Asian Journal of Research in Biochemistry

10(4): 6-14, 2022; Article no.AJRB.90886
ISSN: 2582-0516

Purification of Alcohol Dehydrogenase Enzyme from Chicken Liver and Immobilization Onto Florisil

Havva Ersoz [a], Nuri Gulesci [b*] and Ramazan Bilgin [a]

[a] Faculty of Art and Science, Department of Chemistry (Biochemistry Division), University of Cukurova, 01330, Balcali, Adana, Turkey.
[b] Department of Chemistry and Chemical Processing Technologies, Gumushane Vocational School, Gumushane University, 29100 Gumushane, Turkey.

Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/AJRB/2022/v10i430230

Open Peer Review History:
This journal follows the Advanced Open Peer Review policy. Identity of the Reviewers, Editor(s) and additional Reviewers, peer review comments, different versions of the manuscript, comments of the editors, etc are available here: https://www.sdiarticle5.com/review-history/90886

Original Research Article

Received 12 June 2022
Accepted 17 August 2022
Published 20 August 2022

ABSTRACT

The enzyme alcohol dehydrogenase (ADH) is a dimeric enzyme in which each of its subunits has a Zn^{2+} metal-containing catalytic domain and a cofactor binding domain. This enzyme converts alcohol into an aldehyde. In this article, the activity of the enzyme was investigated by applying the immobilization process directly to the alcohol dehydrogenase enzyme purified and activated florisil from the chicken liver. For this purpose, homogenization of chicken liver was achieved and its supernatants were separated by applying the ultracentrifugation process to the resulting homogenate. Then, % ammonium precipitation, dialysis, and ion exchange chromatography processes were performed, respectively. As a result of these processes, the hepatic alcohol dehydrogenase was purified 150.3 times compared to the coarse homogenate, and the specific activity of the enzyme was determined to be 0.631 U/mg protein. The activity of the enzyme directly immobilized was found to be 0.034 U/mg protein.

Keywords: Alcohol dehydrogenase; florisil; liver; ion exchange chromatography.

**Corresponding author: E-mail: nurigulesci@gmail.com;*

1. INTRODUCTION

Enzymes are biological catalysts that lower the activation energy of the reactions and move the substrate towards the products. Much of the history of biochemistry is the history of enzyme research. The word enzyme means found in yeast [1]. Biological catalysis was first discovered and described in 1700 in studies of gastric secretions and the digestion of meat. Subsequent research was continued in the 1800s with the conversion of starch into sugar with saliva and various plant extracts [1]. In the 1850s, Louis Pasteur concluded that the fermentation of sugar by yeast into alcohol was catalyzed by "ferments." Pasteur argued that these ferments are inseparable from the structures of living yeast cells. In 1897, Eduard Buchner discovered that yeast extracts ferment sugar into alcohol, which is provided by molecules that continue to function when fermentation is removed from the cell. Frederic W. Kühne named these molecules enzymes. The isolation of new enzymes and the investigation of their properties have advanced the science of biochemistry [1].

The use of enzymes for various purposes in technical chemistry and biotechnology led scientists to investigate the possibilities of making these enzymes more economical and useful. It is known that long-term and excessive alcohol consumption causes many different diseases, especially liver diseases. Some of these are irreversible and can be fatal to human life. Especially the liver is a unique organ called the mother of the body. Any disorder in the liver affects all body functions. For this reason, many studies have been done on liver alcohol metabolism for a long time [2,3].

An active form of ADH is generally in a dimeric form in the liver [4]. In studies, the ADH enzyme is expressed by five isozyme classes (ADH, ADH_2, ADH_3, ADH_4, ADH_5) and seven genes (α, β, γ, δ, σ π, χ). Polymorphism has been reported in two of the five classes of encoded ADH subunits (ADH_2, and ADH_3) [5].

Alcohol metabolism contains mainly two pathways, oxidative and non-oxidative pathways. The oxidative pathway takes place in the liver and another in extrahepatic tissues. The oxidative reaction is a catalyzed major pathway by cytosolic alcohol dehydrogenase enzyme. Alcohol dehydrogenase (ADH, EC 1.1.1.1) is a crucial enzyme in this pathway. The zinc-containing enzyme is responsible for the reversible oxidation of alcohol to aldehyde, a highly toxic molecule, by the reduction of NAD^+ to NADH [6].

$$CH_3CH_2OH+NAD^+ = CH_3CHO+NADH+H^+$$

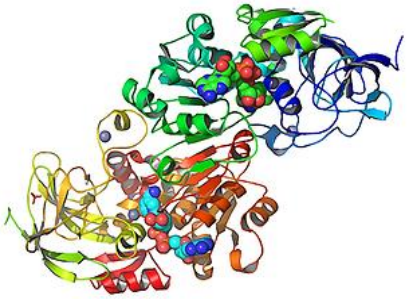

Image 1. Alcohol dehydrogenase (ADH) (E.C 1.1.1.1) [7]

Alcohol (ethanol) is one of the most frequently used and abused chemicals. Many studies have shown that chronic alcohol consumption has toxic effects on hepatic and cerebral functions [8]. In addition, various damages of excessive alcohol consumption on the cardiovascular system have been determined by many researchers [8].

The use of immobilized enzymes provides advantages over the use of free enzymes due to the easy control of reaction conditions, easy removal from the reaction medium, and reusability. Inorganic supports are frequently preferred in immobilization studies due to their high thermal and mechanical stability and non-toxicity [9].

Florisil (magnesium silicate) contains 15% MgO and 85% SiO_2, its pore size is 6-8 nm, and its specific surface area is 170-300 m^2/g. In addition, it is a support with high thermal stability, hydrophilic character, and resistance to microbial attacks and organic solvents [10].

2. MATERIALS AND METHODS

2.1 Chemicals

Florisil, 3-APTES, glutaraldehyde, DEAE-Celulose Column, NADH, and chemicals for protein measurement were obtained from Sigma-Aldrich. All other chemicals were of analytical purity and were obtained from Merck.

2.2 Homogenization and Ultra Centrifugation of Samples

The methods suggested by Lindström et al., [11] were used in the preparation of the homogenate, and by Kessler et al., [12] in the other steps. Fresh chicken liver cut daily was frozen at -19°C overnight. Then, 50 g of the cut chicken liver was taken and kept in the homogenization buffer for 10 minutes.

A solution consisting of 110 mL of 0.1 M phosphate buffer (pH 7.5, 4°C) was used for homogenization. The liver was homogenized with a blender for 4-5 minutes. After homogenate, the samples are filtered and the filtrate was centrifuged at 12.000 rpm for 60 minutes, and the supernatant was separated and combined by centrifugation.

2.3 Precipitation with Ammonium Sulfate

Enzyme extracts were taken into the beaker and cooled to 4°C, the amount of ammonium sulfate required for proper % saturation was calculated and weighed. Ammonium sulfate was added to the enzyme solution. Then the stirring was continued at 4°C for 30 minutes.

The solution was centrifuged at 12,000 rpm for 60 minutes. The precipitate is removed. Then, ammonium sulfate was weighed again for a higher saturation to be added to the supernatant and the precipitation process was repeated as indicated above.

2.4 Preparation of DEAE-Cellulose Chromatograph

25 g of DEAE-cellulose was weighed and stabilized in 400 mL of 1.5 mM pH 6.8 phosphate buffer. The tromb was filtered and washed with the same buffer solution again. DEAE-cellulose stabilized again in 400 mL of 1.5 mM pH 6.8 phosphate buffer and collected supernatants (98 mL hemolysate) were added. The supernatants were mixed in a magnetic mixer at room temperature for 1 hour and left overnight at 4°C. During this time, the enzyme sample was adsorbed on DEAE-cellulose.

The DEAE-cellulose-enzyme precipitate was filled into a column of 2.6x10.5 cm. As a result of this process, 56.2 mL of precipitate was obtained. Then, by washing the colon with a starting buffer solution, most of the hemoglobin was removed.

Alcohol dehydrogenase was eluted from DEAE-cellulose using a 20 mM pH 6.8 phosphate buffer. Fractions of 10 mL were collected with a flow rate of 3.1 mL/min. The fractions with high activity were decoupled together [13].

2.5 Application of Proteins to the DEAE-Cellulose Column

8 mL of enzyme extract containing alcohol dehydrogenase (ADH) was applied to the colon. The samples were taken from the colon at pH 6.8 with a 0.01 M phosphate buffer, and the protein samples were collected in tubes with a volume of 3 mL.

Protein concentration and alcohol dehydrogenase enzyme activity were measured in the collected samples.

The concentration of the NADH solution was prepared to be 0.25 mM. 0.5 mL of 0.4 mM acetaldehyde and 1 mL of NADH solution were added to 1.5 mL of 0.1 M phosphate buffer (pH 7) and prepared blindly.

By adding 100 µL of enzyme solution at 25ºC to this solution, the reduced absorbance at 340 nm was recorded for 3-4 minutes. The activity of the enzyme was calculated in IU/mL.

2.6 Protein Determination

The protein quantity was calculated according to the Lowry procedure. The bovine serum albumin has used as the standard for this experiment. This study was performed at 750 nm in a spectrophotometer [14].

2.7 Preparation of Florisil Support

In the immobilization of alcohol dehydrogenase, the florisil support surface must first be activated [10]. Therefore, the alkylamine derivative of the support was formed using 3-aminopropyltriethoxysilane (3-APTES). The method reported in the literature by Weetall (1976) was used to prepare the alkylamine derivative (silane). The support material was washed with 5% (v/v) nitric acid at 80-90 ºC for 5 minutes. The resulting filler was washed again with water and dried at 120ºC.

The dried support was added to a 4% solution of 3-aminopropyltriethoxysilane in acetone by a volume of 1.0 g and left for 24 hours at 45ºC. It was washed with pure water and dried for 1 night

in an oven at 115ºC. 1 g of 25 mL of 2.5% (v/v) glutaraldehyde solution in a 50 mM pH 7.0 phosphate buffer was added to the silanized support. After waiting for 120 minutes, unreacted glutaraldehyde (GA) was washed off with pure water and dried for 1 hour at 60ºC. After this step, the enzyme was tried to be immobilized directly onto the support [15].

2.8 Covalent Immobilization of Alcohol Dehydrogenase to Florisil

The method of covalent immobilization of alcohol dehydrogenase to florisil over glutaraldehyde, which has been reported in the literature for covalent immobilization of alcohol dehydrogenase over aluminum oxide, is based on [15].

1.0 g of support was used for the immobilization of alcohol dehydrogenase with florisil. The concentration was prepared to be pH 7.0 with a 50 mM phosphate buffer at 1.0 mg/mL. 4.0 mL of the alcohol dehydrogenase solution was added and mixed for 2 hours at room temperature. At the end of immobilization, the immobilized alcohol dehydrogenase sample was thoroughly washed with a buffer solution and the free alcohol dehydrogenase was removed from the medium.

It was determined that the free alcohol dehydrogenase was completely removed by following the absorbance of the filtrate at 280 nm. The determination of protein content in filtrates by Lowry et al., [14] were conducted according to the proposed method. The amount of alcohol dehydrogenase introduced into the medium at the beginning of immobilization was subtracted from the amount of protein in the filtrate and the amount of enzymes bound per 1.0 g of support was calculated. Then, the activity determination for the immobilized enzyme was performed by Vallee et al., [16] were conducted according to the proposed method.

3. RESULTS

To determine the amount of enzyme in the determination of enzyme activity, protein determination was performed. To evaluate the results, a standard protein curve was drawn using bovine serum albumin (BSA) (Fig. 1).

The homogenized liver samples were centrifuged at 12.000 x g for 60 minutes. As a result of centrifugation, the precipitate was decanted and the supernatants were combined. The findings obtained are given in (Fig. 2).

The centrifugation of liver homogenates and the time-dependent variation of absorbance values indicating the enzyme activity of the resulting supernatants are given in (Fig. 3).

The supernatant obtained as a result of ultracentrifuge in precipitation with ammonium sulfate was taken into a beaker and cooled to 4ºC. Ammonium sulfate, necessary for the desired saturation of $(NH_4)_2SO_4$%, was slowly added to the enzyme extract by stirring.

The solution was centrifuged at 12,000 x g for 60 minutes and the precipitate was removed. Then ammonium sulfate was weighed again for a higher $(NH_4)_2SO_4$% saturation and the precipitation process was repeated as indicated above. In the precipitation process, 20%, 30%, 40%, 50%. The concentration of % ammonium sulfate as a result of precipitation with ammonium sulfate. The amount of protein remaining in the solution for the liver is given in (Fig. 4).

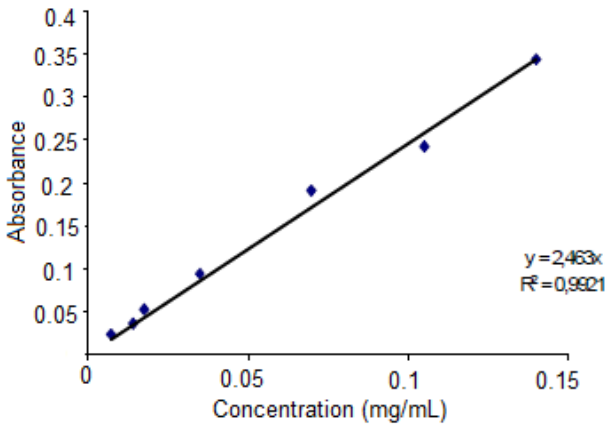

Fig. 1. Standard protein curve

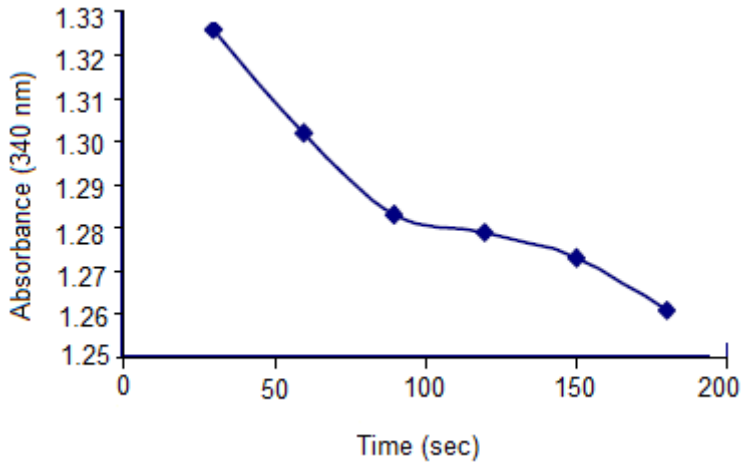

Fig. 2. Enzyme activity in liver homogenates

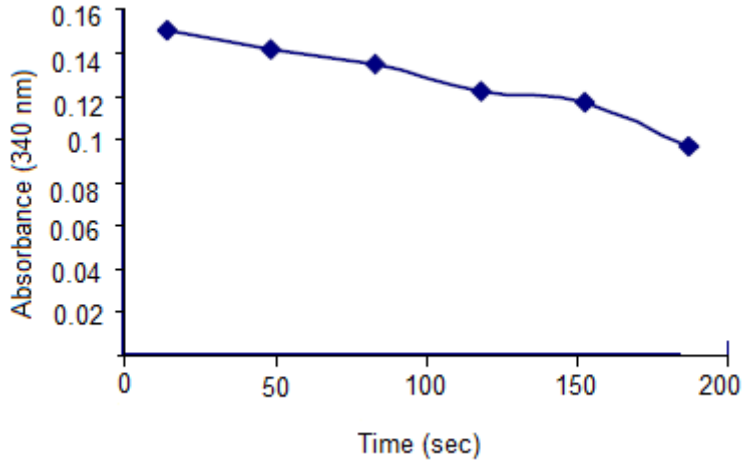

Fig. 3. Enzyme activity of supernatants

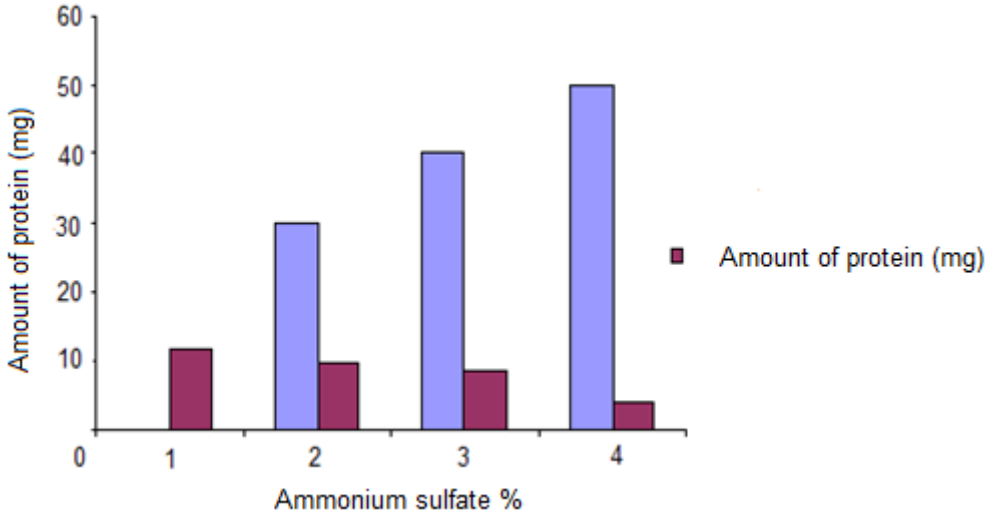

Fig. 4. Amount of protein

The amount of protein and specific activity values of the solution depending on the concentration of % ammonium sulfate as a result of precipitation with ammonium sulfate are shown in Figs. 5 and 6.

The absorbance values read at 280 and 340 nm to determine the protein content for the liver as a result of precipitation with $(NH_4)_2SO_4$ are given in Figs. 7 and 8, respectively.

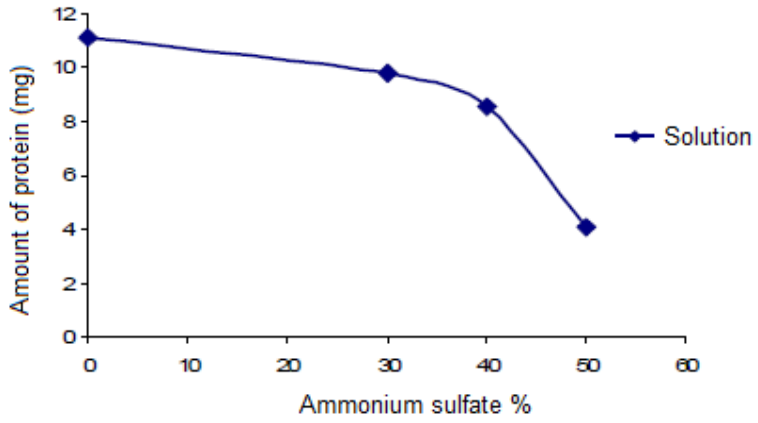

Fig. 5. The amount of protein bound to % ammonium sulfate as a result of precipitation with ammonium sulfate for the liver sample

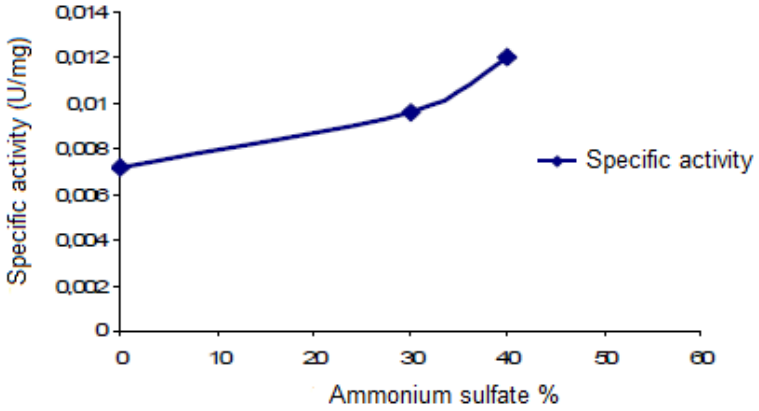

Fig. 6. Specific activity values due to the concentration of % ammonium sulfate as a result of precipitation with ammonium sulfate for a liver sample

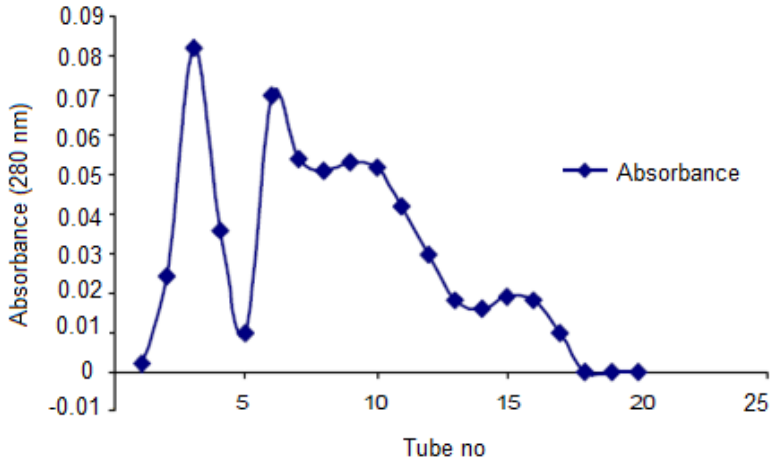

Fig. 7. Results obtained at 280 nm as a result of precipitation with $(NH_4)_2SO_4$

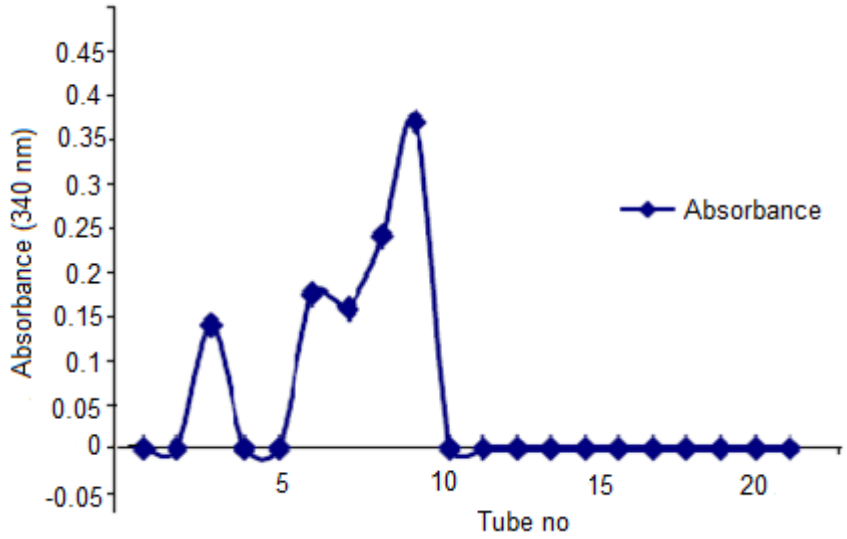

Fig. 8. Results obtained at 340 nm as a result of precipitation with $(NH_4)_2SO_4$

3.1 Direct Immobilization of Alcohol Dehydrogenase to Florisil

In the direct immobilization of alcohol dehydrogenase to florisil, florisil was first washed with HNO_3 and then silenced with 3-APTES. The silane was treated with florisil glutaraldehyde. After the glutaraldehyde treatment, the support was washed until no glutaraldehyde was present in the washing solution. After the glutaraldehyde is completely removed, the support is dried.

Immobilization was performed by using a phosphate buffer with of 50 mM, pH 7.0 in direct immobilization of alcohol dehydrogenase to florisil.

In the direct immobilization of alcohol dehydrogenase florisil (pH 7.0), immobilization was performed using 50 mM phosphate buffer. Immobilization was performed at 25ºC for 2 hours using 4.0 mL of alcohol dehydrogenase solution prepared at a concentration of 1.0 mg/mL per 1.0 g support. The activity of the immobilized alcohol dehydrogenase sample was measured in the presence of 0.025 mM NADH in 0.1 M pH 7.0 phosphate buffer at 25ºC.

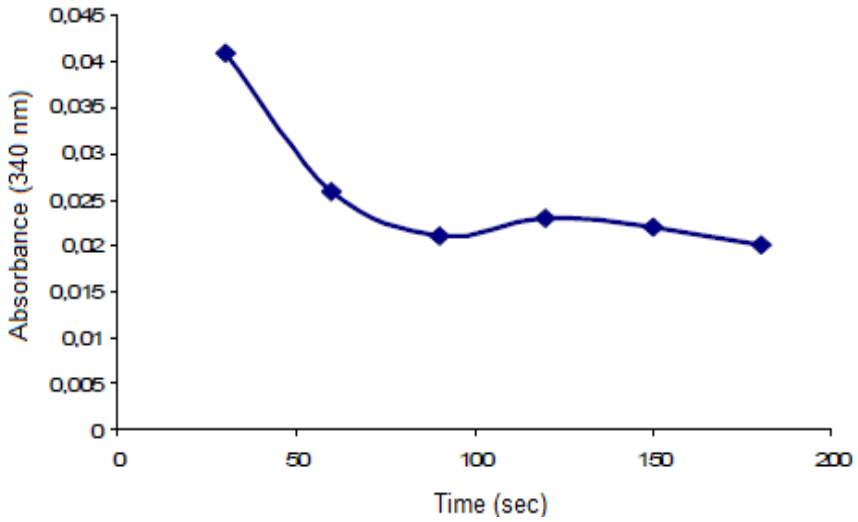

Fig. 9. Time-dependent change of absorbance values showing the activity of alcohol dehydrogenase directly immobilized with florisil

Table 1. The amount of support and calculated activity values for the direct immobilization of alcohol dehydrogenase to activated florisil

Amount of support (mg)	Activity values (U/mL)
25	$3.22.\ 10^{-3}$
50	$1.45.\ 10^{-2}$
100	$3.69.\ 10^{-2}$

4. DISCUSSION AND CONCLUSION

In this study, the enzyme alcohol dehydrogenase, Kessler et al. [17] and Von Bahr et al. [18] by proposed on the method based, it was tried directly from chicken liver on partially purified and activated florisil.

The purification steps are respectively; The liver was purified using homogenization, centrifugation, 40% $(NH_4)_2SO_4$ precipitation, and DEAE-Cellulose chromatography, and activity determinations were made. The activity was determined in the immobilized enzyme directly on the activated florisil. The purpose of choosing chicken liver for the study is that alcohol dehydrogenase, which is found in many living things, has been purified from chicken in a limited number before and that it has not been directly immobilized on florisil, there are limited studies in the literature and the usage areas of alcohol dehydrogenase are limited.

In the alcohol dehydrogenase purification study, the specific activity value was found as 0.0042 U/mg protein as a result of the first homogenization of the sample. In the study of Von Bahr et al. [18] with the liver, the specific activity value was reported as 0.010 U/mg protein after the first homogenization of the sample. In the study of Kessler et al. [17] with the liver, the specific activity value was found as 0.058 U/mg protein as a result of the first homogenization of the sample. The first homogenate was centrifuged at 12,000 rpm for 60 minutes, and the specific activity value was found as 0.0073 U/mg protein in the alcohol dehydrogenase enzyme activity determination made on the supernatant obtained, and the sample was purified 1.74 times. As a result of precipitation with ammonium sulfate applied to the supernatant, the specific activity value was found as 0.012 U/mg protein in the alcohol dehydrogenase enzyme activity determination made in the solution, and the sample was purified 2.9 times. In the study of Kessler et al. [17] with the liver, they found the specific activity value as 0.25 U/mg protein in the alcohol dehydrogenase enzyme activity determination made in the supernatant.

In the precipitation process with different ammonium sulfate for the liver, ammonium sulfate was used in concentrations of 20, 30, 40, and 50%. As a result, the solution concentration with the highest specific activity was found to be 40% ammonium sulfate, and samples with this concentration of 40% were in other steps used.

In the study of Kessler et al. [17] with the liver, they found the specific activity value as 1.2 U/mg protein in the alcohol dehydrogenase enzyme activity determination made in solution as a result of precipitation with ammonium sulfate applied to the supernatant.

Kessler et al. [17] determined that 50% ammonium sulfate was the solution concentration that had the most specific activity in the precipitation process with different ammonium sulfate for the liver. In the study with alcohol dehydrogenase, 40% solution showing the highest specific activity as a result of precipitation with ammonium sulfate applied to the supernatant was applied to the DEAE-Cellulose anion exchange column. In the alcohol dehydrogenase enzyme activity determination made in the eluates taken from the column, the specific activity value was found to be 0.631 U/mg protein, and the sample was purified 150.3 times. Alcohol dehydrogenase was directly immobilized on three different amounts of activated florisil, 25, 50, and 100 mg, and it was determined that the highest activity value was obtained as a result of direct immobilization on 100 mg activated florisil.

DISCLAIMER

The products used for this research are commonly and predominantly used products in our area of research and country. There is no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but the advancement of knowledge.

ETHICAL APPROVAL

This article does not contain any studies done with human or animal participants performed by any of the authors.

ACKNOWLEDGEMENTS

This work was supported by Research Grant from FEF2009YL39 Cukurova University.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. David LN, Michael MC. Lehninger Principles of Biochemistry. Third Edition. 2000;243-244.
2. Li Y, Ding WX. Adipose tissue autophagy and homeostasis in alcohol-induced liver injury. Liver Research. 2017;1-9.
3. Stickel F, Moreno C, Hampe J, Morgan MY. The genetics of alcohol dependence and alcohol-related liver disease. Journal of Hepatology. 2017;66:195–211.
4. Sunderland JR, Tao X, Butrick EE, Keilich LC, Villa CE, Miecznikowski JR, Jain SS. Investigation of liver alcohol dehydrogenase catalysis using an NADH biomimetic and comparison with a synthetic zinc model complex. Polyhedron. 2016;114:145–151.
5. Sayın H, Vural N. Investıgatıon of Some Factors Affectıng Lıver Alcohol Dehydrogenase Actıvıty. J. Fac. Pharm. 2000;29(2);19-32.
6. Demir Y, Şengül B, Ergun B, Beydemir S. Alcohol Dehydrogenase from Sheep Liver: Purification, Characterization and Impacts of Some Antibiotics. Journal of the Institute of Science and Technology. 2017; 7(3):151-160.
7. Hurley TD, Bosron WF, Stone CL, Amzel LM. Structures of three human ß alcohol dehydrogenase variants. Journal of Molecular Biology. 1994;239:415-429.
8. Preedy VR, Richardson PJ. Ethanol induced cardiovascular disease. British Medical Bulletin. 1994;50(1):152-163.
9. Cao L, Schmid RD. Carrier-bound Immobilized Enzymes: Principles, Application and Design. Wiley-VCH Verlag GmbH & Co. KGaA; 2006.
10. Tukel SS, Alptekin O. Immobilization and kinetics of catalase onto magnesium silicate. Process Biochemistry. 2004; 39(12):2149-2155.
11. Lindström JM, Lambert EH. Content of acetylcholine receptor and antibodies bound to receptor in myasthenia gravis, experimental autoimmune myasthenia gravis, and Eaton Lambert syndrome. Neurology. 1978;28(2):130-8.
12. Kessler RJ, Ferrell WJ. The Purification and Properties of an Alcohol Dehydrogenase from Mouse Liver. Int. J. Bıochem. 1974;5:365-374.
13. Asano MM, Ito K, Ikeda H, Sekiguchi S. Purification of Copper- Zinc- Superoxide Dismutase and Catalase from Human Erythrocytes by Copper-Chelate Affinity Chromatography. Journal of Chromatography A. 1986;370:501-507.
14. Lowry OH, Rosebrough NJ, Farr AL, Rondall RJ. Protein Measurement with the Folin Phenol Reagent. J. Biol. Chem. 1951;193:261-275.
15. Costa SA, Tzanov T, Paar A, Gudelj M, Gübitz GM, Paulo AC. Immobilization of Bacillus SF on Alumina for the Treatment of Textile Bleaching Effluents. Enzyme and Microbial Technology. 2001;28:815-819.
16. Vallee BL, Hoch FL. Zinc, a component of yeast alcohol dehydrogenase. National Academiy of Sciences. 1955;41(6):327-338.
17. Kessler RJ, Ferrell WJ. The Purıfıcatıon and Properties Of An Alcohol Dehydrogenase from Mouse Lıver. Pergamon Press. Printed in Great Britain. Int. J. Bıochem. 1974;5:365-374.
18. Von Bahr-Lindström H, Anderson L, Mosbach K, Jörnvall H. FEBS Lett. 1978:89;293-297.

Peer-review history:
The peer review history for this paper can be accessed here:
https://www.sdiarticle5.com/review-history/90886