Trypsin: A Novel Scavenger of Superoxide Anion

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ABSTRACT

Trypsin is a serine protease found in the digestive system of many vertebrates, where it hydrolyses proteins. The superoxide scavenging activity of trypsin was accidentally found. Results showed that trypsin could scavenge superoxide in both intracorporal and extracorporal systems. The value of km of superoxide scavenging presented by the trypsin was 0.0618 mm The rate of superoxide scavenging increased up to 0.103 μM/μg trypsin/min with the increasing of hydrogen peroxide production rate to a maximum of 0.00122 μM/μg trypsin/min. Trypsin has an optimal operating pH of within 7.5-8.5. The scavenging activity of trypsin was accelerated by copper and impeded by chelators of metals. These indubitable results suggested that trypsin is a novel scavenger of superoxide. The rate of superoxide scavenging and hydrogen peroxide production were impacted by the concentration of trypsin or riboflavin, pH or ions. Trypsin might be a potential drug for anti-oxidant stress in human

INTRODUCTION

Reactive oxygen species (ROS) is a class of ubiquitous molecules including superoxide anion (O2−), hydrogen peroxide (H2O2), and hydroxyl radicals [1,2]. ROS regulates critical steps in the signal transduction cascades and many important cellular events, such as protein phosphorylation, gene expression, transcription factor activation, DNA synthesis, and cell proliferation [2,3]. On the other hand, ROS are toxic to cells, due to their damage on cellular components. It was hypothesized that O2− produced by bacterial mammalian pathogens such as E. faecalis might play as a virulence factor [4]. As a result, intracellular defenses against superoxide-mediated damage are robust [5,6].

Protection from ROS may include the production of endogenous enzymes such as catalase, which degrades H2O2 and superoxide dismutase (SOD), which dismutase O2− [7].

Trypsin is a serine protease found in the digestive system of many vertebrates, where it hydrolyses proteins [8]. In our previous works, trypsin was found to be able to scavenge O2− with the concurrent production of H2O2 in the culture of bacteria. The objective of this paper is to characterize this O2− scavenging activities of trypsin, both in vivo and in vitro. Results showed that the activities of trypsin are independent O2− scavenging enzyme in organisms.
METHODS

Bacteria

The Escherichia coli wild type strain (MG1655) used in our works was kindly supplied by Prof. James A. Imlay at Department of Microbiology, University of Illinois, and Urbana. Strain MG1655 was maintained on LB medium and cultured at 37 °C for 48 h. A single colony was cultured in LB liquid medium for an additional 24 h to obtain a suspension of approximately 10⁹ cells per ml. The strain was conserved in glycerol and stored at -20°C until use.

Trypsin Treatment

Trypsin (Bovine, 500 units/mg Crystalline) was purchased from Amersco. Trypsin (100 mg ml⁻¹) was added in the mixtures of O₂⁻ production systems. The mixture was then incubated at 37 °C for 30 min, and the reaction was stopped with 25 µl of soybean trypsin inhibitor (10 mg ml⁻¹, Sigma).

Quantitative Assay of Superoxide Anion

O₂⁻ was produced in the VB₂ (Sigma) solution. O₂⁻ concentration was measured by measuring ferricytochrome c reduction as described by Huycke [4] and Korshunov and Imlay [9].

ESR spectroscopy. Levels of O₂⁻ produced by different strains were determined by electron spin resonance (ESR) spectroscopy with Tiron [10]. Tiron (1,2-dihydroxybenzene-3,5-disulfonic acid, Sigma) not only is a radical scavengers, but also could specific react with O₂⁻ to form the tiron semi quinone, which is detectable by ESR as a four-line first derivative spectrum. The Tiron radical is stable and can be used for quantitation of O₂⁻ production as described by McRae and Thomson [11] and Li, et al. [10]. ESR spectroscopy was performed with a Bruker ER 200 D ESR spectrometer.

Hydrogen Peroxide Production Measurement

H₂O₂ contents were examined by the AR/HRP method reported by Seaver and Imlay [12].

External Factors Treatments

Samples were incubated in 0.5 mM Cu²⁺ or 25 mM EDTA for 0.5 h or 1 mM Diethyldithiocarbamate (DDC) for 1 h at 28 °C in accordance with the method described by Takahama, et al. [13].

Statistics: SPSS for Windows 11.5 was used for statistical analysis. Results are reported as mean ± S.E.M. The significance of differences between superoxide anion affected by EDTA, Cu²⁺ or DDC was determined using one-way analysis of variance (ANOVA). Values are denoted as significant (p<0.05) or highly significant (p<0.01).

RESULTS

The effects of trypsin on O₂⁻ were investigated in chemical VB₂ system, in vitro, or in living bacterial culture, in vivo.

Scavenging Activities of Trypsin in Different Systems

Reproducible results obtained from three or more independent ESR assays suggested that both bacterial cells and VB₂ system produce O₂⁻ (Figure 1B and 1E). Tiron alone in VB₂ control produced weak ESR signal (Figure 1A). LB medium control also produced a small ESR signal in the presence of Tiron (Figure 1D). In our previous works, the amplitude of the Tiron signal was reduced by more than 95% with SOD addition (200 units ml⁻¹), confirming that the ESR spectrum had been derived from O₂⁻ [10]. Either bacterial suspension or VB₂ solution produced no ESR signal after trypsin (0.6 mg ml⁻¹) treatment (Figure 1C and 1F).

Figure 1. The effect of trypsin treatment on ESR spectra in VB₂ system or bacterial culture. A, Control of VB₂ plus Tiron; B, VB₂ system plus Tiron; C, VB₂ system treated with trypsin plus Tiron; D, VB₂ system treated with inactivated trypsin plus Tiron; E, Control of LB culture plus Tiron; F, Bacterial culture plus Tiron; G, Bacterial culture treated with trypsin plus Tiron; H, bacterial culture treated with inactivated trypsin plus Tiron.

Effects of Trypsin Concentration on Scavenging Activities

In the presence trypsin, O₂⁻ scavenging and hydrogen peroxide production were simultaneously observed. When the
concentration of trypsin was lower than 0.4 mg/ml, the curve of hydrogen peroxide production kept consistent in that of \( \text{O}_2^- \) scavenging. Scavenging rate of \( \text{O}_2^- \) remained at a steady but slow-growing performance, while hydrogen peroxide production rate showed an explicit descent with 0.4-1.0 mg/ml trypsin (Figure 2). Kinetic constants, \( K_m \) of trypsin, scavenging \( \text{O}_2^- \) at 37 °C, were determined using a line weaver Burk plot. The value of \( K_m \) presented by the trypsin was 0.0618 mm trypsin concentration was selected to be 0.4 mg/mL in the further works.

![Figure 2. Effect of trypsin concentration on the rate of superoxide scavenging and hydrogen peroxide production.](image)

**Effects of Initial Superoxide Concentration on Scavenging Activities**

The effects of varying initial \( \text{O}_2^- \) concentrations on both \( \text{H}_2\text{O}_2 \) evolution and \( \text{O}_2^- \) scavenging were measured. The initial \( \text{O}_2^- \) concentration was 54.8 μM when VB2 concentration was \( 1.5 \times 10^{-9} \) M in VB2 system determined by cytochrome c assay. \( \text{H}_2\text{O}_2 \) evolution rates increased with \( \text{O}_2^- \) concentration as determined by a double-reciprocal plot (Figure 3). The rate of \( \text{O}_2^- \) scavenging increased up to 0.103 μM/μg trypsin/min with the increasing of \( \text{H}_2\text{O}_2 \) production rate to a maximum of 0.00122 μM/μg trypsin/min when VB2 concentration was \( 2.5 \times 10^{-9} \) M (Figure 4). The curve of \( \text{O}_2^- \) scavenging is not consistent with it of \( \text{H}_2\text{O}_2 \) production. The proportions of \( \text{H}_2\text{O}_2 \) in products increase with \( 3-6 \times 10^{-9} \) M VB2 while reduce with \( 6.5-7.5 \times 10^{-9} \) M VB2.

![Figure 3. Effects of initial VB2 concentration on trypsin activities of superoxide scavenging and hydrogen peroxide production.](image)

![Figure 4. Effects of pH on trypsin activities of superoxide scavenging and hydrogen peroxide production.](image)

**Effects of External Factors on Scavenging Activities**

Effects of pH on the \( \text{O}_2^- \) scavenging activities of trypsin were determined. Results indicated that trypsin has an optimal operating pH of within 7.5-8.5. The rate of \( \text{H}_2\text{O}_2 \) production by the trypsin/ \( \text{O}_2^- \) reaction gradually decreased 6-fold during the increasing of pH from 5 to 10. The rate of \( \text{O}_2^- \) scavenging nearly doubled from pH 5 to 6 and was relatively with \( \text{H}_2\text{O}_2 \) production ratio as pH increased from 6 to 9. Subsequent experiments were carried out at pH 7.0 to approximate physiological conditions.

The trypsin could be inactivated by EDTA. The addition of 25 mM EDTA, a metal chelator, highly significantly inhibited \( \text{O}_2^- \) scavenging activities of trypsin (Figure 5) (p<0.01). The superoxide anion was further reduced by 0.5 mM Cu^{2+} addition in the reaction with trypsin. The addition of 1 mM DDC, a chelator of Cu^{2+}, highly significantly inhibited \( \text{O}_2^- \) scavenging activity (Figure 5) (p<0.01). But the superoxide anion concentration did not pick up to original level. The effects of DDC were significantly weaker than that of EDTA (p<0.05).
DISCUSSION

We confirmed the $O_2^-$ scavenging activities of trypsin in different $O_2^-$ producing systems, including extracorporeal chemical VB$_2$ system, and intracorporal living bacterial culture (Figure 1). No other reactant was necessary for this reaction of $O_2^-$ scavenging by trypsin. In bacterial culture, which meaning the biological concentrations of superoxide, the trypsin could exhibit well $O_2^-$ scavenging activities with the presence of endogenous antioxidant, such as SOD. Trypsin may be effective and competitive under biologically relevant conditions.

In the reaction of $O_2^-$ scavenging by trypsin, H$_2$O$_2$ was observed to be a product. The rate of $O_2^-$ scavenging and H$_2$O$_2$ production were impacted by the concentration of either trypsin or VB$_2$, which represented the initial $O_2^-$ concentration. The optimum concentration of trypsin is 0.4 mg/mL for $O_2^-$ scavenging reaction (Figure 2). In the initial phase of this reaction, the rate of H$_2$O$_2$ production consisted with that of $O_2^-$ scavenging. When VB$_2$ concentration was $2.5 \times 10^{-9}$ M, the proportion of H$_2$O$_2$ production was increased. While when the VB$_2$ concentration beyond $6 \times 10^{-9}$ M, the H$_2$O$_2$ production was significantly drop (Figure 3). The mechanisms of production of H$_2$O$_2$ and the rationale behind it remain unknown.

Results indicated that trypsin has an optimal operating pH of within 7.5-8.5. Both H$_2$O$_2$ production and $O_2^-$ scavenging activity were favored by acidic pH (Figure 4).

In the reactions of trypsin, the Cu$^{2+}$ is a necessary factor for $O_2^-$ scavenging. The addition of EDTA significantly inhibited $O_2^-$ scavenging activities of trypsin (Figure 5), verifying that the reaction was due to reactions with heavy metals. The promotion of trypsin activities by Cu$^{2+}$ confirmed that Cu$^{2+}$ plays important role in the $O_2^-$ scavenging reaction of trypsin. The addition of DDC significantly inhibited $O_2^-$ scavenging activity (Figure 5), further verifying that the Cu$^{2+}$ is an efficient factor in this reaction. The difference of inhibition effects between EDTA and DDC indicated that other metal ions (e.g. Ca$^{2+}$) may be involved in the reactions of trypsin. Considering that the chelation of either EDTA or DDC could easily impair the activities of trypsin, the combination between trypsin and copper ion should not be tight. The interaction between trypsin and calcium ion has been confirmed, while the interaction between trypsin and copper ion has not been clarified. Further works are needed.

Similar to SODs, trypsin scavenges $O_2^-$ and may be components of the cellular defense against $O_2^-$ stress. Trypsin is available in high quantity in pancreases, and can be purified rather easily. Hence it has been used widely in various biotechnological processes. Trypsin is commonly used in biological research during proteomics experiments to digest proteins into peptides for mass spectrometry analysis, e.g. in-gel digestion. While in the future, trypsin can be used for $O_2^-$ scavenging in various conditions.

However, there are still many questions about the mechanisms of $O_2^-$ scavenging by trypsin. Whether trypsin is competitive with native dismutation under biologically relevant conditions (i.e. likely biological concentrations of superoxide and protein) or not? If the answer is yes, the following question is that how does this process compete with the catalytic action of various SOD species. Is this action of trypsin a minor or major process? It is unclear under what circumstances trypsin may act as an SOD mimetic. In what biological systems might such reactions be occurring? Further intracorporal works are urgent needed. Illustration of the novel activities of superoxide scavenging of trypsin should lead us to a new scope on the anti-oxidation mechanisms of trypsin and reveal new insights into mechanism of enzymes.

CONCLUSIONS

Trypsin is confirmed to be an $O_2^-$ scavenger. Scavenging activities should be impacted by either trypsin or initial $O_2^-$ concentration. The optimal pH region of $O_2^-$ scavenging by trypsin is 7.5-8.5. Copper is an effective factor in this reaction. Trypsin might be a potential drug for anti-oxidant stress in human.

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The authors declare that they have no conflict of interest.

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