Partial Purification and Characterization of Alkaline Phosphatase From Goat Kidney

*[1]MUHAMMAD ZABTA, [2] MUHAMMAD ASHRAF, [1]TANVEER SHARIF AND [3]ASMA SAEED

[1]*Faculty of Pharmacy, Bahauddin Zakariya University, Multan, Pakistan.*

[2]*Department of Pharmacy, Faculty of Pharmacy and Alternative Medicine, the Islamia University of Bahawalpur, Pakistan.*

[3]*Department of Biological Sciences, Gomal University Dera Ismail Khan, Pakistan.*

(Received 18th April 2007, revised 24th July 2007)

Summary: Alkaline phosphatase (AP, E.C. 3.1.3.1) was partially purified from goat kidney by ammonium sulphate precipitation, DEAE-trisacryl and gel chromatographic methods. The specific activity of the purified enzyme was 0.1 units/ mg protein. The activity of the enzyme was linear upto 25 minutes with 0.002 units of enzyme. The optimum temperature and pH for enzyme activity were 40 ^{0}C and 11.0 respectively. The enzyme was resistant to heat up to 50 ^{0}C while its activity was irreversibly destroyed at 60 ^{0}C. L-Phenylalanine, zinc chloride, 2-mercaptoethanol, EDTA, and pyridoxal phosphate inhibited enzyme activity and there was no effect of L-leucine and L-tryptophan. Keeping in view these properties, the purified enzyme from goat kidney resembled intestinal alkaline phosphatase.

Introduction

Alkaline phosphatase (AP; E.C. 3.1.3.1) is widely distributed in nature and depending upon the species, has been found in several types of organisms, although in vertebrates, it is primarily a plasma membrane enzyme [1]. It is relatively a non-specific and hydrolyzes a number of phosphomonoesters to yield orthophosphate and alcohol, with a maximum activity at alkaline pH [1-2]. The enzyme has been implicated in numerous physiological functions, all of which involve some aspect of transport, release and accumulation or transfer of phosphate [1].

The existence of three human isoenzymes, each encoded by independent genetic loci [3 - 4] has been proposed [5 - 6]. These isoenzymes include (a) an 'Intestinal isoenzyme' (IAP) localized on the brush border of the mucous membrane with a possible variant in the renal tubules (b) a placental (PLAP) is seen in the serum of pregnant women in the third trimester and (c) a 'tissue nonspecific' (TNSAP), originating from bone, liver, lungs and WBCs [7 - 9]. On the other hand, in several animal species, except higher primates and human, there exist only two types of isoenzymes which are known to differ in their V_{max}, K_m, molecular weight, thermal stability and sensitivity to inhibitors [5, 10 -13]. In this paper, we report some of the properties of partially purified AP from goat kidney.

Results and Discussion

The enzyme was purified using ammonium sulphate precipitation and various chromatographic methods and the purification procedure is summarized in Table 1. The elution pattern of AP from DEAE-trisacryl M® ion exchanger is shown in Fig 1. It is evident that most of the enzyme eluted in the range of 0.25-0.35 M NaCl gradient. A very small amount of enzyme was not bound to the column that might be due to the presence of a different isoenzyme of AP. The specific activity of the purified enzyme was 0.1 units/ mg protein.

Properties of Alkaline Phosphatase

1. Effect of Enzyme Concentration. The influence of enzyme concentration upon the activity was determined by diluting the purified enzyme in various proportions and measuring the activities. These were plotted against enzyme concentration as shown in Fig 2. The relationship between enzyme

*To whom all correspondence should be addressed.

Table-1: Purification of alkaline phosphatase from goat kidney.

Fractions	Activity (U/ ml)	Protein (mg/ ml)	Specific Activity (U/ mg protein)
45-75% Ammonium sulphate fraction	1.071	19.0	0.01
Ultrogel AcA-202® Column chromatography	0.136	1.7	0.08
DEAE-Trisacryl M® Column chromatography	0.1224	1.53	0.08
Ultrogel A_6® Column chromatography	0.045	0.45	0.10

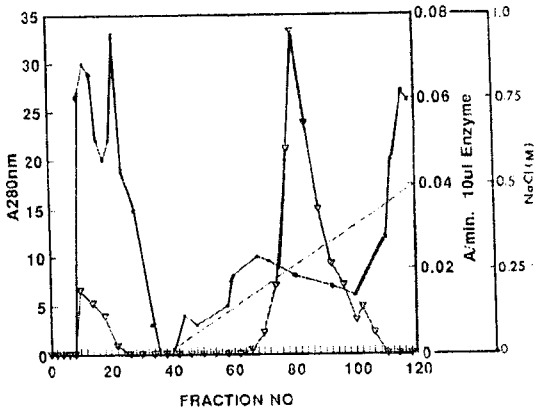

Fig. 1. DEAE-Trisacryl-M® column chromatography of alkaline phosphatase from goat kidney. Column dimension, 1.8 × 15 cm; Equilibrating buffer, 5 mM phosphate, pH 7.5, Flow rate,0.7 m/min; fraction volume, 4 ml. Ordinates: ■------■ protein at 280 nm; ▼-----▼, alkaline phosphatase activity.;....., Sodium chloride (M).

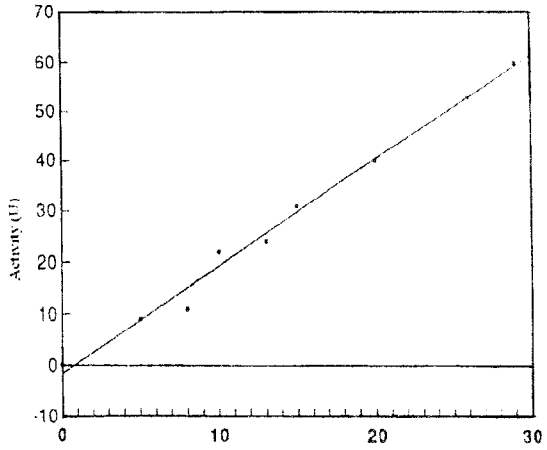

Fig. 2. Effect of enzyme concentrations on the activity of alkaline phosphatase.

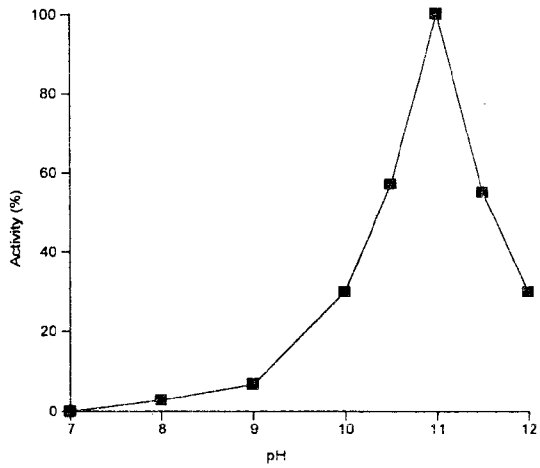

Fig. 3. Effect of pH on the activity of alkaline phosphatase.

concentration and activity was somewhat linear upto 0.003 units.

2. Effect of pH. The effect of pH on the AP was studied by measuring the enzyme activities at different pH values. The optimum pH was found to 11.0 (Fig. 3).

3. Effect of Temperature. The activities of AP were determined at different temperatures. The incubation mixtures were kept for 5-15 min at various temperatures 35-55 °C. As the temperature raised, the activity was increased until it reached the maximum at 40 °C but the enzyme retained its activity upto a temperature of 45 °C Fig 4. at 50 °C, the enzyme appeared to be denatured.

4. Temperature Stability. Temperature stability was studied by incubating of the enzyme solution in required buffer at temperature ranging from 35-60 °C for 5 min or so on. The reaction was stopped by immersion in ice bath and residual activity was assayed as usual. The enzyme was found to be stable between 35-55 °C. At temperature of 55 °C, only 25 % inactivation was

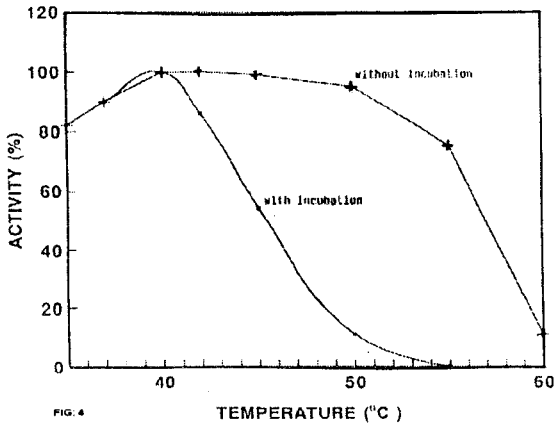

Fig. 4. Effect of temperature on the activity of alkaline phosphatase. Enzyme activity was measured at different temperatures without incubation (□— —□) and with incubation for 5 min and then activity was measured at 37°C (+——+).

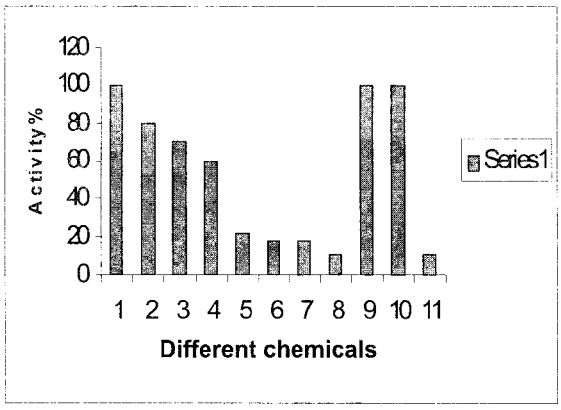

Fig. 5. Effect of different inhibitors on the activity of alkaline phosphatase.
(1) 100% (control) activity obtained without inhibitors (2) 10 mM Sodium fluoride (3) 10 mM Phosphate (4) 10 mM L-Phenylalanine (5) 10 mM Zinc chloride (6) 10 mM Arsenate (7) 10 mM 2-Mercaptoethanol (8) 10 mM EDTA (9) 10 mM Leucine (10) 10 mM L-Tryptophan (11) 2 mM Pyridoxal 5 –Phosphate.

seen Fig 4. At 60 °C, the enzyme was completely denatured.

5. Effect of Inhibitors. The activity of AP was also studied in the presence of different compounds Fig. 5. Pyridoxal 5'-phosphate (PLP) showed the most potent inhibitory activity against AP. PLP at 2 mM concentration inhibited activity by about 85 %. Zinc chloride, arsenate, 2-mercaptoethanol and EDTA (at 10 mM concentration) exhibited strong inhibitions while L-leucine (Leu) and L-Trypophan (Trp) did not shown any effect on the enzyme activity (Fig. 5). Sodium fluoride and phosphate which are the strong inhibitors of high molecular acid phosphatases also inhibited the AP but to small extent.

AP was partially purified from goat kidney and characterized. The characterization of AP has been made by parameters such as heat stability, pH optimum and susceptibility to the inhibition of enzyme activity by certain inhibitors. It should also be noted that the susceptibility of AP to these parameters were easily changeable depending on the experimental conditions employed [2]. Many studies (including ours) on the characteristics of AP have been performed under different conditions, it is therefore, difficult to compare the data quantitatively produced by different investigators [5-14].

The optimum pH was found to be higher as compared to APs from other sources [5-15]. It is difficult to explain why this enzyme showed optimum pH at 11.0 (Fig. 3), although many enzymes denature at this pH. AP from *Thermus thermophilus* showed optimum value at pH 10.5 [16]. The optimum temperature for activity determination was also found to be higher as compared to APs from other sources [5], but was near to AP from *Penicillium chrysogenum* [15]. The enzyme was denatured reversibly upto 55 °C (Fig. 4). After this temperature, denaturation was irreversible and the enzyme lost almost all activity at 60 °C. Thermal stability has been a valuable feature for the differentiation of APs in various organs and species [2, 5, 17]. However, this thermal stability response was pH dependent [5]. Most of the APs from various tissues were quite sensitive to heat stability at 56 °C for 5 min at pH 7.0 [5-18]. At pH 11.0, we have shown that about 60 % activity at 56 °C for 5 min incubation (Fig. 4) is retained and the enzyme is considerably heat-stable. Almost same type of behaviour was observed in IAPs from *Xenopus laevis*, chick and guinea pig [5] and human milk [18].

The inhibition of AP by Zn^{++} (Fig. 5) was according to many findings in the literature [4-5, 19]. In rat hepatoma cells, 1 mM Zn^{++} strongly inhibited AP activation *in vivo*, while at 0.01 mM these metal ions increased AP activity [20]. Zn^{++} has been reported to inhibit the activity AP by competing with Mg^{++} for Mg-binding sites [21]. EDTA (a metal-chelating agent) has inhibited the enzyme strongly, which further supports that this enzyme was also a metalloprotein like others reported [5-16].

The inhibitory effects of phenylalanine (Phe) on the activity of APs is a good indication for distinguishing AP isoenzymes, because bone and liver APs (TNSAP) are more resistant to Phe inhibition as compared to IAP and PLAP [5, 22-23]. The AP from goat kidney was sensitive to inhibition of Phe and thus it might be either IAP or PLAP type. By heat-inactivation experiments it looks more like IAP type. It is known that in several animal species, only two types of isoenzymes (TNSAP and IAL) are present [5]. Possibility of another type of isoenzyme, known as fetal-IAP, has been ruled out because this enzyme was not inhibited by 10 mM Leu while the fetal-IAP is sensitive to Leu inhibition [22]. However, for complete differentiation between isoenzymes of AP from goat kidneys, there is a need of electrophoretic separation of isoenzymes, amino acid sequence and immunological studies.

The inhibition of AP by PLP shows that this enzyme also needs lysine at active site of enzyme [23]. Like most of the APs [5, 24-25], the AP from goat kidney was also inhibited by 2-mercaptoethanol. This may indicate that the enzyme contains at least one disulfide bond for the expression or maintenance of the activity within the structure.

Experimental

Materials and Methods

Chemicals

DEAE-trisacryl M® ion exchange sorbent, Ultrogel A_6® and Ultrogel AcA-202® size exclusion sorbents were obtained from LKB (France). p-Nitrophenylphosphate (PNP) was from Merck (Germany). All other chemicals were of analytical grade.

Partial Purification of Alkaline Phosphatase

Fresh goat kidneys were purchased from the local market. All experiments were carried out at 4 °C unless otherwise mentioned. Fat free kidneys were minced and homogenized (20 % w/ v) in 0.05 M phosphate buffer, pH 7.5. The contents were centrifugation at 8000 x g for 1 hr and the supernatant was brought to 45 % ammonium sulfate saturation. After an hour the suspension was re-centrifuged and supernatant proteins were fractionated with 75 % ammonium sulfate saturation. The suspension was centrifuged and the pallets containing most of the enzyme activity were dissolved into a minimum volume of 0.01 M phosphate buffer, pH 7.5. This solution was passed through a gel filtration column of Ultrogel AcA--202®.

The filtered enzyme was loaded on top of DEAE-trisacyl M® column pre-equilibrated with 0.01 M phosphate buffer, pH 7.5. After washing the column (when absorbance at 280 nm became almost zero), enzyme was eluted by a linear gradient of NaCl (0-0.5M) in phosphate buffer, pH 7.5. Alternate fractions were analyzed for protein and enzyme activities. Fractions containing enzyme were pooled and concentrated by 75 % ammonium sulfate. The concentrated enzyme was passed through a column packed with Ultrogel A_6® and fractions were analyzed for protein and enzyme activities. Fractions containing maximum enzyme activity were pooled and stored at -20 °C until further experiments.

Enzyme Assay

Enzyme activity was measured at 37 °C in 1 ml of reaction mixture containing 50 mM glycine-NaOH buffer, pH 11.0, 5 mM PNP, 0.5 mM $MgCl_2$ and an appropriate amount of enzyme. The production of p-nitrophenol was followed by an increase in absorbance at 405 nm with Shimadzu UV-visible Spectrophotometer equipped thermostatically controlled cell housing (Japan). One unit of enzyme is defined as the amount of enzyme that produces one micromole of p-nitrophenol per minute under the assay conditions. The molar extinction coefficient of p-nitrophenol used was $1.88 \times 10^4\ M^{-1}cm^{-1}$ [26]. The activity over pH range of 7-12, the temperature optimum measured at 5 °C intervals from 35-55 °C and the

effects of inhibitors or metal ions were determined as by Naz *et al.*, [27].

Protein Assay

Protein concentration was determined by Biuret method [28] using bovine serum albumin as standard. Effluents from column chromatography were monitored for the relative amounts of protein by measuring absorbance at 280 mm.

References

1. R. A. Stinson, and J. R. A. Chan, *Adv. Protein Phosphatase,* **4**, 127 (1987).
2. R. A. Stinson, *Clin. Chem.*, **39**, 2293 (1993).
3. Fedde, K. N., C. C., Lane, M. P. Whyte, *Arch. Biochem. Biophysics,* **264**, 400 (1988).
4. S. K Iyer, H. H. Daron and J. L. Aull, *J. Reprod. Fert.* **82**, 657 (1988).
5. T. Yora, and Y. Sakagishi, *Comp. Biochem. Biophysiol.*, **85**, 649 (1986).
6. J. R., Farley, S. L. Hall, D. Ilacas, C. Orcutt, B. E. Miller, C. S. Hill, and D. J. Baylink, *Clin. Chem.*, **40**, 1749 (1994).
7. J. R., Moss, D. W., *Clin. Chem.*, **25**, 2007 (1982).
8. J. Griffith, and J. Black, *Clin. Chem.*, **33**, 2171 (1987).
9. P. M. Crofton, and R. Hume, *Clin. Chem.*, **33**, 1783 (1987).
10. T. T. Kerstin, *J. Vet. Med. Ser. A..*, **35**, 13 (1988).
11. M., K. Kensaku, Sugamoto, K. , Yoshikawa, K. Takaoka, K. Ono, D. C., Morris, H. H. Hsu and H. C. Anderson, *Bone Miner.* **3**, 159 (1987).
12. M. J., Peake, M. Pejakovic, and G. H. White, *J. Clin. Pathol.*. **41**, 202 (1988).
13. T. Mazda, and W. L. Gyure, *Chem. Pharm. Bull.*, **36**, 1814 (1988).
14. D. E., Amacher, D. J. Smith, L. K. Martz and W. E. Haffmann, Enzyme (Basel), **37**, 141 (1987).
15. K., R. Julius Gaspar, J. Kasar, E. Zelinkova and J. Zelnka, *Bilogia* (Bratisi), **42**, 753 (1987).
16. V. Subramanium N. C. H. Bergenhem A. Gafni, and D. G. Steel, *Biochem.*, **34**, 1133 (1995).
17. R. B., McComb, G. N. Bowers, Jr. and S. Posen, Alkaline phosphatase. Plenum Press, New York, p. 394 (1979).
18. N. N., Chuang, *Clin. Chem. Acta,* **169**, 165 (1987).
19. R. P., Cox, Griffin, M. J. Grifin, *Arch. Biochem. Biophys,* **122**, 552 (1967).
20. Sorimachi, K., *J. Biol. Chem.*, **262**, 1535 (1987).
21. G., Cathala, C. Brunel, D. Chappelet-Tordo and M. Luzdunski, *J. Biol. Chem.*, **250**, 6046 (1975).
22. W. H. Fishman, *Clin. Biochem.*, **20**, 387 (1987).
23. Braunstain, A. E., *Enzyme,* **2**, 113 (1960).
24. Y., Ikebana, K. Mansho, K. Takahashi, K. Kato, *J. Biochem.*, **83**, 1471 (1978).
25. Y., Yokata, *J. Biochem.* **83**, 1285 (1987).
26. M., Miura, I. Koyama, H. Matsuzaki, Y. Sakagishi, H. Ikezawa and T. Komoda, *Clin. Chem. Acta,* **171**, 63 (1988).
27. R., Naz, A. Saeed and A. Saeed. *The Protein J.*, **25**, 135 (2006).
28. G., Beisenherz, H. J. Boltze, T. Bucher, R. Czoke, K. H. Garbade, E. Meyer-Arendt and G. Pfleiderer. *Z. Naturforsch.*, **8**, 555 (1953).