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Effects of Ceftazidiine Pentahydrate, Prednisolone, Amikacin Sulfate, Ceftriaxone Sodium and Teicoplanin on Bovine Milk Lactoperoxidase Activity

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Abstract: The antibiotics are substances that kill or inhibit the growth of bacteria. Most antibiotics are modified chemically from original compounds found in nature. The antibiotics were frequently used for antimicrobial therapy. The main objective of this study was to determination of the effects of some antibiotics such as ceftazidime pentahydrate, prednisolone, amikacin sulfate, ceftriaxone sodium and teicoplanin on bovine milk lactoperoxidase activity were investigated in this study. Lactoperoxidase was purified using Amberlite-CG-50 resin, CM Sephadex-C-50 ion-exchange and Sephadex-G-100 gel filtration chromatographies from bovine milk. 2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) was used as a chromogenic substrate for spectrophotometric determination of lactoperoxidase activity. Lactoperoxidase was purified 19 fold with a yield of 24.5%. All of the used antibiotics exhibited inhibitory effects on the enzyme and displayed competitive type of inhibition mechanism. IC_{50} values of ceftazidime pentahydrate, prednisolone, amikacin sulfate, ceftriaxone sodium and teicoplanin were 0.048, 0.053, 0.26, 0.29 and 1.016 mM, respectively. K_i constants were calculated as 0.018±0.0035 mM, 0.019±0.0005 mM, 0.04±0.015 mM, 0.10±0.055 mM and 0.13±0.022 mM for ceftazidime pentahydrate, prednisolone, amikacin sulfate, ceftriaxone sodium and teicoplanin, respectively. Present study showed that ceftazidime pentahydrate exhibited much higher inhibitory effect at lower concentrations compared to other antibiotics used.

Key words: Lactoperoxidase, LPO, enzyme inhibition, antibiotics, bovine milk

INTRODUCTION

Milk is a suitable medium for the growth of many microorganisms, since it contains many necessary nutrients and provides a convenient physical environment. Also, milk supplies an array of defense factors in the form of lactoperoxidase, lactoferrin, lysozyme, immunoglobulins and free fatty acids that protect itself from different microbial infections (Zhang *et al*., 2008). Lactoperoxidase (Donor: Hydrogen peroxide oxidoreductase LPO, E.C.1.11.1.7), an enzyme present in milk, is the heme-containing glycoprotein (Bruck and Harvey, 2003; Boots and Floris, 2006) and is thought to be an important component in the defense against the microbial activity in raw milk. Bovine milk LPO is a basic protein consisting of one polypeptide chain with molecular mass of approximately 80 kDa containing 8-10% carbohydrate (Dumontet and Rousset, 1983; Boscolo *et al*., 2007). Lactoperoxidase, a member of the mammalian peroxidase family, with antibacterial properties is found in the breast secretory epithelial cells, in the salivary, lacrimal glands of mammals and in their secretions, such as milk, saliva, tears (Boscolo *et al*., 2007; Ferrari *et al*., 1997). Lactoperoxidase is a part of an antimicrobial system that catalyes the oxidation of thiocyanate (SCN^-) to the antibacterial hypothiocyanate in a hydrogen peroxide dependent reaction (Tanaka *et al*., 2003). LPO was found in milk together with sufficient amount of thiocyanate and peroxide (Uguz and Ozdemir, 2005; Sisecioglu *et al*., 2010a). In the presence of hydrogen peroxide and thiocyanate, LPO is capable of inhibiting the growth of a wide variety of bacteria, including both Gram-positive and Gram-negative strains. Also LPO is active against fungi, viruses and mycoplasma as well as some types of mammalian tumor cells (Pruitt and Adamson, 1977).

Many drugs or chemicals are known to activate or inhibit several body enzymes catalyzing the metabolic pathways. Systemic antibiotics are usually used in therapeutic and prophylaxis of uncompleted surgical

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infections. Amikacin sulfate and ceftazidime pentahydrate are commonly used for the treatment of Gram negative infections (Farina *et al*., 1999; Norouzi *et al*., 2005). Prednisolone has been available for clinical use as an antirheumatic agent (Brooks *et al*., 1976). Teicoplanin is being used in advanced clinical experimentation to treat severe infections caused by Gram-positive bacteria (Corti *et al*., 1987). Ceftriaxone sodium has excellent antimicrobial activity against Gram-positive and Gram-negative bacteria (Arpacik *et al*., 2004).

Use of antibiotics may worsen LPO activity in milk throughout the lactation period. Because the bovine lactoperoxidase (BLPO) and human LPO is important for immune system (Ihalin *et al*., 2003). Since, they may be used in pregnant patients and also in breast-milk during lactation; it is important to explore the effect of these on LPO activity during lactation. In this study; we have purified the LPO enzyme from bovine milk and investigated *in vitro* effects of the amikacin sulfate, ceftazidime pentahydrate, prednisolone, teicoplanin, ceftriaxone sodium on this enzyme.

MATERIALS AND METHODS

Purification of LPO: Detailed purification methods for bovine milk lactoperoxidase were explained formerly. Firstly, bovine milk was centrifuged at 2500 rpm (4°C, 15 min) to remove fat. Then LPO was purified using the amberlite CG-50 (NH_4^+) Resin, CM Sephadex C-50 and Sephadex G 100 chromatgrapic methods (Ozdemir *et al*., 2001; Sisecioglu *et al*., 2010b). Protein concentration was determined according to the method of Lowry *et al.* (1951) using Bovine Serum Albumin (BSA) as a standard (Gulcin *et al*., 2004). SDS-PAGE was performed under denaturing conditions after LPO purification, according to the Laemmli (1970) procedure explained in previous articles (Beydemir *et al*., 2003; Beydemir and Gulcin, 2004).

Determination of LPO activity: LPO activity was determined by the procedure of Shindler and Bardsley (1975) with a slight modification (Sisecioglu *et al*., 2009). This method is based on oxidation of 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. The absorbance was taken at 412 nm as a function of time in every 15 sec. One unit of LPO activity is defined as the amount of lactoperoxidase oxidising 1 μmol of ABTS substrate per min at room temperature. Molar absorption coefficient of ABTS was used as 32400 M^{-1} cm^{-1} and specific activities are expressed as enzyme units (EU) per mg of protein (Shindler and Bardsley, 1975).

Protein determination: Protein concentration was determined according to the method of Lowry *et al.* (1951). Bovine serum albumin was used as standard protein described previously (Coban *et al*., 2007, 2008; Senturk *et al*., 2009).

Effects of antibiotics on LPO activity: To determine the effects of antibiotics on bovine milk LPO, enzyme activities were measured in the presence of amikacin sulfate (0.116-0.32 mM), ceftazidime pentahydrate (0.0157-0.0785 mM), prednisolone (0.0115-0.08 mM), teicoplamin (0.163-0.785 mM), ceftriaxone sodium (0.144-0.432 mM) Control activity in the absence of antibiotic was taken as 100%. For all of the antibiotics, concentration that produced 50% inhibition known as I_{50} was calculated (Table 2).

For determination of the Ki constant, three different inhibitor concentrations (0.116, 0.213, 0.32 mM for amikacin sulfate, 0.0157, 0.0471, 0.0785 mM for ceftazidime pentahydrate, 0.0115, 0.0347, 0.08 mM for prednisolone 0.163, 0.471, 0.785 mM for teicoplanin and 0.144, 0.288, 0.432 mM for ceftriaxone sodium) were used. In these studies, ABTS was used as a substrate at five different concentrations (0.167-0.5 mM). From the Lineweaver-Burk graph obtained by 1/V versus 1/[S], for each inhibitor, Ki constant and inhibition type were determined (Table 2). The data obtained was analyzed by Student t-test and results are given as X±SD.

RESULTS

LPO was purified in two steps; firstly CM sephadex C-50 ion exchange chromatography and measured Rz ($\lambda_{412}/\lambda_{280}$ nm) of fractions. Rz values 0.7 or higher fractions were pooled. The enzyme obtained from ion exchange chromatography was applied to Sephadex G-100 gel filtration chromatography. Specific activity was calculated for the crude extract and purified enzyme solution, so yielding a purification of 8,7 fold and yield of 2.8 mg (R_z: 0.8) from 1 L bovine milk (Table 1). The degree of purity of the enzyme was controlled with SDS-PAGE (Fig. 1). The standard proteins used for SDS-PAGE were maltose-binding protein (MBP)-β-galactosidase (175 kDa), maltose-binding protein (MBP)-truncated-β-galactosidase (80 kDa), maltose-binding protein (MBP) and chitin binding domain (CBD, 62 kDa), aldolase (46 kDa). As shown in the Fig. 1, bovine lactoperoxidase had a molecular weight about 80 kDa.

For each antibiotic the Lineweaver-Burk graphs were drawn and are shown in Fig. 2. K_i constant was determined as 0.018±0.0035 mM, 0.019±0.0005 mM, 0.04±0.015 mM, 0.10±0.055 mM and 0.13±0.022 mM from

Table 1: Purification steps of lactoperoxidase from bovine milk

Purification steps	Total volume (mL)	Enzyme activity (EU mL^{-1})	Total enzyme activity (EU)	Protein (mg mL^{-1})	Total protein (mg)	Specific activity (EU mg^{-1})	Activity yield (%)	Purification fold
Homogenate	150	2.45	367.50	1.45	217.50	1.68	100.0	1.00
Ammonium sulfate precipitation	28	11.08	310.24	1.80	50.40	6.15	84.4	3.66
CM-Sephadex C-50 column chromatography	240	1.20	288.00	0.11	26.40	10.90	78.4	6.48
Ammonium sulfate precipitation	25	9.72	243.10	0.58	14.50	16.75	66.1	9.97
Sephadex G-100 column chromatography	145	1.15	166.80	0.06	8.70	19.17	45.4	11.41
Ammonium sulfate precipitation and dialyze	20	4.50	90.00	0.14	2.81	32.14	24.5	19.13

Table 2: I_{50} value, K_i constant and inhibition types of amikacin sulfate, ceftazidime pentahydrate, prednisolone, teicoplanin and ceftriaxone sodium for bovine LPO

Purification steps	I_{50} (mM)	Means K_i (mM)	Inhibition type
Ceftazidime pentahydrate	0.048	0.018±0.0035	Competitive
Prednisolone	0.053	0.019±0.0005	Competitive
Amikacin sulfate	0.260	0.040±0.015	Competitive
Ceftriaxone sodium	0.290	0.100±0.055	Competitive
Teicoplanin	1.016	0.013±0.002	Competitive

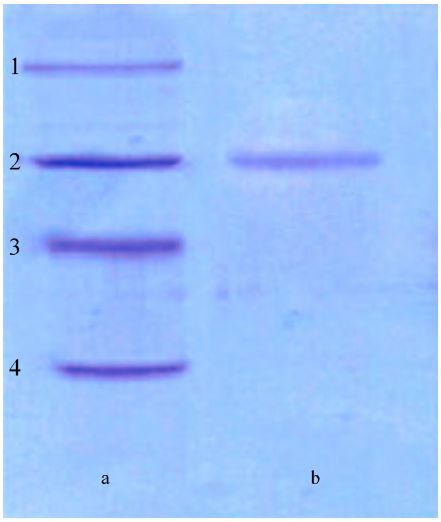

Fig. 1: SDS-PAGE bands of LPO, line a: standard proteins maltose-binding protein (MBP)-β-galactosidase (175 kDa), maltose-binding protein (MBP)-truncated-β-galactosidase (80 kDa), maltose-binding protein (MBP) and chitin binding domain (CBD) (62 kDa), aldolase (46 kDa), line b: Purified LPO from bovine milk

the graphs for ceftazidime pentahydrate, prednisolone, amikacin sulfate, ceftriaxone sodium and teicoplanin (Table 2), respectively. These drugs showed competitive inhibition. Table 2, the inhibitor concentrations causing up to 50% inhibition were determined from Activity (%)-[Antibiotic] graphs (Fig. 2). I_{50} values were calculated as 0.048, 0.053, 0.26, 0.29 and 1.016 mM from the graphs for ceftazidime pentahydrate, prednisolone, amikacin sulfate, ceftriaxone sodium and teicoplanin, respectively (Fig. 3).

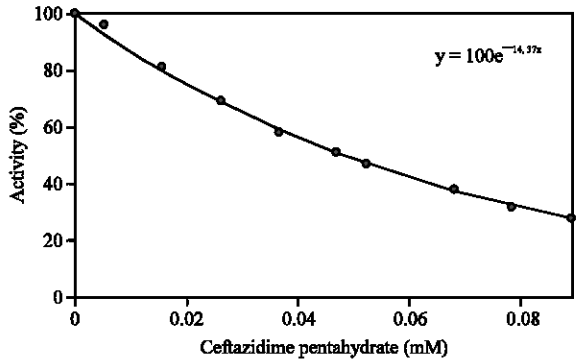

Fig. 2: Activity % vs. [Ceftazidime pentahydrat] regression analysis graphs for bovine milk LPO in the presence of three different ceftazidime pentahydrate concentrations for determination of K_i

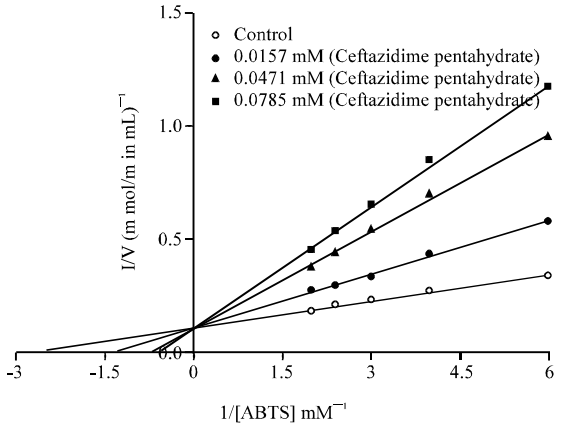

Fig. 3: Lineweaver-Burk graph for different ABTS concentrations and three different ceftazidime pentahydrate concentrations for determination of K_i constant

DISCUSSION

LPO system is one of the important host defense systems in the bovine and human milk. LPO is an oxidoreductase secreted into milk and plays an important role in protecting the lactating mammary gland and intestinal tract of newborn infants against pathogenic

microorganism (Zhang *et al*., 2008; Ozdemir and Uguz, 2005; Lonnerdal and Lien, 2003). LPO catalyzes the oxidation of thiocyanate to produce hypothiocyanate in the presence of hydrogen peroxide, which can kill both Gram-positive and Gram-negative bacteria (Pruitt and Adamson, 1977). LPO in human milk may contribute to the defense against infection already in the mouth and upper gastrointestinal tract. The LPO system in cow milk has been used by the dairy industry in developing countries for decades to preserve microbial quality (Lonnerdal and Lien, 2003). These two lactoperoxidases show the same property and weight (Gothefors and Marklund, 1975).

The decision to use antibiotic medications in breast-feeding mothers and pregnant patients are quite complex. Many chemicals and drugs are known to affect the metabolic pathways by altering normal enzyme activity (Uguz and Ozdemir, 2005; Sisecioglu *et al*., 2009). These chemicals and drugs penetrated breast milk. For example, ceftriaxone entered breast milk quickly and was then slowly eliminated (Kafetzis *et al*., 1983). Ceftazidime is excreted in breast milk at relatively constant levels between days 2 and 4 of therapy (Blanco *et al*., 1983). Owing to the amounts as reflected in the certain concentrations in milk, use of this drug may effective LPO activity. Therefore, it is important to explore the effect of antibiotics on LPO activity during lactation. Inhibitory effects of some drugs on enzymatic activity in LPO have been reported in a few investigations. For example, use of ketamine and bupivacaine which are anesthetic drugs may decrease LPO activity in milk during lactation (Ozdemir and Uguz, 2005).

Amikacin sulfate, ceftriaxone sodium, ceftazidime pentahydrate, teicoplamin and prednisolone are used for medicinal applications in breast-feeding mothers and pregnant patients (Kafetzis *et al*., 1983; Blanco *et al*., 1983; Matsuda, 1984; Petri, 2003). Use of these antibiotics may affect LPO activity in milk throughout the lactation period. No references in the literatures have been found about regarding the effect of these antibiotic drugs on LPO activity. Our investigation showed these antibiotics inhibited bovine LPO *in vitro* and kinetic constants (K_i and I_{50} values) were reported. These drugs showed highly effects on LPO activity. Ceftazidime pentahydrate had the strongest inhibitory effects on LPO, when compared to the other drugs.

In conclusion, according to the results obtained from this study, usage of antibiotic drugs may decrease LPO activity in milk during lactation. Hence, these antibiotics could cause some side effects. For this reason, these antibiotics must be carefully used and their dosages should be very well ordered to decrease the side effects. LPO has antimicrobial activity and contributes to the protective functions of milk. If drug therapy is necessary during lactation, their dosage should be carefully determined to decrease side effects.

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