TÜBİTAK

ORIGINAL ARTICLE

Turk J Med Sci
2006; 36 (5): 301-306
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E-mail: medsci@tubitak.gov.tr

In Vitro Effects of Some Antibiotic Drugs on Bovine Lactoperoxidase Enzyme

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Aim: Owing to the widespread use of antibiotic drugs in both human and animals, we thought it would be important to determine the effect of these on lactoperoxidase (LPO; E.C. 1.11.1.7) activity during lactation. The *in vitro* effects of gentamicin sulfate and ampicillin sodium drugs on bovine LPO enzyme activity in breast-milk were investigated.

Materials and Methods: LPO was purified with Amberlite CG 50 resin, CM Sephadex C-50 ion-exchange chromatography, and Sephadex G-100 gel filtration chromatography from skimmed bovine milk. $R_z(A_{412}/A_{280})$ value for the purified LPO was found to be 0.8. Inhibition or activation effects of the drugs on LPO enzyme were determined using 2,2[1]-azino–bis (3-ethylbenzthiazoline-6 sulfonic acid) diammonium salt (ABTS) as a chromogenic substrate at pH = 6.0.

Results: The purified enzyme showed a single band on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). I50 values of gentamicin sulfate and ampicillin sodium drugs exhibiting inhibition effects were obtained by drawing % Activity- [drugs] graphs. The obtained I50 values of gentamicin sulfate and ampicillin sodium were 3.71 mM and 0.89 mM, respectively. Ki constants of the drugs were found by means of Lineweaver-Burk graphs. The obtained Ki constants for gentamicin sulfate and ampicillin sodium were 4.36 ± 3.122 mM and 0.473 ± 0.099 mM, respectively, and they were competitive inhibitors.

Conclusions: Use of antibiotics may worsen the LPO activity in milk during lactation. If these antibiotic drugs are required during lactation, their dosage should be carefully determined to decrease the side effects.

Key Words: Lactoperoxidase, antibiotic drugs

Sığır Laktoperoksidaz Enzimi Üzerine Bazı Antibiyotiklerin Etkisi

Amaç: Doğum öncesi ve sonrası insan ve hayvanlarda antibiyotiklerin yaygın kullanılması sebebiyle, emzirme esnasında bu antibiyotiklerin laktoperoksidaz enzimi aktivitesi üzerine etkisi olduğu düşünülebilir. Bu amaçla gentamicine sulfate ve ampiciline sodium ilaçlarının sığır laktoperoksidaz enzimi aktivitesi üzerine etkisi incelendi.

Yöntem ve Gereç: Laktoperoksidaz enzimi Amberlite reçinesi, Sephadex G-100 jel filitrasyon ve CM Sephadex iyon değişim kromatografisi kullanılarak yağı alınmış sığır sütünden saflaştırıldı. Saflaştırılmış süttee $R_z(A_{412}/A_{280})$ saflaşma değeri 0,8 bulundu. Enzimi aktivitesi, ve inhibisyon çalışmalarında substrat olarak pH 6 da 2,2[1]-azino–bis (3-ethylbenzthiazoline-6 sulfonic acid) (ABTS) kullanıldı.

Bulgular: Saflaştırılmış enzimin SDS-PAGE elektroferezi yapıldığında tek bant bulundu. Gentamicine sulphate ve ampiciline sodium için yüzde aktivite grafikleri çizilerek I 50 değerleri, hesaplandı. Bu garfiklerden gentamicine sulfatın I 50 değeri 3,71mM bulunurken ampiciline sodium I 50 değeri 0,89 mM olarak hesaplandı. Lineweaver-Burk grafikleri çizilerek her iki ilaç için Ki değerleri tespit edilmiş, gentamicine sulfatın Ki değeri 4,36 ± 3.122 mM, ampiciline sodiumun Ki değeri 0.473 ± 0.099 mM olarak hesaplanmıştır.

Sonuç: Antibiyotik kullanımı emzirme esnasıda sütte bulunan laktoperoksidaz enziminin aktivitesini düşürebilir. Bu antibiyotikleri emzirme esnasında kullanmak gerekirse yan etkileri en aza indirmek için verilecek miktarları hesaplanarak ayarlanabilir.

Anahtar Sözcükler: Laktoperoksidaz, Antibiyotikler

Received: June 12, 2006
Accepted: October 10, 2006

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Introduction

Lactoperoxidase (LPO) is an oxidoreductase secreted into milk and plays an important role in protecting the lactating mammary gland and the intestinal tract of newborn infants against pathogenic microorganisms (1). LPO (donor: hydrogen peroxide oxidoreductase E.C.1.11.1.7) was found in bovine milk and reported (2). Peroxidase activity is present guinea pigs, cows, sheep, pigs, llamas and mice and human milk (3). It is one of the prominent enzymes in milk. LPO has the ability to catalyze the oxidation of halides and pseudohalides such as thiocyanate by hydrogen peroxide to form potent oxidant and bactericidal agents. The enzyme, which catalyzes the oxidation of endogenous thiocyanate (SCN^-) to the antibacterial hypothiocyanate ($OSCN^-$), is a redox enzyme with antibacterial property found in several biological fluids, like milk, tears, and saliva (4,5). Other biological effects of this protein including antitumor activity have been reported. The enzyme has a single polypeptide chain containing 612 amino acid residues, a heme prosthetic group, and four or five carbohydrate chains which occupy approximately 10% of the total mass, and its molecular weight is about 85 kDalton (6,7).

Antibiotics are widely used to deal with various disorders, but there are few reports of their effects on enzyme activities. Some studies found either increases or decreases in mammalian enzyme activities, and the inhibitor or activator effects of some antibiotics have been investigated (8).

Gentamicin sulfate and ampicillin sodium drugs are widely used in the treatment of various disorders in the world. Owing to the widespread use of these drugs in both humans and animals, we aimed to investigate the effect of these on LPO activity in breast-milk during lactation. In this study, the in vitro effect of the antibiotics on LPO enzyme purified from bovine milk was firstly investigated.

Materials and Methods

Purification of LPO

Bovine milk was centrifuged at 2500 rpm at 4 °C for 15 min to remove fat. Amberlite CG 50 NH_4^+ resin (equilibrated with 5 mM sodium acetate pH 6.8) was added at the rate of 22 g/L to the fresh raw skimmed bovine milk (9,10,11). The supernatant was decanted. The resin was washed with distilled water and 20 mM sodium acetate (pH 6.8). The bound protein was eluted with 0.5 M sodium acetate pH 6.8. Solid ammonium sulfate (1st Precipitation; saturation 90%) was gradually added to the green-colored mixture over a period of 30 min while being stirred magnetically and the enzyme solution was dialyzed overnight against 5 mM sodium phosphate buffer pH 6.8.

The clear greenish supernatant as obtained above was loaded to a column of CM Sephadex C-50 (Fluka) (3 x 10 cm) previously equilibrated with 10 mM sodium phosphate buffer (pH 6.8). The column-bounded enzyme was washed with 100 ml of 10 mM phosphate buffer pH 6.8 containing 100 mM NaCl. The enzyme was eluated with linear gradient 100-200 mM NaCl in 10 mM phosphate buffer pH 6.8 and subjected to II ammonium sulfate precipitation (saturation 90%), and thereafter the enzyme solution was dialyzed overnight against 5 mM sodium phosphate buffer pH 6.8.

The dialyzed LPO sample as obtained above was applied to a column of Sephadex G 100 (Fluka) (2.5 x 100 cm). The column-bounded enzyme was eluted with 0.1 M phosphate buffer pH 6.8, and salted out with 90% saturation of 3rd ammonium sulfate precipitation. The enzyme solution was dialyzed overnight against 0.5 M sodium phosphate buffer pH 6.0. Fractions were lyophilized and checked for purity by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel (12) (Figure 1). Protein concentration was determined according to the method given by Lowry using bovine serum albumin as a standard (13).

Determination of LPO Activity

Lactoperoxidase activities were determined by the procedure of Shindler with a slight modification (14). This method is based on oxidation of 2,2-azino–bis (3-ethylbenzthiazoline-6 sulfonic acid) diammonium salt (ABTS) as a chromogenic substrate by means of H_2O_2, and color compound, which occurs during reaction and gives an absorbance at 412 nm. Briefly, 2.8 ml of 1 mM ABTS in phosphate buffer (0.1 M pH = 6.0) was mixed with 0.1 ml of enzyme in phosphate buffer 1 mM pH 6.8 and 0.1 ml of 3.2 mM H_2O_2 solution. The absorbance was taken at 412 nm as a function of time in every 15 sec. One unit of activity is defined as the amount of enzyme catalyzing the oxidation of 1 µmol of ABTS min^{-1} at 298 K (molar absorption coefficient; 32400 M^{-1} cm^{-1})

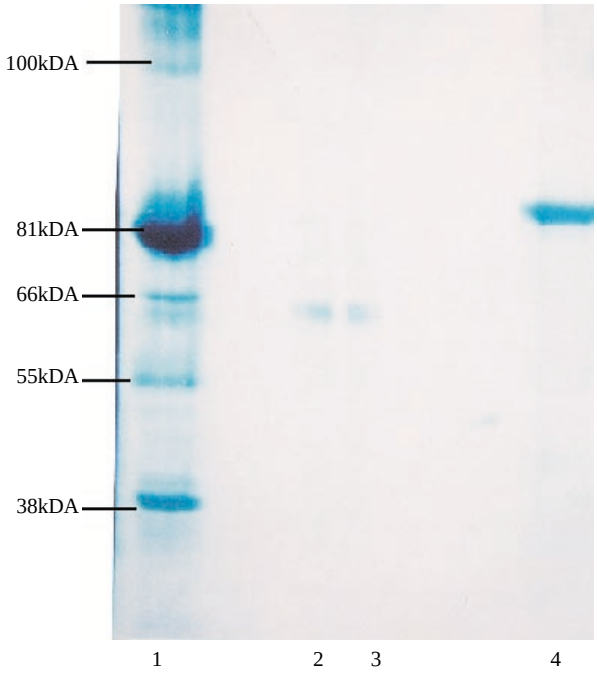

Figure 1. SDS-polyacrylamide gel electrophoresis of bovine LPO.
Lane 1: Standard proteins: yeast hexokinase (100 kDa), rabbit heart creatine phosphokinase (81 kDa), bovine serum albumin (66 kDa), bovine liver glutamic dehydrogenase (55 kDa), bovine spleen deoxyribonuclease (38 kDa). Lanes 2-3 are rainbow trout G6PD. Lanes 4 are purified bovine lactoperoxidase.

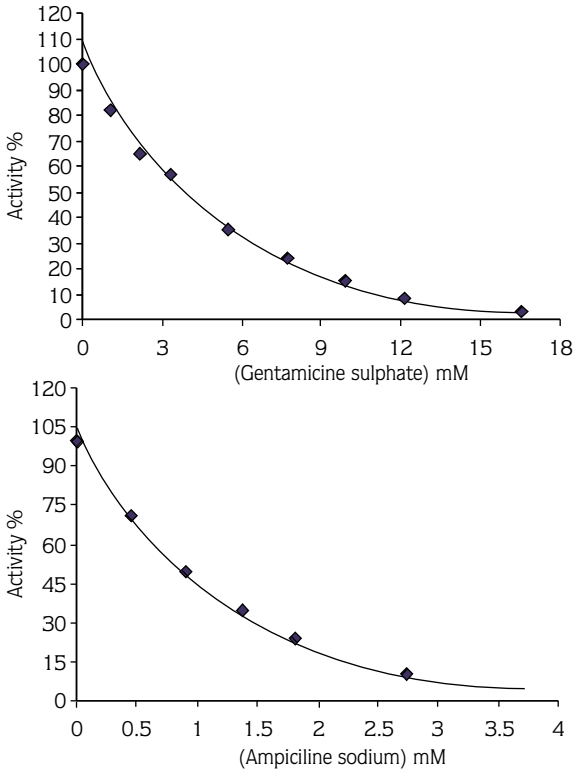

Figure 2. % Activity-[drug] graphs for LPO for two drugs: (A) gentamicin sulfate and (B) ampicillin sodium.

Effects of Antibiotic Drugs on LPO Activity

To determine the effects of the antibiotic drugs on LPO, enzyme activities were measured for gentamicin sulfate (1.1-16.5mM) and ampicillin sodium (0.457-3.57mM) at these cuvette concentrations. Control cuvette activity in the absence of drug was taken as 100%. For each drug an Activity-[Drug] graph was drawn. For these two antibiotics which had an inhibitory effect on the enzyme, drug concentration that produced 50% (I_{50}) was calculated from these graphs (Figure 2).

For determination of the K_i constant, three different inhibitor concentrations (5.10, 7.45 and 15.62 mM for gentamicin sulfate and 0.10, 0.28 and 0.57 mM for ampicillin sodium) were used. In these studies ABTS was used as substrate in five different concentrations (0.06-0.35 mM). The Lineweaver-Burk graphs (1/V-1/[S]) were obtained for each inhibitor; K_i constant and inhibition types were estimated from graphs (Figures 3 and 4). Analysis of data obtained was made by *t*-test and results are given as X ± *SD*.

Results

LPO was eluated by CM Sephadex C 50 ion exchange chromatography and measured R_z (A_{412}/A_{280} nm) of fractions. R_z values 0.7 or higher fractions were pooled. The enzyme obtained from ion exchange chromatography was applied to Sephadex G 100 gel filtration chromatography. As shown in Table 1, specific activity was calculated for crude extract and purified enzyme solution, yielding a purification of 11.2 fold and obtained 6.7 mg (R_z = 0.8) from 1 L bovine milk. Kinetic parameters as optimum pH, K_m and V_{max} were calculated from graphics for ABTS substrate on LPO. Optimum pH value was found by means of activity-pH graphs, and was 6. K_m value at optimum pH was 0.411 mM, V_{max} value at optimum pH was 13.6 µmol/ml min. All purification steps were controlled by SDS-PAGE. Yeast hexokinase, rabbit heart creatine phosphokinase, bovine serum albumin, bovine liver glutamic dehydrogenase and bovine spleen deoxyribonuclease were used as standard. As shown in Figure 1, bovine lactoperoxidase has a molecular weight of about 80000 Da.

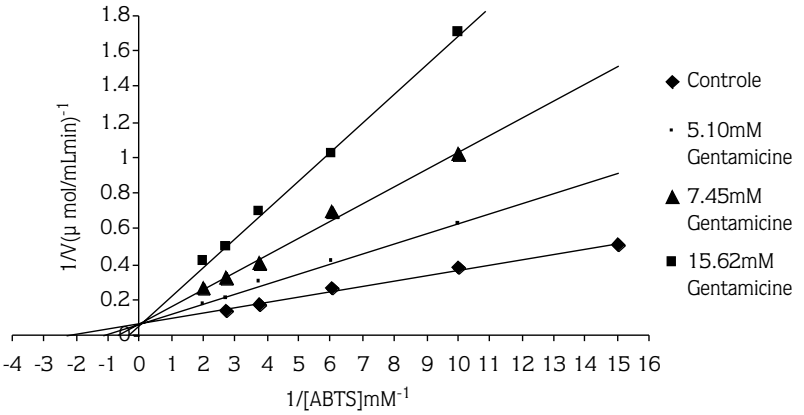

Figure 3. Lineweaver-Burk graph for 5 different substrate (ABTS) concentrations and 3 different gentamicin sulfate concentrations for determination of K_i constant.

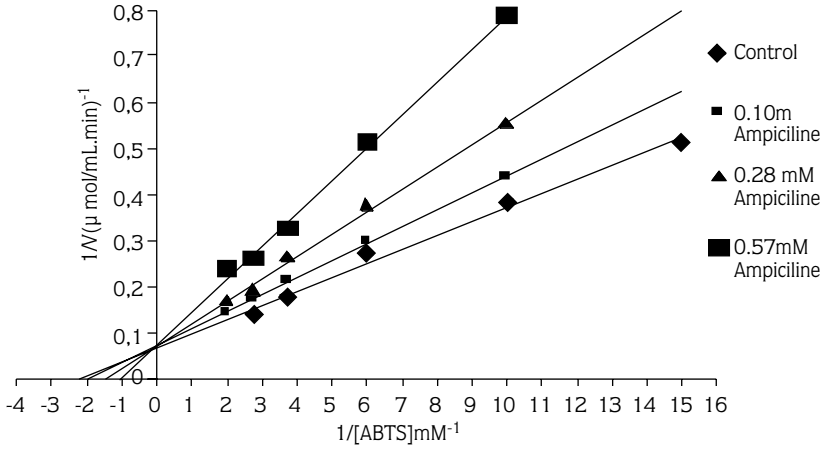

Figure 4. Lineweaver-Burk graph for 5 different substrate (ABTS) concentrations and 3 different ampicillin sodium concentrations for determination of K_i constant.

In order to show inhibition effects, the most suitable parameters are the K_i and I_{50} values. Therefore, in this study, both K_i and I_{50} parameters of these drugs for bovine LPO were determined. As shown in Table 2 and Figure 3, K_i values were calculated from Lineweaver-Burk graphs. K_i constants of gentamicin sulfate, and ampicillin sodium drugs were 4.36 ± 3.122 mM and 0.473 ± 0.099 mM,, respectively. In addition to K_i constants, types of inhibition of the drugs were obtained on Lineweaver-Burk graph. Gentamicin sulfate and ampicillin sodium drugs show competitive inhibition. As shown in Table 2 and Figure 2, the inhibitor concentrations causing up to 50% inhibition were determined from % Activity-[drugs] graphs. The obtained I_{50} values of gentamicin sulfate and ampicillin sodium drugs were 3.71 mM and 0.89 mM, respectively.

Discussion

LPO may exert some protective effects such as antimicrobial activity and removal of toxic H_2O_2 both in the mammary gland and in the gut of infants throughout the lactation period (15). LPO catalyzes the oxidation of thiocyanate to produce hypothiocyanate, which has broad-spectrum antimicrobial activity (4,5,15). Other biological effects of this protein including anti-tumor activity, and immunoregulatory effects have been reported as well. LPO isolated from goat and camel milk

Table 1. Purification steps of lactoperoxidase from bovine milk.

Step	Activity (EU/ml)	Total Volume (ml)	Protein (mg/ml)	Total Protein (mg)	Total Activity (EU)	Specific Activity (EU/mg)	Recovery (%)	Purification (Fold)
Crude homogenate	3.33	150	1.85	277	499	1.80	100	1.00
Ammonium sulfate	15.6	26	197	50.8	405	7.97	82	4.42
CM-Sephadex C50 column	1.29	300	0.09	27.0	387	14.33	77.4	7.96
Ammonium sulfate	12.2	26	0.70	18.2	317	17.41	64	9.67
Sephadex G100 column	1.59	140	0.08	11.2	222	19.8	44	11
Ammonium sulfate and dialysate	5.81	24	0.28	6.7	139.4	20.7	28	11.5

Table 2. I_{50} values, K_i constant, and inhibition types of gentamicin sulfate and ampicillin sodium for bovine LPO.

Inhibitors	[drugs] (mM)	K_i constant(mM)	Mean K_i constant(mM)	Inhibition Type	I_{50} values (mM)
Gentamicin sulfate	0.51	4.28	4.36 ± 3.122	Competitive	3.71
	1.00	4.43			
	1.53	4.37			
Ampicillin sodium	1.53	0.462	0.473±0.099	Competitive	0.89
	3.06	0.471			
	4.59	0.786			

are found to be antibacterial even in the absence of a medium containing thiocyanate-H_2O_2 (15,16). The LPO system is the most significant microbial inhibitor in raw milk (17). LPO is naturally present in raw milk and together with thiocyanate and peroxide constitutes the LPO system.Thiocyanate is more variable in milk and depends on feeding of the animal (FAO, 1999).. Human milk is known to contain several host defense factors including lactoferrin peroxidases and lysozyme (3,17). LPO system is one of the important host defense systems in the oral activity. LPO activity is highest in guinea pigs, cows, sheep, pigs, llamas, and mice and lowest in humans.

Many chemicals and drugs at relatively low dosages affect the metabolism of biota by altering normal enzyme activity, particularly inhibition of a specific enzyme. For example, glutathione reductase enzyme has been inhibited by ofloxacin, levofloxacin, cefepime, and cefazolin [8]. Gentamicin sulfate, an aminoglycoside, is used for treatment of many aerobic Gram-negative infections such as by *Escherichia coli*, *Klebsiella Enterobacter, Proteus,* and *Serratia* and infections by methicillin-resistant *Staphylococci*. The broad–spectrum gentamicin has been widely used in treatment of infectious diseases particularly in dairy animals, including uterine infections in cattle, despite numerous problems, which include its tendency to persist in bovine kidney tissue for several months (18,19). Ampicillin sodium has been very useful in animals and humans in the treatment of infection caused by Gram-positive and -negative bacteria such as *Staphylococcus aureus, Streptococcus viridans, Streptococcus faecalis, Escherichia coli*, *Neisseria catarrhalis,* and *Proteus mirabilis.*

There is no detailed study regarding antibiotics and LPO activity. In this study, LPO activity in antibiotic drug use during lactation *in vitro* was investigated and kinetic constant (K_i and I_{50} values) reported. The study showed that gentamicin sulfate, and ampicillin sodium have

strong inhibitory effects on LPO activity. Since effects of these antibiotics drugs on enzyme activity have not been previously reported, these results are of interest for further researches.

In conclusion, we think that the results of this study must be considered in using antibiotic drugs throughout the lactation period since LPO has antimicrobial activity and contributes to the protective functions of milk. Use of antibiotics may worsen LPO activity in milk during lactation. If these antibiotics drugs are required during lactation, their dosage should be carefully determined to decrease side effects. As in vitro studies are not sufficient for making clinical decisions, investigations on the *in vivo* effects of these drugs on LPO activity is required.

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