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PARTIAL PURIFICATION AND CHARACTERIZATION OF ACID PHOSPHATASE FROM SERA OF OBESE DIABETES MELLITUS PATIENTS

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ABSTRACT : The study aimed to purification of acid phosphatase (ACP) from sera of obesetype 2 diabetes mellitus patients, this study included from thirty T2DM patients and thirty control, purification process was done with several steps included precipitation with inorganic salt $(NH_4)2SO_4$ 30%-80%, dialysis, ion exchange chromatography by DEAE sepharose anion column and size exclusion chromatography by Sepharose 6B.ACP, BMI, FBS, HbA1c, Lipid profile, Urea, Creatinie, Insuline, Homa-IR were determined. Results showed the precipitate and concentrated protein appeared four peaks in ion exchange column. ACP located in the first and second peak with purification fold (21.1), (37.2) yield of enzyme and specific activity (173.3) IU/ml, which obtained a single peakby gel filtration chromatography, the degree of purification (34.1) fold, yield of enzyme (20.5) with specific activity (280) IU/ml. Also, the peak that has the highest enzymatic activity showed single peak after eluted in gel filtration chromatography following steps by using SDS-PAGE Electrophoresis. From this study, it is concluded acid phosphatase which purification from sera of obese T2DM patients have two isoenzymes, also, concluded the purified enzyme had an optimum temperature (50^0C) and optimum pH (5). Purity and molar mass was measured using (SDS.PAGE) electrophoresis showing approximately ~ 56 KD with single band.

Key words : Acid phosphatase, obese-diabetes mellitus patients, purification of acid phosphatase.

INTRODUCTION

Acid phosphatase (EC 3.1.3.2) is one of the hydrolytic enzymes in lysosomes of cells from a variety of tissues. It forms a group of four isoenzymes as prostatic, lysosomal, erythrocytic and macrophagic acid phosphatases with differences originating at the structural level of the gene (Gupta, 2012).

Various forms of acid phosphatase are found in different organs, and their serum levels are used as a clinical diagnosis for many diseases. Acid phosphatase is nonspecific enzyme which cleaves many different phosphate esters (Pushpa Rani *et al*, 2013).

The higher activityof acid phosphatase observed in vasculopathic diabetes, which indicatethat the activation mechanism in these patientsit is not limited to the degradation of mucopolysaccharides and glycoproteins which forms a thick layer on the walls of the affected vessels, but also includes a some different type of lysosomal hydrolases as an unspecified phenomenon, activation of lysosomal enzymes in diabetes, also correlated with presence of vasculopathies and the degree of metabolic decompensation (Bull *et al*, 2002).

Type 2 diabetes is one of the most common diseases in most countries, whether industrial or developing, which is not contagious, but the death rate is high and is ranked fourth or fifth globally (Amos *et al*, 1997).

Adult diabetes is a common description of type 2 diabetes, is a kind of diabetes characterized by high levels of FBS, HbA1C, insulin resistance and relative insulin deficiency (Tanaka *et al*, 2018).

Type 2 diabetes is mainly due to obesity and lack of exercise some individuals are at greater genetic risk than others (Magri *et al*, 2016).

Type 2 diabetes accounts for about 90% of diabetes cases, with the remaining 10% mainly attributed to type 1 diabetes and gestational diabetes (Qibin *et al*, 2012).

Acid phosphatase activity in obese childhood decreased and that was due to fluctuation in phenotype for enzyme when it work as flavor enzyme (Robyt *et al*, 1987).

This study aimed to purify of acid phosphatase (ACP) from sera of obese T2 DM patients and determined molar mass and purity of purified acid phosphatase by SDS-PAGE Electrophoresis.

MATERIALS AND METHODS

Collection of subclinical diabetes mellitus sera

Total of 60 samples were included in this study, divided in two group 30 patients with obese T_2DM and 30 healthy groups. All information was taken for patients and healthy groups inclusive weight, length, BMI, age, familial history. In addition, there was a person responsible for measuring blood pressure by the same scale. The aged for all subjects ranged between 30-60years.

Precipitation by using $(NH_4)_2 SO_4$ concentration 30%-80% (Robyt *et al*, 1987)

The most commonly used method for precipitation of protein is the addition of inorganic saltssuch as $(NH_4)_2SO_4$or K_2SO4, to five ml of the blood sample added graduallyamount of ammonium sulfate in a beaker under stirring and cooling condition (4^0C) after this solution became turbid and precipitated, it was separated by using centrifuge at (4000 rpm) for 30min, the precipitated was dissolved in acetate buffer (50mM, pH=5.0), protein concentration and enzyme activity were determined for each step.

Ion Exchange chromatography

The ion exchange column of DEAE-Sepharose was prepared in a dimension (14 × 1.5 cm), which was washed and equilibrated with acetate buffer (50mM, pH = 5.0), after estimating the column flow rate at 0.5 mL/min, three milliliters were collected in each tube and the optical activity was determined at 280 nm for each fraction and fractions with the highest absorption were collected, protein concentration and acid phosphatase activity of these fractions were determined, then the bound proteins were eluted with 100 ml wash buffer containing a linear gradient of 0.1-1.0 M NaCl.

Gel filtration Chromatography

The sample was obtained from ion exchange chromatography was applied to the top size exclusion gel column (Sepharose 6B) column (20 × 1.5 cm) that equilibrated by (acetate buffer 50mM, pH = 5.0) then eluted with wash buffer, after estimating the column flow rate at 1 mL/min, three milliliters were collected in each tube and the optical activity was determined at 280 nm for each fraction and fractions with the highest absorption were collected, protein concentration and acid phosphatase activity of these fractions were determined, then the bound proteins were eluted with 100 ml wash buffer containing a linear gradient of 0.1-1.0 M NaCl.

Acid phosphatase activitivation assay (Huang *et al*, 2011)

Acid phosphatases (AP) dephosphorylate groups of phosphate esters in acidic conditions, one unit hydrolyzes 1.0 ìmole of substrate (p-nitrophenylphosphate) per minute at pH 4.8 and37°C.

Estimation of protein concentration (Vicki Knowles *et al*, 2013)

Determination of total protein concentration in serum and each purification step was performed by a kit using biuret method provided from a (SPINREACT) company.

SDS-PAGE Electrophoresis (Garfin *et al*, 2003)

The purity and molar mass of the ACP that purified from sera of diabetic patients were carried out by using SDS-PAGE gel electrophoresis.

Effect of pH on enzyme activity

The activity was carried out using the eluted fractions from Sepharose 6B column at optimum reaction condition at several buffers, pH range from3 to 9 (glycine, Na-acetate and Tris- HCl). The rate of reaction was plotted versus the pH to determine the optimum pH for ACP reaction.

Effect of temperature on enzyme activity

The activity of ACP was done at temperature ranges (25, 30, 35, 40, 45, 50, 55) o c, 10 Mm Tris- buffer pH(7.4).

RESULTS AND DISCUSSION

Acid phosphatase partial purification from obese T_2DM

Sera obtained from patients with the same HbA1c level was used in the purification steps included precipitated with ammonium sulfate (30%-80%).

Acid phosphatase precipitated by salting out method which includes added inorganic salt such as $NH_4(SO_4)_2$, which most used because it is easily soluble in water, decreasing protein solubility and precipitation due to protein charge equivalence by $NH_4(SO4)_2$ (Begüm Yenigün *et al*, 2003).

When salt is added to crude solution, hydrophobic interaction between water and protein occurs as a result of high surface tension to. The protein interacts with station by Reducing the surface area in an attetmpt to minimize solvent contact — as demonstrated by folding (folded conformation is more compact than unfolded) and then by self-association leading to precipitation (Sonam Tashi Dolma *et al*, 2014).

Ion exchange by DEAE-sepharose column (an anionic ion exchanger which is characterized by ease of preparation and high selectivity in bio separating, so it has a variety of uses) and size exclusion chromatography by (sepharose 6B risen one of its properties is speed and selectivity in bio separation) were applied in ACP

purification (Chandrakant K Tagad *et al*, 2018).

Results in Fig. 1 showed four protein peaks appeared at 0.2, 0.3, 0.4, 0.5 M NaCl in 50 mMacetate buffer pH=4, acid phosphatase that purified from obese T2DM located only at 0.2 M NaClin fractions (32-38) and 0.3 M NaCl in fractions (44-51).

Gel filtration chromatography was applied using sepharose 6B column for active and concentrated fractions that obtained fromion exchange chromatography.

Results displayed in Fig. 2 -appeared active ACP peak in fraction No. (6-11) with single protein peak by gel filtration chromatography.

Acid phosphatase purification steps as shown in the Table 1.

SDS- PAGE Electrophoresis

Purity andmolar mass of ACP was determined as shown in Fig. 3.

As shown in Fig. 3, ACP that purified from sera of obese T2DM showed the presence give two narrow bands of two isoenzyme with molar mass about 58,000(±1000) Daltons by ion exchange chromatography and one band with molar mass about ~56 kDa by gel filtration chromatography for purified enzyme.

Optimum pH

To order to find out the optimal pH, multiple buffers were used. Sodium acetate, glycine, Tris-HCLbuffer was used. It is well known that the activity of enzymes is strongly influenced by changes inH^+ concentration in the assay mixture; thus, multiple pH gradients (ranging from 3 to 8) were used to determine the optimal pH for ACP activity. Estimated optimal ACP activity at pH 5, however, there was a fluctuations in the ACP activity when pH raised from 4.5 to 5.5.

Optimum temperature

Optimum temperature of purified ACP as shown in the figure below about 50^0C. It is known that the speed of the enzymatic reaction increases with higher temperatures until the optimum reaction temperature reaches and then begins to gradually decrease, due to the denaturation ofenzyme molecule, which causes loss of enzyme activity and this is explained by the change in the geometrical arrangement of the three dimensional structure of enzyme, as high temperatures affect the ionization status of groups on the surface of the enzyme. Generally, the enzyme is unable to perform its active role when the temperature rises to the extent that it affects the conformational structure (Robinson, 2015).

Acid phosphatase is considered an important factor

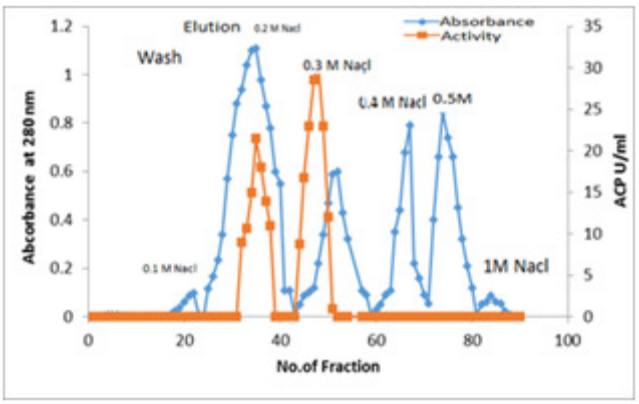

Fig. 1 : Ion Exchange Chromatograph step of ACP purification from sera of obese T2 DM patients

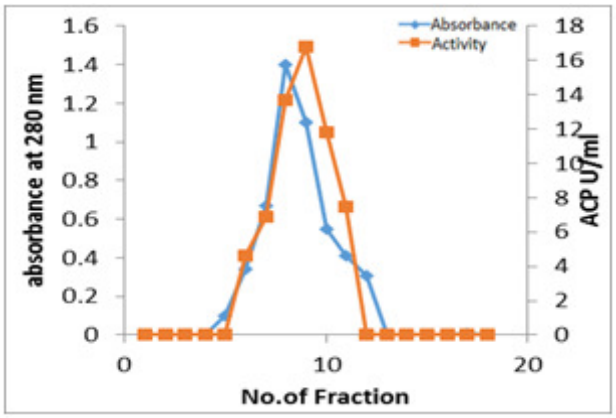

Fig. 2 : Size exclusion Chromatograph step of ACP purification from sera of obese T2 DM.

Fig. 3 : a- crude enzyme b- Participation (NH_4SO_4) c-ion exchange chromatography d-gelfiltration chromatography.

for the growth and development of plants due to its role in hydrolyzes phosphate esters at acidic pH, ACP was extracted and purified from *Erythrina indica* seed to 61 with 5 steps which included; ammonium sulphate fractionation, size exclusion chromatography with ConA-seralose 4B column, acidification to pH 5 and ion exchange chromatography by DEAE-cellulose column.

Acid phosphatase purified from plants *M. uniflorum* with molar mass about 55,000 (±1040) Da. Optimum pH was 5.0. ACP has been found to be relatively thermostable

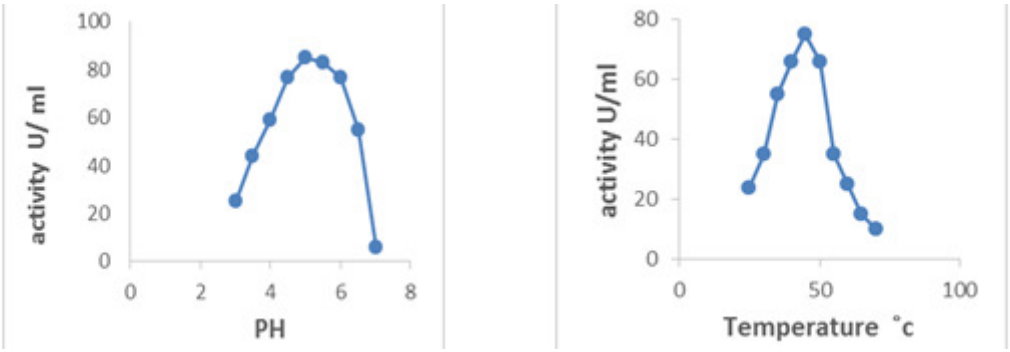

Fig. 4 : pH and temperature optimum of acid phosphatase, respectively

Table 1 : Purification of ACP from sera of obese -T2DM patients.

Steps	Volume (ml)	Total protein conc. (mg/ml)	ACP activity (U/ml)	ACP total activity (U/ml)	Specific activity (IU/mg)	Purification fold	Yield (%)
Crude ACP	10	49.8	409	409	8.21	1	100
Participation ((NH_4SO_4)	5	7.3	33.8	169	23.15	2.8	41.3
Ion exchange Isoenzyme I	5	0.92	21.5	107.5	116.8	14.2	26.2
Ion exchange Isoenzyme II	5	0.88	30.5	152.5	173.3	21.1	37.2
Gel filtration Isoenzyme II	5	0.30	16.8	84	280	34.1	20.5

optimum temperature was 50^{0}C indicating its suitability for food and pharmaceutical applications. Enzyme demonstrated high specificity of substratum (Sushma G Sabharwal, 2017).

The effect of pH on the enzyme activity was calculated by erforming the hydrolysis of pNPP at different pH values in a series of buffers (100 mM) (3.0-6.2). Sodium acetate (pH 5.0) and sodium citrate (pH 4.8) were used as buffers. The pH values for each buffer were 25^{0}C. The pH stability of any phosphatase was tested with 100 mM buffers at a pH range of 3.0-6.2. Buffers used were the same as in pH and temperature optima study. Following 1 h pre-incubation at 25ºC (room temperature), residual phosphatase activity was calculated at 37^{0}C for 10 min by adding pNPP substrate. The thermal inactivation was calculated at the optimum temperature (at pH 5.6) at 37 range C and at each enzyme. Enzymes in sufficient buffers were exposed for up to 150 min at each temperature. Afterwards, aliquots were removed at intervals (15 min) and immediately cooled.

Temperature effect on ACP operation was achieved with temperature range from 0 to 100ºC by using P-nitro phenol phosphate as substrate and citrate buffer with pH 5.6. Optimum temperature for acid phosphatase activity was 50°C (Sudhir Gadgil *et al*, 2016).

CONCLUSION

From this study, it is concluded acid phosphatase which purification from sera of obese T2DM patients have two isoenzymes, also, concluded the purified enzyme had an optimum temperature (50^{0}C) and optimum pH (5). Purity and molar mass was measured using (SDS.PAGE) electrophoresis showing approximately ~ 56 KD with single band.

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