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Inhibitory activity and conformation changes of soybean trypsin inhibitors induced by ultrasound

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Abstract

The inhibitory activities, sulfhydryl bonds and far-UV circular dichroism (CD) spectra of Kunitz and Bowman–Birk soybean trypsin inhibitors (KTI and BBTI) were measured before and after ultrasound treatments. The differences between KTI and BBTI in conformation changes and resistance to ultrasound were observed. The secondary structures of KTI were found to be composed of β-sheet (22.5%), β-turn (16.2%) and random coils (61.4%) while that of BBTI composed of only β-sheet (52.6%) and random coils (47.4%). KTI lost its inhibitory activity more quickly than BBTI, proportionally to the ultrasound amplitudes and sonication durations used. Relevant synchronous phenomena observed included the inactivation of KTI, significant rise in sulfhydryl content and corresponding conformation changes, in which there were dramatic decreases in both β-turn and random coil contents and increase in the β-sheet structure over the entire sonication duration and ultrasonic amplitude scale used by the study. Ultrasound affected the activities and conformations of KTI and BBTI by exerting an influence on their disulfide bonds. For KTI, up to 55% of inhibitory activity could be inactivated, at which about 71.5% of disulfide bonds were altered and the $[\theta]_{200nm}$ value was changed from native -2545 deg cm^2 dmol^{-1} to -1827 deg cm^2 dmol^{-1}. Whereas for BBTI, far-UV CD spectra, β-sheet and random structures were barely affected, only about 5.29% of disulfide bonds were found altered and the $[\theta]_{200nm}$ value was changed only from native -797 deg cm^2 dmol^{-1} to -700 deg cm^2 dmol^{-1}. It is concluded that ultrasound inactivates KTI by inducing a reduction in the disulfide bonds and then changes the conformations.

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Keywords: Trypsin inhibitor; Ultrasound; Inhibitory activity; Conformation

1. Introduction

Trypsin inhibitors (TI) in soybean are classified as small proteins or polypeptides that exhibit inhibitory activity against trypsin and can lead to certain diseases in animals and human, such as pancreatic hypertrophy and hyperplasia which could cause growth inhibitions in rats, mice and chickens [1]. Hence they are considered as the major anti-nutritional factors presented in processed food or forage from soybean. The measures employed to deactivate the soybean trypsin inhibitor (STI) are usually physical or chemical. High temperature heating is an effective physical method for deactivation of STI and the kinetics was well studied [2–4]. But high temperature may lead to browning of the soymilk products and destruction of cystine, arginine and lysine, thus causing nutrient lost of the products. Chemical methods include chemical modifying, reducing, clearing and blocking some of groups in the reactive sites of TI [5–9]. However these methods may result in the

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remaining residual of some chemicals. Ultrasound irradiation, being unique in its physical and chemical effects, is well documented as effective not only in the inactivation but also in the activation of different enzymes, depending on the nature of enzymes and the conditions of treatments [10–14]. Hence this technique can provide with a new synergistic tool in balancing the inactivation of STI while keeping the nutrition quality of soymilk products.

The Kunitz trypsin inhibitor (KTI) and the Bowman–Birk trypsin inhibitor (BBTI) are the two major types of TI found in soybean with different properties and structures. KTI includes several iso-forms with a high degree of homology. In fact, there are great differences between KTI and BBTI in their resistance against physical and chemical deactivation. It is well known that besides a stronger inhibitory activity on trypsin, BBTI also exhibits more resistance against heating, acid and proteolysis by pepsin than KTI owing to its different molecular compositions and conformations. These differences usually make it difficult to deactivate TIs completely during food processing. Hence it is of prime importance to understand the resistance of TIs against physical and chemical factors and the corresponding mechanisms in order to adopt appropriate measures to completely deactivate them. It has been demonstrated that ultrasound irradiation can change the activities of certain peptides, proteins and enzymes, and improve their functions in food significantly by affecting their conformations. Ku et al. [15] found that when heat and ultrasonic hurdles were applied to ascorbate oxidase, considerable reduction in its activity was observed compared to the treatment with other hurdles. Lopez et al. [16] had studied the combined effects of heat and ultrasound on the inactivation of peroxidase, polyphenol oxidase and lipoxygenase over a broad range of temperature, static pressure and ultrasound amplitude, and a synergistic effect, which can substantially reduce the resistances of these enzymes and thus the heat treatment required for their inactivation, was observed in all cases. However few studies had been done on linking the inhibitory activity of STI to its secondary structure affected by ultrasound. The intention of this paper was to study the effects of ultrasound on the inhibitory activities and conformations of KTI and BBTI, thus to verify the relationships between activity and conformation of STIs.

2. Materials and methods

2.1. Ultrasound treatment

Two types of soybean trypsin inhibitors, KTI (type I-S) and BBTI (both purchased from Sigma Chemical Co., Louis, USA) were individually prepared at 200 μg/ml with deionized water (pH 7.0) for ultrasound treatment and circular dichroism (CD) analysis. During the ultrasound treatment, 15 ml of the KTI and BBTI solutions were individually pipetted into a special glass vessel (glass cooling cell with water jacket) and kept at temperature of around 25 ± 3 °C by circulating cooling water to minimize any thermal effect on the TIs. The special glass vessel was composed of an inner container for the sample solutions and an outer jacket for the cooling water connected to circular cooling water system. The circulating water was cooled to about 10 °C with a water bather (mode Julabo F10, Julabo UK Ltd., Glinton Peterborough, UK) and circulated by a console pump. An ultrasonic homogenizer (model 600-W, Cole-Parmer Instrument Company, Vernon Hills, USA) was used to generate ultrasonic wave of 20 kHz and a tapered microtip probe (6 mm in diameter) was immersed into the sample solutions at a distance of 0.5 cm from the container bottom. The volume of the solution in the container was fixed. Various ultrasound outputs were obtained by changing the wave amplitude of the ultrasound generator. The experiments for the effects of ultrasound on STIs were conducted by either changing the amplitude settings (from 25% to 65%) with a fixed processing time or changing the processing time (from 5 min to 20 min) with fixed amplitude setting. To minimize the thermal effects, a 3–3 s on-pulse mode was set. A digital thermometer was used to monitor the temperature changes of the solution.

2.2. Assay of inhibitory activities of KTI and BBTI

The inhibitory activities of KTI and BBTI against trypsin were determined according to Smith et al. [17]. One trypsin activity unit (TU) was defined as the increase of 0.01 absorbance units at 410 nm per min in 10 ml of the reaction mixture. One inhibitory activity unit (TIA) of KTI or BBTI was defined as one trypsin activity unit inhibited under the experiment conditions and procedure described below:

1. Trypsin (EC 3, 4, 21, 4 from porcine pancreas, Sigma Chemical Co., Louis, USA) was prepared at 20 μg/ml with 0.001 M HCl. The substrate solution was prepared at 9.2×10^{-4} mol/l by dissolving 40 mg benzoyl-DL-arginine-*p*-nitroanilide (BAPNA, from Acros Organics Co., Loughborough, UK) in 1 ml dimethyl sulfoxide and then diluting to 100 ml with Tris-buffer (0.05 M, pH 8.2).
2. All the above solutions were pre-warmed to 37 °C before assay.
3. Two milliliters sample trypsin inhibitor solutions with or without ultrasonic treatment, 2 ml trypsin solution and 5 ml BAPNA substrate solution were added into serial test tubes in sequence and the tubes were mixed and placed in a water bath at 37 °C for exactly 10 min. Then 1 ml 30% acetic acid solution was added into each of the tubes to terminate the reaction.
4. Blanks were prepared by adding 2 ml trypsin solution, 1 ml acetic acid solution, 5 ml BAPNA solution and 2 ml trypsin inhibitor solutions with or without ultrasonic treatment in sequence into test tubes.

5. The reacted solutions were filtered through the Whatman No. 2 paper and the absorbance at 410 nm was read on a Hitachi U-2000 UV/V spectrophotometer (Hitachi High-Technologies Corporation, Tokyo, Japan).

All the experiments were carried out in triplicate.

2.3. Measurement of sulfhydryl content

The Beveridge method was adopted to analyze the changes of sulfhydryl contents of KTI and BBTI after ultrasonic irradiation [18]. One milliliter treated sample and 0.1 ml Ellmen's reagent (4 mg/ml, solved in 0.1 M, pH 8.0 NaH_2PO_4–Na_2HPO_4 buffer) were added in sequence into 3.9 ml of NaH_2PO_4–Na_2HPO_4 buffer (0.1 M, pH 8.0, containing 1.2 g/l of NaEDTA). After staining for 5 min, the absorbance at 412 nm was read. Sample and reagent blanks were included for each determination of the triplicate and the sulfhydryl bonds were calculated based on the factor $\varepsilon_{412}(1.39 \times 10^4\ mol^{-1} \times cm^{-1})$.

2.4. Circular dichroism analysis of trypsin inhibitors

The far-UV circular dichroism (CD) spectra of the ultrasound treated KTI and BBTI solutions were measured at 25 ± 0.1 °C on a JASCO J-810 spectra polarimeter (JASCO Corporation, Tokyo, Japan). Two hundred grams per milliliters of treated KTI and BBTI solutions was placed in a 0.1 cm path-length cell and scanned in the far-UV region (from 185 nm to 250 nm) under constant nitrogen purge. The scanning speed was set at 50 nm/min and the accumulation was 3. Each spectrum was collected at an interval of 0.1 nm. Molar ellipticity $[\theta]$ was calculated by employing the formula:

$$[\theta] = \theta/(10\ Cl),$$

where θ is the ellipticity measured by the CD equipment (m deg), C the molar concentrations of the trypsin inhibitors (mol/l), and l the path-length of cell (cm).

The secondary structures of the trypsin inhibitors, including α-helix, β-sheet, β-turn and random coil, were estimated by the Model JWSSE-480 Protein Secondary Structure Estimation Program based on Yang's reference CD spectra [19].

3. Results and discussion

3.1. Changes in the inhibitory activities of KTI and BBTI induced by ultrasound

The differences in the inhibitory characteristics between KTI and BBTI against trypsin have been shown in Fig. 1. The trypsin activity decreased dramatically in the presence of KTI and BBTI. The inhibitions of trypsin by KTI and BBTI followed most of the results reported by Smith et al. [17], Laskowski and Kato [20], and McNiven et al. [21]. But as seen in Fig. 1, in order to attain a 50% inhibitory ratio (the point at which the residual trypsin activity was around 620 TU/mg), the concentrations of KTI and BBTI in this experiment were about 1.7 μg/ml and 1.2 μg/ml, respectively, indicating a stronger inhibitory activity of BBTI than that of KTI. Another difference between the two inhibitors in their inhibitory characteristics was that the trypsin activity could be inhibited almost completely by increasing the concentration of KTI to around 3.6 μg/ml, but that of BBTI was around 9 μg/ml.

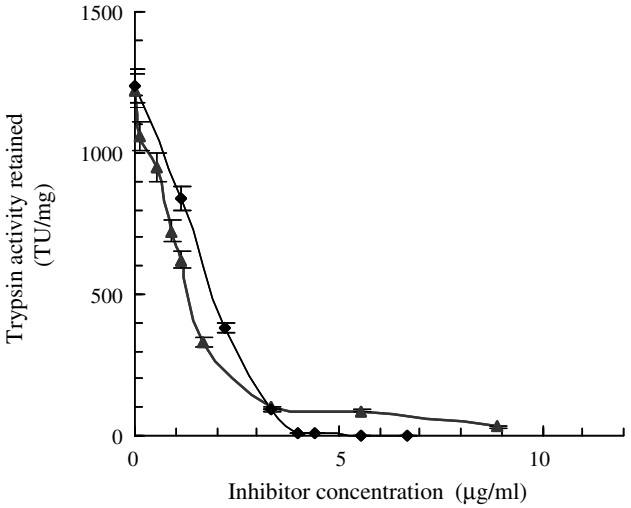

Fig. 1. Inhibition of trypsin activity by KTI (◆) and BBTI (▲). For this and the afterwards Figures, each value is the mean from three assays and vertical bars are the errors at 5%.

The effects of ultrasound irradiation on KTI and BBTI have been shown in Figs. 2 and 3. Fig. 2 showed the effects of ultrasonic amplitudes on the inhibitory activity of the two inhibitors over 9 min, and Fig. 3 showed the effects of sonication durations on the inhibitory activity of the two inhibitors with ultrasound amplitude fixed at 65%, in

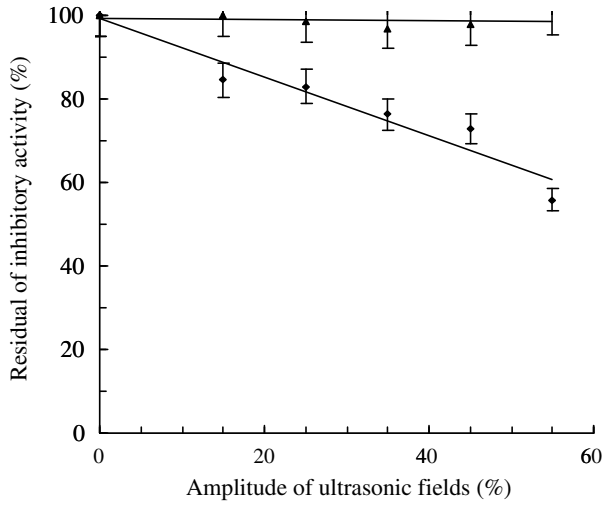

Fig. 2. Effects of ultrasound amplitudes on the inhibitory activity of KTI (◆) and BBTI (▲), with time at 9 min.

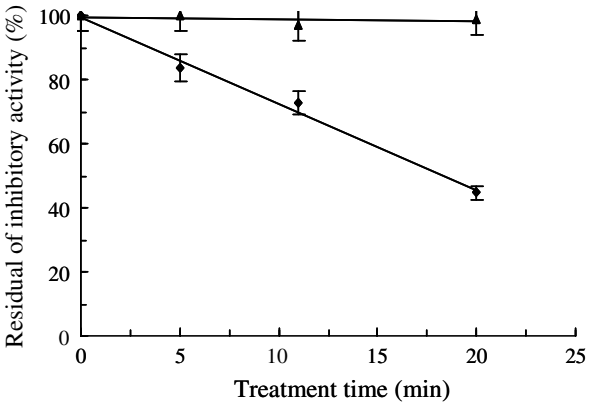

Fig. 3. Effects of sonication durations on the inhibitory activity of KTI (◆) and BBTI (▲), with amplitude at 65%.

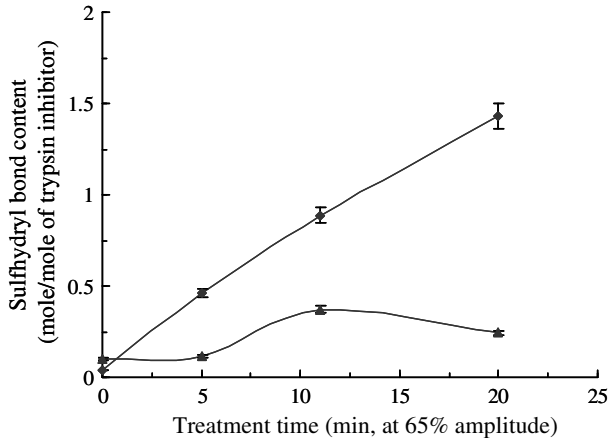

Fig. 4. Effect of ultrasound irradiation on the sulfhydryl bond content of KTI (◆) and BBTI (▲).

which the changes of the KTI and BBTI inhibitory activities were expressed by the residual percentages based on the measured inhibitory activities over their original inhibitory activities. A marked difference between KTI and BBTI in their resistance against ultrasound was observed. The steep decline curves for KTI indicated that it lost its inhibitory activity more rapidly than BBTI under the same ultrasonic conditions. Basically the inactivation of KTI was proportional to the ultrasound amplitudes (i.e. power levels) and sonication durations. When the ultrasound amplitude was raised to 55%, about 45% of the KTI inhibitory activity was found lost. Similarly, as shown in Fig. 3, when the sonication duration was extended to 20 min at fixed amplitude of 65%, about 55% of the KTI inhibitory activity was lost. In contrasted to KTI, the gentle curves for BBTI in Figs. 2 and 3 showed that BBTI lost very few of its inhibitory activity over a wide ultrasonic amplitude scale and sonication durations, indicating that BBTI exhibited a stronger resistance to ultrasound irradiation than KTI.

3.2. Changes in the sulfhydryl content of KTI and BBTI induced by ultrasound

Similar to many other proteins, the composition and state of disulfide bonds of TI play crucial role in stabilizing the native conformation and contribute to the different resistances against environment factors [22]. KTI and BBTI, alike in their different resistances against acidity, enzymatic proteolysis and heating, were found to have prominent differences in their resistance against ultrasound irradiation, accompanying with corresponding synchronous changes in the contents of the sulfhydryl bonds. It was found that changes in the sulfhydryl contents of KTI and BBTI were basically coincident with changes in their inhibitory activities. As shown in Fig. 4, an increase in sulfhydryl bonds of both KTI and BBTI, which was transformed from the reduction of the disulfide bonds of KTI and BBTI, was observed at the initial duration of the ultrasound treatments, with the increasing trend of the sulfhydryl bonds of KTI was more dramatic than that of BBTI. The content of the sulfhydryl bonds of KTI at the 20th minute of irradiation was about 1.43 mol/(mole of KTI), which accounted for almost 71.5% of the original disulfide bonds of KTI; while for BBTI, the maximum content of the sulfhydryl bonds was 0.37 mol/(mole of BBTI) at the 11th minute of the sonication duration, which was accounted for only about 5.29% of the original disulfide bonds of BBTI, indicating the disulfide linkages of KTI were more easily to be broken down under ultrasound irradiation than that of BBTI. This phenomenon indicated that the differences in ultrasound resistance between KTI and BBTI might come not only from their sulfhydryl composite (BBTI contains seven disulfide bonds while KTI contains only two), but also from their disulfide linkages. Free radicals inside the oscillating bubble have been proven as one of the main effects of ultrasound and Friedman et al. [22] considered that free radicals induced from ultrasound irradiation were involved in the transformation of disulfide linkage of STI. Under attack of the free radicals, the disulfide linkage (R–S–S–R′) is transformed to a terminal thiol group (R–SH and R′–SH, sulfhydryl). Hence it is apparent that ultrasound irradiation changed the inhibitory activity of STI by way of inducing the reduction in the disulfide bonds.

3.3. Changes in the secondary conformations of KTI and BBTI induced by ultrasound and their CD spectra

A number of researches had been conducted with CD datum analysis to study the relationship between the functions and the higher order structures of proteins since it was discovered that CD datum reflects faithfully the secondary structures of proteins including α-helix, β-sheet, β-turn and random coils [23–25]. Yet few researches conducted on the CD spectrum of STI were reported. Most information about STI structure came from some X-ray and nuclear magnetic resonance researches. The far-UV spectra of KTI and BBTI and their changes induced by different ultrasound irradiations were shown in Figs. 5 and 6.

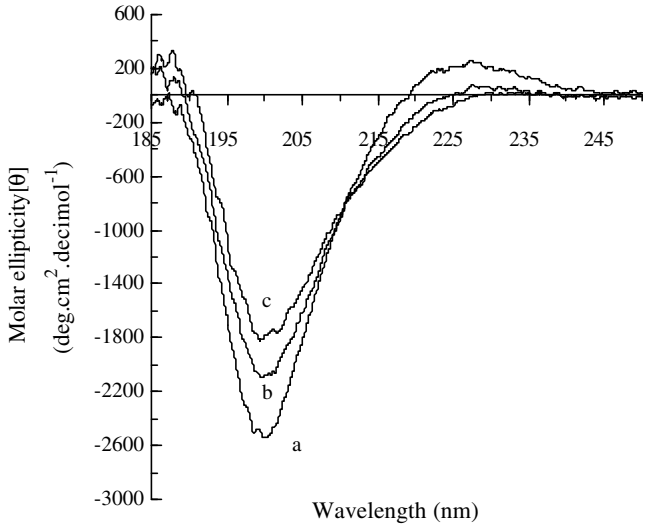

Fig. 5. CD spectrum changes of KTI treated with ultrasound: native (a); with 55% amplitude and 9 min (b); with 65% amplitude and 20 min (c).

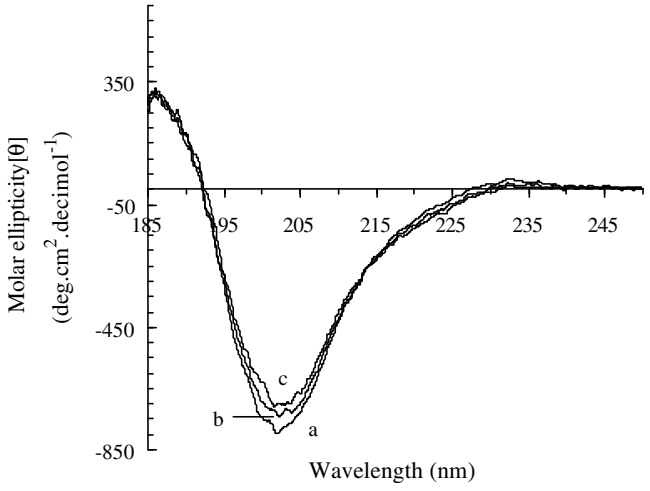

Fig. 6. CD spectrum changes of BBTI treated with ultrasound: native (a); with 55% amplitude and 9 min (b); with 65% amplitude and 20 min (c).

Both native KTI and BBTI showed a single negative peak curve on their far-UV CD spectra. It could be observed that neither native KTI nor native BBTI exhibited a double negative peak which represents the typical α-helix structure. The minimum values of the molar ellipticity at around 200 nm ($[\theta]_{200nm}$,) were -2545 deg cm^2 dmol^{-1} (negative peak) for native KTI and -797 deg cm^2 dmol^{-1} for native BBTI, which represents the characteristics of the random coil and β-sheet structure. This indicated that the β-sheet and random coil structure dominated in the secondary structures of KTI and BBTI. The calculated results by computer software based on Yang's reference showed that the percentages of the secondary structure of native KTI with a molecular of 21.5 kDa were 22.5% for β-sheet, 16.2% for β-turn and 61.4% for random coil, respectively. Native BBTI, with a less molecular weight of 7.95 kDa and exhibiting a more simple structure than KTI, composed of only 52.6% for β-sheet and 47.4% for random coil (Tables 1 and 2). No α-helix structure (a structure characterized by a negative peak at around 222 nm) was found in KTI or BBTI, which was in basic agreement with the works of Meester, Song, and Tetenbaum [26–28].

The effects of ultrasound on the CD spectrum of KTI and BBTI were clearly observed in Figs. 5 and 6. Matching the dramatic changes in the inhibitory activity and the sulfhydryl bond content (refers to Figs. 2–4), the CD spectrum of KTI exhibited a dramatic change under ultrasound, and the negative peak at 200 nm increased gradually with the increases of both sonication duration and amplitude (Fig. 5). The value of $[\theta]_{200nm}$ was found changed from -2545 deg cm^2 dmol^{-1} at the native state to -1827 deg cm^2 dmol^{-1} at the treatment of 65% ultrasound amplitude for 20 min. Among the four secondary structures, β-sheet and β-turn of KTI were affected dramatically by ultrasound irradiation. As shown in Tables 1 and 2, the β-sheet of KTI was observed to have increased from native 22.5% to maximum 50.7%, β-turn decreased from native 16.2% to zero by the treatment of 55% ultrasound amplitude and 9 min irradiation, while the random coil was changed insignificantly. In contrast, the CD spectrum of BBTI (Fig. 6) remained unchanged basically under various ultrasonic treatments; matching with its unchanged inhibitory activity and sulfhydryl bond content (Figs. 2–4). The value of $[\theta]_{200nm}$ was found changed only from native -797 deg cm^2 dmol^{-1} to -700 deg cm^2 dmol^{-1} at the treatment of 65% ultrasound amplitude for 20 min. Tables 1 and 2 showed that its secondary structures (β-sheet and random coil) remained unchanged basically in various

Table 1
Changes of the secondary structure of KTI and BBTI in different ultrasound amplitudes (at sonication time of 9 min)

Treatment time	KTI					BBTI				
	α-Helix (%)	β-Sheet (%)	β-Turn (%)	Random (%)	Total (%)	α-Helix (%)	β-Sheet (%)	β-Turn (%)	Random (%)	Total (%)
0	0	22.5	16.2	61.4	100	0	52.6	0	47.4	100
15%	0	26.6	13.6	59.8	100	0	52.7	0	47.3	100
25%	0	30.4	13.1	56.6	100	0	53.0	0	47.0	100
35%	0	31.7	12.4	55.9	100	0	52.6	0	47.4	100
45%	0	34.2	10.9	54.9	100	0	53.1	0	46.9	100
55%	0	50.7	0	49.3	100	0	53.1	0	46.9	100

Table 2
Changes of the secondary structure of KTI and BBTI in different sonication times (at ultrasound amplitude of 65%)

Treatment time	KTI					BBTI				
	α-Helix (%)	β-Sheet (%)	β-Turn (%)	Random (%)	Total (%)	α-Helix (%)	β-Sheet (%)	β-Turn (%)	Random (%)	Total (%)
0	0	22.5	16.2	61.4	100	0	52.6	0	47.4	100
5 min	0	28.1	13.1	58.8	100	0	52.7	0	47.3	100
11 min	0	31.6	11.5	57.0	100	0	52.6	0	46.1	100
20 min	0	35.2	10.8	54.0	100	0	53.6	0	46.4	100

ultrasound treatments. It has been known that, when applied to liquids, the effective mechanisms induced by ultrasound usually include: cavitation (formation and violent collapse of bubbles), thermal effect, dynamic agitation and shear stresses, micro-streaming, and generation of free radicals inside the oscillating bubble. Because the sample temperatures in this research were kept within a relative lower and stable range (25 ± 3 °C) over all the experiments, the influence of the thermal effect was minimized. The inactivation of STI induced by ultrasound and its different effects on KTI and BBTI were reasonably accounted from other mechanisms such as cavitation and free radical generation. Further studies are necessary in order to understand how the physical and chemical effects generated during acoustic cavitation cause the observed changes. From all of these relevant synchronous phenomena observed, including the increase of sulfhydryl contents in KTI, the corresponding conformation changes in the β-turn, β-sheet and random coil content, and the inactivation of KTI, a close relationship between the secondary conformations and the inhibitory activity of the STI could be inferred. The different presentations to ultrasound irradiation between KTI and BBTI might be due to their differences in disulfide bonds and secondary conformation reliability. Ultrasound inactivates KTI by causing the reductions in the disulfide bonds and then the conformations changed.

4. Conclusion

The inactivation effects of ultrasound irradiation on STI and the different influences on KTI and BBTI resulted from other mechanisms rather than the thermal effect of ultrasound. The differences in the disulfide bonds and the secondary conformations between KTI and BBTI account for the differences in the resistance against ultrasound. The relevant synchronous phenomena observed in this study, including the inactivation of KTI, the conformation changes and the increase in the sulfhydryl content of KTI, indicated that the inhibitory activity of STI could be affected by ultrasound which exerts an influence on the disulfide bonds. The findings further showed that, compared to that of BBTI, the disulfide linkages (R–S–S–R′) of KTI was more easily broken down, transformed to terminal thiol group (R–SH and R′–SH) and finally caused the secondary conformations changed.

Acknowledgements

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