Potent Inhibitory Effects of Some Phenolic Acids on Lactoperoxidase

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ABSTRACT: Lactoperoxidase (LPO) plays a key role in immune response against pathogens. In this study, we examined the effects of some phenolic acids on LPO. For this purpose, bovine milk LPO was purified 380.85-fold with a specific activity of 26.66 EU/mg and overall yield of 73.33% by using Amberlite CG-50 H^+ resin and CNBr-activated Sepharose-4B-L-tyrosine-sulfanilamide affinity chromatography. After purification, the *in vitro* effects of phenolic acids (tannic acid, 3,4-dihydroxybenzoic acid, 3,5- dihydroxybenzoic acid, chlorogenic acid, sinapic acid, 4-hydroxybenzoic acid, vanillic acid, salicylic acid, and 3-hydroxybenzoic acid) were investigated on LPO. These phenolic acids showed potent inhibitory effect on LPO. K_i values for these phenolic acids were found as 0.0129 nM, 0.132 μM, 0.225 μM, 0.286 μM, 0.333 μM, 2.33 μM, 10.82 μM, 0.076 mM, and 0.405 mM, respectively. Sinapic acid and 4-hydroxybenzoic acid exhibited noncompetitive inhibition; 3,4-dihydroxybenzoic acid showed uncompetitive inhibition, and other phenolic acids showed competitive inhibition. © 2016 Wiley Periodicals, Inc. J. Biochem. Mol. Toxicol. 30:533–538, 2016; View this article online at wileyonlinelibrary.com. DOI 10.1002/jbt.21819

KEYWORDS: **Lactoperoxidase; Bovine Milk; Phenolic Acids; Inhibition**

INTRODUCTION

Lactoperoxidase (LPO) (E.C.1.11.1.7) is a mammalian peroxidase and plays an important role against pathogens [1]. Primarily LPO was isolated from bovine milk and usually found in secretions from human mammary, salivary, lacrimal glands, and secretory glands [2]. It preferentially oxidizes thiocyanate ions (SCN^-) to hypothiocyanate ions ($OSCN^-$) at the expense of hydrogen peroxide (H_2O_2) [3]. $OSCN^-$ oxidizes the -SH groups of vital enzymes such as hexokinase, glyceraldehyde-3-phosphate dehydrogenase in bacteria and cause to lose biological functions of these enzymes. The resulting bacterial cytoplasmic membranes are damaged structurally, glucose, purine, pyrimidine and amino acid uptake protein, DNA and RNA synthesis is inhibited [4]. So, *LPO* system plays an important role in the immune defense *system*. Additionally, *LPO* system has wide applications area for example preservative in food as well as in oral care, shelf-life, cosmetic, and other products [5, 6].

Phenolic acids are the most important natural substances because of their biological, pharmacological, and medicinal properties. They have anticarcinogenic, antibacterial, antiviral, anti-inflammatory actions as well as powerful antioxidant activity. Because of these properties, phenolic acids are extensively studied [7]. Up to now, many studies have been done to examining the interaction of enzymes with phenolic acids. Recent studies have been reported that some different phenolic acids showed a significant inhibitory effect on the activity of many enzymes such as carbonic anhydrase [8], glucose-6-phosphate dehydrogenase, and 6-phosphogluconate dehydrogenase [9]. For example, in recent study, the effects of ellagic acid, gallic acid, ferulic acid, caffeic acid, quercetin, *p*-coumaric acid, *p*-hydroxybenzoic acid, and syringic acid on human carbonic anhydrase I (hCAI) and II (hCAII) were examined and it has been shown that these phenolic acids had strong inhibitory effect on activity of hCAI and hCAII isozymes [8]. In another study, Adem et al. [9] examined the effects of different phenolic compounds on glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase.

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They reported that caffeic acid, ellagic acid, ferulic acid, and sinapic acid had inhibition effects on activities of both enzymes but chlorogenic acid, p-coumaric acid, and syringic acid did not exhibit an effect on the activity of two enzymes [9]. In addition, Sarikaya et al. [7] investigated the inhibitory effects of ellagic acid, gallic acid, ferulic acid, caffeic acid, p-coumaric acid, and syringic acid on bovine LPO activity. According to this study findings, phenolic acids exhibit strong inhibitory effect on bovine LPO enzyme [7].

However, no reports could be found in literature on the effects of tannic acid, 3,4-dihydroxybenzoic acid, 3,5- dihydroxybenzoic acid, chlorogenic acid, sinapic acid, 4-hydroxybenzoic acid, vanillic acid, salicylic acid, and 3-hydroxybenzoic acid on purified LPO from bovine milk. In this study, we aimed to investigate the inhibitory effects of a series of simple phenolic acids on LPO, which are widely used antioxidant food additives, prodrugs, or drugs. These phenolic acids are important bioactive compounds and they have been associated a variety of pharmacological activities. For this aim, LPO enzyme was purified from bovine milk then K_i constants and inhibition types were firstly determined for these phenolic acids.

MATERIAL AND METHODS

Chemicals and Instruments

Bovine milk was obtained from the local dairy. CNBr-activated Sepharose-4B-L-tyrosine, sulfanilamide, Amberlite CG-50- NH_4^+ resin, BSA (lyophilized powder), chemicals for electrophoresis, phenolic acids, and other chemicals were obtained from Sigma–Aldrich Co. (Sigma-Aldrich Chemie GmbH Export Department Eschenstrasse 5, 82024 Taufkirchen, Germany). Standard protein markers for electrophoresis were obtained from Thermo Scientific (Vilnius, Lithuania).

Measurement of LPO Activity

Shindler and Bardsley's [10] method was used with a slight change for checking of activity of LPO. This method is based on the oxidation of ABTS as a chromogenic substrate by H_2O_2, which results in a product that absorbs at 412 nm. Briefly, the 2.8 mL of 1 mM ABTS in 0.1 M phosphate buffer, pH 6.0, was mixed with 0.1 mL of enzyme sample in 1 mM phosphate buffer, pH 6.0, and 0.1 mL of 3.2 mM H_2O_2 solution. The absorbance was measured at 412 nm as a function of time in every 1 min during 3 min. The one unit of activity is defined as the amount of enzyme catalyzing the oxidation of 1 μmol of ABTS min^{-1} at 25°C (molar absorption coefficient 32,400 M^{-1} cm^{-1}) [11].

Purification Procedure of LPO from Bovine Milk

Bovine milk was centrifuged at 3000 × g at 4°C for 15 min to remove fat from bovine milk. Amberlite CG 50 NH_4^+ resin was added at a rate of 4.4 g/150 mL. Then, the resin was washed with distilled water and sodium acetate solution (0.5 mM, pH 6.8). The bound proteins were eluted with sodium acetate solution (2 M, pH 6.8). The eluate was applied to the Sepharose-4B-L-tyrosine-sulfanilamide affinity column to obtain the purified LPO. All of the purification steps made accordingly by Atasever et al. [12].

SDS-PAGE-Protein Determination

The enzyme purity was checked by SDS-PAGE [13]. Protein concentration was measured according to the method of Bradford using bovine serum albumin as a standard [14].

In Vitro Inhibition Studies

LPO activity was measured in the presence of different concentrations of phenolic acids (Figure 1). A control sample without inhibitor was taken as 100% and for each inhibitor, an activity%-[inhibitor] graphs were drawn. IC_{50} values were obtained from these graphs. For determination of the K_i constant, three different phenolic acid concentrations were used. ABTS was also used as a substrate at five different concentrations (0.166–0.5 mM). K_i constant obtained from the Lineweaver–Burk graph ($1/V - 1/[S]$), and all inhibition type was found for all phenolic acids. Analysis of data obtained was made by *t*-test and they are given as $X \pm SD$.

RESULTS

The LPO enzyme was purified from bovine milk 380.85-fold with a specific activity of 26.66 EU/mg and overall yield of 73.33% by using Amberlite CG-50 H^+ resin and CNBr-activated Sepharose-4B-L-tyrosine-sulfanilamide affinity chromatography (Table 1). The purified enzyme from affinity chromatography gave a single band in SDS-PAGE (Figure 2). Then purified enzyme was used in inhibition studies. Inhibitory effects of some phenolic acids on enzyme activity

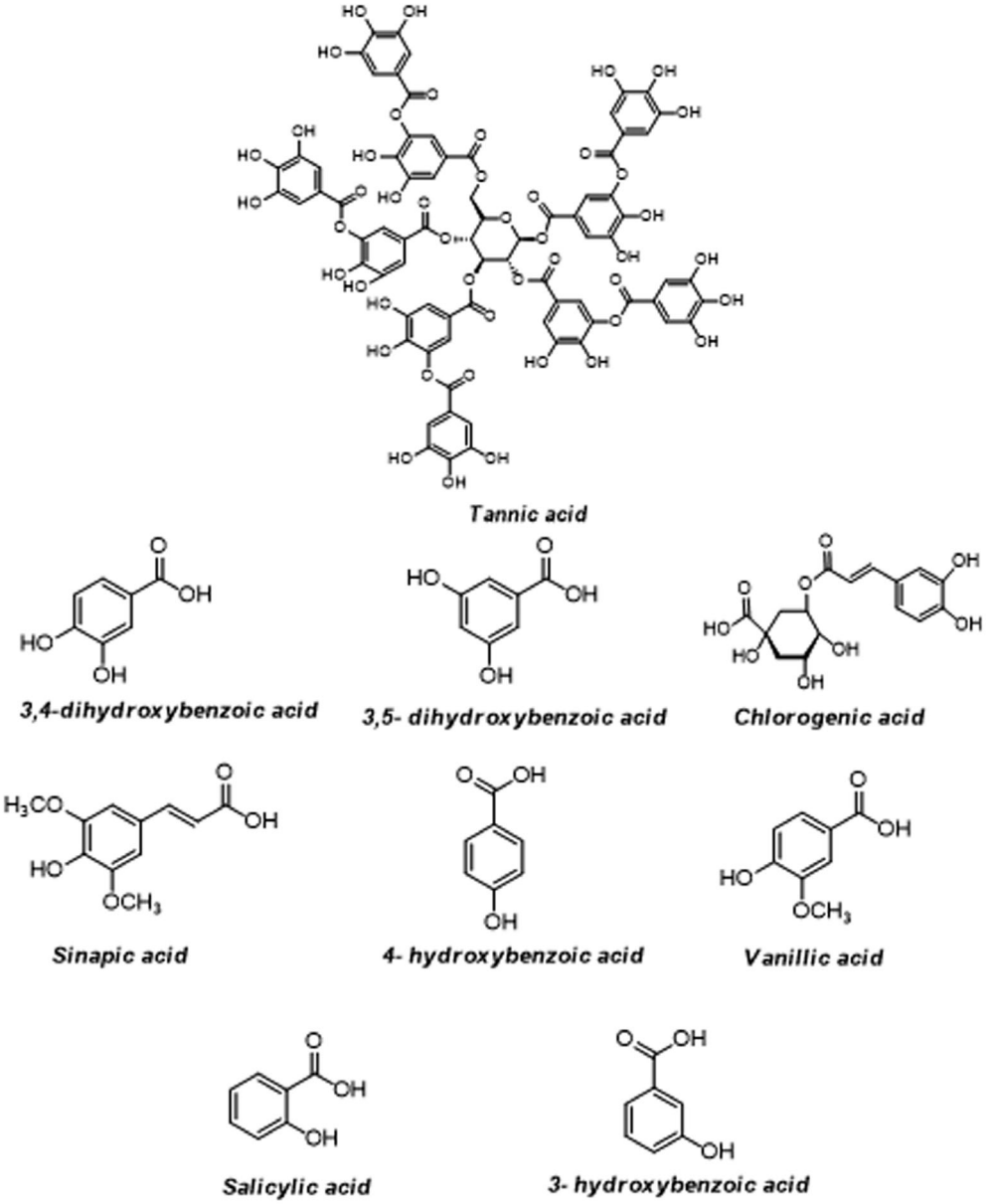

FIGURE 1. The molecular structures of phenolic acids used in this study.

were tested under *in vitro* conditions; IC_{50} values were calculated using activity%-[inhibitor] graphs. IC_{50} values for tannic acid, chlorogenic acid, sinapic acid, 3,5-dihydroxybenzoic acid, 3,4-dihydroxybenzoic acid, 4-hydroxybenzoic acid, vanillic acid, 3-hydroxybenzoic acid, and salicylic acid were determined as 0.0049 nM, 0.220 μM, 0.521 μM, 0.650 μM, 0.895 μM, 6.930 μM, 69.31 μM, 0.201 mM, and 0.232 mM, respectively. K_i values were calculated using the Lineweaver–Burk curves (Figure 3) and were given in Table 2. As shown in Table 2, pure LPO was inhibited by tannic acid, 3,4-dihydroxybenzoic acid, 3,5-dihydroxybenzoic acid, chlorogenic acid, sinapic acid, 4-hydroxybenzoic acid, vanillic acid, salicylic acid, and 3-hydroxybenzoic acid, and the K_i constants were in the range of 0.0129 nM to 0.405 mM. Sinapic acid and 4-hydroxybenzoic acid exhibited noncompetitive inhibition effect, 3,4-dihydroxybenzoic acid showed uncompetitive inhibition effect and other phenolic acids exhibited competitive inhibition effect (Table 2).

DISCUSSION

LPO is one of the important proteins in bovine whey. It has a natural antibacterial defense agent that

TABLE 1. Summary of Purification of Lactoperoxidase from Bovine Milk

Purification Steps	*Activity (EU/mL)*	*Protein (mg/mL)*	*Total Volume (mL)*	*Total Activity (EÜ)*	*Total Protein (mg)*	*Specific Activity (EU/mg)*	*Purification Fold*	*Yield %*
Crude homogenate from Amberlite CG-50-NH_4^+ resin	1.00	14.00	60	60	840	0.07	1	100
Purified LPO from Sepharose-4B-L-tyrosine sulfanilamide affinity chromatography	4.40	0.165	10	44	1.65	26.66	380.85	73.33

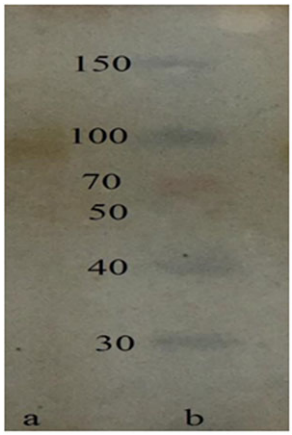

FIGURE 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of purified bovine milk lactoperoxidase. Lane b: Standard proteins (kDa). Lane a: Purified bovine milk lactoperoxidase enzyme.

catalysis the oxidation of thiocyanate ions (SCN^-) into hypothiocynate ($OSCN^-$) at the expense of hydrogen peroxide (H_2O_2) [15]. LPO has no antibacterial effect on its own but the resulting chemical compound ($OSCN^-$) has an antibacterial effect. So, the LPO system exerts an antimicrobial effect by the $OSCN^-$-mediated oxidation of essential protein and enzyme sulfhydryl groups [16]. The LPO system plays an important role in the innate immune system by killing bacteria in milk and mucosal secretions therefore enzyme system may have significant in therapeutic applications. Hence, protection the activity of the LPO enzyme has a vital role for immune systems of mainly newborns and all mammals [7]. In this study, we aimed to investigate the inhibitory effects of some phenolic acids on purified bovine milk LPO enzyme.

Phenolic acids are the most important groups of bioactive compounds. There is an increasing awareness and interest in the antioxidant behavior and potential health benefits associated with simple phenolic acids [17]. However, various harmful effects of phenolic compounds have been mentioned in some conducted studies [18]. Therefore, we have to be careful when used phenolic acid-containing food or drugs. Numerous investigations were reported effects of these molecules on different enzymes. For example, the effects of some phenolic compounds (ellagic acid, gallic acid, ferulic acid, caffeic acid, quercetin, p-coumaric acid, syringic acid, catechol, and epicatechin) on bovine LPO activity were investigated by Sarikaya et al. [7]. According to the results of this study, all phenolic compounds showed inhibition effect on LPO enzyme. In this study, we were investigated the *in vitro* effects of tannic acid, 3,4-dihydroxybenzoic acid, 3,5-dihydroxybenzoic acid, chlorogenic acid, sinapic acid, 4-hydroxybenzoic acid, vanillic acid, salicylic acid, and 3-hydroxybenzoic acid on bovine LPO activity. For this aim, LPO was purified from bovine milk by using Amberlite CG-50 H^+ resin and CNBr-activated Sepharose-4B-L-tyrosine-sulfanilamide affinity chromatography.

Our investigation showed all phenolic acids inhibited bovine milk LPO. Salicylic acid and 3-hydroxybenzoic acid were weak inhibition effect on LPO activity. K_i values of salicylic acid and 3-hydroxybenzoic acid were found as 0.076 and 0.405 mM, respectively. Tannic acid (K_i of 0.0129 nM) was the most effective inhibitor detected in this study. Furthermore, 3,4-dihydroxybenzoic acid (K_i of 0.132 μM), 3,5-dihydroxybenzoic acid (K_i of 0.225 μM), chlorogenic acid (K_i of 0.286 μM), sinapic acid (K_i of 0.333 μM), 4-hydroxybenzoic acid (K_i of 2.33 μM), and vanillic acid (K_i of 10.82 μM) were the other best inhibitors. 3,4-Dihydroxybenzoic acid exhibited uncompetitive inhibition, and sinapic acid and 4-hydroxybenzoic acid exhibited noncompetitive inhibition. The others phenolic acids showed competitive inhibition. According to these results, sinapic acid and 4-hydroxybenzoic acid caused to inhibition by binding to enzyme somewhere other than active site and 3,4-dihydroxybenzoic acid caused to inhibition by only binding to enzyme–substrate complex. Other phenolic acids lead to inhibition by binding to the active site of the enzyme.

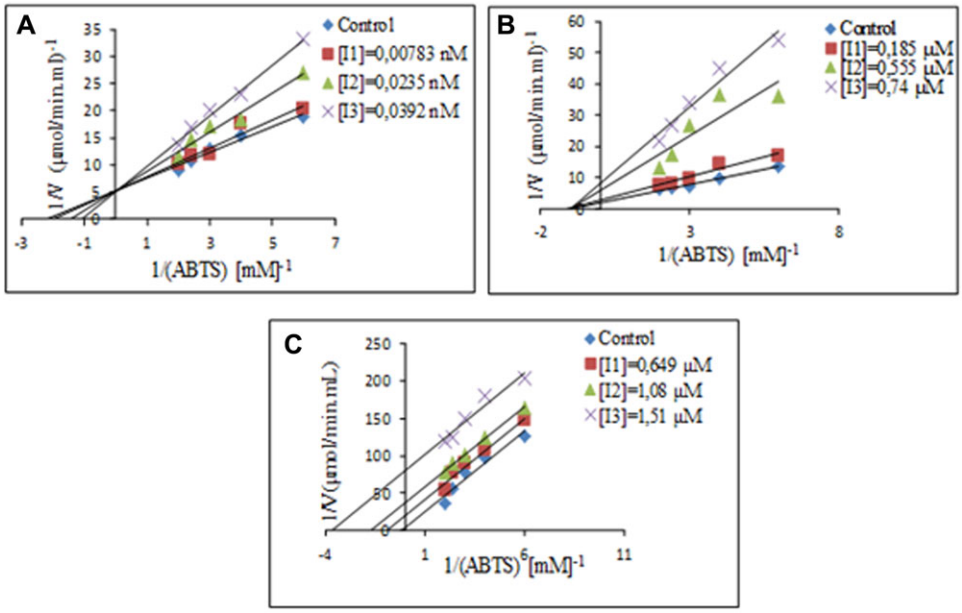

FIGURE 3. (A) Lineweaver–Burk graph of tannic acid using three different tannic acid concentrations for determination of K_i and inhibition type. (B) Lineweaver–Burk graph of sinapic acid using three different sinapic acid concentrations for determination of K_i and inhibition type. (C) Lineweaver–Burk graph of 3,4-dihydroxybenzoic acid using three different 3,4-dihydroxybenzoic acid concentrations for determination of K_i and inhibition type.

TABLE 2. K_i Values and Inhibition Types for Phenolic Acids Used in this Study

Phenolic Acids	*K_i*	*Inhibition Type*
Tannic acid	0.0129 ± 0.0204 nM	Competitive
3,4-Dihydroxybenzoic acid	0.132 ± 0.045 μM	Uncompetitive
3,5-Dihydroxybenzoic acid	0.225 ± 0.0792 μM	Competitive
Chlorogenic acid	0.286 ± 0.084 μM	Competitive
Sinapic acid	0.333 ± 0.157 μM	Noncompetitive
4-Hydroxybenzoic acid	2.33 ± 0.129 μM	Noncompetitive
Vanillic acid	10.82 ± 1.962 μM	Competitive
Salicylic acid	0.076 ± 0.0204 mM	Competitive
3-Hydroxybenzoic acid	0.405 ± 0.138 mM	Competitive

CONCLUSION

The conducted study shows that these phenolic acids reduced LPO activity. LPO activity and thiocyanate content in milk are very significant throughout lactation [19]. So, LPO has a vital role for the innate immune system. Thus, decrease in the enzyme activity means that the immune system is weakened. Because of this, we must be careful in the use of phenolic acids.

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