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RESEARCH ARTICLE

Lactoperoxidase, an antimicrobial enzyme, is inhibited by some indazoles

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ABSTRACT

Lactoperoxidase (LPO) has bactericidal and bacteriostatic activity on various microorganisms and it creates a natural antimicrobial defense system. So, LPO is one of the essential enzyme in biological systems and the protection of the LPO activity is extremely important for the immune system. Because of these features, the protection of the activity of the LPO has vital importance for the health of the organisms. Also, LPO is used in various sectors from cosmetics industry to agriculture industry due to its broad antimicrobial properties. Therefore, the identification of inhibitors and activators of the LPO is becoming increasingly important. In present study we aimed to investigate the inhibitory effects of some indazoles [1H-indazole (1a), 4-Bromo-1H-indazole (2a), 6-Bromo-1H-indazole (3a), 7-Bromo-1H-indazole (4a), 4-chloro-1H-indazole (5a), 6-chloro-1H-indazole (6a), 7-chloro-1H-indazole (7a), 4-fluoro-1H-indazole (8a), 6-fluoro-1H-indazole (9a), 7-fluoro-1H-indazole (10a)] on bovine milk LPO. Indazole derivatives are heterocyclic organic molecules with a wide range of biological activity. For this aim, bovine milk LPO was purified using Sepharose-4B-L-tyrosine-5-amino-2-methyl benzenesulfonamide affinity chromatography method. Then, the potential inhibitory effects of indazoles on LPO activity were investigated. K_i values were calculated for each indazole molecule. K_i values were ranging from 4.10 to 252.78 μM for 1a to10a. All of the indazole molecules we studied showed strong inhibitory effect on LPO activity. Also we determined inhibition types of the indazoles to clarify the mechanisms of inhibition.

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Antimicrobial; bovine milk; inhibition; indazoles; lactoperoxidase; purification

Introduction

Lactoperoxidase (LPO, E.C. 1.11.1.7) is an important milk-protein with oxidoreductase activity. Peroxidase isolated from the milk was called LPO (Reiter and Harnulv 1984), and it was the first enzyme discovered in milk (Arnold 1881). The main functions of the enzyme are to catalyze the oxidation of molecules in the presence of hydrogen peroxide and to aid in the production of molecules with a wide range of antimicrobial activity. Pseudohalogens, thiocyanates or halogens function as the secondary substrates for the enzyme and demonstrate a similar antimicrobial activity (Reiter and Perraudin 1991). LPO is mostly found in the breast tissue, saliva and lachrymal glands of mammalians, including human, bovine, buffalo, goat, sheep, lama, cow, camel and mouse (Koksal *et al.* 2017a).

Hydrogen peroxide/thiocyanate/LPO is defined as the LPO system. The antibacterial activity of the LPO system is due to the short-lived oxidation products (HOSCN, $OSCN^-$) resulting from the oxidation of thiocyanate in the catalysis of LPO. The main active oxidation product, hypothiocyanate (OSCN), specifically reacts with free -SH groups. These -SH groups are found in enzymes with vital precautions such as hexokinase, glyceraldehyde-3-phosphate dehydrogenase in bacteria. Hypothiocyanate oxidizes the -SH groups of these enzymes (such as hexokinase, glyceraldehyde-3-phosphate dehydrogenase) to sulfenyl thiocyanate and sulfenic acid and these situation causes these enzymes to lose their biological functions. In the bacteria that are affected by the LPO system, besides the oxidation of the SH groups, the cytoplasmic membrane of the bacterium is also damaged, resulting in extracellular potassium ion, amino acid and polypeptide leaks (Reiter and Harnulv 1984, Wolfson and Sumner 1993, Dionysius *et al.* 1992). After this, the intake of carbohydrates, purines, pyrimidines and aminoacids by the bacteria and the synthesis of protein, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) are blocked (Figure 1). As a result, bacteria are dying or growth and proliferation is prevented (Dionysius *et al.* 1992). LPO system is therefore, safe for human health.

The biological importance of LPO involves its role in providing a natural defense system against the invasion of microorganisms. In addition to such antiviral activity, it is also known to protect animal cells from various types of damage and peroxidative effects (Reiter and Harnulv 1984, Wolfson and Sumner 1993). LPO is also an important agent of the defense system against pathogenic microorganisms in the gastrointestinal tract of infants. LPO enzyme serves as an innate component of the non-immune biological defense system in mammalians, and it catalyzes oxidation of the thiocyanate ion to antibacterial hypothiocyanate (Kumar *et al.* 1995).

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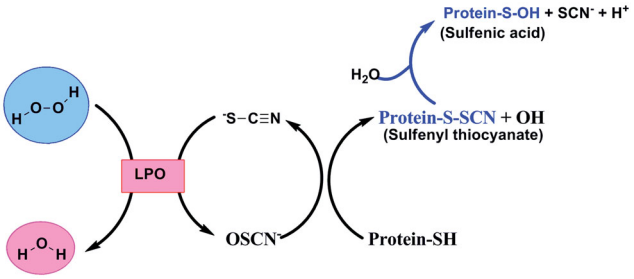


Figure 1. Catalytic reaction of LPO and the mechanism of antibacterial action of LPO system.

Due to these functional properties LPO is an important enzyme in many industrial applications such as dairy, cosmetic, pharmaceutical, veterinary and agricultural industries (Urtasun *et al.* 2017).

As LPO has several potential areas of application which are expected to grow, various researchers are currently conducting purification studies to decrease the costs associated with purification of LPO (Morrison and Hultquist 1963, Shin *et al.* 2001, Ozdemir and Uguz 2005, Boots and Floris 2006) and also extensive studies are being conducted to identify novel inhibitors of LPO enzyme (Koksal *et al.* 2016a,b, 2017a,b).

In present study we aimed to investigate the inhibitory effects of some indazole derivatives [1H-indazole (1a), 4-Bromo-1H-indazole (2a), 6-Bromo-1H-indazole (3a), 7-Bromo-1H-indazole (4a), 4-chloro-1H-indazole (5a), 6-chloro-1H-indazole (6a), 7-chloro-1H-indazole (7a), 4-fluoro-1H-indazole (8a), 6-fluoro-1H-indazole (9a), 7-fluoro-1H-indazole (10a)] on bovine milk LPO enzyme activity and assess their potential to be used as inhibitors. For this aim, LPO enzyme was purified from bovine milk then K_i constants and inhibition types were firstly determined for these indazole molecules. No previous study in the literature so far investigated the inhibitory effects of these molecules on bovine milk LPO. Indazoles are an important class of heterocyclic compounds with a wide range of application as biologic and pharmaceutic agents. They have been extensively studied due to their interesting chemical and biological characteristics. Indazole belongs to the azoles family, which includes carbon, hydrogen and a nitrogen atom. Indazole has a heterocyclic structure made up of benzene and pyrazole rings. Indazole derivatives have a wide range of biological activities. For instance, indazole derivatives show vasorelaxant and anti-aggregator activities by stimulating NO release and increasing cGMP levels. Recent medical research and development studies resulted in the production of indazole derivatives for the treatment of osteoporosis, inflammatory diseases and neurodegenerative disorders (Gaikwad *et al.* 2015).

In our study, we found that bovine milk LPO activity was reduced by very low concentrations of these indazole molecules. Since LPO has a crucial role for the immunity system, the inhibition of this enzyme means that the immune system is weakened. According to our results, we can say that we should be careful in terms of LPO inhibition when using indazole-containing drugs.

Material and methods

Chemicals and instruments

Bovine milk was obtained from the local dairy. CNBr-activated Sepharose-4B, L-tyrosine, 5-amino-2-methylbenzenesulfonamide, Amberlite CG-50-$NH4^+$ resin, BSA (lyophilized powder), chemicals for electrophoresis, 1H-indazole, 4-Bromo-1H-indazole, 6-Bromo-1H-indazole, 7-Bromo-1H-indazole, 4-chloro-1H-indazole, 6-chloro-1H-indazole, 7-chloro-1H-indazole, 4-fluoro-1H-indazole, 6-fluoro-1H-indazole, 7-fluoro-1H-indazole 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

LPO activity was measured using the procedure based on modifications of Shindler and Bardsley method (Shindler and Bardsley 1975). This method depends on the principle of oxidation of 2, 2'-azino-bis (3-ethylbenztiazolin-6-sulfonic acid) (ABTS) chromogenic substrate and monitoring of the absorbance increase at 412 nm. One enzyme unit was defined as the amount of enzyme catalyzing the oxidation of 1 μmol ABTS per minute at 25 °C (Koksal *et al.* 2017a).

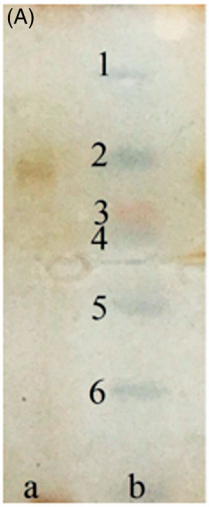
Purification procedure of LPO from bovine milk

One liter of fresh bovine milk was firstly centrifuged at 2.700 rpm, 4 °C for 15 min and centrifugation was repeated for three times to completely remove butter. Amberlite CG50 NH_4^+ resin was added at a proportion of 4.4 g/150 ml. Then, the resin was washed with distilled water and sodium acetate solution (0.5 mM, pH 6.8). Bound proteins were washed with sodium acetate solution (2 M, pH 6.8). To purify LPO, the obtained solution was applied on Sepharose-4BL-tyrosine-5-amino-2-methylbenzenesulfonamide affinity column and purification process were performed according to our previous work (Koksal *et al.* 2016a, 2017a). In purification process, Bradford method was used for quantitative protein detection (Bradford 1976). The purity of the enzyme was checked by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970, Demir and Beydemir 2015). In SDS-PAGE only a single band was observed for bovine milk LPO.

In vitro *inhibition studies*

The inhibitory effects of some indazoles on LPO enzyme activity purified from bovine milk were tested in triplicate at each concentration by using the procedure based on modifications of Shindler and Bardsley method (Shindler and Bardsley 1975). LPO activity was measured in the presence of five different fixed substrate concentrations and three different indazole concentrations for each indazole molecule. Lineweaver-Burk plots were drawn and the K_i value and inhibition type for each indazole molecule was determined from these graphs. Data were analyzed by *t*-test, and the

(A)



(B)

Purification steps	Total Volume (mL)	Activity (EU/mL)	Protein (mg/mL)	Total Activity (EU)	Total Protein (mg)	Specific Activity (EU/mg)	Yield %	Purification Fold
Crude Homogenate from Amberlite CG-50-NH_4^+ resin	10.00	4.36	13.00	43.60	130.0	0.33	100	1.00
Purified LPO from Sepharose-4B-L-tyrosine-5-amino-2-methyl benzenesulfonamide Affinity Chromatography	3.00	5.04	0.03	15.12	0.09	168.00	34.67	509.09

Figure 2. (A) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of purified bovine milk LPO. Lane a: Purified bovine milk LPO enzyme, Lane b: Standard proteins [150(1), 100(2), 70(3), 50(4), 40(5), 30(6) kDa]. (B) Purification results of LPO from bovine milk.

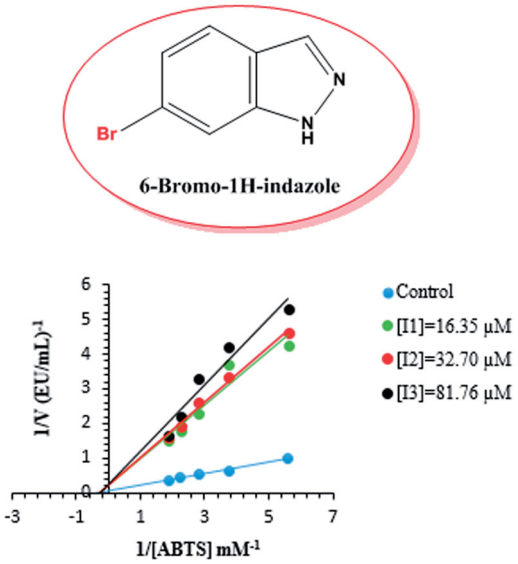


Figure 3. Lineweaver-Burk graph of 6-Bromo-1H-indazole.

Table 1. K_i values and inhibition types for indazoles used in this study.

Molecules	K_i (µM)	Inhibition type
1a	83.74 ± 30.38	Competitive
2a	50.75 ± 21.26	Competitive
3a	4.10 ± 1.96	Noncompetitive
4a	61.04 ± 29.33	Noncompetitive
5a	184.09 ± 59.48	Competitive
6a	252.78 ± 27.85	Noncompetitive
7a	31.97 ± 10.31	Competitive
8a	24.45 ± 0.51	Noncompetitive
9a	55.36 ± 19.88	Noncompetitive
10a	36.11 ± 7.92	Noncompetitive

results were given as $X \pm SD$ (Koksal *et al.* 2016a,b, Koksal *et al.* 2017a).

Results

LPO enzyme was purified from bovine milk with a specific activity of 168.00 EU/mg, 509.09-fold and a yield of 34.67% by using Amberlite CG-50 H^+ resin and CNBr-activated Sepharose-4B-L-tyrosin-5-amino-2-methylbenzenesulfonamide affinity chromatography. Purity of enzyme was checked with SDS-PAGE (Figure 2). Then the purified enzyme was used for inhibition studies. The inhibitory effects of 1a–10a molecules on enzyme activity were tested *in vitro*; K_i values were calculated using Lineweaver-Burk diagrams. K_i values were found as 83.74 ± 30.38 µM for 1a; 50.75 ± 21.26 µM for 2a; 4.10 ± 1.96 µM for 3a; 61.04 ± 29.33 µM for 4a; 184.09 ± 59.48 µM for 5a; 252.78 ± 27.85 µM for 6a; 31.97 ± 10.31 µM for 7a; 24.45 ± 0.51 µM for 8a; 55.36 ± 19.88 µM for 9a; 36.11 ± 7.92 µM for 10a. The results clearly indicated that the indazole molecules had effective LPO inhibition. According to the results, 3a molecule had the strongest inhibitory effect (Figure 3) whereas 6a molecule had the weakest inhibitory effect and 3a, 4a, 6a, 8a, 9a and 10a exhibited noncompetitive inhibition effect, other molecules exhibited competitive inhibition effect (Table 1).

Discussion

The biological importance of LPO enzyme involves its role in providing a natural defense system against the invasion of microorganisms. Bovine milk, on the other hand, is the only milk that has antimicrobial agents. In addition to such an antiviral activity, it was shown to provide protection from various kinds of damage and peroxidative effects in animal cells (Reiter and Harnulv 1984, Reiter and Perraudin 1991, Wolfson and Sumner 1993, De Wit and Hooydonk 1996). LPO, the most important enzyme in milk (Pakkanen and Aalto 1997, Scammell 2001), is an oxidoreductase secreted in milk and it plays significant roles against pathogenic microorganisms and defense in the gastrointestinal system of infants. LPO enzyme functions as an innate component of the mammalian non-immune biological defense system (Kumar *et al.* 1995).

LPO has an extensive area of application to reduce microflora in milk and cheese (Reiter 1985). LPO enzyme, purified from various animal resources, has a significant role in suppression of bacterial growth and supporting inhibition of

bacteria. Bacterial growth inhibition by bovine LPO is attributed to the peroxidase system, which includes H_2O_2 and thiocyanate (Jacob *et al.* 1998). LPO system has a natural antimicrobial activity in breast milk. LPO also has bacteriostatic effects on gram positive and gram-negative bacteria. Antibacterial studies performed with LPO enzyme purified from camel milk indicated that LPO-thiocyanate and peroxide system results in a significant level of inhibition of pathogenic bacteria. Therefore, LPO has several areas of application. It is particularly used in dairy industry for the protection of milk during transfer to milk processing facilities (Barrett *et al.* 1999, Sisecioglu *et al.* 2010b). Besides the dairy industry, LPO is also important in cosmetics, pharmaceuticals, veterinary and agricultural industries (Seifu *et al.* 2005).

As LPO has several areas of application, various researchers are currently conducting purification studies and identification of inhibitors and activators of LPO. Until now, it has been determined that various substances are LPO inhibitors. For example, hydrasines (Kumar *et al.* 1995), thiocarbamide compounds (Doerge 1986), Sulfanilamides (Atasever 2013), propofol and its derivatives (Sisecioglu *et al.* 2009, Koksal *et al.* 2014), some anesthetic drugs (Ozdemir and Uguz 2005), some bacteria species (Uguz and Ozdemir 2005), some phenolic acid compounds and phenolics (Sarikaya *et al.* 2015, Pruitt and Kamau 1985), avermectins (Koksal *et al.* 2016b), adrenaline, melatonin, serotonin and norepinephrine (Sisecioglu *et al.* 2012), fungi and bacteria (Sisecioglu *et al.* 2010a), hydrasines (Kumar *et al.* 1995) and some thiocarbamide compounds (Doerge 1986) were tested and reported as LPO inhibitors in the literature. But, there is not any research in the literature that investigated the inhibitory activity of indazole derivatives on LPO. In this study we investigated the *in vitro* inhibitory effects of some indazole derivatives [1H-indazole (1a), 4-Bromo-1H-indazole (2a), 6-Bromo-1H-indazole (3a), 7-Bromo-1H-indazole (4a), 4-chloro-1H-indazole (5a), 6-chloro-1H-indazole (6a), 7-chloro-1H-indazole (7a), 4-fluoro-1H-indazole (8a), 6-fluoro-1H-indazole (9a), 7-fluoro-1H-indazole (10a)] on bovine milk LPO enzyme activity.

The world's best-selling drugs are nitrogen-containing heterocyclic compounds. This is due to their widely common structure, which forms the backbone of several biological molecules and pharmaceutic products. Among them, indazole is a molecule with biological, agricultural and industrial areas of application. The biological and pharmaceutical properties of substituted for indazoles provide them with a range of applications in new drug development. Indazole derivatives are crucially important in several heterocyclic chemical reactions. Thus, these reactions and synthesis gain medical attention (Gaikwad *et al.* 2015). Indazole derivatives are pharmacologically important as they form the basic molecular structure of several drug molecules. For example, granisetron is used as an antiemetic for cancer chemotherapy, a 5HT3 receptor antagonist and an anti-inflammatory. Indazole ring includes two nitrogen atoms which may be functionalized at different positions with a high level of selectivity. The uniformity of indazole ring, side-ring length and assembling at different positions can allow generation of many indazole derivatives, which may then present new molecules with biological and therapeutic characteristics (Gaikwad *et al.* 2015).

In this study, LPO was purified from bovine milk by using Amberlite CG-50 H^+ resin and CNBr-activated Sepharose-4B-L-tyrosin-5-amino-2-methylbenzenesulfonamide affinity chromatography. Then, we investigated the inhibitory effect of some indazoles on this pure enzyme. In our study, the inhibitory effect of indazoles was determined by K_i values. K_i is the dissociation constant of the enzyme-inhibitor complex. Low K_i value means strong inhibition effect. The K_i values were found by Lineweaver-Burk graphs to be 83.74 ± 30.38, 50.75 ± 21.26, 4.10 ± 1.96, 61.04 ± 29.33, 184.09 ± 59.48, 252.78 ± 27.85, 31.97 ± 10.31, 24.45 ± 0.51, 55.36 ± 19.88 and 36.11 ± 7.92 μM for 1a–10a, respectively. According to K_i values we obtained, 3a molecule had the strongest inhibition on the bovine milk LPO enzyme. These results showed that when compared to the results of inhibition studies previously done on the bovine LPO enzyme, 3a molecule is a stronger inhibitor of LPO than norepinephrine (Sisecioglu *et al.* 2010a), ellagic acid, gallic acid, ferulic acid, quercetin, p-coumaric acid, syringic acid, catechol, epicatechin (Sarikaya *et al.* 2015) and avermectins (Koksal *et al.* 2016b) whereas 3a molecule is a weaker inhibitor of LPO than propofol (Sisecioglu *et al.* 2009), melatonin, seratonin (Sisecioglu *et al.* 2010b), caffeic acid (Sarikaya *et al.* 2015), 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 (Koksal *et al.* 2016a) and some secondary sulfonamides (Koksal *et al.* 2017b). According to the results we obtained, bromine and fluorine bonded indazole molecules exhibited a much stronger inhibitory effect than 1H-indazole on LPO activity. In the fluorinated indazole derivatives, only 7-fluoro-1H indazole had stronger inhibitory effect than 1H-indazole. When we look at bromine bound indazole molecules we have seen that the 4-bromo-1H indazole molecule is a much stronger inhibitor of 6-bromo-1H indazole and 7-bromo-1H indazole. 3a, 4a, 6a, 8a, 9a, 10a exhibited noncompetitive inhibition. The others molecules showed competitive inhibition. According to these results, 3a, 4a, 6a, 8a, 9a, 10a caused to inhibition by binding to enzyme somewhere other than active site and other molecules lead to inhibition by binding to the active site of the enzyme. As can be seen, the different order of binding of fluoro, chloro and bromo to the 1H-indazole molecule caused different inhibitory effects of these molecules on LPO activity.

Conclusions

The present study showed that the investigated indazole derivatives decreased the activity of LPO. The presence of LPO system, as one of the significant enzymes in milk, is crucial for the formation of antibacterial hypothiocyanite and LPO plays a vital role in the innate immune system. Hence, decreased enzyme activity reflects an impairment in the immune system. Based on the results of our study, we can say that indazoles may be strong inhibitor candidates for LPO and that caution should be exercised when using medicines containing indazole derivatives. In addition, today, LPO is used in the production of canned foods, beauty products and optic solutions, as well as pharmaceuticals, veterinary

and agricultural industries. For this reason, we hope that the results of our study may also be useful in industrial applications of LPO.

Disclosure statement

No potential conflict of interest was reported by the authors.

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