Subtilisin QK, a Fibrinolytic Enzyme, Inhibits the Exogenous Nitrite and Hydrogen Peroxide Induced Protein Nitration, *in Vitro* and *in Vivo*

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Subtilisin QK, which is newly identified as a fibrinolytic enzyme from *Bacillus subtilis* QK02, has the ability of preventing nitrotyrosine formation in bovine serum albumin induced by nitrite, hydrogen peroxide and hemoglobin *in vitro* verified by ELISA, Western-blot and spectrophotometer assay. Subtilisin QK also attenuates the fluorescence emission spectra of bovine serum albumin in the course of oxidation caused by nitrite, hydrogen peroxide and hemoglobin. Furthermore, subtilisin QK could suppress the transformation of oxy-hemoglobin to met-hemoglobin caused by sodium nitrite, but not the heat-treated subtilisin QK. Compared with some other fibrinolytic enzymes and inactivated subtilisin QK treated by phenylmethylsulfonylfluoride, the ability of inhibiting met-hemoglobin formation of subtilisin QK reveals that the anti-oxidative ability of subtilisin QK is not concerned with its fibrinolytic function. Additionally, nitrotyrosine formation in proteins from brain, heart, liver, kidney, and muscle of mice that is intramuscular injected the mixture of nitrite, hydrogen peroxide and hemoglobin is attenuated by subtilisin QK. Subtilisin QK can also protect Human umbilical vein endothelial cell (ECV-304) from the damage caused by nitrite and hydrogen peroxide.

Keywords: Fibrinolysis, Hemoglobin, Hydrogen peroxide, Nitrotyrosine, Protein nitration

Introduction

Microorganisms are important sources of thrombolytic agents, because they have some advantages in large quantity of production and oral administration for thrombotic diseases such as the acute myocardial and cerebral infarctions.

Subtilisin QK, produced by *Bacillus subtilis* QK02 that screened from the fermented soybean by fibrin plate method, has comparatively high fibrinolytic activity on both heated plasma and fibrin plate and is highly homologous to nattokinase (NK) (Ko *et al.*, 2004). NK (or designated subtilisin NAT), a serine protease produced from *Bacillus natto* (Sumi *et al.*, 1987; Fujita *et al.*, 1993), has approximately four-time stronger fibrinolytic activity than plasmin in clot lysis assay, *in vivo* (Fujita *et al.*, 1995b). The oral administration of NK produced a mild and frequent enhancement of the fibrinolytic activity in plasma, as indicated by the fibrinolytic parameters and the production of tissue plasminogen activator (t-PA), which suggests NK represents a possible drug applied not only in the treatment but also in the prevention of thrombotic diseases (Sumi *et al.*, 1990). Then it is confirmed that NK is absorbed from the rat intestinal tract and cleaves fibrin in plasma after intra-duodenal administration of the enzyme (Fujita *et al.*, 1995b). Besides its direct effect to dissolve thrombus, the potentiated fibrinolysis of NK depends on its ability to inactivate plasminogen activator inhibitor 1 (PAI-1) by limited proteolysis of its reactive site (Urano *et al.*, 2001). Moreover, it was reported that NK showed enhanced thrombolytic activity near the vessel wall (Suzuki *et al.*, 2003a) and suppress intimal thickening after vascular injury (Suzuki *et al.*, 2003b).

Reperfusion through thrombolysis by using thrombolytic agents is a standard way to treat impending acute myocardial infarction, but reperfusion itself may lead to accelerated and additional myocardial injury through the oxidative stress. This is referred to as the "reperfusion injury". Since reperfusion injury is initiated by the treatment of myocardial infarction, it is of importance to limit the extent of the injury (Wang *et al.*,

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2002). The cardiovascular disease was associated with progressive changes in the production of free radicals and radical-derived reactive species. These intermediates react with all major cellular constituents and may serve several physiological and pathophysiological functions (Turko *et al.*, 2002). Vascular endothelial cells are crucial both to the integrity of the vessel wall and to modulation of vascular resistance by releasing nitric oxide (NO). However, they are subject to the deleterious effects of oxidant stress originating in bloodstream, underlying tissues, and oxidants generated within the cells (James *et al.*, 2003).

Protein tyrosine nitration produced by the reaction of nitrogen and oxygen species was used as a suitable marker of radical mediated tissue damage (Fisarkova, 2002; Schopfer *et al.*, 2003). Incubation of human hemoglobin (Hb) with nitrite and hydrogen peroxide was found to induce auto-nitration and nitration of another protein (Grzelak *et al.*, 2001).

Based on these considerations, we have researched the relationship between subtilisin QK, a new fibrinolytic enzyme, and some proteins (BSA and Hb, *in vitro* and tissues proteins, *in vivo*) under the oxidative stress condition.

Material and Methods

Materials BSA, sodium nitrite (NaNO_2) and hydrogen peroxide (H_2O_2) were purchased from Roche Co.. All solvents and other reagents were the highest purity and commercially available. Anti-nitrotyrosine monoclonal antibody (mouse) and goat anti-mouse IgG-Ap antibody were purchased from CAYMAN CHEMICAL Co. Hb, benzamidine, aprotinin, leupeptin, pepstatin A and PMSF were purchased from Sigma Co. Human umbilical vein endothelial cell (ECV-304 cell line) was purchased from CCTCC (Number GDC-023) in China. The solution of subtilisin QK (0.2 mg/mL, in 50 mM sodium phosphate buffer, pH 7.0) was prepared from the lyophilized powder of subtilisin QK (41,000 IU/mg) purified from *Bacillus subtilis* QK02 (Ko *et al.*, 2004).

ELISA and Western-blot assay ELISA, spectrophotometer, and Western-blot (Bian *et al.*, 2003) were all used for the determination of nitrotyrosine formation in BSA oxidized by nitrite, hydrogen peroxide, and Hb in the absence or at the presence of subtilisin QK. Western-blot was done as follows: BSA (2 mg/mL) in 0.1 M phosphate buffer (pH 7.4) was incubated at 37°C with Hb (25 μM), NaNO_2 (1 mM), H_2O_2 (1 mM) and subtilisin QK (0, 5, 20, 40 μg) for 30 min. 50 μL reaction aliquots was mixed with 50 μL sample loading buffer. Equal amounts of proteins were loaded into the gel for each experimental sample. Separated proteins were transferred to PVDF membrane and the membrane was incubated with the anti-nitrotyrosine monoclonal antibody. The nitrated BSA was detected by goat anti-mouse alkaline phosphatase (AP)-conjugated IgG antibody.

Intrinsic fluorescence spectroscopy assay Intrinsic Fluorescence Spectroscopy assay was used to detect structural changes of BSA that was oxidized by nitrite, hydrogen peroxide, and Hb with or without subtilisin QK. Intrinsic Fluorescence Spectroscopic experiments

were carried out at 37°C using LS-55 luminescence spectrometer (PerkinElmer Life Sciences, Shelton, CT). BSA solution at a final concentration of 1 mM, was incubated with Hb (50 mg), NaNO_2 (100 mM), H_2O_2 (100 mM), in the absence or at the presence of subtilisin QK (1 or 2 mg) for 30 minutes at 37°C. In the measurement of fluorescence quenching, the protein solution was excited at 280 nm, and the emission spectra were recorded from 300 to 420 nm. The excitation and emission slits were 10 nm and 6 nm, respectively, and the scan speed was 100 nm/min.

Spectrophotometer assay Oxy-hemoglobin solution was prepared from fresh blood of mice (body weight, 25-30 g) as previously described (Ana *et al.*, 1998). Optical absorption spectra were recorded in a dual-wavelength, double-beam spectrophotometer (UV-2401PC, SHIMADZU Co., Tokyo, Japan). The optical absorption spectra of oxy-hemoglobin solution from the mouse demonstrate the specific wavelengths at 415, 541 and 577 nm (data not shown). The 20 μL of 50 mM sodium nitrite (50 mM sodium phosphate buffer, pH 7.0) solution and 200 μL of Hb solution were poured into an absorption vessel with 2 mL of 50 mM sodium phosphate buffer (pH 7.0), intimately mixed and record every 2 minutes until the completion of the reaction. The spectral time course for oxy-hemoglobin solution with NaNO_2 exhibited that the spectra were decreased in the absorption at $\lambda = 541$ and 577 nm and increased at $\lambda = 630$ nm (data not shown). These variations in the absorption at $\lambda = 541$, 577 and 630 nm indicates the existence of met-hemoglobin (Abdu *et al.*, 1998; Privalle *et al.*, 2000).

Animal test Male mice (body weight, 25-30 g) were obtained from Wuhan University animal center. Mice were divided into three groups (every group contains ten mice); the normal group was not injected, the oxidation group was intramuscular injected with 0.5 ml of solution I (NaNO_2 ; 5 mM, H_2O_2 ; 5 mM, Hb; 25 μM), and the treatment group was injected with solution I containing subtilisin QK (40 μg , 1600 IU). Injection was performed three times at intervals of two hours. 2 hours after the last injection, each group of mice was killed and the brain, heart, lung, liver, kidney, spleen and skeletal muscle between the ribs were quickly isolated, the tissues were frozen in liquid nitrogen immediately for further analysis. Frozen tissues were pulverized with a pestle and mortar that contained liquid nitrogen. For protein extraction, these tissues were homogenized at 4°C in 20 mM Tris-HCl buffer (pH 7.4) containing protease inhibitors (final concentration: 1mM benzamidine, 0.01 KU aprotinin, 10 $\mu\text{g}/\text{ml}$ leupeptin, 10 $\mu\text{g}/\text{ml}$ pepstatin A, 0.2 mM PMSF). Each sample was homogenized and sonicated on ice. The homogenates were centrifuged at $10,000 \times g$ for 15 min at 4°C, and the supernatant was used for detection of nitrotyrosine. Nitrotyrosine was detected by ELISA and Western-blot using anti-nitrotyrosine monoclonal antibody (Bian *et al.*, 2003).

Cellular assay ECV-304 cells were cultured in RPMI-1640 medium (GIBCO/BRL) containing 10% (v/v) fetal bovine serum (FBS) and double antibiotics in 24-well plates. When the cells reached confluence (about 8×10^5 cells/well) on the plastic plate, the cells were washed three times with FBS-free RPMI-1640 and subjected to oxidation test. Cell injury was initiated by addition of growth culture containing NaNO_2 and H_2O_2 (0.5 mM, respectively) into each well, with or without subtilisin QK (5 μg , 200 IU or 10

μg , 400 IU). After 24 h, cells were inspected and calculated. Cell viability was determined by using colorimetric MTT assay (Claise *et al.*, 1999). Experiment was repeated three times and values were averaged.

Statistical analysis Data are expressed as mean $\pm$ SEM. Results were analyzed by using one-way ANOVA, and statistical significance assumed when P values were <0.05 .

Results

Subtilisin QK inhibits tyrosine nitration in BSA To test whether subtilisin QK has an effect on BSA tyrosine nitration, BSA was incubated with different concentrations of subtilisin QK in Hb/ $\text{NO}_2^-/\text{H}_2\text{O}_2$ system. As shown Fig. 1, nitrotyrosine level in BSA was significantly decreased due to addition of subtilisin QK. When 40 μg of subtilisin QK was added into the reaction mixture, the absorption of the reaction mixture at 630 nm was close to the background hemoglobin absorption in ELISA assay (Fig. 1A) and complete inhibition of tyrosine nitration in BSA was exhibited in Western-blot assay (Fig. 1B). Similar results were also obtained in spectrophotometer assay (data not shown).

Fluorescence emission spectra of tyrosine and tryptophan residues in BSA The basic information contained in fluorescence measurements relates to the molecular environment of the chromophore. Fluorescence of tryptophan residues is very sensitive to the changes in their vicinity, thus it is widely used to study variations of the molecular conformations of proteins. BSA contains two tryptophan residues, one can evaluate the interactions between protein and dendrimer molecules by studying changes of fluorescence spectra (Gelamo *et al.*, 2002). The wavelength of the fluorescence maximum for BSA was at 348 nm, which indicated that the tryptophan residue (Trp134) is in contact with bound water molecules. Commonly used method to study the environment of tryptophans is to measure the shift in the wavelength of emission maximum. The shift in the position of emission maximum corresponds to the changes of the polarity around chromophore molecule. The red shift indicates that tryptophans are, on average, more exposed to the solvent, whereas the blue shift is a consequence of transferring tryptophan residues into a more hydrophobic environment (Klajnert *et al.*, 2003).

To further examine the role of subtilisin QK in inhibiting BSA oxidation by Hb, NaNO_2 and H_2O_2 , we observed the fluorescence emission spectra of tyrosine and tryptophan residues in BSA. Illumination with 280 nm light excites both of tyrosine and tryptophan residues (Fan *et al.*, 1999; Evgenia *et al.*, 2001). When excited at this wavelength, BSA exhibits a strong fluorescence emission peak at 345 nm and the interaction of oxidants with BSA quenches this fluorescence emission peak, causing about 65.4 % decrease in the intensity, but the addition of subtilisin QK (2 mg) significantly decreased

Fig. 1. Effect of subtilisin QK on the BSA tyrosine nitration in Hb/ $\text{NO}_2^-/\text{H}_2\text{O}_2$ system. (A) ELISA analysis (B) Western blot assay; 1: control (without subtilisin QK); 2: addition of 5 μg subtilisin QK; 3: addition of 20 μg subtilisin QK; 4: addition of 40 μg subtilisin QK; BSA (2 mg/ml) in 0.1 M phosphate buffer (pH 7.4) was incubated with Hb (25 μM), NaNO_2 (1 mM), and H_2O_2 (1 mM) under the condition of addition of subtilisin QK (0, 5, 20, 40 μg) for 30 min at 37°C. In Fig. 1A, hemoglobin absorbance background was abstracted.

the conformational changes of BSA caused by the oxidants, as shown by a little change in the fluorescence emission intensity (22.8%) (Fig. 2A). The slight blue shift of emission maximum (3 nm) was observed in the oxidized BSA (Fig. 2A). As the blue shift is a consequence of transferring tryptophan residues into a more hydrophobic environment, we measured the illumination at 295 nm light, which excites tryptophan residues. When excited at this wavelength, the intensity quenching (by about 51.7%) was obtained, which is largely decreased by subtilisin QK (16.2%) (Fig. 2B).

These results revealed that the interaction of oxidants with BSA altered the microenvironment around tryptophan and tyrosine residues of the protein, but subtilisin QK could protect BSA from the structural alteration induced by oxidative stress.

Subtilisin QK prevent the Met-hemoglobin formation To further evaluate the effect of subtilisin QK on the protein nitration, we have compared it with hemoglobin and other proteins. Oxy-hemoglobin was oxidized to met-hemoglobin by nitrite and appearance of met-hemoglobin represented as the decrease in absorbance at 577 nm (Fig. 3. line 7) and the increase at 630 nm and 406 nm (Depto *et al.*, 1998). Subtilisin QK significantly attenuated the decrease in absorbance at 577 nm (Fig. 3. line 2, 3) and the increase at 630 nm and 406 nm (data not shown). In order to further investigate the nature of this function of subtilisin QK, some enzymes and inactivated form of subtilisin QK were tested here. As demonstrate in Fig. 3, plasmin (fibrinolytic enzyme), urokinase (fibrinolytic enzyme),

Fig. 2. Fluorescence emission spectra of BSA excited at 280 nm (A) and 295 nm (B) in the course of oxidation by Hb, NaNO_2 , and H_2O_2 , in absence or presence of subtilisin QK. 1; BSA, 2; BSA + oxidants + subtilisin QK (2 mg), 3; BSA + oxidants + subtilisin QK (1 mg), 4; BSA + oxidants: BSA oxidation was performed by addition of Hb (50 mg), NaNO_2 (100 mM), and H_2O_2 (100 mM) for 25 min at 37°C . $\lambda_{\text{exc}} = 280$ nm. E_{x} slit = 10 nm. E_{m} slit = 6 nm. Scan speed = 100 nm min^{-1} . Emission spectrum = 300–420 nm.

thrombin (coagulative enzyme, serine protease), and trypsin (serine protease) could not inhibit met-hemoglobin formation. PMSF could completely inhibit the fibrinolytic activity of subtilisin QK (Ko *et al.*, 2004), but could not inhibit the anti-oxidant activity of subtilisin QK for oxy-hemoglobin (Table 1). In addition, heat-treating could completely inhibit both fibrinolytic (Ko *et al.*, 2004) and the anti-oxidant activity of subtilisin QK (Table 1).

These results revealed that anti-oxidant function of subtilisin QK was not concerned with its fibrinolytic activity, and might implicate in its secondary structure.

Fig. 3. The anti-oxidant properties of subtilisin QK on HbO_2 at $\text{HbO}_2/\text{NaNO}_2$ system ($\lambda = 577$ nm). 1: oxy-hemoglobin, 2: oxy-hemoglobin + NO_2^- + subtilisin QK (1600 IU, 40 μg), 3: oxy-hemoglobin + NO_2^- + subtilisin QK (800 IU, 20 μg), 4: oxy-hemoglobin + NO_2^- + thrombin (10 NIH), 5: oxy-hemoglobin + NO_2^- + trypsin (40 μg), 6: oxy-hemoglobin + NO_2^- + plasminogen (1.3 U) + urokinase (1600 IU), 7: oxy-hemoglobin + NO_2^- (control), 8: oxy-hemoglobin + NO_2^- + urokinase (1600 IU). All experiments were performed in the total volume 2.2 mL containing of NaNO_2 (4.5×10^{-4} M), oxy-hemoglobin (6.8×10^{-5} M) and phosphate buffer (0.05 M, pH 7.0). The similar results were obtained at $\lambda = 541$ and 630 nm (data not shown).

Effect of subtilisin QK on the protein nitration in some tissues of mouse, *in vivo* In order to investigate the effect of subtilisin QK on the protein nitration, *in vivo*, we injected Hb, NaNO_2 and H_2O_2 with or without QK into crus muscle of mice three times at intervals of two hours, two hours after injection, each group of mice was killed and samples of some tissues was prepared as described in materials and methods. The detection of nitrotyrosine formation in samples by ELISA using nitrotyrosine monoclonal antibody (Fig. 4) revealed that subtilisin QK could significantly inhibit nitrotyrosine formation in brain, heart, liver, kidney and muscle tissues, but not in lung and spleen.

Mice were divided into three groups of normal, oxidation and treatment groups as described in the part of Methods. QK (1600 IU, 40 μg) effectively prevented the protein nitration in brain, heart, liver, kidney, and muscle. The values are averages of ten samples in every group. This indicated that subtilisin QK could prevent the protein nitration induced by Hb, NaNO_2 and H_2O_2 , *in vivo*.

The effect of subtilisin QK on the human umbilical vein endothelial cell (ECV-304 cell line) damaged by NaNO_2 and H_2O_2 We investigated whether subtilisin QK could protect endothelial cell from the cytotoxicity of exogenous oxidants. ECV-304 cell line, which was isolated from human umbilical vein at 1996, has several advantages over HUVEC (Sian 1996). Our experiment revealed that subtilisin QK could prevent ECV-304 cells from cell death caused by exogenous nitrite and hydrogen peroxide (Fig. 5). To quantitatively evaluate the protection effect of subtilisin QK, the cell

Table 1. Effect of PMSF and heat-treating on the inhibition of subtilisin QK against the met-hemoglobin formation by nitrite

Test group	Subtilisin QK	PMSF	ΔOD_{630nm}	Inhibition (%)
1	+	+	0.025 ± 0.001	70.9
2	-	+	0.064 ± 0.003	25.5
3	+	-	0.028 ± 0.003	67.4
4	+ (Heated)	-	0.082 ± 0.005	4.6
5 (control)	-	-	0.086 ± 0.001	0

Table 2. The effect of subtilisin QK on ECV-304 cell viability

Test group	Growth medium	NaNO ₂ + H ₂ O ₂ (mM)	Subtilisin QK (IU)	Living Cells ($\times 10^5$ cells)	Viability (%)
1	+	0	0	7.9 ± 0.2	100.0
2	+	0.5	0	0.64 ± 0.08	8.2
3	+	0.5	200	6.4 ± 0.3	81.0
4	+	0.5	400	7.9 ± 0.3	100.0

Fig. 4. ELISA for nitrotyrosine in tissue homogenates from brain, heart, lung, liver, kidney, spleen and muscle of mice subjected to oxidation stress.

viability was investigated by using MTT method, and subtilisin QK completely inhibited ECV-304 cell death caused by the exogenous nitrite and hydrogen peroxide when 10 μ g protein (400 IU) was added (Table 2).

These results indicate that subtilisin QK in blood could not only dissolve thrombus, but also protect BSA, free hemoglobin and the endothelial cells from cytotoxicity of NO₂⁻ and H₂O₂.

Discussion

It is well known that intravenous administration of urokinase, streptokinase and tissue plasminogen activator has been widely used for thrombosis therapy, but these agents have some disadvantages such as bleeding and high cost. But the fibrinolytic enzymes from microorganism living in the healthy food had many advantages: oral administration, long half-life in plasma, high affinity to fibrin, increasing tissue plasminogen activator, inactivating PAI-1, and so on (Sumi *et al.*, 1987; Fujita *et al.*, 1993; Fujita *et al.*, 1995a; Fujita *et al.*, 1995b; Urano *et al.*, 2001; Suzuki *et al.*, 2003a; Suzuki *et al.*, 2003b;

Fig. 5. The protection effect of QK on human umbilical vein endothelial cells (ECV-304) from oxidant stress. A: control (growth medium), B: NaNO₂ (0.5 mM) + H₂O₂ (0.5 mM) + subtilisin QK (200 IU, 5 μ g), C: NaNO₂ (0.5 mM) + H₂O₂ (0.5 mM), D: NaNO₂ (0.5 mM) + H₂O₂ (0.5 mM) + subtilisin QK (400 IU, 10 μ g). At 24 h after the addition of oxidant and subtilisin QK into growth medium, cells were taken photography. We can see in Fig. 5, the cells in panel A are all stucked to the cell culture plate and grow homogeneously, but all the cell in panel C floated and collected under the oxidative stress. Panel B and C indicate cells are protected by QK from oxidant stress.

Ko *et al.*, 2004). Subtilisin QK was newly isolated and identified from the culture broth of *Bacillus subtilis* QK02 which has the highest fibrinolytic activity among the strains screened from the fermented soybean, and its fibrinolytic activity was 41.000 IU/mg (Ko *et al.*, 2004).

In order to develop it as a pharmacological agent, we have researched its function in blood and found that it could inhibit the nitration of BSA and oxy-hemoglobin. Serum albumin is

one of the most available and extensively studied proteins. It is the major component of blood plasma in many species, accounting for about 60% of its total protein content and providing about 80% of the blood osmotic pressure. It is the carrier of many low-polar metabolites and drugs in the blood (Ercelena *et al.*, 2003). In the presence of hydrogen peroxide, hemoglobin oxidized nitrite, which resulted in BSA nitration. BSA nitration results in conformational and functional alteration (Bian *et al.*, 2003).

Subtilisin QK could inhibit nitrotyrosine formation and conformational changes in BSA caused by hemoglobin, nitrite and hydrogen peroxide (Fig. 1, 2). Here, hemoglobin acts as a nitrite reductase to produce nitric oxide (NO) from nitrite, a major bio-available pool of NO, and itself was oxidized to met-hemoglobin (Bian *et al.*, 2003; Cosby *et al.*, 2003). Numerous pathologies may involve toxic side effects of met-hemoglobin-derived free heme and heme derived iron. In support, hemoglobin in plasma, when oxidized to met-hemoglobin by oxidants, causes oxidative modification of LDL, which in turn induces endothelial cytotoxicity primarily caused by lipid hydroperoxides (Balla *et al.*, 2000; Jeney *et al.*, 2002).

As demonstrated in Fig. 3 and Table 1, subtilisin QK significantly inhibited the met-hemoglobin formation induced by nitrite, it did not concern with its fibrinolytic function. From these results, we assumed that subtilisin QK could prevent proteins in tissues and cells, especially heme containing protein rich tissues and vascular endothelial cells, from exogenous oxidative stress. Heme containing proteins such as hemoglobin and myoglobin are widely distributed in body, especially heart, liver and muscles (Bian *et al.*, 2003). Vascular endothelial cells are also highly vulnerable to oxidative injury, because they are in close contact with leukocytes generated ROS and its endogenous NO (Shimpuku *et al.*, 2000; James *et al.*, 2003).

Nitration of tyrosine in biological conditions represents a pathological event that is associated with several neurodegenerative diseases, such as amyotrophic lateral sclerosis, Parkinson's disease and Alzheimer's disease (AD). Increased level of nitrated proteins have been reported in AD brain (Castegna *et al.*, 2003). Our results showed that subtilisin QK could significantly inhibit nitrotyrosine formation in brain, heart, liver, kidney, and muscle *in vivo*.

The lumen of blood vessels is covered with a single layer of vascular endothelial cells, which are always in direct contact with the constituents of blood and serve as a barrier enabling selective permeability of substances. Activated leukocytes release the reactive oxygen species (ROS) for biophylaxis. Vascular endothelial cells are highly vulnerable to oxidative injury, because they are in close contact with leukocytes (Shimpuku *et al.*, 2000). And these ROS can react with NO generated by endothelial cells to form peroxynitrite, which is highly reactive and harmful to cell proteins, lipids, and nucleic acids (James *et al.*, 2003). But our results indicated that subtilisin QK could protect ECV-304 cells from exogenous

oxidation stress (Fig. 4, 5 and Table 2).

Although it is still unclear how subtilisin QK inhibits protein nitration and exerts its protective function in endothelial cells and rat tissues, these results implicate the fibrinolytic enzymes from *Bacillus* such as subtilisin QK and NK could reduce the oxidative stress caused damage besides their fibrinolytic activity.

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