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ORIGINAL ARTICLE

Interactions of melatonin and serotonin with lactoperoxidase enzyme

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Abstract

Melatonin is the chief secretory product of the pineal gland and is synthesized enzymatically from serotonin. These indoleamine derivatives play an important role in the prevention of oxidative damage. Lactoperoxidase (LPO; EC 1.11.1.7) was purified from bovine milk with three purification steps: Amberlite CG-50 resin, CM-Sephadex C-50 ion-exchange, and Sephadex G-100 gel filtration chromatography, respectively. LPO was purified with a yield of 21.6%, a specific activity of 34.0 EU/mg protein, and 14.7-fold purification. To determine the enzyme purity, SDS-PAGE was performed and a single band was observed. The R_z (A_{412}/A_{280}) value for LPO was 0.9. The effect of melatonin and serotonin on lactoperoxidase was determined using ABTS as chromogenic substrate. The half-maximal inhibitory concentration (IC_{50}) values for melatonin and serotonin were found to be 1.46 and 1.29 μM, respectively. Also, the inhibition constants (K_i) for melatonin and serotonin were 0.82 ± 0.28 and 0.26 ± 0.04 μM, respectively. Both melatonin and serotonin were found to be competitive inhibitors.

Keywords: *Melatonin; serotonin; lactoperoxidase; enzyme purification; inhibition; LPO*

Introduction

The pineal hormone melatonin (*N*-acetyl-5-methoxytryptamine), an indoleamine, is the chief secretory product of the pineal gland. It is synthesized enzymatically from serotonin (5-hydroxytryptamine) by the sequential action of serotonin *N*-acetyltransferase and hydroxyindole-*O*-methyltransferase[1]. It is also produced in other organs and found in all body fluids after its release from the pineal. It is known that melatonin influences a variety of biological processes including circadian rhythm and neuroendocrine, cardiovascular, and immune functions, as well as thermoregulation[2–4]. Additionally, this molecule functions in protecting cell components such as nuclear DNA, membrane lipids, and cytosolic proteins from free radical damage[5,6].

Serotonin (5-hydroxytryptamine) has been implicated in the control of many physiological (cardiovascular, respiratory, and thermoregulatory) and behavioral (feeding and sexual behavior, circadian rhythm, sleep–wake cycle, aggression, learning, and pain sensitivity) functions that could be disturbed by depression[7,8]. It is a biogenic amine belonging to the most common neurotransmitters in nature. Serotonin is a well-established neurotransmitter produced and activated in nervous tissues and the digestive tract[9]. In humans, serotonin modifies body temperature, blood pressure, sexual behavior, and mood. It is also involved in several interactions of the immune system[10]. It has been reported that serotonin is implicated in many brain functions, including those related to pleasure and the intake of food, depression, suicide, anxiety, and Parkinson's and Alzheimer's diseases[8,11].

Milk contains a variety of compounds that protect the neonate as well as the milk itself from a host of deleterious microorganisms. One of those compounds is the enzyme lactoperoxidase[12]. Lactoperoxidase (LPO; EC 1.11.1.7) is a heme-containing glycoprotein with a single chain of 612 residues that has a molecular mass of approximately 78 kDa and a carbohydrate content of about 10%[13]. Lactoperoxidase catalyzes the oxidation of halides and pseudohalides at the expense of hydrogen peroxide, and generates products with a wide antimicrobial activity. Hence, it catalyzes the inactivation of a wide range of microorganisms[14–16]. The other members of the mammalian peroxidase family include eosinophil peroxidase (EPO), thyroid peroxidase (TPO), and myeloperoxidase (MPO). LPO, EPO, and MPO contribute to the nonimmune host defense system by oxidizing halide and pseudohalide

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ions to produce potent antimicrobial agents[17]. LPO is present and active in many secretory fluids in various parts of the body including milk, tears, and saliva[15]. It was reported that LPO carries out this function in the above exocrine secretions, while EPO and MPO play similar roles in the phagosomes of eosinophils and neutrophils, respectively, during engulfment of microorganisms. On the other hand, TPO is an intracellular membrane-bound protein, which is involved in catalysis of the iodination and coupling of thyroglobulin moieties in the biosynthesis of thyroid hormones thyroxine and triiodothyronine[17]. Bovine milk LPO has a high activity, more than other types of LPO; therefore, most research has been carried out on bovine LPO. Also, the quantity of LPO in human milk is less than in other sources such as bovine milk LPO. The objective of this study was to evaluate the *in vitro* effects of melatonin and serotonin on lactoperoxidase purified from bovine milk.

Materials and methods

Chemicals

Melatonin, serotonin, Sephadex G-100, CM-Sephadex C-50, 2,2′-azino-bis(3-ethylbenzthiazoline-6 sulfonic acid) (ABTS), Coomassie Brilliant Blue R-250, and standard proteins (egg albumin, bovine albumin, and β-galactosidase) were obtained from Sigma Aldrich Chemie GmbH. Amberlite CG-50 resin was purchased from Fluka Chemie GmbH.

Determination of lactoperoxidase activity

Lactoperoxidase activity was determined by the procedure of Schindler with a slight modification[18]. This method is based on the oxidation of ABTS as a chromogenic substrate with hydrogen peroxide. The formed colored compound gives an absorbance at 412 nm. As a typical procedure, 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 6.0) and 0.1 mL of H_2O_2 solution (3.2 mM) and the absorbance was taken at 412 nm as a function of time every 15 s during 3 min[19].

Lactoperoxidase activity unit

One unit of activity is defined as the amount of enzyme catalyzing the oxidation of 1 μmol of ABTS min^{-1} at 298K (molar absorption coefficient: 32,400 M^{-1} cm^{-1})[19,20].

Protein determination

Protein concentration was determined according to the method of Lowry *et al.*[21]. Bovine serum albumin (BSA) was used as standard protein[22,23].

Purification of lactoperoxidase

Bovine milk was centrifuged at 5000 × *g* using the centrifuge maximum at 48°C for 15 min to remove fat. First, Amberlite CG-50 resin was equilibrated with sodium phosphate buffer (5 mM, pH 6.8), then added in the proportion of 22 g/L to the fresh, raw, skimmed bovine milk[21,24]. The supernatant was decanted and the resin was washed with distilled water, then equilibrated with 20 mM sodium phosphate buffer (pH 6.8). The bound protein was eluted with 0.5 M sodium phosphate buffer (pH 6.8). To the green-colored mixture was gradually added solid ammonium sulfate (precipitation I, saturation 90%) over a period of 30 min while it was being stirred magnetically, and the enzyme solution was dialyzed overnight against 5 mM sodium phosphate buffer (pH 6.8).

The clear greenish supernatant obtained above was loaded onto a column of CM-Sephadex C-50 (3 × 10 cm) previously equilibrated with 10 mM sodium phosphate buffer (pH 6.8). The column-bound enzyme was washed with 100 mL of 10 mM phosphate buffer (pH 6.8) containing 100 mM NaCl. The enzyme was eluted with a linear gradient of 100–200 mM NaCl in 10 mM phosphate buffer (pH 6.8) and subjected to ammonium sulfate precipitation (precipitation II, saturation 90%). Thereafter the enzyme solution was dialyzed overnight against sodium phosphate buffer (5 mM, pH 6.8)[19,20].

Lactoperoxidase enzyme obtained from the CM-Sephadex C-50 column was applied to a column of Sephadex G-100 (2.5 × 100 cm). The column-bound enzyme was eluted with 0.1 M phosphate buffer (pH 6.8), and salted out with ammonium sulfate precipitation (precipitation III, 90% saturation). The enzyme solution was dialyzed overnight against phosphate buffer (0.5 M, pH 6.0). Fractions were lyophilized and checked for purity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)[25–27].

Ammonium sulfate precipitation

Lactoperoxidase enzyme obtained from the CM-Sephadex C-50 column was subjected to ammonium sulfate fractionation and the precipitate in the 0–90% saturation range was collected by centrifugation for 60 min at 15,000 × *g*. The precipitate was suspended in ~2 mL phosphate buffer (0.5 M, pH 6.0)[28].

SDS-PAGE

SDS-PAGE was performed under denaturing conditions after LPO purification, according to Laemmli's procedure[29]. The stacking and running gels comprised 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) bromphenol blue, 0.5 mL of β-mercaptoethanol, and 3.85 mL of water. A 20 μg aliquot of enzyme (50 μL) was added into 50 μL of sample buffer and the mixture was heated in a boiling water bath for 3 min and then cooled[30].

LPO samples were loaded into each space of the stacking gel. LPO was analyzed separately by PAGE. Initially, an electric potential of 80 V (Hoefer Scientific Instruments, SE 600) was applied until the bromphenol dye reached the running gel. Then it 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[31,32].

Results and discussion

Lactoperoxidase enzyme (LPO) is a member of the mammalian peroxidase family. It catalyzes the oxidation of thiocyanate and halides. As reported in the literature, LPO, EPO, and TPO are monomeric proteins, while MPO is a covalently linked dimer of two identical halves, each consisting of two polypeptide chains of 108 and 466 amino acid residues as a result of a posttranslational deletion of six amino acid residues[17]. LPO has been intensively studied over the years, and is present and active in many secretory fluids in various parts of the body. The effect of increasing concentrations of melatonin (0.5–2.5 μM) and serotonin (0.5–2.15 μM) on LPO enzyme activity was determined.

First, LPO was purified from bovine milk using CM-Sephadex C-50 ion exchange chromatography. Then the enzyme obtained from CM-Sephadex C-50 ion exchange was applied to Sephadex G-100 gel filtration chromatography (Table 1). Specific activity was determined for each purification step. Kinetics parameters K_m and V_{max} were calculated by a Lineweaver–Burk plot for the ABTS substrate.

The K_m value was found to be 0.358 mM; on the other hand, the V_{max} value was calculated to be 18.86 μmol/mL/min. The purification of LPO was controlled by SDS-PAGE. Maltose-binding protein (MBP)–β-galactosidase (fusion of MBP and β-galactosidase, 175 kDa), MBP–paramyosin (fusion of MBP and paramyosin, 80 kDa), MBP–chitin binding domain (CBD) (fusion of MBP and CBD, 58 kDa), and CBD–Mxe Intein–2CBD (fusion of CBD and Mxe Intein followed by two CBDs, 46 kDa) were used as standard proteins. LPO purified from bovine milk, when subjected to SDS-PAGE electrophoresis, exhibited only one band of LPO, as shown in Figure 1, column b.

There is no detailed study regarding the effect of melatonin and serotonin on LPO activity. In the present study, melatonin and serotonin were investigated for their inhibitory effects on LPO, and kinetics constants K_i and IC_{50} were evaluated. The results obtained from the present study clearly showed that melatonin and serotonin had strong inhibitory effects on LPO activity.

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 concentration required to inhibit LPO activity of the purified proteins by 50% (IC_{50}) and inhibition constants K_i were determined for each compound. The IC_{50} and K_i values were used to compare the inhibitory potentials of melatonin and serotonin. To determine the K_i value as well as the inhibition type, at least three different melatonin or serotonin concentrations were selected. At each melatonin or serotonin concentration, enzyme activity was measured in the presence of various substrate concentrations. The relationship of K_i and IC_{50} for a given compound varies, depending on the assay conditions and the compound's mechanism of inhibition. In this study, K_i and IC_{50} parameters for melatonin and serotonin as inhibitors of bovine LPO were determined. The inhibitor concentrations causing up to 50% inhibition were determined from activity (%) vs. [melatonin/serotonin] plots. As can be seen in Figures 2 and 4, IC_{50} values for melatonin and serotonin were determined as 1.46 and 1.29 μM, respectively. In addition, K_i values were calculated from Lineweaver–Burk plots (Figures 3 and 5). K_i constants for melatonin and serotonin were 0.82 ± 0.28 and 0.26 ± 0.04 μM, respectively. Both melatonin and serotonin exhibited competitive inhibition. These results showed that lactoperoxidase had affinity to serotonin more than to melatonin. In comparison, in a previous study, *in vitro* effects of ketamine and bupivacaine as analgesic agents were determined on LPO activity[18]. K_i constants for both anesthetic drugs were found to be 19 and 15 μM, and IC_{50} values were 290 and 155 μM, respectively.

The binding and structural studies of bovine lactoperoxidase with three aromatic ligands, acetylsalicylic acid (ASA), salicylhydroxamic acid (SHA), and benzylhydroxamic acid (BHA), were studied by Singh and co-workers[33]. This study showed that all three compounds bind to lactoperoxidase at the substrate binding site on the distal heme side. The binding of ASA occurs without perturbing the position of the conserved heme water molecule W-1, whereas both SHA and BHA displace it by the hydroxyl group on hydroxamic acid moieties. The acetyl group carbonyl oxygen atom of ASA forms a hydrogen bond with W-1, which in turn makes three other hydrogen bonds, one each with heme iron, His-109 N2, and Gln-105 N2. In contrast, in complexes of SHA and BHA, the OH group of the hydroxamic acid moiety in both complexes interacts with the heme iron directly. The OH is also hydrogen-bonded to His-109 N2 and Gln-105 N2. The plane of the benzene ring of ASA is inclined at 70.7° from the plane of the heme moiety, whereas the aromatic planes of SHA and

Table 1. Purification scheme of lactoperoxidase obtained after the application of different purification steps.

Purification step	Total volume (mL)	Enzyme activity (EU/mL)	Total enzyme activity (ABTS unit)	Protein (mg/mL)	Total protein (mg)	Specific activity (EU/mg)	Activity yield (%)	Purification-fold
Homogenate	180	3.83	689	1.65	297	2.3	100	1.00
Ammonium sulfate precipitation	32	16.30	522	1.88	60.2	8.7	75.8	3.7
CM-Sephadex C-50 column chromatography	275	1.72	473	0.1	27.5	17.2	68.7	7.4
Ammonium sulfate precipitation	30	13.52	407	0.72	21.6	18.8	59.1	8.1
Sephadex G-100 column chromatography	160	2.06	330	0.07	11.2	29.43	47.9	12.7
Ammonium sulfate precipitation and dialysis	23	6.48	149	0.19	6.21	34.0	21.6	14.7

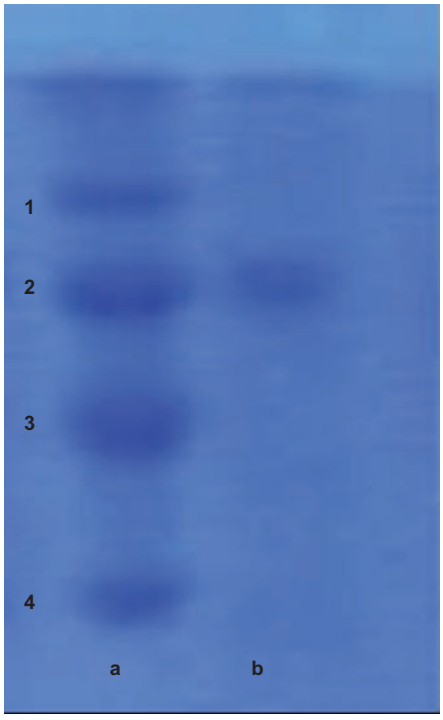

Figure 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) bands of lactoperoxidase (LPO) purified from bovine milk. Column a: standard proteins, 1: maltose-binding protein (MBP)–β-galactosidase (fusion of MBP and β-galactosidase, 175 kDa); 2: MBP–paramyosin (fusion of MBP and paramyosin, 80 kDa); 3: MBP–chitin binding domain (CBD) (fusion of MBP and CBD, 58 kDa); 4: CBD–Mxe Intein–2CBD (fusion of CBD and Mxe Intein followed by two CBDs, 46 kDa). Column b: purified LPO from bovine milk.

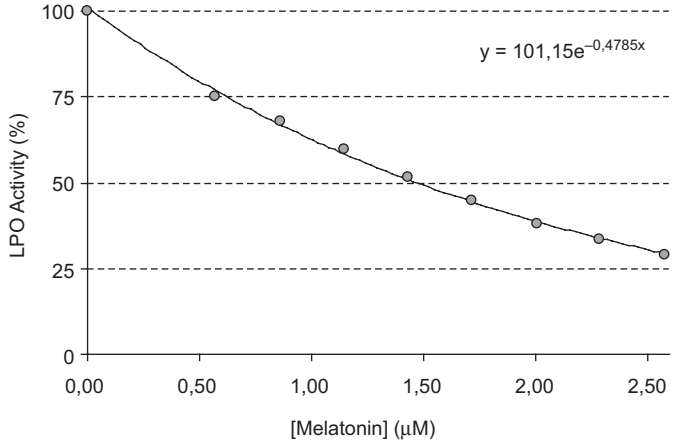

Figure 2. The effect of different concentrations of melatonin (0.5–2.5 mM) on lactoperoxidase obtained from bovine milk.

BHA are nearly parallel to the heme plane. The mode of ASA binding provides information about the mechanism of action of aromatic substrates, whereas the binding characteristics of SHA and BHA indicate the mode of inhibitor binding[33]. In the same manner, melatonin has a carbonyl group and serotonin has also a phenolic hydroxyl group. Both molecules can bind the heme group of lactoperoxidase.

In conclusion, the results showed that melatonin or serotonin had greater inhibition on LPO than other anesthetic

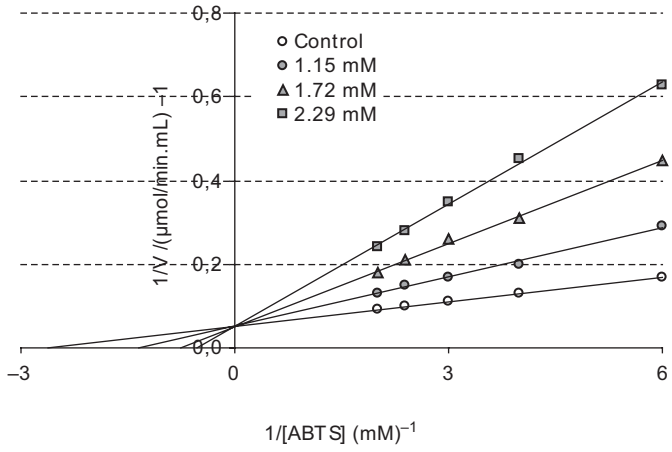

Figure 3. Lineweaver–Burk plot for different ABTS concentrations and three different melatonin concentrations for determination of K_i constant.

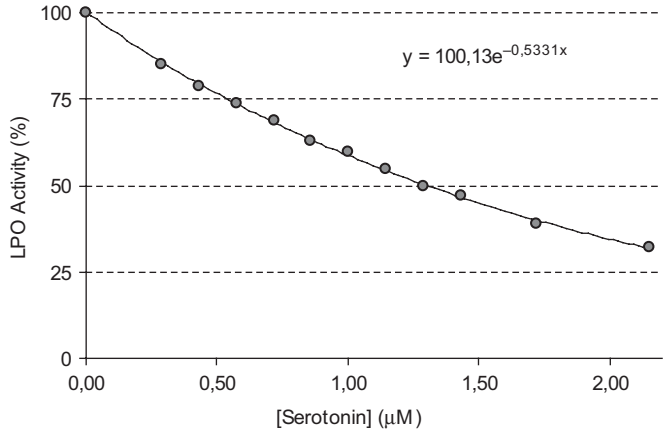

Figure 4. The effect of serotonin at different concentrations (0.5–2.15 mM) on bovine milk lactoperoxidase.

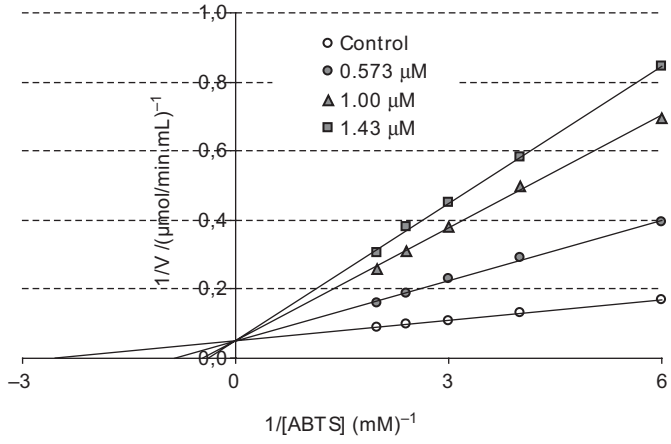

Figure 5. Lineweaver–Burk plot for different ABTS concentrations and three different serotonin concentrations for determination of K_i constant.

drugs such as ketamine and bupivacaine[20,21]. Melatonin and serotonin showed *in vitro* inhibition of LPO activity. According to the results obtained from the present study, both compounds were found to be marked LPO inhibitors, and could cause some side effects in lactation periods.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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