



Inactivation of soybean trypsin inhibitor by dielectric-barrier discharge (DBD) plasma



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ABSTRACT

Soybean trypsin inhibitor (STI) is considered as one of the most important anti-nutritional factors in soybeans. The objective of this study was to investigate the impacts and underlying mechanisms of dielectric-barrier discharge (DBD) plasma on STI activities. The results shown that DBD plasma treatment significantly induced the inactivation of STI in soymilk and Kunitz-type trypsin inhibitor from soybean (SKTI) in a model system. After exposure to DBD plasma at 51.4 W for 21 min, the STI activities of soymilk were reduced by 86.1%. After being treated by DBD plasma, the intrinsic fluorescence and surface hydrophobicity of SKTI were significantly decreased, while the sulfhydryl contents were increased. It is assumed that DBD plasma-induced conformational changes and oxidative modification might contribute to the inactivation of SKTI. In summary, DBD plasma technology is a potential alternative to heat treatment for the inactivation of anti-nutritional substances in food legumes.

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1. Introduction

Soybean (*Glycine max* (L.) Merr.) is one of the most economical and nutritious food resources in the world, especially in the developing countries. In comparison to many of today's major food sources, soybeans are rich in protein, lipids, carbohydrates, dietary fiber, vitamins, minerals, and a number of phytochemicals. For their unique nutritional-profile and health-promoting effects, soybeans and soy based foods are widely consumed in the world (Isanga & Zhang, 2008; Tidke, Ramakrishna, Kiran, Kosturkova, & Ravishankar, 2015). However, like other legumes, soybeans contain a plethora of antinutritional factors, including protease inhibitors, lectins, urease, oligosaccharides, lipoxygenase, phyto-oestrogens and phytate (Liener, 1994). The presence of anti-nutritional substances in soybeans can significantly reduce the digestion and absorption of essential nutrients and cause adverse health effects to human and animal health. Among them, trypsin inhibitor is considered as one of the most important anti-nutritional factors in grain soybeans. Soybean trypsin inhibitor (STI) belongs to the

family of serine protease inhibitors, which can reduce the biological activities of proteolytic enzymes, such as trypsin and chymotrypsin. STI can interfere with protein digestion and may cause pancreatic disorders (Weizman, 2004). It was reported that pancreatic hypertrophy and hyperplasia were induced in rats fed either soy protein concentrate or direct concentrate of STI (Smith, Wilson, Allen, & Berry, 1989).

To ensure the safety and nutritional quality of soybean foods, it is necessary to reduce or eliminate these anti-nutrients by using proper processing methods and techniques. Currently, soybean foods are usually be subjected to various thermal treatment (such as roasting, microwaving, and boiling) before being consumed by human beings or animals, in order to inactivate or reduce the activities of anti-nutritional compounds (Andrade, Mandarino, Kurozawa, & Idaet, 2016; Chen, Xu, Zhang, Kong, & Hua, 2014). Nevertheless, thermal treatment process can lower the contents of some essential nutrients and induce the formation of hazardous compounds in foods (van Boekel et al., 2010). Therefore, non-thermal processing techniques have been extensively studied for their applications in food industry. In recent years, non-thermal treatments, including high hydrostatic pressure (van der Ven, Matser, & van den Berg, 2005), ultrasound (Huang, Kwok, & Liang, 2008), and instantaneous controlled pressure (Haddad & Allaf, 2007), have been used to inactivate trypsin inhibitors in soybean and soy-based foods.

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Over the last several years, low temperature plasma technology has drawn a considerable attention and shows a huge application potential in the field of food industry (Shaw, Shama, & Iza, 2015; Thirumud, Sarangapani, & Annapure, 2015). The term “plasma” was first introduced by Irving Langmuir to describe an ionized gas in 1927 (Thirumud et al., 2015). Plasma, often recognized as the fourth state of matter after solids, liquids, and gases, is estimated to constitute more than 99% of the visible matter in the universe. Plasma is ionized gas, which is composed of numerous reactive species, including positive ions, negative ions, electrons, free radicals, gas atoms, excited and ground state atoms and/or molecules, and photons (visible and UV), (Shaw et al., 2015; Thirumud et al., 2015). Plasmas are usually divided into two main categories: high temperature plasma (equilibrium plasma) and low temperature plasma (non-equilibrium plasma). For high temperature plasma, the electrons, ions, and neutral species are in an equilibrium or near equality state. The low temperature plasma can be further divided into thermal plasma, also known as quasi-equilibrium plasma (typically 100–150 °C), and cold plasma, also called non-equilibrium plasma or non-thermal plasma (<60 °C) (Huang & Tang, 2007).

Due to its strong thermodynamic non-equilibrium nature, low gas temperature, and presence of reactive chemical species with a high selectivity, cold plasma has received great attention in recent years and has been used in a wide variety of applications, including biology, medicine, and food security. For food industry, cold plasma technology has shown great potential for microorganism inactivation (including bacterial cells, spores, yeasts, and moulds) in raw agricultural products and foods (Niemira, 2012), modification of food components (including whey protein, rice flour, and starch) (Han, Manolach, Denes, & Rowelld, 2011; Misra, Pankaj, Segat, & Ishikawa, 2016), food packaging (Pankaj et al., 2014), and seed germination (Li et al., 2014), and so on.

Numerous studies have been reported that cold plasma treatment can induce inactivation of enzymes, such as lysozyme, polyphenol oxidase, peroxidase, lipase, and α -amylase, in various foods and model systems (Misra et al., 2016). However, studies in the effects of cold plasma on soybean trypsin inhibitor are still limited. Therefore, the objective of this work was to investigate the effects of dielectric-barrier discharge (DBD) plasma treatment on the activities of STI in soymilk and Kunitz-type trypsin inhibitor from soybean (SKTI) in a model system. In addition, the working mechanisms of DBD plasma were also determined by measuring changes in the intrinsic fluorescence spectra, free sulfhydryl contents and surface hydrophobicity of SKTI after exposure to DBD plasma.

2. Materials and methods

2.1. Materials

Soybeans were obtained from a local market in Zhengzhou (Henan, China) and were stored at 4 °C until use. Kunitz-type trypsin inhibitor from soybean (SKTI) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). N-benzoyl-dl-arginine-p-nitroanilide hydrochloride (BAPNA), Tris base, dimethylsulfoxide (DMSO), and bovine pancreatic trypsin were obtained from Beijing Huamaike Biotechnology Co., Ltd. (Beijing, China). Cysteine (Cys), 1-anilino naphthalene-8-sulphonate (ANS), and 4,4'-dithiodipyridine (DTDP) were supplied by Aladdin Reagent Co., Ltd. (Shanghai, China). All other reagents were analytical grade and used without further purification.

2.2. Preparation of soymilk

Soymilk was prepared according to the method developed by Chen et al. (2014). Briefly, soybeans (20 g) were soaked in

deionized water (200 mL) for 14 h at 4 °C. After being rinsed with deionized water for three times, the swollen beans were mixed with deionized water (200 mL) and blended for 5 min at low speed with a household blender (model QH-900B1, Joyoung Co., Ltd., Hangzhou, China). The soy slurry was filtered through four layers of cheese cloth and then a 200-mesh sieve. The filtrate was designated as raw soymilk.

2.3. Experimental apparatus and DBD plasma treatment

Schematic diagram of experimental apparatus to generate DBD plasma is shown in Fig. 1. The experimental apparatus consists of a high voltage alternating current power source (CTP-2000 K), a DBD plasma reactor (DBD-50) and a quartz dish (Nanjing Suman Electronics Co., Ltd., China). The DBD plasma reactor consists of two steel electrodes of 50 mm diameter, a upper quartz plate (ϕ 102 mm $\times$ 1 mm), and a lower quartz plate (ϕ 90 mm $\times$ 1 mm). The air gap distance between quartz plates is set to 10 mm. The reactor dish is made by quartz with a outer diameter of 70 mm, inner diameter of 50 mm, a wall thickness of 9 mm and a depth of 4 mm, which is placed between the two quartz plates. The DBD plasma was generated between the upper electrode and the sample solutions.

SKTI was dissolved in deionized water at initial concentration of 0.01 mg/mL. For DBD cold plasma treatment, the quartz dish containing 3 mL of soymilk or SKTI solution was placed in the center of the two quartz plates. After being treated at given operating conditions, the samples were collected for further analysis.

2.4. Measurement of inhibitory activities of STI

The activities of STI in soymilk and SKTI were determined according to the methods described previously (Chen et al., 2014; Huang et al., 2008). The substrate solution was prepared by dissolving 15.0 mg BAPNA in 0.5 mL dimethyl sulfoxide and then diluting to 50 mL with Tris-HCl buffer (0.05 M, pH 8.2, 0.02 M CaCl_2). 2 mL of soymilk samples or SKTI solution, 5 mL of BAPNA solution, and 1 mL of bovine trypsin solution (0.2 mg/mL in 0.04 mM HCl) were mixed in test tubes. The reaction mixtures were incubated in a water bath at 37 °C for exactly 10 min and terminated by adding 1 mL of 30% (v/v) acetic acid solution. In the control group, the soymilk samples or SKTI solution was replaced by 1 mL of deionized water. The absorbance at 410 nm was measured using a UV-visible spectrophotometer (UV-2450, Shimadzu, Japan). One unit of trypsin activity (TU) is defined as the increase in the absorbance of 0.01 at 410 nm under assay conditions. The trypsin inhibitor unit (TIU) was calculated as the difference in the absorbance between the standard test-tube without soymilk samples or SKTI and the sample test-tube with soymilk samples or SKTI. The percentage of residual inhibitory activity of STI was expressed as the ratio between the TIU amounts of the assay and the untreated sample, multiplied by 100%.

2.5. SDS-PAGE analysis of SKTI samples

After DBD plasma treatment, the SKTI samples were mixed with one part of 5 $\times$ sample buffer and then held in boiling water bath for 5 min. The samples were separated using 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, the gels were stained with 0.1% (w/v) Coomassie Brilliant Blue R-250 (40% methanol and 10% acetic acid) for 30 min and then destained overnight. Protein bands in the gel were visualized with the ChemiDoc XRS⁺ imaging system (Bio-Rad) and analyzed using Image Lab[™] software (version 5.2, Bio-Rad).

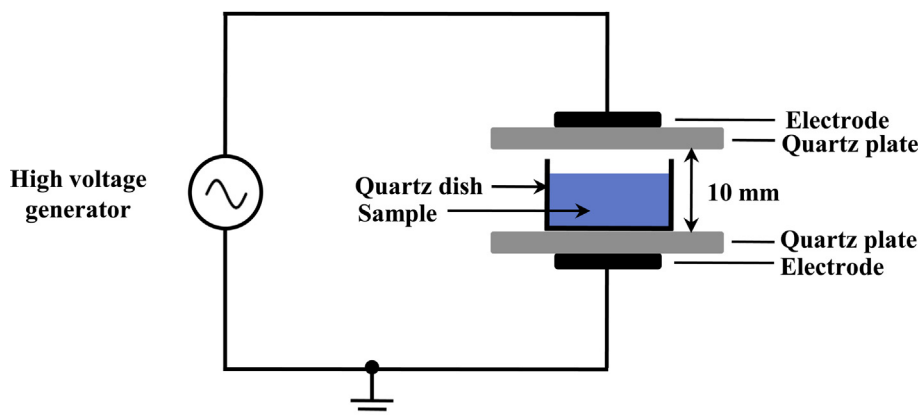


Fig. 1. Schematic diagram of the experimental apparatus for DBD plasma preparation.

2.6. Quantification of protein sulfhydryls with DTDP

Protein sulfhydryls were measured according to the 4, 4'-dithiodipyridine (DTDP) methods described in the previous reports (Hansen, Østergaard, Nørgaard, & Winther, 2007; Liu, Hong, & Deng, 2013) with slight modifications. Briefly, protein samples were diluted with Sodium phosphate buffer (100 mM NaH_2PO_4 , 0.2 mM EDTA, pH 7.0 adjusted with NaOH). 0.7 mL of diluted protein samples was incubated with 0.1 mL of 4 mM DTDP reagent (in acetonitrile) for 5 min at room temperature. Then, the absorbance at 324 nm was measured. Cysteine was used as standard for the calibration curve and the results were expressed as $\mu\text{mol SH/g}$ protein ($\mu\text{mol/g pro.}$).

2.7. Intrinsic fluorescence measurements

The protein concentration of SKTI samples were measured using BCA Protein Assay Kit (Thermo Scientific Pierce, Rockford, USA) and then the samples were diluted with phosphate buffer solution of pH 7.2 to finally yield a working solution of 10 $\mu\text{g/mL}$. Fluorescence emission spectra of different DBD plasma-treated SKTI samples were recorded using a Hitachi F-7000 fluorescence spectrophotometer (Hitachi High-Technologies Co. Ltd., Tokyo, Japan) with a 1 cm quartz cuvette at room temperature. Fluorescence emission spectra were recorded in the range of 290–450 nm ($\lambda_{\text{ex}} = 280 \text{ nm}$) at scan speed of 240 nm/min with slit widths of 5 nm for both excitation and emission (Li & Geng, 2016). The fluorescence intensity was expressed in arbitrary units (A.U.).

2.8. Measurement of protein surface hydrophobicity (PSH) index

The surface hydrophobicity of SKTI samples was determined with fluorescent probe 1,8-anilinonaphthalene-8-sulfonate (ANS) with previously reported methods (Alizadeh-Pasdar & Li-Chan, 2000; Sun et al., 2015). SKTI samples were diluted with the PBS buffer (10 mM, pH 7.2) to a serial concentration of 0.2–1.0 mg/mL. An aliquot (50 μL) of ANS solution (8 mM ANS in 10 mM phosphate buffer, pH 7.0) was added to 4 mL of each dilution and the mixtures were incubated for 10 min in dark. Then, the relative fluorescence intensity of ANS in SKTI solutions was measured on a Hitachi F-7000 spectrofluorometer (Tokyo, Japan) at 390 nm (excitation) and 470 nm (emission). The excitation and emission slit widths were set to 2.5 nm. The index of surface hydrophobicity was determined with the slope method reported by Alizadeh-Pasdar and Li-Chan (2000).

2.9. Statistics

All experiments were conducted at least three times. Data reported are mean values $\pm$ standard deviations. The data were statistically analyzed using the IBM SPSS for Windows (version 22.0, SPSS Inc. Chicago, IL, USA) and the means were compared by independent samples *t*-test to evaluate significant differences. A level of $*p < 0.05$, and $**p < 0.01$, was regarded as statistically significant.

3. Results and discussion

3.1. DBD plasma treatment resulted in inactivation STI in soymilk

Firstly, the influences of DBD plasma treatment on trypsin inhibitor activities of soymilk were investigated. As shown in Fig. 2A, DBD plasma treatment could effectively induce time-dependent inactivation of trypsin inhibitor in soymilk. After being treated at 51.4 W for 27 min, the trypsin inhibitor activities of soymilk were mostly almost completely destroyed (Fig. 2A). The input power also significantly affected the efficacy of DBD plasma on inactivation of trypsin inhibitor. With increasing input power, the residual inhibitory activities of trypsin inhibitor in soymilk were also significantly decreased (Fig. 2B). These results indicated that DBD plasma could effectively inactivate STI activities of soymilk in a time- and input power-dependent way.

3.2. DBD plasma treatment resulted in inactivation of SKTI

SKTI was chosen as a standard soybean trypsin inhibitor to investigate the working mechanisms of DBD plasma. As indicated in Fig. 3A, SKTI dramatically inhibited the hydrolytic activity of trypsin and the inactivation of trypsin was promoted by the increasing amount of SKTI, which were consistent with the results of previous research (Huang et al., 2008). When the concentration of SKTI was set up at 10 $\mu\text{g/mL}$, the activity of trypsin decreased by 93.8% ($p < 0.01$). In order to attain a high inhibitory ratio, the concentration of SKTI used in following study was set at 10 $\mu\text{g/mL}$.

It was observed that DBD plasma treatment caused a dramatic reduction in the inhibitory activities of SKTI (Fig. 3B and C). With increasing treatment time from 30 to 210 s, the inactivation efficiency of DBD plasma was also reinforced. As indicated in Fig. 3B, the inhibitory activities of SKTI were reduced by 95.6% ($p < 0.01$) after the exposure to DBD plasma at 51.4 W for 210 s. The input power was another important factor affecting the performance of DBD plasma. As the input power increased from 2.9 to 123.7 W, the inhibitory activities of SKTI were also significantly decreased (Fig. 3C). After being treated at 123.7 W for 90 s, the residual

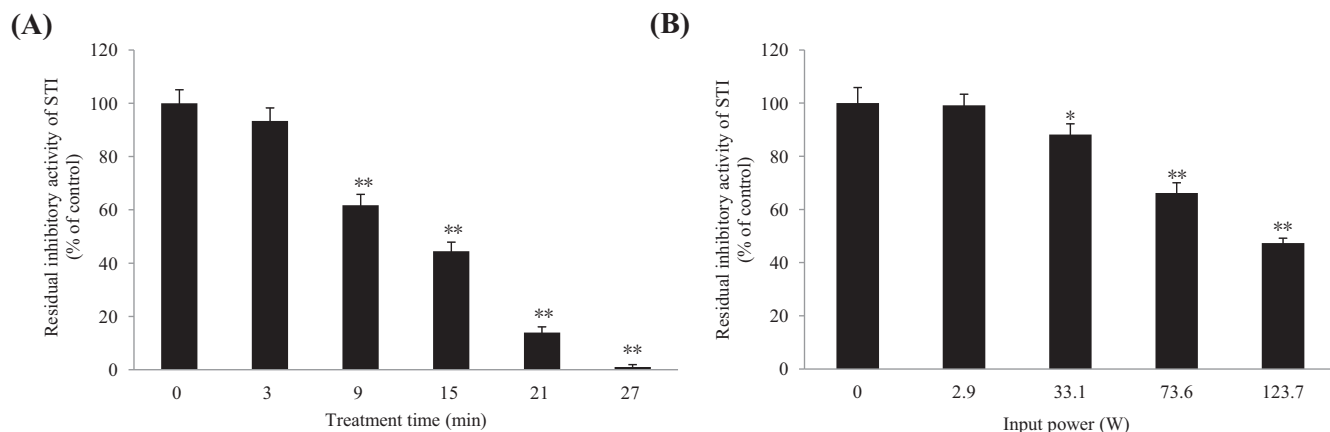


Fig. 2. Effects of DBD plasma treatment on residual STI activities of soymilk. Soymilk was treated by DBD plasma at 51.4 W for different time interval (A) or at various input power for 90 s (B). Residual STI activities of soymilk were measured as described in the Materials and Methods section. Each value represents the mean $\pm$ SD ($n = 3$). * $p < 0.05$, ** $p < 0.01$ versus the control group.

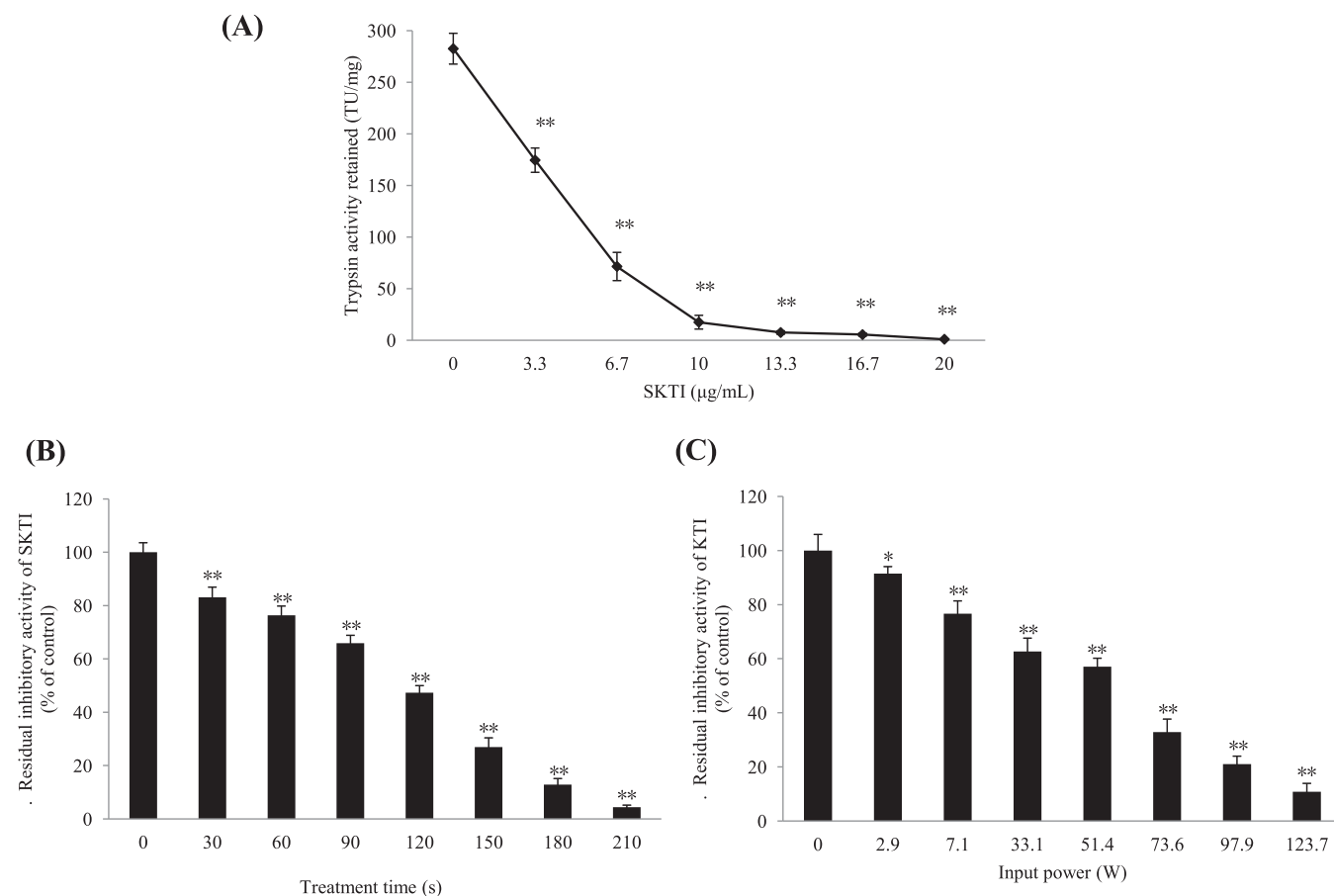


Fig. 3. Residual inhibitory activities of SKTI after DBD plasma treatment. (A) Inhibitory characteristic of KTI against bovine trypsin. (B) Residual inhibitory activity of SKTI after DBD plasma treatments at 51.4 W for varying time periods. (C) Residual inhibitory activity of SKTI after DBD plasma treatments at various input power for 90 s. Each value represents the mean $\pm$ SD ($n = 3$). * $p < 0.01$, ** $p < 0.01$ vs. the control group.

inhibitory activity of SKTI against trypsin was decreased by 89.2% ($p < 0.01$). These results indicated that DBD plasma could destroy the inhibitory activities of SKTI in a time- and input power-dependent manner.

3.3. SDS-PAGE profiles of SKTI after DBD plasma treatment

The SDS-PAGE profile of SKTI was shown in Fig. 4A and B. According to the report of Roychaudhuri, Sarath, Zeece, and

Markwell (2004), the molecular weight of SKTI is about 21.5 kDa. Therefore, the protein bands between 18.4 and 25.0 kDa in Fig. 4A and B are ascribed as SKTI. SDS-PAGE analysis indicated that no obvious lower molecular weight fragment or cross-linked protein bands with greater molecular weight were observed after exposure to DBD plasma (Fig. 4A and B). However, after being treated at 51.4 W for 240 s or 175.6 W for 90 s, the bands of SKTI faded, indicating that degradation of SKTI took place (indicated by the arrow in Fig. 4A and B).

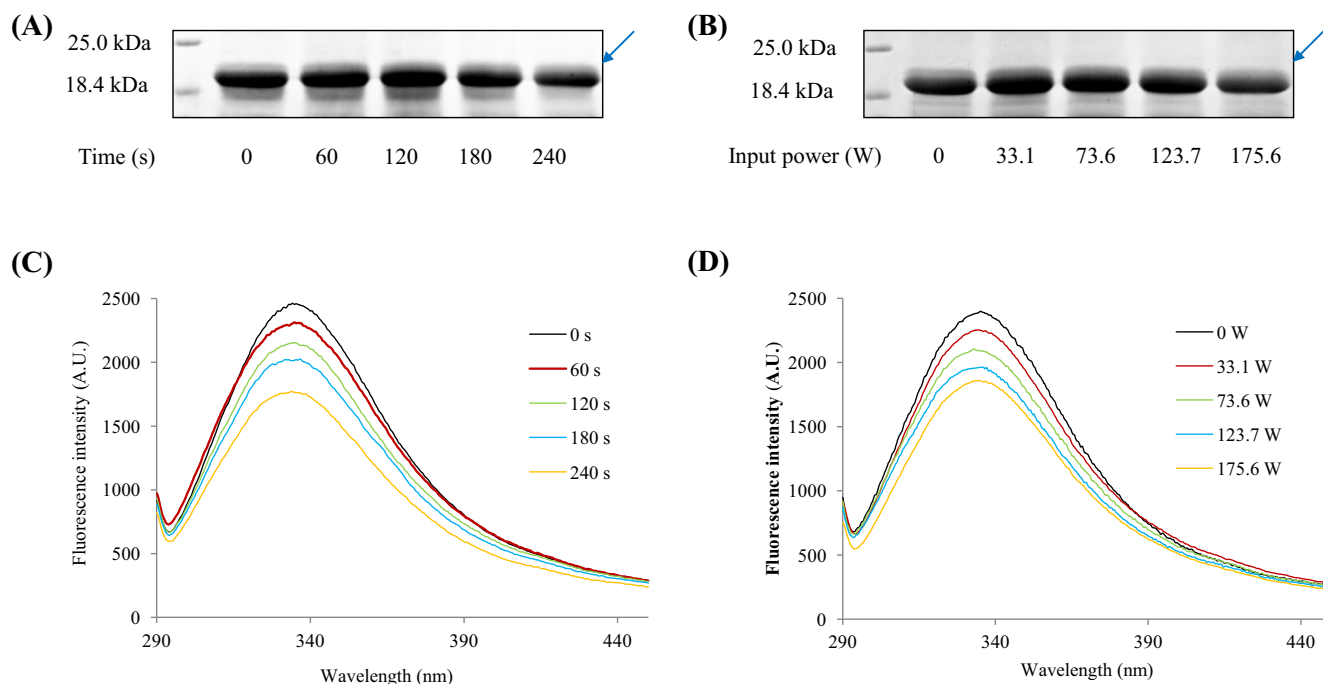


Fig. 4. SDS-PAGE profile and fluorescence emission spectra of SKTI after DBD plasma treatment. SKTI were treated by DBD plasma at 51.4 W for different time intervals (A and C) or at various input power for 90 s (B and D). SDS-PAGE and measurement of fluorescence emission spectra were carried out as described in the Materials and Methods section.

It has reported that various biologically relevant reactive oxygen and nitrogen species are generated during plasma treatment, such as hydrogen peroxide (H_2O_2), ozone (O_3), peroxyxynitrite (ONOO^-), hydroxyl radical ($\text{OH}^\cdot$), superoxide anion (O_2^-), and singlet oxygen ($^1\text{O}_2$) in liquids (Priya Arjunan & Morss Clyne, 2011). These reactive species are able to cause oxidation in both amino acid side chains and protein backbones, thereby resulting in oxidative degradation or cross-linking of proteins (Misra et al., 2016; Zhang et al., 2015). For the low sensitivity of coomassie staining and the low levels of cross-linked and degraded products, there was no obvious changes in the SDS-PAGE profile of DBD plasma-treated SKTI samples. It is supposed that the reactive species-induced oxidative modifications may result in the inactivation of SKTI. As the major components of food, proteins are significantly affected by various processing conditions. Therefore, further research is needed to investigate the influences of DBD plasma on the nutritional and functional properties of food proteins.

3.4. DBD plasma treatment induced changes intrinsic fluorescence of SKTI

The proper folding and the maintenance of its native conformation are essential for the integrity and biochemical activity of a protein. The intrinsic fluorescence of proteins, originating from aromatic amino acids, including phenylalanine (Phe), tyrosine (Tyr), and tryptophan (Trp) residues, has been extensively used to obtain information on structural and dynamic properties of proteins (Ghisaidoobe & Chung, 2014). In this study, the influence of DBD plasma on conformational changes of SKTI was investigated by measuring the intrinsic fluorescence spectra of DBD plasma-treated SKTI at $\lambda_{\text{ex}} = 280 \text{ nm}$ and the results indicated that DBD plasma treatment could induce a distinct decrease in the relative peak fluorescence intensities of SKTI, depending on the treatment time and input power-dependant used. As depicted in Fig. 4C, after being treated at 51.4 W for 240 s or at 175.6 W for 90 s, the peak fluorescence intensities of SKTI were decreased by 28.4% and 22.5%, respectively. Trp is the dominant intrinsic fluorophore and

the intensity of Trp residues are recognized to be sensitive to the microenvironment of proteins (Ghisaidoobe & Chung, 2014). As an aromatic and hydrophobic amino acid, Trp prefers to be buried in protein hydrophobic cores. When Trp residues are exposed into a high-polar aqueous environment, which quite often is the case for unfolded states, the emission intensity will decrease (Alston et al., 2004). Therefore, the decrease in fluorescence intensity of SKTI indicated that the local environment of Trp residues in SKTI was changed to more polar after DBD plasma treatment.

Furthermore, a small red shift of maximum emission wavelength was observed after DBD plasma treatment. After being treated at 51.4 W for 240 s, the maximum emission wavelength of SKTI was shifted from 334 nm to 336 nm (Fig. 4C). Unfolding of a protein always accompanied a red shift in the fluorescence spectrum of buried tryptophan residues in the range 345–355 nm (Alston et al., 2004; Ervin, Sabelko, & Gruebele, 2000). The observed red shift suggested that the fluorophores were replaced from the less-polar interior of SKTI to solvent exposed regions upon unfolding after DBD plasma treatment (Duy & Fitter, 2006). Taken together, these data indicate that the DBD plasma treatment induce unfolding of SKTI and the conformational changes of SKTI may be related to the decrease in the trypsin inhibitory activities.

3.5. DBD plasma treatment induced increase in the free sulfhydryl content of SKTI

The composition and status of disulphide bonds play a key role in determining the structure, inhibitory function, and resistance to thermal and chemical denaturation of SKTI (Roychaudhuri et al., 2004; Tetenbaum & Miller, 2001). In this work, the total free sulfhydryl groups were measured to evaluate the effects of DBD plasma on the covalent interactions of SKTI. When compared to the control group without DBD plasma treatment ($4.16 \mu\text{mol/g pro.}$), the free SH-group contents of SKTI were increased significantly ($p < 0.01$) by 26.2%, 48.4%, and 66.1%, respectively after DBD plasma treatment for 120, 180, and 240 s at 51.4 W (Fig. 5A). The free SH-group contents of SKTI were also influenced by input

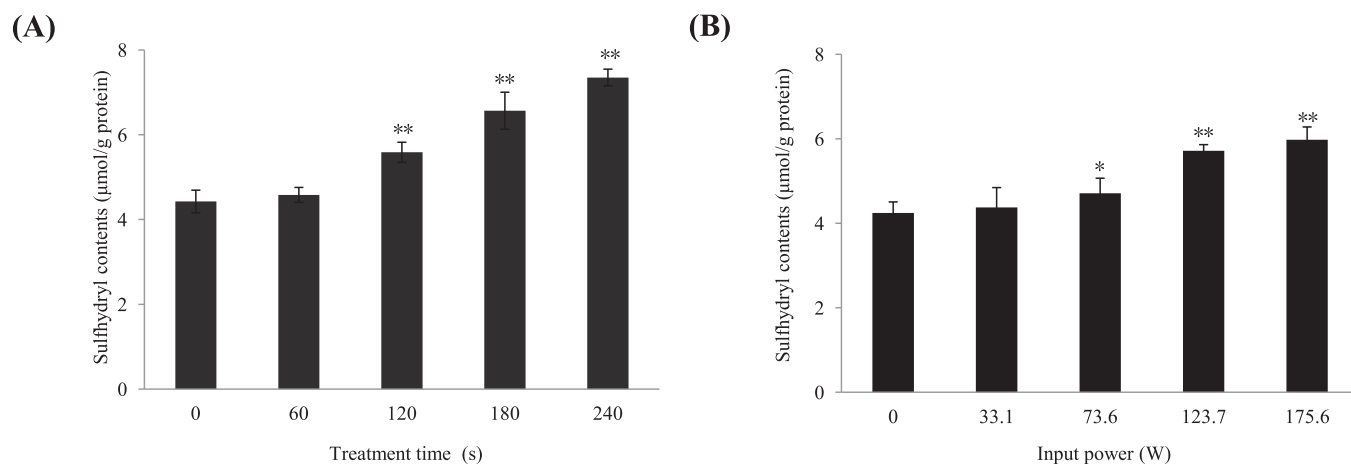


Fig. 5. Changes in free sulfhydryl group content of DBD plasma-treated SKTI solutions. SKTI were treated by DBD plasma at 51.4 W for different time intervals (A) or at various input power for 90 s (B). SDS-PAGE was carried out as described in the Materials and Methods section. Each value represents the mean $\pm$ SD ($n = 3$). * $p < 0.05$, ** $p < 0.01$ versus the control group.

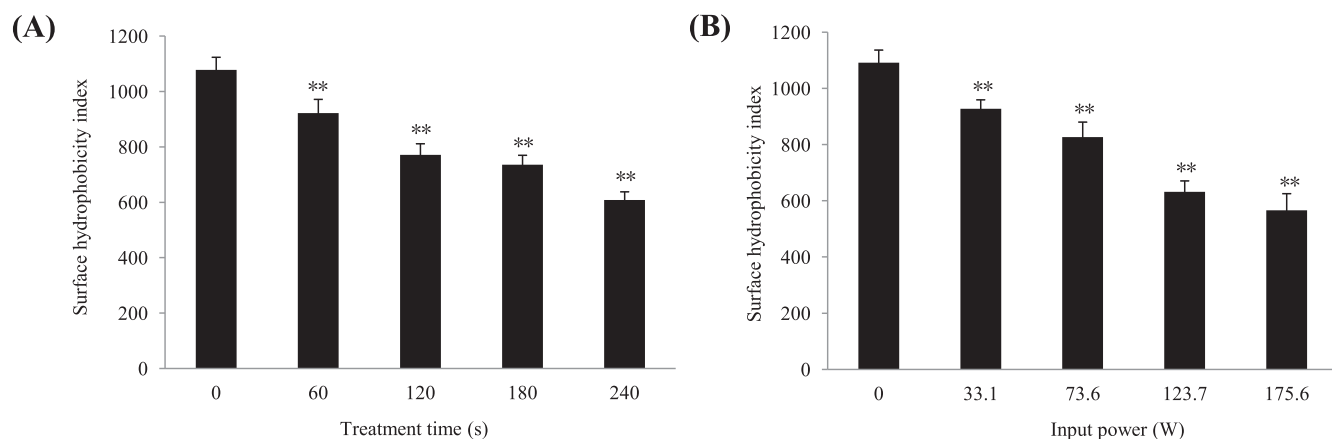


Fig. 6. Effects of DBD plasma treatment on the surface hydrophobicity of SKTI. SKTI were treated by DBD plasma at 51.4 W for different time intervals (A) or at various input power for 90 s (B). SDS-PAGE was carried out as described in the Materials and Methods section. Each value represents the mean $\pm$ SD ($n = 3$). * $p < 0.05$, ** $p < 0.01$ versus the control group.

power. As shown in Fig. 5B, the free SH-group contents of SKTI were increased significantly with increased input power. After being treated with DBD plasma at 73.6, 123.7, and 175.6 W for 90 s, the free SH-group contents of SKTI were increased significantly ($p < 0.01$) by 10.9%, 34.5%, and 40.7%, respectively. These results are in agreement with a previous report of Huang et al. (2008), who reported that the contents of sulfhydryl groups in SKTI were increased dramatically after ultrasound treatment. It has been reported that there are three kinds of Kunitz-type trypsin inhibitor (Tia, Tib, and Tic) in soybean seeds, which all consist of 181 amino acid residues with 4 Cys and 2 Met and have a single reactive site at the same position (Arg₆₃-Ile₆₄). SKTI contains two intramolecular disulfide bridges (Cys₃₉-Cys₈₆ and Cys₁₃₆-Cys₁₄₅) (Kim et al., 1985; Song & Suh, 1998). Although the disulfide bonds are not located near the active site of SKTI, the breakdown of disulfide bonds may result in conformational changes in SKTI, which is necessary to sustain inhibitory activity of SKTI (Tetenbaum & Miller, 2001). According to the previous research, cold plasma can cause the cleavage of disulfide bonds involved in the structure of polypeptides and proteins (Xia & Cooks, 2010), which result in the inactivation of proteins, such as

arginine vasotocin (Motrescu et al., 2011) and rabbit anti-RpoH antibody (Lackmann et al., 2015).

As reported by previous studies, some strong oxidizing agents can convert one disulfide bond into 2 cysteic acids, in an irreversible way (Lu & Chang, 2010). The free radicals inside the oscillating bubble also play an important role in ultrasound irradiation-induced reduction of disulfide bonds (Huang et al., 2008). Therefore, it is speculated that the DBD plasma produced-reactive species may be involved in changes in the disulfide linkage of SKTI by transforming the disulfide linkage (R-S-S-R') into a terminal thiol group, such as R-SH and R'-SH (Huang et al., 2008; Priya Arjunan & Morss Clyne, 2011; Rehman, Jawaid, Uchiyama, & Kondo, 2016).

3.6. DBD plasma treatment decreased the surface hydrophobicity of SKTI

The effects of DBD plasma on the surface hydrophobicity of SKTI was investigated by ANS binding method. As a fluorescent dye that binds to hydrophobic surfaces of proteins with high affinity, ANS has been widely used to study the hydrophobic character of

proteins (Latypov et al., 2008). As illustrated in Fig. 6, DBD plasma treatment exhibited a notable influence on surface hydrophobicity index of SKTI. After being treated at 51.4 W for 240 s or 175.6 W for 90 s, the surface hydrophobicity indices of SKTI were decreased significantly ($p < 0.01$) by 43.6% and 48.1%, respectively.

The function of a protein is determined by its specific conformation. Surface hydrophobicity is an important surface-related property of proteins, which is significantly correlated with the conformations and solubility of protein. The conformational changes of protein are often associated with changes in surface hydrophobicity (Chao, Ma, & Stadtman, 1997; Wagner, Sorgentini, & Añón, 2000). It may be hypothesized that DBD plasma treatment-induced decrease in surface hydrophobicity plays an important role in the inactivation of SKTI.

4. Conclusions

The results of the present study suggested that DBD plasma treatment could induce the inactivation of STI in soymilk and SKTI in a model system. The DBD plasma treatment also resulted in significant decrease in the surface hydrophobicity and intrinsic fluorescence of SKTI, as well as increase in free sulfhydryl contents. These results indicate that DBD plasma-induced conformational changes and oxidative modification might contribute to the inactivation of SKTI. In summary, the results of this study indicate the potential application of cold plasma technology in food processing industry for reducing anti-nutritional attributes of soybean and other legumes. However, many further investigations are still required to reveal the impacts of cold plasma treatment on the nutritional value and sensorial qualities of foods. In addition, the safety of cold plasma-treated foods should be fully evaluated and the regulatory standards should be established before the widespread application of cold technique in food industry.

Declaration of interest

The authors have no conflicts of interest to declare.

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References

- Alizadeh-Pasdar, N., & Li-Chan, E. C. Y. (2000). Comparison of protein surface hydrophobicity measured at various pH values using three different fluorescent probes. *Journal of Agricultural and Food Chemistry*, 48, 328–334.
- Alston, R. W., Urbanikova, L., Sevcik, J., Lasagna, M., Reinhart, G. D., Scholtz, J. M., & Pace, C. N. (2004). Contribution of single tryptophan residues to the fluorescence and stability of ribonuclease Sa. *Biophysical Journal*, 87, 4036–4047.
- Andrade, J. C., Mandarino, J. M. G., Kurozawa, L. E., & Ida, E. I. (2016). The effect of thermal treatment of whole soybean flour on the conversion of isoflavones and inactivation of trypsin inhibitors. *Food Chemistry*, 194, 1095–1101.
- Chao, C. C., Ma, Y. S., & Stadtman, E. R. (1997). Modification of protein surface hydrophobicity and methionine oxidation by oxidative systems. *Proceedings of the National Academy of Sciences of the United States of America*, 94, 2969–2974.
- Chen, Y. M., Xu, Z. C., Zhang, C. M., Kong, X. Z., & Hua, Y. F. (2014). Heat-induced inactivation mechanisms of Kunitz trypsin inhibitor and Bowman-Birk inhibitor in soymilk processing. *Food Chemistry*, 154, 108–116.
- Duy, C., & Fitter, J. (2006). How aggregation and conformational scrambling of unfolded states govern fluorescence emission spectra. *Biophysical Journal*, 90, 3704–3711.
- Ervin, J., Sabelko, J., & Gruebele, M. (2000). Submicrosecond real-time fluorescence sampling: application to protein folding. *Journal of Photochemistry and Photobiology B: Biology*, 54, 1–15.
- Ghisaidoobe, A. B. T., & Chung, S. J. (2014). Intrinsic tryptophan fluorescence in the detection and analysis of proteins: a focus on Förster resonance energy transfer techniques. *International Journal of Molecular Sciences*, 15, 22518–22538.
- Haddad, J., & Allaf, K. (2007). A study of the impact of instantaneous controlled pressure drop on the trypsin inhibitors of soybean. *Journal of Food Engineering*, 79, 353–357.
- Han, Y., Manolach, S. O., Denes, F., & Rowell, R. M. (2011). Cold plasma treatment on starch foam reinforced with wood fiber for its surface hydrophobicity. *Carbohydrate Polymers*, 86, 1031–1037.
- Hansen, R. E., Østergaard, H., Nørgaard, P., & Winther, J. R. (2007). Quantification of protein thiols and dithiols in the picomolar range using sodium borohydride and 4,4'-dithiodipyridine. *Analytical Biochemistry*, 363, 77–82.
- Huang, H. H., Kwok, K. C., & Liang, H. H. (2008). Inhibitory activity and conformation changes of soybean trypsin inhibitors induced by ultrasound. *Ultrasonics Sonochemistry*, 15, 724–730.
- Huang, H., & Tang, L. (2007). Treatment of organic waste using thermal plasma pyrolysis technology. *Energy Conversion and Management*, 48, 1331–1337.
- Isanga, J., & Zhang, G. N. (2008). Soybean bioactive components and their implications to health—a review. *Food Reviews International*, 24, 252–276.
- Kim, S. H., Hara, S., Hase, S., Ikenaka, T., Toda, H., Kitamura, K., & Kaizuma, N. (1985). Comparative study on amino acid sequence of Kunitz-type soybean trypsin inhibitor, Ti^a, Ti^b, Ti^c. *Journal of Biochemistry*, 98, 435–448.
- Lackmann, J. W., Baldus, S., Steinborn, E., Edengeiser, E., Kogelheide, F., Langklotz, S., ... Bandow, J. E. (2015). A dielectric barrier discharge terminally inactivates RNase A by oxidizing sulfur-containing amino acids and breaking structural disulfide bonds. *Journal of Physics D: Applied Physics*, 48, 494003.
- Latypov, R. F., Liu, D. J., Gunasekaran, K., Harvey, T. S., Razinkov, V. I., & Raibekas, A. A. (2008). Structural and thermodynamic effects of ANS binding to human interleukin-1 receptor antagonist. *Protein Science*, 17, 652–663.
- Li, X. R., & Geng, M. J. (2016). Probing the binding of procyanidin B3 to trypsin and pepsin: A multi-technique approach. *International Journal of Biological Macromolecules*, 85, 168–178.
- Li, L., Jiang, J. F., Li, J. G., Shen, M. C., He, X., Shao, H. L., & Dong, Y. H. (2014). Effects of cold plasma treatment on seed germination and seedling growth of soybean. *Scientific Reports*, 4, 5859.
- Liener, I. E. (1994). Implications of antinutritional components in soybean foods. *Critical Reviews in Food Science and Nutrition*, 34, 31–67.
- Liu, X. R., Hong, W. W., & Deng, Y. H. (2013). Determination of thiol content in thiolated chitosan by 4,4'-dithiodipyridine. *Shenyang Yaoke Daxue Xuebao*, 30, 120–125.
- Lu, B. Y., & Chang, J. Y. (2010). Rapid and irreversible reduction of protein disulfide bonds. *Analytical Biochemistry*, 405, 67–72.
- Misra, N. N., Pankaj, S. K., Segat, A., & Ishikawa, K. (2016). Cold plasma interactions with enzymes in foods and model systems. *Trends in Food Science & Technology*, 55, 39–47.
- Motrescu, I., Ogino, A., Tanaka, S., Fujiwara, T., Kodani, S., Kawagishi, H., ... Nagatsu, M. (2011). Mechanism of peptide modification by low-temperature microwave plasma. *Soft Matter*, 7, 4845–4850.
- Niemira, B. A. (2012). Cold plasma decontamination of foods. *Annual Review of Food Science and Technology*, 3, 125–142.
- Pankaj, S. K., Bueno-Ferrer, C., Misra, N. N., Milosavljević, V., O'Donnell, C. P., Bourke, P., ... Cullen, P. J. (2014). Applications of cold plasma technology in food packaging. *Trends in Food Science & Technology*, 35, 5–17.
- Priya Arjunan, K., & Morss Clyne, A. (2011). Hydroxyl radical and hydrogen peroxide are primarily responsible for dielectric barrier discharge plasma-induced angiogenesis. *Plasma Processes and Polymers*, 8, 1154–1164.
- Rehman, M. U., Jawaid, P., Uchiyama, H., & Kondo, T. (2016). Comparison of free radicals formation induced by cold atmospheric plasma, ultrasound, and ionizing radiation. *Archives of Biochemistry and Biophysics*, 605, 19–25.
- Roychaudhuri, R., Sarathi, G., Zeece, M., & Markwell, J. (2004). Stability of the allergenic soybean Kunitz trypsin inhibitor. *Biochimica et Biophysica Acta (BBA) – Proteins and Proteomics*, 1699, 207–212.
- Shaw, A., Shama, G., & Iza, F. (2015). Emerging applications of low temperature gas plasmas in the food industry. *Biointerphases*, 10, 029402.
- Smith, J. C., Wilson, F. D., Allen, P. V., & Berry, D. L. (1989). Hypertrophy and hyperplasia of the rat pancreas produced by short-term dietary administration of soya-derived protein and soybean trypsin inhibitor. *Journal of Applied Toxicology*, 9, 175–179.
- Song, H. K., & Suh, S. W. (1998). Kunitz-type soybean trypsin inhibitor revisited: refined structure of its complex with porcine trypsin reveals an insight into the interaction between a homologous inhibitor from *Erythrina caffra* and tissue-type plasminogen activator. *Journal of Molecular Biology*, 275, 347–363.
- Sun, C. C., Wu, T., Liu, R., Liang, B., Tian, Z. J., Zhang, E. Q., & Zhang, M. (2015). Effects of superfine grinding and microparticulation on the surface hydrophobicity of whey protein concentrate and its relation to emulsions stability. *Food Hydrocolloids*, 51, 512–518.
- Tetenbaum, J., & Miller, L. M. (2001). A new spectroscopic approach to examining the role of disulfide bonds in the structure and unfolding of soybean trypsin inhibitor. *Biochemistry*, 40, 12215–12219.
- Thirumud, R., Sarangapani, C., & Annapure, U. S. (2015). Cold plasma: A novel non-thermal technology for food processing. *Food Biophysics*, 10, 1–11.
- Tidke, S. A., Ramakrishna, D., Kiran, S., Kosturkova, G., & Ravishankar, G. A. (2015). Nutraceutical potential of soybean: review. *Asian Journal of Clinical Nutrition*, 7, 22–32.
- van Boekel, M., Fogliano, V., Pellegrini, N., Stanton, C., Scholz, G., Lalljie, S., ... Eisenbrand, G. (2010). A review on the beneficial aspects of food processing. *Molecular Nutrition & Food Research*, 54, 1215–1247.

- van der Ven, C., Matser, A. M., & van den Berg, R. W. (2005). Inactivation of soybean trypsin inhibitors and lipoxygenase by high-pressure processing. *Journal of Agricultural and Food Chemistry*, 53, 1087–1092.
- Wagner, J. R., Sorgentini, D. A., & Añón, M. C. (2000). Relation between solubility and surface hydrophobicity as an indicator of modifications during preparation processes of commercial and laboratory-prepared soy protein isolates. *Journal of Agricultural and Food Chemistry*, 48, 3159–3165.
- Weizman, Z. (2004). Vegetarian diet and exocrine pancreatic function using fecal tests. *Journal of Pediatric Gastroenterology & Nutrition*, 39, 212–213.
- Xia, Y., & Cooks, R. G. (2010). Plasma induced oxidative cleavage of disulfide bonds in polypeptides during nanoelectrospray ionization. *Analytical Chemistry*, 82, 2856–2864.
- Zhang, H., Xu, Z. M., Shen, J., Li, X., Ding, L. L., Ma, J., ... Chu, P. K. (2015). Effects and mechanism of atmospheric-pressure dielectric barrier discharge cold plasma on lactate dehydrogenase (LDH) enzyme. *Scientific Reports*, 5, 10031.