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The Effects of Various Divalent Cations on the Enzyme Activity of Bovine Intestinal Alkaline Phosphatase

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Alkaline phosphatases have binding sites for Zn^{2+} and Mg^{2+}, on which enzyme activity is dependent. In this experiment, we tested for the effects of Mg^{2+}, Zn^{2+}, Co^{2+} and Ca^{2+} on the enzymatic conversion of synthetic pNPP substrate to nitrophenol by bovine intestinal alkaline phosphatase. Different pNPP concentrations were used to determine the V_{max} and K_m of the reaction for each cation. Higher enzyme activities were observed for the Co^{2+} and Ca^{2+} tests when compared with the positive control, in which no divalent cations were added, but only at low (< 1.5 mM) substrate concentrations. The apparent increase in enzyme activity could be attributed to the displacement of Zn^{2+} from its binding site by Co^{2+}; this possibly led to a more stable enzyme-substrate complex and a higher release rate of phosphate from the phosphoenzyme complex, after hydrolysis of the phosphomonoester bond. Enzyme activity appeared to be inhibited in the assay buffer with Zn^{2+}. No appreciable activity was observed in the negative control containing EDTA.

In experiment A3 (6), an unexplained decrease in enzyme activity (observation 3) was reported: as enzyme concentration increased, enzyme activity decreased. Observation 5 stated that the activity rate was 20 times faster in Tris than in glycine buffer at the same pH. Because divalent cations can have significant effects on enzyme activity, we wondered whether the observations could be the result of contaminating cations. For example, an inhibitory cation could be responsible for the unexpected decrease in specific activity as enzyme activity increased in A3, while an enzyme-activating cation could be present in the Tris buffer to cause the increase in activity.

Some alkaline phosphatases have binding sites for Zn^{2+} and Mg^{2+}. The presence of certain divalent cations can have either stimulatory or inhibitory effects on these enzymes (www.worthington_biochem.com/manual/BAP.html). Cobalt and zinc cations, for instance, can act as positive allosteric effectors to some alkaline phosphatases (2).

An interesting venture would thus be to determine whether the ions, namely Mg^{2+}, Zn^{2+}, Co^{2+}, and Ca^{2+}, have a profound effect on the activity of BIAP, as divalent cations are generally required for optimal enzyme activity. We hypothesize that these four cations will stimulate the activity of BIAP, acting as positive allosteric effectors to increase the rate of the enzymatic reaction. As well, the cations may be able to act as a substitute for Mg^{2+}.

MATERIALS AND METHODS

Enzyme, substrate, and cations. Alkaline phosphatase (2 mg/ml; bovine intestinal, B-grade, CalBiochem) and pNPP (2 mg/ml; Sigma Chemical Company) were prepared by dissolving each reagent separately in de-ionized distilled water, supplied by Mrs. Ehleen Hinze (Department of Microbiology and Immunology, UBC). The divalent cations used in this study were in the form of inorganic salts: $MgCl_2$, $CoCl_2$, $ZnCl_2$, and $CaCl_2$; each of which was dissolved in 40.0 ml of glycine buffer (10 mM; pH 10) to give a final concentration of 2 mM. The salts were provided by Mr. Addy Po (Department of Microbiology and Immunology, UBC).

Standard Enzyme Assay. The assay that determined the effects of different salts on the activity of BIAP was performed according to the protocol of Experiment A3 (6) with the following modifications. De-ionized distilled water replaced the use of distilled water in the assay. Different volumes of pNPP (0.05 ml, 0.1 ml, 0.3 ml, 0.5 ml, 1.0 ml) were used to determine the V_{max} and K_m of the BIAP reaction for each salt. $MgCl_2$ was sequentially replaced with $CoCl_2$, $ZnCl_2$, and $CaCl_2$. No salt was added for the positive control; similarly, only EDTA (8 mM; bovine pancreas, Sigma Chemical Company) was included in the assay for the negative control. The assay was repeated once for each of the salts tested.

RESULTS

All the divalent cations, with the exception of Mg^{2+}, appeared to decrease the catalytic efficiency of BIAP, as quantified in terms of V_{max}, when compared with the positive control (Table 1). The addition of 2 mM Mg^{2+} may have increased the V_{max} of the enzyme by ~11%. When EDTA was added, the catalytic activity diminished to an essentially zero level.

Table 1. V_{max} , K_m , and K_a values of the bovine intestinal alkaline phosphatase using different cofactors.

Cofactor	V_{max} (units/ml)	K_m (mM)	K_a (M^{-1} s^{-1})
Control	0.96	1.43	0.67
Mg^{2+}	1.07	1.65	0.65
Zn^{2+}	0.57	2.78	0.21
Co^{2+}	0.79	0.47	1.68
Ca^{2+}	0.73	0.63	1.16
EDTA	0.01	0.02	0.50

The presence of additional Co^{2+} and Ca^{2+} decreased the K_m of the enzyme reaction by 67% and 56%, respectively. Mg^{2+} and Zn^{2+} increased the K_m of the enzyme reaction by 15% and 94%, respectively. When EDTA was added, the K_m dropped to nearly zero.

The specificity constant K_a, which defines the rate of reaction at low concentrations of substrate, was calculated to be significantly higher than the positive control for the reactions in which Co^{2+} and Ca^{2+} were added. Specificity improved by factors of 2.5 and 1.7, respectively. Specificity was largely unaffected by Mg^{2+}, but was impaired by EDTA and especially by Zn^{2+}.

DISCUSSION

Of the cations examined, Co^{2+} appeared to have enhanced the activity of BIAP best, at least at low substrate concentrations, when the enzyme reaction was ran in the presence of Mg^{2+} and Zn^{2+} that were packaged with the enzyme. The Ca^{2+} cation produced similar effects. However, these observations were different from those of most reported alkaline phosphatases that contain two Zn^{2+} and one Mg^{2+} binding sites per unit (4). The observed increase in BIAP activity could, in theory, be attributed to the displacement of Zn^{2+} by Co^{2+} (or Ca^{2+}) from the binding sites. Such displacement possibly led to a more stable enzyme-substrate complex, thereby enabling BIAP to be more active catalytically. A related chemical explanation could be that dissociation of the product phosphate (P_i) is generally thought to limit enzyme turnover at high pH (http://pps98.cryst.bbk.ac.uk/assignment/projects/grembecka/chapter33.html). The stimulatory effects of Co^{2+} could thus be attributed to facilitating the dissociation of P_i from the phosphoenzyme complex better than Zn^{2+}. Because Co^{2+} is less electrophilic than Zn^{2+}, Co^{2+} could have bound less tightly to P_i than Zn^{2+}, allowing more enzyme to be available in the reaction. Furthermore, in many Zn^{2+} requiring enzymes, such as carboxypeptidase A and carbonic anhydrase, Zn^{2+} can be replaced by Co^{2+} without loss of much activity.

However, from a more practical perspective, neither Co^{2+} nor Ca^{2+} could improve the overall catalytic efficiency of BIAP since they both resulted in lower ultimate V_{max} values than the positive control enzyme reaction with no additional cofactors. In other words, it could be said that the activity at higher substrate concentrations is being traded off for enhanced activity at lower substrate concentrations. This, however, is usually impractical since small amounts of enzyme are added to vastly larger amounts of substrate.

The presence of Zn^{2+} resulted in the second lowest V_{max}, highest K_m, and lowest K_a, suggesting that Zn^{2+} had an overall negative effect on the enzyme activity of BIAP. The low V_{max} indicated that the presence of Zn^{2+} might have inhibitory effects on the ability of the enzyme to catalyze the reaction; the high K_m suggested that the presence of Zn^{2+} resulted in a lowered affinity of the enzyme for the substrate. The low K_a value indicated that the specificity of BIAP for pNPP was decreased in the presence of Zn^{2+}.

Based on these observations, it might be possible to suggest the following model to describe the effects that Zn^{2+} had on BIAP enzyme kinetics. Because increased concentrations of substrate appeared unable to reconstitute the V_{max} to higher levels, it was unlikely that Zn^{2+} was a competitive inhibitor that bound to the substrate binding site. The zinc ion might have bound to another site on the enzyme and induced conformational changes that reduced both the catalytic efficiency of the enzyme and the ability for the enzyme to recognize and bind pNPP. For example, it might have bound to the site on the enzyme to which Mg^{2+} normally binds. Even though a low concentration of Zn^{2+} is required for catalysis (for this reason, a small amount of Zn^{2+} is packaged with the enzyme), if Zn^{2+} is present in excessive amounts, it may displace the Mg^{2+} ion at the active site, thus counteracting the stabilizing effects of Mg^{2+} (5).

The ability of Zn^{2+} to impair the activity of alkaline phosphatases via the induction of conformational changes has been observed before, as in the case of the rat matrix-induced alkaline phosphatase (3).

The V_{max} of the negative control, EDTA, was the lowest. EDTA inactivated the activity of BIAP by chelating the magnesium ion and the two required zinc ions originally present at the enzyme's active site (5). Without these zinc ions, BIAP could not have catalyzed the conversion of pNPP to nitrophenol and phosphate even at high pNPP concentrations, thereby resulting in the experimentally low V_{max}. Furthermore, the chelation could have induced a conformational change in the enzyme that inhibited the reaction. An interesting observation is that the binding affinity between BIAP and pNPP in the presence of EDTA was the highest (inferred from the low K_m of 0.02 mM) when compared with the enzymes treated with the divalent cations. The K_m for the negative control was determined to be the lowest, but since BIAP was rendered ineffective by EDTA, the enzyme could not have converted substrate into product efficiently despite the increase in binding affinity. This may be interpreted as irreversible binding of the enzyme to the substrate; BIAP might have bound pNPP, but with no subsequent catalysis and no dissociation of product. The K_a, which was calculated to be ~34% less than that of the positive control, can be interpreted in one of two ways. One could say that the enzyme treated with EDTA bound the substrate strongly and permanently, without significant loss in substrate specificity; or perhaps, the K_a was in fact meaningless in this case as the V_{max} and K_m values were negligible.

ACKNOWLEDGEMENTS

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REFERENCES

1. **Bugg, T.** 1997. An introduction to enzyme and coenzyme chemistry, Blackwell Science Ltd., Oxford, London.
2. **Cathala, G., and C. Brunel.** 1975. Bovine kidney alkaline phosphatase. Purification, subunit structure, and metalloenzyme properties. J. Biol. Chem. 250:6040-5.
3. **Ciancaglini, P., J. M. Pizauro, M. J. Grecchi, C. Curti, and F. A. Leone.** 1989. Effect of Zn(II) and Mg(II) on phosphohydrolytic activity of rat matrix-induced alkaline phosphatase. Cell Mol. Biol. 35:503-10.
4. **Dong, G., and J. G. Zeikus.** 1997. Purification and characterization of alkaline phosphatase from *Thermotoga neapolitana*. Enzyme Microbiol. Technol. 21:335-40.
5. **Ensinger, H. A., H. E. Pauly, G. Pfleiderer, and T. Stiefel.** 1978. The role of Zn(II) in calf intestinal alkaline phosphatase studied by the influence of chelating agents and chemical modification of histidine residues. Biochim. Biophys. Acta. 527:432-41.
6. **Ramey, W. D.** 2002. Experiment A3, Properties of Alkaline Phosphatase at different substrate concentrations, different enzyme concentrations and different reaction pH, p. A3:1-5. *In* Microbiology 421 Manual of Experimental Microbiology. University of British Columbia, Vancouver.

APPENDIX

Table A1. Observations and results of the standard assay with $CoCl_2$
(The symbols, *, §, and β, refer to sample calculations.)

2mg/ml pNPP (ml)	Time (s)	Raw Data		Average (A_{420})	For the Michaelis-Menten Plot (Figure B)		For the Lineweaver-Burk Plot (Figure C)	
		Trial 1 (A_{420})	Trial 2 (A_{420})		Enzyme Activity (unit/ml)	[pNPP] (mmol/L)	1/ enzyme activity	1/[pNPP]
0.05	**10**	0.031	0.037	*0.034	§0.125	β0.0869	8.00	11.5
	20	0.046	0.051	0.0485				
	30	0.061	0.064	0.0625				
	40	0.076	0.076	0.076				
	50	0.089	0.089	0.089				
	60	0.101	0.101	0.101				
0.1	**10**	0.044	0.06	0.052	0.195	0.174	5.12	5.75
	20	0.068	0.084	0.076				
	30	0.09	0.106	0.098				
	40	0.11	0.13	0.12				
	50	0.131	0.151	0.141				
	60	0.14	0.171	0.1555				
0.3	**10**	0.091	0.116	0.1035	0.435	0.521	2.30	1.92
	20	0.139	0.166	0.1525				
	30	0.193	0.212	0.2025				
	40	0.242	0.257	0.2495				
	50	0.287	0.3	0.2935				
	60	0.334	0.34	0.337				
0.5	**10**	0.11	0.15	0.13	0.530	0.869	1.89	1.15
	20	0.161	0.212	0.1865				
	30	0.223	0.272	0.2475				
	40	0.287	0.33	0.3805				
	50	0.338	0.384	0.361				
	60	0.386	0.438	0.412				
1.0	**10**	0.139	0.196	0.1675	0.636	1.74	1.57	0.575
	20	0.21	0.269	0.2395				
	30	0.295	0.34	0.3175				
	40	0.364	0.408	0.386				
	50	0.426	0.474	0.45				
	60	0.48	0.532	0.506				

Sample Calculations

* **Average Absorbance Value** (A_{420}) = $\frac{\text{Trial 1 } A_{420} + \text{Trial 2 } A_{420}}{2}$

$= \frac{0.031 + 0.037}{2} = \mathbf{0.034}$

§ E**nzyme Activity** (units/ml) = $\frac{\Delta A}{\Delta t} \times \frac{103}{20{,}000} \times \frac{N_v}{E_v}$

Where (6):

1 unit	=	1μmole of nitrophenol product formed per minute
Δ A	=	change in absorbance at a wavelength of 420 nm
Δ t	=	time in minutes for the observed change in absorbance.
20,000	=	the molar extinction coefficient of p-nitrophenol in Spectronic 20 tube (the absorbance caused by one molar solution of p-nitrophenol). It is derived by making a standard curve which relates the concentration of nitrophenol in molar units to the concentration in absorbance units and then using this standard curve to predict the absorbance of a one molar solution.
103	=	a correction to change the extinction coefficient from liter per mole to milliliters per micromole
N_v	=	total assay volume (ml) at the time of the absorbance reading
E_v	=	volume of enzyme (ml) in assay

$\frac{\Delta A}{\Delta t}$ = *slope of "Absorbance vs. Time" (Figure A1) = 0.0806*

N_v = *3.1 ml*

E_v = *0.1 ml*

Enzyme Activity *(units/ml)* = $0.0806 \times \frac{103}{20{,}000} \times \frac{3.1}{0.1} = \mathbf{0.12493\ units/ml}$

β **[pNPP] (mmol/L) in 3.1 ml of enzyme assay:**

- *pNPP = 371.1 g/mol*
- *pNPP stock solution = 2 mg/ml*
- *2 mg/ml x 0.05ml (added to assay) = 0.1 mg = 0.0001 g*

[pNPP] = $0.0001g \times \frac{1\ mmole}{0.3711\ g} \times \frac{1}{0.0031\ L} = \mathbf{0.0869\ mmol/L}$

◆ V_{max} and K_m Calculations:

Construct the Lineweaver-Burk plot (Figure A3) using the following equation:

$$\frac{1}{V_o} = \frac{K_m}{V_{max}} \times \frac{1}{[S]_o} + \frac{1}{V_{max}}$$

$$(y = m \chi + b)$$

where: Y axis intercept = $1/ V_{max}$

X axis intercept = $-1/ K_m$

⇨Y axis intercept = 1.267

⇨ X axis intercept = -b/slope = -2.11

$$V_{max} = \frac{1}{\text{Y-intercept}} = \frac{1}{1.267} = \textbf{0.789 units/ml}$$

$$\boldsymbol{K_m} = \frac{-1}{\text{X-intercept}} = \frac{-1}{-2.11} = \textbf{0.474 mM}$$

◆ K_a Calculation:

K_a = specificity constant ($M^{-1} s^{-1}$) . It defines the rate of reaction at low concentrations of substrate. The bigger the K_a of the enzyme for a substrate, the more specific the enzyme is for that substrate.

$$K_a = \frac{k_{cat}}{K_m} = \frac{V_{max}/[\text{enzyme}]}{K_m}$$

⇨[enzyme] is constant in all samples, so it can be left out when calculating K_a using k_{cat}. (since [BIAP] is unknown in our BIAP extract)

$$\therefore K_a \cong \frac{V_{max}}{K_m}$$

k_{cat} = catalytic constant. It is the "turnover number" that represents the maximum number of catalytic cycle that the active site can undergo per unit time (s^{-1}).

$$k_{cat} = \frac{V_{max}}{[\text{enzyme}]}$$

K_a of BIAP under the influence of 2 mM Co^{2+} = *0.79/0.47* = ***1.68***

Figure A1.

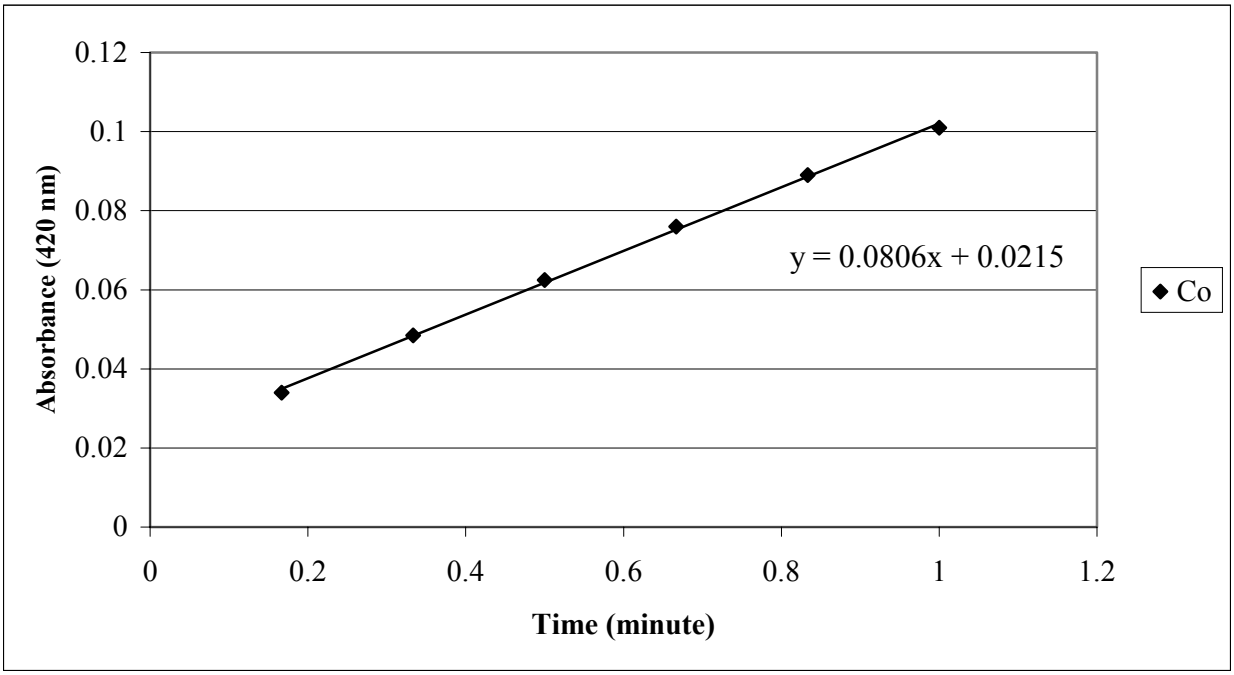

Figure A1. Absorbance of nitrophenol at 420 nm at 10 second intervals for a period of 1 minute. The change in absorbance from the standard assay of BIAP with 2 mM $CoCl_2$ in 10 mM glycine buffer (pH 10) was determined to be 0.0806.

Figure A2.

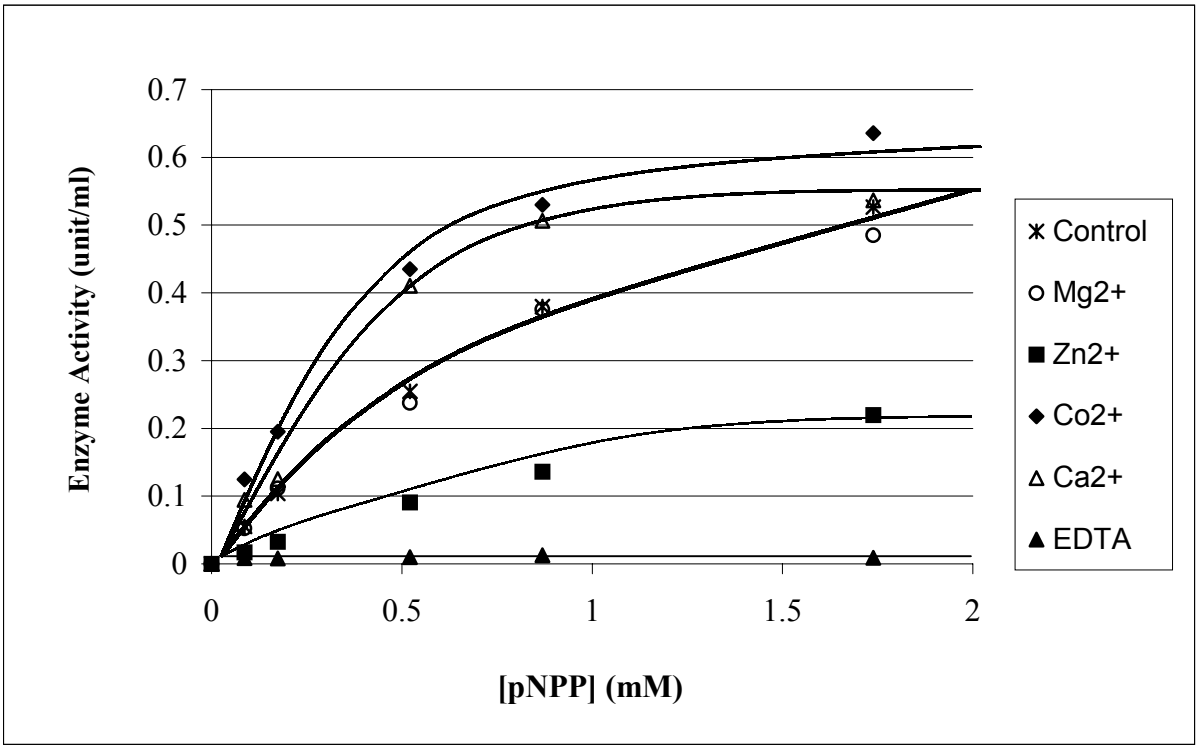

Figure A2. Michaelis-Menten Plot. Effects of divalent cations on the enzyme activity of BIAP. BIAP was assayed for 1 minute in 3.1 ml of activity assay system containing 10 mM glycine buffer (pH 10), five different concentrations of pNPP substrate, with 2 mM Mg^{2+} (○), 2 mM Zn^{2+} (■), 2 mM Ca^{2+} (Δ), 2 mM Co^{2+} (♦), 8 mM EDTA (▲), and no divalent cations added as control (*). The values of enzyme activity represent the mean of two trials. The V_{max} and K_m of BIAP in these assay conditions were determined using the Lineweaver-Burk plot (Figure C).

Figure A3.

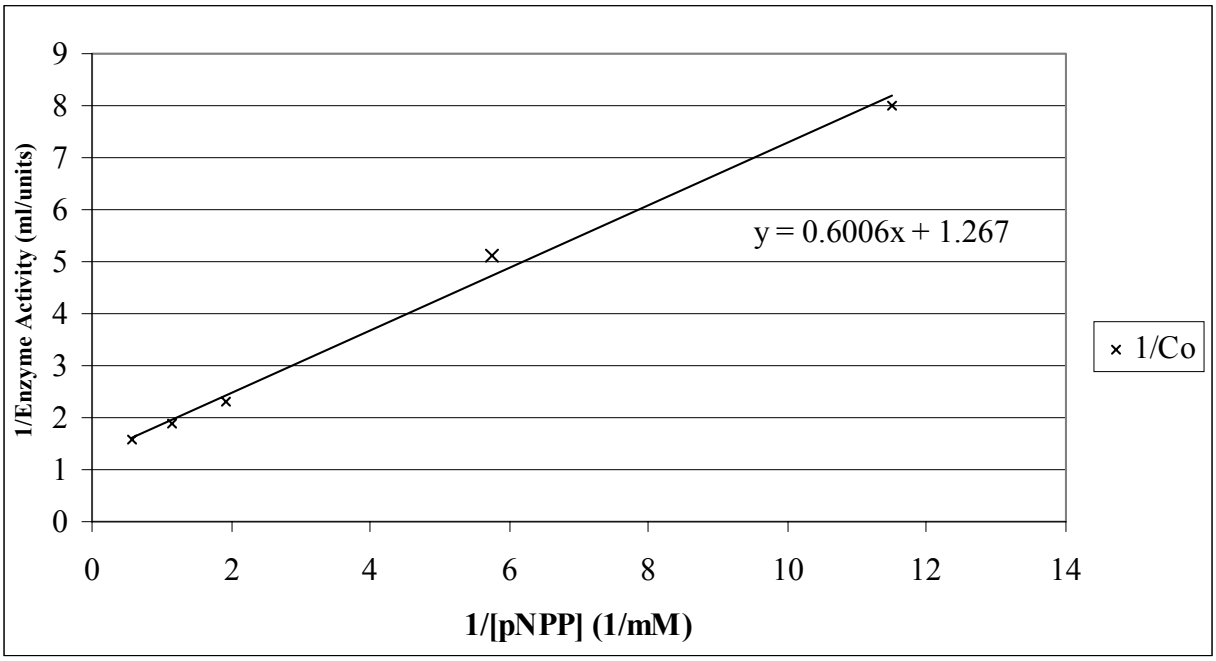

Figure A3. Lineweaver-Burk Plot. The V_{max} and K_m values of BIAP under the influence of 2 mM Co^{2+} in 10 mM glycine buffer (pH 10) over a period of 1 minute were determined to be 0.789 units/ml and 0.474 mM, respectively.