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Kinetic Investigation on Lactoperoxidase upon Interaction with Lead ion

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Abstract— **Lactoperoxidase (LPO) which is an enzyme of the mammalian peroxidase family , is known as an antibacterial enzyme, and it can be used as a biopreservative agent in food, feed specialties, cosmetics and related products.**

Lead (Pb), a heavy metal with no known physiological function in human body, is considered as one of the most hazards that affect all biological systems through exposure from air, water, and food source.

The aim of this project was to study the effect of Pb on the Lpo activity isolated from bovine milk in vitro.

Bathwise chromatography on phosphocellulose was used for partial purification of LPO from bovine milk by using a linear gradient of Nacl from 0 to 0.5 M. The purified enzyme had specific activity of 1.1 U/mg protein.

LPO activity was determined in the absence and Presence of different concentrations of Lead acetate, and Lineweaver-Burk double reciprocal plot was drawn according to the data obtained.

Pb^{2+} inhibited LPO activity progressively up to 0.8 mM concentrations where about 85% of the enzyme activity was lost. The inhibition was found to be non- competitive with respect to 2, 2´- azion- bis (3-ethylbenez- thiazoline-6- sulfonic acid (ABTS). Glutathione (1.2, 12 mM) or β - mercaptoethanol (1.2 mM) protected the enzyme inhibition, and protection by glutathione was concentration dependent. The data suggest a conformational change in the enzyme due to Pb^{2+} binding caused enzyme inactivation and sulfhydryl groups on the enzyme molecule probably are involved in the inhibition of the enzyme by Pb^{2+}.

Index Terms— **Enzyme, Lactoperoxidase, Lead, inhibition, non- competitive**

I. Introduction

Lactoperoxidase (LPO) is one of the most prominent enzymes in bovine milk [1]. It enters to whey during the cheese manufacturing process. LPO catalyzes the oxidation of thiocyanate (SCN-) by using hydrogen peroxide (H2O2), and generates hypothiocyanate (OSCN-).

The LPO enzyme together with SCN- and H2O2 is called lactoperoxidase system (LPO-S). This LPO-S has been of interest during two recent decades due to potent antibacterial properties of its product, OSCN- [2].

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Many applications have been recognized for the LPO-S, including in cosmetic, drug, medicine and food industries [3]-[5].The most widely recommended application of the LPO- S in food industries is in the dairy products for the preservation of raw milk during storage and transportation from spoilage [6].

The LPO- S may also be a useful additive for infant formula because of low concentration of LPO in human milk [7].

Efficiency of the LPO- S as preservative agent is dependent on the catalytic activity of the LPO enzyme in the LPO- S. optimum condition for the LPO enzyme has been reported, but inhibitors of the enzyme are not completely known. Among environmental pollutants, heavy metals have been known a group of elements effect on catalytic activities of enzyme[8].

Sat IG studied the effect of heavy metals on peroxidase from Jerusalem artichoke turbers and reported that some heavy metals such as iron, cobalt, strontium, zinc, mercury, nickel and aluminum inhibited peroxidase activity.

They showed that the enzyme activity decreased rapidly with increasing metal concentrations and the enzyme was completely inhibited even at low concentrations of the metal.

These metal ions can affect enzymes activities through several ways as follows binding to the protein- active groups of the enzyme. Biding to enzyme- substrate complex, and form a complex with the enzyme substrate [8].

Among heavy metals, Lead (Pb) has received attention in recent decades because of it has been recognized as one of the most widely distributed toxic metals in the world, and its levels found in air, food, water and soil is widely dependent to the degree of industrial development, urbanization and lifestyle facto [9].

The effect of Pb on the enzymes activities have been reported by many investigators Pandya etal in their study showed that Pb inhibits 17- β - hydroxy steroid oxidoreductase and uridine diphosphate-glucuronyltransferas activities of rats liver [10].

Pb also interfere withbiosynthesis of heme by inhibiting the activity of three enzymes δ - aminolevulinic acid synthetase δ - aminolevulinic acid dehydratase , and ferrochelatase [11].

It has been indicated that Pb+2 inhibit δ - aminolevulinic acid dehydratase non-competitively, and sulfhydryl groups on the enzyme have been involved in Pb+2 binding [12]. Although some kinetics parameters of the bovine milk LPO have been well investigated [13], but effect of metal ions on the catalytic activity of the enzyme is less studied.

Since LPO enzyme can be used in many industrial products as a preservative agent, and its antibacterial properties in dependent on the catalytic activity of the enzyme, investigation of the effect of Pb, which is a widely distributed metallic pollutant of our environment, seems to be of great importance.

In this study , the effect of Pb^{2+} ion on LPO activity was investigated.

II. Materials And Methods

A. Materials

2,2´-azino-bis(3-ethlbenz-thiazoline-6-sulfonic acid (ABTS) diammonium salts, phosphocellulose, hydrogen peroxide (30% solution), lead acetate, ammonium sulfate, glutathione, and β-mercaptoethanol (BME) were obtained from sigma (U.S.A). All other chemicals were of analytical grade.

B. Enzyme Purification

a.Skimmed milk preparation

Fresh cow's milk was centrifuged at 2000 ×g for 20 min at room temperature for preparing skimmed milk. The upper layer (fats) was separated and was discharged.

b. Whey preparation

Samples were used for preparing whey, by adding rennet to skimmed milk (20 mg/lit).

c.. Ammonium sulfate preparation

The whey sample was participated by adding ammonium sulfate (360 g/L) to the whey sample during 3 hours while it was on an electrical magnetic mixture and was mixing slowly. It was centrifuged at 12000 ×g for 15 min and the pellet obtained was dissolved in minimum volume of 50 mM phosphate buffer with pH 7.

d. Cation-exchange chromatography

The enzyme was then purified on phosphocellulose using 50 mM phosphate buffer with pH = 7 and a linear gradient of NaCl from 0 to 0.5 mM. Enzyme was eluted in phosphate buffer containg 0.4 mM of NaCl. The eluted enzyme was precipitated by adding ammonium sulfate (360 g/L), and the pellet obtained by centrifugation of 12000 × g for 15 min was dissolved in minimum volume of the same buffer. The enzyme was dialyzed over night against 100 volume of the same buffer [14].

C. Enzyme Assay

LPO activity was measured in 0.1M phosphate buffer (pH = 6), containing 0.6 mM ABTS and 0.1 mM H_2O_2 as described by Barrett et al [15]. The reaction was started by the addition of the enzyme, and absorbance was recorded at 412 nm during 2 min.

D. Enzyme Inhibition

The inhibition of LPO by pb^{2+} was done in the assay mixture containing different concentrations of pb^{2+} at 25 °C. The incubation time was 5 min. the effect of sulfhydryl compounds on the enzyme inhibition by pb^{2+} was investigated by incubating the enzyme solution (2 µg/ml) in the presence of pb^{2+} (0.8 mM) and either glutathione (1.2, 12 mM), or β-mercaptoethanol (1.2 mM) for 5 min at 25 °C followed by measurement of the enzyme activity. Protein concentration was determined according to the method of Lowry et al [16].

III. Result

A. Enzyme Purification

LPO from bovine milk was purified (800 fold) using the procedure described in the methods and had a purity index (A 412 nm/ A280 nm) of about 0.7 with a yield of 52% with specific activity of 30 U/mg protein.

B. Effect of Lead

Pb^{2+} inhibited bovine milk LPO activity progressive ly up to 0.8 mM where about 85% inhibition was achieved 5 min after addition of pb^{2+} (Fig 1).

Lineweaver- Burk reciprocal plot of LPO in the presence of two fixed concentration of pb^{2+} is shown in Fig 2. The inhibition was non-competitive with respect to 2, 2`-azino-bis (3-ethylbenz-thiazoline-6-sulfonic acid (ABTS) as substrate. The presence of glutathione or β-mercaptoethanol decreased pb^{2+} -induced enzyme inhibition, and the protection of the inhibition of the enzyme by glutathione was concentration dependent (Fig 3).

Fig. 1

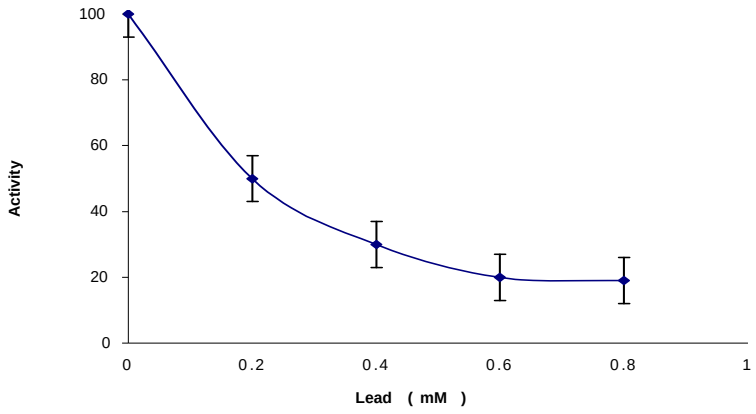

Fig. 2

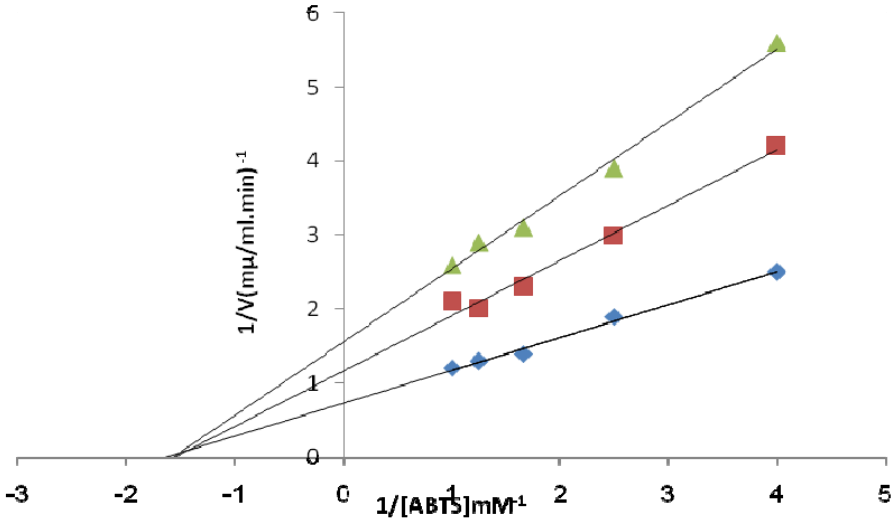

Fig. 3

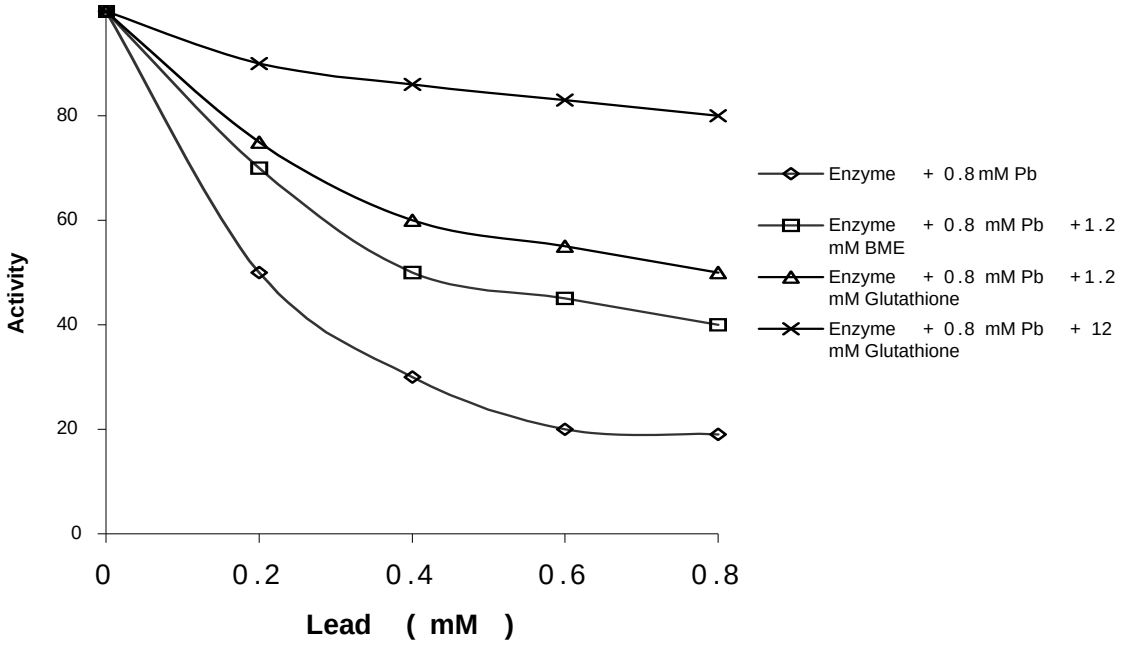

Fig 1. The effect of different concentrations of pb^{2+} on bovine milk LPO activity. Lead acetate was added to the assay mixture at 25 °C and after 5 min the enzyme activity measured as described in methods.

Fig 2. Lineweaver-Burk reciprocal plot of the bovine milk LPO in the presence of two fixed concentrations of pb^{2+} as an inhibitor

Fig 3. the effect of different concentrations of pb^{2+} on LPO activity in the absence and presence of 1.2 mM glutathione, 12 mM glutathione, and 1.2 mM β-mercaptoelhanol (BME).

Lead acetate was added to the assay mixture at 25°C and after 5min the enzyme activity measured as described in methods.

IV. Discussion

Lactoperoxidase system (LPO-S) has been of interest during recent two decades for its antibacterial properties [2].

This system can be used as a preservative agent in various products including, drug, cosmetic, and food products [3]-[5].

Lead (pb), which is widely distributed in the world, has been known as one of the most toxic substances, that affects all biological systems [17].

The toxicity of pb is primarily induced by inhibition of enzymes activities and / or replacement of essential trace elements of metalloproteins.

In this project, the effect of pb^{2+} on purified bovine milk LPO was studied in vitro. The enzyme was partially purified having specific activity of 30 U/mg protein.

Pb^{2+} inhibited the enzyme activity reversibly, and according to the inhibitory pattern of the enzyme in the presence of two different concentrations of pb^{2+}, the inhibition was found to be non-competitive with respect with 2, 2´-azion-bis (3-ethylbenz-thiazoline-6-sulfonic acid (ABTS) as substrate.

It means that pb^{2+} binds to a place other than active site of the enzyme, and this binding occurs both with free enzyme (E) form, and enzyme-substrate (E-S) complex.

The interaction of pb with some enzymes have been studied.

Hande et al investigated the effect of pb^{2+} on some antioxidant enzymes and showed that pb^{2+} inhibits sulfhydryl groups-containing enzymes through binding of pb^{2+} to the sulfhydryl groups on the enzyme molecule [18].

For clarifying the reacting groups on the LPO molecule, effect of pb^{2+} on the enzyme activity in the presence of sulfhydryl groups-containing compounds was studied.

Glutathione and / or β-mercoptoethanol protected of the enzyme inhibition by pb^{2+}, showing that sulfhydryl groups on the LPO molecule were involved in the pb-induced enzyme inhibition.

The protection of glutathione against enzyme inhibition was concentration dependent, showing that there is a competition between sulfhydryl groups on the glutathione molecules and the slfhydryl groups on the LPO molecules for binding to pb^{2+}.

Unfortunately using of pb-coated containers, especially in food industries, are still common.

Our in vitro experiments showed that pb^{2+} inhibits LPO activity, and pb-induced LPO inhibition can reduce by the addition of sulfhydryl groups containing substances to the medium.

As mentioned above, LPO-s can be used in food products as a biopreservative agent, and the LPO efficiency is dependent on the presence of pb in the medium.

It is suggested that the elimination of pb from food containers must be seriously considered for better efficiency of the LPO-s as a biopreservative agent**.**

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