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Effect of pulsed electric fields (PEF) on protease and antioxidant activity of selenium modified fibrinolytic enzyme nattokinase produced by *Bacillus* natto cells

Khalid Amin[1], Xiangbo Zeng[1], You Ying[1], Yang Hu[1], Sun He[1], Lv Bo[1], Naveed Ahmad[2], Muhammad Ishaq[1], Hansong Yu[1*]

[1]Food Science and Engineering College, Jilin Agricultural University, 2888 Xincheng Street, Changchun, Jilin, 130118, China

[2]College of Life Sciences, Jilin Agricultural University, 2888 Xincheng Street, Changchun, Jilin, 130118, China

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Abstract

Nattokinase is a serine protease of the subtilisin family which exhibits a strong fibrinolytic activity and works by inactivating plasminogen activator inhibitor 1 (PAI-1). In this study selenium was enriched in nattokinase, as it has many functions incorporated with selenoprotein like as an antioxidant which protects the body from oxidative stress. Also, effect of pulsed electric field (PEF) on protease and antioxidant activity of selenium enriched nattokinase was determined. SDS-PAGE was used to determine the molecular weight and purity of enzyme. Antioxidant activity was evaluated by different spectrophotometric methods like, DPPH, OH radical scavenging, FRAP and T-AOC assay. Optimization of PEF parameters revealed that 2 kV/cm of electric field strength, 10 min electroporation time, 20 µs of pulse width and 1 Hz frequency were suitable conditions for cell vitality and nattokinase production. The vitality of selenium enriched cells was higher most (9.34×10^7) at optimized PEF treatment. After PEF treatment, the overall antioxidant activity was significantly ($P < 0.05$) increased. However the DPPH activity increased from 0.38±0.03 mg/mL (NK1) to 0.107±0.01 mg/mL (NK4) of EC_{50} values. The improvement of ·OH radical scavenging activity was observed from 60.63±5.2 U/mg (NK1) to 104.8±5.6 U/mg (NK4). The antioxidant activity by T-AOC and FRAP assays after PEF treatment was improved from 32.8±4.0 U/mg and 682.63±70.6 µg TE/mg (NK1) to 63.3±3.5 U/mg and 844.46±81.05 µg TE/mg respectively. From this study, it was concluded that selenium enrichment by pulsed electric field can analogously enhance the antioxidant and protease activity of novel fibrinolytic enzyme nattokinase.

* **Corresponding Author:** Hansong Yu ✉ yuhansong@163.com

Introduction

Selenium is an essential non-metal micronutrient which cannot be synthesised by the human body. We need to get selenium as micronutrient from its supplemented forms or natural food sources. It has strong antioxidant (free radicals scavenging) capacity, where free radicals are one of the major causes of thrombosis. It exists in different oxidative and chemical forms (-2, 0, +4, +6 valent, selenite, selenate, elemental Se, selenomethionine/selenocysteine, etc) in nature where it is more toxic in selenite form (Biswas, 2011; Li *et al.*, 2017). Due to the promising health-promoting properties of selenium, it is believed that it can be considered as a functional food additive and that's why it has attracted the attention for its speciation and biotransformation. A study reported that probiotics enriched with selenium have many health friendly effects on the host, like, anti-carcinogenic, anti-pathogenic, anti-oxidative, anti-mutagenic and anti-inflammatory effects (Pophaly *et al.,* 2014). Biogenic selenium nanoparticles (SeNPs) have very low, negligible or even no cytotoxicity as compared with selenate, selenite, SelPlex and nano Sel. Moreover extracted biogenic SeNPs by *Lactobacillus casei*393 had no cytotoxicity on the growth and proliferation of a human normal epithelial cell (NCM460) at a concentration of 25 µg or less Se/mL (Nagy 2016; Xu *et al.*, 2018).

Seleniumand zinc are the functional and structural constituents of several enzymes, hormones, metalloproteins, and microelements which are involved in several biological processes of living organisms (Andreini 2012; Navarro *et al.*, 2008). Organic complexes are the most bio available forms of selenium for human. In selenium deficient states, the biomass enriched with Se4+ can be used as an alternative to commercial pharmaceutical supplements (Lacour, 2004; Dumnot, 2006; Pankiewicz *et al.*, 2008). Bacteria is mainly consists of polysaccharides and peptides on its surface bound metal ions which take part in micro precipitation, chelation, complexion and ion exchange. Extent of these mechanisms depends on the functional group of cells, surface charge, type and concentration of metal ions. After saturation of available binding sites on the cell wall, metal ions are accumulated in the protoplast. As soon as some of the binding sites become free, they are filled again with external metal ions until the state of equilibrium is achieved (Monachese 2012; Mrvčić *et al.*, 2012).

Electroporation is one of the relatively easy, non-toxic and cheap techniques used for the introduction of specific macromolecules into the cells. Application of Pulsed Electric Field (PEF) causes short-term loss of cell membrane integrity and formation of pores with sizes big enough to allow some molecules to enter cytoplasm but small enough to seal after some period of time. Pulsed electric field technology (PEF) can be applied for enrichment of bacteria in ions. In a cell subjected to PEF, induced trans-membrane tension facilitates the formation of pores in the membrane and leads to an increase in its permeability (Dellarosa 2016; Traffano *et al.*, 2016). The effects of this process depend on voltage applied, e.g., in the range of voltage from 0.5 to 1 V arising of new pores with diameter of several nanometers is observed. Permeability of cell membrane increases secretion of compounds inside a cell or accumulation of ions in a protoplast. At the optimized parameters of the process electroporation is reversible (Barba *et al.*, 2015). Electric field strength of 0.7–3.0 kV/cm may cause irreversible membrane breakage and destruction of cellular structures. Cell loses an ability to maintain its natural physiological processes. Phenomenon of electroporation was used, among others, by Pankiewicz *et al* for enrichment of Saccharomyces cerevisiae cells in metal ions (Gehl 2003; Pankiewicz *et al.*, 2015). The aim of the study was to optimize the parameters of PEF in order to maximize a simultaneous accumulationof selenium and zinc in Bacillus Natto cells.Also determine the effect of (PEF) on protease and antioxidant activity of nattokinase.

Materials and methods

Reagents

Casein, Sodium Selenite, folin phenol reagent, tyrosin

and Trichloroacetic acid (TCA) were bought from Sigma Aldrich. DPPH, ABTS (2, 2-azino-bis (3-ethylbenzthiazoline)-6-sulfonic acid) trolox and some antioxidant commercial assay kits were bought from Nanjing Jiancheng Bioengineering Institute, PR. China. All other reagents used were of analytical grade and commercially available.

Biomass cultivation and optimization of selenium concentration

A strain of *Bacillus* Natto was used in this work. For the preparation of inoculums and culture medium sterilization was performed at 121 °C for 20 min. The strain was activated and cultured at 37 °C and 170 rpm in a culture medium with optimized pH 7.4, having 0.5% NaCl, 1% Glucose, 1% peptone and 0.3% beef extract (w/v) in the fixed concentrations. Bacteria were passed three times in culture medium for activation. Then inoculums was prepared by transferring 2% (v/v) of activated bacteria to 80 mL of sterile medium in 250 mL Erlenmeyer flasks for 24 hours incubation. Optimization of selenium concentration in the medium was performed by culturing of Natto Bacillus at different concentrations of Na_2SeO_3 (0, 1, 5, 15, 30, 60 and 120 mg/L) for 18 hours. The growth of cells and protease activity of nattokinase under different selenium stress were evaluated by OD600 and casein lysis activity respectively.

Optimization of PEF parameters

After 18 hours of culture under optimized selenium concentration, cultures were treated with PEF by using a laboratory electroporator. The cultures were then incubated for additional 18 h and the protease activity of supernatant was evaluated. Nattokinase NK1 (without added selenium and without PEF treatment) and NK2 (with optimized added selenium and without PEF treatment) respectively were used as control for determination of protease activity under PEF treatment with different parameters. PEF parameters optimization for protease activity of selenium enriched nattokinase (5 mg/L) was carried out by electric field strength, electroporation time and pulse width. The culture previously incubated for 18 h was exposed to various electric field strengths (0.1 - 3.5 kV/cm), while time 10 min, pulse width 10 µs and frequency 1 Hz were constant. The electroporation time was optimized in the range of 5–30 min, at optimal electric field strength (2.0 kV/cm) and pulse width 10 µs with 1Hz frequency. Pulse width was optimized in the range of 10 – 120 µs with previously optimized PEF parameters (2.0 kV/cm, 15 min and 1Hz).

Determination of cell vitality

Cell vitality was determined by platedilution method according to the American Public Health Association (Hausler *et al*., 1972). The optimized PEF parameters were applied for volatility determination under different selenium concentration in medium. Culture NB1 (without selenium added to the medium and without PEF treatment) was used as control for determination of cells volatility under optimized PEF exposure. Colonies of microorganisms were diluted 8 times with 0.8% sterile NaCl. Then 1 cm^3 of each was taken and placed in Petri dishes in two replicates. The diluted bacteria were flooded with sterile agar medium. Cultures were incubated for 48 h at 37 °C. The Petri dishes with two consecutive dilutions on which grew from25 to 250 colonies were selected for reading. The total number of microorganisms (L) in 1 cm^3 of the sample was calculated according to the formula:

$$L = \frac{C}{N1 + 0.1N2} \times d$$

Where, C is sum of colonies on all dishes selected for counting, N1 is number of dishes from the first calculated dilution, and N2 is number of dishes from second calculated dilution, d dilution ratio corresponding to the first (lowest) dilution.

Production and purification of nattokinase

For the production of nattokinase, fermentation medium with pH7.4, having Glucose 2%, Soy Peptone 2.5%, KH2PO4 0.1%, K2HPO4 0.4%, MgSO4 0.05%, CaCl2 0.02% and optimized selenium concentration (5mg/L of Na_2SeO_3) was used. 2% (v/v) of the starter culture was inoculated in fermentation medium with and without added selenium and incubated at 170rpm

for 60 h. Supernatant of crude enzyme was obtained by centrifugation at 9500g at 4 ℃ for 30 min. Then the crude enzyme was salted out and dialyzed in a dialysis bag (35 kDa) after activity determination. The dialyzed enzyme was concentrated by lyophilization, re-suspended in phosphate buffer (pH 7) and subjected to Sephadex G-75 column (1.5cm × 35cm) for further purification. The column was equilibrated with phosphate buffer (pH 7) and eluted at a flow rate of 1 mL/min. Fraction that has the enzyme with highest activity was collected and lyophilized.

Determination of protease activity

Protease activity of nattokinase was assayed according to the previous method (Anson *et al.*, 1938) with slight modifications. Briefly, 1 mL of enzyme solution was added to 5ml of casein (1% w/v in 50mM potassium phosphate buffer, pH 7.5) and incubated at 37°C for 10 min reaction. Immediately after 10 min, 5ml of tri chloro acetic acid (TCA 0.4M) was added and incubated for additional 30 min. Then the mixture was filtered followed by the addition of 5 ml Na_2CO_3 (500mM) to 2 mL filtrate 1 mL phenol reagent. This mixture was incubated again at 37 °C for 10 min and absorbance was measured at 660nm. Standard curve was drawn using different concentrations of L-tyrsoin (0-100 micro gram) standard. One unit of NK activity was defined as the amount of enzyme that produce 1 µg of tyrosin per minute per mL (U/ml) at 37°C. Molecular weight and enzyme's purity were analyzed by SDS-PAGE. The protein content of the samples was determined by using BSA protein assay reagent Kit (BCATM Protein Assay Kit, Thermo SCIENTIFIC) using bovine serum albumin as standard.

Antioxidant activity

DPPH scavenging activity

The DPPH radical scavenging activity of nattokinase was determined by the previous method (Brand *et al.*, 1995). Briefly, 1 mL of different concentrations (0.1 – 0.5 mg/ml) of enzyme solution was added to 2 mL of DPPH ethanol solution (0.2 mM). Reaction mixture was stirred on magnetic stirrer and kept in dark for 30 min at room temperature and absorbance was measured at 517 nm. The percent DPPH activity was measured according to the formula.

$$\text{DPPH scavenging activity (\%)} = \frac{\text{Acontrol} - \text{Asample}}{\text{Acontrol}} \times 100$$

Concentrations of sample which eliminate 50% of DPPH free radical represent fifty percent elimination concentration (EC50). EC50 was measured graphically by plotting curves of elimination percentages on Y-axis against elimination concentrations on X-axis in. Results were expressed as EC50 mg/ml.

•OH radical scavenging activity

This assay was carried out according to the principal of Fenton reaction which produce ·OH free radical. Commercial assay kit (Nanjing Jiancheng Bioengineering Institute, PR. China) was used for this assay. Instructions provided by the kit were followed to operate this analysis. All the samples were allowed for one minute reaction followed by the addition of chromogenic agent and TCA mixture. Absorbance was measured at 550 nm after 20 minutes. Hydroxyl radical scavenging activity was measured as Unit/mg of protein and one unit is defined as the amount of enzyme that deplete 1 mM H_2O_2 concentration in the reaction mixture per minute at 37°C.

Total antioxidant capacity (T-AOC)

The T-AOC assay was based on the principle Fe^{3+} reduction to Fe^{2+}. Commercial assay kit bought from Nanjing Jiancheng Bioengineering Institute, PR. China was used for this assay. Instructions provided by the kit were followed for this analysis. Enzyme mixture of 0.5 ml was analyzed for T-OAC assay and activity was measured as Unit/mg of protein. One unit of total antioxidant activity was defined as the amount of enzyme increase the absorbance of 0.01 of reaction mixture per minute at 37°C.

Ferric reducing ability of plasma (FRAP)

This assay was carried out by modifying previous method (Benzie *et al.*, 1996) with slight modifications. Briefly working solution for FRAP assay was prepared by adding 2.5 mL of TPTTZ

solution (10 mM TPTZ in 40 mM HCl) and 2.5 mL of 20 mM FeCl3-6H2O solution with 25 mL of 300 mM acetate buffer and the mixture was maintained by incubation at 37°C. Then 200 µL of enzyme mixture was mixed with 2.8 mL of maintained working solution and incubated for 30 minutes in dark at room temperature. Trolox was used as standard and absorbance was measured at 593 nm. FRAF antioxidant activity was measured according to standard curve expressed as micrograms of trolox equivalents (TE) per/mg of protein.

Statistical analysis

All results are expressed as mean ± S.D. with triplicate analysis. Differences between means of each group were assessed by one-way analysis of variance using Statistix 8.1 software. P-value of than 0.05 was considered statistically significant.

Results

Selenium effect on growth and activity of strain

The effect of different selenium stresses (0, 1, 5, 15, 30, 60, 120 mg/L) on growth of cells and nattokinase activity is shown in Table 1. Results indicated that selenium significantly improved the growth and activity of strain at optimized amount as we increase the concentration from 0 to 5 mg/L. Higher concentrations (>5 mg/L) inhibited the cell growth.

When highest concentration (120 mg/L) of Na_2SeO_3 was applied, about 25% of reduction was observed in cell growth compared cells cultured in normal medium. Cells were grey after culture at optimized concentration (5 mg/L) of selenium, but turned into red at higher concentrations. Conversion of strain color from grey to red may be due to the biogenic reduction of selenium.

Table 1. Growth of Natto *Bacillus* strain and its protease activity at different concentrations of sodium selenite (Na_2SeO_4).

Na_2SeO_4	0mg/L	1mg/L	5mg/L	15mg/L	30mg/L	60mg/L	120m/L
OD600	0.72 ± 0.03^{bc}	0.78 ± 0.02^{ab}	0.85 ± 0.03^{a}	0.71 ± 0.06^{bc}	0.65 ± 0.07^{cd}	0.62 ± 0.03^{de}	0.55 ± 0.04^{e}
ActivityU/ml	58.73 ± 1.66^{bc}	64.26 ± 3.06^{ab}	71.16 ± 4.28^{a}	54.46 ± 4.96^{c}	36.25 ± 5.86^{d}	22.71 ± 4.1^{e}	13.24 ± 3.03^{f}

The protease activity of enzyme produced during unexposed medium to selenium was 59.63 ± 1.66^{bc} initially. This activity was significantly increased to 70.26 ± 4.28^{a} after addition of selenium in the concentration of 5 mg/ l. However, the higher amounts resulted in the decline of protease activity. It may because selenium facilitated the growth of Natto *Bacillus* cells at optimized amount, thus caused the production of nattokinase with better protease activity and vice versa in the case of higher amount selenium.

Table 2. Influence of selenium concentration in medium and optimized PEF (2.0 kV/cm, 10 min, 10 µs and 1Hz) treatment on vitality of Nattobacillus.

Selenium Conc (mg/L)	Vitality (CFU/mL) Without PEF	Vitality (CFU/mL) With PEF (2kV/cm, 10µs, 10min)
NB1	1.51×10^7	-
1	4.85×10^7	5.71×10^7
5	6.93×10^7	9.34×10^7
15	8.10×10^6	7.18×10^6
30	5.32×10^6	2.91×10^6

Control culture NB1 without PEF treatment and without added selenium.

Optimization of PEF parameters

Selenium concentration of 5 mg/L in medium was assumed as the potential optimal amount for production of nattokinase during optimizing PEF parameters. Results demonstrated that pulsed electric field with electric field strength of 0.1, 0.2 and 2 kV/cm had positive effect on overall protease activity. The highest protease activity was achieved at electric

field strength of 2 kV/cm shown in Fig. 1A. When the value of this parameter was raised from 2 kV/cm, decline in the protease activity was noted. As compared to controls NK1, NK2 (without treated with PEF), a high drop of protease activity was observed at electric field strength of 3 – 3.5 kV/cm. Statistically significant changes were observed in the protease activity after applying PEF to the medium. The study on time of eletroporation exposure was conducted in the range of 5 – 30 min is shown in Fig. 1B.

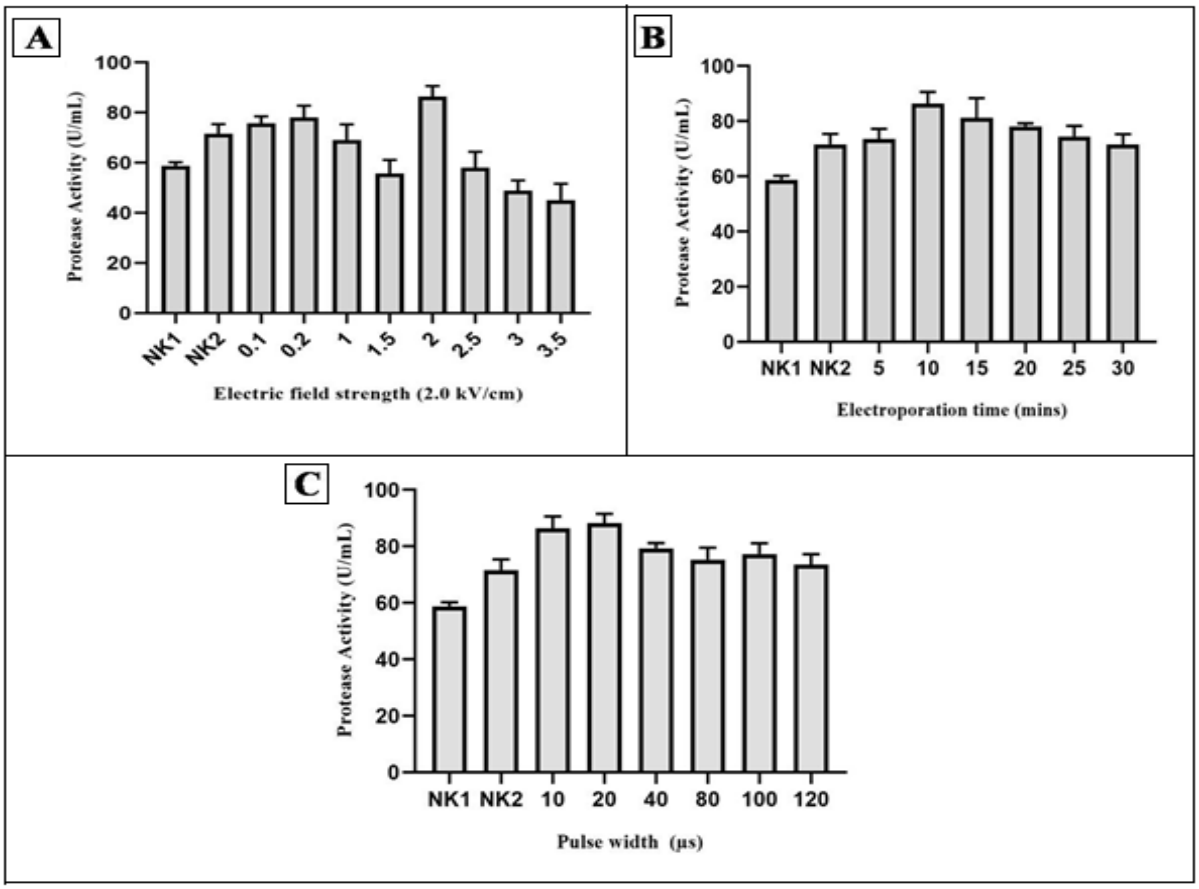

Fig. 1. (A) Protease activity of nattokinase exposed to different electric field strengths during electroporation ranging from 0.1 - 3.5 kV/cm with pulse width of 10 µs, field frequency 1 Hz and time of exposure to PEF 10 min. (B) Protease activity of nattokinase exposed to PEF with different electroporation time in the range of 5–30 min, at optimal electric field strength (2.0 kV/cm) and pulse width of 10 µs with 1 Hz frequency. (C) Protease activity of nattokinase exposed to PEF with different pulse width ranging from 10 – 120 µs with previously optimized PEF parameters (2.0 kV/cm, 10 min and 1Hz).

The extension of eletroporation time from 5 – 30 significantly increased the protease activity other parameters used were 2kV/cm, 10 µs and 1 Hz. The activity was highest after 10 min of treatment as compared to controls. Hence time exposure of 10 min to PEF treatment was decided as optimal. Pulse width was optimized in subsequent step while setting other parameters as 2 kV/cm, 10 min and 1 Hz shown in Fig. 1C. The highest activity was achieved when pulse width of 20 µs was applied. The overall activity of PEF treated samples with different pulse width was significantly higher than controls. Hence it was evaluated that applying pulsed electric field to selenium enriched medium for nattokinase production have positive effect on the protease activity.

The overall optimized PEF parameters in this analysis was recognized as electric field strength of 2 kV/cm, electroporation time 10 min, pulse width 20 µs and requency 1 Hz.

Effect of PEF treatment on volatility of biomass

The effect of pulsed electric field on cell volatility under different selenium concentrations was determined. In contradiction with control cultures (NB1), the increase of cell volatility was observed in electroporated cultures together with increase in selenium supplementation up to optimized amount (Table 2). The nonlinear dependence of cell upon concentration of selenium in the medium was significant both in cultures treated with PEF and non-treated. Cell volatility at concentration of 5 mg/L of selenium exposed to optimized PEF treatment was dramatically raised to 9.34×10^7 CFU/mL as compared to unexposed cells to PEF at the same concentration (6.93×10^7 CFU/mL).

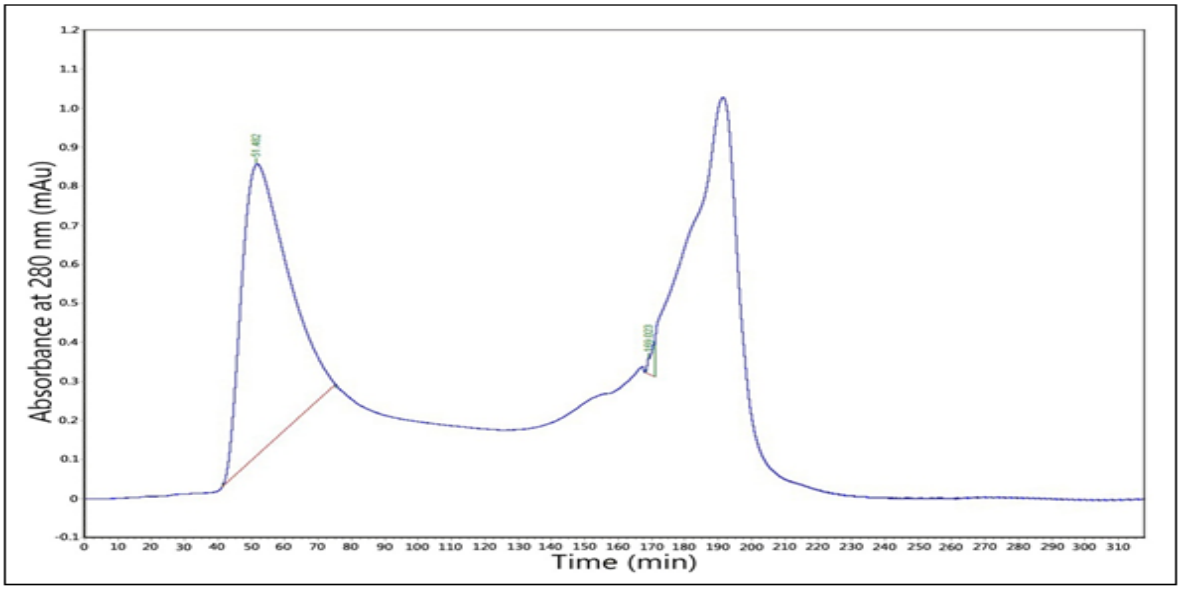

Fig. 2. Elution peaks of Sephadex G-75 gel filtration chromatography at different time intervals. Nattokinase was purified with Sephadex G-75 column after ammonium sulfate precipitation and dialysis.

This volatility was also higher than cells exposed and unexposed to optimize PEF, supplemented with 1 mg/L of selenium. However the volatility of cells not exposed to PEF and supplemented with 15 and 30 mg/L of selenium was dropped by 8.10×10^6 CFU/mL and 5.32×10^6 CFU/mL. At 15 and 30 mg/L of selenium concentration, the volatility of cells was further dropped by 7.18×10^6 CFU/mL and 2.91×10^6 CFU/mL respectively when they were exposed to PEF. This is because of the higher concentration of selenium than optimized amount. These results indicated that at optimized concentration of selenium and optimized PEF conditions, the volatility of cells enhance. However at optimized PEF treatment and higher amount of selenium the volatility of cells degrades.

Properties of nattokinase

All the control and sample (NK1, NK2, NK3) of crude Nattokinase was purified by ammonim sulfate salting out and Sephadex G-75. Where NK3 is selenium enriched nattokinase with optimized amount (5 mg/L) and treated with optimal PEF parameters. The highest activity was achieved by at 80% (w/v) of $(NH_4)_2SO_4$. Then it was dialyzed, concentrated by Lyophilization and applied to Sephadex G-75 gel filtration column. Two fractions were separated by the column among which the protein concentration and protease activity of peptides in peak A was higher most (Fig. 2).

The molecular weight and enzyme purity of the enzyme was determined by SDS-PAGE shown in Fig. 3. The enzyme was homogeneously purified where the purity was observed in SDS-PAGE by a single band of purified enzyme. The molecular weight of nattokinase produced by nattobacillus was 44 kDa as analyzed in SDS-PAGE.

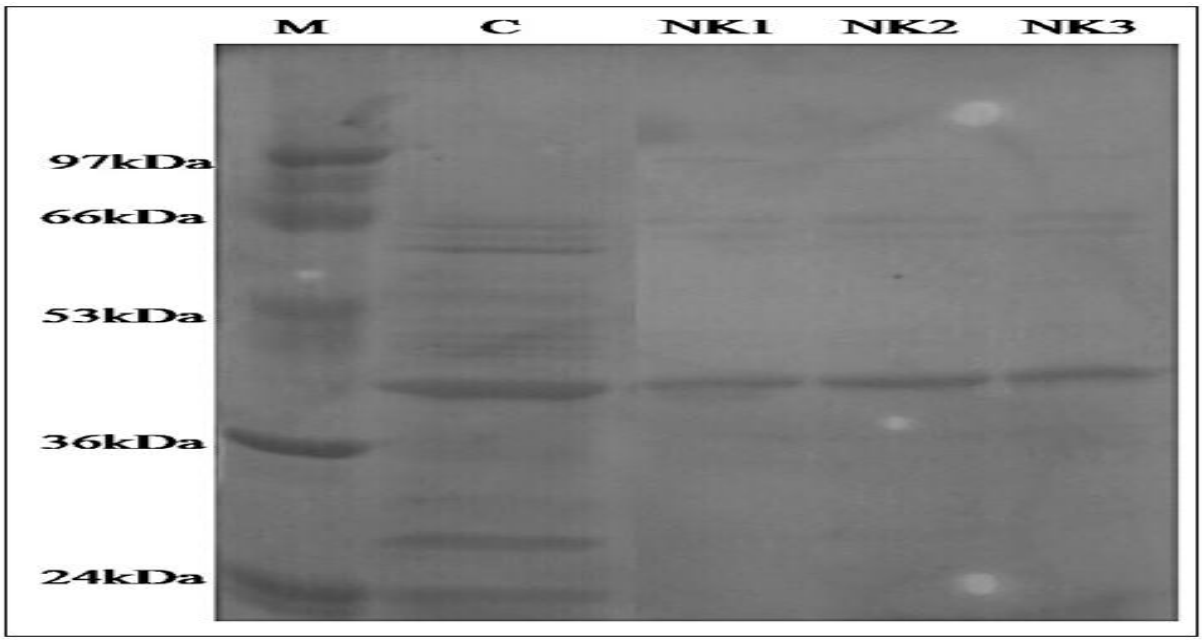

Fig. 3. Molecular weight and nattokinase enzyme purity. M is marker, C is crude enzyme, NK1 is nattokinase without added selenium and without PEF, NK2 is nattokinase with added selenium and without PEF while NK3 is nattokinase with added selenium and PEF treated.

Antioxidant activities

Influence of optimized PEF (2.0 kV/cm, 10 min, 20 µs and 1Hz) treatment on antioxidant activity of selenium enriched nattokinase was determined after purification. The DPPH scavenging activity of purified was determined after different treatments. Nattokinase without selenium supplementation and without PEF treatment (NK1), selenium enriched without PEF (NK2) and selenium enriched with PEF (NK3, NK4, NK5) were evaluated. Results indicated that DPPH scavenging capacity of enzyme increased after selenium enrichment and further increased after PEF treatment. EC_{50} (concentration which scavenge 50 % of DPPH) values are shown in Fig 4A. The DPPH EC_{50} value of NK1 was significantly higher than NK2. However the EC_{50} value of NK2 was higher than NK3 and NK4. Lowest EC_{50} value was observed in NK4 which indicated that applying the PEF with optimized parameters significantly increased the DPPH scavenging activity of selenium enriched nattokinase. Hydroxyl radical scavenging activity assayed according to kit instruction. As shown in Fig 4B, ·OH radical scavenging activity of NK4 was significantly higher than control NK1 and NK2. NK4 activity was also higher than NK3 and NK5 which indicated that selenium amount higher or lower cause depletion of OH radical scavenging activity. The same results were observed for total antioxidant activity (T-AOC) and ferric reducing ability of plasma (FRAP) antioxidant activity shown in Fig 4C and 4D. It was concluded that PEF treatment can significantly improve the antioxidant activity of selenium enriched nattokinase.

Discussion

Selenium is an essential trace element for humans which plays important role in antioxidant defense, anticancer, detoxification, and improving immune Function (Xu *et al.*, 2018). Sodium selenite and selenium methionine are usual utilized as dietary supplement.

However, sodium selenite is the most toxic form of selenium. Although organic selenium possessed properties which are desirable, such as lower toxicity, better palatability and less environmental pollution, the cost of producing them is relatively high. SeNPs attracted more attention due to several advantages including chemical stability, biocompatibility, and low toxicity (Zhang 2001; Zhang *et al.*, 2007). Selenium concentration was optimized for culture medium where we found that selenium higher than

optimized amount caused reddish color in bacterial culture. As we increased the amount of Na_2SeO_3 from 5mg/L to 120mg/L, red color intensity rise which indicates that nattobacillus have the ability to reduce Se (IV), to non-soluble Se(0) by the process of detoxification (Pophaly *et al.*, 2014).

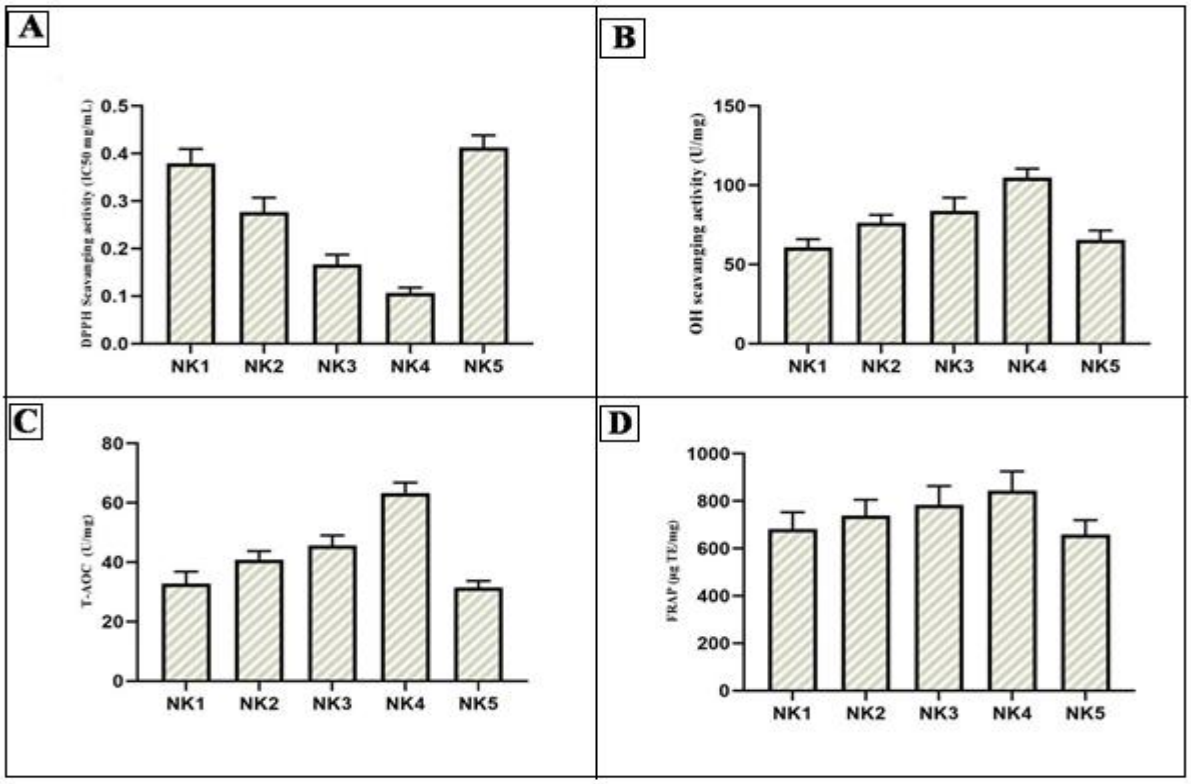

Fig. 4. Where A shows the DPPH antioxidant activity, B shows the OH radical scavenging activity, C shows the total antioxidant activity (T-AOC) and D shows the ferric reducing ability of plasma (FRAP). Control NK1 is nattokinase without added selenium and without PEF treatment, while control NK2 is nattokinase with optimized added selenium and without PEF treatment. NK3, NK4 and NK5 are nattokinases with 1, 5 and 15 mg/mL added selenium with exposed to PEF.

Pulsed electric field was optimized for the production of selenium enriched nattokinase with better protease activity. Different parameters of PEF were analyzed and optimized where electric field strength of 2 kV/cm, electroporation time 10 min, pulse width 20 μs and frequency 1 Hz were the potentially optimal conditions. Pulsed electric field treatment on *Bacillus subtilis* protease enhanced its proteolytic activity when the PEF treatment was carried out in milk (Bendicho *et al.*, 2005). Another study revealed that the biomass content in the range of 0.2–0.3 g/g d.m obtained the highest magnesium accumulation of in *S.cerevisiae* cells treated with PEF at the field strength of 4.0 kV/cm (Pankiewicz *et al.*, 2010). Another study was conducted for determining the effect of pulsed electric field which resulted that application of PEF significantly affected the growth of L. acidophilus LA-K and L. delbrueckii ssp. bulgaricus LB-12.

The parameters of PEF they used in this study were electric field strength of 1.0 kV/cm, pulse width of 3 μs, and pulse period of 0.5 s. They concluded that PEF treatment can be recommended for culture pretreatment to enhance the desirable properties and attributes of LAB used for the production of different probiotic and functional foods (Seratlić *et al.*, 2013).

The effect of pulsed electric field on cell volatility under different selenium concentrations was determined. In contradiction with control cultures (NB1), the increase of cell volatility was observed in

electroporated cultures together with increase in selenium supplementation up to optimized amount.
In 2008 Loghavi *et al* analysed the effect of PEF on cell growth, volatility and metabolic activity of Lactobacillus acidophilus. He demonstrated that the PEF frequency of 45 Hz and 90 Hz had no significant influence on cell volatility (Loghavi *et al.*, 2008).

In 2017 Małgorzata *et al.* revealed that at optimized conditions of PEF (15 min exposure, 2.0 kV/cm electric field strength, 1 Hz frequency and 20 μs pulse width), the volatility of Lactobacillus rhamnosus B 442 Cells and magnesium accumulation was maximum (Góral *et al.*, 2017).

The molecular mass and enzymes purity were analyzed where MW was 44 kDa and the purity of enzyme was shown by single band in SDS-PAGE. Molecular weight of nattokinase or fibrinolytic enzyme depends on the Bacillus specie ranging from 25 – 46.5 kDa. Some species produce nattokinase or fibrinolytic enzyme of lower molecular weight (26-32 kDa and some species produce high molecular weight (43-46 kDa). In this study, we achieved nattokinase with MW of 44 kDa which is similar to fibrinolytic enzyme produced by *Bacillus* sp. from Korean traditional food Chungkookjang (45 kDa), soybean paste (44 kDa), and fermented shrimp paste (43-46 kDa) (Wang *et al.*, 2009).

The human body antioxidant defenses are vital for eliminating harmful free radicals and other reactive oxygen species which can cause oxidative damage to cell membrane lipids, cellular proteins, DNA and enzymes (Ndhlala *et al.*, 2010). In this study influence of optimized PEF (2.0 kV/cm, 10 min, 20 μs and 1Hz) treatment on antioxidant activity of selenium enriched nattokinase was determined after purification. Results indicated that the treatment of selenium enriched fermentation medium with PEF for the production of nattokinase significantly improve the antioxidant activity.

It is important to note that there are some researches about the improvement of antioxidant activity of peptides by pulsed electric field. The DPPH scavenging activity of GSH was increased from 81% to 97% by applying pulsed electric field with with 9.74 kV/cm electric field intensity and 2549 Hz frequency (Wang *et al.*, 2014).

The DPPH scavenging activity of KCHQP was significantly increased by PEF treatment from 89% to 93%.

The parameters of PEF used were 15kV/cm, 1800Hz and it was revealed that may be the zeta potential reduction caused the improvement of antioxidant activity during electroporation treatment (Liang *et al.*, 2017). These results indicated that PEF treatment enhance the antioxidant activity. However the specific reasons for improvement of antioxidant activity by PEF are still unclear. In addition these related researches investigated only the effects of PEF on peptide's chemical oxidation reactions.

Conclusion

The present study was successfully carried out for evaluation of effect of pulsed electric field on antioxidant activity of selenium enriched nattokinase. Parameters of pulsed electric field and selenium concentration for enrichment of fermentation medium were optimized.

It was observed that the overall antioxidant activity of selenium enriched nattokinase, after exposure to electroporation was improved which may because of the enhancement of cell growth and volatility.

This study will provide theoretical basis for further improvement of overall status of nattokinase to make it more suitable and stable for oral use during cardiovascular problems.

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Declaration of Interest

All the authors declare that they have no conflict of interest.

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