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Purification and Characterization of Lactoperoxidase from Goat Milk, Investigation of Inhibition Effects of Cefotaxime Sodium

Metin Tansu UĞUZ[1]✉, Mehmet Muhsin EROL[2]
[1]Kahramanmaraş Sütçü İmam Üniversitesi SHMYO Kahramanmaraş, [2]Emin Paşa Ortaokulu Akahramanmaraş
[1]https://orcid.org/ 0000-0002-3557-1677, [2]https://orcid.org/0009-0009-3660-0271
✉: metinuguz@ksu.edu.tr

ABSTRACT
İt has been shown in the literature that LPO is important for natural immunity. The purpose of this study is to purify the lactoperoxidase (LPO) enzyme from goat milk and to investigate the inhibition property of cefotaxime sodium on the enzyme. In this study, the LPO enzyme was purified from goat milk and the inhibition effect of cefotaxime sodium was examined. Enzyme purification processes were carried out by various chromatographic methods and the enzyme inhibitory effect was analyzed with Lineweaver-Burk graphs. İnhibition of cefotaxime sodium on LPO was determined as competitive inhibition. The enzyme was purified by first partially purifying milk using Amberlite CG-50 H+ resins, CM-Sephadex C-50 ion-exchange chromatography, and Sephadex G-100 gel filtration chromatography. The 2.2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) substrate was used to determine the enzyme's Km and Vmax values. Enzyme purity was determined using SDS-PAGE electrophoresis. A specific activity of 7.21 EU mg-1 of protein per liter was detected. The LPO was purified 13.35 times from 1 liter of defatted milk, and 1.9 mg of enzyme was obtained. ABTS substrate (ε412nm=32400M-1 cm-1) was used for the enzyme-activity assays.

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Laktoperoksidaz Enziminin Keçi Sütünden Saflaştırılması ve Karakterizasyonu, Cefotaxime Sodyumun İnhibisyon Özelliklerinin Araştırılması

ÖZET
LPO' nun doğal bağışıklık için önem arzettiği literatürlerde gösterilmiştir. Bu çalışmanın amacı, keçi sütünden laktoperoksidaz (LPO) enzimini saflaştırmak ve sefotaksim sodyumun enzim üzerindeki inhibisyon özelliğini araştırmaktır. Bu çalışmada, keçi sütünden LPO enzimi saflaştırılmış ve sefotaksim sodyumun inhibisyon etkisi incelenmiştir. Enzim saflaştırma süreçleri çeşitli kromatografik yöntemlerle gerçekleştirilmiş ve enzim inhibitör etkisi Lineweaver-Burk grafikleri ile analiz edilmiştir. Sefotaksim sodyumun enzim üzerindeki inhibisyonu yarışmalı inhibisyon olarak belirlenmiştir. Enzimin keçi sütünden saflaştırılması için, Amberlite CG-50 H+ reçinesiyle kısmi saflaştırma yapılarak, CM-Sephadex C-50 iyon değişim kromatografisi, Sephadex G-100 jel filtrasyon kromatografisi, sırasıyla çalışıldı. ABTS (3-ethylbenzothiazoline-6-sulphonicacid) substratı için Km ve Vmax değerleri ile optimum pH, optimum sıcaklık belirlendi. Enzim saflığı SDS-PAGE elektroforezi ile tespit edildi. Enzimin bir litre keçi sütünden spesifik aktivitesi 7.21 EÜ mg-1 protein miktarı belirlendi. LPO saflaştırma işlemleri sonucunda 1 litre yağı alınmış taze sütten 13.35 kat saflaştırıldı ve 1.9 mg (Rz=0.7) elde edildi. Enzim saflaştırılması sırasında aktivite tayinleri için ABTS substratı (ε412nm=32400M-1 cm-1) kullanıldı.

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INTRODUCTION

The enzyme lactoperoxidase (LPO) (E.C.1.11.1.7) is available found in liquid form in milk, saliva, tears, and gastric juices (Prince & Ratner, 2000; Kumar & Bhatla, 1995; Amornkul & Henning, 2007; Boots & Floris, 2006). Raw milk is a thick white nutritious substance produced by the secretory glands of mammals. It is at the primary level in the food chain of young individuals. Milk contains large amounts of compounds that protect against infections. One of the important proteins is the LPO enzyme (Wolf et al., 2000). Studies have shown that the LPO enzyme is important for immune system activities. It creates a barrier that protects babies against infections after birth. In studies, it has been stated that the LPO system has been tested at different concentrations and has antibacterial and antifungal activity. (Uğuz & Özdemir, 2005). They form a defensive barrier that protects the young from microorganisms in the postnatal period. The structure of milk contains many compounds that have the ability to protect itself against infections. One of these structures is the LPO enzyme (Wolf et al., 2000). The use of the LPO system is important against the risk of encountering reasons that cannot prevent milk spoilage, such as transportation difficulties in the cold chain, power outages, and device maintenance problems, especially in countries with weak economies (Seifu et al., 2005). Milk and its products used as a food source can quickly deteriorate due to microbial contamination. Some preservatives are added to milk to prevent this negative process. The LPO system is one of them. LPO is a natural preservative system found in fresh raw milk. Approximately 30 mg-1 of LPO is found in bovine milk, and its concentration is stable during lactation. In the goat milk LPO system, oxidation of thiocyanate to antibacterial hypothiocyanite may help protect the mammary gland from involution (Zou et al., 2021). LPO does not have an antimicrobial effect on its own; it requires the presence of hydrogen peroxide. Thus, it turns into hypothiocyanate and has an antibacterial effect. Lactoperoxidase is responsible for the oxidation reaction of many organic molecules, as well as catalyzes its antimicrobial activity with compounds with phenol and thiol aromatic rings. Not only do secondary products such as halide and thiocyanate formed in the metabolic reactions of lactoperoxidase change the compounds, but also chemical change can occur with different compounds. Lactoperoxidase can show its catalytic activity in the lactating mammary glands of mothers as well as in the gastrointestinal tract of babies. Researchers have shown that the LPO system has antibacterial and antifungal properties at different concentrations. (Uğuz & Özdemir, 2005). Although it has a bacteriostatic effect on Lactobacilli settling in the intestinal flora, it also has a bactericidal effect on some pathogenic bacteria such as Esherichia coli and Pseudomonas. (Roger et al., 1994). Recently, the desire to understand the role of milk chemicals in biochemical mechanisms has been the focus of the attention of researchers (Y.S. Qin et al., 2021). LPO, thiocyanate ion (SCN^-) into the antibacterial hypothiocyanate ion ($OSCN^-$) (Figure 1), and this is important for the natural immune system. (Wolfson & Summer, 1993; Kumar & Bhatla, 1995; Shin et al, 2001; Reiter & Harnulv, 1984). The source of LPO activity is the secondary products formed as a result of the reaction it catalyzes. The antibacterial effect is due to the reaction of the hypothiocyanate formed with the protein thiol groups. The effect of LPO on bacteria is that the sulfhydryl -SH groups undergo oxidation, and thus, the ions cannot be transported through the bacterial membrane structure (Sisecioglu et al., 2010a). The LPO system acts as a strong and effective inhibitor together with hydrogen peroxide and thiocyanate ions (Reiter & Harnulv, 1984). LPO thus suppresses enzymes used in bacterial defense (Hoogendorn et al., 1977; Pruitt et al., 1982). LPO enzyme is a single polypeptide chain protein with a molecular weight of 80–85 kDa and 612 amino acids, isoelectric pH of 9.2, carrying a heme group. (Thomas & Bozeman, 1991; Paul & Ohlsson., 1985; Elegamy et al., 2010; Hashimoto et all, 2005). The best-known substrates of enzymes used in metabolic reactions are Guaiacol, (2.2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and catechol (Shindler and Bardsley, 1975; Daniel et al., 1997; Brück et al., 2001). Despite this, there are few studies in the literature on the purification of LPO from goat milk and the inhibition of the enzyme by cefotaxime sodium. İn addition, the fact that this study was conducted for the first time in a geographical region, that antibiotics have many side effects, and that the LPO enzyme inhibition activity of cefotaxime sodium is known shows the importance of the study. Research has shown that immunity weakens as a result of increased bacterial resistance (Sisecioglu et al., 2010a). At the end of the study conducted to check whether cefotaxime sodium has an inhibitory effect on LPO, it was determined that cefotaxime sodium inhibited the enzyme . The aim of the study is to purify the LPO enzyme from goat milk with a good yield and to investigate the inhibition effect of cefotaxime. It will contribute to the literature since there is no study in the literature on goat milk LPO. It is reported that LPO will not cause any adverse effects on consumer health (Sarkar and Misra, 1994).

LPO
$SCN^- + H2O2 \rightarrow OSCN^- + H2O$
Figure 1 Enzymatic reaction for Lactoperoxidase
Şekil 1. Laktoperoksidaz'ın enzimatik reaksiyonu

MATERIAL and METHOD

Purifying the LPO Enzyme from Goat's Milk

First, 1 L of fresh raw goat's milk was centrifuged 3 times at 2700 rpm for 15 min each time, until the oil was completely removed (Kumar & Bhatla, 1995). Then the weakly acidic cation exchanger Amberlite CG 50 H_4^+ resin (equilibrated with 5 mm of sodium acetate, at pH =6.8) was added at a ratio of 22 g L^{-1} of milk (Doumonted & Rousset, 1983; Kumar & Bhatla, 1995). The milk mixture was mixed with a magnetic stirrer in the refrigerator for 1 hour. It was allowed to rest for 30 minutes. Then, the eluate was washed with distilled water at pH 6.8- and 20-mM sodium acetate until the absorbance decreased below 0.01 at 280 nm. It was eluted using 500 mM sodium acetate at pH 6.8 with the aid of a Buchner Funnel. Protein and enzyme activities of the extracts were measured, and the extracts were stored in the refrigerator at 4°C. $(NH_4)_2SO_4$ solution was slowly added to the eluate to precipitate. The residue formed by centrifugation at 12,000 rpm at 5°C for 1 hour was dissolved in 25 mL of 5 mM sodium phosphate buffer at pH 6.8. Dialysis was performed overnight in the refrigerator in a 5 mM sodium phosphate buffer of pH 6.8. The supernatant was equilibrated using 10 mM sodium phosphate buffer pH 6.8 and placed in a CM-Sephadex C-50 column. For the washing process, 100 mL of 10 mM pH 6.8 sodium phosphate buffer containing 100 mM NaCl was used. The enzyme was eluted with a linear gradient of 100-200 mM NaCl in 10 mM sodium phosphate buffer at pH 6.8 followed by a 90% saturated ammonium sulfate precipitate II treatment. The enzyme solution was then dialyzed overnight in 5mM Sodium phosphate buffer at pH 6.8. The eluate was collected in 10 mL fractions. RZ value by looking at absorbance at 280 nm and 412 nm against the blank (RZ = A412/A280). Eluates with Rz values greater than 0.75 were combined. The LPO sample was dialyzed. Then it is placed on the Sephadex G-100 column. The enzyme bound to the column was eluted with 0.1 M sodium phosphate buffer at pH 6.8 and removed with 90% saturated ammonium sulfate solution III. The enzyme solution was dialyzed overnight with 0.5 M sodium phosphate buffer at pH 6.0. (Sisecioglu et al., 2010a; Koksal et al., 2017).

Determining Protein Concentration

Protein concentration, enzyme activity, specific activity, and purification rates were determined according to the Lowry method using bovine serum albumin as the standard (Lowry et al., 1951, Boroujeni et al., 2024, Kandasamy et al., 2014).

Determining LPO Activity

LPO activity was measured according to the Shindler procedure. Enzyme activity can be measured by monitoring the absorbance of the resulting-colored compound at 412 nm. (Shindler & Bardsley, 1975, Koksal & Alim, 2020). Working procedure: 2.8 mL of 1 mM ABTS in 0.1 M sodium phosphate buffer at pH 6.0, pH 6.0, and 0.1 mL of LPO enzyme in 1 mM sodium phosphate buffer were mixed. Then 0.1mL of 3.2 mM H_2O_2 solution was added. The absorbance at 412 nm was then measured every 15 seconds for 3 minutes.

Kinetic Studies

To calculate K_m and V_{max} criteria, 5 different (0.2 mL, 0.5 mL, 0.8 mL, 1.1 mL, and 1.5 mL) ABTS substrate concentrations were measured for enzyme activity at pH 6.0 at room temperature. For each concentration, the total volume was adjusted to 2.8 mL. 0.1 mL enzyme solution and 0.1 mL H_2O_2 were added to each tube and absorbance values were measured with a spectrophotometer at 412 nm. Each measurement was repeated twice. Values of 1/V-1/[S] were determined and a Lineweaver-Burk plot was plotted using least squares regression line parity. K_m and V_{max} values were calculated from this graph and presented in Table 1. LPO enzyme activity was calculated similarly. To determine the optimum temperature of the enzyme, 5 substrate concentrations were measured at temperatures ranging from 5-80°C, and these temperatures were achieved using a water bath. To determine the K_m and V_{max} values, first, the optimal pH and temperature values were measured using ABTS activity in 5 substrate concentrations: 0.2 mL, 0.5 mL, 0.8 mL, 1.1 mL, and 1.5 mL. For each concentration, the total volume used, including the buffer solution, was 2.8 mL. Then, 0.1 mL of enzyme solution and 0.1 mL of H_2O_2 were added to each tube, and absorbance values were measured at 412 nm using a spectrophotometer. Each measurement was repeated twice. The 1/V-1/[S] values were calculated, and a Lineweaver-Burk graph was plotted. (Fatemeh et al., 2016).

Table 1. K_i, K_m ve V_{max} values of three different drug concentrations obtained from Lineweaver-Burk graphs
Çizelge 1. Lineweaver-Burk grafiklerinden elde edilen üç farklı ilaç konsantrasyonunun K_i, K_m ve V_{max} değerleri

Drug	[Drug] M	K_i values(M)	Mean K_i (M)	İnhibition form	K_m	V_{max}
	0.1	0.012			1.4	5.89
Cefotaxime	0.2	0.014	0.015	Competitive	2.0	5.89
	0.3	0.02			2.13	5.89

Investigating The Effect of Cefotaxime Sodium on Enzyme Activity

A water-soluble drug was used to investigate the inhibition effect of the purified LPO enzyme. To measure the inhibitor concentration (I_{50}) value of the drug due to the ABTS substrate At room temperature and optimal pH, saturated ABTS (26.67×10^{-2} mM) and drug without H_2O_2 were added at different concentrations. Activity percentage-concentration graphs were drawn, assuming the enzyme activity was 100% without drug addiction. For this purpose, activity percentage was calculated by adding six different volumes of the drug to the fixed substrate concentration of ABTS, and I_{50} values causing 50% inhibition were determined for each drug used (Figure 3). Then, activity values were studied at five different ABTS substrate concentrations and three different drug concentrations. Inhibition types and K_i values were found by drawing Lineweaver-Burk graphs. (Sarıkaya et al., 2015).

RESULTS

Partial purification of the enzyme was done by adding Amberlite CG-50 H^+, a weakly acidic cation exchanger, to goat milk. (Köksal et al., 2017). The purification steps of the LPO enzyme are given in Table 2, respectively.

Table 2. Purification steps of lactoperoxidase from goat milk
Çizelge 2. Keçi sütünden laktoperoksidazın saflaştırma basamakları

Step	Activity, (EÜmL^{-1})	Total volume, (mL)	Protein (mg)	Total Protein, (mg)	Total Activity, (EÜmL^{-1})	Specific Activity, (EÜmg^{-1})	Recovery (%)	Purification (fold)
Crude homogenate	0.76	120	1.40	168	91.2	0.54	100	1.00
Ammonium sulfate	3.81	20	1.58	28.6	76.2	2.66	83.5	4.92
CM Sephadex C-50 column	0.25	240	0.05	13.2	60.0	4.54	65.8	8.40
Ammonium sulfate	1.98	20	0.35	7.0	39.6	5.65	43.4	10.46
Sephadex G-100 column	0.25	90	0.04	3.6	22.5	6.25	24.6	11.57
Ammonium sulfate and dialyse	0.98	14	0.14	1.9	13.7	7.21	15.0	13.35

LPO enzyme activity was measured by a modification of the Shindler and Bardsley method based on measuring at 412 nm the increased absorbance of the colored compound formed by oxidation of the ABTS chromogenic substrate by H_2O_2 (Shindler & Bardsley, 1975). Enzyme activity measurement results in 1 enzyme unit; It was defined as "the amount of enzyme that catalyzes the oxidation of 1 µmol ABTS in 1 minute at 20°C. " Six purification steps were performed, at the end of which the measured activity increased from 0.54 EU m g^{-1} to 7.21 EU mg^{-1}. The enzyme was purified 13.35-fold. After purification, the yield value was 15%. Using the obtained 1/V-1/[S] values, the Lineweaver-Burk graph was drawn (Figure 2), and K_m and V_{max} values were determined.

According to ABTS usage, the V_{max} value was found to be 5.89 EU mL^{-1} and the K_m value was 2. For kinetic studies of the LPO enzyme, Lineweaver-Burk graphs were drawn by measuring spectrophotometric at six different ABTS substrate concentrations and three different drug concentrations. Inhibition types and K_i values were determined. Lineweaver-Burk graphs were created with the help of the obtained 1/V-1/[S] values (Figure 2). For these values, it was found that 1g of cefotaxime sodium had a significant inhibitory effect, the K_i value of 1g of cefotaxime sodium for ABTS was 0.015 M. The results showed that 1 g of cefotaxime sodium significantly inhibited the LPO enzyme. According to these values, cefotaxime (1g Cefotaxime Sodium) shows a competitive inhibition effect. The activity-concentration graphs for the ABTS substrate were plotted against the values obtained in the kinetic study using 1 g of cefotaxime sodium, and the I_{50} values were determined. For this purpose, activity values were calculated by adding 1g of cefotaxime sodium at various concentrations of ABTS (26.67×10^{-2} mM) and H_2O_2 (10.67×10^{-2} mM) at constant substrate concentrations. Thus, the reaction rate was found to be dependent on ABTS. Figure 3 shows this graph.

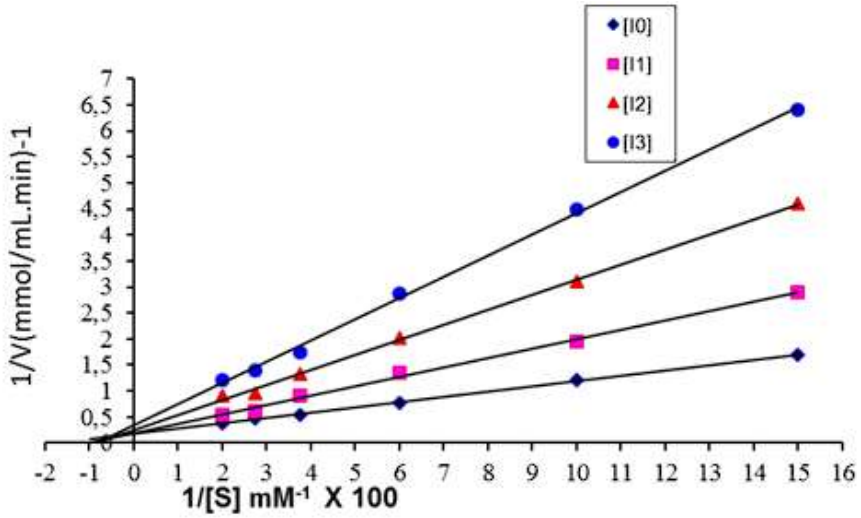


Figure2. Lineweaver-Burk graphics for three different Cefotaxime Sodium (1g Cefotaxime sodium) concentrations and different ABTS concentrations

Şekil 2. Üç farklı Sefotaksim Sodyum (1g Sefotaksim sodyum) konsantrasyonu ve farklı ABTS konsantrasyonları için Lineweaver-Burk grafikleri

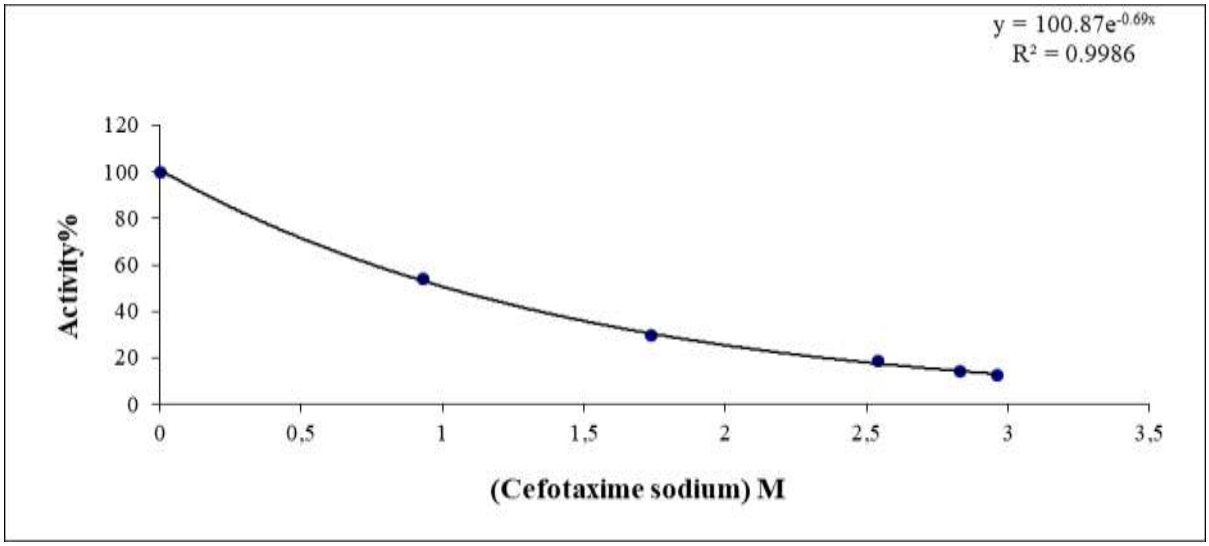


Figure3. Activity %Concentration analysis graphs for Goat Milk LPO of Cefotaxime Sodium

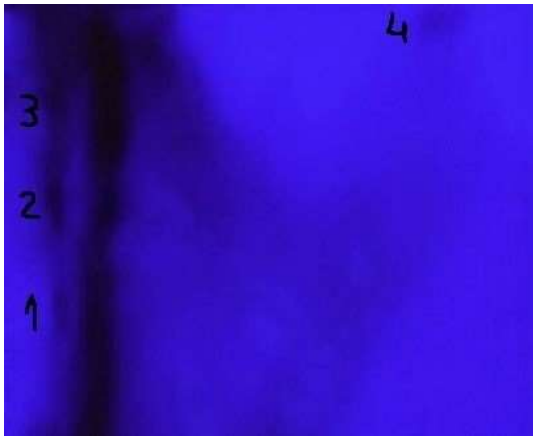
Şekil 3. Sefotaksim Sodyumun Keçi Sütü LPO'su için Aktivite %Konsantrasyon analiz grafikleri

According to this result, the I_{50} value for cefotaxime sodium is 0.93 M. After the chromatographic steps, SDS-polyacrylamide gel electrophoresis was performed to determine the purity of the purified lactoperoxidase enzyme. Presented in Figure 4. Thus, LPO was compared with markers with different molecular weights

DISCUSSION

Purification of the investigated LPO enzyme from goat milk is not frequently seen in the literature. İn previous studies, LPO has been purified from goat milk by different methods. A single-step purification process was performed using affinity chromatography. (Koksal et al., 2017). Antimicrobial inhibition studies are being investigated due to the high side effects of antibiotics, which are generally used in some bacterial diseases, and their antibacterial, antifungal, and antiviral effects. (Gobbetti et al., 2004). The Schindler and Bardsley method are based on the oxidation of the ABTS substrate by H_2O_2 and the increase in the absorbance of the resulting colored compound. Many researchers said that they used this method themselves. (Sisecioglu et al., 2011; Ozdemir & Uguz, 2005). The enzyme was first purified to a total of 5mg from 1 liter of bovine milk using Amberlite CG-50 H^+ resins, CM-Sephadex C-50 ion exchange chromatography, and Sephadex G-100 gel filtration chromatography. While the average lactoperoxidase concentration determined by purification processes in the goat milk sample was

1.9 mgL^{-1} LPO (Rz = 0.75), it was determined that the total protein in the human milk sample was 0.77 ± 0.38 mgL^{-1} (Kouichirou Shin et al., 2001). The difference in the amount of pure lactoperoxidase we obtained may be due to regional and racial characteristics. (Kumar & Bhatla, 1995).



Figüre 4. SDS- PAGE for the purity of the enzyme lactoperoxidase from the purified goat milk
1. Bovine Carbonic Anhydrase (M.W.29 kdal), **2.** Chicken Egg Albumin (M.W. 45 kdal), **3.** Bovine Serum Albumin (M.W. 66 kdal), **4.** Goat milk LPO (M.W. 80-85 kdal).
Şekil 4. Saflaştırılmış keçi sütündeki laktoperoksidaz enziminin saflığı için SDS-PAGE
1. Sığır Karbonik Anhidraz (M.W.29 kdal), 2. Tavuk Yumurtası Albümini (M.W. 45 kdal), 3. Sığır Serum Albümini (M.W. 66 kdal), 4. Keçi sütü LPO (M.W. 80-85 kdal).

The specific activity of LPO obtained as a result of purification steps and dialysis was found to be between 0.54 EU mg^{-1} and 7.21 EU mg^{-1}. Koksal and her colleagues used the affinity chromatography method to purify LPO from different milk sources and purified 53-fold LPO with a yield of 49.5%. (Koksal et al., 2017) In our study, LPO was obtained in pure form with 13.35 times 15% yield. The increase in the number of purification steps reduces the efficiency. Thus, LPO was obtained with lower efficiency.

İnhibition types and K_i values were found by drawing Lineweaver-Burk graphs (Koksal et al., 2017; Sisecioglu et al., 2010). It was found that the Lactoperoxidase enzyme showed maximum activity at pH: 6 and 50 ^{0}C (Sisecioglu et al., 2010b). According to ABTS usage, the V_{max} value was found to be 5.89 EU mL^{-1} and the K_m value was 2. K_m value was found as 0.358 µmol, also Vmax values were calculated as 18.86 EU mL^{-1}. (Sisecioglu et al., 2009). K_i constant values for melatonin and serotonin were calculated as 0.82 ± 0.28 and 0.26 ± 0.04 µM, respectively. Inhibition of melatonin and serotonin has been demonstrated. For this purpose, activity values were calculated by adding 1 g cefotaxime sodium at different concentrations of ABTS and H_2O_2. For this, the H_2O_2 concentration was taken above saturation, while the ABTS substrate concentration was taken below saturation. (Sisecioglu et al., 2011; Ozdemir & Uguz, 2005). In their study, Kalın and his colleagues investigated the enzyme inhibition of cefazolin on LPO, which they purified from bovine milk.

In previous studies, LPO enzyme from large animal production was purified by ion exchange chromatography and gel filtration chromatography. Specific activity was determined in its purification step. LPO has many applications. The shelf life of pasteurized milk in transportation and storage may be increased, especially if it is added to milk in milk processing units. (Barrett et al., 1999). Purification of the LPO enzyme from goat's milk has seldom been discussed in the literature. This study was conducted because LPO enzyme activity varies in various bacterial strains. It has been reported that thiocyanate, the precursor of hypothiocyanate found in animal cell secretion, is known as the substrate with antimicrobial activity for lactoperoxidase. (Thomas et al., 1991; Reiter & Perraudin 1991). Similarly, the inhibitory effect of LPO activity on melatonin and serotonin was determined (Sisecioglu et al., 2010; Barrett et al, 1999). Clinical research has demonstrated that numerous side effects arise from the unnecessary use of antibiotics. The most significant of these is that overuse diminishes the natural immunity of the immune system.

CONCLUSION

Since the purification of lactoperoxidase (LPO) enzyme from goat milk and the study on the inhibitory activity of cefotaxime sodium on the enzyme have not been widely reported in the literature, this study gains importance. Studies on cefotaxime sodium, which inhibits the enzyme, are also rare, but it has been shown to inhibit the lactoperoxidase enzyme. In addition, since some species are routinely treated with antibiotics, the antimicrobial effects of the tested drug have also been expressed. Due to the antimicrobial activity of LPO, ongoing human

studies will provide more information on the physiological roles of LPO. Less use of antibiotics will protect the immune system's health. Although the study on goat milk lactoperoxidase was conducted regionally, the results were found to be consistent with the studies of other researchers. The findings of this study showed the kinetic parameters and competitive inhibition of cefotaxime on LPO. However, more research should be done on lactoperoxidase to know its physiological effects in human milk in detail. Since the current study showed that cefotaxime sodium inhibits the LPO enzyme, a more comprehensive study of the LPO enzyme is recommended.

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Contribution Rate Statement Summary of Researchers

The authors declare that they have contributed equally to the article.

Conflict of Interest

The authors of the article declare that there is no conflict of interest between them.

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