Food Chemistry 136 (2013) 864–870

Contents lists available at SciVerse ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

One-step purification of lactoperoxidase from bovine milk by affinity chromatography

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ARTICLE INFO

Article history:
Received 28 March 2012
Received in revised form 13 July 2012
Accepted 30 August 2012
Available online 8 September 2012

Keywords:
Lactoperoxidase
LPO
Affinity chromatography
Enzyme purification
Inhibition
Kinetics

ABSTRACT

Sulphanilamide was determined to be a new inhibitor of lactoperoxidase (LPO) with an IC_{50} of $0.848.10^{-5}$ M. The K_i for sulphanilamide was determined to be $3.57.10^{-5}$ M and sulphanilamide showed competitive inhibition, which makes it a suitable ligand for constructing a Sepharose 4B-L-tyrosine affinity matrix. The affinity matrix was synthesised by coupling sulphanilamide as the ligand and L-tyrosine as the spacer arm to a cyanogen bromide (CNBr)-activated-Sepharose 4B matrix. Lactoperoxidase was purified 409-fold from the synthesized affinity matrix in a single step, with a yield of 62.3% and a specific activity of 40.9 EU/mg protein. The enzyme activity was measured using ABTS as a chromogenic substrate (pH 6.0). The degree of LPO purification was monitored by SDS–PAGE and its R_z (A_{412}/A_{280}) value. The R_z value for the purified LPO was found to be 0.7. Maximum binding was achieved and K_m and V_{max} values were determined.

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1. Introduction

Milk contains a variety of constituents that protect the neonate and the milk itself from a host of deleterious microorganisms. One such constituent is lactoperoxidase (LPO) (Ueda, Sakamaki, Kuroki, Yano, & Nagata, 1976). LPO is an oxidoreductase secreted into milk, which plays an important role in protecting the lactating mammary gland and the intestinal tract of newborn infants against pathogenic microorganisms (Golhefors & Marklundi, 1975; Kumar & Bhatla, 1995). LPO is found in bovine milk (Dumonte & Rousst, 1983; Wolfson & Sumner, 1993). LPO is one of the prominent enzymes in milk. LPO catalyses the oxidation of halides and pseudohalides, such as thiocyanate, by H_2O_2 to form potent oxidant and bactericidal agents. LPO, which catalyses the oxidation of endogenous thiocyanate (SCN^-) to the antibacterial hypothiocyanate ($OSCN^-$), is a redox enzyme with antibacterial properties found in several biological fluids, such as milk and saliva (Cals, Mailliart, Brignon, Anglade, & Dumas, 1991; Jacob, Anthony, Sreekumar, & Haridas, 2000; Jacob, Manoj, & Harridas, 1998). LPO consists of a single polypeptide chain containing 612 amino acid residues, a haeme prosthetic group, and four or five carbohydrate chains, which constitute approximately 10% of the total mass, resulting in its molecular weight of approximately 85 kDa (Elagamy, Ruppanner, Ismail, Champagne, & Assaf, 1992; Reiter & Harnulv, 1984; Sisecioglu, Gulcin, Cankaya, Atasever, & Ozdemir, 2010).

Purification of LPO, using different purification techniques, has been the focus of many research groups (Ozdemir, Aygul, & Kufrevioglu, 2001; Ozdemir, Hacibeyoglu, & Uslu, 2002). CM-Sephadex ion-exchange chromatography (Ozdemir, Hacıbeyoglu, & Kufrevioglu, 2003; Uguz & Ozdemir, 2005), Sephadex G-100 gel filtration chromatography (Ozdemir & Uguz, 2005; Shin, Hayasawa, & Lönnerdal, 2001), hydrophobic affinity chromatography on Phenyl-Sepharose CL-4B (Langbakk & Flatmark, 1984) and Toyopearl-SP cation-exchange chromatography (Mecitoglu & Yemenicioglu, 2007) have all been used for the purification of LPO from bovine milk. In addition, cation-exchange chromatography and immunoaffinity chromatography with coupled IgG were used for the purification of LPO from whey (Shin et al., 2001). LPO was purified using reverse micelles-assisted extraction from whey (Nandini & Rastogi, 2010). All of this research shows that LPO can be purified using very time-consuming complicated methods.

Sulphanamide compounds ($R\text{-}SO_2\text{-}NH_2$) contain an acidic nitrogen moiety, histidine and imidazole, which are heterocyclic aromatic imines (Drew, 2000). Sulfonamide is used to treat a variety of bacterial diseases in humans and other species and promote growth in food-producing animals (Supuran, Scozzafava, & Clare, 2002). The sulphanamides constitute an important class of drugs, and are pharmacological agents that possess antibacterial, antiglaucoma, diuretic, hypoglycemic and antithyroid activity (Supuran et al., 2002). A large number of structurally novel sulphanamide

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http://dx.doi.org/10.1016/j.foodchem.2012.08.072

derivatives have been reported to show substantial protease inhibitor properties. Sulphanamide compounds are very important inhibitors of carbonic anhydrases (Supuran, Scozzafava, & Casini, 2003). Therefore, these compounds have been used as ligands in affinity chromatography to purify these enzymes (Arslan, Nalbantoglu, Demir, Ozdemir, & Kufrevioglu, 1996).

The fundamental principle of affinity chromatography is the utilisation of the exceptional property of biologically active substances to form stable, specific and reversible complexes (Cuatrecasa, 1970). The selective isolation and purification of enzymes and other biologically important macromolecules by "affinity chromatography" exploit this unique biological property of these proteins to bind ligands specifically and reversibly. The protein to be purified is passed through a column containing an insoluble polymer or gel to which a specific competitive inhibitor or ligand has been covalently attached (Cuatrecasa, 1970).

Cross-linked dextran (Sephadex) has many of these desirable features. The beaded agarose derivatives (Sepharose) are even more desirable because of their very loose network. A gentle method has been developed for coupling proteins and small molecules to these carbohydrate derivatives, using cyanogen halides. Specific agarose adsorbents prepared by this basic procedure have now been used successfully to purify various enzymes, antibodies, chemically synthesised peptides and thyroxine-binding serum protein. The number of chemically modifiable groups (carboxamides) in polyacrylamide beads is far greater than the number of groups that can be substituted on agarose granular gels by the cyanogen bromide method (Porath & Flodin, 1959). Chemical compounds containing primary aliphatic or aromatic amines can be coupled directly to agarose beads after activation of the latter with cyanogen bromide at alkaline pH (Porath, 1968). Therefore, it was very simple to couple sulphanilamide to CNBr-activated-Sepharose 4B in our study following these previously published methods. Thus far, there have been no reports on the purification of LPO with a sulphanilamide compound using CNBr-activated-Sepharose 4B affinity chromatography.

Our work is the first report of the kinetic properties of sulphanilamide on LPO. Sulphanilamide was found to be a strong and competitive reversible inhibitor of LPO. The aim of this study was to evaluate the *in vitro* effect of sulphanilamide on LPO purified from bovine milk and to develop a protocol for the purification of LPO to extend this purification method to different peroxidases.

2. Materials and methods

2.1. Chemicals and materials

Fresh bovine milk was obtained from the local dairy. CNBr-activated-Sepharose 4B, L-tyrosine, sulphanilamide, Amberlite CG – 50 – NH_4^+ resin protein assay reagents and chemicals for electrophoresis were purchased from Sigma–Aldrich Co. (Sigma–Aldrich Chemie GmbH Export Department Eschenstrasse 5, 82024 Taufkirchen, Germany). All other chemicals were of analytical grade and obtained from Merck and Sigma–Aldrich Co.

2.2. Inhibition kinetics of sulphanilamide

The effects of sulphanilamide on LPO (R_z: 0.7) purified from bovine milk, using different chromatographic techniques, were previously determined (Jacob et al., 2000). In our study, LPO activity was measured in the presence of different concentrations of sulphanilamide (0.03–0.15 mM). A control sample without sulphanamide was taken as 100% and an activity-[Sulphanilamide] plot was drawn. For the determination of the K_i, three different sulphanamide/concentrations (0.03, 0.093 and 0.155 mM) were used. ABTS was also used as a substrate at five different concentrations (0.066–0.36 mM). Lineweaver–Burk plots (1/V-1/[S]) were obtained for sulphanilamide; the K_i and the inhibition type were calculated from these plots (Lineweaver & Burk, 1934). The data obtained were analysed by *t*-test and the results are given as X ± SD.

2.3. Determination of LPO activity

LPO activities were determined by the procedure of Shindler and Bardsley (1975) with a slight modification (Jacob et al., 2000). 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, 2.8 ml of ABTS (1 mM) 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 H_2O_2 solution (3.2 mM). The absorbance was measured at 412 nm as a function of times every 15 s.

To obtain K_m and V_{max} values at pH 6.0, the enzyme activity was measured at 412 nm for five different substrate concentrations at room temperature. For this purpose, 0.2, 0.3, 0.5, 0.8 and 1.1 ml of the substrate stock solution were mixed with the appropriate buffer solution to a final volume of 2.8 ml. Then, 0.1 ml of enzyme and 0.1 ml of H_2O_2 were added, and the enzyme activity was determined.

One unit of enzyme is defined as the amount of enzyme catalysing the oxidation of 1 μmol of ABTS min^{-1} at 298 K (Molar absorption coefficient, 32400 $M^{-1}cm^{-1}$). Finally, K_m and V_{max} values were obtained from Lineweaver–Burk plots. Quantitative protein determination was determined according to Bradford method (1976).

2.4. Preparation of Sepharose 4B-L-tyrosine-sulphanamide affinity matrix

The affinity matrix was synthesised by coupling sulphanilamide as the ligand and L-tyrosine as the spacer arm to CNBr-activated-Sepharose 4B, following the previously published procedure with a slight modification (Arslan et al., 1996; Cuatrecasa, 1970). CNBr-activated-Sepharose 4B was transferred to a beaker by washing it with cold $NaHCO_3$ buffer (0.1 M, pH 10). L-tyrosine was coupled to CNBr-activated Sepharose 4B. The reaction was stirred for 90 min. To remove excess of L-tyrosine from the Sepharose 4B-L-tyrosine gel, the mixture was washed with distilled and deionised water. The affinity gel was obtained by diazotisation of sulphanilamide and coupling of this compound to the Sepharose 4B-L-tyrosine matrix. For this purpose, sulphanilamide (20 mg) was suspended in 10 ml of ice-cold water. Then, 1 M HCl was added to 70 mg of sodium nitrite and 5 ml of ice-cold water. After 10 min of reaction, the diazotised sulphanilamide was poured into 40 ml of the Sepharose 4B-L-tyrosine suspension. The pH was adjusted to 9.5 with NaOH (1 M), and then stirred for 3 h at room temperature. The coupled red Sepharose gel was washed with water (1 l) and Tris-sulphate (200 ml, 0.05 M) at pH 7.5.

2.5. Preparation of bovine milk

Bovine milk was centrifuged at 2500×*g* at 4 °C for 15 min to remove fat. Amberlite CG 50 NH_4^+ resin [equilibrated with 5 mM sodium acetate solution (pH 6.8)] was added at a rate of 4.4 g/150 ml to the fresh raw skimmed bovine milk (Ozdemir et al., 2003; Uguz & Ozdemir, 2005). The supernatant was decanted. The resin was washed with distilled and deionised water and sodium acetate solution (20 mM, pH 6.8). The bound proteins were eluted with 0.5 M sodium acetate solution at pH 6.8.

2.6. Purification of LPO from affinity column

The eluate was applied to the Sepharose 4B-L-tyrosine-sulphanamide affinity column and equilibrated with phosphate buffer

(10 mM, pH 6.8). The affinity gel was washed with phosphate buffer (400 ml, 25 mM, pH 6.8). The bovine LPO enzyme was eluted with a solution of 1 M NaCl/25 mM Na_2HPO_4 (pH 6.8), measuring the $R_z(A_{412}/A_{280})$ of the fractions. The enzyme solution was dialysed overnight against sodium phosphate buffer (0.5 M, pH 6.8). The fractions were lyophilised and checked for purity by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) (Laemmli, 1975). In addition, protein concentration was determined according to the Bradford method described previously (Pourtois et al., 1991; Sisecioglu, Uguz, Cankaya, Ozdemir, & Gulcin, 2011).

2.7. SDS–PAGE

SDS–PAGE was performed after LPO purification according to the procedure of Laemmli (1975). The stacking and running gels contained 3% (w/v) and 10% (w/v) acrylamide, respectively, and 0.1% (w/v) SDS. The electrode buffer was 0.025 M Tris/0.2 M glycine (pH 8.3). The sample buffer was prepared by mixing 0.65 ml of Tris–HCl (1 M, pH 6.8), 3 ml of 10% (w/v) SDS, 1 ml of neat glycerol, 1 ml of 0.1% (w/v) bromophenol blue, 0.5 ml of β-mercaptoethanol and 3.85 ml of water. A 20 mg aliquot of enzyme (50 ml) was added to 50 ml of sample buffer, and the mixture was heated in a boiling water bath for 3 min. The cooled LPO sample was loaded into each lane of the stacking gel. LPO was analysed separately by polyacrylamide gel electrophoresis. Initially, an electrical potential of 80 V (Hoefer Scientific Instruments SE 600) was applied until the bromophenol blue dye reached the running gel. Then, the electrical potential was increased to 200 V for 3–4 h. Gels were stained for 1.5 h in 0.1% (w/v) Coomassie Brilliant Blue R-250 in 50% (v/v) methanol and 10% (v/v) acetic acid, and destained with methanol/acetic acid.

2.8. Binding capacity for LPO on Sepharose 4B-L-tyrosine-sulphanilamide matrix

Purified LPO was applied to an affinity column containing 1 g of Sepharose 4B-L-tyrosine-sulphanilamide affinity matrix. The binding capacity was determined by measuring the amount of eluted LPO or dried gel-containing LPO from this column at different temperatures, pH values and ionic strengths (Arslan et al., 1996). The column capacity was determined according to the following procedure: 1 ml of prepared gel was equilibrated with equilibration buffer, poured into a 1×10 cm column and saturated with LPO obtained from affinity chromatography.

Unbound enzyme was washed away and eluted with washing buffer. Then, bound LPO was eluted with elution buffer. The amount of eluted protein was measured according to the Bradford method. Simultaneously, the gel was dried and weighed, and the LPO binding capacity was determined as mg protein/g gel.

3. Results and discussion

Lactoperoxidase has been identified as an antimicrobial agent in milk, saliva and tears. LPO is a natural antibacterial defence agent that catalyses the oxidation of thiocyanate ions (SCN^-) by hydrogen peroxide (Kumar & Bhatla, 1995; Sisecioglu, Cankaya, Gulcin, & Ozdemir, 2009; Wolfson et al., 1993). LPO has been recognised as an effective antimicrobial agent for many years and used extensively as an antibacteriostatic agent in reducing microflora in milk and in cheese-making (Reiter, 1985). LPO is a glycoprotein, consisting of a single polypeptide chain with a molecular weight of 78 Da (Golhefors & Marklundi, 1975; Jacob et al., 2000).

LPO has 15 half-systemic residues and a much higher isoelectric point (pH 9.2) than most other whey proteins. The carbohydrate content of LPO is approximately 10%, which occurs at four or five potential binding sites. The enzyme contains a haem, with one iron molecule per molecule of LPO. The conformation of the protein is stabilised by a strongly chelated calcium ion. In addition to its use as an antibacterial agent, other applications of LPO in cosmetics, ophthalmic solutions, dental and wound treatment, and as anti-tumour and anti viral agents, have been found (Atamer et al., 1999; Kussendrager & van Hooijdank, 2000; Pourtois et al., 1991). The goal of many research groups has been to study LPO inhibitors (Ozdemir & Uguz, 2005; Ozdemir et al., 2001; Shin et al., 2001; Uguz & Ozdemir, 2005). The inhibitory effects of different chemicals were studied on bovine LPO and most of them inhibited the enzyme (Ozdemir et al., 2002, 2003; Ozdemir & Uguz, 2005; Sisecioglu et al., 2011). An enzyme inhibitor is a molecule that binds to enzymes and decreases their activity. Inhibitor binding is either reversible or irreversible. Many drug molecules are enzyme inhibitors; therefore their discovery and improvement is

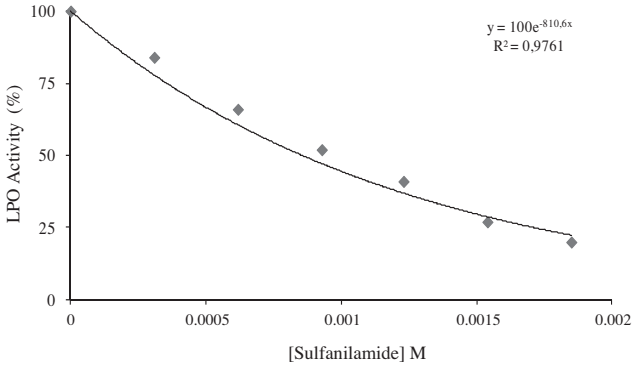


Fig. 1. Activity (%)-[sulphanilamide] plot for bovine milk LPO in the presence of 6 different sulphanilamide concentrations.

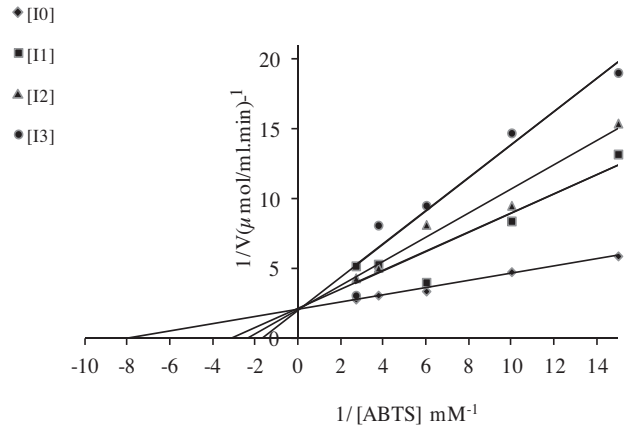


Fig. 2. Lineweaver–Burk plot for five different substrate (ABTS) concentrations and 3 different sulphanilamide concentrations for determination of K_i([I_1]: $3.09.10^{-5}$ M, [I_2]: $9.28.10^{-5}$ M, [I_3]: $15.5.10^{-5}$ M, [I_o]: Control).

Table 1
I_{50} value, K_i constant and inhibition type of sulphanilamide for bovine LPO (LPO: lactoperoxidase).

Kinetic properties	Inhibitor (*p*-aminobenzenesulphanilamide)
K_i constants (M)	$3.01.10^{-5}$
	$3.80.10^{-5}$
	$3.90.10^{-5}$
Mean K_i constant (M)	$3.57.10^{-5}$
I_{50} value (M)	$8.48.10^{-4}$
Inhibition type	Competitive

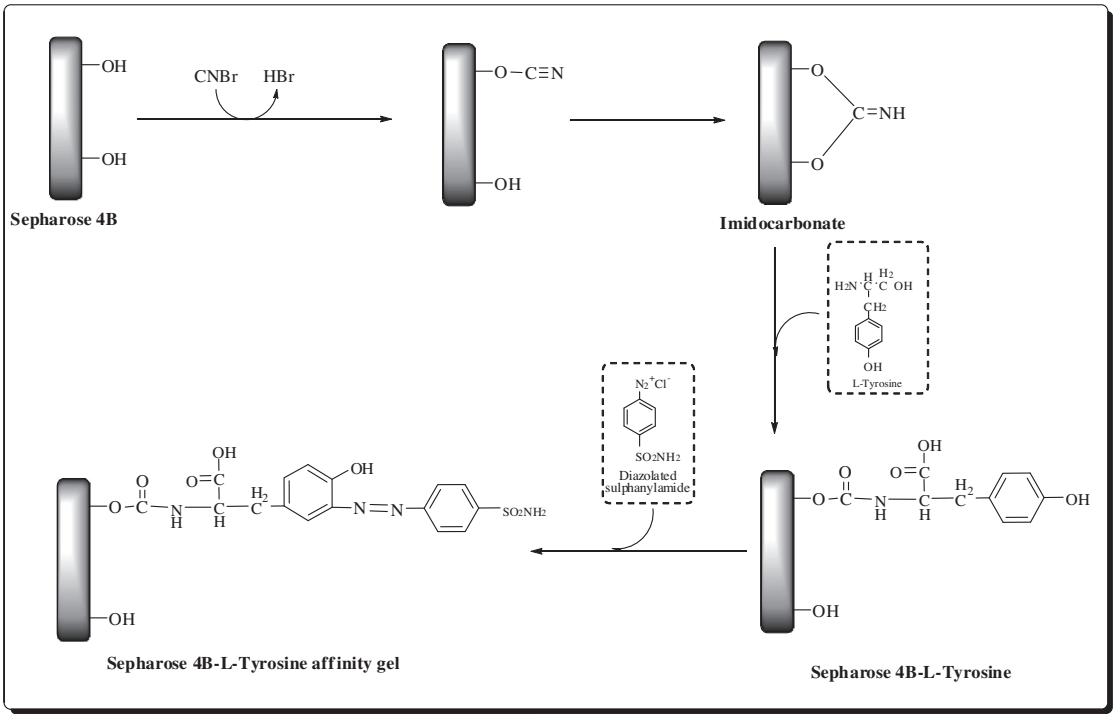


Fig. 3. Synthesis of Sepharose-4B-L-tyrosine- sulphanilamide affinity matrix.

an active area of research in biochemistry and pharmacology (Segel, 1993; Shapiro & Vallee, 1991). Sulphanamide is the basis of several groups of drugs (Daniel & Caroline, 1994; Drew, 2000).

Sulphanamide antibiotics, typified by sulphamethazine (4-amino-*N*-(4,6 dimethyl-2-pyrimidinyl) benzenesulphanamide) are widely used in veterinary practise. Residual sulphanamide in milk and meat products is of regulatory concern because sulphamethazine is a thyroid carcinogen in rodents and sulphanamide-induced hypersensitivity reactions, including hypothyroidism, have been reported in humans. It was reported that sulphamethazine and other primary arylamines inhibit iodination reactions catalysed by thyroid peroxidase (TPO) and the closely related LPO. The apparent K_i values for sulphamethazine inhibition of TPO- and LPO-catalysed iodide ion oxidation were 0.42 and 0.11 mM, respectively (Daniel & Caroline, 1994). The concentration required for 50% inhibition (IC_{50}) and inhibition constant (K_i) values are often reported in the literature, but direct comparison of these values is not possible. The relationship of K_i and IC_{50} for a given compound varies, depending on the assay conditions and the inhibitory mechanism of the compound. The aim of our study was to determine the kinetic properties of sulphanilamide on bovine LPO. The K_i and IC_{50} values are the most suitable parameters to show the effect of inhibition. Until now, a detailed report on the inhibition kinetics of sulphanilamide on LPO has not been published. In this study, both K_i and IC_{50} parameters for the sulphanamide as an inhibitor of bovine LPO were determined. K_i was determined to be $3.55.10^{-5}$ M. The inhibitor concentration causing up to 50% inhibition was determined from an activity (%)-[sulphanilamide] plot (Fig. 1). The obtained IC_{50} value was 0.848 mM. The K_i value was calculated from a Lineweaver and Burk plot (1934) and the sulphanilamide showed competitive inhibition (Fig. 2, Table 1). Reversible or competitive inhibitors bind to enzymes with non-covalent interactions, such as hydrogen bonds, hydrophobic interactions and ionic bonds (Segel, 1993; Shapiro & Vallee, 1991). Multiple weak bonds between the inhibitor and the active site combine to produce strong and specific binding. In contrast

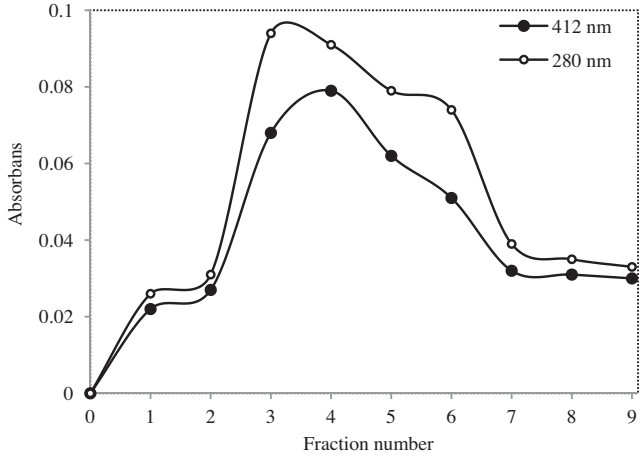


Fig. 4. Purification of LPO by Sepharose 4B-L-tyrosine- sulphanilamide affinity gel chromatography. The column (1.3 × 10 cm) was eluted by 1 M NaCl / 25 mM Na_2HPO_4 (pH 6.8) at 20 ml/h flow rate for fraction volume 2 ml (Fractions having $R_z(A_{412}/A_{280})$ values of 0.7 or higher were pooled.

to substrates and irreversible inhibitors, reversible inhibitors generally do not undergo chemical reactions when bound to the enzyme and can be easily removed by dilution or dialysis. We found sulphanilamide to be a reversible inhibitor of LPO, making it a suitable ligand for the Sepharose 4B column. Some research groups purify LPO using different purification methods, starting from different sources. The first method of enzyme purification uses the precipitation technique based on salt concentration. Ammonium sulphate is a common solution used in this method (Ozdemir et al., 2001, 2003).

Chromatographic methods include ion-exchange, bio-affinity and hydrophobic affinity chromatography. Recently, it has been reported that ion-exchange is used for molecular charge. In contrast, bio-affinity is used for biomolecular interaction (Nandini & Rastogi,

Table 2
Purification steps of LPO from bovine milk by Sepharose 4B-L-tyrosine- sulphanilamide affinity column (LPO: lactoperoxidase).

Purification steps	Total volume (ml)	Enzyme activity (EU/ml)	Total enzyme activity (EU)	Protein (mg/ml)	Total protein (mg)	Specific activity (EU/mg)	Yield (%)	Purification fold
Crude homogenate from Amberlite CG – 50 – NH_4^+ resin	50	1.01	50.5	10.0	500	0.1	100	1.00
Purified LPO from Sepharose 4B-L-tyrosine-Sulphanilamide column and dialyses	7	5.50	31.5	0.11	0.77	40.9	61.3	409

2010; Shin et al., 2001). The initial preparation for such methods includes lysing the cells and centrifugation to obtain a clarified supernatant. Gel filtration is a method of chromatography based on the molecular weight of the targetprotein. The purification of LPOis time-consuming and requires many different techniques. The purpose of this research was to develop a method for the purification of LPO from bovine milk. In this study, affinity chromatography was used for LPO purification. Affinity chromatography is a method of separating biochemical mixtures based on highly specific interactions such as interactions occurring between antigen and antibody, enzyme and substrate, or receptor and ligand (Voet & Voet, 1995). The affinity matrix was synthesised by coupling sulphanamide, as the ligand, and L-tyrosine, as the spacer arm, to commercially available CNBr-activated Sepharose 4B to purify LPO from bovine milk. The effect of pH on the coupling of ^{14}C-alanine to activated Sepharose was previously reported in the literature (Cuatrecasa, 1970). The following procedure was integrated and modified into our study. In that study, packed Sepharose 4B was mixed with water and treated with CNBr. It was then washed, and activated and added to L-alanine in cold distilled water. Finally, this sample was mixed and added rapidly to beakers containing buffer. After 24 h, the suspensions were thoroughly washed. The aliquots were hydrolysed and the amount of unlinked L-alanine was determined (Cuatrecasa, 1970).

As shown in Fig. 3, the affinity column material was synthesised by means of two consecutive reactions. Sepharose 4B was chosen as a matrix because of its better flow properties than those of other matrices. L-tyrosine was chosen as a spacer arm to prevent nonspecific binding. Finally, sulphanamide, a specific and strong inhibitor of LPO, was diazotised and then coupled to the phenol ring of L-tyrosine. Bovine milk was centrifuged to remove fat. Amberlite CG 50 NH_4^+ resin (equilibrated with 5 mM sodium acetate solution pH 6.8) was added at a rate of 4.4 g /150 ml to the fresh raw skimmed bovine milk (Ozdemir et al., 2001, 2003).

The supernatant was decanted. The resin was washed with distilled and deionised water and sodium acetate solution (20 mM, pH 6.8). The bound proteins were eluted with sodium acetate solution (0.5 M, pH 6.8). The eluate was applied to the Sepharose 4B-L-tyrosine-sulphanamide affinity column and equilibrated with phosphate buffer (10 mM, pH 6.8). The newly synthesised affinity gel was washed with phosphate buffer (400 ml, 25 mM, pH 6.8). The bovine LPO enzyme was eluted with a solution of 1 M NaCl / 25 mM Na_2HPO_4 (pH 6.8), and the R_z (A_{412}/A_{280}) of the fractions was measured. Fractions having R_z values of 0.7 or higher were pooled (Fig. 4). Thereafter the enzyme solution was dialysed overnight against sodium phosphate buffer (0.5 M, pH 6.8). The fractions were lyophilised and checked for purity by SDS–PAGE (Laemmli, 1975; Uguz & Ozdemir, 2005). As shown in Table 2, the specific activity was calculated for each purification step and purified enzyme solution. LPO was purified 409-fold from the newly synthesised affinity matrix in a single step, resulting in 0.11 mg (R_z: 0.7) of LPO obtained from 150 ml of bovine milk with a yield of 62.3% and a specific activity of 40.9 EU/mg protein. A great deal of research has reported on the purification of LPO, most of which has used different techniques. The purification of LPO with these tech-

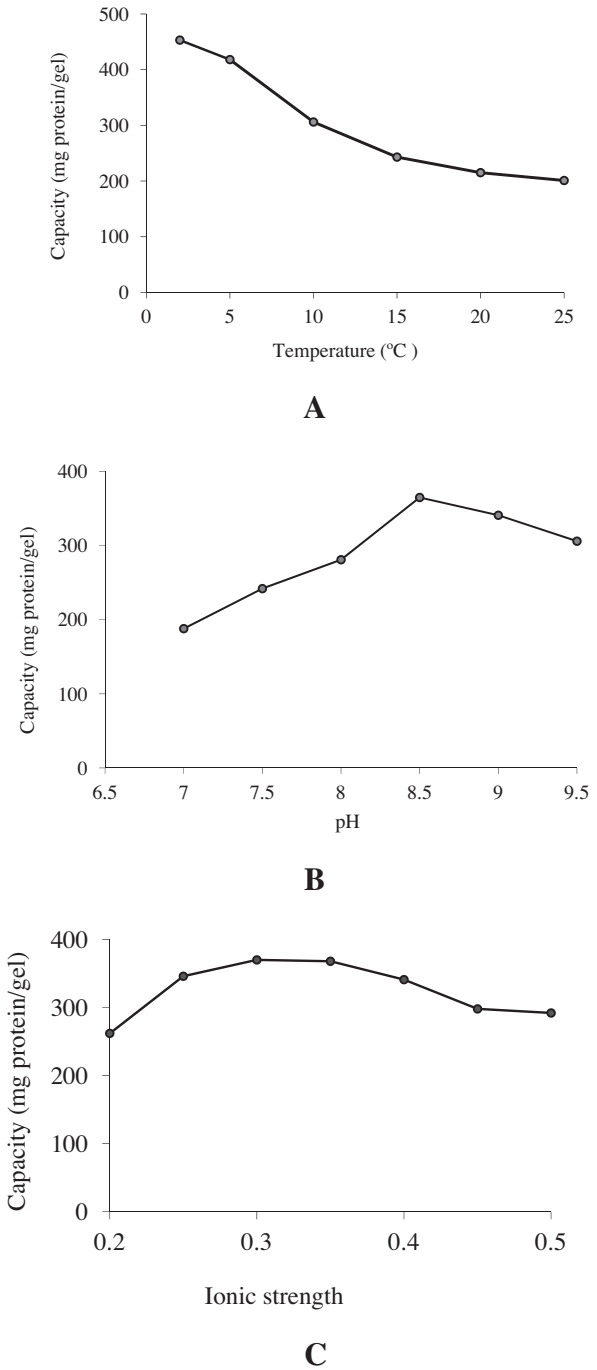


Fig. 5. A. Binding capacity of the Sepharose 4B-L-tyrosine- sulphanilamide affinity gel for LPO at different temperatures with constant pH of 6.8 and ionic strength. B.: Binding capacity of the Sepharose 4B-L-tyrosine- sulphanilamide affinity gel for LPO at different pH's with constant temperature of 4°C and ionic strength. C.: Binding capacity of the Sepharose 4B-L-tyrosine- sulphanilamide affinity gel for LPO at different ionic strengths with constant temperature of 4°C and a pH (6.8).

niques gave different results. For example, LPO was purified 31-fold with a yield of 64%, 10-fold with a yield of 45% and 13.2-fold with a yield of 29% (Nandini & Rastogi, 2010; Shin et al., 2001. We have previously performed several studies on the purification, inhibition and antibacterial properties of LPO (Ozdemir et al., 2002, 2001, 2003;Uguz & Ozdemir, 2005; Ozdemir & Uguz, 2005; Sisecioglu et al., 2011). In our previous studies, we used time-consuming chromatographic methods to purify LPO from milk and obtained very low yields, ranging from 20–30%, specific activities ranging from approximately 10–30 EU/mg proteins and about approximately 10–20-fold purification. Our present method provides 400-fold purification for LPO and is superior to both our previous studies and those reported in the literature. The binding capacity of the affinity matrix for LPO was determined at different temperatures (Fig. 5A), pH (Fig. 5B) and ionic strengths (Fig. 5C). Maximum binding was achieved at 4 °C, with a pH between 8.5 and 9.0 and an ionic strength of approximately 0.25 M. The binding capacity for LPO under optimum conditions was 395 mg protein/g gel.

The kinetic parameters, such as K_m and V_{max}, were calculated by a Lineweaver–Burk plot, using ABTS as a substrate for the purified LPO. K_m is the concentration of substrate at which half of the enzyme active sites are filled. V_{max} is the maximum rate of an enzyme-catalysed reaction, i.e. when the enzyme is saturated by the substrate. K_m and V_{max} were calculated from plots using ABTS as a substrate for LPO. The optimum pH was found to be 6.0. The K_m value at the optimum pH was found to be 0.14 mM, and V_{max} was found to be 0.55 μmol/min.ml.

The purity of LPO was monitored by SDS–PAGE. As shown in Fig. 6, MBP (maltose-binding protein-β-galactosidase, 175 kDa), MBP (maltose- binding protein)-paramyosin (fusion of MBP and paramyosin, 80 kDa), MBP-CBD (chitin-binding domain, fusion of MBP and chitin-binding domain, 58 kDa) and CBD-Mxe Intein-2CBD (fusion of the chitin-binding domain and the Mxe Intein, followed by two chitin-binding domains, 46 kDa) were used as standards. LPO has a molecular weight of approximately 80 kDa. The purified LPO migrated to a similar distance on the SDS–PAGE gel as LPO purified from other sources (Ozdemir et al., 2002, 2001).

The results of this study clearly demonstrate the kinetic properties of sulphanilamide on LPO and its competitive inhibition. In addition, there is no literature concerning the purification of LPO with sulphanilamide compounds, using CNBr-activated-Sepharose 4B. We are the first to demonstrate a successful purification method for LPO, using a Sepharose 4B-L-tyrosine-sulphanilamide affinity matrix in a single step. The purification method developed for LPO can be applied several times and proves to be significantly less time-consuming with lower cost. In light of our results, this method shows promise toward guiding the development of purification methods for other peroxidases.

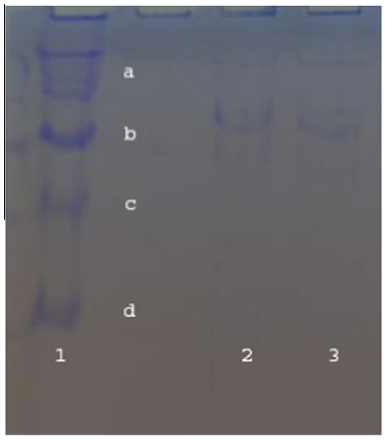


Fig. 6. SDS–PAGE band of LPO. Column 1: Standard proteins a: MBP (maltose-binding protein-β-galactosidase, 175 kDa), b: MBP (maltose-binding protein)-paramyosin (fusion of MBP and paramyosin, 80 kDa), c: MBP-CBD (Chitin-binding domain, fusion of MBP and chitin-binding domain, 58 kDa), d: CBD-Mxe Intein-2CBD (Fusion of the chitin-binding domain and the Mxe Intein, followed by two chitin-binding domains, 46 kDa). Columns 2 and 3: Purified LPO from bovine milk by Sepharose 4B-L-tyrosine-sulphanilamide affinity column chromatography (LPO: Lactoperoxidase, SDS–PAGE: Sodium dodecylsulphate–polyacrylamide gel electrophoresis).

References

Arslan, O., Nalbantoglu, B., Demir, N., Ozdemir, H., & Kufrevioglu, O. I. (1996). A new method for the purification of carbonic anhydrase isozymes by affinity chromatography. *Turkish Journal of Medical Sciences, 26*, 163–166.

Atamer, M., Kocak, C., Cimer, A., Odabasi, S., Tamucay, B., & Yamaner, N. (1999). Some quality characteristics of Kasar cheese manufactured from milk preserved by activation of lactoperoxidase/thiocyanate/hydrogen peroxide (LP) system. *Milchwissenschaft, 54*, 553–556.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry, 72*, 248–251.

Cals, M. M., Mailliart, P., Brignon, G., Anglade, P., & Dumas, B. R. (1991). Primary structure of bovine lactoperoxidase, a fourth member of a mammalian heme peroxidase family. *European Journal of Biochemistry, 198*, 733–739.

Cuatrecasa, P. (1970). Protein purification by affinity chromatography. *The Journal of Biological Chemistry, 245*, 3059–3065.

Daniel, R. D., & Caroline, J. D. (1994). Inhibition of peroxidase-catalyzed reactions by arylamines: Mechanism for the anti-thyroid action of sulfamethazine. *Chemical Research and Toxicology, 7*, 164–169.

Drew, J. (2000). Drug discovery: A historical perspective. *Science, 287*, 1960–1964.

Dumonte, C., & Rousst, B. (1983). Identification, purification, and characterization of a non-heme lactoperoxidase in bovine milk. *Journal of Biological Chemistry, 258*, 14166–14172.

Elagamy, E., Ruppanner, R., Ismail, A., Champagne, C. P., & Assaf, R. (1992). Antibacterial and antiviral activity of camel milk protective proteins. *Journal of DairyResearch, 59*, 169–175.

Golhefors, L., & Marklundi, S. (1975). Lactoperoxidase activity in human milk and in saliva of newborn infants. *Infection and Immunity, 11*, 1210–1215.

Jacob, B. M., Anthony, K. E., Sreekumar, B., & Haridas, M. (2000). Thiocyanate mediated antifungal and antibacterial property of goat milk. *Life Sciences Including Pharmacology Letter, 66*, 2433–2439.

Jacob, B. M., Manoj, N. K., & Harridas, M. (1998). Antibacterial property of goat milk lactoperoxidase. *Indian Journal of Experimental Biology, 36*, 808–810.

Kumar, R., & Bhatla, K. L. (1995). Purification, crystallization and preliminary X-ray crystallographic analysis of lactoperoxidase from buffalo milk. *Acta Crystallographica D, 51*, 1094–1096.

Kussendrager, K. D., & van Hooijdank, A. C. M. (2000). Lactoperoxidase: physico-chemical properties, occurrence, mechanism of action and applications. *British Journal of Nutrition, 84*, 19–25.

Laemmli, D. K. (1975). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, 227*, 680–685.

Langbakk, B., & Flatmark, T. (1984). Lactoperoxidase from human colostrum. *FEBS Letters, 174*, 300–303.

Lineweaver, H., & Burk, D. (1934). The determination of enzyme dissociation constants. *Journal of the American Chemical Society, 56*, 658–666.

Mecitoglu, C., & Yemenicioglu, A. (2007). Partial purification and preparation of bovine lactoperoxidase and characterization of kinetic properties of its immobilized form incorporated into cross-linked alginate films. *Food Chemistry, 104*, 726–733.

Nandini, K. E., & Rastogi, N. K. (2010). Single step purification from whey involving reverse Micelles-assisted extraction and its comeration with reverse micellar extraction. *Biotechnology Progress, 26*, 763–771.

Ozdemir, H., Hacıbeyoglu, H. I., & Kufrevioglu, O. I. (2003). Effects of the water-miscible organic solvents on lactoperoxidase purified from creek-water buffalo milk. *Biochemistry-Moscow, 68*, 329–333.

Ozdemir, H., & Uguz, M. T. (2005). In vitro effects of some anaesthetic drugs on lactoperoxidase enzyme activity. *Journal of Enzyme Inhibition and Medicinal Chemistry, 20*, 491–495.

Ozdemir, H., Aygul, I., & Kufrevioglu, O. I. (2001). Purification of lactoperoxidase from bovine milk and investigation of the kinetic properties. *Preparative Biochemistry and Biotechnology, 31*, 125–134.

Ozdemir, H., Hacibeyoglu, H. I., & Uslu, H. (2002). Purification of lactoperoxidase from bovine milk and investigation of the kinetic properties. *Preparative Biochemistry and Biotechnology, 32*, 143–155.

Porath, J., & Flodin, P. (1959). Gel filtration: A method for desalting and group separation. *Nature, 183*, 1657–1659.

Porath, J. (1968). Sweden: Molecular sieving and adsorption. *Nature, 218*, 834–838.

Pourtois, M., Binet, C., Van Tieghem, N., Courtois, P. R., Vandenabbeele, A., & Thirty, L. (1991). Saliva can contribute in quick inhibition of HIV infectivity. *AIDS, 5*, 598–600.

Reiter, B. (1985). Protective proteins in milk. *International Dairy Federation Bulletin, 191*, 2–35.

Reiter, B., & Harnulv, G. (1984). Lactoperoxidase antibacterial system natural occurrence, biological functions and practical applications. *Journal of Food Protection, 47*, 724–732.

Segel, I. H. (1993). *Enzyme kinetics: Behavior and analysis of rapid equilibrium and steady-state enzyme systems*. Wiley-Interscience. New Edition.

Shapiro, R., & Vallee, B. L. (1991). Interaction of human placental ribonucleic with placental ribonuclease inhibitor. *Biochemistry, 30*, 2246–2255.

Shin, K., Hayasawa, H., & Lönnerdal, B. (2001). Purification and quantification of lactoperoxidase in human milk with use of immuno adsorbents with antibodies against recombinant human lactoperoxidase. *American Journal of Clinical Nutrition, 73*, 984–989.

Shindler, J. S., & Bardsley, W. (1975). Steady-state kinetics of lactoperoxidase with ABST as chromogens. *Biochemistry and Biophysics Research Communication, 67*, 1307–1312.

Sisecioglu, M., Cankaya, M., Gulcin, I., & Ozdemir, H. (2009). The inhibitory effect of propofol on bovine lactoperoxidase. *Protein Peptide Letters, 16*, 46–49.

Sisecioglu, M., Gulcin, I., Cankaya, M., Atasever, A., & Ozdemir, H. (2010). The effects of norepinephrine on lactoperoxidase enzyme. *Scientific Research and Essays, 5*, 1351–1356.

Sisecioglu, M., Uguz, M. T., Cankaya, M., Ozdemir, H., & Gulcin, I. (2011). Effects of ceftazidimepentahydrate, prednisolone, amikacinsulfate, ceftriaxone sodium and teicoplanin on bovine milk lactoperoxidase activity. *International Journal of Pharmacology, 7*, 79–83.

Supuran, C. T., Scozzafava, A., & Clare, B. W. (2002). Bacterial protease inhibitors. *Medicinal Research Reviews, 22*, 329–372.

Supuran, C. T., Scozzafava, A., & Casini, A. (2003). Carbonic anhydrase inhibitors. *Medicinal Research Reviews, 23*, 146–189.

Ueda, T., Sakamaki, K., Kuroki, T., Yano, I., & Nagata, S. (1976). Molecular cloning and characterization of the chromosomal gene for human lactoperoxidase. *European Journal of Biochemistry, 243*, 32–41.

Uguz, M. T., & Ozdemir, H. (2005). Purification of bovine milk lactoperoxidase and investigation of antibacterial properties at different thiocyanate mediated. *Applied Biochemistry andMicrobiology, 41*, 397–401.

Voet & Voet (1959). John Wiley and Sons.

Wolfson, L. M., & Sumner, S. A. (1993). Antibacterial activity of the lactoperoxidase system: A review. *Journal of Food Protection, 56*, 887–892.