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Purification, Characterization and Some Kinetic Properties of Fructose 1,6 Bisphosphate Aldolase from Human Placenta

İnsan Plasentasından Fruktoz 1,6-Bisfosfat Aldolaz'ın Saflaştırılması, Tanımlanması ve Bazı Kinetik Özellikleri

Neşe Hayat AKSOY, PhD

Department of Biochemistry, Faculty of Medicine, Hacettepe University, Turkey

Pakize DOĞAN, Prof. Dr.

Department of Biochemistry, Faculty of Medicine, Hacettepe University, Turkey

Corresponding Author

Pakize Doğan, Prof. Dr.

Department of Biochemistry, Faculty of Medicine, Hacettepe University
Ankara/Turkey
E-mail: pakize@hacettepe.edu.tr

ABSTRACT

Fructose–1,6–bisphosphate aldolase (E.C. 4.1.2.13) is a major glycolytic enzyme found in most cells. Fructose-1,6-bisphosphate aldolase reversibly catalyses the cleavage of fructose 1,6-bisphosphate into the triose phosphates: D-glyceraldehyde phosphate and dihydroxyacetone phosphate. In this study, we wanted to examine the presence of aldolase in healthy human placenta and then to purify the enzyme.

We also determined the optimum conditions (time, pH, and temperature) of enzyme assay measurements. With this procedure, we determined the specific activity of placental aldolase as 831.90 mU/mg protein and aldolase was purified about 40.5 fold from healthy human placenta. It was demonstrated that the molecular weight of human placental aldolase was 160 kD.

In this study, substrate kinetics were also examined. Enzymatic assays were performed and substrate kinetic properties were detected and Vm value of healthy placental FBPA was determined as 1769.513 ± 200.322, and Km as 20.003 ± 4.497 mM.

Keywords: *Fructose 1,6 bisphosphate aldolase, Purification, Placenta, Kinetics*

ÖZET

Fruktoz–1,6–bisfosfat aldolaz (E.C. 4.1.2.13) pek çok hücrede bulunan major bir glikolitik enzimdir. Fruktoz–1,6–bisfosfat aldolaz geri dönüşümlü olarak fruktoz–1,6–bisfosfat'ın iki trioz fosfata; D-gliseraldehit 3-fosfat ve dihidroksiaseton fosfat'a bölünmesini katalizler.

Bu çalışmada amacımız sağlıklı insan plasentasında aldolaz enziminin varlığını incelemek, ve enzimi saflaştırmaktır. Bu çalışmaya ek olarak enzim aktivitesi tayini için kullanılan yöntemin optimum şartlarını (zaman, pH, ve sıcaklık) da tayin etmektir. Bu işlemde plasental aldolaz'ın spesifik aktivitesi 831.90 mU/mg protein olarak bulunmuş ve enzim sağlıklı insan plasentasından 40.5 kere saflaştırılmıştır. Moleküler ağırlığı 160 kD olarak tespit edilmiştir. Çalışmada sübstrat kinetiği de incelenmiştir. Enzimatik assay ve sübstrat kinetiği özellikleri incelendiğinde Vm değerinin 1769.513 ± 200.322 mM ve Km değerinin ise 20.003 ± 4.497 mM olduğu bulunmuştur.

Anahtar Kelimeler: *Fruktoz 1,6 bisfosfat aldolaz, saflaştırma , plasenta, kinetikler*

INTRODUCTION

Fructose-1,6-bisphosphate aldolase (D Fru-1,6-bisphosphate D glyceraldehyde-3-P-lyase; FBPA; E.C. 4.1.2.13) catalyses the reversible cleavage of fructose-1,6-bisphosphate (FBP) to dihydroxyacetone phosphate (DHAP) and glyceraldehyde-3-phosphate (GAP) in the glycolytic pathway of prokaryotic and eukaryotic organisms. FBPA is also an essential enzyme in the reversible gluconeogenesis and the fructose metabolic pathways (1–9).

Three major tissue-specific types of aldolase isozymes are known in higher organisms commonly referred to as: Type A, excreted from the classical muscle and red blood cells enzyme, Type B, from liver, kidney and small intestine, Type C, from neuronal tissues, brain and smooth muscle (1–6, 8, 10, 14).

Aldolases are classifed as Class I and Class II aldolases depending on their requirement of divalent ions in catalysis. For their catalytic activity, Class I Aldolases are not depend on cations and found in higher animals and plants. Class I Aldolases form a "Schiff Base" with the substrate and are homotetramers (40.000 Da) (1-3). Class II aldolases are homodimers (80 kDa), found in bacteria and fungi and require a metal cofactor such as Zn+2 for catalysis (1–3, 7–14).

Purification and definition the characteristics and properties of human placental FBPA have not been reported previously. In our study, we wanted to examine the presence of aldolase in healthy human placenta and then to purify the enzyme. We also determined the optimum conditions (time, pH, temperature) of enzyme assay method. Consequently, advanced kinetic researchs had been optimised.

MATERIALS AND METHODS

Materials: All reagents used were of analytical grade and were obtained from Sigma Co. (St Louis, MO, USA).

Purification Procedures

Step 1. Preparation, extraction and homogenization of tissues

Human placental aldolases were purified according to Penhoet method (15). All purification procedures were carried out at +4ºC.

The fresh placenta is obtained from Hacettepe University, Gynaecology and Obstetrics Department. Tissues were washed, and perfused with cold buffer containing; 50mM Tris-HCl, 5 mM EDTA and 4 mM mercaptoethanol and isotonic

NaCl (0,95% ml), pH=7.5 at 4ºC. They were weighed, minced into small pieces and suspended in 3 volumes (v/w) of the buffer at +4ºC. Then the sample was homogenized for about 10 strokes by using a Teflon homogenizer in an ice bath and centrifuged for 1 hour at 14.000 × g, and +4ºC. The precipitate was discarded and the clear supernatant was removed for ammonium sulphate fractionation.

Step 2. Ammonium sulphate [$(NH4)_2SO_4$] fractionation

In order to obtain 45% ammonium sulphate [$(NH4)_2SO_4$] concentration, 26.20 g of solid [$(NH4)_2SO_4$] was added to 100 ml of supernatant and waited for 1 hour period then centrifuged at 14 000xg for 1 hr, at +4ºC, supernatant was obtained and precipitate was discarded. At the second step of [$(NH4)_2SO_4$] fractionation (65%); for each 100 ml supernatant fluid, 12.5 g [$(NH4)_2SO_4$] was added and the procedure was repeated. For, this time, supernatant was discarded, the precipitate was collected, and dissolved in 10 mM Tris-HCl-EDTA buffer containing 1 mM EDTA, pH= 7.5 and any insoluble material is discarded by centrifugation.

Protein (18) and activity (16) determinations of the precipitates and supernatants were performed at all along the line of extraction and fractionation.

Chromotographic Procedures

Step 3. Sephadex G–25 gel filtration for desaltation

For the desaltation, the dissolved precipitate solution obtained after 65% ammonium sulfate [$(NH4)_2SO_4$] application was passed through a column of Sephadex G-25. The column was equilibrated with running buffer. The sample volume was 20 ml and flow rate range was 2 ml/min. At the end of the application all samples have been desalted (Data not shown).

Step 4. Substrate elution from phosphocellulose column

The desalted fraction was applied to a phosphocellulose column for purification of aldolase. The fractions were absorbed at 0.5 ml/min onto a column of phosphocellulose pre-equilibrated with buffer. The column was washed with the buffer until the A280 decreased to baseline, (narrow red band visible on the column and OD of the efluent at A280 nm drop below 0.1) and then the substrate elution was carried out with stock buffer containing 2.5 mM Fru–1,6-P2. After this process, enzyme was eluted with 50 mM Tris-HCl, 5 mM EDTA pH 7.5 buffer, containing 1 mM substrate.

Protein and enzyme activity determinations were performed at all along the line of gel filtration and phosphocellulose column chromatography stages.

Polyacrylamide Gel Electrophoresis

SDS-PAGE with polyacrylamide gel was performed according to the modified Laemmli method (17). Purified enzyme was applied to SDS-PAGE electrophoresis inorder to determine the molecular weight and purity. Samples were run on a 7.5 % (w/v) acrylamide separating gel and a 4.5 % stacking gel. Proteins on gels were stained with Coomassie Brilliant blue R–250.

Enzyme Assay

The quantitative estimation of aldolase activity present in the extracts are determined by measuring the rate of cleavage of fructose 1,6 bisphosphate spectrophotometrically. A unit of aldolase activity was defined as that amount of enzyme which catalyses the cleavage of 1 μmole of substrate per minute at 37ºC under conditions of assay. The specific activity was defined as the number of activity units per milligram of protein. The ubstrate cleavage rate was determined spectrophotometrically by measuring

hydrazone formation of product with dinitrophenyl hydrazine (DNPH) (16).

Protein Determination

During the purification of FBPA procedure, protein determination was performed at 280 nm, corrected for nucleic acid impurities according to Warburg and Christian (19). The A280 method takes advantage of the absorbance of light at 280 nm by the amino acids Tyr and Trp.

Amount of the protein in the samples was determinated with Lowry Method that slightly modificated (18). The reactions result in a strong blue color, read at 750 nm. Determination was run triplicate for all samples.

Kinetic Studies

All experimental conditions were adjusted at 37ºC, and pH= 7.4 which were optimum enzyme assay conditions (16).

Non-linear regression analysis module of Systat 11 statistic program was used to make the calculation of kinetic parameters. Substrate affinity constants (Km values) were determined from the Henri–Michaelis–Menten equation using a nonlinear least squares regression computer program. Substrate (fructose 1,6 bisphosphate) concentrations were selected as 0.0, 0.9, 3.0, 5.0, 7.0, 9.0 mM. Vm (maximal reaction rate or maximal velocity at saturating substrate levels) and Km (Michaelis-Menten constant) constants were calculated from reciprocal Lineweaver-Burke plot of the initial velocity/substrate concentration data by the using 1/(V) vs 1/(S) values (20).

RESULTS

With this modified method (15) enzyme was purified about 40.5 fold from healthy human placenta. Elution graphic from phosphocellulose column of healthy human placental aldolase was projected at Figure 1.

Specific activity of placental aldolase was determined as 831.90 mU/mg protein. Summary of the purification steps are presented at Table 1.

Pure enzyme samples were applied to SDS-PAGE electrophoresis for control of purity and in order to determine the molecular weight (17). We demonstrated that the purity of enzyme samples and also verified molecular weight of human placental aldolase was 160 kD (Figure 2).

Samples were run on a 7.5% (w/v) acrylamide separating gel and a 4.5% stacking gel. The subunit molecular mass was determined as 40 kDa. Therefore it was observed that the human placental aldolase was a homotetramer (Figure 3).

pH optimization

The effect of pH on the aldolase activity was determined by using 50 mM FBP with the following buffers (50 mM) at the indicated pH; acetate buffer (pH 5.0 and 5.5), phosphate buffer (pH 6.0 and 6.5), Tris–HCl buffer (pH 7.0–8.5). Then, the standard enzyme assay described previously was used. The optimum pH determined as 7.4.The optimum pH obtained was used for determining thermal properties and other parameters.

Optimization of Temperature

To assess the optimum temperature, enzymatic assay was exercised between 10–60ºC. The optimum temperature determined as 37ºC for the sample by measuring enzyme activities under standard assay conditions (data was not shown).

Optimal incubation time determination

Incubation time of enzyme assay method were performed between 0-60 minutes, by 5 minutes increments at 37ºC. The optimum incubation time determined as 30 minutes under standard assay conditions (data was not shown).

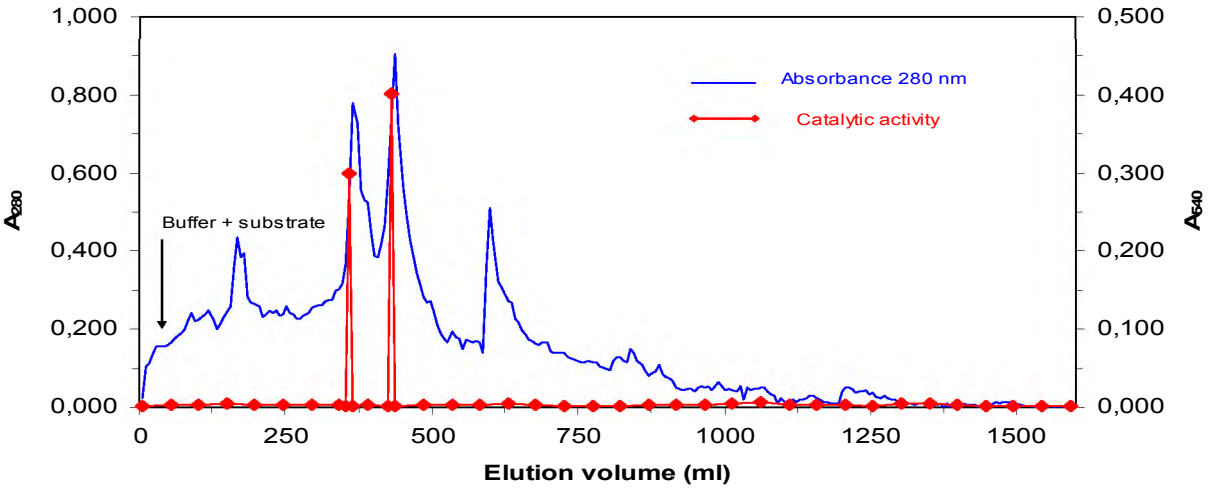

Figure 1: *Elution graphic of FBPA from phosphocellulose column (2.5 x 50 cm) which obtained from healthy human placenta. FBPA eluted as a double peaks with 50 mM Tris-HCl, 5 mM EDTA pH 7.5 buffer, containing 1 mM FBP (Fraction size is 2 ml).*

Table 1: Purification steps of Fructose 1,6 bisphosphate aldolase from healthy human placenta

Purification Steps	Total volume (ml)	Protein (mg/ml)	Total protein (mg)	Specific activity (mU/mg protein)	Total activity (U)	Purification (fold)
14.000 × g supernatant	3110	16.7	52154	20.54	1071.74	1
45% Ammonium Sulphate Fract.	3382	9.98	33752	78.35	2644.72	3.8
65% Ammonium Sulphate Fract.	98.3	51.13	5026	78.24	393.24	3.8
Sephadex G-25 Column	185	27.02	5000	83.96	419.45	4.0
Phosphocellulose Column	6	6.4	38.4	831.90	321.41	40.5

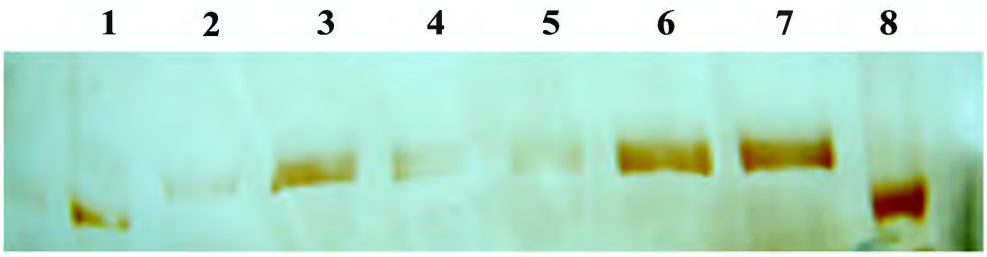

Figure 2: *SDS-PAGE of healthy and gestational diabetic human placental aldolases.*
Line 1 and 8: carbonic anhydrase, (molecular weigth is 29 kDa)
Line 2 and 5: rabbit muscle aldolase, (subunit molecular weigth is 40 kDa)
Line 3 and 4: human placental aldolase (Applied (10 µL), one sample per well),
Line 6 and 7: human placental aldolase (Applied (20 µL), one sample per well)

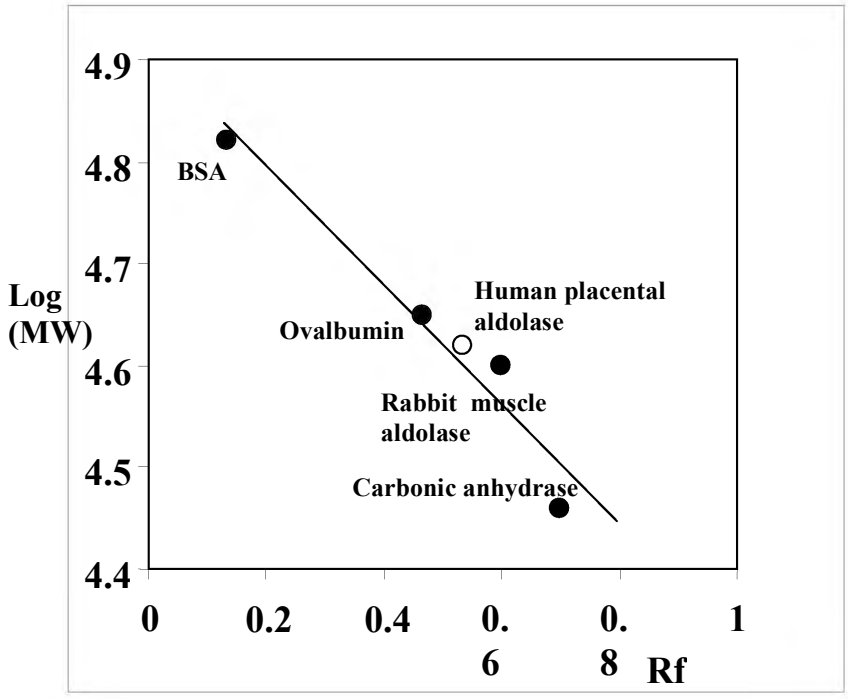

Figure 3: *Molecular weight distribution of FBPA. Standard Rf-Log MW graph of aldolase from human placenta.*

Optimization of Hydrazone Stability Time

To study the time stability of hydrazone formation, at the last stage of assay, the tubes were incubated for different periods of time at 37ºC. Enzyme activity was measured at 0, 5, 10, 30, 45, and 60th minutes, after formation of hydrazone. The optimum stability time determined between 0-5 minutes for samples under standard assay conditions.

Substrate Kinetics of Healthy Human Placental Aldolase

According to our results, for total substrate 0-60mM FBP concentrations, Vm was determined as 1769.513 ± 200.322 and Km was 20.003 ± 4.497 mM.

The Michaelis-Menten equation means the relationship between the initial velocity and substrate concentration. A plot of V (velocity) vs substrate (Michaelis-Menten plot) is hyperbolic for many enzymes (20). The Michaelis-Menten plot for total substrate concentrations which describes the relevance between the initial velocity (Vo) and substrate concentrations was shown at Figure 4.

In consideration of the Michaelis-Menten plot was appeared with two phases, so new diagrams were formed for low (0.1-3.0 mM) (Figure 5) and high (3.0-50.0mM) (Figure 6) concentrations of FBP. With reference to plots it was observed that the graphic of high concentration was closer to a hyperbolic diagram.

The Lineweaver-Burk (LB) plot that appertaining total FBP concentrations was shown at figure 7. With reference to plot, Vm was 1769.513±200.322 and Km was 20.003±4.497 mM for human placental FBPA. Lineweaver-Burk plot was appeared to be nonlinear and appropriate to negative cooperativity (20).

It was observed that, LB diagram line of total substrate concentrations was appeared to be down. New diagrams were performed for the phases that separate this point. At low concentrations of substrate, value of Km of healthy placental aldolase was determined as 3.048±1.39 mM and value of Vm was determined as 636.103 ± 196.165.

At high concentrations of substrate, Vm 1885.457 ± 292.48 and Km 23.063 ± 6.845 mM.

The Lineweaver-Burk plot for low substrate concentrations was demonstrated at figure 8, and for high concentrations was illustrated in figure 9.

In accordance with the presented graphics, high substrate concentration range (3–50 mM) has been determined as the linear substrate concentration zone. So, advanced kinetic studies will be performed at this linear zone.

Hill plot was graphed for to understand exactly the "biphasic" manner, which was related to the concentrations of substrate, of FBPA (Figure 10).

As can be seen at Hill plot diagram, there was a conspicuous biphasic character of FBPA for different substrate concentrations. Enzyme points out different kinetic features for varied substrate concentrations. That is, enzyme has different affinity for low and high concentrations of fructose 1,6 bisphosphate.

This situation was displayed previously in Lineweaver-Burk diagram (LB). According to LB plot, we obtained two different Km values. While at high substrate concentrations Km was 23.063 ± 6.845 mM, at low concentrations Km was found as 3.048 ± 1.39 mM.

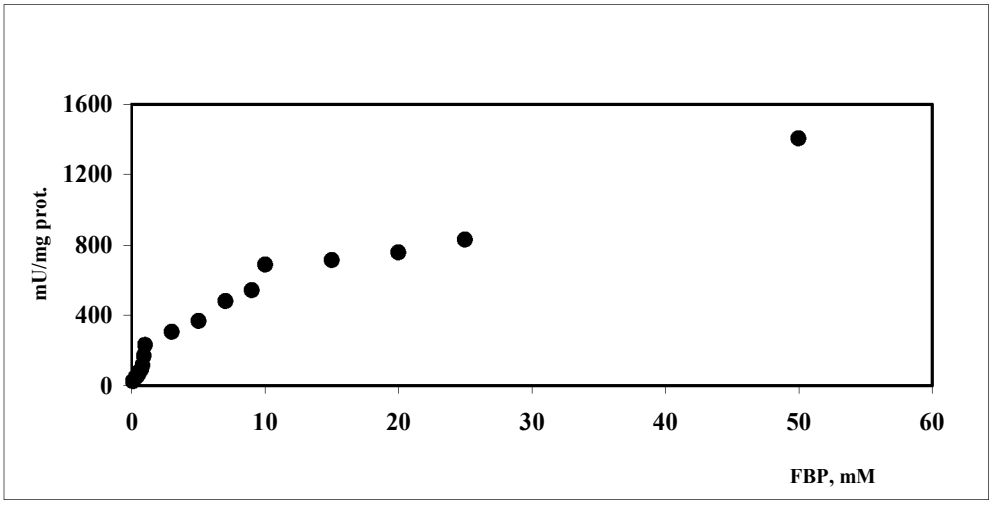

Figure 4: *Effect of total FBP concentrations on healthy human placental FBPA (Michaelis-Menten plot for FBP)*

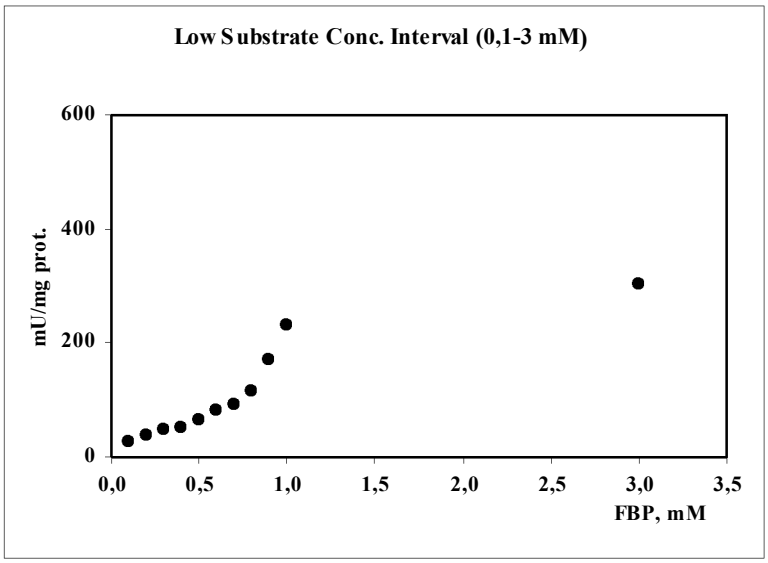

Figure 5: *Effect of low FBP concentrations (0,1–3 mM) on healthy human placental FBPA (Michaelis-Menten plot for 0,1–3 mM FBP)*

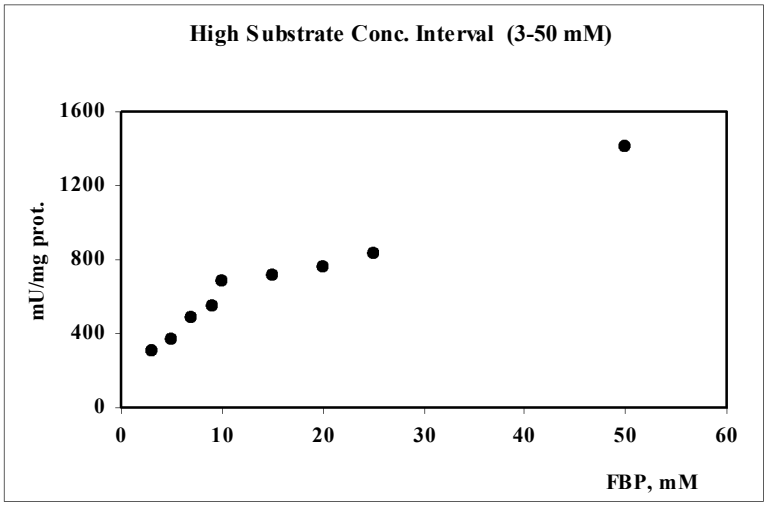

Figure 6: *Effect of high FBP concentrations (3–50 mM) on healthy human placental FBPA (Michaelis-Menten plot for 3–50 mM FBP)*

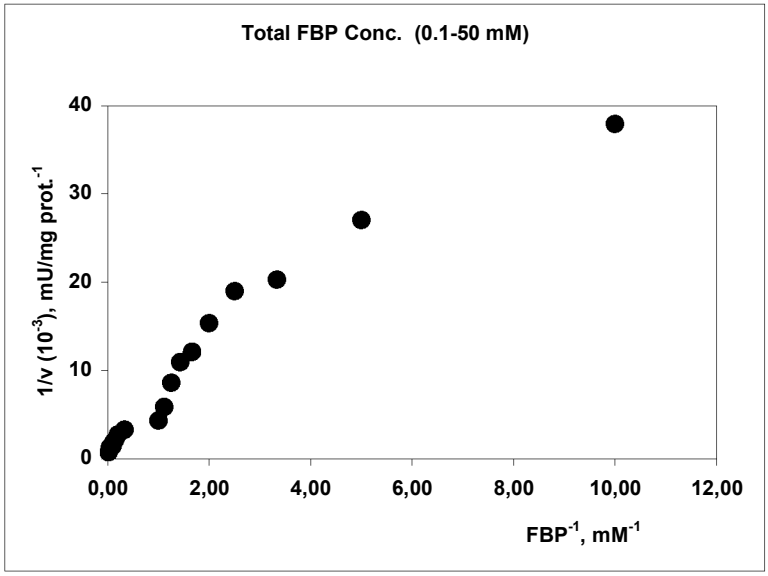

Figure 7: *Lineweaver-Burk plot of effect of total FBP concentrations on healthy human placental FBPA*

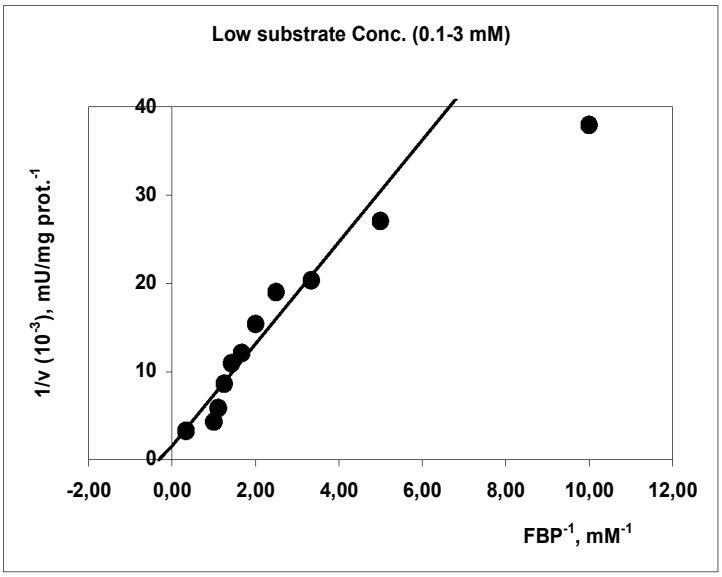

Figure 8: *Effect of low FBP concentrations (0,1–3 mM) on healthy human placental FBPA (Lineweaver-Burk diagram for 0.1–3 mM FBP)*

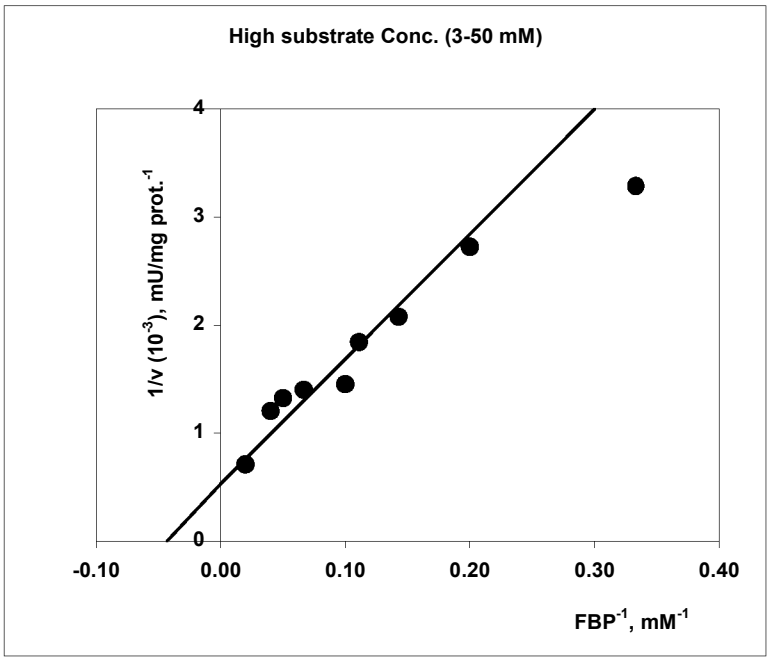

Figure 9: *Effect of high FBP concentrations on healthy human placental FBPA (Lineweaver-Burk diagram for 3–50 mM FBP)*

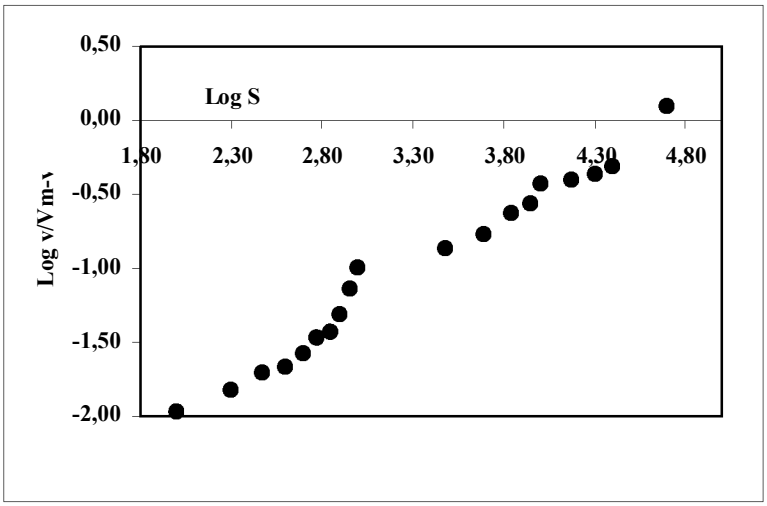

Figure 10: *Hill plot of healthy human placental aldolase for total substrate concentrations*

DISCUSSION

In this study, with the determined procedure (15), aldolase was purified 40.5 fold from healthy human placenta. The different steps of purification of placental aldolase is summarized in table 1. Phosphocellulose elution graphic of enzyme were shown in figure 1.

SDS-Page electrophoresis gave a pure single band when the pure enzyme was subjected to electrophoresis. The band was found to migrate identically with rabbit muscle aldolase under identical conditions. As a result of electrophoretic tests it was determined that molecular weight of human placental aldolase is 160 kDa and homotetramer, and similar to rabbit muscle.

When temperature, pH, and time optimizations were performed for placental aldolase it was observed that the optimum pH was 7.4. temperature was 37ºC. Additionaly incubation and hydrazone stability times of enzymes were optimized.

Aldolases, particularly Class I aldolases, are crucial enzymes in glycolysis. Aldolase enzymes are subjects which are under investigation with regard to their substrate cleavage mechanisms. FBPA has been investigated in many tissues (4-7, 10-12). Until now, there is no knowledge in literature about the presence and purification of aldolase from human placenta.

In this study we principally showed the presence of aldolase in human placenta. Then, enzyme was purified with a modified method based on Penhoet (15). We found that the specific activity of the enzyme was 831.90 mU/mg protein.

Enzymes from tissues were eluted with substrate bounding to column. Pure two separate peaks were determined at eluation graphic profiles. We believe in that this difference from model literature, is characteristic of placental aldolase.

In this study we also searched thoroughly the kinetic properties of purified placental aldolase.

For healthy placental aldolase, the Michaelis-Menten plot was appeared with more than one phases. Therefore new diagrams were formed for low (Fig. 5) and high (Fig. 6) concentrations of healthy human placental FBP. With reference to

the Lineweaver-Burk plot, that related total FBP concentrations, Vm was 1769.513±200.322 and Km was 20.003±4.497 mM for healthy human placental FBPA.

The LB plots were seemed to be nonlinear and convenient to negative cooperativity. In accordance with presented graphics, high substrate concentration range (3–50 mM) has been determined as linear substrate concentration zone. Then, advanced kinetic experiments had been performed at this linear zone. In this sudy, it was determined that Km value was 20 mM for fructose 1,6-bisphosphate. The apparent catalytic parameters of class I FBP aldolases have wide variations. The calculated Km values of characterized class I; aldolase A from human muscle is 52 μM, liver type aldolase B is 1.6 μM and brain type aldolase C is 10.7 μM for fructose 1,6-bisphosphate (21).

Interesting part of our study is to observe two different Km and Vm values at high and low concentrations of substrate Fructose 1,6-bisphosphate. Hill plot of healthy placental aldolase support that enzyme points out different kinetic features for varied substrate concentrations. When two Km values were compared to each other it was determined that the affinity of enzyme for its substrate is increased when the concentration of Fructose 1,6-bisphosphate is low. It seems that, Fructose 1,6-bisphosphate aldolase is an important enzyme of glycolysis and energy metabolism for placental cells. There may be a modification in the ezyme structure of placental cells for low concentrations of fructose 1,6-bisphosphate. Previuos studies showed that human muscle, liver and brain type aldolases have monophasic character for fructose 1,6-bisphosphate (21). Therefore, this phenomena seems to be specific for placental aldolase.

Further studies are under consideration about the kinetic properties of inhibitors of the human placental aldolase. We believe that determination of kinetic features of placental aldolase will give more knowledge for to understand the differences between other tissues and placental aldolases. These kinetic properties will clarify the characterization of enzyme structure.

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