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Effect of ultrasonic pre-treatment on Ara h 1 in peanut sprouts

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ABSTRACT

Ara h 1 is the most abundant sensitizing protein in peanuts; it has high thermal stability and is difficult to degrade. The peanut sprout is a high-quality, natural food that has various beneficial effects and lower allergenicity than peanut seeds. In this study, ultrasonication (US) of peanut sprouts was used to alter their Ara h 1 content. We determined that the optimal parameters for the US process were 35 °C temperature, 30 min duration, 240 W power, and 100 kHz frequency. After 5 days of germination, the protease activity of the control (blank) group increased to 262.39 ± 0.10 U, whereas that of the US group increased to 290.1 ± 0.25 U. We also investigated the effects of US on Ara h 1 protein composition, structure, and related gene expression during germination. ELISA results showed that after 5 days of germination, Ara h 1 content in the blank group decreased from 20.63 ± 0.31 ppm to 3.35 ± 0.42 ppm, whereas in the US group, they decreased to below the detection limit. SDS-PAGE bands between 50 and 70 kDa from peanut sprout extracts gradually became lighter in both groups. The band almost disappeared at day 5 of germination in the US group, indicating that US reduced the Ara h 1 content of peanut sprouts, consistent with the ELISA results. The expression of the *Ara h 1* gene in peanut seeds was 173.92 ± 26.37. In the BK control group, it decreased to 0.49 ± 0.17 on the fourth day and increased slightly to 0.75 ± 0.09 on the fifth day. In the US group, it decreased to 1.37 ± 0.28 on the first day, dropped sharply to 0.00 on the third day, and increased slightly to 0.04 ± 0.01 on the fourth and fifth days. Protein structure results showed that the α-helix structure of Ara h 1 decreased after US, whereas the content of β-fold structures increased. The surface hydrophobicity decreased, and the secondary and tertiary structures of Ara h 1 were loose.

1. Introduction

Peanut (*Arachis hypogaea*) is a very nutritious and economically important crop worldwide. It contains healthy fatty acids, high levels of fiber and protein, and numerous compounds that can directly scavenge free radicals [1]. The peanut sprout is a type of sprout vegetable that grows into an edible seedling after peanut seed germination. It is more easily absorbed by the human body and has more functions than peanut seeds, including antioxidant and anti-aging properties [2,3].

In recent years, researchers have shown great interest in the peanut sprout. They have studied the resveratrol, total phenolic, and flavonoid contents of peanut seeds and sprouts, as well as their antioxidant capacity. The anti-inflammatory effects of peanut sprouts have been linked to their polyphenol content and antioxidant capacity [3]. Studies of caffeic acid enrichment in peanut sprouts [4] have shown that total phenolics and flavonoids increase greatly after germination [5]. Lee studied microcapsules containing powdered peanut sprout extract placed into milk or yogurt [6–8], and Yang studied the anti-allergenic effects of peanut sprouts [9].

Although the peanut seed has a high nutritional value, it can also cause a severe allergic reaction. Peanut allergy is considered to be among the most severe food allergies [10], with a high allergic rate and reaction severity [11,12]. A large body of research has focused on reducing peanut allergens. The application of pulsed UV light to peanuts and peanut butter was reported to effectively reduce the IgE binding of peanut extracts and peanut butter [13]. Gamma irradiation also reduced the IgE binding of whole peanut protein extract and Ara h 6 due to loss of the latter's α-helix structure [14]. Another study reported that pressures above 300 MPa (400, 500, and 600 MPa) significantly reduced the immunoreactivity of the purified peanut allergen Ara h 2 in extracts

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[15].

Ara h 1 is considered to be the primary peanut allergen, and it comprises the major fraction of peanut protein [16]. Ara h 1 is a 7S globulin of the vicilin protein family; it has a molecular weight of ~ 63 to 65 kDa and an isoelectric point (pI) of 4.55, and it constitutes 12% to 16% of peanut protein [17]. Li found that Ara h 1 immune response was reduced by one third after five to seven days of peanut germination [18]. Yu used ultrasonication (US) to increase resveratrol and reduce the allergenicity of peanut sprouts [19].

US is a novel technology that has recently found wide application in food processing, but to date there have been few reports of Ara h 1 reductions in peanut sprouts following ultrasound treatment. Accordingly, we investigated the effects of ultrasound on the Ara h 1 content of peanut sprouts. To the best of our knowledge, this is the first study to report decreased Ara h 1 in peanut sprouts after ultrasound treatment of peanut seeds.

2. Materials and methods

2.1. Peanut seeds and chemicals

Seeds of the peanut cultivar Fuhua 23 were supplied by the Liaoning Academy of Agricultural Sciences in mid-October 2018. The chemicals and solvents used in this study were of analytical or high-performance liquid chromatography (HPLC) grade and were purchased from Sinopharm Chemical Reagent Co., Ltd. (Beijing, China) or Solarbio (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China). The kit used to measure Ara h 1 content was purchased from Chundu Biotech Co., Ltd. (Wuhan, China). The kits used for quantitative polymerase chain reaction (qPCR) were purchased from Sinogene Biotech Co., Ltd. (Beijing, China) and Thermo Fisher Scientific Inc. (Waltham, MA, USA).

2.2. US pre-treatment

Fresh peanuts were shelled in advance, and all processing implements and surfaces were washed and sanitized with 1% sodium hypochlorite before use. Peanut kernels were weighed into a set of beakers (100 seeds per beaker) and surface-sanitized in 500 ml of 1% sodium hypochlorite solution for 15 min. After washing and rinsing the seeds with sterilized deionized water, the samples were sonicated in a US cleaner bath (KQ-300VDE, Kunshan Ultrasonic Instrument Co., Ltd., Kunshan City, China) for 30 min at 35 °C, 100 kHz, and 240 W. After US treatment, peanuts were imbibed for 6–8 h at 25 °C in the dark. Control kernels were treated identically but were not subjected to US treatment.

2.3. Peanut germination

The samples were divided into two groups: the US group (US) and the blank group (BK). For each group, peanut kernels were placed on a ceramic tray and germinated in the dark in a growth chamber (BD-ZGX-400G-4P, Nanjing Beidi Instrument Co., Ltd., Nanjing City, China) at 27 °C for 5 days. Sterile water was changed every 12 h, and samples were collected every 24 h. After germination, one part of the sample was used immediately for measurements, and the remainder was freeze-dried (FreeZone 2.5L + CentriVap, Labconco Corporation, USA) and stored at −80 °C (MDF-U54V, Sanyo, Japan).

2.4. Protease activity measurement

Protease activity in the peanut sprouts was measured according to the method of Miao et al. [20]. One gram of fresh peanuts/peanut sprouts was combined with 8 ml 0.05 M Tris HCL buffer (pH 7.4, containing 1% PVP, 10 mM β-mercaptoethanol, and 1 mM EDTA) and ground in an ice bath. The mixture was centrifuged (10000 rpm, 4 °C) (TGL20M, Hunan Xiangyi Laboratory Instrument Development Co., Ltd., China) for 30 min, and the protease activity of the supernatant was measured. One milliliter of pre-made 2% casein substrate solution was mixed with 1 ml of the enzyme extract. The mixture was kept at 40 °C for 20 min, then moved to a 90 °C water bath for 5 min to inactivate the enzymes (HH-4, Changzhou Guohua Electric Appliance Co., Ltd., China). Next, 3 ml of 0.4 M trichloroacetic acid (TCA) solution was added to react with the unreacted protein at room temperature for 15 min. Protease activity was calculated by measuring the change in OD_{275nm}, and a mixture to which TCA solution had been added before the reaction was used as the blank. One unit of enzyme activity was defined as a 0.01 per hour increase in the OD value at 275 nm (UV-5100, Shanghai Metash Instruments Co., Ltd., China).

2.5. Enzyme-linked immunosorbent assay (ELISA) inhibition assay

Concentrations of Ara h 1 in peanut sprouts and extracts were measured according to the protocol of the sandwich ELISA kit based on the intensity of a blue to yellow color change measured at 450 nm with a microplate reader. To measure the Ara h 1 concentration of the sample, the kit included a set of calibration standards used to produce a standard curve of OD versus Ara h 1 concentration. The concentration of Ara h 1 in the samples was then determined by comparing the sample ODs to the standard curve.

Ground peanut/peanut sprout samples were mixed with a 20 × volume of sample buffer, extracted at 60 °C for 10 min, and centrifuged at 2500 g and 4 °C for 10 min (SHZ-88, Jintan District Baita Jinchang Experimental Instrument Factory, China). A sample of the supernatant (10 μl) was combined with 40 μl of sample diluent in a well, 100 μl of HRP-conjugate reagent was added, and the mixture was incubated for 60 min at 37 °C. Each well was then aspirated and washed, and the process was repeated four times for a total of five washes. After inverting the microplate and blotting it on clean paper towels, 50 μl each of chromogen solution A and chromogen solution B were added to each well, gently mixed, and incubated for 15 min at 37 °C in the dark. Stop solution (50 μl) was then added to each well, followed by 100 μl of HRP-conjugate reagent, and the mixture was incubated for 60 min at 37 °C. The OD at 450 nm was then measured on a microplate reader within 15 min.

The defatted peanut powder in each sample was calculated using standard curves developed with purified Ara h 1 using the ELISA procedure described above. The final results were calculated as PPM Ara h 1 and were expressed as the mean ± standard deviation of three replications.

2.6. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE)

2.6.1. Degreasing treatment

Peanut kernels contain as much as 43%–52% fat. To reduce interference in subsequent experiments, the freeze-dried, powdered peanuts/peanut sprouts were defatted, and the protein was extracted. One gram of powder from each treatment was mixed with 10 ml acetone and stirred at 4 °C for 2 h. Mixtures were then centrifuged at 3000 g (TGL-10G, Shanghai Anting Scientific Instrument Factory Co., Ltd., China) for 10 min, and the precipitate was obtained. The precipitate typically required three to four rounds of degreasing until the supernatant was transparent. The precipitate was then naturally air dried in the fume hood.

2.6.2. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was used to separate proteins extracted from the degreased powder using an SDS Kit obtained from Solarbio (Sinopharm Chemical Reagent Co., Ltd, Beijing, China). The SDS-PAGE method was modified from that described in Meng et al. [21] and used a Mini Protein Tetra System (BioRad, Hercules, CA, USA). Each electrophoresis sample (2.0 mg) was placed in a 1.5-mL centrifuge tube. Soluble protein extracts

were mixed with an equal volume of sample buffer containing 1% β-mercaptoethanol. The mixture was boiled for 5–10 min for denaturation. Electrophoresis was performed on 13% acrylamide gels for 40 min at 80 V, followed by 3 h at 120 V. Protein marker standards were used to identify and estimate the major allergens and their molecular masses. To quantify the peanut major allergen Ara h 1, gels were scanned and analyzed with a Molecular Imager (AI600UV, GE Analytical Instruments, USA). The major allergens and their relative composition were calculated based on the band intensity and total area of their subunits.

2.7. Extraction of peanut sprout proteins

2.7.1. Degreasing treatment

Degreasing was performed as described in Long et al. [15] with modifications. One gram of peanut or peanut sprout powder from each treatment was mixed with 10 ml of acetone, stirred at 4 °C for 2 h, and then centrifuged at 3000 g for 10 min to collect the precipitate. The above steps were repeated three times, and the precipitate was dried in the fume hood overnight.

2.7.2. Peanut protein extraction

Degreased peanut powder and protein extraction buffer (pH 8.0, 50 mM Tris HCl buffer) were mixed at a mass to volume ratio of 1:10, magnetically stirred at 4 °C for 2 h, and then centrifuged at 10,000 g and 4 °C for 30 min. The supernatant was collected and stored at −20 °C for future use.

2.7.3. Fractional precipitation by ammonium sulphate

Peanut protein extract was measured and precipitated with 80% and 100% ammonium sulfate as described previously [22]. SDS-PAGE electrophoresis was used to analyze the supernatant and the precipitated solution. Then 100% precipitated complex solution was placed into a dialysis bag with a molecular weight cutoff of 3.5 kDa for dialysis treatment. The dialysate was Tris HCl buffer (pH 8.0, 50 mM). After dialysis, the material was stored at −20 °C for further use.

2.7.4. Purification of peanut allergen protein Ara h 1

Anion exchange chromatography of peanut sprouts was performed with an AKTA pure chromatography system (GE) and a DEAE Fast Flow column (GE Healthcare Company). The column was equilibrated until the baseline became smooth. Then a protein sample (5% of the column volume) was loaded onto the column. By continuously increasing the concentration of sodium chloride (dissolved in Tris-HCl as an elution buffer) to 0.4 M at a flow rate of 1.0 ml/min, the eluted proteins were monitored at 280 nm and then collected in the receptor machine. SDS-PAGE was subsequently performed to identify the components.

2.7.5. Identification of Ara h 1 by UV scanning

The purified protein samples were scanned with a UV spectrophotometer (TU-1810, Beijing Purkay General Instrument Co. Ltd., China) with a wavelength range of 200–450 nm and a spectral interval of 1 nm.

2.7.6. Analysis of Ara h 1 protein structure by fourier transform infrared spectroscopy

The purified Ara h 1 was scanned by infrared spectroscopy (TU-1810, Thermo Fisher Technology Co., Ltd, USA) to analyze changes in its secondary structure. The purified protein was diluted to 0.5 mg/ml and pressed flat into the sample chamber for spectral scanning. The scanning band was 4000–525 cm^{-1}, the resolution was 4 cm^{-1}, and the sample was scanned 16 times.

2.8. Analysis of Ara h 1 by quantitative polymerase chain reaction (qPCR)

Actin was used as the reference gene to measure the expression of *Ara h 1*. Fresh peanuts/peanut sprouts (50–100 mg) were homogenized in liquid nitrogen, mixed with 1 ml TRIzol, and kept for 5 min at room temperature. Then 200 μl of chloroform was added and the mixture was shaken vigorously for 15 sec. After standing for 5 min at room temperature, it was centrifuged for 10 min at maximum speed. The supernatant (400 μl) was mixed with 400 μl isopropanol, allowed to stand for 5 min, and centrifuged for 10 min. The supernatant was discarded, and the pellet was washed once with 75% ethanol, air dried for 5 min, dissolved in 20–50 μl DEPC-treated water, and kept at −80 °C.

3. Results and discussion

3.1. Effect of US treatment on peanut sprout length

As shown in Fig. 1, sprout length was higher in the US group than in the BK group. After 5 days of germination for sprout preparation, germinated sprout lengths of the BK and US groups were 27.26 ± 0.19 mm and 32.25 ± 0.25 mm, respectively. US technology has been shown to accelerate the imbibition process of several legume grains [23]. Our results were consistent with a previous study by Chiara et al. in which US treatment of seeds increased their water uptake without altering seed morphology or seed coat wettability, although it did induce slight chemical modifications to the outer part of the seed [24]. US also reduced the duration of the imbibition process by approximately 50% and promoted higher germination rates in paddies, which were beneficial for the commercial malting of grains [25]. Therefore, US can improve peanut seed vigor and germination rate.

3.2. Effect of ultrasound treatment on the protease activity of peanut sprouts

Changes in protease activity during seed germination are shown in Table 1. Protease activity increased significantly during germination; it rose from 75.74 ± 0.17 U to 262.39 ± 0.10 U in the BK group and from 89.15 ± 0.08 U to 290.1 ± 0.25 U in the US group. Protease activity was lowest in fresh seeds of both groups and was consistently higher in the US group. This result is caused mainly by the acoustic cavitation effect of the US energy, in which the expansion and collapse of microbubbles causes transient high temperatures and pressures, which strengthen the heat and chemical effect [26].

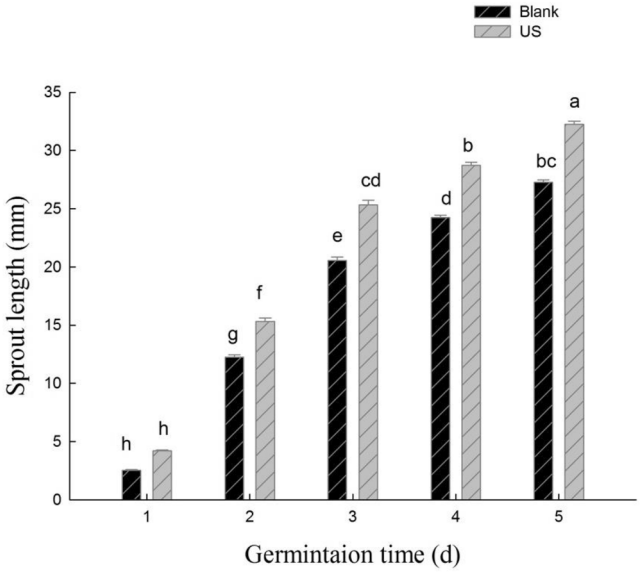

Fig. 1. Peanut sprout length at different germination times.

Table 1
Protease activity of peanut sprouts at different germination times.

Germination times (d)	Protease activity in peanut sprouts (U/g)	
	BK	US
0	75.74 ± 0.17 [a*]	89.15 ± 0.08[b]
1	94.40 ± 0.18 [a]	98.83 ± 0.10[b]
2	112.64 ± 0.05 [a]	159.99 ± 1.14[b]
3	143.19 ± 0.05 [a]	168.56 ± 0.07[b]
4	208.69 ± 0.04 [a]	229.34 ± 0.06[b]
5	262.39 ± 0.10 [a]	290.1 ± 0.25[b]

BK: Peanuts or peanut sprouts with no treatment
US: Peanuts or peanut sprouts that received ultrasonic pretreatment
*Each value is the mean ± SD of three replicates. Within a column, means followed by different lowercase letters are significantly different ($p \leq 0.05$).

3.3. Result of ELISA analysis

We used ELISA to investigate changes in Ara h 1 content during peanut germination after treatment at different frequencies. As shown in Table 2, the Ara h 1 content was highest in seeds and decreased gradually with increasing germination time. After 4 days of germination, the Ara h 1 content in the blank group decreased to 3.43 ± 0.51 ppm. The Ara h 1 content of the 45 kHz US group decreased to 3.15 ± 0.51 ppm, and that of the 80 kHz US group dropped to 2.19 ± 0.34 ppm. Only the 100 kHz US group had an Ara h 1 content below the detection limit after 4 days of germination.

3.4. Effect of ultrasound treatment on protein components in peanut sprouts

There was a clear band at ~ 35 kDa that became significantly lighter after US treatment, indicating that ultrasound treatment can degrade part of the protein (Fig. 2). The bands in the blank group between 35 and 48 kDa did not change significantly between 1 and 3 days, whereas these bands in the US group became gradually lighter (Fig. 2A). The band color change between 20 and 25 kDa was not obvious, but the color was darkest on the first day and gradually lightened between 2 and 5 days, indicating that the protein macromolecule was hydrolyzed into smaller protein molecules during the growth process of peanut. The molecular weight of the target protein Ara h 1 is ~ 63.5 kDa. In Fig. 2B, the bands between 63 and 75 kDa gradually lightened during germination, and the color of these bands almost disappeared after 4 d in the US group, indicating that ultrasound treatment significantly reduced Ara h 1 content, consistent with the ELISA results. Protein is hydrolyzed into smaller proteins during the germination process, and US cavitation accelerates the speed of this hydrolysis, causing the protein content to decrease sharply [27].

We also performed anion exchange chromatography, which separated the target protein from other proteins based mainly on their different isoelectric points. Ara h 1 is an acidic protein and is therefore suitable for anion exchange chromatography. The chromatographic purification results from the four samples are shown in Fig. 3. Compared with the ammonium sulfate precipitation solution, the other proteins were significantly reduced after purification. Nonetheless, a 35-kDa protein was always present throughout the whole process. According to previous studies, this protein may be derived from the processing of Ara h 1 precursor protein, which is degraded to form a subunit during continuous processing.

The purified Ara h 1 protein samples were scanned at 200–500 nm, and the results are shown in Fig. 4. All samples had clear characteristic peaks at 280 nm, whereas peaks were not detected at other wavelengths, and the scanning results were consistent with the literature. Based on the combined results of electrophoresis and wavelength scanning, the purified sample was confirmed to be Ara h 1.

Table 2
Ara h 1 content of peanuts and peanut sprouts measured by ELISA.

Germination time (d)	Arah1 content			
	BK	US-45	US-80	US-100
0	20.63 ± 0.31[j*]			
1	12.29 ± 0.98 [i]	10.56 ± 0.28 [h]	10.36 ± 0.53 [h]	7.64 ± 0.23 [g]
2	7.38 ± 0.15 [g]	6.98 ± 0.55 [g]	5.37 ± 0.51[f]	5.18 ± 0.31 [ef]
3	4.31 ± 0.08 [de]	5.43 ± 0.68[f]	4.28 ± 0.13[de]	3.25 ± 0.13[c]
4	3.43 ± 0.51 [cd]	3.15 ± 0.51 [bc]	2.19 ± 0.34[b]	0 [a]
5	3.35 ± 0.42 [cd]	2.59 ± 0.83 [bc]	2.79 ± 0.28 [bc]	0 [a]

BK: Peanuts or peanut sprouts with no treatment
US-45: Peanuts or peanut sprouts that received 45 kHz ultrasonic pretreatment
US-80: Peanuts or peanut sprouts that received 80 kHz ultrasonic pretreatment
US-100: Peanuts or peanut sprouts that received 100 kHz ultrasonic pretreatment
*Each value is the mean ± SD of three replicates. Within a column, means that do not share a lowercase letter are significantly different ($p \leq 0.05$).

3.5. Ara h 1 protein structure in peanut sprouts assessed by fourier transform infrared spectroscopy

Infrared spectroscopy can reveal chemical bond information for a molecule. In the secondary structure of proteins, the α-helix structure is associated with the tight type of protein molecule, and β-folding, β-corners, and random coils reflect a loosening of the protein molecule. The corresponding relationships between the sub-peaks and protein secondary structures are as follows: 1650–1660 cm^{-1} is α-helical, 1660–1700 cm^{-1} is β-corner, 1610–1640 cm^{-1} is β-fold, and 1640–1650 cm^{-1} is random coil.

The experimental results are shown in Fig. 5. The peanut protein amide I band is mainly produced by stretching vibrations of carbonyl (C = O) bonds. After US processing, amide I underwent a slight blue shift because of the cavitation and heat effects of the US that destroy the intramolecular hydrogen bonds and cause the blue shift phenomenon. There was an absorption peak at 2400 cm^{-1}, which weakened after ultrasound and gradually disappeared after germination. It may be that the peptide bond was broken under the high intensity ultrasound, leading to different changes in protein structure.

3.6. Results of qPCR analysis

The results of qPCR analysis are shown in Fig. 6. The RNA had good integrity, showing two bands corresponding to 28S and 18S on the denaturing gel; the brightness of 28S was about twice that of 18S. The 5S bands extracted by the RNA kit were weak, and the denaturing gel showed no obvious bands. As can be seen from Fig. 7, the expression level of the *Ara h 1* gene in peanut seeds was relatively high, with a relative expression level of 173.92 ± 26.37. In the normal peanut germination process, the expression of *Ara h 1* decreased sharply as germination time increased, decreasing to 0.49 ± 0.17 on the fourth day and slightly increasing to 0.75 ± 0.09 on the 5th day. Nonetheless, the expression of *Ara h 1* was much lower during germination than in ungerminated peanut seeds. In the US group, the expression of *Ara h 1* in peanut seeds decreased to 1.37 ± 0.28 on the first day of germination, dropped sharply to 0.00 on the third day, and increased slightly to 0.04 ± 0.01 on the fourth and fifth day. In conclusion, the *Ara h 1* expression in peanut seeds after ultrasonic induction was significantly lower than that in the BK group, which fully explained the reason for the rapid increase in sensitization protein content on the first day of peanut germination after US induction. By comparing the expression of *Ara h 1* with the trend in sensitization protein, we found that the decrease in *Ara h 1* gene expression was significantly positively correlated with the content of sensitization protein. The expression of *Ara h 1* was slightly

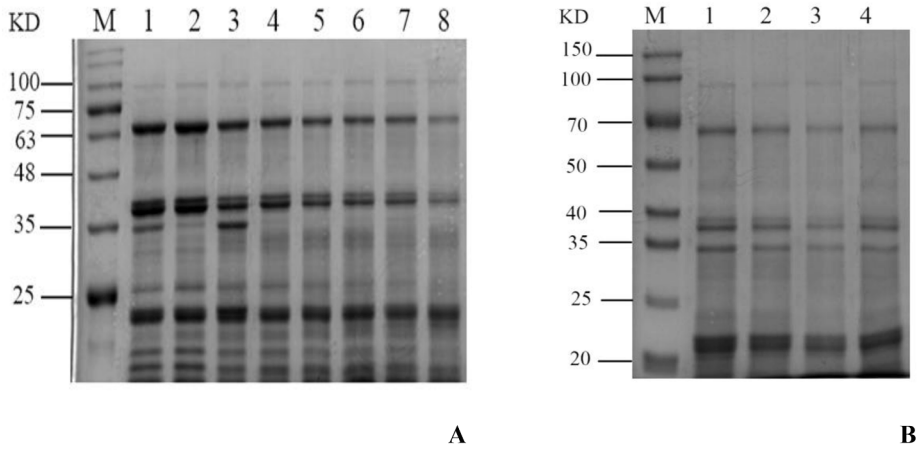

Fig. 2. SDS-PAGE analysis of the blank and ultrasonicated groups.

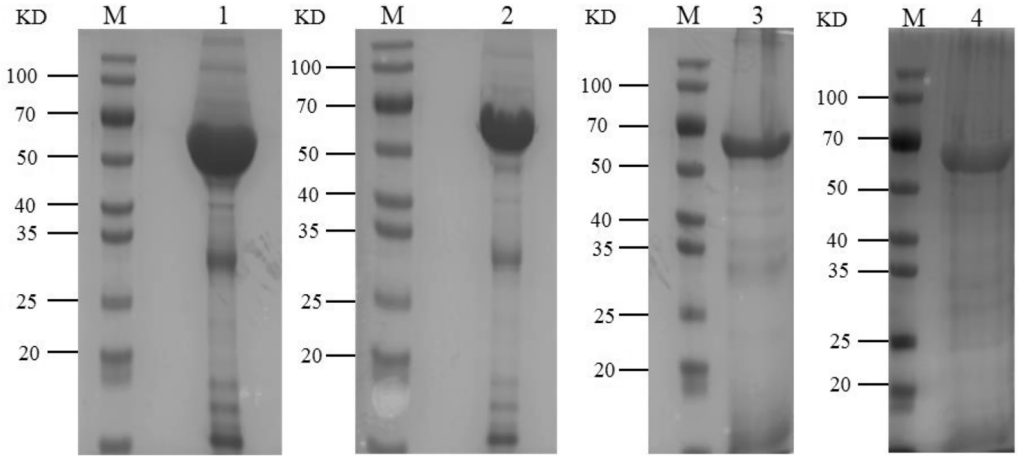

Fig. 3. Electrophoretogram of anion exchange chromatography.

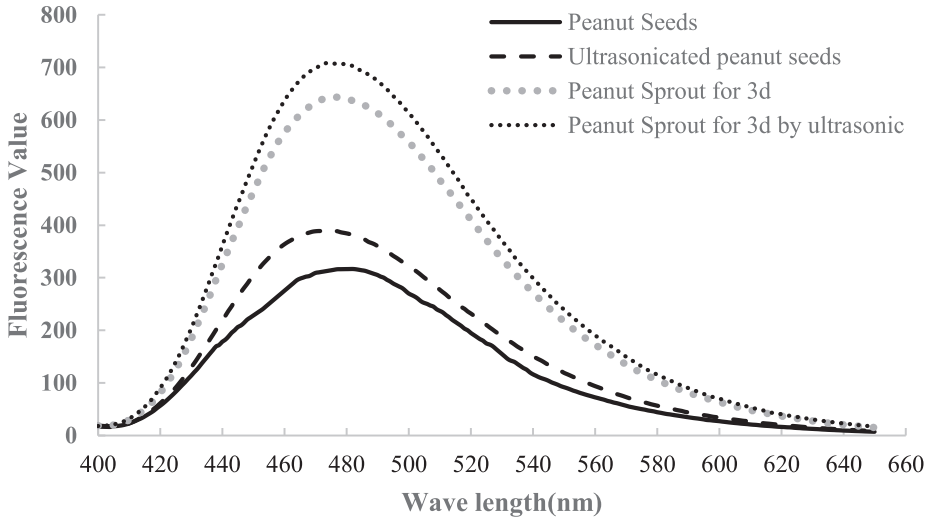

Fig. 4. Wavelength scanning of purified proteins.

increased at the later stage of peanut germination, perhaps reflecting protein hydrolysis at the early stage of germination and protein production in the later stage.

4. Conclusion

US had profound effects on Ara h 1 in peanut sprouts. A sharp decline in Ara h 1 content occurred when peanut seeds were exposed to post-harvest stress by US and germinated. The reasons are as follows. First,

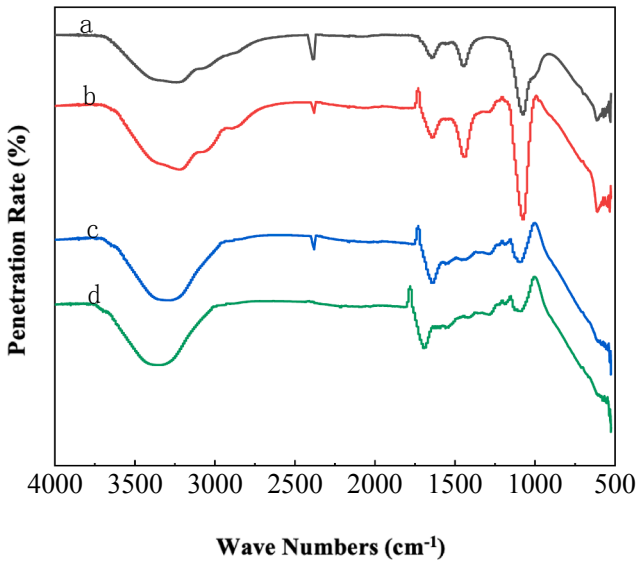

Fig. 5. FT-IR spectra of Ara h 1 in peanut sprouts under different treatments.

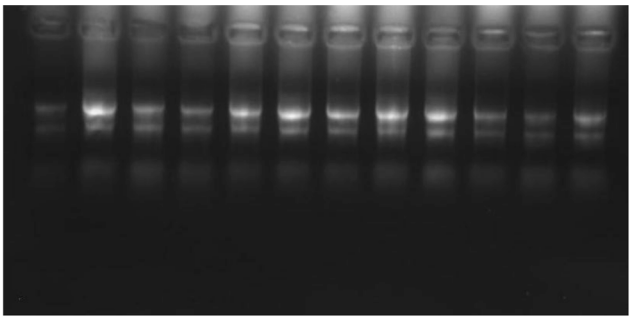

Fig. 6. RNA extraction.

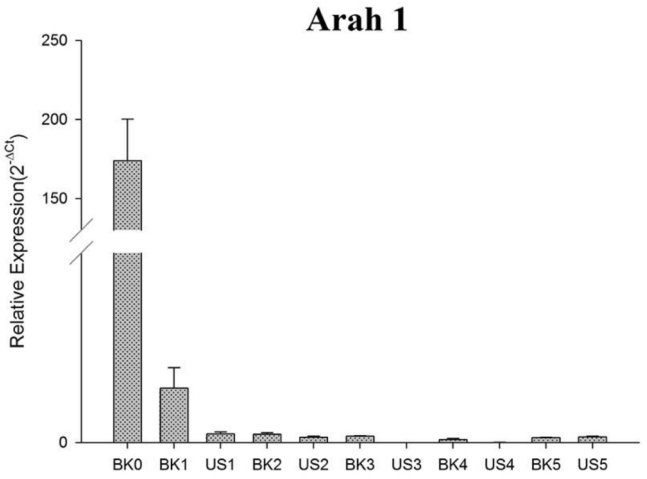

Fig. 7. Expression of *Ara h 1*.

protease enzyme activity increased significantly during germination. Second, cavitation and the heat effect from US destroyed the intramolecular hydrogen bonds and resulted in the blue shift phenomenon. It may be that peptide bonds were broken under high intensity ultrasound, leading to different changes in protein structure. Finally, the expression level of *Ara h 1* in peanut seeds after US induction was significantly lower than that in the BK group. In addition, US can improve peanut seed vigor and germination rate. Therefore, US treatment has the potential for application during the commercial production of peanut sprouts.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

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