow 2HO^-$

anodic reaction : $Ag+Cl^- \longrightarrow AgCl + e^-$

overall reaction : $4Ag+O_2+2H_2O+4Cl^- \longrightarrow 4AgCl+4HO^-$

Figure 2.2 shows the diffusion barriers of oxygen while it passes from reaction medium to the cathode.

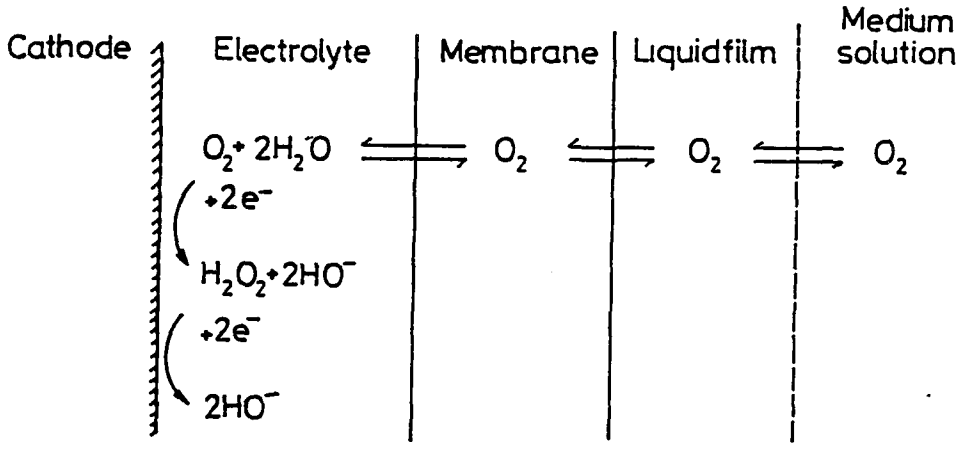


Figure 2.2 Diffusion barriers of oxygen.

If there is a high oxygen pressure differential due to the dissolved oxygen concentration between the two faces of teflon membrane, the oxygen diffusion will be faster from the membrane to the cathode and the higher oxygen concentration will be determined on the oxygenmeter because of the higher current present (Dinçkaya and Telefoncu, 1994).

2.2.5 Preparation of enzyme electrode based on ascorbate oxidase immobilized in gelatine

In this study YSI dissolved oxygen probes were used as basic sensor. The reason of this selection can be explained that reaction;

$$O_2 + 2\text{L-Ascorbate} \xrightarrow{\text{Ascorbate Oxidase}} 2\text{-Dehydro-L-Ascorbate} + 2H_2O$$

According to above reaction, it can be suggested that the amount of expended oxygen can be related to L-ascorbate. As a result of this effect, on the membrane of dissolved oxygen probe, a gelatine-ascorbate oxidase enzyme layer crosslinked with glutaraldehyde, was formed.

The preparation procedure of ascorbate oxidase electrode immobilized in gelatine is as follows;

Table 2.3. The preparation procedure of ascorbate oxidase electrode.

Process Number	Procedure
1	10 mg ascorbate oxidase and 10 mg gelatine are dissolved in 300 µl phosphate buffer (pH:7.5, 50 mM) by using a water bath at 38°C.
2	Dissolved oxygen probe's membrane was treated with 0.5% SDS (in phosphate buffer; pH:7.5, 50 mM)
3	200 µl of ascorbate oxidase gelatine mixture was easily spread on the membrane of the dissolved oxygen probe.
4	It was allowed to stand for one hour at 4°C.
5	After one hour it was allowed in 2.5% glutaraldehyde (in phosphate buffer; pH:7.5, 50 mM)
6	It was washed with bidistilled water for couple of times, then waited in a working buffer at the working temperature.

The enzyme electrode based on ascorbate oxidase immobilized in gelatine is washed with bidistilled water after using and stored at 4°C in a flask which contains the some amount of bidistilled water in it. The electrode should not contact with water. Water provides a moisture medium therefore the dryness of the gelatine-ascorbate oxidase layer is prevented.

Figure 2.3 shows the preparation steps of enzyme electrode based on ascorbate oxidase immobilized in gelatine.

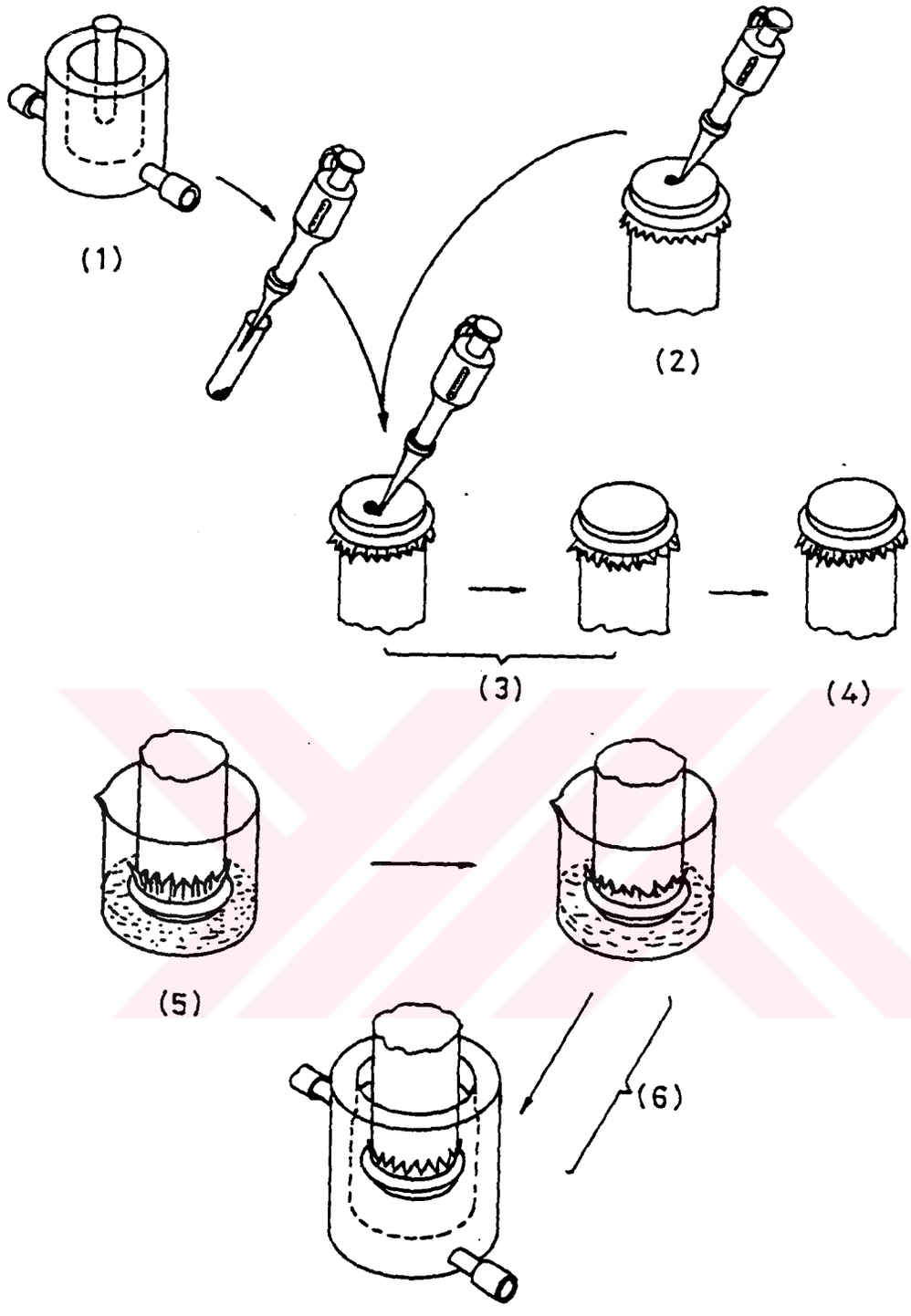


Figure 2.3 The preparation steps of enzyme electrode based on ascorbate oxidase immobilized in gelatine.

Figure 2.4 shows the scheme of the bioactive layer of ascorbate oxidase electrode.

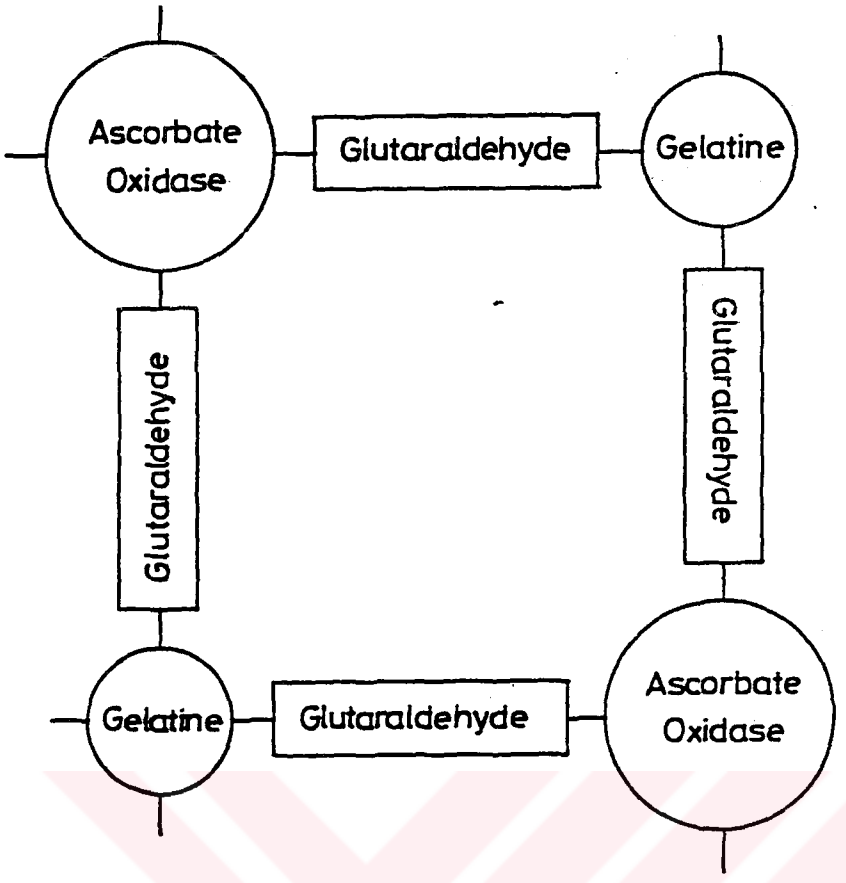


Figure 2.4 The scheme of the bioctive layer of ascorbate oxidase electrode

Figure 2.5 shows the molecular structure of the bioatcive layer of ascorbate oxidase electrode.

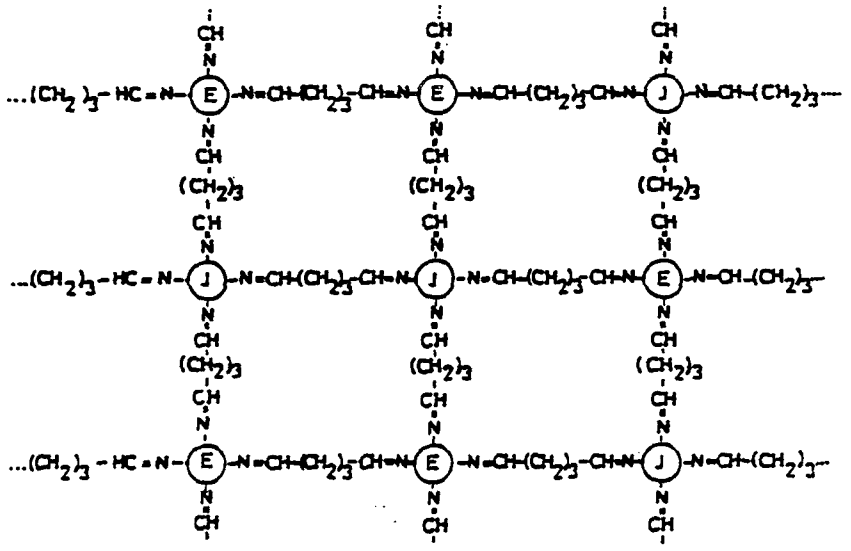


Figure 2.5 The molecular structure of the bioactive layer of ascorbate oxidase electrode.

2.2.6 The working principle of ascorbate oxidase electrode based on dissolved oxygen probe

The reactions occured on the bioactive layer are shown in figure 2.6

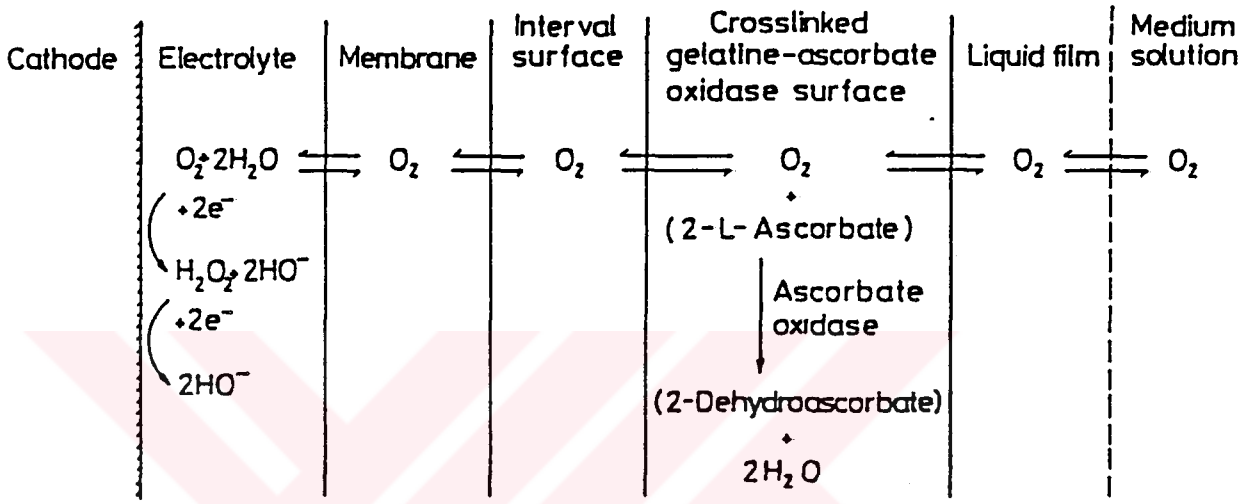


Figure 2.6 The reactions occured on the bioactive layer.

When there is no substrate in the reaction medium, dissolved oxygen concentration in interval surface was recorded on oxygenmeter as mg/ml. This concentration value is more lower than the dissolved oxygen concentration in the reaction cell as the bioactive layer causes obstacles for the diffusion of O_2.

As the substrate was added to reaction medium some of the oxygen diffused from reaction cell to the cathode, would be lost because of an enzymatic reaction occurs on the bioactive layer. As a result of the diffusion of L-ascorbic acid added to the reaction medium, dissolved O_2 concentration of interval surface will be reduced and a new equilibrium will be formed in a short period of time.

In the presence of the substrate, the dissolved oxygen concentration will be lower than the absence of the it. Related to increase of substrate

concentration, more oxygen will be lost on bioactive layer so for dissolved oxygen concentration of interval surface more lower values will be obtained. Result in increasing of substrate concentration more higher ΔDO values will be obtained on the oxygenmeter and so by using the linear range of curves which are obtained between L-ascorbic acid concentration and ΔDO values the determination of L-ascorbic acid was realed.

2.2.7 Measurement system for the determination of L-ascorbic acid by ascorbate oxidase electrode.

The system used in the measurements is given in figure 2.7.

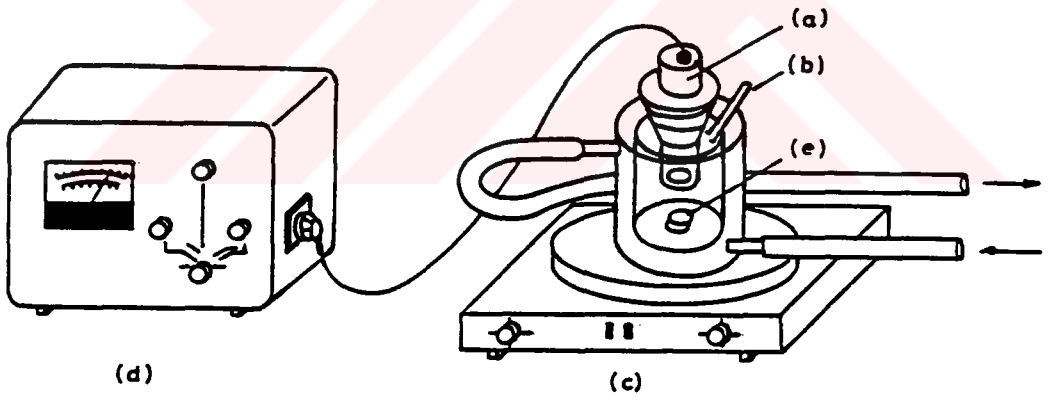


Figure 2.7 Details of the measurement system.
a: enzyme electrode. b:injection valve. c:magnetic stirrer. d:YSI 54A Model oxygenmeter. e:working buffer.

Before the measurements, the electrode was incubated in the working buffer for 30 minutes at working temperature. Oxygenmeter was calibrated and then the working buffer was put into the reaction cell. The

magnetic stirrer was fixed as 1000/min. speed. After incubation by 10 minutes for electrode polarization ascorbate oxidase electrode was put into the reaction cell and the measurement was done. The enzyme electrode was equilibrated approximately after twenty second and kept constant in a certain oxygen concentration. At this time, standard or sample was injected into the reaction cell. The dissolved oxygen concentration, related to L-ascorbic acid concentration, changed. In the 20x-60 seconds the electrode was equilibrated.

When the measurements were completed, the ascorbate oxidase electrode was washed with bidistilled water and stored in the working buffer at the working temperature until another measurement.

A measurement, containing electrode polarization, enjection, reading, electrode washing takes 15 minutes.

2.2.8 The optimization of the enzyme electrode based on ascorbate oxidase immobilized in gelatine

In this part of the work, it was aimed to get the optimum electrode responses for determination of L-ascorbic acid by the using biosensor developed. During the preparation of bioactive layers, various mixtures containing different ratios of ascorbate oxidase, gelatine and glutaraldehyde (crosslinked agent) were used. After the obtaining standard curve, optimization of the enzyme electrode based on ascorbate oxidase immobilized in gelatine was carried out.

2.2.8.1 The effect of enzyme amount on the electrode response

The experiments were carried out in phosphate buffer (pH:7.5, 50 mM) at 35°C. In order to investigate the effect of enzyme amount to electrode response the amount of gelatine (10 mg) and glutaraldehyde percent (2.5%) were kept constant, however the amount of enzyme were changed.

The amount of enzyme were selected as follows;

a) 10 mg gelatine + 5 mg enzyme, (2.9 mgenzyme/cm^2)
b) 10 mg gelatine + 10 mg enzyme, (5.9 mgenzyme/cm^2)
c) 10 mg gelatine + 15 mg enzyme, (8.8 mgenzyme/cm^2)

The prepared enzyme-gelatine mixture were spreaded on the electrode surface. By using the electrodes, the measurements were carried out in the concentration range between 0.05-1.6 mM for L-ascorbic acid.

2.2.8.2 The effect of gelatine amount on the electrode response

For preparation of ascorbate oxidase electrode, the most suitable enzyme amount was selected as 10 mg (5.9 mgenzyme/cm^2). By keeping the amount of enzyme constant and changing the amount of gelatine, the electrode response were examined. The measurements were done in the phosphate buffer (pH:7.5, 50 mM)

The amount of gelatine were selected as follows;

a) 10 mg enzyme+5mg gelatine (5.9 mgenzyme/cm^2, 2.95 mggelatine/cm^2)
b) 10 mg enzyme+10mg gelatine (5.9 mgenzyme/cm^2, 5.9 mggelatine/cm^2)
c) 10 mg enzyme+20mg gelatine (5.9 mgenzyme/cm^2, 11.8 mggelatine/cm^2)

The measurements were carried out in the concentration range between 0.05-1.6 mM for L-ascorbic acid by using, the each electrode.

2.2.8.3 The effect of glutaraldehyde (crosslinked agent) ratio on the electrode response

The experiment assays were carried out in the phosphate buffer (pH:7.5, 50 mM). For the best electrode response, the amount of the enzyme and gelatine was selected as [10 mg (5.9 mgenzyme/cm^2)] and [10 mg (5.9 mggelatine/cm^2)] respectively. In order to examine the effect of

glutaraldehyde ratio on the electrode response, the amount of the enzyme and the gelatine were kept constant whereas the glutaraldehyde ratios were changed as 2.5%, 5.0%, and 10% respectively.

The effect of glutaraldehyde ratio on the electrode response was examined in the L-ascorbic acid concentration range between 0.05-1.6 mM.

2.2.8.4 Determination of optimum pH

In this work, phosphate buffer (pH:5.5, 6.0, 6.5, 7.0, 7.5, 8.0; 50 mM),glycine buffer (pH:8.5, 9.0, 9.5, 10.0; 50 mM), and citrate buffer (pH:3.0, 3.5, 4.0, 4.5 and 5.0; 50 mM) were used and the most suitable pH for the optimum electrode response was investigated. As the optimum pH for the free ascorbate oxidase from various plants was 7.5 (Uchiyama et al., 1993) the experiments were carried out at this pH. The effect of the immobilization method used for preparation of a bioactive layer on the optimum pH of ascorbate oxidase was investigated. For this purpose, buffers having different pH values were used. Before the measurements, the electrode was incubated in the above buffers for 30 minutes at 35°C.

2.2.8.5 Determination of the appropriate buffer system

In this part, various buffer systems at pH:7.5 which was the optimum value for ascorbate oxidase were used to get the highest electrode response. Potassium phosphate (pH:7.5, 50 mM), sodium phosphate (pH:7.5, 50 mM), Tris-HCl (pH:7.5, 50 mM) and Na_2HPO_4/citric acid buffers were used after they are saturated with oxygen at room temperature. The working temperature was selected as 35°C. The assays were carried out at 0.05-1.6 mM L-ascorbic acid concentration range. Before the measurements, the enzyme electrode was incubated in the above buffers for 30 minutes at 35°C.

2.2.8.6 Determination of the appropriate buffer concentration

The effects of the buffer concentration on electrode response was searched. The potassium phosphate buffer was determined as the most suitable buffer system for the enzyme electrode based on ascorbate oxidase immobilized in gelatine. 25, 50 and 100 mM potassium phosphate buffers were respectively prepared. Measurements were carried out in the phosphate buffers (pH:7.5) mentioned above for the L-ascorbic acid concentration range of 0.05-1.6 mM at 35°C. Before the measurements, the enzyme electrode was incubated in the above buffers for 30 minutes at 35°C.

2.2.8.7 Determination of the optimum temperature

The experiments were carried out at 15, 20, 25, 30, 35, 40 and 45°C from lower to higher degrees respectively to investigate the effect of the temperature on electrode response by using phosphate buffer (50 mM, pH:7.5) and standard curves (at 0.05-1.6 mM substrate concentration range) were drawn for each temperature value.

2.2.9 The characterization of the enzyme electrode based on ascorbate oxidase immobilized in gelatine

2.2.9.1 Determination of L-ascorbic acid concentration range

After the optimum conditions such as pH,temperature, the effects of the enzyme and the gelatine amount and the glutaraldehyde percent on electrode response were determined the standard curve were drawn. The experiments were carried out in the phosphate buffer (pH:7.5, 50 mM) at 35°C.

2.2.9.2 Reproducibilty of the results

In this study a phosphate buffer (pH:7.5, 50 mM) was used and the experiments were done at 35°C. 11 measurements were carried out for

the same L-ascorbic acid standard containing 0.4 mM L-ascorbic acid. Standard deviation (S.D) and variation coefficient (C.V) were calculated.

2.2.9.3 Substrate specificity

By using oxalic acid,L-aspartik asit, L-glutamic acid, sucsinic acid, citric acid, glycolicacid, D(+) glucose, D(-) fructose, hydroquinone, 8-hydroxyquinoline and catechol which were all thought to give the same electrode response as L-ascorbic acid as standard solutions the experiments were done in the phosphate buffer (pH:7.5, 50 mM) at 35°C. All the standard solutions were prepared in the phosphate buffer. For the determination of substrate specificity of enzyme electrode based on ascorbate oxidase immobilized in gelatine 0.4 and 1.0 mM of standard solutions and L-ascorbic acid were used.

The electrode response obtained for L-ascorbic acid, was accepted as 100% and compared with the standards.

2.2.9.4 Interference effects of some substances on L-ascorbic acid determination

In this work, the ammount of L-ascorbic acid was determined by using ascorbate oxidase electrode in the presence of some interferent substances in reaction medium and the effect of these substances on electrode response depended on L-ascorbic acid concentration was investigated. For this purpose hydroquinone (0.4 mM), fructose (250 mM), glucose (200 µg/ml), citric acid (250 µg/ml), tartaric acid (60 µg/ml), and oxalic acid (50 µg/ml), were used. The experiments were carried out in the phosphate buffer (pH:7.5, 50 mM) at 35°C. The electrode response obtained for 0.8 mM of L-ascorbic acid was accepted as 100% and the electrode responses of samples which contain L-ascorbic acid (0.8 mM) and other interferent substances were compared with this value.

2.2.9.5 Determination of storage stability

Experiments were carried out in the phosphate buffer (pH:7.5, 50 mM) at 35°C. During a three months period, an experiment was done in every twenty days. The enzyme electrode based on ascorbate oxidase immobilized in gelatine was not used except this purpose and other days the electrode was stored in cold room at 4°C. During the storage period, changes in electrode response related to the L-ascorbic acid concentrations (0.05; 0.2; 0.8 mM) were determined.

2.2.10 Determination of L-ascorbic acid contents of some fruit extracts and vitamin-C tablets by using an enzyme electrode based on ascorbate oxidase immobilized in gelatine

2.2.10.1 Determination of L-ascorbic acid in vitamin-C tablets

For this purpose Ca-Sandoz (1000 mg ascorbic acid/tablet) and Redoxan (500 mgascorbic acid/tablet) tablets were used. The solution of each tablet was prepared in the phosphate buffer (pH:7.5, 50 mM) by containing 0.6 mM L-ascorbic acid after they were powdered. This ascorbic acid concentration was used in the experiments. The ascorbic acid content of the solutions was determined both by using the enzyme electrode and the modified DCIP method. In addition, the results obtained by standard addition was examined.

2.2.10.2 Determination of L-ascorbic acid in some fruit extracts

For this purpose oranges and lemons were used. After the fruits (100 g) were peeled, they were homogenized with a blender. The homogenate was filtered thorugh chese-cloth to remove the cellulose residue and then the filtrate was centrifuged at 10000 rpm for 5 minutes. The supernatant obtained was kept at 4°C in dark and mearusements were carried out at the sdmeday.

The amount of L-ascorbic acid of each extract prepared was determined by using ascorbate oxidase and DCIP methods. The two methods were compared with each other. In addition, the accuracy of the results obtained by standard addition was examined. Standard deviations and variation coefficients were calculated.

3. RESULTS AND DISCUSSION

3.1 Protein Standard Curve

The protein amount of preparate and fraction which was obtained from the isolation and the partially purification of ascorbate oxidase, was determined by using the protein standard curve given below. This curve was obtained by the modification of the method suggested by Lowry et al., (Lowry et al., 1951). Figure 3.1 shows the relation between the absorbance and the protein concentration.

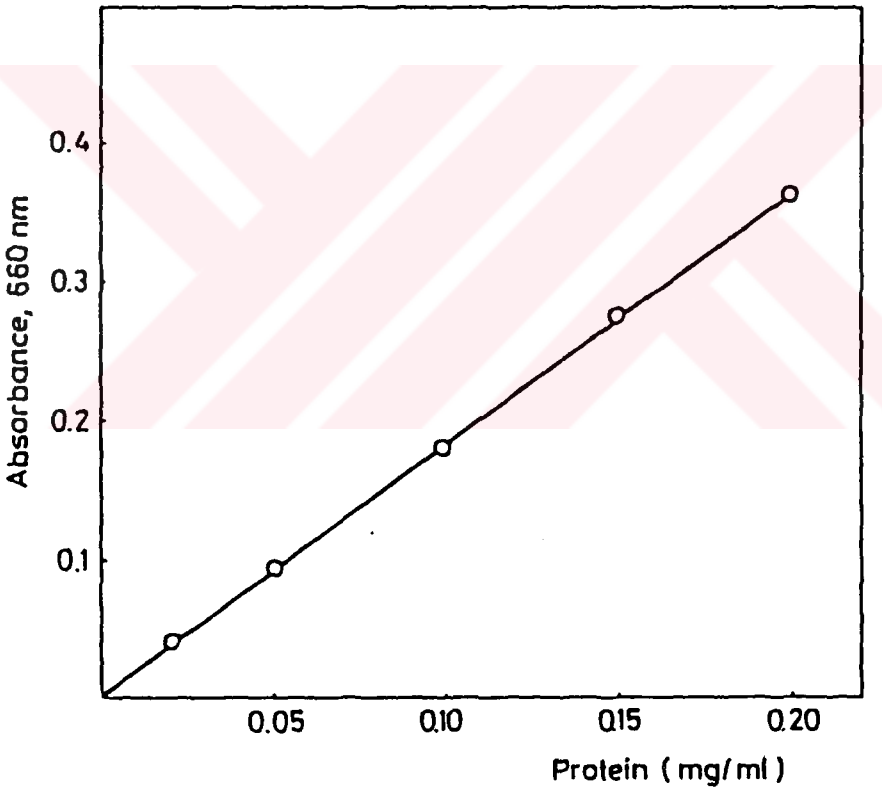


Figure 3.1. The protein standard curve

3.2 The Standard Curve of Enzymatic Acticity

The standard curve shown in Figure 3.2 was drawn by using the modified DCIP method in order to determine the activity of ascorbate oxidase.

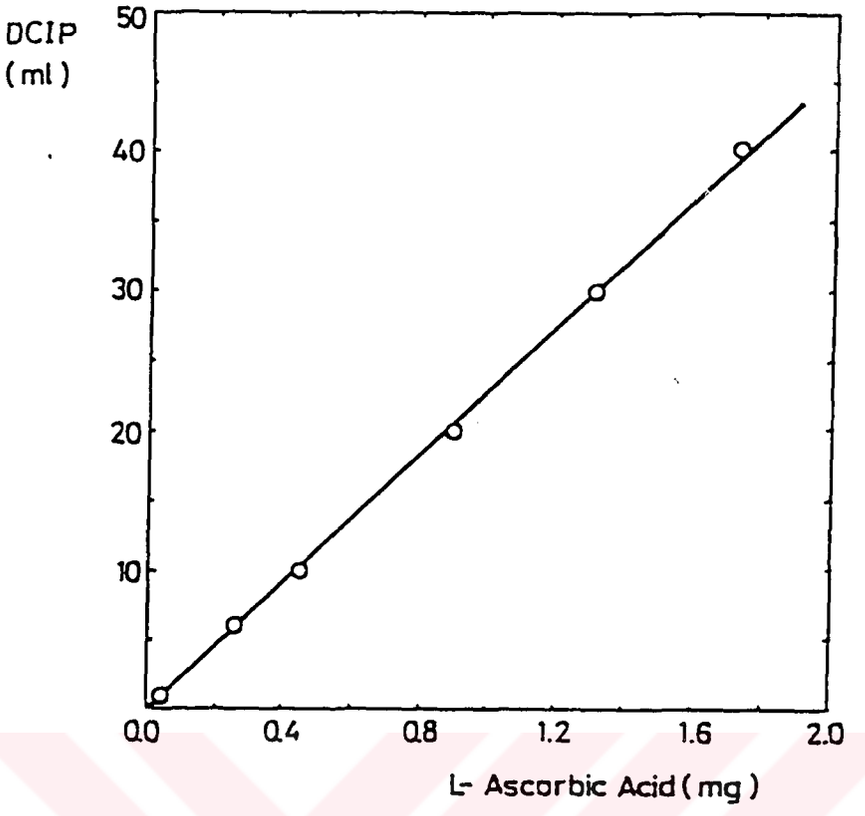


Figure 3.2 The standard curve of enzymatic activity

When 2.0×10^{-4} M DCIP solution for titration is used, a linear graph was obtained which shows a linearity in the L-ascorbic acid concentration range between 0.044-1.744 mg.

Ascorbate oxidase convert one mole of L-ascorbic acid to one mole of dehydro-L-ascorbic acid and one mole of H_2O. The enzymatic reaction and formulas of activity determination were given in section 2.2.2.

3.3 Data of Isolation and Partially Purification of Ascorbate Oxidase

In this work, it is aimed to obtain an enzyme which is soluble in water, and is mainly based on the preparation of enzyme electrodes by using partially purified ascorbate oxidase.

To isolate and partially purify the ascorbate oxidase, mince by using a blender homogenization, centrifugation, ammonium sulphate precipitation (80%), dialysis and lyophilisation were subjected to extract which is obtained at various steps respectively, After all of these steps, ascorbate oxidase was obtained as partially purified. Finally the activity and protein amount of ascorbate oxidase preparate were determined.

After the isolation and partially purification, 320 mg solid preparate of ascorbate oxidase were obtained for 350 g of cucumber (*Cucumis sativus* L.)

The protein amount of ascorbate oxidase was found as 83.5% and the spesific activity as 5.03 U/mgprotein. The activity of solid preparate was determined as 4.2 U/mg solid preparate and total activity of partially purified ascorbate oxidase preparate from cucumber (*Cucumis sativus* L.) was determined as 1344 U.

3.4 Results of Optimization of Enzyme Electrode Based On Ascorbate Oxidase Immobilized in Gelatine

3.4.1 The effect of enzyme amount on the electrode response

The amount of gelatine and the percentage of glutaraldehyde were kept constant whereas the amount of enzyme was changed as 5, 10 and 15 mg respectively. By using 10 mg of gelatine and 2.5% of glutaraldehyde and the enzyme in an amount given above, the enzyme electrodes based on ascorbate oxidase immobilized in gelatine were prepared.

Figure 3.3 shows the relation between the amount of enzyme used and electrode responses.

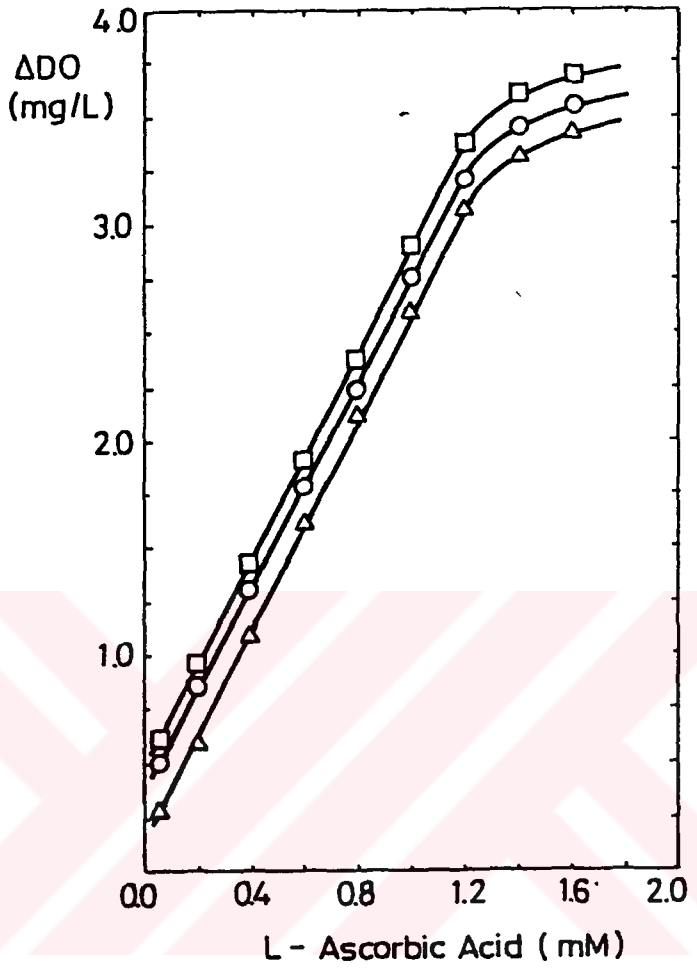


Figure 3.3 The effect of enzyme (ascorbate oxidase) amount on the electrode response (Phosphate buffer; pH:7.5, 50 mM; T:35°C -Δ-Δ-: 2.9 mgenzyme/cm^2 (12.4 U/cm^2), -O-O-: 5.9 mgenzyme/cm^2 (24.8 U/cm^2), -□-□-: 8.8 mgenzyme/cm^2 (37.2 U/cm^2). For all the enzyme electrodes the amount of gelatine was 5.9 mggelatine/cm^2 and the percentage of glutaraldehyde was 2.5%)

When the amount of the enzyme on the bioative layer increase as 2.9 mgenzyme/cm^2 (12.4 U/cm^2), 5.9 mgenzyme/cm^2 (24.8 U/cm^2), and 8.8 mgenzyme/cm^2 (37.2 U/cm^2) an increase was observed for the electrode response. although the amount of enzyme was doubled from 2.9 mgenzyme/cm^2 to 5.9 mgenzyme/cm^2 and three fold from 2.9 mgenzyme/cm^2 to 8.8 mgenzyme/cm^2, the electrode responses were not increased as much. When the amount of enzyme used at the beginning is doubled the eletcrode response was calculated as 10.8% and when it's three fold the electrode response was calculated as 16.9 %.

Although the best electrode response is obtained by 8.8 mgenzyme/cm^2 this amount causes some disadvantages in membrane preparation and membrane stability. As a result 5.9 mgenzyme/cm^2 was found as the most suitable amount for the enzyme and it was observed that, the biactive layer could easily be formed and stable.

3.4.2 The effect of gelatine amount on the electrode response

In this work, the effect of gelatine amount on the electrode response was defined in order to prepare the enzyme electrode based on ascorbate oxidase immobilized in gelatine.

For this purpose, the ascorbate oxidase amount was kept constant as 5.9 mgenzyme/cm^2 (24.8 U/cm^2) which was found as the ideal enzyme amount. In contrast, the amount of gelatine was changed as 5, 10 and 20 mg respectively.

Figure 3.4 shows the relation is between the used gelatine amount and electrode responses.

When the amount of gelatine is increased the electrode response gets decreased. In general, increased amount of gelatine leads to thicker diffusion barriers. When the amount of gelatine is decreasede from 11.8 mggelatine/cm^2 to 5.9 mggelatine/cm^2, a slight increase on the electrode response was observed whereas it was decreased from 11.8 mggelatine/cm^2 to 2.95 mggelatine/cm^2 higher electrode responses were recorded.

As the bioactive layer gets thinner the diffusion barrier decreases, therefore higher electrode responses occur but the bioactive layer becomes unstable and gets broken very easily in a few days time. As the preparation of the electrode was quite simple and the electrode responses obtained were high enough, 5.9 mggelatine/cm^2 was preferred as the most suitable gelatine amount.

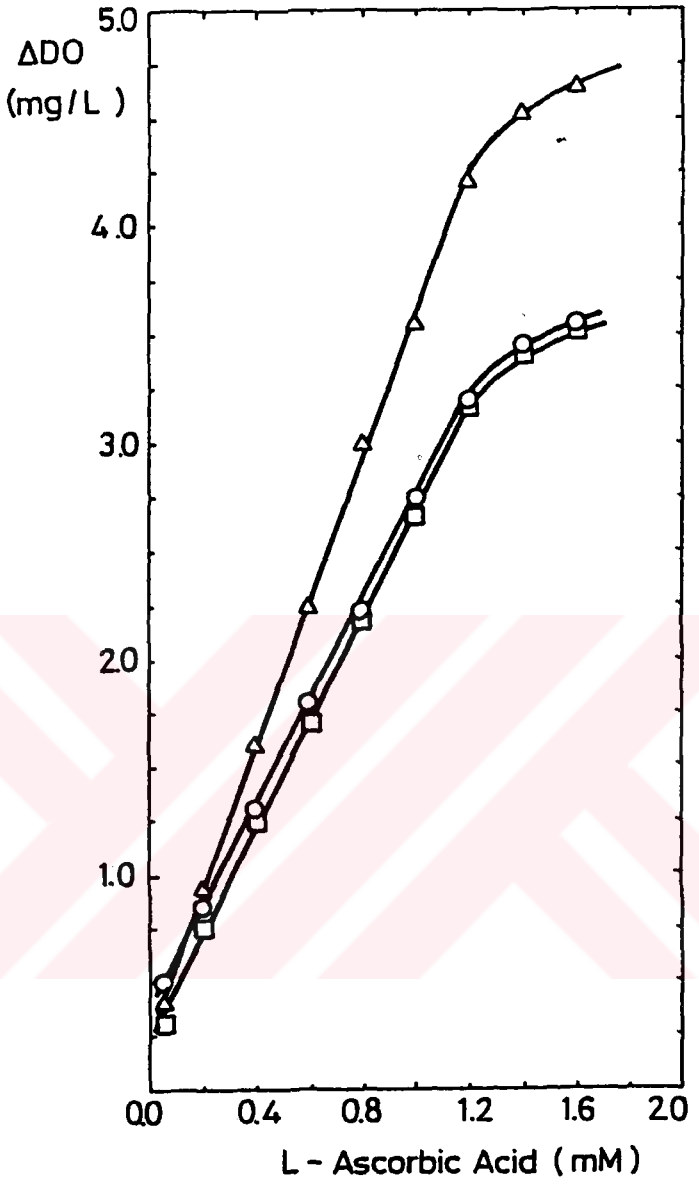


Figure 3.4 The effect of gelatine amount on the electrode response (Phosphate buffer; pH:7.5, 50 mM); T:35°C. -Δ-Δ-: 2.95 mggelatine/cm^2, -O-O-:5.9 mggelatine/cm^2, -□-□-: 11.8 mggelatine/cm^2. For all the enzyme electrodes the amount of ascorbate oxidase and the percentage of glutaraldehyde were kept constant as 5.9 mgenzyme/cm^2 (24.8 U/cm^2) and 2.5%, respectively).

3.4.3 The effect of percentage of glutaraldehyde on the electrode response

After both the amount of enzyme and gelatine were determined as

10 mg (24.8 U/cm^2), 10 mg (5.9 mggelatine/cm^2), respectively, each of the newly prepared enzyme electrodes were treated with 2.5, 5.0 and 10.0 % glutaraldehyde, therefore bioactive layers were formed.

Figure 3.5 shows the effect of percentage of glutaraldehyde on the electrode response.

When the percentage of glutaraldehyde was increased, a slight decrease was observed for the electrode response due to more enzyme-enzyme and enzyme-gelatine cross-links that caused steric hindrance which would lead to an activity loss for the ascorbate oxidase.

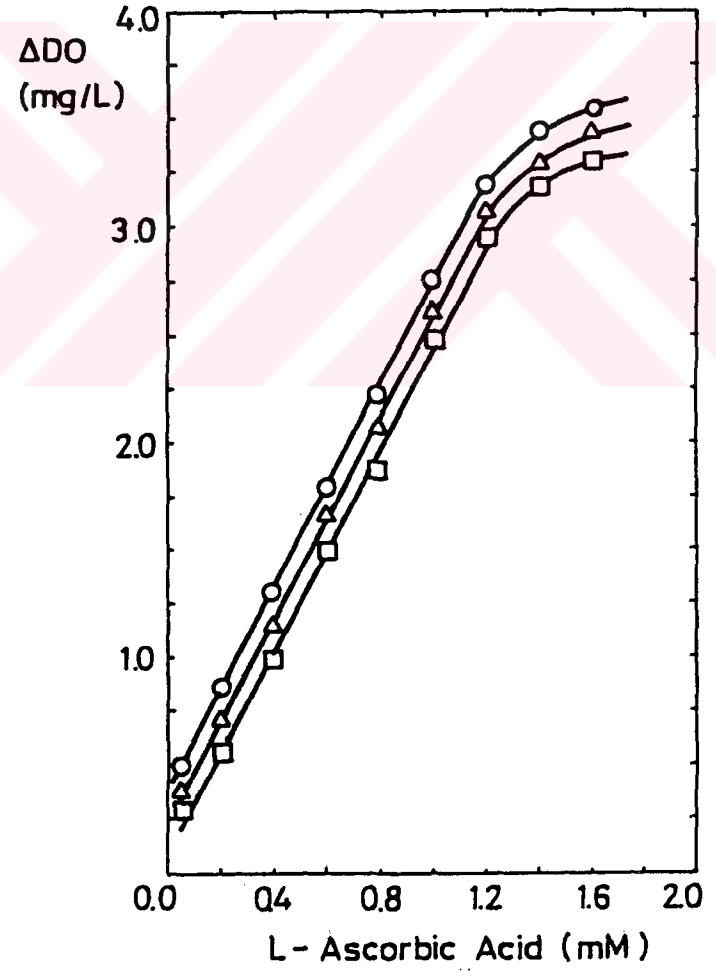


Figure 3.5 The effect of percentage of glutaraldehyde on the electrode response (Phosphate buffer; pH:7.5, 50 mM); T:35°C, -O-O-: 2.5%, -Δ-Δ-:5.0 %, -□-□-: 10.0 %. For all the enzyme electrodes the amount of enzyme and gelatine were kept constant as 5.9 mgenzyme/cm^2 (24.8 U/cm^2) and 5.9 mggelatine/cm^2 respectively.)

In addition, increasing the cross-links numbers, connected with glutaraldehyde concentration, results in the formation of a more tighter cage-shaped structure on the bioactive layer which makes the diffusion from outer medium to interval surface difficult and as a result, the electrode response decreases.

In case of using 1.25% glutaraldehyde, the bioactive layer was easily decomposed due to few number of cross links formed.

3.4.4 The optimum pH

As mentioned in chapter 2.2.8.4, pH:7.5 was found to be the optimum value for the enzyme electrode based on ascorbate oxidase immobilized in gelatine. Therefore, it was understood that the immobilization method used, was the most suitable one for the enzyme as it did not change the optimum pH.

Figure 3.6 shows the results obtained for the determination of the optimum pH value for the enzyme electrode.

According to the figure 3.6, when pH was incerased from 7.5 to 8.0 and decreased from 7.5 to 5.5, 5.0% and 11.5% decreased enzyme activities were observed, respectively. In case of using glycine buffer (pH:10.0) it was determined that the activity loss was 17.5%. In contrast, when a citrate buffer was used in acidic range, the activity, at pH:5.0 and pH:3.0 were found as 33.0% and 87.5% respectively. As a result, it was determined that the activity loss in the acidic range was higher, which could also be thought as a disadvantage from buffertype. Consequently, pH:7.5 was selected as the most suitable pH value as the working buffer.

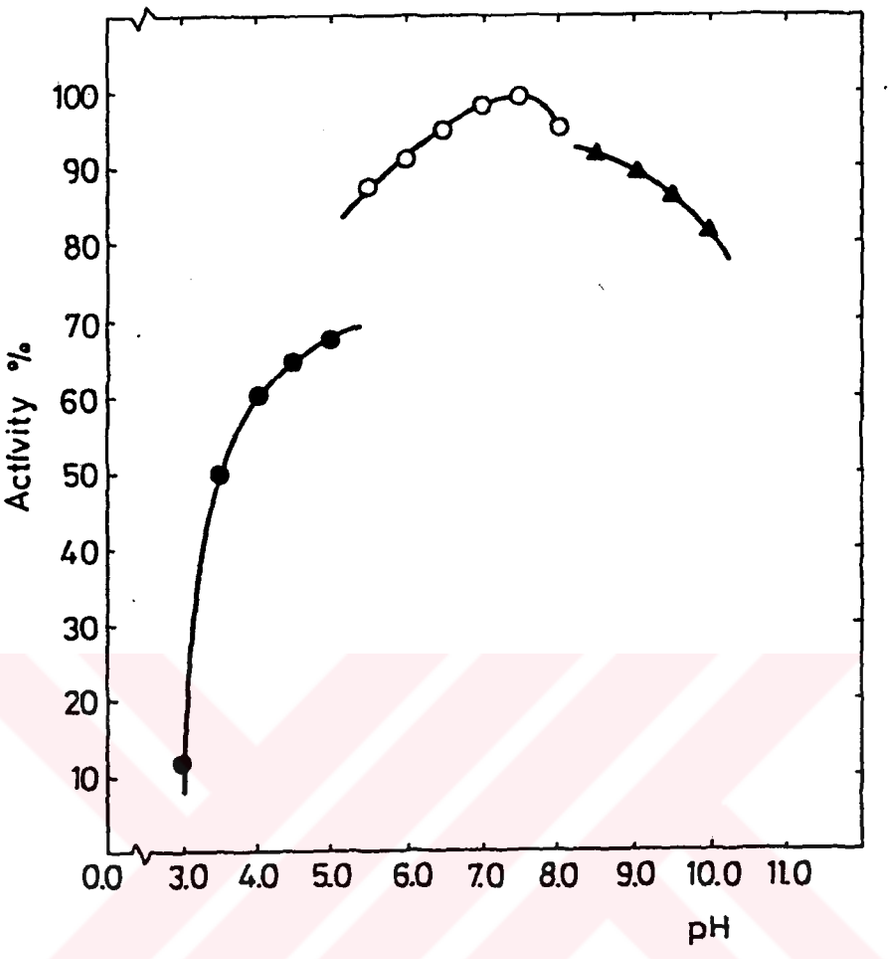


Figure 3.6 Determination of the optimum pH (Phosphate buffer; pH:7.5, 50 mM; T:35^{0}C. The amount of ascorbate oxidase, gelatine and the percentage of glutaraldehyde were kept constant as 5.9 mgenzyme/cm^{2} (24.8 U/cm^{2}), 5.9 mggelatine/cm^{2} and 2.5%, respectively).

3.4.5 The appropriate buffer system

After the optimum pH value for working buffer was found as 7.5, potassium phosphate, sodium phosphate, Tris-HCl and Na_2HPO_4/citric acid were used in order to determine the most suitable buffer system.

Figure 3.7 shows the effects of the buffer types on the electrode response. The highest electrode response was observed with potassium phosphate buffer. When other buffer systems were used, lower electrode

responses were recorded. The lowest responses were obtained in case of Tris-HCl system.

It was found that the electrode response was decreased due to the interactions between the enzyme molecule and the compounds of the buffer systems. It was thought that these interactions could lead to lower the activity. As a result, the potassium phosphate buffer was selected as the most suitable system as the highest electrode responses were obtained by using it.

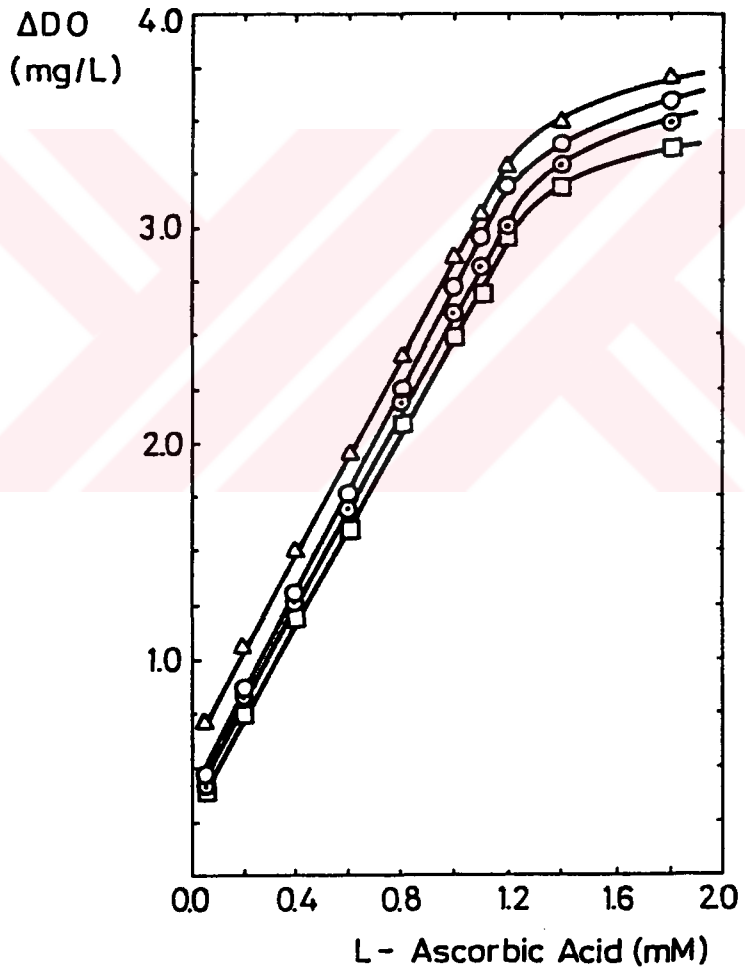


Figure 3.7 The effect of the buffer system on the electrode response (Concentrations of buffers were 50 mM; pH:7.5; T:35°C, -Δ-Δ-: potassium phosphate, -O-O-: sodium phosphate, -⊙-⊙-: sodium phosphate/citric acid, -□-□-: Tris/HCl. The amount of enzyme, gelatine and the percentage of glutaraldehyde were kept constant as 5.9 mgenzyme/cm^2 (24.8 U/cm^2), 5.9 mggelatine/cm^2 and 2.5%, respectively).

3.4.6 The optimum buffer concentration

Results obtained for the determination of optimum buffer concentration were shown in figure 3.8.

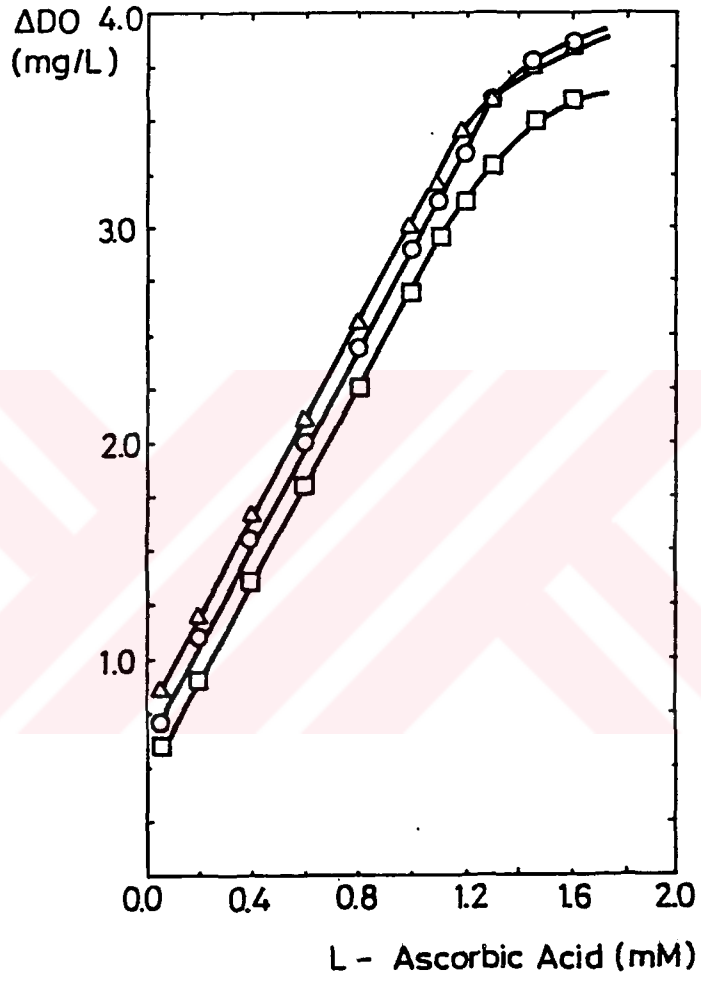


Figure 3.8 The effect of the buffer concentrations on the electrode response. (Phosphate buffer, pH:7.5; T:35°C, -Δ-Δ-: 25 mM, -O-O-: 50 mM, -□-□-: 100 mM. The amount of ascorbate oxidase, gelatine and the percentage of glutaraldehyde were kept constant as 5.9 mgenzyme (24.8 U/cm^2), 5.9 mggelatine/cm^2 and 2.5% respectively).

According to the figure 3.8 when the buffer concentration was increased the electrode response was decreased. This can be explained in terms of the increasing effects of the ionic strength on enzymatic acticity as negative. As the buffer capacity of 50 mM buffer concentration was

higher than the 25 mM one it was prefered for the determination of ascorbic acid in natural samples. Moreover, it was observed that the increase in buffer concentration from 25 mM to 50 mM decreased the electrode response only by 5.0% and it was thought that this decrease was not important for getting sensitive results. Therefore, 50 mM phosphate buffer was used for all measurements through out this work.

3.4.7 The optimum temperature

To determine the optimum temperature for enzyme electrode based on ascorbate oxidase immobilized in gelatine, the experiments were carried out at 15, 20, 25, 30, 35, 40 and 45°C respectively. The standard curves drawn were given at figure 3.9.

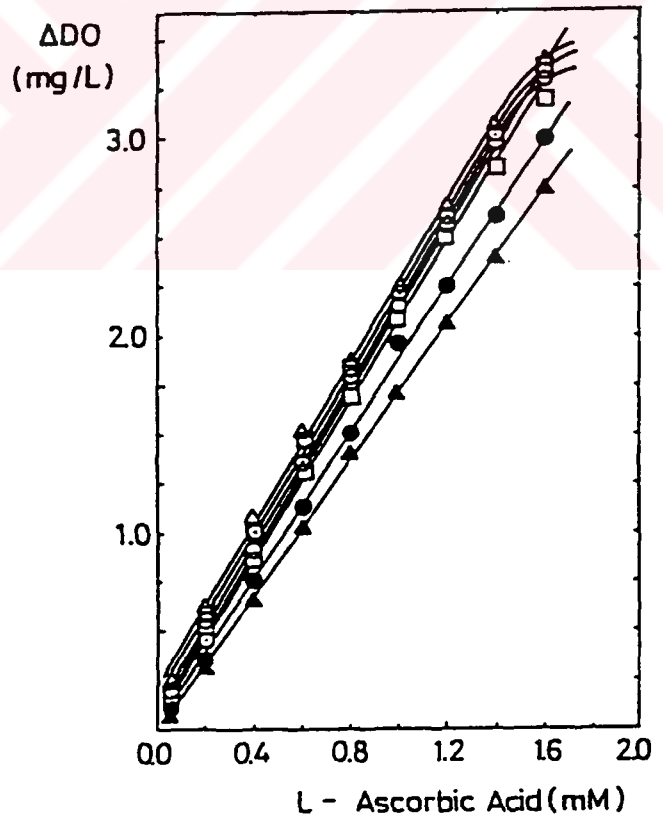


Figure 3.9 The effect of temperature on the electrode response (Phosphate buffer; pH:7.5, 50 mM, -▲-▲-: 15°C, -●-●-: 20°C, -□-□-: 25°C, -O-O-: 30°C, -Δ-Δ-: 35°C, -⊡-⊡-: 40°C, -◨-◨-: 45°C. The amount of ascorbate oxidase, gelatine and the percentage of glutaraldehyde were kept constant as 5.9 mgenzyme/cm^2 (24.8 U/cm^2), 5.9 mggelatine/cm^2 and 2.5% respectively).

According to the figure 3.9 the most highest electrode responses were observed at 35°C. Below and above 35°C, decreases for electrode responses were recorded. Deviation from linearity at high concentrations occured at temperatures such as 35 and 40°C. Since higher enzyme activity observed at these temperatures, the deviation from linearity can be due to the insufficient amount of oxygen which was a co-substrate of the enzyme. On the other hand at lower temperatures, for high ascorbic acid concentrations, the standard curve showed no deviation from linearity as the concentration of the dissolved oxygen was higher.

Figure 3.10 shows the relation between the working temperature and the electrode response with ascorbic acid concentration.

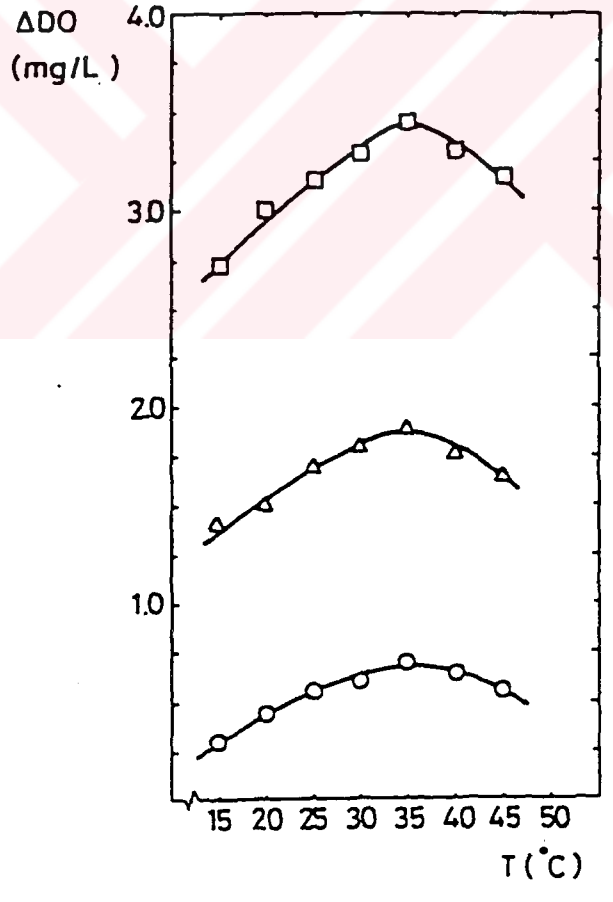


Figure 3.10 Changes in the electrode response related to the temperature at different ascorbic acid concentrations. (Phasphate buffer; pH:7.5, 50 mM, -O-O-: 0.2 mM, -Δ-Δ-: 0.8 mM, -□-□-: 1.6 mM. The amount of ascorbate oxidase, gelatine and the percentage of glutaraldehyde were kept constant as 5.9 mgenzyme/cm^2 (24.8 U/cm^2), 59 mggelatine/cm^2 and 2.5%, respectively).

concentration results in the decrease in substrate amount diffused through the bioactive layer.

3.5 Characterization of The Enzyme Electrode Based On Ascorbate Oxidase Immobilized In Gelatine

3.5.1 Determination of the detection limits for L-ascorbic acid

The data obtained for the determination of the detection limits for L-ascorbic acid were given in figure 3.12.

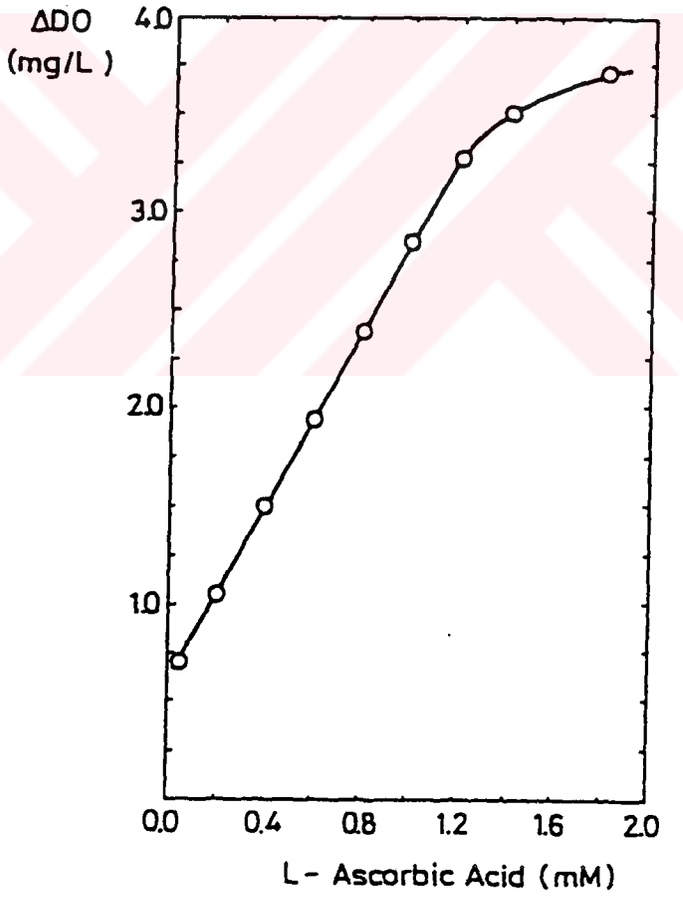


Figure 3.12 Determination of the detection limits for L-ascorbic acid. (Phosphate buffer; pH:7.5, 50 mM, T:35°C. The amount of ascorbate oxidase, gelatine and the percentage of glutaraldehyde were kept constant as 5.9 mgenzyme/cm^2 (24.8 U/cm^2), 5.9 mggelatine/cm^2 and 2.5%, respectively).

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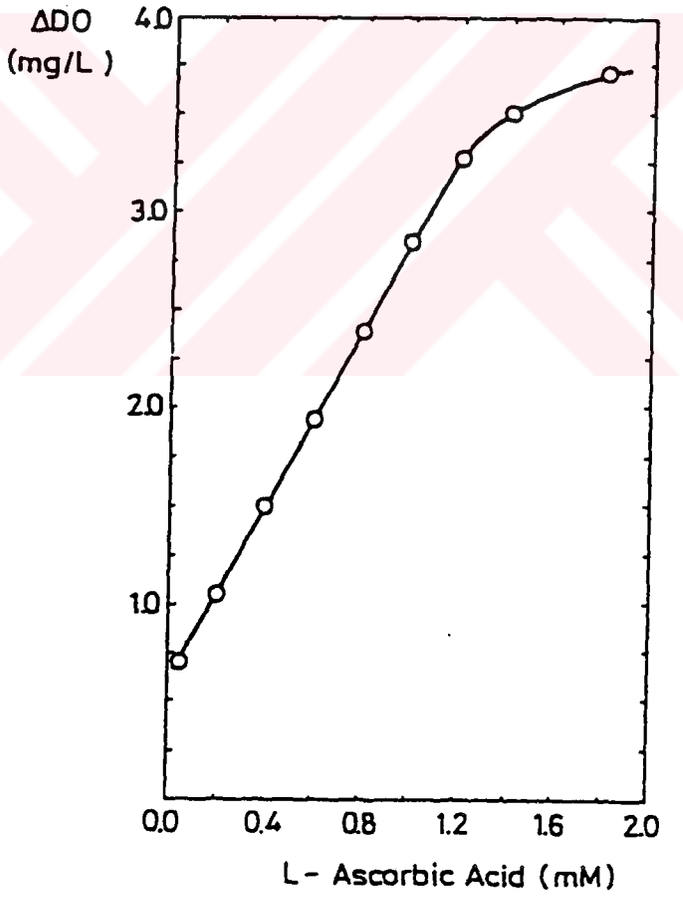

Figure 3.12 Determination of the detection limits for L-ascorbic acid. (Phosphate buffer; pH:7.5, 50 mM, T:35°C. The amount of ascorbate oxidase, gelatine and the percentage of glutaraldehyde were kept constant as 5.9 mgenzyme/cm^2 (24.8 U/cm^2), 5.9 mggelatine/cm^2 and 2.5%, respectively).

A linear standard curve was obtained in the L-ascorbic acid concentration range between $5x10^{-5}$ M and $1.2x10^{-3}$ M. At higher concentrations than $1.2x10^{-3}$, the standard curve was showed a deviation from linearity. In the catalysed by ascorbate oxidase oxygen which was a co-substrate, behaved as the limiting reagent, therefore, a deviation from linearity was observed for high ascorbic acid concentrations.

Figure 3.12 shows the base standard curve obtained by applying the optimum conditions for the enzyme electrode based on ascorbate oxidase immobilized in gelatine.

So far, approximately 200 measurements for carried out by using the enzyme electrode prepared. Among these measurements, some were performed at extreme conditions at which optimization experiments were carried out. However, a standard curve for the sensitive ascorbic acid determination could obtained as shown in figure 3.13.

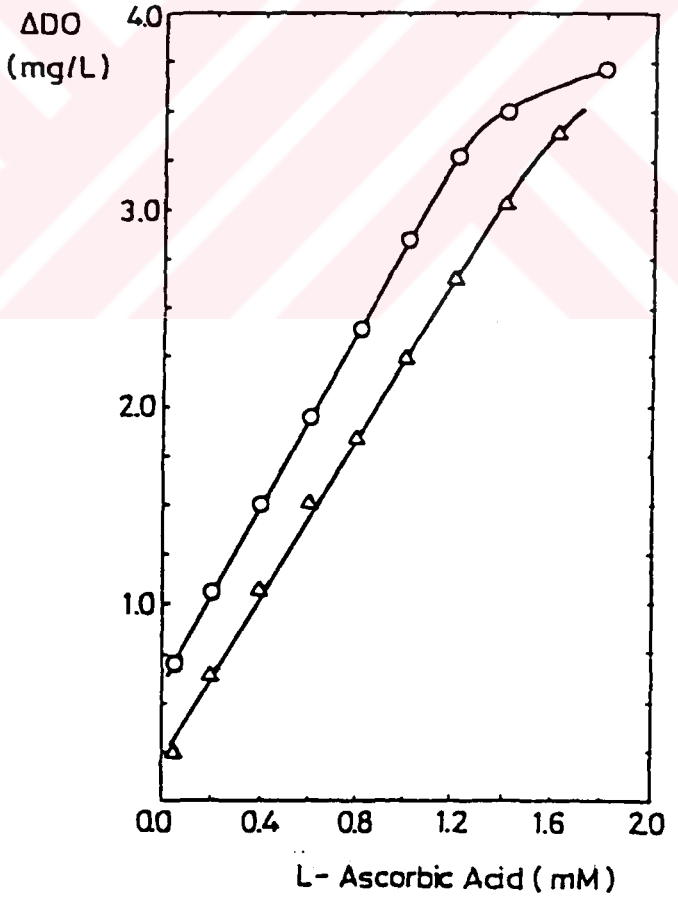

Figure 3.13 The effect of the way of handling the enzyme electrode on electrode response. (Phosphate buffer; pH:7.5, 50 mM, T:35°C, -O-O-: The new prepared ascorbate oxidase electrode, -Δ-Δ-: After approximately 200 measurements. The amount of ascorbate oxidase, gelatine and the percentage of glutaraldehyde were kept constant as 5.9 mgenzyme/cm^2, (24.8 U/cm^2), 5.9 mggelatine/cm^2 and 2.5%,respectively).

3.5.2 Reproducibility

In this part, 11 measurements were carried out for 11 identical ascorbic acid samples (0.4 mM) and L-ascorbic acid concentrations corresponding to the electrode responses obtained, were calculated. In addition, the standard deviation and variation coefficient (%) were calculated.

Table 3.1 shows the reproducibilty of the analysis results for L-ascorbic acid samples (0.4 mM).

Table 3.1 Reproducibility of the results for L-ascorbic acid samples(0.4 mM).

Standard No	Concentration of L-Ascorbic acid (mM)
1	0.408
2	0.400
3	0.400
4	0.400
5	0.400
6	0.400
7	0.400
8	0.400
9	0.400
10	0.400
11	0.396
Average	0.399
Standard deviation	0.001
Variation coefficient(%)	0.251

3.5.3 Substrate specificity

The substrate specificity of the enzyme electrode was given in table 3.2.

Table 3.2 The substrate specificity of ascorbate oxidase electrode*.

Substrate	(%) Relative Activity	
	**	***
L-Ascorbic acid	100.0	100.0
Oxalic acid	0.0	0.0
L-Aspartic acid	0.0	0.0
L-Glutamic acid	0.0	0.0
Sucsinic acid	0.0	0.0
Citric acid	0.0	0.0
Glycolic acid	0.0	0.0
Glucose	0.0	0.0
Fructose	0.0	0.0
Hydroquinone	2.2	7.5
8-Hydroxyquinoline	0.0	0.0
Catechol	0.0	1.9

* Working conditions : (Phosphate buffer; pH:7.5, 50 mM, T:35^{o}C. The amount of ascorbate oxidase, gelatine and the percentage of glutaraldehyde were kept constant as 5.9 mgenzyme/cm^2 (24.8 U/cm^2), 5.9 mggelatine/cm^2 and 2.5 %, respectively).

** The substrate concentrations were 0.4 mM

*** The substrate concentrations were 1.0 mM

In this part only hydroquinone (0.4 and 1.0 mM) and catechol (1.0mM) responded to the enzyme electrode but these responses were not important for the substrate specificity of the enzyme electrode. Hydroxyl groups or structures of these substances might effect the active side of the enzyme.

3.5.4 Interference effects of some substances on L-ascorbic acid determination

The effect of some substances usually existed in vegetable materials on the electrode response arose from the ascorbic acid concentration were determined.

The data obtained were given in table 3.3.

Table 3.3 Interference effects of the some substances on L-ascorbic acid determination.*

Interferent	Concentration	% Response
Citric acid	1.30 mM	103.0
Fructose	250 mM	103.0
Glucose	1.10 mM	98.8
Hydroquinone	0.40 mM	108.0
Oxalic acid	0.55 mM	96.0
Tartaric acid	0.40 mM	102.0

* The experiments were carried out in the phosphate buffer (pH:7.5, 50 mM), T:35°C. The amount of ascorbate oxidase, gelatine and the percentage of glutaraldehyde were kept constant as 5.9 mgenzyme/cm^2 (24.8 U/cm^2), 5.9 mggelatine/cm^2 and 2.5%, respectively).

For 0.8 mM L-ascorbic acid electrode response obtained was accepted as 100% and the electrode responses of samples which contain L-ascorbic acid (0.8 mM) and other interferent substances were compared with this value.

3.5.5 Storage stability

Storage stability measurements revealed that, even after 3 months storage period, the decrease in electrode responses was determined as 58%, 21% and 15% for 0.05, 0.2 and 0.8 mM L-ascorbic acid respectively.

Figure 3.14 shows the effect of storage time on the storage stability of the enzyme electrode.

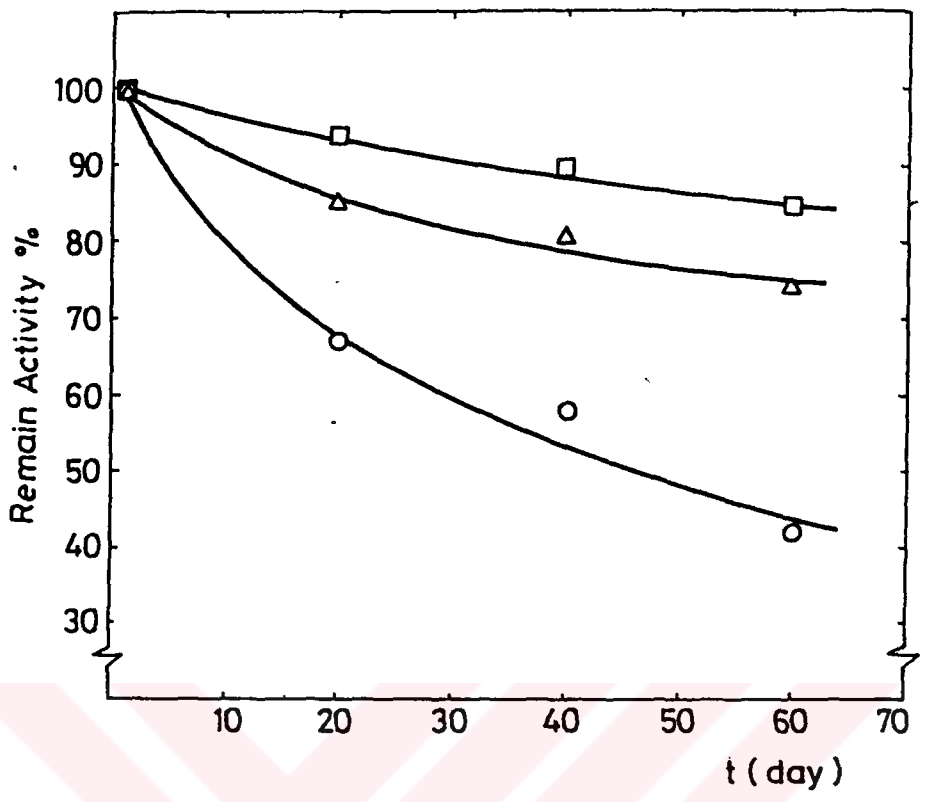

Figure 3.14 The effect of storage time on the storage stability of ascorbate oxidase enzyme electrode. (Phosphate buffer; pH:7.5, 50 mM, T:35°C, -O-O-: 0.05 mM, -Δ-Δ-: 0.2 mM, -□-□-: 0.8 mM. The amount of ascorbate oxidase, gelatine and the percentage of glutaraldehyde were kept constant as 5.9 mgenzyme/cm^2 (24.8 U/cm^2), 5.9 mggelatine/cm^2 and 2.5%, respectively).

The decreases observed for various L-ascorbic acid standards could only not be explained in terms of storage period. It was observed that the increases in concentrations would results in smaller decreases in electrode responses. At lower concentrations, the diffuision of L-ascorbic acid from reaction cell to the bioactive layer was slow compared to higher concentrations. At lower L-ascorbic acid concentrations, the back diffusion ratio of the L-ascorbic acid from the bioactive layer to the reaction cell was bigger compared to higher L-ascorbic acid concentrations, therefore the concentration differences in the dissolved oxygen at interval surface was found smaller compared to lower L-ascorbic acid concentrations.

3.6 Data for The Determination of L-Ascorbic Acid in Vitamin-C Tablets and Fruit Extracts by Ascorbate Oxidase Enzyme Electrode

3.6.1 Determination of L-ascorbic acid in vitamin-C tablets

Results obtained for the determination of L-ascorbic acid in vitamin-C tablets were given in table 3.4.

Table 3.4 Determination of L-ascorbic acid in vitamin-C tablets*.

Method	Sample	L-Ascorbic Acid Content of Tablets (mg/tablet)	Found L-Ascorbic Acid Content (mg/tablet)	Recovery (%)
Ascorbate oxidase Enzyme electrode	Ca-Sandoz	1000	992.5	99.3
	Redoxan	500	497.8	99.6
DCIP	Ca-Sandoz	1000	1015	101.5
	Redoxan	500	510	102.0

* The avarage of 5 analysis

According to the table 3.4, the ascorbic acid amount determined by using the enzyme electrode 0.3%-0.6% smaller than the tablet's original content. Whereas, in case of DCIP method, the amount of L-ascorbic acid was found 1.5%-2.0% larger. As a result, it can be suggested that the obtained data by using both method very sensitive and positive. In DCIP method, higher L-ascorbic acid levels were determined due to the additives present in the vitamin-C tablets. It was understood that better results were obtained by the ascorbate oxidase electrode in comparison to DCIP method. In addtion the method is very practical, useful and not time-consuming.

3.6.2 Determination of L-ascorbic acid in some fruit extracts

The obtained results which were found with both of two methods (Ascorbate oxidase electrode and DCIP methods) have been shown in table 3.5.

Table 3.5 Determination of L-ascorbic acid in fruit juices by using the two different methods

Fruit juices	By Ascorbate oxidase electrode			By DCIP		
	The average	n=5		The average	n=5	
	(mg/100 g)	S.D	C.V(%)	(mg/100 g)	S.D	C.V(%)
Orange	39.19	0.00204	2.35	31.15	0.0153	2.42
Lemon	26.42	0.00167	2.76	25.50	0.0158	3.04

The table 3.5 shows that the results obtained for Lemon juices by using the two methods mentioned above are almost similar however there is a controversy for orange juices.

If two methods were compared with each other in terms of standard deviation and variation coefficient, it can be suggested that the ascorbate oxidase enzyme electrode method was more sensitive than DCIP method. In addition for orange and lemon juices, L-ascorbic acid determined by ascorbate oxidase enzyme electrode were found to be more accurate than DCIP method.

Finally, for L-ascorbic acid determination the interference effects arose from the contents of fruit juices were investigated. For this purpose, the standard addition was used for both methods. Table 3.6 shows the results obtained.

Table 3.6 Determination of L-ascorbic acid by ascorbate oxidase enzyme electrode and DCIP methods by using standard addition.

Sample	Method	L-Ascorbic acid content of sample (mg/100 g)	Sample+Adding L-Ascorbic Acid (mg/100 g)	Found L-Ascorbic acid (mg/100 g)	(%) Recovery
Orange	Ascorbate oxidase Enzyme electrode	39.19	46.23	47.55	102.85
	DCIP	31.15	31.63	29.48	93.20
Lemon	Ascorbate oxidase enzyme electrode	26.42	33.46	33.91	101.34
	DCIP	25.50	25.97	24.97	96.14

As a result of this work the method developed by the ascorbate oxidase enzyme electrode was found to be more advantageous in comparison to other methods known in the literature so far, it was determined that the method is sensitive, economic, practical and less time-consuming. Moreover it can easily be conditions providing that no expensive instruments were necessary. Consequently, it can be suggested that the method developed would be an original and useful procedure for L-ascorbic acid determination.

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CURRICULUM VITAE

Personel
B.Sc.Erol Akyılmaz
Ege University, Faculty of Science
Department of Biochemistry
35100 Bornova, İZMİR-TÜRKİYE
Phone : 0232-3884000/1775
Fax : 0232-3881036

Private Address
275/5 Sokak
No:3, D:6
Manavkuyu, Bornova
TÜRKİYE

Citizenship	:	Turkish Republic
Date of Birth	:	Febr. 11, 1971
Marital Status	:	Single
Place of Birth	:	Dörtyol, Hatay-TÜRKİYE

Academic Background

Degree Obtained or Academic Status	Department/Branch	University	Period
B.Sc	Chemistry	Univ. of Ege	1989-1993
M.Sc.	Chem./Biochem.	Univ. of Ege	1993-

Participation to Scientific Meetings

1. Biochemistry Summer School "Membrane Proteins"	May 14-20, 1995 Çeşme, İzmir-TÜRKİYE
2. XIII.National Biochemistry Congrees (With International Participation)	March 26-30, 1996 Antalya-TÜRKİYE