Int. J. Chem. Sci. : 3 (1), 2005, 16–22

THE INHIBITORY EFFECT OF ALCOHOLS ON ALKALINE PHOSPHATASE ACTIVITY

R. SARIRI[a]*, A. GHANADZADESH[b], E. LIALI[a] and R.H. SAJEDI[a]

[a]Department of Biology and[b] Department of Chemistry
The University of Guilan, RASHT, IRAN

ABSTRACT

The inhibition of calf alkaline phosphatase by a group of alcohols was studied in the presence of p–nitrophenol phosphate (pNPP), one of its normal substrates. The results showed that ethanol was a more potent inhibitor than other alcohols, i.e. propanol and butanol and the bifunctional alcohol, ethylene glycol. However, none of the alcohols reached 50% inhhibition of enzyme activity. This is a low inhibition rate when compared with inhibition of the same enzyme by glutathione. From the Lineweaver Burk plots of the enzyme in the presence and absence of inhibitors, it was also found that all alcohols were reversible non–competitive inhibitors.

Key words : Alkaline phosphatase, Inhibitory effect.

INTRODUCTION

Alkaline phosphatase (EC 3.1.3.1) is a dimeric protein containing Zn and Mg ions with a molecular weight of 140 KD. Unlike many enzymes that origin from cytoplasm, alkaline phosphatase is located in the cellular membrane by PGI anchor and, therefore, is not easily released from the cell wall. It is a nonspecific phosphomonesterase that function through a phosphoseryll intermediate to librate a free inorganic phosphate from phosphoesters or to transfer the phosphoryl group to other alcohols[1,2].

$$\text{Orthophosphoric monoester} + H_2O \longrightarrow \text{alcohol} + H_3PO_4$$

There are several sources of alkaline phosphatase from bacteria to mammals that differ in how easily they can be inactivated. *Bacterial alkaline phosphatase (BAP)* is the most active of the enzymes, but also the most difficult to destroy at the end of phosphorylation reaction[3]. *Calf intestinal alkaline phosphatase (CIP)* is purified from bovine intestine and is most used in diagnostics and as a reagent in molecular biology assays such as enzyme–linked immunosorbent assays[4]. Although less active than BAP, CIP can be effectively destroyed by protease digestion or heat i.e. at 75°C for 10 minutes in the presence of 5 mM EDTA. It is widely used in nonradioactive detection techniques, hybridization and sequencing system[5]. Calf intestine

*Corresponding author, Fax : 0098 131 3220912, e–mail: sariri@guilan.ac.ir

alkaline phosphatase has a molecular weight of 140000 with an isoelectric point of 5.7 and optimum pH of 9.8.

Various isozymes of alkaline phosphatase are elevated in plasma during different physiological disorders such as bone and kidney carcinoma[6]. Fishman et al.[7] were first to report on the neoplastic ectopic production of an alkaline phosphatase isozyme, that was indistinguishable from placental alkaline phosphatase, named placental–like alkaline phosphatase (PLAP). Other scientists have also demonstrated that PLAP is associated with various malignant neoplasms, most commonly ovary, colonic and lung carcinomas[7]. It has been reported that increased serum concentrations of PLAP are found in 35–40% of patients with ovarian cancer[7–11]. A more recent investigation has suggested that elevated serum alkaline phosphatase may enable early diagnosis of ovarian cancer[12].

Like many other enzymes, the biological activity of alkaline phosphatase is affected by a variety of inorganic and organic compounds such as inorganic phosphate, Cu (II) and Ni (II), denaturing agents (SDS, urea), metal ion chelators (EDTA, EGTA), and thiol–reducing agents (dithiothreitol [DTT], MCE)[13–15]. However, the inhibition of the enzyme by hydrozyl compounds, although of prime importance in alcohol toxification, has not been reported. In this research, the effect of some alcohols on the activity of calf alkaline phosphatase upon one of its substrates, p–nitrophenyl phosphate (pNPP) was studied.

MATERIALS AND METHODS

Materials

Mot of the chemical reagents used were of analytical grade and used as supplied by the manufacturer. Calf alkaline phosphatase, para–nitrophenyl phosphate (pNPP disodium salt hexahydrate) and glutathione were purchased from Sigma Chemical Company. Glycine, magnesium chloride, zinc chloride, glycerol, methanol, ethanol, n–propanol, n–butanol and ethylene glycol were from Merck.

Experimental methods

Electrophoresis : Although the presence of other proteins with no alkaline phosphatase activity may not interfere with the results, but these types of impurities may act as retarder, inhibitor or even activator. Therefore, before any activity measurement, the purity of enzyme was tested using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE)[12]. In a typical electrophoresis experiment, vertical slab gel unit was assembled using a 1.0 mm spacer. The separating gel (10% T, 2.7% C) was prepared by mixing appropriate amounts of acrylamide, ammonium persulphate and N, N, N', N–tetramethylene diamine TEMED, and pipetting into the slabs. The separating gel was overlaid with the stacking gel (4% T, 2.7% C) and left to polymerize while the proper comb was inserted. 20 µL of different concentrations of alkaline phosphatase solution were added to each well and high molecular weight protein mixture was also used as standard. The electrophoresis run continued for 3 hours at 25 amperes until the

sample reached 1 cm above the bottom. The gel was stained with colloidal brilliant blue and de–stained with acetic acid–methanol mixture.

Enzyme assay : Alkaline phosphatase activity was measured spectrophotometrically using p–nitrophenyl phosphate (pNPP disodium salt hexahydrate) as the substrate. Unless otherwise indicated, reaction mixtures (final volume 1.0 mL) contained 100 mM glycine–NaOH (pH 11.0), 2 mM $MgCl_2$, 4mM pNPP and 10 unit of the enzyme. The reaction was carried out at 50^oC and terminated by the addition of 40 mL of 10 M NaOH. The extent of hydrolysis was determined by measuring the absorbance due to the librated p–nitrophenol at 410 nm. Figure 1 shows the chemical structure of the enzyme substrate and the simplified enzymatic reaction that leads to p–nitrophenol liberation with an absorption maximum at 410 nm. Blank reaction mixture without the enzyme were also included to correct for the rate of non–enzymatic hydrolysis of pNPP.

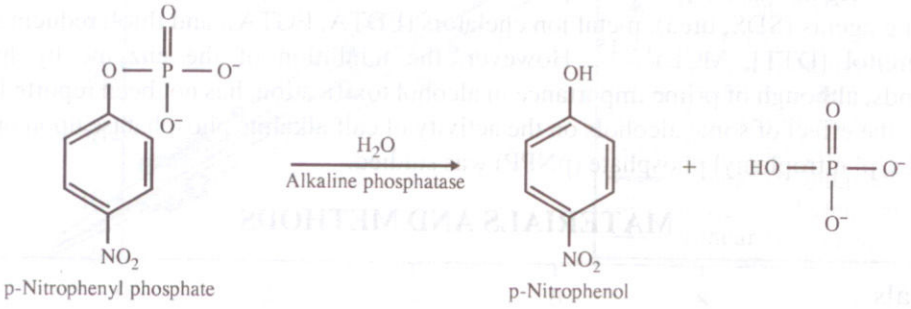

Figure 1. Hydrolysis of pNPP to produce p–nitrophenol

Enzyme inhibition : Various concentrations (10 mM–1.0 M) of each inhibitor were used in order to study effect of inhibits on the enzymatic activity of alkaline phosphatase. The same enzymatic assay was used with a constant concentration of substrate i.e. 4 mM. The decrease in activity (%) was then plotted against inhibitor concentration in mM.

RESULTS AND DISCUSSION

Figure 1 is the rate of enzymatic reaction in the presence and absence of various alcoholic inhibitors. It is seen that in all cases the maximum rate V_{max} has been decreased. Figure 2 shows that the highest inhibition rate in the case of all alcohols was observed when the concentration of inhibitor was 100 mM. Beyond this concentration, the activity started to increase again and reached almost to the original value at concentration of about 700 mM. This is interesting finding that has not been observed for cysteine[13]. In fact, there is an optimum alcohol concentration for inhibition of alkaline phosphatase. This finding is important when the serum level of alkaline phosphatase is used as an indication of alcohol toxification or when the change in enzyme activity is followed for diagnosis of ovarian cancer especially in the case of alcoholic patients[8–10, 12, 14].

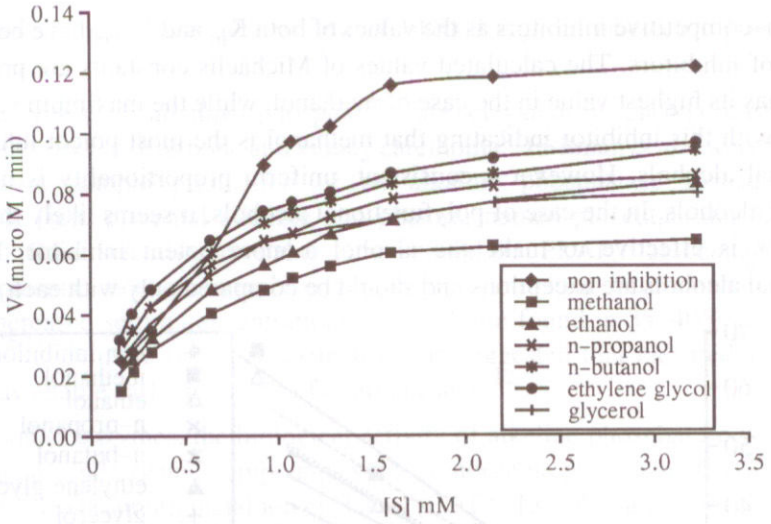

Figure 1. The rate of alkaline phosphatase activity in the presence and absence of 100 mM of various inhibitors

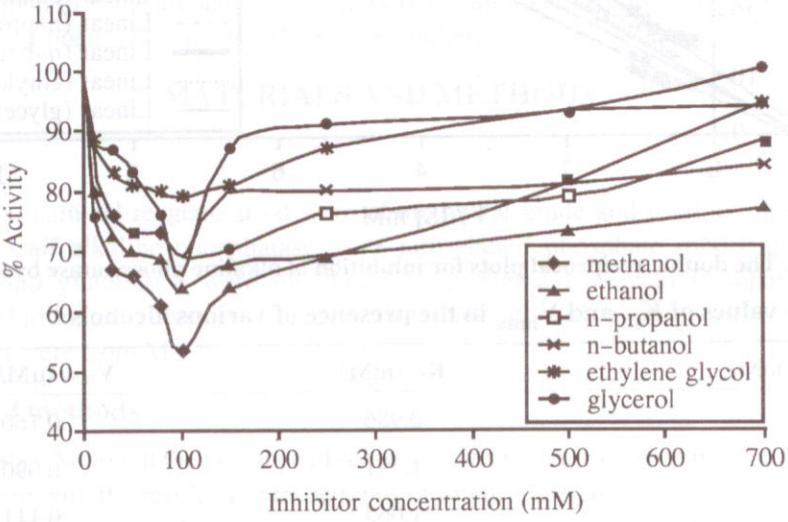

Figure 2. The effect of inhibitor concentration on the activity of alkaline phosphatase

Another important point found from these results was that although the inhibitory effect of small monofunctional alcohols such as methanol was much higher than higher alcohols and bifunctional alcohols, but none of them could inhibit 50% of the enzymatic activity. This is a low inhibition rate when compared with inhibition of the same enzyme by thiol compounds such as cysteine[13]. The hydroxyl group in alcohols could act in a similar way to the – SH group in cysteine, a potent inhibitor of alkaline phosphatase, but with milder degree of inhibition. The kinetic data obtained from Lineweaver–Burk plots (Figure 3), indicates that all alcohols are

reversible non–competitive inhibitors as the values of both K_m and V_{max} have been changed in the presence of inhibitors. The calculated values of Michaelis constants are presented in the Table 1. K_m has its highest value in the case of methanol, while the maximum velocity is at its lowest value with this inhibitor indicating that methanol is the most potent inhibitors among monofunctional alcohols. However a consistent, uniform proportionality is not seen when comparing all alcohols. In the case of polyfunctional alcohols, it seems likely that the number of OH groups is effective to make the alcohol a more potent inhibitor. However, the monofunctional alcohols are exceptions and should be compared only with each other.

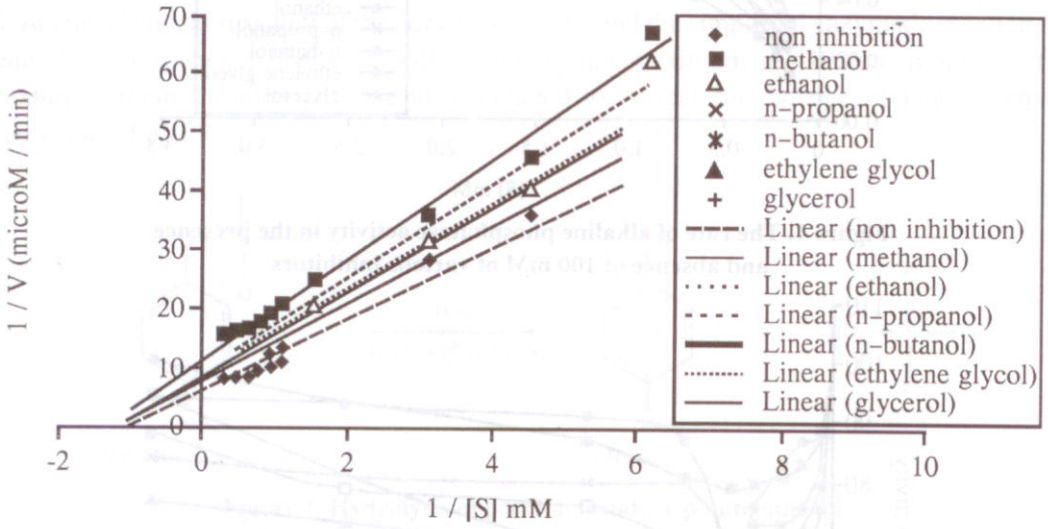

Figure 3. The double reciprocal plots for inhibition of alkaline phosphatase by alcohols.

Table 1. The values of K_m and V_{max} in the presence of various alcohols

Inhibitor (100 mM)	K_m (mM)	V_{max} (μM/min)
Non	0.986	0.160
Methanol	1.291	0.090
Ethanol	1.065	0.111
Propanol	1.104	0.125
Butanol	1.147	0.142
Ethylene Glycol	1.080	0.156
Glycerol	1.213	0.135

CONCLUSION

According to the results obtained from this study, we conclud that the hydroxyl group present in alcohols, especially in monofuntional alcohols could act as inhibitor on the biological activity of alkaline phosphatase. The kinetics of inhibition showed that all alcohols are reversible non–competitive inhibitors .The value of Michaelis constants obtained from double reciprocal plots showed that methanol with a K_m of 1.291 mM is the most potent inhibitor followed by three functional alcohol, glycerol (K_m = 1.213 mM). However, the order of inhibition strength does not follow a consistent uniform pattern.

Considering that the change of alkaline phosphatase in human serum has been used in many diagnostic purposes, it can be suggested that in alcohol toxification and alcohol over loss this type of early diagnosis could seriously be interfered.

REFERENCE

1. H. Fernely, "Mammalian Alkaline Phosphatases", *The Enzymes, 3rd Ed. 4,* P. Boyer, Academic Press, NY, (1971) p. 417.
2. R. L. Mellgren, et al., Dephosphorylation of phosphoproteins by *Escherichia coli* alkaline phosphatase, Biol. Chem., **252**, 6082 (1977).
3. T. Reid, and I. Wilson, *E. coli* Alkaline Phosphatase, *The Enzymes, 3rd Ed.,* P. Boyer, Academic Press, NY, (1971) p. 373.
4. Manson, M.M. (Ed). "*Immunochemical Protocols*". Humanan Press, Totowa, N.J., (1992) p. 480.
5. E. Jablonski, E. W. Moomaw, R. H. Tullis and J. L. Ruth, Preparation of oliginucleotide–alkaline phosphatase conjugates and their use as hybridization probes. *Nucleic Acids Res.*, **14**, 6115, (1986).
6. T. Kuwana and S. B. Rosalki, Intestinal variant alkaline phosphatase in plasma in disease. *Clin. Chem.*, **19**, 209, (1990).
7. W. H. Fishman, N. I. Ingis, L. I. Stolbach and M. J. Kant. A serum alkaline phosphatase isozyme of human neoplatic cell origin, *Cancer Res.*, **28**, p. 150, (1968).
8. M. E. DeBore and D. E. Poller, Multicenter evaluation of human placental alkaline phosphatase as a possible tumor–associated antigen in serum, *Clin. Chem.*, **34**, 1995, (1988).
9. I. Vergote, M. Onsrud and K. Nustad. Placental alkaline phosphatase as a tumor marker in ovarian cancer, *Obster Gynecology*, **69**, 228, (1987).

10. J. Fisken, R.C.F. Leonard, G. Shaw, A. Bowman and J. E. Roulston. Serum placental like alkaline phosphatase (PLAP): a novel combined enzyme linked immunoassay for monitoring ovarian cancer, *J. Clin. Pathol.*, **42**, 40, (1989).
11. H. A. Muensch, W. C. Maslow, F. Azama, M. Betrand, Dehwurst and B. Harman, Placental like alkaline phosphatase. *Cancer*, **58**, 1689 (1986).
12. A. Ben–Arie, Z. Hagay, H. Ben–Hur, M. Open and R. Dgani. Elevated serum alkaline phosphatase may enable early diagnosis of ovarian cancer. *Eur. J. Obstetrics & Gynecology and Reproductive Biology*, **86**, 69, (1999).
13. S. Agus, R. Cox and M. Griffin, Inhibition of alkaline phosphatase by cysteine and its analogues, *Biochim, Biophys. Acta*, **118**, 363, (1966).
14. W. Withold, G. Geurgescu, H. Khakzad, H. Vosberg, H. W. Mueller–Gaertiner and H. Reinauer. Efficacy of simultaneous determination of bone alkaline phosphatase mass concentration in serum and urinary excretion of pyrimidinium cross–links for detection of bone metastases, *Clin. Biochem.*, **28**, 511, (1995).

Accepted: 2.2.05