P- ISSN 1991-8941 E-ISSN 2706-6703 *Journal of University of Anbar for Pure Science (JUAPS)* Open Access
2007,(1), (3) :58-69

STUDYING THE EFFECTS OF SOME COMPOUNDS AND IONS ON *Escherichia coli* ALKALINE PHOSPHATASE ACTIVITY

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ARTICLE INFO

Received: 3 / 5 /2007
Accepted: 12 / 8 /2007
Available online: 19/7/2022
DOI: 10.37652/juaps.2007.15558

Keywords:
Compounds ,
Ions ,
Escherichia coli ,
Alkaline Phosphatase.

ABSTRACT

The effect of some compounds and ions such as EDTA,Urea,KCN,2-Mercaptoethanol,$BeCl_2$ and L-Phenyl alanine on the activity of Alkaline phosphatase extracted from *Escherichia coli* has been studied by using p-NitroPhenyl Phosphate as a substrate at a different concentrations (1.5×10^{-1},3.0×10^{-2},4.5×10^{-3}) M.,and the alkaline glycine regulating solution pH=10.5.Inhibition constant K_i and type of inhibition(competitive or noncompetitive)using Dixon plot for competitive & noncompetitive inhibition as well as the percentage of inhibition have all been calculated & I_{50} for different compounds.

Introduction:

Alkalinephosphatase(ALP): Orthophosporic monoester phosphohydrolase, 3.1.3.1;Phosphatases are enzymes which catalyse the spliting off of phosphoric acid from monophosphoric esters . Purified ALP from different sources exhibits three types of activity ;hydrolytic, phosphotransferase, pyrophosphatase.[1] Alkaline phosphatase is present in most tissues,the richest sources being osteoblasts in the bone ,bile canaliculi in the liver,small intestinal epithelium,proximal tubules in the kidney.[2] Alkaline phosphatase is a substrate nonspecific phosphomonoesterase,which exists in almost all living forms,from bacteria to human.It is an enzyme well known for its clinical and diagnostic applications [3] .However,the physiological role of the enzyme has not been fully elucidated.The crystal structure of the *Escherichia coli* alkaline phosphatase was first solved by [4] and refined to 2.0 A resolution [5],but the structure of the enzyme from other sources is not yet available.This enzyme cleaves phosphate from phosphomonoesters nonspecifically, or transfers the phosphoryl group to other alcohols. *E. coli* alkaline phosphatase is dimmeric and each monomer has two Zn^{2+} and one Mg^{2+} in its active site. The two zinc ions are 0.4 nm as shown in Fig. (1) [6]. Although Mg^{2+} is close to Zn^{2+} it does not directly participate in the catalytic step [7].

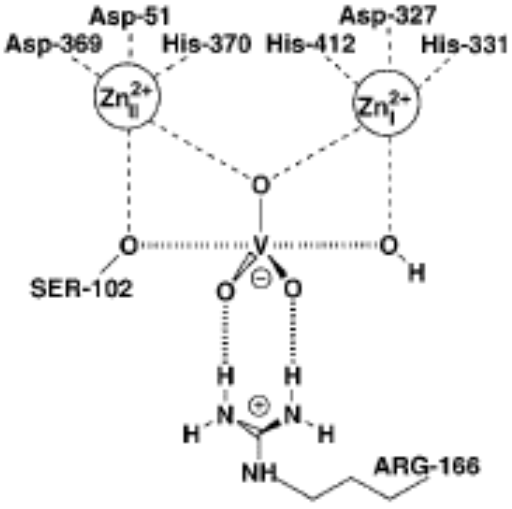

Figure 1: Active site of *E.coli* ALP

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When phosphate, Zn^{2+} and Mg^{2+} are present, the coordination is as follows:

- for Zn1: His 412, His 331, Asp 327, one oxygen of phosphate;
- for Zn $^{2+}$: His 370, Asp 51, Asp 369, one oxygen of the phosphate;
- for Mg^{2+}: Thr 155, Asp 51, Glu 322 and three water molecules.

In contrast to other metallophosphatases, the reaction mechanism involves a covalent intermediate: phosphorylated Ser. 102 (Figure 2). [8]

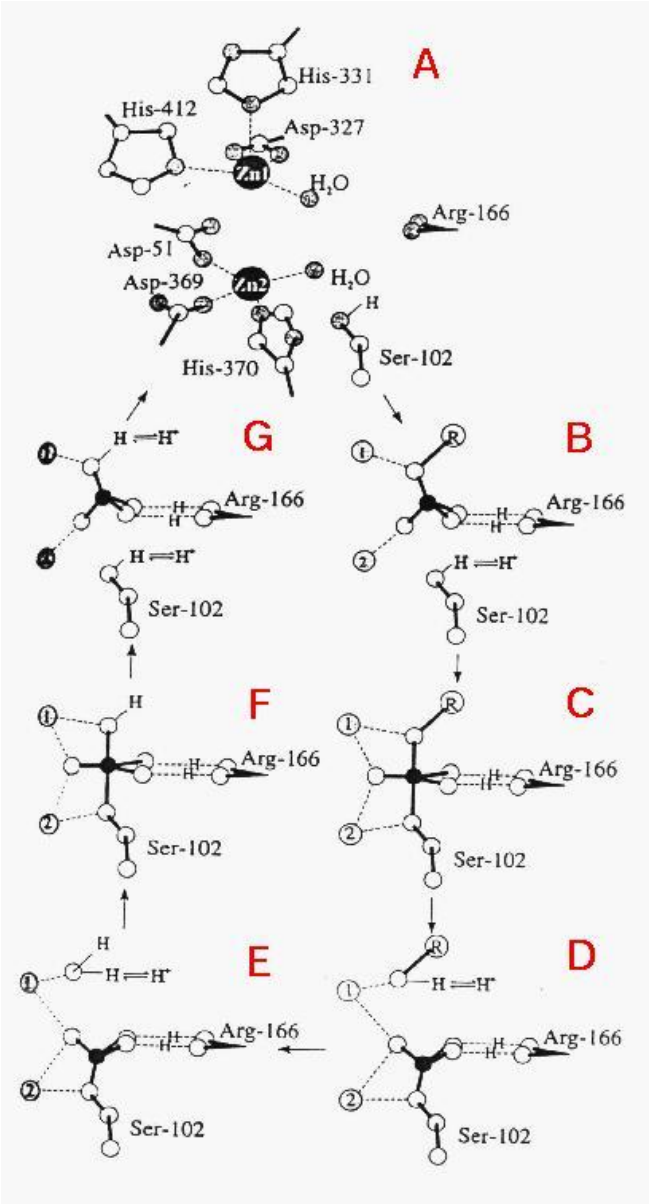

Figure 2. The two-step mechanism of alkaline phosphatase.

Molecular weight: 80,000 [8]. 86,000 [9]. 89,000 [10].Composition: *E. coli* alkaline phosphatase is a dimeric, zinc and magnesium containing protein[10][11]. Though the subunits are believed to be coded by the same gene [12], they develop molecular heterogeneity after translation [11][13]. Fernley, 1973 [14] and Chappelet-Tordo et al. 1974 [15] indicated that there are two active sites, only one is functional at a time. Two Zn^{2+} are needed for activity. A stable apoenzyme can be reactivated with Zn^{2+} , Mn^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Cd^{2+} and Hg^{2+} have been substituted for Zn but only Co restores significant activity [8]. Magnesium does not activate the apoenzyme but enhances activity of the enzyme containing two gram atoms of zinc [10] .Bloch and Schlesinger,1974 [16] reported on kinetic studies. Bock and Sheard, 1975 [17] indicate that the *E. coli* enzyme binds phosphate tightly over a wide range of pH forming complexes that may be intermediate in the hydrolytic action.

Any substance that reduced the velocity of an enzyme-catalyzed reaction can be considered to be an "inhibitor" The inhibition of enzyme activity is one of the major regulatory devices of living cells,and one of the most important diagnostic procedures of the enzymologist.Inhibition studies often tell us something about the specificity of an enzyme,the physical and chemical architecture of the active site,and the kinetic mechanism of the reaction.In our everyday life,enzyme inhibitors can be found masquerading as drugs,antibiotics,presevatives,poisons and toxins[18]. The alkaline phosphatase was inhibited by chelating agents and inorganic phosphate.Other compounds caused competitive inhibition for *E.coli* ALP activity such as Tungstate , $MoO4^{2-}$, Chromate [19] , Vanadate , Thioglycolate [20] , Arsenate [21], Phosphate [22]as well as other inhibitors of unknown

mechanism like L-cysteine [20].In a study carried out by [23]on chicken epiphyseal cartilage it was found that ALP activity was inhibited competitively by Vanadate.In another study by [24] to know the effect of some compounds on the activity of *E.coli* ALP it was found that phosphonate compounds,phosphonoacetic acid (PAA) and mercaptomethylphosphonic acid (MMP) inhibit the activity of the enzyme competitively.A. Larsson and G. Hasselgren,1979 [25] studied the activity of ALP enzyme in Rat tooth.They found that Diphosphonate,D-Penicillamine,and sodium fluoride inhibited the activity of the enzyme.

In a spectrophotometric study using p-Nitrophenyl phosphate as a substrate to measure the activity of the enzyme in mosquito,Culex tarsalis Coquillett,[26] found that Dithiotheitol,2-Mercaptoethanol,and poly vinylpyrollidone(PVP) ;inhibited ALP activity, NaF,several alternative cations Ca^{2+}, Ba^{2+}, Fe^{2+},Cu^{2+},and EDTA.They also found that inhibited the activity of ALP enzyme extracted from human uterine myoma.I.was inhibited by Zn^{2+},phosphate,fluoride,EDTA[27].

Magnesium activited ALP and showed protective effect towards inhibition by EDTA and Zn^{2+} [28].In a study carried out by [29] to measure the activity of ALP in vascular smooth muscle of Rat and Dog by using (p-NPP) as a substrate,it was found that magnesium, fluoride, vanadate and EDTA inhibited the activity of the enzyme.To measure the activity of liver ALP,Otani R,et al,1978 [30]discovered that inorganic phosphate, amino acid, EDTA, and Levamisole inhibited the activity of the enzyme [31].Hasunuma K,Ishikawa T.1977, [32] found that ALP activity extracted from *Neurospora crass* was stimulated by EDTA, $MgCl_2$,, $CoCl_2$, $MnCl_2$. When studying the activity of ALP enzyme extracted from guinea pig thymus ,[33] found that the enzyme activity was effectively inhibited by EDTA,Zn^{2+},Histidine and Urea and therefore resembling the inhibition characteristics of Alkaline phosphatase in the placenta and kidney,but not that in the liver and intestine.In a study conducted on Carcinoembryonic proteins in gastric carcinoma by Kojima J,et al,1979, [34] it was found that L-leucine,L-phenyl alanine and EDTA inhibited the activity of the enzyme.Studies of the ALP in the sperm,testes and accessory sex glands of bulls showed that ALP was inactivated strongly up to (84-97%) by Urea,L-arginine and EDTA. [35]

EXPERIMENTAL

Partial purified *E.coli* alkaline phosphatase and P-Nitrophenyl phosphate (disodium salt) were purchased from Sigma-Aldrich.Other compounds were purchased from E.Merck .

Measuring the activity of Alkaline phosphatase [36]

Principle:

Alkaline phosphatase (ALP) hydrolyate decomposes p-Nitrophenyl phosphate (pNPP)at pH buffer Alkaline (pH=9-10)to produce (p-Nitro phenol) and release the phosphate group.

The substrate (pNPP) and the solution regulating Alkaline glycine(buffer ,pH=10.5) incubate with disolved enzyme solution for 30minutes in a waterbath at 37 °C tempreture . Soduim hydroxide (NaOH) is added to stop the reaction and control the regulating solution.Absorption spectra of the produced p-Nitro phenol at maximum wave length is λmax=400 nm,while for the substrate it is at maximum wave length is λmax=300 nm.

Procedure:

1-Test sample

a-0.5ml of Alkaline glycine regulating solution is placed in the test tube with 0.5ml of substrate(pNPP).The solution then is mixed well. The tube is incubated for 5 minutes in a waterbath at 37°C tempreture .

b-0.1ml of enzyme solution is added into the tube and steadily mixed,then brought back to the waterbath for 30minutes .Incubation time is accurately measured.

c-After the incubation time, 10ml of sodium hydroxide (0.02N)are added into the tube to stop the reaction.The mixture is then well steered.

2-Control sample

a-The work is done as shown in test tube item (a).

b-0.1 ml distilled water is added instead of enzyme solution and mixed verywell.The tube is left in a waterbath at 37 °C tempreture for 30minutes.

c-After incubation time 10ml of sodium hydroxide (0.02N)are added into the tube.

3-At the same time absorption for both the test tube and controll tube is measured by using spectrophotometer at a constant wave length

λmax=400 nm.

Results and Discussion

Amount of the realsed p-Nitro phenol = AT - AC

Where:

AT= Absorption of Test tube

AC= Absorption of Controll tube.

To calculate the activity of the enzyme a series of standard solution was prepared as shown in table (1). Absorption of each prepared tube is measured at wave length 400nm by using NaOH (0.02N) as a reference.

The relationship between the measured absorption for each prepared concentration and the corresponding activity for each prepared cocentration is drawn from this realationship.The values of the activity of Alkaline phosphatase measured is calculated according to Beer-Lambert law as shown in fig.(3).

Dixon equation:

1-Competitive inhibition:

$$1/V=(K_m/V_{max}.K_i.S) [I] +(1/V_{max})\{1+K_m/ S\}\text{----- 1}$$

2-*Noncompetitive* inhibition:

$$1/V=(1+K_m / S /V_{max}.K_i) [I]+(1/V_{max})\{1+K_m/ S\}\text{--2}$$

were :

V= Velocity (activity) Vmax=Maximal velocity Ki = Inhibition constant

Km=Michaelis constant S=Substrate concentration I=Inhibitor concentration

Alkaline phosphatases (ALP) (orthophosphate monoester phosphohydrolases [alkaline optimum];(EC 3.1.3.1.) are classically described as homodimeric nonspecific metalloenzymes which catalyze phosphomonesterase reactions [37]. The fact that they are widely found in nature, from bacteria to mammals indicates that APs are included in fundamental biochemical processes [38]. Despite the fact that their physiological function is not clear, their induced production under inorganic phosphate starvation in many species (especially procaryotic organisms) indicates that they play a vital role in the phosphate metabolism. In mammals, they are linked with transport mechanisms. [39].The importance of enzymes in biological systems cannot be overstated. Enzymes are used everyday and make human life

possible. The enzyme being studied in this experiment is alkaline phosphatase. This enzyme catalyze p-Nitro-Phenyl Phosphate to p-Nitrophenol and inorganic phosphate [40]. This experiment is interesting in observing and proving reaction rates of enzyme catalyzed reactions that can be altered by inhibitory compounds.Many researches have shown the role of some bivalent ions on the structure and function of *E.coli* alkaline phosphatase. For this reason were interested to assay the effect of (EDTA, Urea, KCN,2-Mercaptoethanol, $BeCl_2$,L-Phenyl alanine) at various concentrations,on the activity of ALP from *E.coil*.Although ALP that has been long associated with mineralization process.We tested the effect of these compounds and powerful competitive and Noncompetitive inhibitors on *E.coli* ALP activity.The effect of these inhibitors can be attrbutied to their accumalation on the surface of the enzyme or their reaction with its active site.In both cases these inhibitors isolate the enzyme from its substrate.The effect of inhibitor might be temporary or it might be perminant [41].Results of the study have shown that EDTA inhibited *E.coli* ALP reversibily .It blocks the active site in the enzyme via chelating agents mechanism on Zn^{2+} ion that is very necessary for the work and activity of the enzyme.This confirmes with the results arrived at by [42][43][44]. Fig.(4),(10) Illustrates the inhibition of EDTA for *E.coli* ALP activity.Urea inhibits the activity of the enzyme via blocking the active site.This result confirms with the results arrived at by [45].They found that the activity of ALP extracted from human choronic villi is being inhibited by Urea,EDTA , L-(+)ascorbic acid and L-amino acid. Fig.(5),(11) Illustrates the inhibition of Urea for *E.coli* ALP activity. Pottasium cyanide(KCN)inhibits the activity of the enzyme reversibly.This result confirms with the results arrived at by [42] . They found that metal ion-complexing agents,like KCN,EDTA inactivate alkaline phosphatase of pig kidney.This inactivation is reversible at low concentrations of the complexing agent and irreversible at hight concentrations. Fig.(6),(12) Illustrates the inhibition of KCN for *E.coli* ALP activity.It is found that the activity of *E.coli* ALP is Competitively inhibited by L-phenyl alanine.This confirms with the results of [46] when they studied the activity of this enzyme in rat small intistine.They found that this activity was completely inhibited by 20 mM L-phenyl alanine,10 mM L-cysteine and 3mM EDTA. [47] Fig.(7),(13) Illustrates the inhibition of L-phenyl alanine for *E.coli* ALP activity.When studying the effect of the activity of *E.coli* ALP by 2-Mercaptoethanol,it was found that this material inhibited the activity of the enzyme.This conforms with the results of [48]when they studied the activity of the enzyme extracted from rat small intistine. They found that some compounds inhibited the activity of the enzyme via 2-Mercaptoethanol , Theophylline , phenyl alanine and,ethylenediaminetetraacetic acid (EDTA).Fig.(8)&(14) Illustrates the inhibition of 2-Mercaptoethanol for *E.coli* ALP activity.When studying the effect of Be^{2+} on the activity of *E.coli* ALP,it was found that this ion irreversibley inhibited the enzyme activity.It blocks the active site of the enzyme.This confirms with results arrived at by[49]when studying the activity of ALP extracted from bovine fetal epiphyseal cartilage.

They found that ALP activity is inhibited irreversibly by Be^{2+}, EDTA, EGTA, ethane-1-droxydiphonate, dichloromethanediphosphonate , L-cysteine , phenyl- ethylsulphonylfluoride Fig.(9)&(15) Illustrates the inhibition of Be^{2+} for *E.coli* ALP activity.Table (3),(4)&(5) : Include also the type of inhibition,percentage of inhibition and inhibition constant (K_i),Conc. that inhibtion 50% of enzyme activity (I_{50}).

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Table (1) :Represents standard solutions prepared to measure the ALP activity

Tube No.	Working standard (ml)	p-Nitro phenol (µ mole)	0.02N, NaOH (ml)	ALP activity Equivelent to I.U (µ mole/min./L.)
1	0.1	0.006	11.0	2.0
2	0.2	0.012	10.9	4.0
3	0.3	0.018	10.8	6.0
4	0.4	0.024	10.7	8.0
5	0.5	0.030	10.6	10.0
6	1.0	0.060	10.1	20.0
7	2.0	0.120	9.1	40.0

Table (2) : The types and concentraions of inhibitors

Material	Concentration mM					
EDTA	0.025	0.050	0.1	0.15	0.2	0.25
Urea	0.01	0.02	0.03	0.04	0.05	0.06
KCN	0.002	0.004	0.006	0.008	0.01	0.02

$BeCl_2$	L-phenyl alanine	2-Mercapto ethanol
0.004	0.002	0.0006
0.008	0.004	0.0012
0.012	0.006	0.0024
0.016	0.008	0.0048
0.020	0.01	0.0095
0.024	0.02	0.018

P-Nitrophenyl phosphate(p-NPP) as a substrate at a different concentratuions ($1.5*10^{-1}$,$3.0*10^{-2}$,$4.5*10^{-3}$) M.

Table (3):The Effect of some compounds and ions on the *E.coli* ALP activity with values of inhibition constant (K_i).

No.	Material	Type of effect	Inhibition constant K_i ,Mole
1.	EDTA	Noncompetitive	0.05×10^{-3}
2.	Urea	Noncompetitive	0.035×10^{-3}
3.	KCN	Noncompetitive	0.0075×10^{-3}
4.	L-phenyl alanine	Competitive	0.0015×10^{-3}
5.	2-Mercaptoethanol	Noncompetitive	0.005×10^{-3}
6.	Be^{2+}	Noncompetitive	0.0045×10^{-3}

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Table (4): Percentage of inhibition of E.coli ALP enzyme by different Inhibitors

Conc. Of Substrate p-NPP	Inhibitors	Inhibition Percentage %
$1.5*10^{-1}$ M	EDTA	79.39%
$3.0*10^{-2}$ M		80.51%
$4.5*10^{-3}$ M		86.28%
$1.5*10^{-1}$ M	Urea	38%
$3.0*10^{-2}$ M		39.22%
$4.5*10^{-3}$ M		52%
$1.5*10^{-1}$ M	KCN	45.5%
$3.0*10^{-2}$ M		52.79%
$4.5*10^{-3}$ M		56.30%
$1.5*10^{-1}$ M	L-phenyl alanine	46%
$3.0*10^{-2}$ M		47.87%
$4.5*10^{-3}$ M		51.26%
$1.5*10^{-1}$ M	2-Mercaptoethanol	68.88%
$3.0*10^{-2}$ M		69.66%
$4.5*10^{-3}$ M		79.36%
$1.5*10^{-1}$ M	Be^{2+}	80.44%
$3.0*10^{-2}$ M		81.56%
$4.5*10^{-3}$ M		82.99%

Table (5): The conc. of I50 that inhibition of E.coli ALP enzyme activity by different inhibitors

No.	Compounds and Ions	Conc. Of I_{50} mM
1.	EDTA	0.094
2.	Urea	٠.٠٢٩٣
3.	KCN	٠.٠٠٦٢
4.	L-phenyl alanine	٠.٠٠٧٣
5.	2-Mercaptoethanol	٠.٠٤٩
6.	Be^{2+}	٠.٠١٢٦

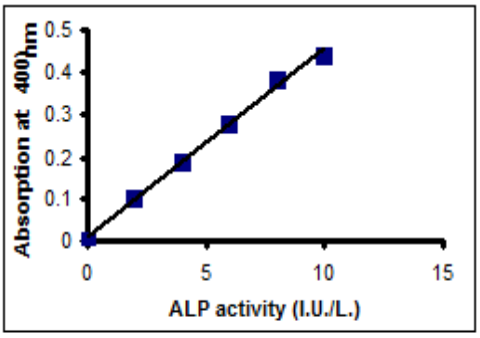
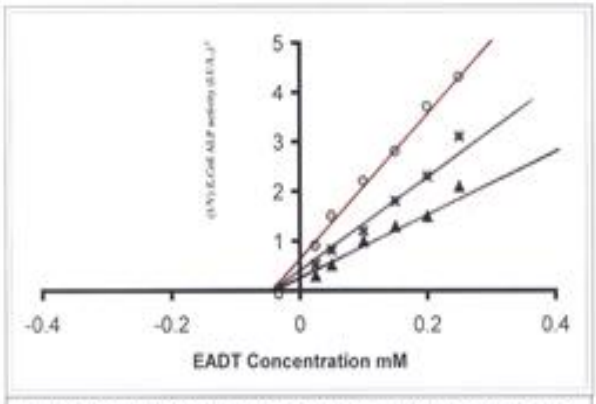
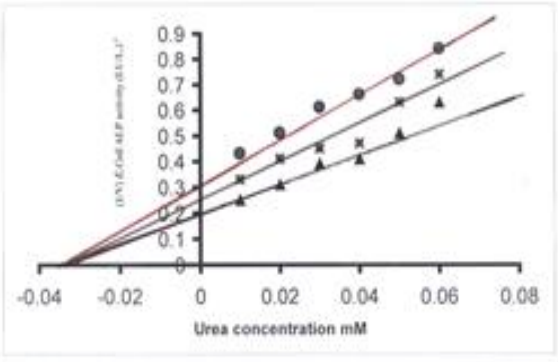


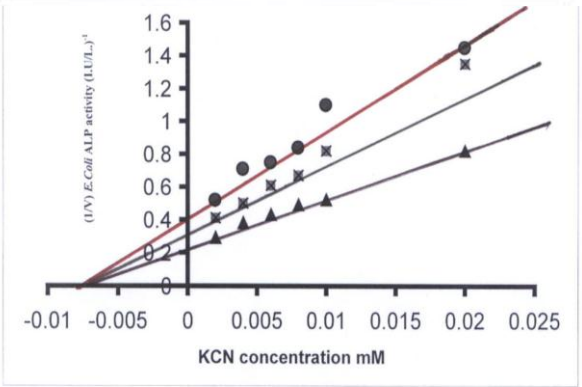
Fig. (3):Standard curve for measuring alkaline phosphatase activity at 400nm



Fig(4):Inhibition of *E.Coli* ALP activity by EDTA at different concentration of substrate (p-NPP) ▲- $1.5*10^{-1}$ M ■ -$3.0*10^{-2}$ M ●- $4.5*10^{-3}$ M



Fig(5):Inhibition of *E.Coli* ALP activity by Urea at different concentration of substrate (p-NPP) ▲- $1.5*10^{-1}$ M ■ -$3.0*10^{-2}$ M ●- $4.5*10^{-3}$ M



Fig(6):Inhibition of *E.Coli* ALP activity by KCN at different concentration of substrate (p-NPP) ▲- $1.5*10^{-1}$ M ■ -$3.0*10^{-2}$ M ●- $4.5*10^{-3}$ M

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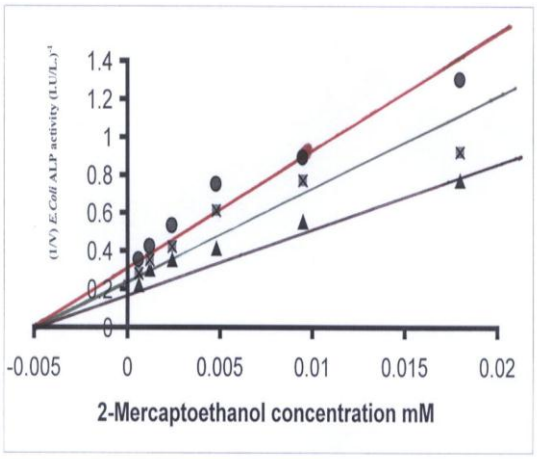
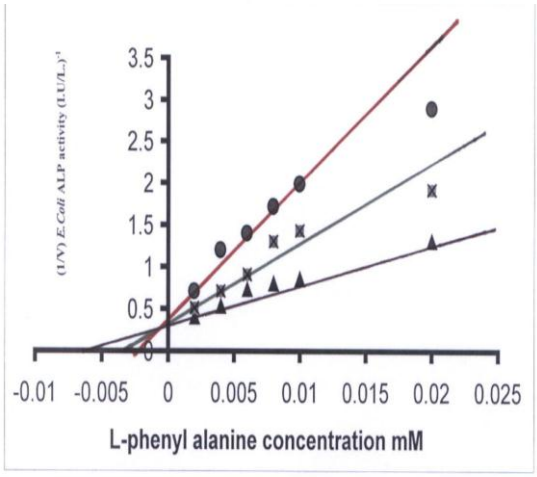
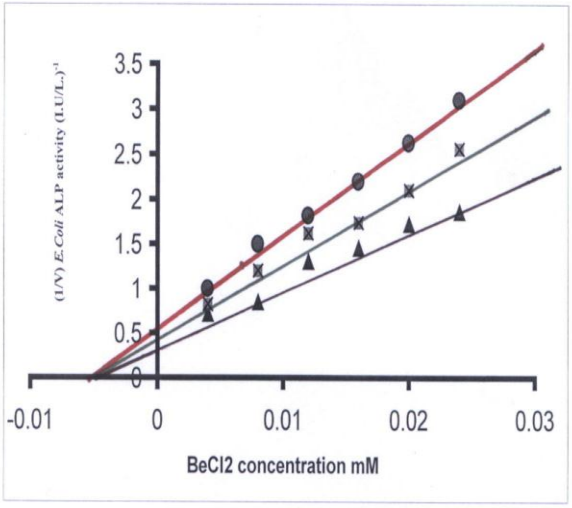


Fig(7):Inhibition of *E.Coli* ALP activity by 2-Mercaptoethanol at different concentration of substrate (p-NPP) ▲- $1.5*10^{-1}$ M ■ -$3.0*10^{-2}$ M ● - $4.5*10^{-3}$ M



Fig(8):Inhibition of *E.Coli* ALP activity by L-Phenyl alanine at different concentration of substrate (p-NPP) ▲- $1.5*10^{-1}$ M ■ -$3.0*10^{-2}$ M ● - $4.5*10^{-3}$ M



Fig(9):Inhibition of *E.Coli* ALP activity by $BeCl_2$ at different concentration of substrate (p-NPP) ▲- $1.5*10^{-1}$ M ■ -$3.0*10^{-2}$ M ● - $4.5*10^{-3}$ M

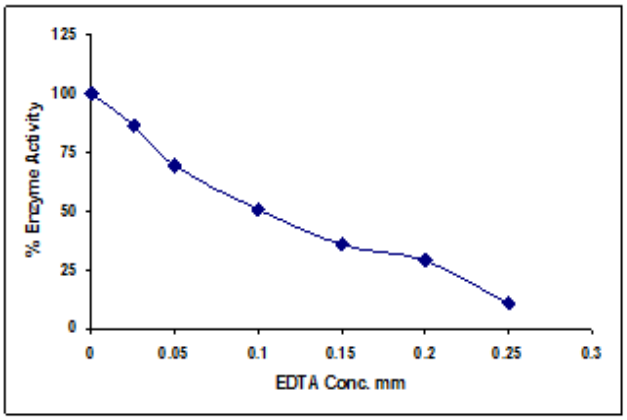


Fig.(10):Activity % vs EDTA concentration regression analysis graphs for *E.coli* ALP in the presence of 6 different EDTA concentrations.

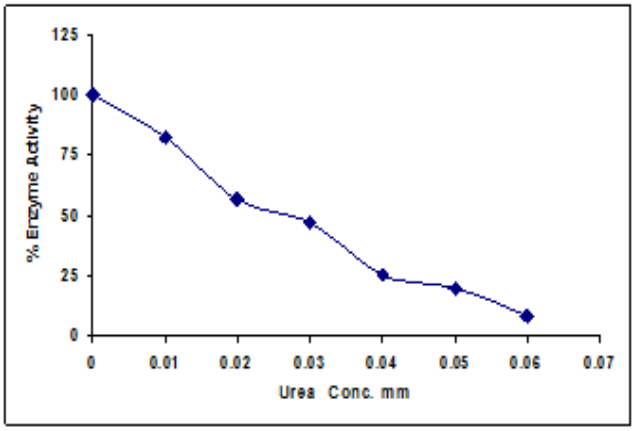


Fig.(11):Activity % vs Urea concentration regression analysis graphs for *E.coli* ALP in the presence of 6 different Urea concentrations.

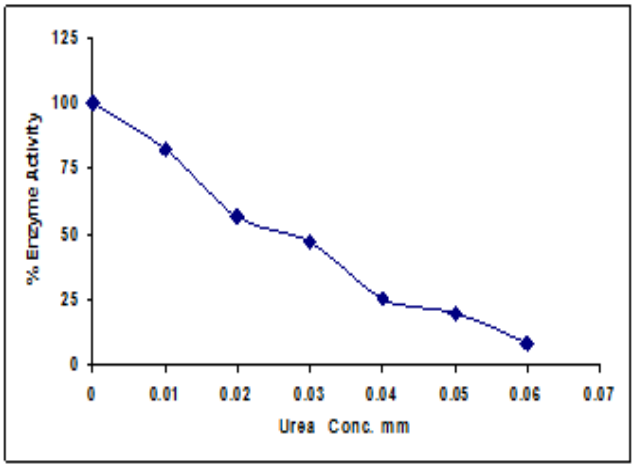


Fig.(11):Activity % vs Urea concentration regression analysis graphs for *E.coli* ALP in the presence of 6 different Urea concentrations.

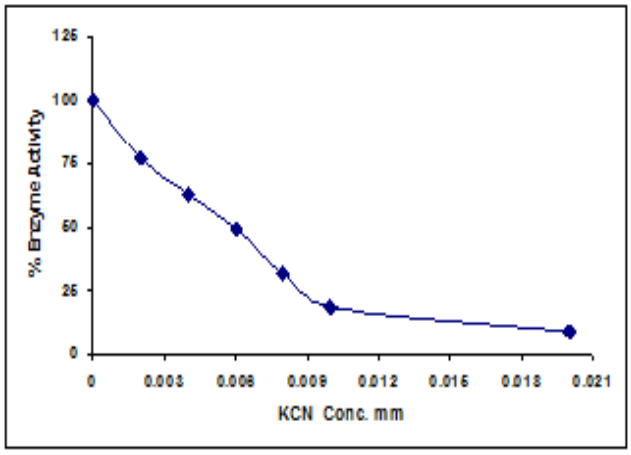


Fig.(12):Activity % vs KCN concentration regression analysis graphs for *E.coli* ALP in the presence of 6 different KCN concentrations.

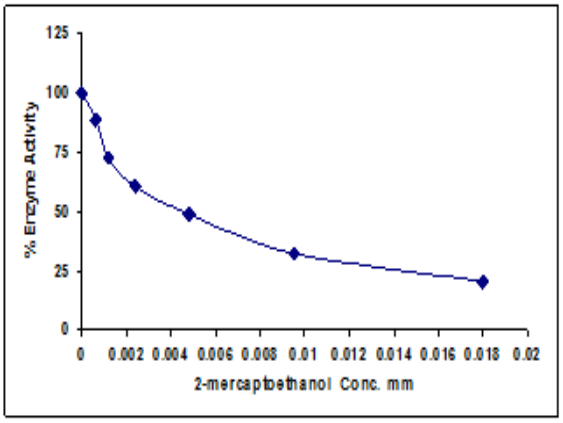


Fig.(13):Activity % vs 2-Mercaptoethanol concentration regression analysis graphs for *E.coli* ALP in the presence of 6 different 2-Mercaptoethanol concentrations.

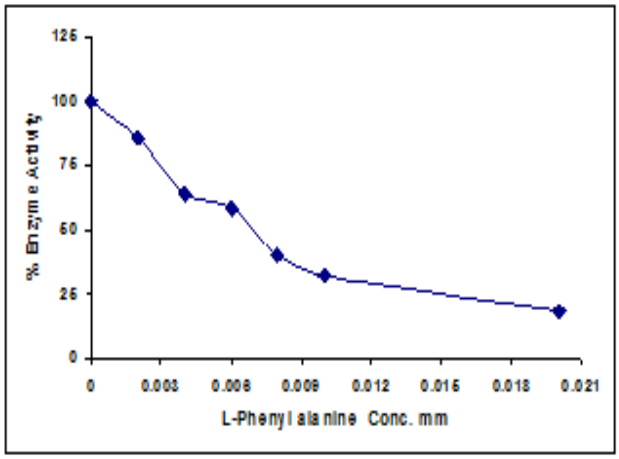


Fig.(14):Activity % vs L-Phenyl alanine concentration regression analysis graphs for *E.coli* ALP in the presence of 6 different L-Phenyl alanine concentrations.

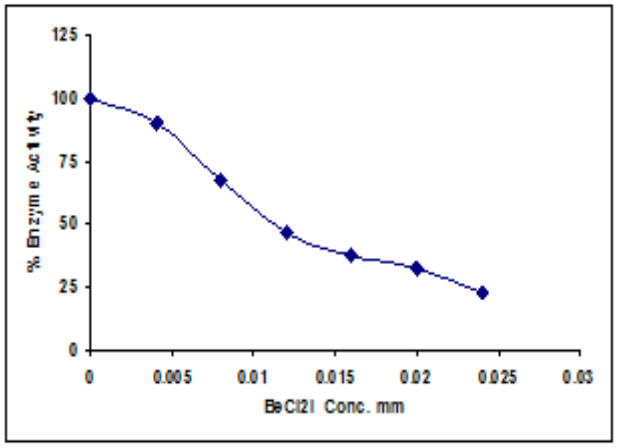


Fig.(15):Activity % vs $BeCl_2$ concentration regression analysis graphs for *E.coli* ALP in the presence of 6 different $BeCl_2$ concentrations.

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P- ISSN 1991-8941 E-ISSN 2706-6703 *Journal of University of Anbar for Pure Science (JUAPS)* Open Access
2007,(1), (3) :58-69

دراسة تأثير بعض المركبات والأيونات على فعالية أنزيم الفوسفاتيز القاعدي من بكتيريا القولون

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الخلاصة

تم دراسة تأثير بعض المركبات والأيونات مثل EDTA و Urea و KCN و 2-Mercaptoethanol و $BeCl_2$ و L-Phenyl alanine على فعالية أنزيم الفوسفاتيز القاعدي المستخلص من بكتريا الاشيريشيا القولون *Escherichia coli* باستخدام p-NitroPhenylPhosphate(p-NPP) كمادة أساس وبتراكيز مختلفة (1.0×10^{-1} , 3×10^{-2} , 4.0×10^{-3}) مولاري والمحلول المنظم الكلايسين القاعدي pH =10.5.وتم حساب ثابت التثبيط K_i ونوعية التثبيط (تنافسي أو غير تنافسي) باستخدام معادلة دكسون للتثبيط التنافسي والتثبيط الغير تنافسي وكذلك تم حساب النسبة المئوية للتثبيط، كما تم حساب الثابت الذي يثبط ٥٠% من فعالية الأنزيم I_{50} للمركبات أعلاه بتراكيز مختلفة وباستخدام تركيز ثابت من المادة الأساس (3×10^{-2}) مولاري .