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Trypsin: A Novel Scavenger of Superoxide Anion

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Research Article

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Abbreviations: DDC: Diethyldithiocarbamate; ESR: Electron Spin Resonance; H_2O_2: Hydrogen Peroxide; ROS: Reactive Oxygen Species; O_2 Superoxide anion; SOD: Superoxide Dismutase.

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 ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), 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 $O_2^{\cdot-}$ 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 H_2O_2 and superoxide dismutase (SOD), which dismutase $O_2^{\cdot-}$ [7].

Trypsin is a serine protease found in the digestive system of many vertebrates, where it hydrolyses proteins [8]. In our pervious works, trypsin was found to be able to scavenge $O_2^{\cdot-}$ with the concurrent production of H_2O_2 in the culture of bacteria. The objective of this paper is to characterize this $O_2^{\cdot-}$ scavenging activities of trypsin, both *in vivo* and *in vitro*. Results showed that the activities of trypsin are independent $O_2^{\cdot-}$ scavenging enzyme in organisms.

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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 10^9 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 $O_2^{\cdot-}$ 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

$O_2^{\cdot-}$ was produced in the VB_2 (Sigma) solution. $O_2^{\cdot-}$ concentration was measured by measuring ferricytochrome c reduction as described by Huycke [4] and Korshunov and Imlay [9].

ESR spectroscopy. Levels of $O_2^{\cdot-}$ 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 $O_2^{\cdot-}$ 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 $O_2^{\cdot-}$ 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

H_2O_2 contents were examined by the AR/HRP method reported by Seaver and Imlay [12].

External Factors Treatments

Samples were incubated in 0.5 mM Cu^{2+} 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, Cu^{2+} 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 $O_2^{\cdot-}$ were investigated in chemical VB_2 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 VB_2 system produce $O_2^{\cdot-}$ **(Figure 1B and 1E).** Tiron alone in VB_2 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 $O_2^{\cdot-}$ [10]. Either bacterial suspension or VB_2 solution produced no ESR signal after trypsin (0.6 mg ml^{-1}) treatment **(Figure 1C and 1F).**

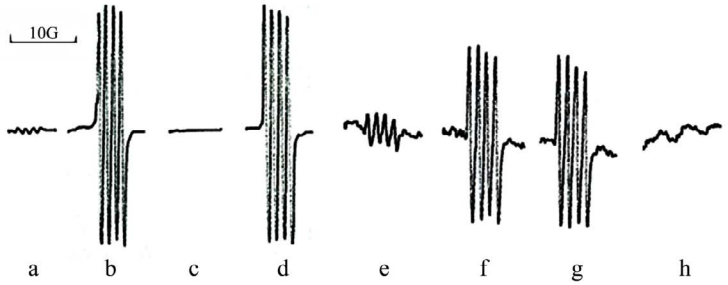

Figure 1. The effect of trypsin treatment on ESR spectra in VB_2 system or bacterial culture. A, Control of VB_2 plus Tiron; B, VB_2 system plus Tiron; C, VB_2 system treated with trypsin plus Tiron; D, VB_2 system treated with inactivated trypsin plus Tiron; E, Control of LB culture plus Tiron; F, Bacterial 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, $O_2^{\cdot-}$ 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 $O_2^{\cdot-}$ scavenging. Scavenging rate of $O_2^{\cdot-}$ 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, Km of trypsin, scavenging $O_2^{\cdot-}$ at 37 °C, were determined using a line weaver Burk plot. The value of km presented by the trypsin was 0.0618 mm trypsin concentration was selected to be 0.4 mg/mL in the further works.

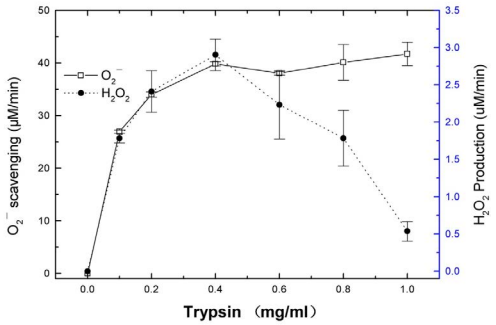

Figure 2. Effect of trypsin concentration on the rate of superoxide scavenging and hydrogen peroxide production.

Effects of Initial Superoxide Concentration on Scavenging Activities

The effects of varying initial $O_2^{\cdot-}$ concentrations on both H_2O_2 evolution and $O_2^{\cdot-}$ scavenging were measured. The initial $O_2^{\cdot-}$ concentration was 54.8 μM when VB_2 concentration was 1.5 × 10^{-9} M in VB_2 system determined by cytochrome c assay. H_2O_2 evolution rates increased with $O_2^{\cdot-}$ concentration as determined by a double-reciprocal plot **(Figure 3).** The rate of $O_2^{\cdot-}$ scavenging increased up to 0.103 μM/μg trypsin/min with the increasing of H_2O_2 production rate to a maximum of 0.00122 μM/μg trypsin/min when VB_2 concentration was 2.5^{-6} ×10^{-9} M **(Figure 4).** The curve of $O_2^{\cdot-}$ scavenging is not consistent with it of H_2O_2 production. The proportions of H_2O_2 in products increase with 3-6 × 10^{-9} M VB_2 while reduce with 6.5-7.5 × 10^{-9} M VB_2.

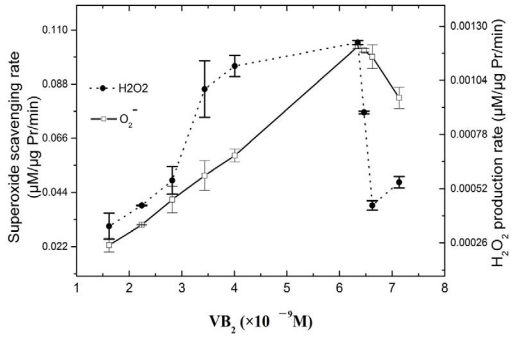

Figure 3. Effects of initial VB_2 concentration on trypsin activities of superoxide scavenging and hydrogen peroxide production.

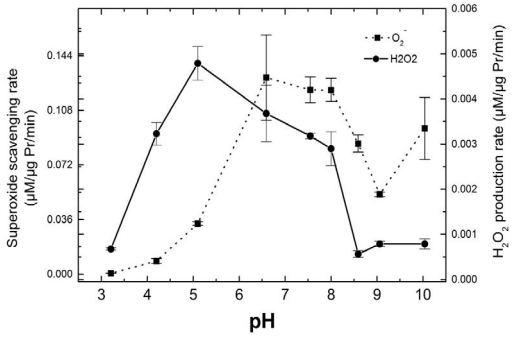

Figure 4. Effects of pH on trypsin activities of superoxide scavenging and hydrogen peroxide production.

Effects of External Factors on Scavenging Activities

Effects of pH on the $O_2^{\cdot-}$ scavenging activities of trypsin were determined. Results indicated that trypsin has an optimal operating pH of within 7.5-8.5. The rate of H_2O_2 production by the trypsin/ $O_2^{\cdot-}$ reaction gradually decreased 6-fold during the increasing of pH from 5 to 10. The rate of $O_2^{\cdot-}$ scavenging nearly doubled from pH 5 to 6 and was relatively with H_2O_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 $O_2^{\cdot-}$ 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 $O_2^{\cdot-}$ 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 ($p<0.05$).

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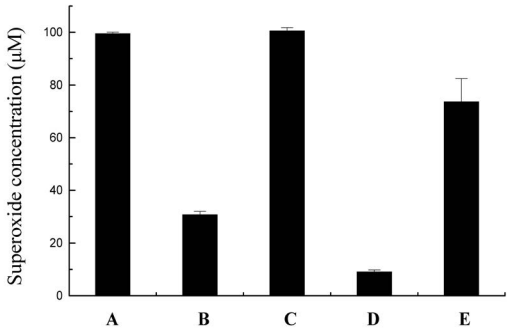

Figure 5. Effects of EDTA, DDC and Cu^{2+} on trypsin activity of superoxide scavenging. A, Control mixture of VB_2 system; B, Mixture with Tyrpsin; C, Mixture with trypsin plus EDTA; D, Mixture with trypsin plus Cu^{2+}; E, Mixture with trypsin plus DDC.

DISCUSSION

We confirmed the $O_2^{\cdot-}$ scavenging activities of trypsin in different $O_2^{\cdot-}$ producing systems, including extracorporeal chemical VB_2 system, and intracorporal living bacterial culture **(Figure 1).** No other reactant was necessary for this reaction of $O_2^{\cdot-}$ scavenging by trypsin. In bacterial culture, which meaning the biological concentrations of superoxide, the trypsin could exhibit well $O_2^{\cdot-}$ 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 $O_2^{\cdot-}$ scavenging by trypsin, H_2O_2 was observed to be a product. The rate of $O_2^{\cdot-}$ scavenging and H_2O_2 production were impacted by the concentration of either trypsin or VB_2, which represented the initial $O_2^{\cdot-}$ concentration. The optimum concentration of trypsin is 0.4 mg/mL for $O_2^{\cdot-}$ scavenging reaction **(Figure 2).** In the initial phase of this reaction, the rate of H_2O_2 production consisted with that of $O_2^{\cdot-}$ scavenging. When VB_2 concentration was $2.5^{-6} \times 10^{-9}$ M, the proportion of H_2O_2 production was increased. While when the VB_2 concentration beyond 6×10^{-9} M, the H_2O_2 production was significantly drop **(Figure 3).** The mechanisms of production of H_2O_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_2O_2 production and $O_2^{\cdot-}$ scavenging activity were favored by acidic pH **(Figure 4).**

In the reactions of trypsin, the Cu^{2+} is a necessary factor for $O_2^{\cdot-}$ scavenging. The addition of EDTA significantly inhibited $O_2^{\cdot-}$ 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 $O_2^{\cdot-}$ scavenging reaction of trypsin. The addition of DDC significantly inhibited $O_2^{\cdot-}$ 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 $O_2^{\cdot-}$ and may be components of the cellular defense against $O_2^{\cdot-}$ 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 $O_2^{\cdot-}$ scavenging in various conditions.

However, there are still many questions about the mechanisms of $O_2^{\cdot-}$ 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 $O_2^{\cdot-}$ scavenger. Scavenging activities should be impacted by either trypsin or initial $O_2^{\cdot-}$ concentration. The optimal pH region of $O_2^{\cdot-}$ 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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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

REFERENCES

1. Barth C, et al. The Timing of Senescence and Response to Pathogens is Altered in the Ascorbate-Deficient Arabidopsis Mutant vitamin c-1. Plant Physiol. 2004;134:1784-1792.
2. Hoidal JR. Reactive Oxygen Species and Cell Signaling. Am J Respir Cell Mol Biol. 2001;25:661-663.
3. Demidchik V, et al. Free oxygen radicals regulate plasma membrane Ca^{2+} and K^{+} permeable channels in plant root cells. J Cell Sci. 2003;116:81-88.
4. Huycke MM, et al. Augmented production of extracellular superoxide by blood isolates Enterococcus faecalis. J Infect Dis. 1996;173:743-746.
5. Fridovich I. Superoxide anion radical, superoxide dismutases, and related matters. J Biol Chem. 1997;272:18515-18517.
6. Gort SA and Imlay JA. Balance between Endogenous Superoxide Stress and Antioxidant Defenses. J Microbiol. 1998;180:1402-1410.
7. Katsuwon J and Anderson AJ. Catalase and Superoxide Dismutase of Root-Colonizing Saprophytic Fluorescent Pseudomonads. Appl Environ Microbiol. 1990;56:3576-3582.
8. Kühne W. Uber das. Trypsin (Enzym des Pankreas). Verhandlungen des naturhistorisch-medicinischen Vereins zu Heidelberg, new series. 1877;1:194-198.
9. Korshunov S and Imlay JA. Detection and Quantification of Superoxide Formed within the Periplasm of Escherichia coli. J Bacteriol. 2006;188:6326-6334.
10. Li X, et al. Extracellular Superoxide Anion Production contributes to virulence of Xanthomonas oryzae pv. oryzae. Can J Microbiol. 2009;55:110-116.
11. McRae DG and Thompson JE. Senscence-dependent changes in superoxide anion production by illuminated chloroplasts from bean leaves. Planta. 1983;158:185-193.
12. Seaver LC and Imlay JA. Are respiratory enzymes the primary sources of intracellular hydrogen peroxide? J Biol Chem. 2004;279:48742-48750.
13. Takahama U, et al. Reduction of Exogenous Cytochrome c by Neurospora crassa Conidia: Effects of Superoxide Dismutase and Blue Light. J Microbiol. 1982;152:151-156.