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

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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.

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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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