Archives of Oral Biology 85 (2018) 70–78

Contents lists available at ScienceDirect

Archives of Oral Biology

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

Reactivation of peroxidase activity in human saliva samples by polyphenols

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ARTICLE INFO

Keywords:
Inflammation
Saliva
Lactoperoxidase
Hypothiocyanite
Polyphenols
Salivary peroxidase

ABSTRACT

Objectives: The enzyme lactoperoxidase (LPO), which is released into several body fluids like saliva, is an essential part to maintain the oral bacterial homeostasis by catalysing the oxidation of thiocyanate (SCN^-) to hypo-thiocyanite (^-OSCN). The formation of unreactive redox intermediates (like Compound II) leads to a decreased pseudo-halogenating enzyme activity, which is associated with a higher risk for oral infections. According to former studies with *bovine* LPO selected flavonoids were tested in respect to their potential to reactivate the enzymatic activity in a more physiological, human salivary system.

Design: Saliva samples from healthy donors were collected and characterized by using several gel staining methods and immunoblotting. Afterwards kinetic measurements were performed by applying the TNB-assay to evaluate the pseudo-halogenating salivary peroxidase (SAPX) activity. The measurements were performed in the presence of excess H_2O_2 to simulate pro-inflammatory conditions. Moreover selected flavonoids or an ethanolic extract of *Tormentillae rhizoma* were applied to test their regenerating effect on the LPO-derived ^-OSCN production.

Results: Despite the complex protein composition of the collected saliva samples, an SAPX-derived pseudo-halogenating activity could be identified. The ^-OSCN regenerating effects of the tested polyphenols were completely comparable to previous *in vitro* experiments with *bovine* LPO. Thus, we could show that phenolic substances are suitable to regenerate the peroxidase activity in human saliva samples after H_2O_2-induced inactivation.

Conclusion: The studies provide new insights into the effect of pharmaceutical relevant polyphenols on salivary peroxidase activity and thus, suggest this enzyme as a new target for the prevention and therapy of oral inflammatory diseases.

1. Introduction

Lactoperoxidase (LPO) is a heme containing protein, which belongs to the immunological relevant chordata peroxidases (Zamocky et al., 2015). In the oral cavity LPO-analogue salivary peroxidase (SAPX) is released into the primary saliva secretion via acinus cells of the salivary gland (Ihalin, Loimaranta, & Tenovuo, 2006). In the presence of thiocyanate (SCN^-) and hydrogen peroxide (H_2O_2) LPO/SAPX catalyzes the formation of antimicrobial hypothiocyanite (^-OSCN) that inhibits microbial growth by entering bacterial biofilms and reacting with free thiol groups (Day, 2012, 2015; Day, 2012, 2015; Furtmuller et al., 2002; Hawkins, 2009). SCN^-, which is mainly obtained by food, enters the epithelium from the blood stream via sodium-iodide-symporter and is released by different ion channels like CFTR (cystic fibrosis transmembrane conductance regulator) or calcium-activated chloride channel (Chandler & Day, 2012). H_2O_2 is formed after spontaneous or enzymatic (superoxide dismutase) conversion of superoxide anion ($O_2\cdot^-$), which is released by membrane-associated enzymes like NADPH-oxidase (NOX) or dual oxidase (DUOX-2) (Cross & Segal, 2004; Donko, Peterfi, Sum, Leto, & Geiszt, 2005; Harper, Xu, McManus, Heidersbach, & Eiserich, 2006). Furthermore H_2O_2 could enter the lumen after release from several pathogens like *Lactobacilli* or *Streptococci* during oral infections or neutrophil granulocytes in case of oral bleeding or exudation (Chandler & Day, 2015; Fabian, Hermann, Beck, Fejerdy, & Fabian, 2012; Hoogendoorn & Moorer, 1973).

As illustrated in Fig. 1, the catalytic cycle of LPO/SAPX can be

Abbreviations: ABTS, 2,2′, azinobis(3-ethylbenzothiazoline-6-sulfonic acid); ANOVA, analysis of variance; bLPO, *bovine* lactoperoxidase; CFTR, cystic fibrosis transmembrane conductance regulator; DMSO, dimethyl sulfoxide; DUOX-2, dual oxidase; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); ECL, electrochemiluminescence; IgG, immunoglobulin G; LPO, lactoperoxidase; MPW, millipore water; NOX, NADPH-oxidase; PAS, periodic acid-Schiff's reagent; PRP, proline-rich proteins; PVDF, polyvinylidene fluoride; SAPX, salivary peroxidase; TBS, tris-buffered saline; TMB, 3,3′,5,5′-tetramethylbenzidine; TNB, 5-thio-2-nitrobenzoic acid

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http://dx.doi.org/10.1016/j.archoralbio.2017.09.037
Received 22 December 2016; Received in revised form 8 September 2017; Accepted 30 September 2017

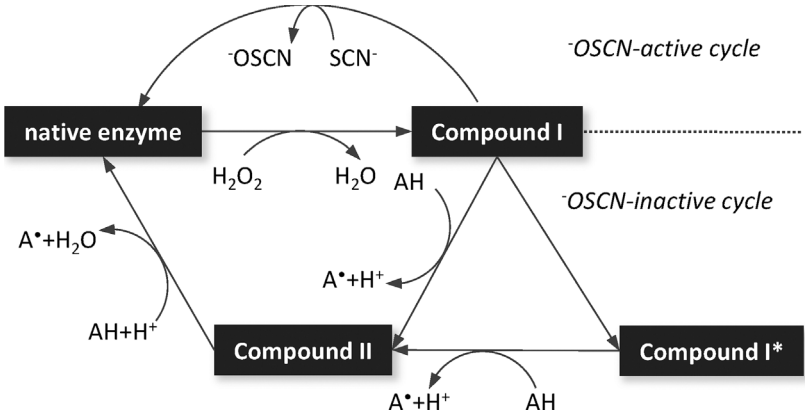

Fig. 1. Catalytic cycle of LPO. In the halogenation cycle ($^{-}$OSCN-active cycle) the native enzyme is oxidized to Compound I in the presence of H_2O_2. The reconstitution of the native enzyme can either occur via the two electronic oxidation of SCN^{-} to $^{-}$OSCN or by two consecutive one electronic reductions via the formation of Compound II in the so-called peroxidase cycle. As the resolution of Compound II is the rate determining step to reform the native enzyme only in the presence of suitable one electron donors the $^{-}$OSCN-active cycle can be continued. Furthermore a spontaneous transition from Compound I to Compound I* is possible, which can be also transferred to Compound II by one electron-donating substrates. AH and A· indicate oxidizable substances and the substance radicals, respectively.

divided into a $^{-}$OSCN-active halogenation and an $^{-}$OSCN-inactive peroxidase cycle (Furtmuller et al., 2006). Both reactivities require the primary activation of the ferric enzyme to Compound I by two-electronic H_2O_2 reduction (Furtmuller et al., 2006). In the halogenation cycle this activated enzymatic state two-electronically oxidizes SCN^{-} to $^{-}$OSCN under the regeneration of the native enzyme (Furtmuller et al., 2002, 2006). Alternatively the native enzyme can be regenerated via two consecutive one-electron reactions whereby Compound II is formed as an enzymatic redox intermediate (Furtmuller et al., 2006). In the absence of the natural substrate SCN^{-} the halogenating cycle can be also disrupted by the spontaneous transition of Compound I to Compound I* that can also lead to the formation of Compound II in the presence of one-electron donating substances (Furtmuller et al., 2002). As the resolution of Compound II is the rate-determining step in the peroxidase cycle this redox intermediate can accumulate in the absence of suitable substrates, leading to a disturbed formation of $^{-}$OSCN (Flemmig, Rusch, Czerwinska, Rauwald, & Arnhold, 2014). The pseudo-halogenating LPO/SAPX activity is known to be an important part of the oral bacterial homeostasis and thus was shown to be an important indicator for oral health and hygiene (Koch & Strand, 1979; Tenovuo & Anttonen,1980; Tenovuo, Mansson-Rahemtulla, Pruitt, & Arnold, 1981). In fact, an impaired salivary peroxidase activity, as e.g. observed at oral inflammation or xerostomia, is associated with higher risk of caries, gingivitis or periodontitis (Hugoson, Koch, Thilander, & Hoogendoorn, 1974; Lagerlof & Oliveby, 1994; van Steenberghe, Van den Eynde, Jacobs, & Quirynen, 1994).

The accumulation of Compound II is not an irreversible process and can be overcome in the presence of several naturally occurring substances. In former studies especially 3,4-dihydroxylated polyphenols like flavonoids were shown to be potent regenerators of the pseudo-halogenating activity of *bovine* LPO after H_2O_2-mediated enzyme inactivation (Gau, Furtmuller, Obinger, Arnhold, & Flemmig, 2015; Gau et al., 2016). These aspects indicate SAPX as an interesting new target for naturally occurring polyphenolic substances as well as for those plant extracts, which are known for their antibacterial properties and are already used to treat slight oral infections (ESCOP Scientific Committee, 2013; Hänsel & Sticher, 1965). Yet the stated previous studies were only performed in the presence of commercially available *bovine* LPO. Therefore in the current study we extended our peroxidase activity measurements to enriched human saliva although the amino acid sequence of human SAPX and *bovine* LPO is quite similar (at least 85 %) (Sharma et al., 2013). After establishing the test system selected polyphenolic substances were tested in order to get information about the transferability of the data obtained with *bovine* LPO to the *human* saliva system.

These results show a suitable method to address the pseudo-halogenating peroxidase activity in human saliva samples and also provide options to influence the salivary $^{-}$OSCN-formation in the presence of suitable Compound II-resolving aromatic peroxidase substrates. Thus this study may also give new insights into the mode of action of pharmaceutical relevant substances like natural anti-inflammatory oral care products.

2. Materials and methods

2.1. Materials

The chemical reagents and biochemicals used in this study were obtained at the highest grade available from following sources: Coomassie brilliant blue G250; laemmli sample buffer; 2-mercaptothenanol, tris/glycine (TG buffer) or tris/glycine/SDS buffer (TGS buffer) and 4–20% precast polyacrylamide gel were purchased from Bio-Rad Laboratories GmbH, Munich, Germany. Luteolin and quercetin were acquired from Biopurify Phytochemicals Ltd., Chengdu, China. Tormentil (*Potentilla erecta*) rhizome was bought from Alfred Galke GmbH, Gittelde, Germany. Ethanol, methanol and Tween 20 were purchased from Carl Roth GmbH + Co. KG, Karlsruhe, Germany. Rabbit anti-LPO monoclonal antibody was bought from Abcam, Cambridge, USA. Gout anti-rabbit secondary antibody (HRP conjugated) was obtained from Dianova GmbH, Hamburg, Germany. Hydrochloric acid and acetic acid were purchased from Merck Chemicals GmbH, Darmstadt, Germany. Paraffin pellets were acquired from Ivoclar Vivadent GmbH, Ellwangen, Germany. A prestained protein standard (Spectra multicolor broad range protein ladder) was obtained from Thermo Fisher Scientific, Waltham USA. The electrochemiluminescence reagent (ECL reagent) was purchased from GE Healthcare Europe GmbH, Freiburg, Germany. All other chemicals and enzymes were bought from Sigma Aldrich, Germany.

2.2. Preparation of human saliva

For electrophoretic experiments human saliva samples (10–100 ml per sample) were obtained from three healthy donors (indicated with A–C) 2 h after the last meal. In the same way four healthy donors (indicated with 1–4) are compared in case of kinetic measurements. For both experimental settings the salivation was stimulated by chewing paraffin gum. Both female and male donors at the age of 24–33 were included, none of them being smokers. After collecting, the samples were centrifuged at 25,000 × *g* for 15 min at 4 °C. Afterwards the supernatants were dialyzed against phosphate buffer (10 mM, pH 7.4) for 24 h with a molecular weight cut off of 30 kDa. In case of kinetic measurements the samples were always freshly prepared. We took care that the sample volumes after collection were similar to be able to compare the results of the peroxidase activity measurements. For gel electrophoretic experiments the dialyzed samples were afterwards lyophilized and stored at −20 °C for a maximum of 7 days.

The whole protein content of the saliva samples was determined at 280 nm by using a NanoDrop ND-1000-spectrophotometer, peQlab