

# Influences of Peroxidase on Lysozyme Activity

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It is well known that many antimicrobial proteins in saliva interact with each other. The purpose of the present study was to investigate the interactions of lysozyme with peroxidase in the aspects of enzymatic activity *in vitro*. The interactions of lysozyme with peroxidase were examined by incubating hen egg-white lysozyme (HEWL) with bovine lactoperoxidase (bLP). The influence of peroxidase system on lysozyme was examined by subsequent addition of potassium thiocyanate and hydrogen peroxide. Lysozyme activity was determined by turbidity measurement of a *Micrococcus lysodeikticus* substrate suspension. Peroxidase activity was determined with an NbsSCN assay. The Wilcoxon signed rank test was used to analyze the changes of enzymatic activities compared with their controls. bLP at physiological concentrations enhanced the enzymatic activity of HEWL ( $P < 0.05$ ) and its effect was dependent on the concentration of peroxidase. However, HEWL did not affect the enzymatic activity of bLP. Thiocyanate did not affect the enzymatic activity of HEWL, either. The addition of potassium thiocyanate and hydrogen peroxide did not lead to additional enhancement of the enzymatic activity of HEWL. The changes of hydrogen peroxide concentration in the peroxidase system did not affect the enzymatic activity of HEWL. Collectively, despite an *in vitro* nature of our study, the results of the present study provide valuable information on the interactions of lysozyme and peroxidase in the aspects of enzymatic activity in oral health care products and possibly in the oral cavity.

Key words: Lysozyme, Peroxidase, Interaction

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## I. INTRODUCTION

Many of the antimicrobial defense systems in saliva are common to all exocrine secretions such as tears, milk, and seminal, vaginal, and gastrointestinal fluids.<sup>1-4)</sup> Especially lysozyme, lactoferrin, and peroxidases which are the main oral innate defense factors are present in measurable concentrations in all these secretions. *In vitro*, these proteins are known to (1) limit bacterial or fungal growth, (2) interfere with bacterial glucose uptake or glucose metabolism, and (3) promote aggregation and, thus, the elimination of bacteria. These antimicrobial agents are mainly synthesized in, and secreted via, the major or minor salivary glands, but a smaller amount enters the oral cavity from tissue fluid or polymorphonuclear leukocytes via the

gingival crevicular fluid.<sup>2,3)</sup>

Two species of peroxidase are present in human saliva. Salivary peroxidase is secreted by the salivary glands, whereas myeloperoxidase emerges from leukocytes reaching the oral cavity. Peroxidase provides antimicrobial activity and protection of oral tissues from oxygen toxicity through oxidation of SCN<sup>-</sup> and consumption of H<sub>2</sub>O<sub>2</sub>.<sup>4,5)</sup> Lysozyme provides its antimicrobial activity by a muramidase-dependent mode and a cationic-dependent or structure-related bactericidal mechanisms.<sup>6,7)</sup> Adsorbed peroxidase and lysozyme molecules incorporated as pellicle components display bactericidal activity as well as their enzymatic activities and reduce the adherence of *Streptococcus mutans* to the HA surface.<sup>8-10)</sup> These antimicrobials, either alone or in combination with other antimicrobial molecules, have been incorporated in oral health care products to restore the antimicrobial capacity of saliva.<sup>11)</sup>

It is well known that many antimicrobial proteins in saliva interact *in vitro* with each other. The interactions result in additive, synergistic, or inhibitory effects on mutans streptococci, lactobacilli, or fungi.<sup>3,12,13)</sup> For example, such interactions have been reported between sIgA and peroxidase,<sup>14)</sup> lactoferrin and peroxidase,<sup>15,16)</sup> lactoferrin and lysozyme,<sup>17)</sup> and lysozyme and histatins.<sup>18)</sup> Although these observations are from *in vitro* experiments, it is likely that such concerted effects exist also *in vivo* in mixed saliva, where all the components are simultaneously present. Thus, salivary antimicrobial factors form a network in which no single component is necessary for the overall antimicrobial capacity of the host's defense system.

Although many reports have investigated interactions between various kinds of antimicrobials present in saliva, there is no information about the interactions between peroxidase and lysozyme in the aspects of enzymatic activity. In the present study, we have investigated the interactions of lysozyme with peroxidase system *in vitro*.

## II. MATERIALS AND METHODS

### 1. Peroxidase and Lysozyme

Bovine lactoperoxidase (bLP, Sigma-Aldrich Chemical Co., St Louis, MO, USA) and hen egg-white lysozyme (HEWL, Sigma-Aldrich Chemical Co., St. Louis, MO, USA) dissolved in 66 mM potassium phosphate buffer (pH 6.24) served as sources of peroxidase and lysozyme, respectively.

### 2. Peroxidase activity

Peroxidase activity was determined by measuring the rate of oxidation of 5-thio-2-nitrobenzoic acid (Nbs) to 5,5-dithiobis(2-nitrobenzoic acid) (Nbs)<sub>2</sub> by OSCN<sup>-</sup> ions generated during the oxidation of SCN<sup>-</sup> by bLP.<sup>19)</sup> Peroxidase activity was expressed as units/mL. The potassium phosphate buffer was used as a blank.

### 3. Lysozyme activity

Lysozyme activity was determined by the turbidimetric method.<sup>20)</sup> Samples were placed in a suspension of *Micrococcus lysodeikticus*, starting OD<sub>450</sub> = 0.65 - 0.70, at 37°C for 5 min. The lysozyme activity was calculated using the equation described below. The potassium phosphate buffer was used as a blank.

$$\text{Lysozyme activity (units/mL)} = \frac{(\Delta A_{450\text{nm}}/\text{min Test} - \Delta A_{450\text{nm}}/\text{min Blank}) (\text{df})}{(0.001) (0.1)}$$

df: dilution factor

0.001: change in absorbance at A<sub>450nm</sub> as per the unit definition

0.1: volume (in mL) of enzyme used

### 4. Influence of peroxidase on lysozyme activity

The effects of peroxidase on lysozyme activity were examined by incubating 500 μL of bLP (a final concentration of 12.5 μg/mL) with 500 μL of HEWL

(a final concentration of  $10 \mu\text{g/mL}$ ) for 10 min at room temperature (RT). The incubated mixture was placed in a suspension of *M. lysodeikticus* and an incubated mixture of buffer with HEWL was used as a control. Either an incubated mixture of peroxidase with buffer, or an incubated buffer alone was used as a blank. The effect of peroxidase concentration on lysozyme activity was also examined using different concentrations bLP solutions (6.25, 12.5, 25.0, 50.0  $\mu\text{g/mL}$ ).

#### 5. Influence of lysozyme on peroxidase activity

The effects of lysozyme on peroxidase activity were examined by incubating 500  $\mu\text{L}$  of bLP (a final concentration of  $12.5 \mu\text{g/mL}$ ) with 500  $\mu\text{L}$  of HEWL (a final concentration of  $10 \mu\text{g/mL}$ ) for 10 min at room temperature (RT). To 300  $\mu\text{L}$  of reaction mixture for NbsSCN assay, 15  $\mu\text{L}$  of KSCN (a final concentration of 4.2 mM  $\text{SCN}^-$ ) and 15  $\mu\text{L}$  of sample solution were added, and reaction was initiated by the addition of 15  $\mu\text{L}$  of  $\text{H}_2\text{O}_2$  (a final concentration of 50  $\mu\text{M}$ ). An incubated mixture of buffer with bLP was used as a control. For the blank reaction, an incubated mixture of HEWL with buffer, or an incubated buffer alone was used.

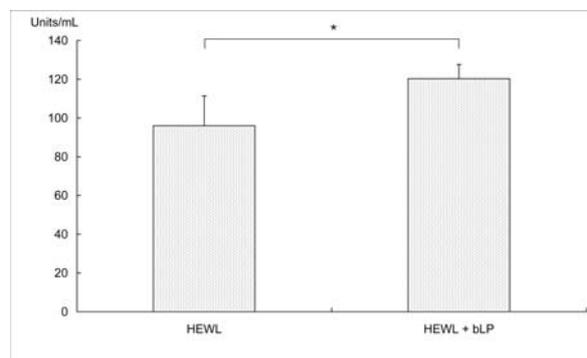


Fig. 1. Influence of peroxidase on lysozyme activity. Bovine lactoperoxidase (bLP) at  $12.5 \mu\text{g/mL}$  enhanced hen egg-white lysozyme (HEWL) activity. The assay was performed 7 times.  $P < 0.05$

#### 6. Influence of $\text{SCN}^-$ on lysozyme activity

The effect of  $\text{SCN}^-$  on lysozyme activity was examined by using different concentrations  $\text{SCN}^-$  solutions (final concentrations of 4.2 mM to 100 mM). The mixture of buffer with HEWL was used as a control. Either an incubated mixture of  $\text{SCN}^-$  with buffer, or an incubated buffer alone was used as a blank.

#### 7. Influence of the peroxidase system on lysozyme activity

The effect of peroxidase system on lysozyme activity was examined by comparing lysozyme activities of HEWL (a final concentration of  $10 \mu\text{g/mL}$ ), HEWL with bLP (a final concentration of  $12.5 \mu\text{g/mL}$ ), HEWL with bLP and KSCN (a final concentration of 4.2 mM  $\text{SCN}^-$ ), and HEWL with bLP, KSCN, and  $\text{H}_2\text{O}_2$  (a final concentration of 50  $\mu\text{M}$ ). The effect of  $\text{H}_2\text{O}_2$  concentration was also examined by comparing lysozyme activities between mixtures containing HEWL, bLP, and KSCN with and without  $\text{H}_2\text{O}_2$  at the concentrations of 25, 50, and 100  $\mu\text{M}$ .

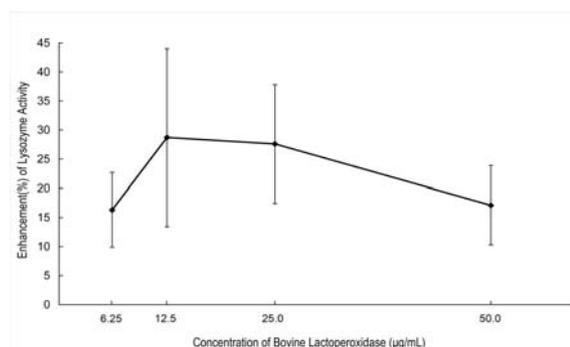


Fig. 2. Influence of peroxidase on lysozyme activity according to peroxidase concentration. Hen egg-white lysozyme (HEWL) activity was measured following pre-incubation of HEWL with different levels of bovine lactoperoxidase (bLP at concentrations of 6.25, 12.5, 25.0, and 50.0  $\mu\text{g/mL}$ ). Enhancement % of lysozyme activity compared with its control was determined. The assay was performed 7 times.

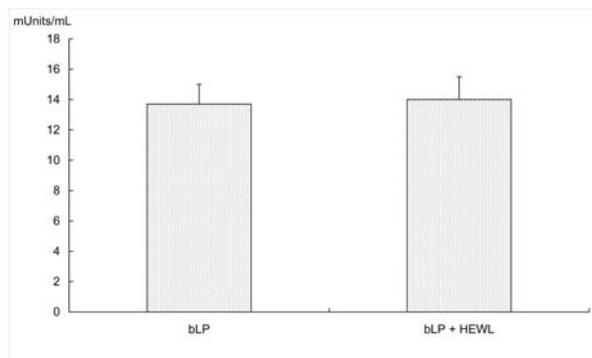


Fig. 3. Influence of lysozyme on peroxidase activity. Hen egg-white lysozyme (HEWL) at 10.0  $\mu\text{g}/\text{mL}$  did not affect bovine lactoperoxidase (bLP) activity. The assay was performed 7 times.

### 8. Statistics

The Wilcoxon signed rank test was used to analyze the changes of lysozyme and peroxidase activities compared with their controls. *P*-values less than 0.05 were considered statistically significant.

## III. RESULTS

### 1. Influence of peroxidase on lysozyme activity

bLP at 12.5  $\mu\text{g}/\text{mL}$  enhanced the enzymatic activity of HEWL ( $P < 0.05$ ) and the percentage of enhancement was 6.9 - 48.8% (mean,  $27.4 \pm 16.8\%$ ) (Fig. 1). Its effect was increased by the increase of peroxidase concentration from 6.25  $\mu\text{g}/\text{mL}$  to 12.5  $\mu\text{g}/\text{mL}$  and reached the plateau between 12.5 and 25.0  $\mu\text{g}/\text{mL}$  (Fig. 2).

### 2. Influence of lysozyme on peroxidase activity

HEWL at 10  $\mu\text{g}/\text{mL}$  did not affect the enzymatic activity of bLP ( $P = 0.446$ ) (Fig. 3).

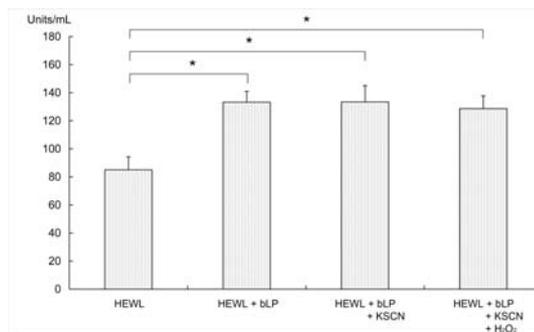


Fig. 4. Influence of the peroxidase system on lysozyme activity. Hen egg-white lysozyme (HEWL) activity was measured following pre-incubation with bovine lactoperoxidase (bLP), bLP and KSCN, and bLP, KSCN, and H<sub>2</sub>O<sub>2</sub>. The assay was performed 7 times.

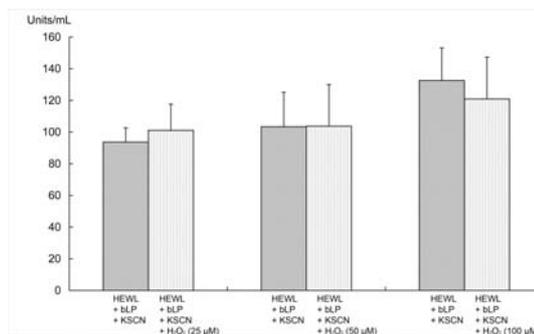


Fig. 5. Influence of the peroxidase system on lysozyme activity according to H<sub>2</sub>O<sub>2</sub> concentration. Hen egg-white lysozyme (HEWL) activity was measured following pre-incubation of HEWL with bLP, KSCN, and different levels of H<sub>2</sub>O<sub>2</sub> (at concentrations of 25, 50, and 100  $\mu\text{M}$ ). The lysozyme activity was compared with its control without H<sub>2</sub>O<sub>2</sub>. The assay was performed 7 times.

### 3. Influence of SCN<sup>-</sup> on lysozyme activity

SCN<sup>-</sup> at the range of 4.2 to 100 mM did not affect the enzymatic activity of HEWL (data not shown).

#### 4. Influence of the peroxidase system on lysozyme activity

The addition of KSCN or H<sub>2</sub>O<sub>2</sub> did not lead to additional enhancement of the enzymatic activity of HEWL (Fig. 4). The changes of H<sub>2</sub>O<sub>2</sub> concentration in the peroxidase system did not affect the enzymatic activity of HEWL (Fig. 5).

#### IV. DISCUSSION

Oral antimicrobial factors work in the same milieu – saliva, gingival fluid, and dental plaque – at the same time, and may interact with each other.<sup>3)</sup> Previous studies suggested that many of the salivary antimicrobial agents interact with each other, in most cases in a synergistic or additive way.<sup>3,12,13)</sup> Lysozyme was reported to enhance the inhibitory effects of the peroxidase system on glucose metabolism of *S. mutans*.<sup>21)</sup> Lysozyme and lactoferrin showed synergistic antistaphylococcal properties<sup>22)</sup> and additive bactericidal effect against *S. mutans*.<sup>17)</sup> The additive effects of lysozyme and histatins on coaggregation between *Porphyromonas gingivalis* and *Streptococcus mitis* were also suggested.<sup>18)</sup> Regarding peroxidase, IgA was suggested to enhance the antimicrobial effect of the lactoperoxidase system against *S. mutans*.<sup>14)</sup> Lactoferrin and lactoperoxidase system showed additive inhibitory effect on the viability of *S. mutans*.<sup>16)</sup>

Because the previous studies usually focused on the bacteriostatic and bactericidal effects on cariogenic or periopathogenic microorganisms, not the effects on enzymatic activity, our aim was to investigate the interaction of lysozyme and peroxidase in the aspects of enzymatic activity. Our results showed that bLP enhanced the enzymatic activity of HEWL and its effect was dependent on the concentration of peroxidase in certain ranges. The concentrations of bLP and HEWL were at the physiological ranges in human saliva. However, HEWL did not affect the enzymatic activity of bLP. The previous studies showed that anions enhance

lysozyme action and that the physiological concentrations of various anions present in saliva may be sufficient to trigger lysis of oral microorganisms by lysozyme.<sup>23-25)</sup> However, different ranges of KSCN did not affect the enzymatic activity of lysozyme in the present study. The increase of lysozyme activity by peroxidase was not further affected by the complete peroxidase system composed by subsequent addition of KSCN and hydrogen peroxide.

There have been attempts to enhance or restore saliva's own antimicrobial capacity by commercially available oral health care products. This idea seems sound to add physiological salivary antimicrobial agents into the mouth that lacks saliva-mediated protection in patients with dry mouth. The antimicrobial host proteins most widely used in these products are lysozyme, lactoperoxidase, and lactoferrin.<sup>11)</sup> Although the lack of appropriate control makes it impossible to rule out the placebo effect, some studies have shown Biotene<sup>®</sup> alone or with Oralbalance<sup>®</sup> gel containing salivary antimicrobial agents to relieve subjective symptoms in patients with dry mouth.<sup>26,27)</sup> We are not sure that the results obtained *in vitro* can be extended to *in vivo*. However, lysozyme might enhance the inhibitory effects of the peroxidase system on *S. mutans* in these products. bLP might increase the enzymatic activity of lysozyme in these products. In addition, natural lysozyme and peroxidase in residual saliva of patients can interact with each other and with antimicrobials in the products.

Our results showed that peroxidase enhances the enzymatic activity of lysozyme *in vitro*. There might be heterotypic complex formation between the molecules. However, the mechanism of the interactions needs further study. In addition, the oral cavity provides an environment for molecular interactions on surfaces as well as in solution. Lysozyme and peroxidase retain their enzymatic activities on tooth surfaces.<sup>10)</sup> When proteins adsorb to a surface, they undergo conformational changes,<sup>28,29)</sup> an event which almost certainly results in a modification of the enzymes' active sites.<sup>10)</sup>

Indeed, such changes are reported to induce an increase or decrease in enzymatic activities of  $\alpha$ -amylase, lysozyme, and glucosyltransferase immobilized onto HA or enamel surfaces.<sup>30-33)</sup> Since lysozyme and peroxidase molecules in commercial products and host-derived antimicrobial salivary molecules exist simultaneously in whole saliva and pellicles of patients with salivary hypofunction, interactions between these molecules may occur. Such interactions may modify the antimicrobial activity of the innate salivary defense molecule in distinct ways in solution or on surface phase.<sup>34)</sup>

Collectively, despite an *in vitro* nature of our study, the results of the present study provide valuable information on the interactions of lysozyme and peroxidase in the aspects of enzymatic activity in oral health care products and possibly in the oral cavity. Further studies are needed to investigate the mechanism of enhancement and the interactions on the surfaces.

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## 국문요약

# Peroxidase가 Lysozyme 활성에 미치는 영향

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이상구 · 김형일 · 고흥섭

타액에 존재하는 여러 항균물질은 상호작용을 통하여 부가적이거나 상승작용을 나타내고 때로는 저해작용을 나타내는 것으로 알려져 있다. 본 연구는 *in vitro* 상에서 lysozyme과 peroxidase 사이의 상호작용을 효소활성 측면에서 조사하고자 시행되었다. Lysozyme과 peroxidase의 상호작용은 hen egg-white lysozyme (HEWL)과 bovine lactoperoxidase (bLP)를 혼합하는 방법으로 조사되었고, peroxidase system이 lysozyme에 미치는 영향은 potassium thiocyanate와 hydrogen peroxide를 추가적으로 첨가하는 방법으로 조사되었다. Lysozyme 활성은 *Micrococcus*

*lysodeikticus* 기질용액의 혼탁도 변화를 측정하는 방법으로 측정되었고, peroxidase 활성은 NbsSCN 법으로 측정되었다. Wilcoxon signed rank 법을 이용하여 lysozyme과 peroxidase 효소활성 변화를 대조군과 비교하였다. 생리적 농도범위에서 bLP는 HEWL의 효소활성을 증가시켰으며 ( $P < 0.05$ ), 그 효과는 bLP의 농도증가에 따라 영향을 받았다. 하지만 HEWL는 bLP의 효소활성에 영향을 주지 못하였다. Thiocyanate는 HEWL의 효소활성에 영향을 주지 못하였고, potassium thiocyanate와 hydrogen peroxide를 추가한 peroxidase system도 HEWL 효소활성의 추가적인 상승을 유도하지는 못하였으며, hydrogen peroxide의 농도변화도 영향을 미치지 못하였다. 본 연구의 결과는 타액 항균 물질을 포함하고 있는 구강건강용품이나 구강에서 lysozyme과 peroxidase의 상호작용을 이해하는데 필요한 중요한 정보를 제공해준다.

주제어: Lysozyme, Peroxidase, 상호작용

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