

An Alkaline Phosphatase Lacking Wheat Germ Agglutinin Binding Sites; Useful Enzyme for Lectin Assays with Comparable Activity to the Calf Enzyme

Mohammad Morad Farajollahi^{*1}, David B. Cook² and Colin H. Self²

¹Dept. of Biochemistry, Lorestan University of Medical Sciences, Khorramabad, Iran; ²Dept. of Clinical Biochemistry, University of Newcastle upon Tyne, NE2 4HH, U.K.

ABSTRACT

Despite the availability of various alkaline phosphatase (ALP) isoenzymes, the calf enzyme is being used in current enzyme assays as the detector enzyme. The glycosylation pattern of this enzyme makes it a suitable ligand for binding to wheat germ agglutinin lectin (WGA). As a result of this property, the enzyme cannot be used as a conjugate with this lectin, and the calf enzyme conjugates can not be used when lectin is on the solid phase in lectin based ELISA systems. The purpose of this study was to evaluate the activity and lectin binding property of an ALP from the hepatopancreas of shrimp *Pandalus borealis* as an alternative for the calf enzyme. While the problem of non-specific binding to WGA lectin was circumvented with the shrimp enzyme, the activity of the new studied enzyme was seen to be higher than the calf enzyme. Studies of the effect of magnesium ion concentration on both enzymes demonstrated a major effect of the cation on shrimp enzyme and a relatively minor effect on the calf ALP. Conjugation of the shrimp ALP with streptavidin can be used in enzyme amplification technique in lectin based ELISA using WGA. *Iran. Biomed. J.* 6 (4): 105-109, 2002

Keywords: Shrimp alkaline phosphatase, Enzyme amplification, Lectin based assay

INTRODUCTION

Alkaline phosphatases (ALP) (EC 3.1.3.1) are intrinsic plasma membrane enzymes found on the membranes of almost all animal cells. These enzymes, which hydrolyze a broad class of phosphomonoester substrates, are dimeric Zn-metalloproteins [1-3]. The membrane form of the enzyme is tetrameric, but is converted to a soluble, dimeric form by treatment with phosphatidylinositol specific phosphatase C or by extracting with n-butanol at slightly acid pH [4-6]. The molecular weight of the soluble, dimeric mammalian ALP is in the range of 130,000-170,000 Dalton [7, 8]. Both for the mammalian and *E. coli* enzymes at least two subunits must be associated before the enzyme can exhibit catalytic activity [5, 9-11]. In higher mammals, three distinct groups of isoenzymes: placental, intestinal, and hepatic/renal/skeletal are recognized [12].

Calf intestine ALP is one of the mammalian ALP which is widely used in enzyme assays including

amplification techniques [13]; however the non-specific binding of the enzyme to wheat germ agglutinin (WGA) lectin made it unusable for lectin assays in which wheat germ agglutinin lectin is used. ALP has been detected in most animal species and the existence of the enzyme in non-mammalian vertebrates and invertebrates has been reported [14-18]. The present work deals with the use of an ALP from the hepatopancreas of shrimp *Pandalus borealis*. This enzyme is a dimeric protein with a molecular weight of 155,000 Dalton and may be dissociated into subunits, without losing its activity [19, 20].

The aims of the present report is to characterize the new ALP and use it as a detector in lectin binding assays in which lack of non-specific binding of detector enzyme to WGA lectin is crucial.

MATERIALS AND METHODS

WGA and NADH (NAD free) was obtained from

* Corresponding Author;

Boehringer; ADH, diaphorase and INT Violet from BDH; Shrimp ALP (*Pandalus borealis*) from Amersham Life Sciences; calf ALP from Biozyme Laboratories Ltd., sulfosuccinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (Solfo-SMCC), N-succinimidyl-s-acetylthioacetate (SATA), and dimethylformamide (DMF) were obtained from Pierce (Chester, UK), streptavidin from Sigma and Maxisorb plates from Nunc company.

Methods applied for preparing the streptavidin shrimp ALP conjugate. The conjugate was prepared using the two-step maleimide procedure. At first, the maleimide activated alkaline phosphatase (MAAP) was prepared using the cross linker, Solfo-SMCC. This ALP derivative is reactive towards sulphydryl (-SH) groups. The MAAP can be used to prepare ALP conjugates directly from proteins, peptides, and other ligands that contain free -SH groups. SATA (N-succinimidyl-S-acetylthioacetate) reagent was used for generating free -SH group on the streptavidin molecule.

Assessment of streptavidin shrimp ALP conjugate. Maxisorb plates were coated with 5 μ g/ml biotinylated BSA in 0.1 M bicarbonate buffer pH 9.6 at 4°C overnight. The plates were then washed with Tween-20 solution and incubated with a solution of streptavidin shrimp ALP conjugate (1:2000) in 0.05 M Tris-HCl at room temperature for 10 minutes. After washing the plates, pNPP substrate was added to the plates and OD was measured at 405 nm (after 10 minutes incubation).

Comparison of calf and shrimp ALP. A) Binding to WGA. Plates were coated with 0.1 ml of 10 μ g/ml lectin (WGA) in 0.1 M carbonate buffer, pH 9.6 at 4°C overnight and washed three times with 0.1% Tween-20 in demineralized water. Then, the plates were incubated with 0.0321 U/ml of both shrimp ALP and calf ALP in parallel in 0.05 M Tris-HCl buffer, pH 7.5 at room temperature for 2 h and washed three times with 0.1% Tween-20 in demineralized water. Two series of plates that were not incubated with lectin (blank) were used to show the binding of the enzymes to plastic surface. All the plates were then incubated with 2 mg/ml pNPP in 0.05 M DEA buffer, pH 9.6 including 1 mM MgCl₂ and OD was measured at 405 nm after one hour.

B) Effect of Mg²⁺ concentration on activity of shrimp ALP. Fifty μ l (0.3 U/ml) shrimp ALP in 0.05 M Tris-HCl, pH 8.5 was added to the microwell plates. MgCl₂ and then pNPP was added in the same buffer and plates were incubated at room temperature for 20 minutes. OD was measured at 405 nm.

C) Effect of the concentration of zinc and magnesium and the combination of both cations on shrimp-ALP activity. Eight series of MgCl₂ solutions (initial concentrations from 0-24 mM) were prepared in 0.05 M DEA buffer, pH 9.0. Zn²⁺ solution was prepared in 12 series from 0.0 to 800 μ M. Fifty μ l of magnesium solutions was added to each series of equal zinc concentration solution. Then, 50 μ l of 0.04 U/ml shrimp ALP was added to all wells in the same buffer. Finally, 2 mg/ml pNPP in the same buffer was added to all plates and incubated at room temperature for 30 minutes. OD was measured at 405 nm.

D) Effect of pH on activity of shrimp ALP. A series of 5-ml tubes including 0.1 M DEA buffer with 6 mM MgCl₂ at various pHs from 7.8-9.8 was prepared. Shrimp-ALP was added to the tubes to give an activity of 0.02 U/ml. Afterwards, 50 μ l of each solution containing the enzyme was added to the plates. At the same time, 50 μ l of 0.08 mg/ml NADP in demineralized buffer was added to the plates, and incubated at room temperature for 10 minutes. Finally, 100 μ l of amplification reagent (59.21 U ADH, 1.97 U Diaphorase and 184 μ L ethanol in 10 ml 0.1 M phosphate buffer pH 7.5 including 1 mM INT-violet) was added to the plates and incubated at room temperature for 5 minutes. OD was then measured at 495 nm.

RESULTS

Comparison of calf and shrimp ALP in binding with WGA. Figure 1 compares shrimp and calf ALP binding to WGA coated plates as well as blank plates. It is evident that shrimp ALP neither bound to the WGA coated nor uncoated (blank) plates, while calf ALP bound to both WGA coated plates and to some extent to uncoated plates. These results demonstrate that the shrimp ALP exhibits no binding to the lectin. Furthermore, the low affinity of shrimp ALP to the plastic plates, in comparison

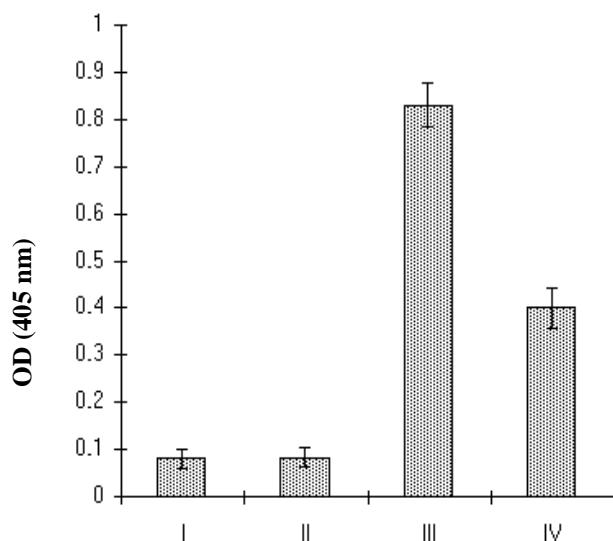


Fig. 1. Comparison of calf and shrimp alkaline phosphatase in binding to wheat germ agglutinin lectin coated plates; (I), Non-specific binding of shrimp ALP to the uncoated plates; (II), Non-specific binding of shrimp ALP to the WGA coated plates; (III), Binding of calf ALP to the WGA coated plates; (IV), Non-specific binding of calf ALP to the uncoated plates. Standard deviation is indicated on the Figure (n= 6).

with the value shown in the figure for the calf enzyme, demonstrates structural differences.

The effect of magnesium ion on the activity of the both enzymes is shown in Figure 2. The activity of shrimp ALP was higher by increasing the magnesium concentration, and leveled off at 30 mM. The activity of shrimp ALP was enhanced about 10 times in the presence of 30 mM Mg^{2+} . However, the activity of calf ALP was increased at concentrations between 0-1.5 mM. Furthermore, the activity of calf ALP in the absence of magnesium ion is about 82% of its activity than in the presence of 1.5 mM magnesium (which had the highest influence on the activity of calf ALP). On the other hand, the activity of shrimp ALP in the absence of magnesium ions is only 8% of its activity in the presence of 30 mM magnesium.

Figure 3 shows the combined effect of Zn^{2+} and Mg^{2+} on the activity of shrimp ALP. Unlike magnesium ion, zinc ion lowers the activity of the enzyme at concentration higher than 0.5 μ M. From 25 μ M zinc ion towards higher concentration of the cation, the activity of shrimp ALP is close to zero.

At these concentrations, the addition of magnesium ion did not result in activation of the enzyme.

In general, the presence of zinc ion in the enzyme solution inactivate, while the presence of magnesium ion activates the enzyme.

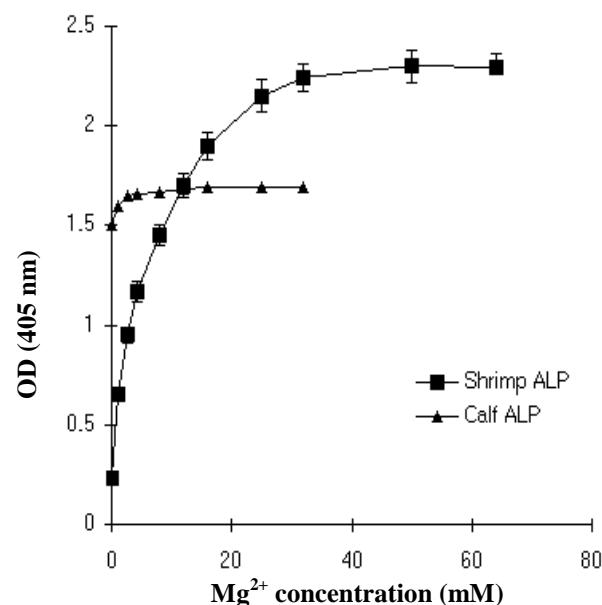


Fig. 2. Effect of magnesium ion concentration on activity of shrimp and calf ALP in DEA buffer with pNPP substrate. Standard deviation is indicated on the figure (n= 6).

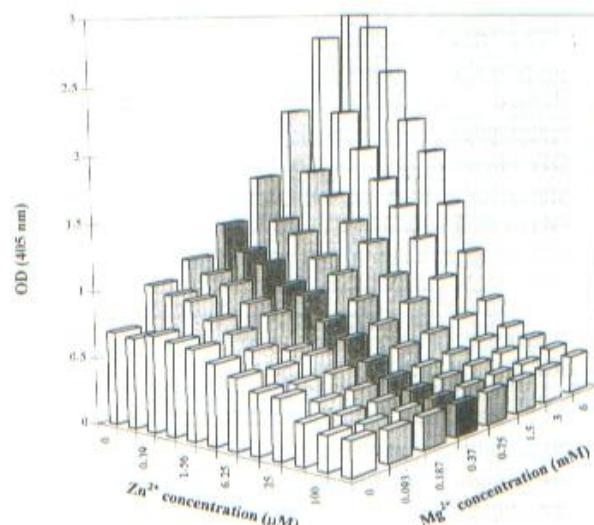


Fig. 3. Effect of magnesium, zinc and their combined effect on the activity of shrimp ALP.

The effect of pH on the activity of shrimp ALP shown in Figure 4. Like other ALP, shrimp ALP activated in alkaline solutions. The highest activity of shrimp ALP was determined between pH 8.7-9.3 with a maximum at pH 9.0. This optimum pH for shrimp ALP activity is close to that of calf ALP.

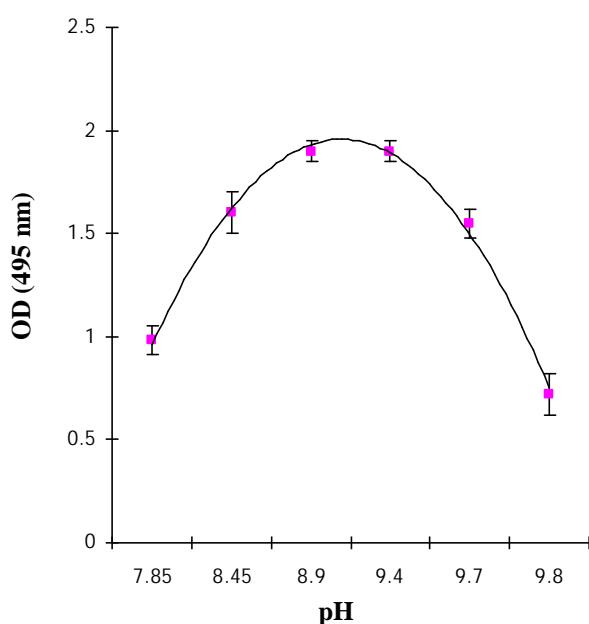


Fig. 4. Effect of pH on the activity of shrimp ALP ($n = 5$).

The results obtained from the assessment of streptavidin -(shrimp) ALP prepared in our lab showed that binding of the conjugate to the biotinylated BSA coated plates was successful. The OD obtained from binding of the conjugate to the biotinylated BSA coated plates was 1.6 while the OD of the blank ELISA well was 0.08.

DISCUSSION

Differences in binding properties between shrimp ALP and calf ALP are clearly shown in Figure 1, presumably demonstrates the difference of glycosylation of the enzymes. The observed non-specific binding of calf ALP to plastic surface, in comparison with the shrimp ALP, might be due to the structural differences of the two enzymes.

Binding of shrimp ALP from *P. borealis* to Con A Sepharose suggests that this enzyme is a glycoprotein [10]. However, results presented here propose a different pattern of glycosyl components associated with this enzyme compared with other ALP such as the calf enzyme.

Interaction and effect of ions especially magnesium and zinc are another aspects of ALP activity in general. The effect of both ions (magnesium and zinc) on the calf ALP has been studied in detail previously [2, 3, 21, 22]. The

comparison of the effect of magnesium ion concentration on the activity of the both enzymes in the present study demonstrate a major effect of the cation on the shrimp ALP and a relatively minor effect on the calf ALP. In general, speaking the activity of calf ALP in the absence of magnesium ion is about 82% of its activity in the presence of 1.5 mM magnesium (which had the highest influence on the activity of calf ALP). However, the activity of shrimp ALP in the absence of magnesium ions is only 8% of its activity in the presence of 30 mM magnesium. These data clearly demonstrate that the fully activated shrimp enzyme needs higher magnesium concentrations than that of the calf enzyme.

The unusual stimulatory effect of magnesium ions on the activity of shrimp ALP is interesting. In our opinion, this effect may be related to the fact that shrimps live in oceans, which contain high concentrations of the cation.

Combined effect of both magnesium and zinc cations on the activity of shrimp ALP revealed that the addition of zinc ion more than 25 μ M in DEA buffer inactivates the enzyme. The similar observations were reported in a study on the human placental enzyme [23]. This inactivation effect was not influenced by the addition of magnesium, which had activated the enzyme in the absence of zinc ion. The effect of zinc and magnesium on shrimp ALP from *P. borealis* studied by Ragnar [19] revealed the importance of zinc ion on the activation of dialyzed enzyme. The enzyme was activated up to 70 % in the presence of 25 μ M zinc ion and 1 mM magnesium. However, our studies were carried out with undialyzed enzyme.

The optimum pH of shrimp ALP for NADP substrate in DEA buffer was found to be about 9.0, which is lower than that of calf ALP.

In an experiment comparing the activity of two enzymes, it was observed that the activity of shrimp ALP in the DEA buffer appears to be about two times higher than calf ALP (data not shown).

The successful conjugation of shrimp ALP to the streptavidin permits using this enzyme conjugate in lectin assays using WGA. In addition, the lack of glycan specific binding site for WGA allows conjugating the enzyme to the WGA lectin and using it in lectin assays as detector. In this case, the conjugate can be used to take advantage of enzyme amplification technique for obtaining higher sensitivities.

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