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Purification and Characterization of Alkaline Phosphatase from Water Buffalo Liver *(Bubalus bubalis*).

Doaa A. Darwish*, Mahmoud A. Ibrahim, and Abdel-Hady M. Ghazy.

Molecular Biology Department, National Research Centre, Dokki, Giza, Egypt.

ABSTRACT

Two forms of alkaline phosphatases (EC 3.1.3.1) designated P1 and P2 isoenzymes were purified to homogeneity from the liver of water buffalo after anion exchange chromatography on DEAE-cellulose column and gel filtration on Sephacryl S-300 column. The native molecular weights of the two isoenzymes P1 and P2 are 188 ± 4 kDa and 194 ± 3 kDa and both of them exhibited homotrimeric structure. The purified isoenzymes displayed their maximum activity at pH 10. Both P1 and P2 isoenzymes showed isoelectric point (pI) value at pH 5.6-5.8. MnCl2 increased the AP activity of P1and P2 isoenzymes while $ZnCl^{+2}$ was potent inhibitor of both P1 and P2 isoenzymes. The enzyme was sensitive to EDTA and the thiol compounds in the following order: DTT > GSH > cysteine > β mercaptoethanol. The P2 isoenzyme was more sensitive to β-mercaptoethanol and phenylalanine than P1.

Keywords: Alkaline phosphatases, water buffalo, liver, properties and purification

Abbreviations: AP: alkaline phosphatase; BSA: Bovine serum albumin; PAGE: Polyacrylamide gel electrophoresis; p*I*: isoelectric point; *p*-NPP*: p*-nitrophenylphosphate; BCIP: Bromo-4-chloro-3-indolyl phosphate; ADP: adenosine diphosphate; CTP: cytosine triphosphate.

Corresponding author

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INTRODUCTION

The alkaline phosphatase, (AP) is one of nonspecific ectophosphomonoesterases. AP is designated as metalloenzyme that consists of a group of true isoenzymes, encoded by four different gene loci: tissue-nonspecific, (or liver/bone/kidney (L/B/K)) and tissue- specific AP, intestinal (IAP) and placental (PLAP) and germ cell isoenzymes [1, 2, 3, 4 and 5]. The tissue-nonspecific AP is present nearly in all species, from bacteria to man, with a high degree of homology [1]. The wide distribution of APs in nature shows that these enzymes are included in fundamental biochemical processes [6]. AP from various mammalian tissues have been purified by different methods and commercialized for different purposes. They have been purified and studied in details from mammalian tissues or organs such as human placenta [7 and 8], human liver [9], calf intestine [10], dog intestine [11], sheep kidney [12], bovine kidney [13] and swine kidney [14]. The bacterial alkaline phosphatases are 20-30 fold less active than the corresponding mammalian enzymes [15 and 16]. For labelling purposes, APs from eukaryotic origins are used as colourimetric enzymes because of their high specific activities [16]. AP is also used greatly as a reagent in most molecular biological procedures such as the deletion of phosphate from the end of a DNA or RNA fragment, the production of hybrid proteins to study gene expression, the linking of the enzyme to antibodies for use in an enzyme-linked immunosorbent assay (ELISA) and many types of bioassays, and enzyme apply to activate a phosphorescent substrate at the non-radioactive detection of DNA and RNA [15]. Immunoassays such as ELISA are extremely important in process monitoring and quality control in many biotechnological industries, including pharmaceutical industries. So AP is need to improve economical and production methods at large-scale [17]. The water buffalo is one of the important domestic mammal's animals and it is a common source of meat in Egypt. Therefore, this work describes a simple purification procedure and introduces some properties of AP from the water buffalo liver as a mammalian locally available rich source in Egypt.

MATERIALS AND METHODS

Liver materials

Fresh liver from water buffalo liver *(Bubalus bubalis*) was obtained from a local slaughter-house and stored at -40° C.

Chemicals

DEAE-cellulose, Chemicals for molecular weight marker kits for gel filtration and Sephacryl S-300 were purchased from Sigma Chemical Co. All other chemicals used were of high analytical grade.

Alkaline phosphatase assay

Alkaline phosphatase activity was monitored by spectrophotometric detection of the the released *p*-nitrophenol from *p*-NPP at 37° C [18]. Assay reaction mixture (500µl) contained 1M diethanolamine-HCl pH 10 containing 1 mM $MgCl_2$, 4 mM (*p*-NPP) and appropriate dilution enzyme was incubated at 37° C for 20 min. assay was ended by adding 500µl of 1N NaOH and the liberated *p*-nitrophenol absorbance was estimated at 405 nm. One Unit of enzyme activity represents1 µ mol of *p*-nitrophenol per min produced under the described conditions.

In case of using other phosphorylated compounds, the liberated phosphorus was evaluated according to the method of Josse [19]**.** The terminated colour of respective reaction (1 ml) was carried out for 10 minutes in 2 ml containing 0.3 ml of 5N H_2SO_4, 0.3 ml of 2.5% ammonium molybdate, 0.3 ml of 3% $NaHSO_4$ in 1% p-methylaminophenol sulfate and 1.1 ml water. The developed blue colour was measured at 660 nm. The specific activity to the control (containing 1 ml distilled H_2O instead of the sample and treated similarly) was calculated. The constructed standard curve covering the range of 0.1-1.1 µ mol using KH_2PO_4 as standard was used for determine phosphorous concentration.

Purification of the alkaline phosphatase

Crude extract Preparation

All procedures were carried out at 4° C. Water buffalo liver was minced, with two volumes of distilled water (2 ml / gm tissue) then one volume of n-butanol (1 ml / gm tissue) was blended, using omni-mixer (SorvallDupont Instruments) [14], and stored at -20° C overnight then centrifuged at 12.000 xg for 30 min at 4° C. This aqueous phase containing the enzyme was saved and termed n-butanol extract. Prechilled acetone one volume was added to the n-butanol extract. The sediment was assembled by centrifugation at 12.000 xg for 30 min at 4°C, washed three times with acetone and dried under vacuum.

DEAE-cellulose Chromatography

Dissolved acetone powder in 0.05M Tris-HCl buffer (pH 7.6) containing 1 mM $MgCl_2$ was chromatographed on DEAE-cellulose column (22 x 1.6 cm i.d.) equilibrated with the same buffer. Proteins were eluted at a flow rate of 60 ml / h with a linear NaCl gradient (0 to 1 M). The fractions containing AP activity were pooled and concentrated by sucrose

Determination of native molecular mass

Gel filtration was carried out to estimate the molecular mass of the native AP on a Sephacryl S-300 column equilibrated with 0.05 M Tris-HCl buffer pH 7.6 containing 1mM $MgCl_2$ at a flow rate of 30 ml / h.

Protein estimation and SDS-PAGE

Native gel electrophoresis was carried out with 7% PAGE according to Smith, [20]. The molecular mass of the purifed enzyme was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions on a 12% polyacrylamide gel according to the method of Laemmli [21], and the subunit molecular weight of the purified enzyme was detected by SDS-PAGE (Weber and Osborn) [22]**.** The proteins were visualized by 0.25% Coomassie brilliant blue R-250. Focusing of the purified AP was carried out, on 5 % polyacrylamide gel vertical slabs [23]. Broad pI kit was used as isoelectric point (pI) markers.

Activity staining for alkaline phosphatase

Staining of AP activity was performed in native 7% PAGE using 5-Bromo-4-chloro-3-indolyl phosphate (BCIP) as substrate principally described by Baechtel *et al*. [24], and non-denatured 12% SDS-PAGE in the absence of β-mercabtoethanol. After completion of the electrophoresis, the gel was incubated in 40 ml of 0.1M glycine-NaOH PH 10 containing 4mg BCIP (0.27mM) at 37˚C. After the appearance of the blue colour bands in the gel itself, the BCIP solution was removed and the gel was fixed in 30% methanol solution and dried.

Protein determination

Protein was determined according to the method of the dye binding assay [25] using BSA as a standard protein.

RESULTS AND DISCUSSION

Alkaline phosphatases purification from water buffalo liver

Purification scheme of the AP from the water buffalo liver is presented in Table (1). The specific activity in the n-butanol extract was determined to be 0.77 unit / mg protein. The established purification procedure of water buffalo liver AP seems to be convenient method. The n-butanol extraction was a successful step since it removed most of the proteins. The present study was focused primarily to purify of membrane enzymes by tissue homogenization followed by n-butanol extraction. This n-butanol create low solubility in aqueous solutions (9.1% v / v in water at 25°C) with a great solvating capacity for lipid and different hydrophobic material and has only acceptable denaturing effect as compared to other organic solvents [26].

The acetone precipitation step removed 56% of the protein content and retained 94% of the water buffalo liver AP activity.

Two major peaks of AP activity were detected by chromatography on the DEAE-cellulose column of eluted with 0.05 M and 0.1 M NaCl (Fig. 1). The eluted fractions containing the activity of enzyme were pooled separately and concentrated by sucrose. The concentrated material of each peak was loaded onto a sephacryl S-300 column Fig. 2 (A and B), which reveal presence of a single peak. Increasing specific activities of P1 and P2 (8.13 units / mg protein and 13.8 units / mg protein) were observed with (17.1 % and 24.5%) yield respectively comparable to that of rat liver 27% [27], and human liver 7.4% and 6% [28, 29]

The enzyme elution volume (Ve) estimated to be 195 ml and 186 ml respectively from the sephacryl S-300 column Fig. 2 (A and B).The apparent molecular mass of the native AP was calculated to be 186 ± 2.7 kDa and 191 ± 3.3 kDa for P1 and P2 respectively more or less similar to that from beef brain (190 kDa and 180 kDa) [30, 31], from bovine kidney (172 kDa) [32], from rat liver (154 kDa) [27] and human liver (146 kDa) [29].

Electrophoretic analyses of water buffalo liver alkaline phosphatases

The native and denatured 12% SDS-PAGE proven the purity of the water buffalo liver alkaline phosphatases and the molecular weights of native purified proteins P1 and P2 was estimated to be 188 ± 4 kDa and 194 ± 3 kDa Fig. 3 (A and B). Their subunit molecular weight was found to be 63 ± 2 kDa. These results indicate that both P1 and P2 isoenzymes are homotrimeric proteins composed of three identical subunits. The majority of the studied alkaline phosphatases are dimers while that from the metacestode *Echinococcus multilocularis* was composed of three identical subunits of 80 kDa [33]. Samples of the purified P1 and P2 were electrofocused and presented in (Fig. 4) The p*I* values of both P1 and P2 isoenzymes were calculated from a calibration curve to be at pH 5.6-5.8, while that of human liver AP was 4 [29]. The water buffalo liver AP isoenzymes (p*I*) values explain the binding of the enzyme to the DEAE-cellulose column at pH 7.6, where the net charge on the enzyme should be negative.

The AP activity was detected on 7% native PAGE and native 12% SDS-PAGE. P2 isoenzyme migrated faster than P1 isoenzyme due to its higher negative charge on the 7% native PAGE (Fig. 5A). The isoenzyme shape of different purification steps affirmed that the water buffalo liver contain two distinct AP isoenzymes. P1 isoenzyme displayed lower molecular weight (188 kDa) and migrated in the SDS-PAGE faster than P2 isoenzyme (194 kDa) (Fig. 5B, 5C and 5D), and their activity bands appeared at the same locus on the 12% SDS-polyacrylamide gel. Moreover, the staining of the isoenzymes activity during the different purification steps from the liver indicates that the n-butanol extraction and the acetone precipitation did not alter the native enzyme.

Substrate specificity

The hydrolytic activity of the purified water buffalo liver AP toward various phosphorylated compounds is summarized in (Table 2). The rate of *p*-NPP hydrolysis was taken as 100%. Similar affinity of P1 and P2 toward the various substrates was found as in the following order; creatine phosphate > ADP > CTP > Fructose-1,6 diphosphate > ATP > phosphoenol pyruvate > glucose-6-phosphate > glucose-1- phosphate > AMP > UMP. (Table 2).

Enzyme Kinetics

The purified water buffalo liver AP, P1 and P2 isoenzymes were incubated with *p*-NPP and the most susceptible substrate creatine phosphate, ADP and CTP. The representations of Lineweaver-Burk plots Fig. 6 (A and B) for *p*-NPP and creatine phosphate and Fig. 7 (A and B) for ADP and CTP were detected the Michaelis-Menten constants (Km), which evaluated to be 0.22, 3.0, 2.6 and 2.6 mM of *p*-nitrophenylphosphate, creatine phosphate, ADP and CTP respectively for the isoenzyme P1. The Km values were found to be 0.21, 2.27, 2.5 and 2.2 mM of *p*-nitrophenylphosphate, creatine phosphate, (ADP) and (CTP) respectively for the isoenzyme P2. These results suggest that the liver alkaline phosphatase may be involved in the dephosphorylation of creatine phosphate for creatinine production.

pH optimum

The enzyme activity of P1 and P2 isoenzymes toward *p*-NPP were measured in 1 M diethanolamine-HCl buffer was active only in the range between pH 8.0 to 10.8 with a maximal activity around pH 10 (Fig. 8) Which is very consistent with that of the swine kidney alkaline phosphatase pH 10 [14].

Effect of various metals

In order to check the effect of metal ions on the purified water buffalo liver P1 and P2 isoenzymes alkaline phosphatase activity spectrophotometric assay was carried out using 2 and 4 mM of each cation and *p*-NPP as a control test which was taken as 100 % relative activity at 37° C Table (3). The activity of the isoenzyme P1 was increased about 2.5 fold and that of the isoenzyme P2 about 1.7 fold in the presence of 2 mM MnCl2. In contrast, CuCl2 was moderate inhibitor and ZnCl2 was potent inhibitor of both P1 and P2 isoenzymes. The other test cations exhibited nonsignificant effect.

Effect of various inhibitors

The purified water buffalo liver alkaline phosphatase P1 and P2 isoenzymes were preincubated with 2 and 4 mM of each inhibitor for 5 min at 37° C and the remaining activity was assayed. Tables (4and 5) show the inhibition % and the residual activity of each isoenzyme P1 and P2 with each inhibitor respectively. The metal chelator EDTA and the thiol compounds; DTT, GSH and cysteine were potent inhibitors of P1 and P2 isoenzymes. The P2 isoenzyme was more sensitive to β-mercaptoethanol and phenylalanine than P1.

In conclusion, the present study provides a simple and reproducible method for production of highly purified AP from the water buffalo liver suitable for application in ELISA, immunoblotting and different conjugation reactions with proteins or nucleotides.

Table 1: A typical purification scheme of the water buffalo liver alkaline phosphatase isoenzymes es P1 and P2.

Purification steps	Total protein (mg)	Total Activity (unit)	Specific Activity	Yield (%)	Fold Purification
N-Butanol extract	650	502	0.77	100.0	1.0
Acetone fraction	285	474	1.66	94.4	2.15
DEAE-Cellulose fraction					
P1	55.3	130.5	2.36	26	3.06
P2	60.0	224	3.73	44.6	4.84
Sephacryl S-300 fraction					
P1	10.58	86	8.13	17.1	10.56
P2	8.91	123	13.8	24.5	17.92

Table 2: Substrate specificity of water buffalo liver alkaline phosphatase isoenzymes P1 and P2.

Substrate	Relative activity (%)	
	P1	P2
p-nitophenylphosphate (*p*-NPP)	100.0	100.0
Creatine phosphate	3915.8	5033.0
Adenosine diphosphate (ADP)	2340.2	3150.0
Cytosine triphosphate (CTP)	2134.6	2653.4
Fructose-1,6-diphosphate	643.9	817.0
Adenosine triphosphate (ATP)	323.4	397.7
Phosphoenol pyruvate	216.8	255.7
Glucose-6-phosphate	186.0	233.0
Glucose-1 phosphate	143.9	202.3
Adenosine monophosphate (AMP)	132.7	152.3
Uridinemonophosphate (UMP)	128.0	149.5

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Table 3: Effect of metals on water buffalo liver alkaline phosphatase isoenzymes P1 and P2

Reagent	Final concentration (mM)	Residual activity (%)	
		P1	P2
control		100	100
$MnCl_2$	2	245.1	173.6
	4	227.48	189.07
$MgCl_2$	2	128.2	130.4
	4	128.6	128.49
$CoCl_2$	2	114.7	112.3
	4	129.3	114.4
$CaCl_2$	2	106.16	112
	4	105.4	123.6
$NiCl_2$	2	99.68	109
	4	111.28	131
$CuCl_2$	2	76.69	86.59
	4	77.1	86.59
$ZnCl_2$	2	46.81	49.54
	4	42.42	45.49

Table 4: Effect of inhibitors on water buffalo liver alkaline phosphatase P1 isoenzyme.

Inhibitor	Final Concention (mM)	Inhibition (%)	Residual activity (%)
Control	-----	0.0	100.0
Cysteine	2	73.96	26.03
	4	79.62	20.37
Ethylenediamine-tetra-acetic acid (EDTA)	2	73.31	26.68
	4	78.5	21.45
Dithiotheritol (DTT)	2	72.4	27.55
	4	74.9	25.05
Glutathione reduced form (GSH)	2	33.76	66.23
	4	49.67	50.32
β-Mercaptoethanol	2	7.407	92.59
	4	9.912	90.08
Phenylalanine	2	3.26	96.73
	4	14.7	85.92

Table 5: Effect of inhibitors on water buffalo liver alkaline phosphatase P2 isoenzyme

Inhibitor	Final Concentration (mM)	Inhibition (%)	Residual activity (%)
Control	-----	0.0	100.0
Ethylenediamine-tetra-acetic acid (EDTA)	2	72.7	27.28
	4	73.4	26.53
Cysteine	2	72.5	27.49
	4	75.6	24.38
Dithiotheritol (DTT)	2	70.6	29.32
	4	73.0	26.96
Glutathione reduced form (GSH)	2	41.5	58.43
	4	52.7	47.26
β-Mercaptoethanol	2	38.9	61.0
	4	72.7	27.28
Phenylalanine	2	18.36	81.63
	4	26.7	73.25

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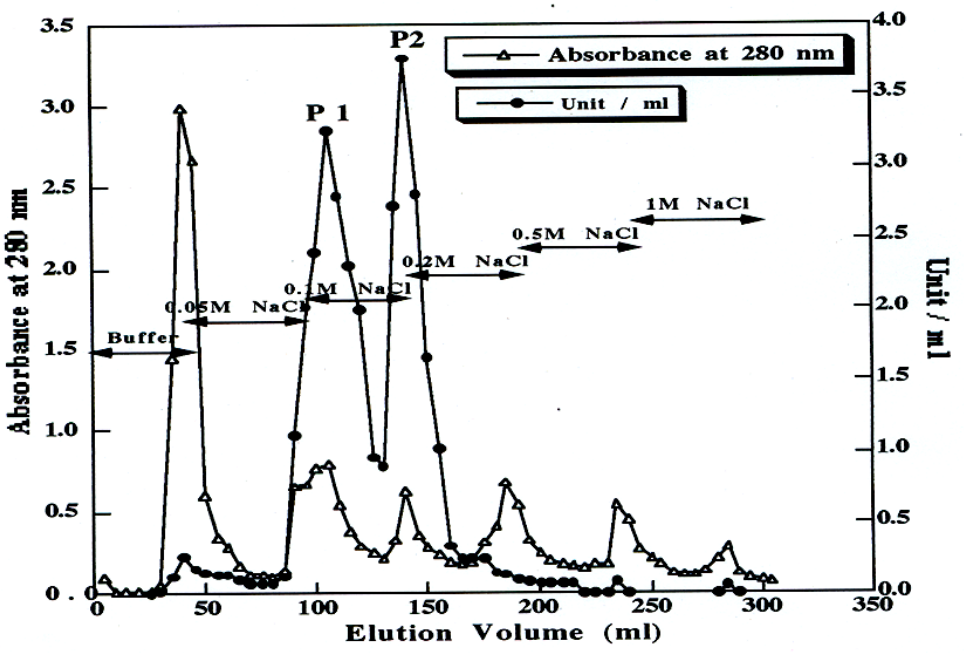

Fig. (1): A typical elution profile of water buffalo liver alkaline phosphatase on DEAE-cellulose column (22 cm X 1.6 cm i.d.) previously equilibrated with 0.05 M Tris-HCl buffer pH 7.6 containing 1 mM MgCl2. The proteins were eluted by NaCl ranging from 0 to 1 M in the equilibration buffer. 5 ml fractions were collected at a flow rate of 60 ml / h.

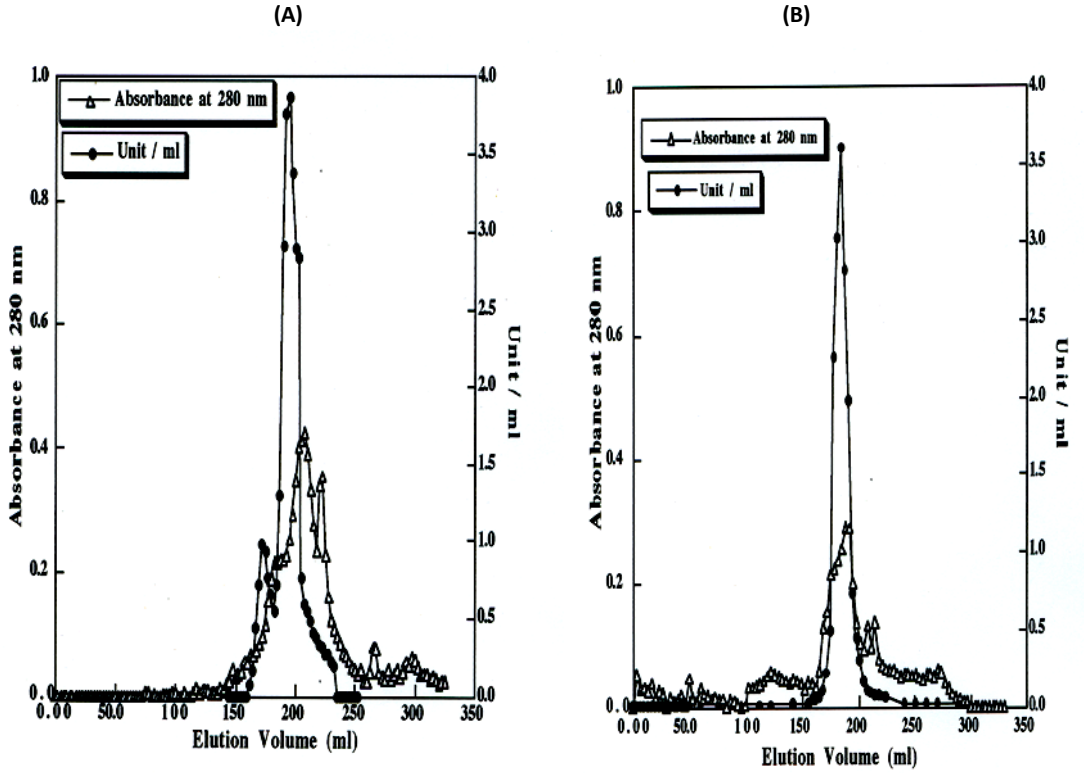

Fig. (2): (A: P1) and (B: P2): Typical elution profiles for the chromatography of the concentrated pooled DEAE-cellulose fractions containing the isoenzyme of water buffalo liver alkaline phosphatase on Sephacryl S-300 column (130 cm X 1.8 cm i.d.) previously equilibrated with 0.05 M Tris-HCl buffer pH 7.6 containing 1 mM MgCl2. The proteins were eluted by the same buffer. 3 ml fractions were collected at a flow rate of 30 ml / h.

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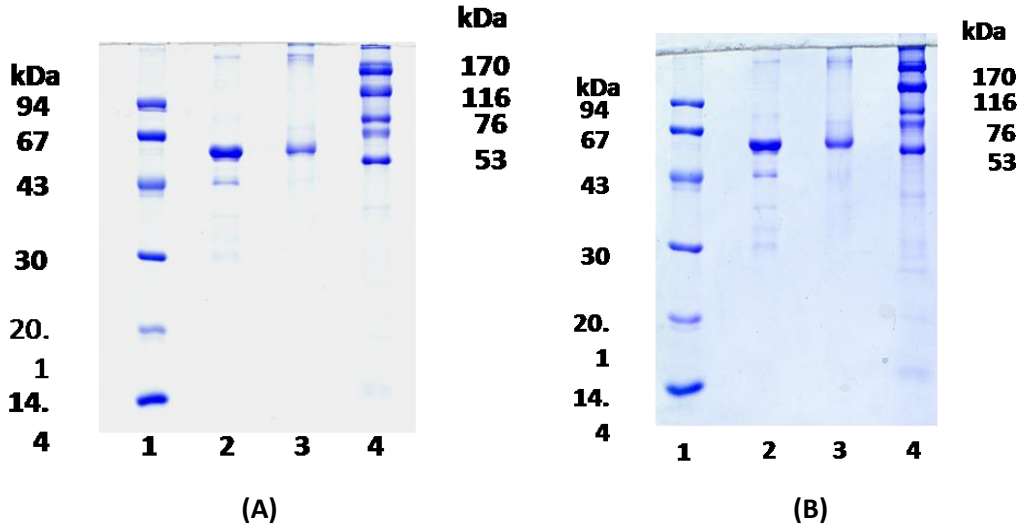

Fig. (3): (A: P1) and (B: P2) 12% SDS-polyacrylamide gel electrophoresis (PAGE) of the water buffalo liver alkaline phosphatase: [1] low molecular weight marker proteins, [2] denatured purified isoenzyme for subunit determination, [3] non-denatured purified isoenzyme and [4] high molecular weight marker proteins.

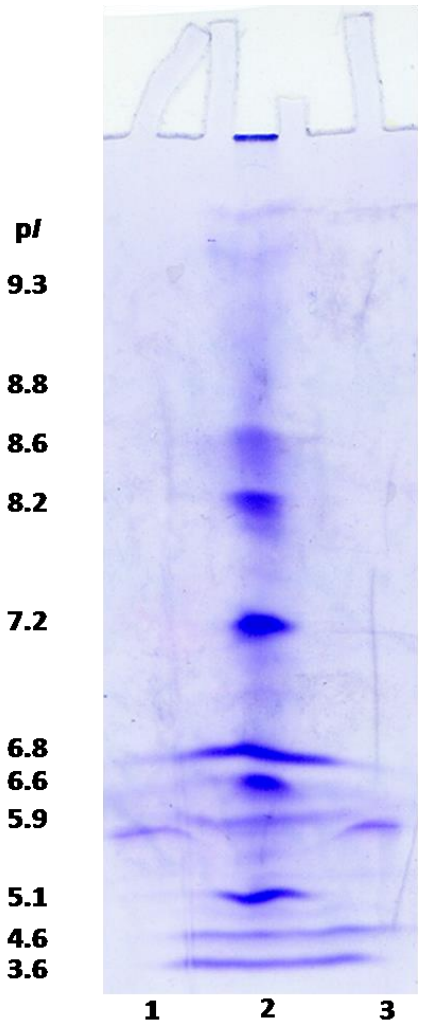

Fig. (4): Isoelectrofocusing; [1] the purified water buffalo liver alkaline phosphatase P1. [2] Isoelectric point (*pI*) marker proteins and [3] the purified water buffalo liver alkaline phosphatases P2.

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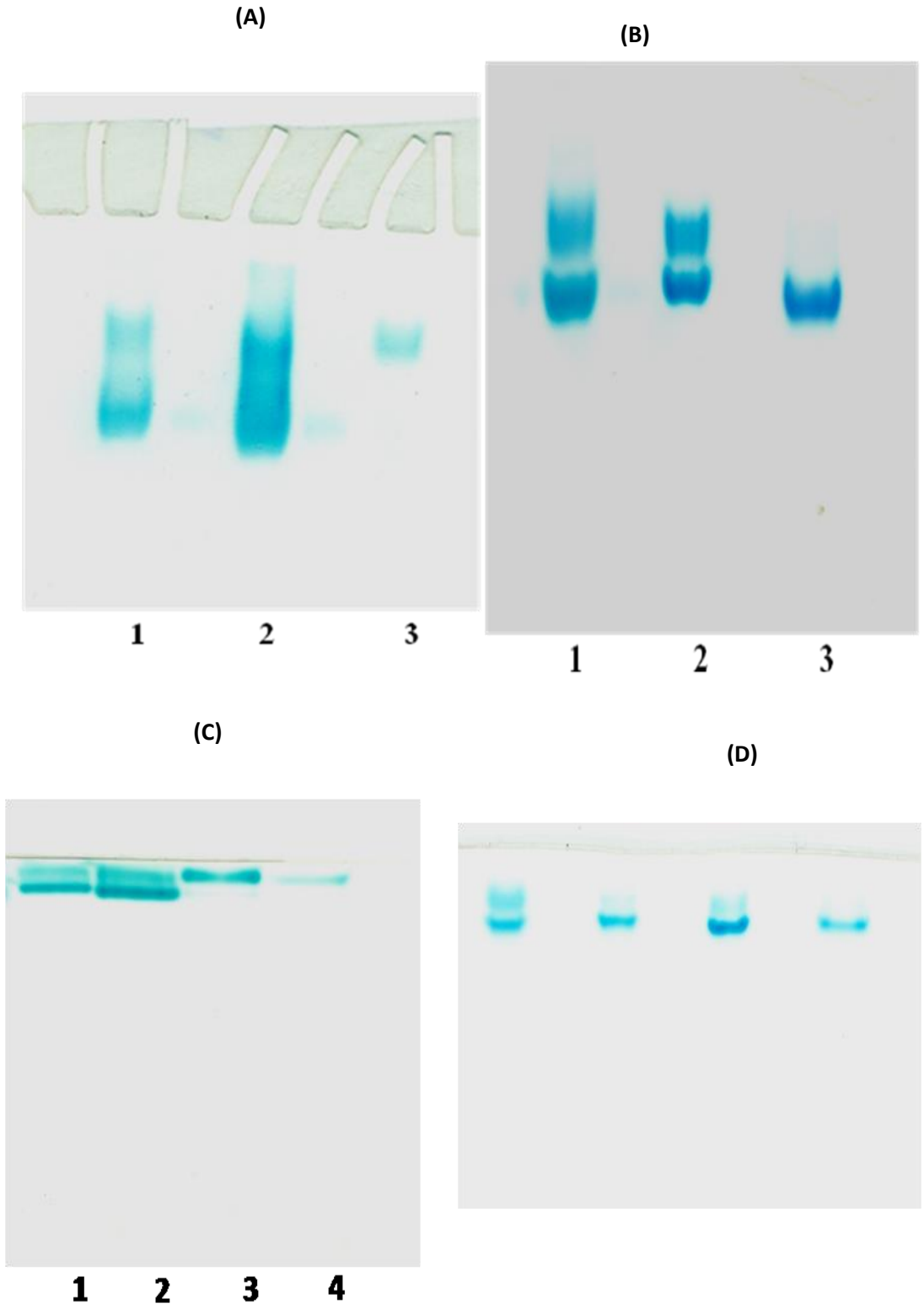

Fig. (5): (A: P1) and (B: P2) 7% Native polyacrylamide gel electrophoresis (PAGE) of the water buffalo liver alkaline phosphatases activity: [1] n-butanol extract, [2] acetone fraction [3] Sephacryl S-300 fraction isoenzyme. (C and D) 12% SDS-polyacrylamide gel electrophoresis (PAGE) of the buffalo liver alkaline phosphatases (C: P1), and (D: P2) activities: [1] n-butanol extract, [2] acetone fraction, [3] DEAE-cellulose fraction and [4] Sephacryl S-300 fraction isoenzyme.

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(A) (B)

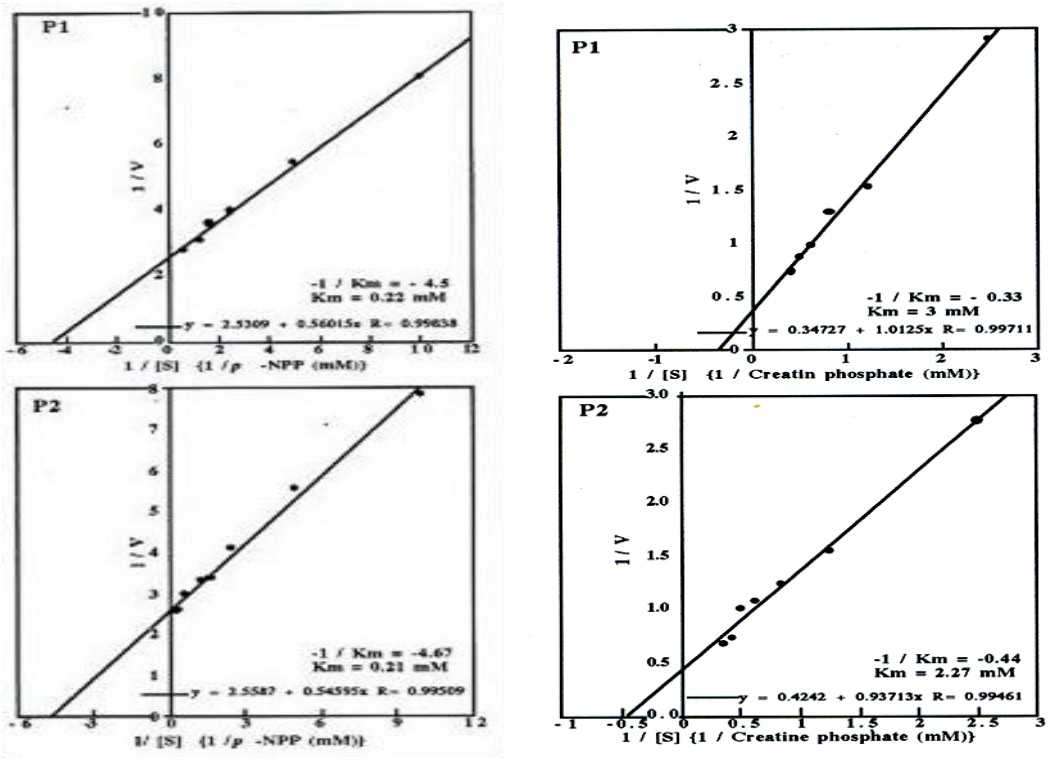

Fig. (6): (A): *p*-nitophenylphosphate (*p*-NPP)) and (B): creatine phosphate) Lineweaver-Burk plots relating the purified water buffalo liver alkaline phosphatase P1 and P2 isoenzymes reaction velocity to *p*-nitophenylphosphate (*p*-NPP) and creatine phosphate concentration in mM.

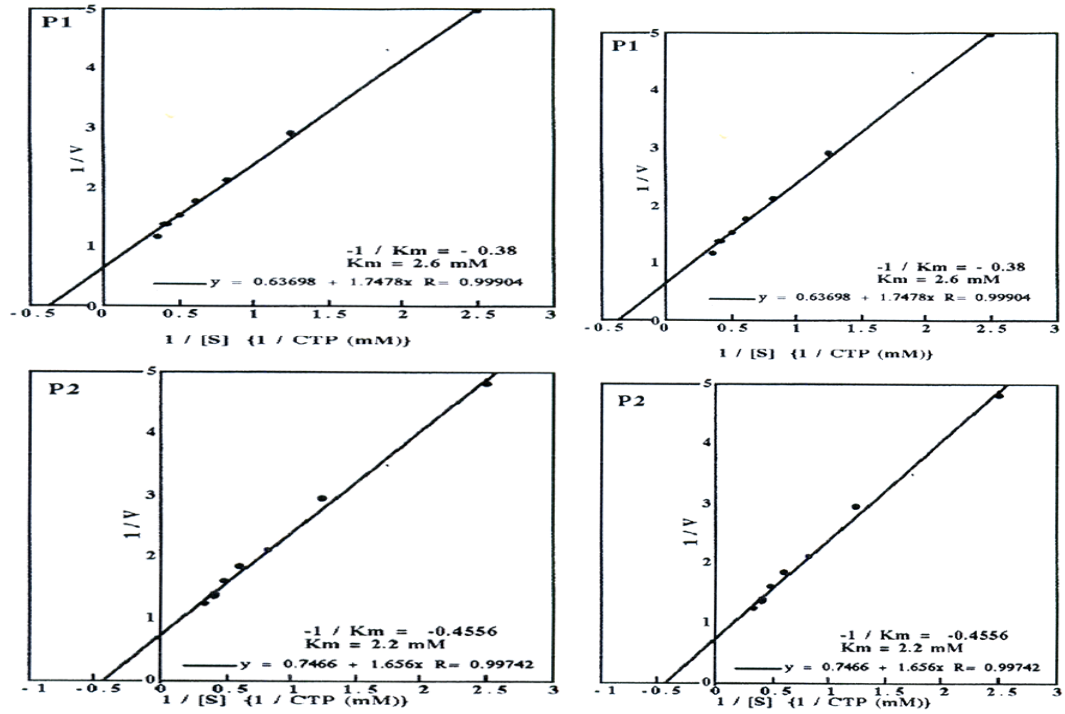

Fig. (7): (A): adenosine diphosphate (ADP) and (B): cytosine triphosphate (CTP)) Lineweaver-Burk plots relating the purified water buffalo liver alkaline phosphatase P1 and P2 isoenzymes reaction velocity to adenosine diphosphate (ADP) and cytosine triphosphate (CTP) concentration in mM.

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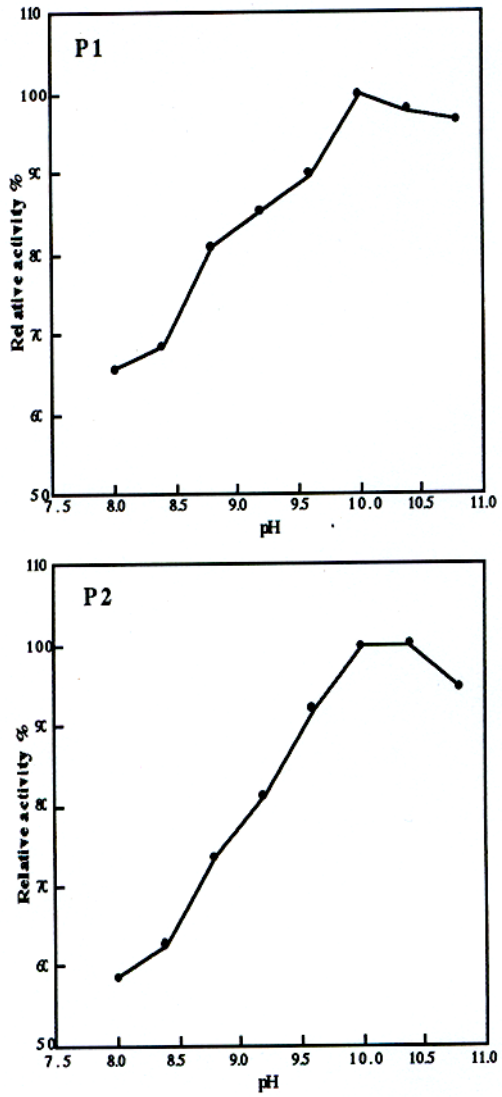

Fig. (8): Effect of pH on the purified water buffalo liver alkaline phosphatase P1 and P2 isoenzymes.

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