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Assessment activity and Partial purification of Alkaline phosphatase (AIP) from sera of patients with celiac disease.

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ABSTRACT

Celiac disease (CD) is an immune disease caused by intolerance to gluten which is found in wheat and barley . Alkaline phosphatase enzyme (EC : 3.1.3.1) was partial purified from sera of patients with celiac disease by using two techniques: The first, gel filtration chromatography packed with sephdex G 50 and the second technique by ion exchange chromatography with DEAE cellulose. Initially the gel filtration results giving 39.32% yield and 2 – 3 fold purification followed by ion exchange and it was obtained 64.07% yield and 0.613 fold purification for the first iso- enzyme (I) . The significant differences ($P < 0.01$) showed in activity of AIP for patients as compared to healthy women and the highest total activity was 0.206 U and the lowest 0.0337 U. The purification by ion exchange was illustrated a clear distinct peaks for three alkaline phosphatase iso-enzymes .

Keywords: Celiac disease , AIP purification , Gel filtration, Ion exchange

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INTRODUCTION

Celiac disease (CD), also known as Gluten – sensitive enteropathy or celiac sprue is an autoimmune inflammatory disease of small intestine that is precipitated by the ingestion of gluten, a component of wheat protein [1]. Historically, CD was first described by Dr. Samuel Gee in 1888 who reported poor growth, abnormal stools and abdominal distension as common symptoms in children, then the ability to perform perioral mucosal biopsies was established in 1950, when a typical small bowel mucosal abnormality in patients with CD was observed [2]. Although CD is defined by the small intestine injury and resulting malabsorption and it has been recognized to be a multisystem disorder been recognized to be a multisystem disorder that may affect other organs, such as the nervous system, bones, skin, heart and liver [3]. Alkaline phosphatase (AIP), also referred to as ortho phosphoric monoester phosphor hydrolase (EC : 3.1.3.1.) is generic term that describes a group of catalytic proteins sharing the capacity to hydrolyze phosphate esters in Alkaline medium [5]. AIP enzyme is a metalloenzyme and – its containing two classes of zinc (II) ions and magnesium (II) ions, the active form of the enzyme is transported to the periplasmic space and it occurs in three forms that have been designated isoenzymes 1, 2 and 3 [6]. The isoenzymes of AIP differ by the presence of an NH₂ – terminal arginine residue on the subunits of isoenzyme 1 and the absence of the same on isoenzyme 3, with isoenzyme 2 representing a hetero dimer of two types of chains [7]. The serum level of AIP is compounded of isoenzymes arising from bone, liver, intestine and during pregnancy, from the placenta, AIP is located mainly in the brush border of intestinal mucosa [8]. Faiza and Nada were purified AIP enzyme from bacteria of *Escherichia coli* C90 using an ion exchange column packed with DEAE – cellulose as matrix, and were absorbed by native gel results a clear distinct bands for the three Alkaline phosphatase isoenzymes [9]. The extracts of AIP enzyme from different tissues of organs and various biological systems exhibit variable but the characteristic kinetic properties that serve as basis for distinguishing AIP isoforms [10]. The purpose of this investigation was estimation of the activity of the serum levels of Alkaline phosphatase and the identification of isoenzymes from female adult with celiac disease by using Gel filtration chromatography and ion exchange column.

MATERIALS AND METHODS

Subjects

Thirty female patients with celiac disease and thirty healthy women as control group their age ranged (20-50) year. They patients were referred to Al-Uarmook hospital in Baghdad, Iraq. Five milliliters of samples of venous blood were taken and it left for 10 minutes at room temperature. After blood coagulation, the sera were separated by centrifugation at 3000 rpm for 10 minutes, then sera stored at -20 until being used.

Enzyme assay

Serum alkaline phosphatase activity (AIP) (EC:3.1.3.1) have been determined according to the method of kind and king [11]. It was assayed spectrophotometry by following the release of 4-nitrophenol from 4-nitrophenylphosphate. Total enzyme activity [U] was calculated by multiplying the measured activity or rate, times the volume all divided by 1000.

$$\text{Total activity [U]} = (\text{Enzyme activity} * \text{volume}) / 1000.$$

Specific activity (u/mg) is calculated by dividing the value obtained above (Total activity) with protein in mg.

Determination of protein concentration

The total protein amount was estimated in serum by using a kit provided from a company (Syrbio) depending on Biuret method [12]. The amount of total protein in mg was calculated from the concentration multiplied by the actual volume used all divided by 1000.

Purification of AIP enzyme

Enzyme purification process had been done by using the following steps :

Precipitation of enzyme by ammonium sulfate we added 0.8gm of inorganic salt : ammonium sulfate with saturation ratio (40%) gradually to the 5ml of crude serum in a beaker and the mixture was mixed gently on a magnetic stirrer at 4°C for one hour till the turbid solution . The precipitated proteins were separated by centrifugation at 3500 rpm for 10minutes to split the precipitate from leach [13] . finally the precipitate was dissolved in a less amount of buffer with pH (9.5) that contain (100mM Tris-HCl, 100mM NaCl , 5mM MgCl₂ , 0.05% Tween 20) . The AIP activity , protein conc. and specific activity were estimated before and after ammonium sulfate precipitation .

Dialysis of enzyme

The solution (crude serum precipitated) was dialyzed against Tris-buffer by putting 2ml of crude serum precipitated in to a tightly wrapped cellophane bag from bottom and then it was wrapped tightly also from its top . The pipe was left in to a container that contain a buffer solution at 4c for 24 h with constant stirring by using a stirrer . The final volume of the resultant solution after the finishing of dialysis process was concentrated by sucrose and it was calculated. The AIP activity , Protein concentration and specific activity were estimated .

Gel filtration chromatography

Two ml of concentrated enzyme solution obtained from dialysis step was added gently on the surface of sephadex G50 column with dimensions of (1.5 x 20 cm) equilibrated previously with buffer AIP (pH 9.8) . To pass the concentrated enzyme through the column we use the same buffer of AIP with flow rate of 2 ml/min . Twenty fractions were collected , each fraction contains 2ml . Enzyme activity and total protein concentration was also determined in each fraction and all fractions were pooled and kept at 4°C for the last step of purification by ion exchange .

Ion exchange chromatography

The Diethylaminoethyl Cellulose (DEAE_Cellulose) was packed with column with dimensions of (2 x 20 cm) . Two ml of enzyme concentrated was applied to ion exchange chromatography that equilibrated previously with small volume of AIP buffer , then column was washed with an equal volume of the same buffer to displace unbinding protein (wash) , while attached proteins were stepwise eluted AIP buffer with gradual concentrations of sodium chloride (0.1-0.4 M respectively) . All fractions were eluted at flow rate (2ml/4min) . AIP activity and total protein for each fraction was determined .

Statistical analysis

The data were processed with the statistical analysis system _SAS (2012) program . This analysis was used to show the mean and standard error deviation of variable and the significance differences between mean values were estimated by student T- Test with probability (P< 0.05)= significant [14] .

RESULTS AND DISCUSSION

The results showed the AIP activity enzyme in sera of women with celiac disease was increased significantly (P < 0.01) comparison with healthy women as shown in Table (1) . The activity of AIP of thirty healthy women reached to 65.31 U/L comparison with sera of patients With celiac disease was increased significantly to 109.15 UI/L .

Table (1) : The comparison of AIP enzyme activity between healthy women and patients with celiac disease in serum

Group	Subjects	AIP activity (UIL) (Mean ± SE)	P – Value
Patients	30	109.15 ± 6.27	5.027**
Healthy	30	65.31 ± 3.52	
** (P < 0.01)			

The AIP enzyme was partially purified by using purification two techniques of sera patients with celiac disease. The amount of protein in each sample, total activity , specific activity, fold purification and yield are indicted in Table (2) . The purification fold was also calculated from the original sample which was the specific activity value designated 1 fold ; the rest of the sample were Calculated as component of the crude serum specific activity. To Calculate the yield and crude serum were consumed to contain all proteins (Enzyme) in the sample ; therefor the total activity it showed was considered of 100% yield . The yield of all other samples from percentage yield of crude serum total activity . The results indicated the purification fold was reached to 1.76 with recovery of 58.25% in dialysis step while the purification degree was increased to 2.3 fold with recovery 39.32% from the crude serum by using gel filtration chromatography on a sephadex G 50 column . We used the Propitiation technique of enzyme by ammonium sulfate salts , it was widely used for fractionation of protein and it was expensive way for concentrating a protein extract [15] . Total activity was decreased with most purification steps while the purification degree of the first isoenzyme (I) was 0.613 fold , the second isoenzyme (II) was 0.176 fold and the third (III) was increased to 0.833 fold .

Table (2) : Purification steps of serum Alkaline phosphatase(ALP) from patients with celiac disease

Purification step	late (ml)	Protein conc. (mg/ml)	Total protein (mg)	Activity U/L	Total Activity (Units)	Specific activity (U/mg)	Purification folds	Yield(%)
Crude serum		9500	47.5	41.30	0.206	0.0043	1	100
Amm onium sulphate ppt.		8120	16.24	53.81	0.107	0.0066	1.53	51.94
Dialysis		7900	15.8	60.21	0.120	0.0076	1.76	58.25
Gel filtration		3800	7.6	40.57	0.081	0.010	2.3	39.32
Ion Exchange								
Iso.I		25000	50	66	0.132	0.0026	0.613	64.07
Iso.II		22000	44	16.85	0.0337	0.00076	0.176	16.35
Iso.III		9800	19.6	35.14	0.0702	0.00358	0.833	34.11

Purification by using Gel filtration Sephadex G 50 column

The results showed the AIP enzyme have a single peak by using gel filtration column and the activity of AIP was 40. 57 UIL with fraction five . The protein concentration was measured at 540 nm and the highest protein content values were obtained in fractions 4 and 12 ; fraction 4 shows the highest protein content was 4.06 gm / dl as shown in figure (1) . They were pooled for further purification using DEAE cellulose .

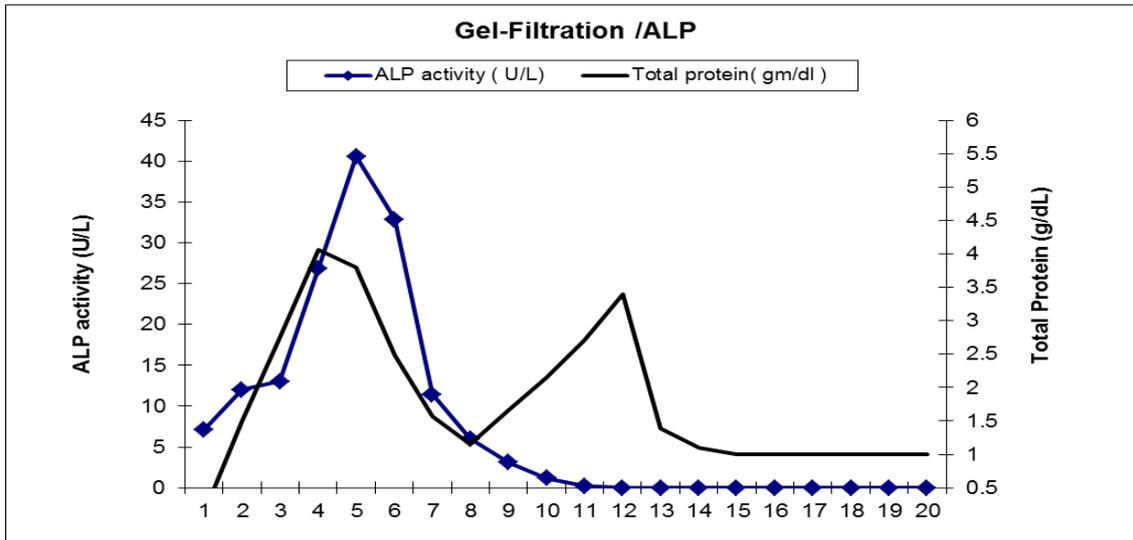


Figure (1) : Elution of the gel – filtration chromatography of sephadex G 50 Fractions plot of protein content values and AIP activity with sera of celiac disease .

Purification by using DEAE cellulose chromatography (Ion exchange)

Both AIP activity and protein content of 44ml fraction eluted by using the ion exchange chromatography and the values obtained were used to plot the graph (figure 2) . Sample 5, 12 and 20 show the highest AIP activity and they reached to 66 UI/L , 16.85 and 35.14 respectively . The fraction with the highest protein content was eluted between 6 and 15 ml .

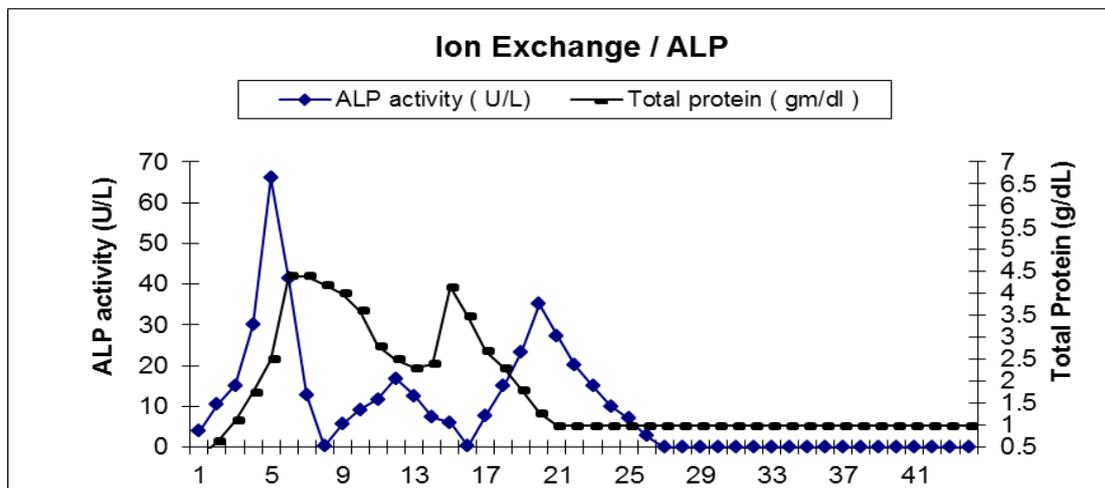


Figure (2) : Elution of the ion exchange chromatography of DEAE cellulose fractions plot of protein content values and AIP activity .

The two – stage chromatographic technique, which involved separation using gel filtration followed by ion exchange column, yielded highly the specific activity of Alkaline Phosphatase from patients with celiac disease comparable to the control. Increased of the activity of AIP contributed to the stress oxidative that increased in AIP activity [16] . There is evidence that gluten protein ingestion induced an increased oxidative stress due to over production of free radicals, that the reactive oxygen and nitrogen species were happened [17]. In this study, the elevated activity of AIP enzyme from sera with celiac disease was agreement with the other study that which report to have a raised alkaline Phosphatase with 67 patients have abnormal liver biochemistry and coeliac disease [18]. The three major isoenzymes observed by using ion exchange column which differ by a single arginine residue at the amino terminus [19] . The isoenzymes are probably due to post

– translation modification arising during processing of a precursor from of the enzyme as it is transported to the periplasmic space [20].

CONCLUSION

our study illustrated that clear significant differences ($P < 0.01$) between the activity of AIP with patient women of celiac disease and healthy group , the highest specific activity was 0.010 U/mg and the folds purification of enzyme increased to 2.3 fold by gel filtration while it decreased to 0.176 by ion exchange technique.

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