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Rice protein radicals: growth and stability under microwave treatment

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The change of protein structure in a microwave field is often presumed to be caused by free radicals. This study addressed whether protein in a microwave field would generate free radicals, and which factors affect the formation and stability of the free radicals. Electron paramagnetic resonance (EPR) spectroscopy was used to investigate the signal of radicals in microwave-treated rice protein. The results revealed carbon-centred radicals in the treated rice protein samples. Spectral analysis of the rice protein revealed the presence of radicals not only in the thermally treated samples, but also in native samples of rice protein. The growth of radicals was strongly influenced by the water activity. Under microwave radiation, the intensity of the protein radicals increased as the water activity decreased and the microwave power increased. The intensity and growth rate increased with the radiation time. During storage time, the signal of the radicals dropped to 85% of the original value within 1 h. However, the radical signal gradually increased to its original level by the fifth day. Then, the EPR signal continued to decline at a slow pace until the intensity decreased to 75% of the original level. Based on the heating characteristics of oven heating, a temperature-controlled program during microwave heating was designed to compare the intensity of radicals after the two heating methods. The results indicated that microwave treatment induced much stronger radical signals, and the increase of the radical growth rate under microwave heating mostly occurred at 80–100 °C.

Introduction

As a Chinese staple food, rice is the most basic source of grain food in the daily diet. The main constituents of rice are starch and protein, with contents of about 80% and 8%, respectively.[1,2] Rice protein is an important source of protein in the diet, and has the characteristics of high nutritional value and low allergenicity. Many regions in the world choose rice protein as a nutritional supplement for infants.[3,4] During grain processing, the drying process in particular significantly influences the quality of the grain.[5,6] Microwave drying has a broad potential in the drying process of grain,[7,8] but during the process of microwave heating, free radicals were generated.

The study of the free radicals in food using EPR was mainly focused on the starch and grain material. Ciesielski *et al.*[9–12] found that the unpaired electrons during the pyrolysis of starch were not uniformly distributed in the crystalline and amorphous regions, and the results showed that the formation of free radicals was caused by chemical bonds such as C–H, O–H, C–O. Meanwhile, the type and quantity of free radical generation were affected by the different nature and the number of electrons in the outer layer of each metal element such as alkaline earth metals (K, Mg) and transition metals (Fe, Cu) after heating treatment. Due to the large ionic radius, K^+ had easier access and stronger interaction to starch, and the formation of carbon radicals in starch was promoted. While the interaction of Mg^{2+} and starch was weak, and inhibit the generation of free radicals. The formation mechanism of free radicals could be changed by changing the oxidation state due to the existence of various valence states of Cu^{2+} and Fe^{3+}.[13] Powder grain materials such as the corn flour, potato flour, cassava flour, wheat flour were investigated the free radicals after heating treatment.[14] The free radicals were also detected in raw grain powder without heating, and increased during the heating process. However, the studies on free radicals produced by food macromolecules were mainly focused on traditional air heating, radiation, ultraviolet, ozone process, and the research object were pure starch system, such as potato starch, corn starch of processing method.

With the wide application of microwave technology, the safety of microwave processing has received much attention in recent years, and preliminary research has indicated that microwaves can induce free radicals in rice starch.[15] However,

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there has been no systematic study on the effects of microwave processing conditions and material properties on the formation of protein free radicals. Most of the relevant studies have focused only on pure starch,[13,16] and did not incorporate more complex food systems. In food processing, research on the type and content of protein free radicals is relatively scarce.

In this study, the growth of protein radicals in food materials in a microwave field was studied using rice protein. The formation of radicals in rice protein with different levels of water activity (especially low levels of water activity) was explored under a microwave frequency (2450 kHz). The type and signal intensity of free radicals were detected by an electronic spin resonance instrument, and the growth of protein free radicals in the microwave field was inferred. The growth of free radicals was compared under oven heating and microwave heating, which has a direct relevance for microwave cooking.

Materials and methods

Materials

Native rice protein purchased from Golden Agriculture Biotech Co., Ltd, Jiangxi, was used for the investigation. The purity of the protein was 95%.

To adjust the water activity in the protein sample, four kinds of saturated solutions ($MgCl_2$, K_2CO_3, NaCl and KCl) were prepared and kept in separate air-tight containers for 2 weeks to reach equilibrium. Water activities of the samples were measured with an FA-st lab water activity meter (GBX, Romans sur Isere, France).

Methods

Water regulating method. Regulating method for Aw < 0.8: the saturated solutions of $MgCl_2$, K_2CO_3, KCl and NaCl were placed in four glass dryers, respectively (salt crystals were present on the bottom of each dryer). 20 samples of 2 g protein were weighed and arranged in the dryers to reach equilibrium over 2 weeks, and the seam portion of each dryer was wrapped with parafilm to ensure that the water activity of the protein was in the state of equilibrium. They were then stored at 25 °C, and the water activity was determined every 2 d after 1 week until the water activity remained constant.

Regulating method for Aw > 0.8: 100 g protein samples were weighed onto a stainless steel plate, uniformly sprayed with ultra-pure water from a spray bottle, and mixed completely in the spraying process. They were then stored at 4 °C, and the water activity was determined every 2 d after 1 week until the water activity remained constant.

Determination of sample dielectric parameters. The system for testing the electromagnetic properties included vector network analysers, a temperature probe, an open-ended cable and testing software to estimate dielectric properties. Each sample was well mixed before measuring. The test frequency range was 2.3–2.5 GHz.[17] Each sample was measured five times and the average reported.

Electron paramagnetic resonance (EPR) measurements. EPR measurements of the samples before and after microwave treatment were performed with a Bruker EMX-8/2.7 B spectrometer (Karlsruhe, Germany) operating in the X band (9.85 GHz) with a modulation frequency of 100 kHz. EPR spectra were recorded at room temperature with a modulation amplitude of 0.6 mT, microwave power of 20 mW, and receiver gain of 3.17×10^5.

The *g* value was measured using a standard mark (Bruker, gs = 1.9800). Hs is the value of the magnetic resonance field for the standard and Hx is the value of the sample. The gx value was calculated using the formula

$$h\nu = \mathrm{gs}\beta\mathrm{Hs} = \mathrm{gx}\beta\mathrm{Hx}$$

$$\mathrm{gx} = \mathrm{gsHs/Hx}.$$

In the equations, h is the Planck constant ($h = 6.626 \times 10^{-34}$ J s), β is the Bohr magneton, and ν is the frequency of the microwave added perpendicular to the magnetic field.[18]

The peak-to-peak height of each spectrum was calculated as the signal intensity. The EPR data acquisition and processing were conducted by WinEPR (Bruker, Germany).

Irradiation with microwaves. The samples were irradiated with a microwave synthesiser (MultiSYNTH, Milestone, Italy) at a frequency of 2450 MHz. 1.0 ± 0.005 g of protein sample was uniformly distributed in a quartz test tube (inner diameter = 12 mm) without a lid. The microwave power could be set in the range 0–400 W g^{-1} based on the expected heating effect and the samples were detected by a paramagnetic resonance spectrometer. Each experiment was conducted three times.

Oven heating. In the oven heating process, 1.0 ± 0.005 g protein samples were accurately weighed in a quartz tube with an inner diameter of 1.2 cm, the oven temperature was set to 100 °C, and the temperature was changed to 140 °C after heating for 10 min. During the heating process, the samples were measured at 2, 4, 6, 8, 10, 15, 20, 25, and 30 min by EPR. Each experiment was conducted three times.

Determination of protein oxidation. Carbonyl group was determined by the colorimetric method using DNPH.[19] Sulfhydryl group was determined by Ellman's reagent.[20]

Storage method of samples. Sixty to eighty milligram samples were gathered after the microwave irradiation in a nuclear magnetic tube, then transferred into a sealed container under air-tight and light-free conditions at room temperature (20 °C). The change in the EPR signal was intensively measured 1 d after microwave treatment, and the time points for the detection of the EPR measurement were 0.25 h, 0.5 h, 1 h, 2 h, 10 h, 24 h, 5 d, 10 d, 30 d, 60 d, 90 d and 120 d.

Statistical analysis. The spectral analysis and statistical calculations were performed using Origin Pro 8 SR1 (Origin Lab Corporation, USA) and Microsoft® Office Excel 2013 software.

Results and discussion

Dielectric properties of rice protein

The dielectric properties of the materials are described by the dielectric constant ε', the dielectric loss ε'', and the loss tangent δ. The dielectric constant is a measure of the electromagnetic

energy stored in the material, while the dielectric loss measures the material's ability to convert electricity into heat form.[21] Protein is a macromolecular system that has a weak ability to store and transform electromagnetic energy, and it is thus classified as an organic material with a low dielectric constant.

The dielectric properties of the protein samples at different water activities (Aw) were shown in Table 1. The results showed that the ε' for rice protein was less than 4 and the ε'' was less than 1, *i.e.* the samples were relatively weak microwave-absorbing materials. It must be noted that the gradual evaporation of moisture during the heating process lowered the capacity of the material to absorb electromagnetic waves, and the dielectric constant of the samples decreased.

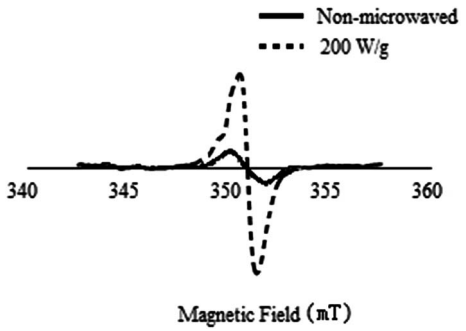

Fig. 1 EPR spectrum of rice protein before and after 3 min of microwave treatment.

Identification of radicals

As shown in Fig. 1, the EPR spectrum of the original rice protein had a low signal-to-noise ratio compared with the treated samples. The numbers and types of protein free radicals after microwave treatment were identified. Comparison of the *g* values and the peak shape for the protein samples and the standard showed that the calculated *g* values were all in the range of 2.0041–2.0054, which is typical for carbon radicals. Therefore, it can be determined that the radicals formed in the rice protein by microwave treatment in this study were carbon-centred radicals, which is consistent with other reports.[15,22]

Effect of water activity on the radicals of non-microwaved samples

EPR analysis detected a radical signal in the non-microwaved protein samples, indicating that the protein sample itself contained a certain number of free radicals. Water molecules, controlled by the water activity in this work, in the microwave field affect not only the dielectric properties and microwave absorption properties of the sample, but also the growth of free radicals, because when the water activity is low, water molecules may act as both initiators and quenchers of free radicals.

The EPR results for the protein radicals with different Aw values (Fig. 2) indicated that the radical content of the rice protein was higher and more active in the lower-Aw than in the high-Aw samples. As the water activity of the original protein was extremely low, the water molecules had a weak quenching effect on the protein radicals formed, whereas the high water activity of the high-Aw samples allowed the water molecules to quench the protein radicals and reduce the free radical content.

Effect of microwave power on the evolution of protein radicals

Previous work had clearly confirmed that heat can induce the growth of radicals in starches from various origins.[9,11,12,23] In this study, microwaves were used as a source of energy contributing to heat generation and radical reactions in protein molecules.[24] Therefore, it was reasonable to assume that the electromagnetic field would affect the progress of radical growth.

Fig. 3 showed that the higher the microwave power, the higher the intensity of the radicals. Below 80 W g^{-1}, the free radical signal was weak, and the growth trend was not obvious. The low radical count at low microwave power may be due to either the water activity in the protein samples having a strong ability to quench radicals, or the electromagnetic energy not being converted into sufficient heat and chemical energy to initiate the formation of free radicals. Above 80 W g^{-1}, the free radical growth in the protein samples with water activity 0.42 increased particularly significantly. Though the free radicals in the protein samples with water activities of 0.54–0.98 displayed a growth trend, the growth rate was about half that of the Aw = 0.42 samples. This indicated that when the water activity was less than 0.5, the priming effect of water molecules on the free radicals was greater than the quenching effect in the microwave field, and more free radicals could be generated. Moreover, when the microwave power was higher than 160 W g^{-1}, the colour of the rice protein samples turned brown. This suggests

Table 1 Dielectric properties of rice protein as a function of water activity

Water regulating method	Aw	Dielectric constant ε'	Dielectric loss ε''	Loss tangent tan δ
Untreated sample	0.26	2.19 ± 0.04	0.13 ± 0.03	0.05 ± 0.01
Saturated solution of K_2CO_3	0.42	2.28 ± 0.05	0.15 ± 0.03	0.06 ± 0.01
Saturated solution of NaCl	0.64	2.38 ± 0.06	0.25 ± 0.03	0.11 ± 0.02
Saturated solution of $MgCl_2$	0.69	2.64 ± 0.04	0.47 ± 0.03	0.17 ± 0.01
Saturated solution of KCl	0.78	2.69 ± 0.05	0.49 ± 0.02	0.18 ± 0.01
Spraying water	0.86	2.79 ± 0.07	0.50 ± 0.04	0.18 ± 0.03
Spraying water	0.94	2.94 ± 0.05	0.73 ± 0.06	0.25 ± 0.05
Spraying water	0.98	3.99 ± 0.08	0.92 ± 0.07	0.26 ± 0.06

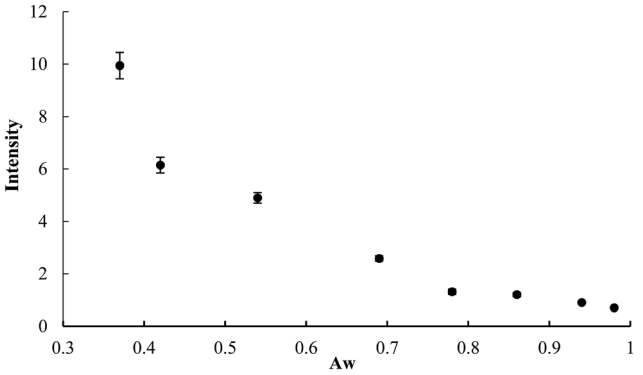

Fig. 2 Effects of water activity on intensity of protein radicals.

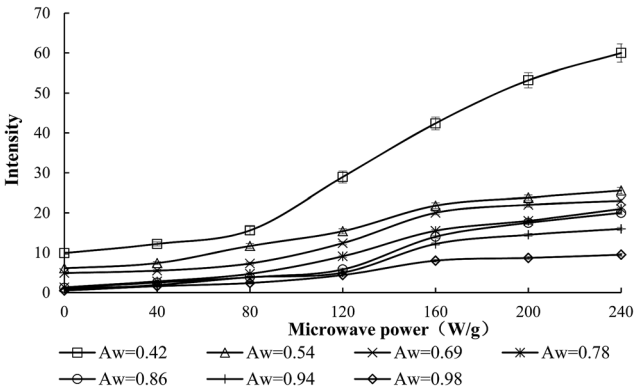

Fig. 3 Growth curve of rice protein radicals under microwave radiation at 40–240 W g^{-1} for 4 min.

that protein degradation may have occurred, and the absolute error in the determination of the free radical count increased with the microwave power.

Effect of time and water activity on formation of radicals

The microwave power was set to 80 W g^{-1} and 160 W g^{-1}, which provide better resolution to discriminate signals in the spectra. The time of the microwave treatment was set as 2, 4, 6 and 8 min.

Comparison of the signal intensity of the proteins treated by microwaves at 80 W g^{-1} and 160 W g^{-1} indicated that the number of radicals did not double with the microwave power (Fig. 4). The signal intensity tripled in the protein samples with Aw = 0.42, irradiated for 6 min, after doubling the microwave power. Moreover, the growth of free radicals was not obvious during the microwave heating between 0 and 2 min, and there was almost no generation of stable free radicals. After microwave heating for 2 min, free radical signals were significantly enhanced. The results under 160 W g^{-1} showed that the content of free radicals underwent rapid growth between 4 and 6 min, and the intensity of the free radicals weakened with increasing water activity.

Microwave photon energy is low, only 0.0016 eV, while the lowest energy of chemical bond can reach 0.44 eV. It can be known that the quantum energy of microwave is far lower than the bond energy of chemical bond, although it can not directly depend on the quantum energy to disconnect the chemical bond, but it can affect the chemical structure of the molecule. Until now, it is found that as the time of microwave heating to rise, there was an increase in the solvent (water) exposure of the hydrophobic core residues, and protein disulphide bonds were broken by microwave irradiation to create an increase first and a decrease later in free sulfhydryl contents with time, thereby inducing subunit disaggregation.[25]

Water is a kind of polar molecule, and there is an electric dipole interaction between the molecules to form hydrogen bonds, which has a strong dielectric response in microwave field. The original cluster structure of water is affected when in the external magnetic field, the dynamic hydrogen bond network system of water molecular cluster is destroyed, some large association clusters of water molecules become smaller, which has "activation" function, and produce amount of $\cdot O^{2-}$. The radical character is active, which can react with amino acid residues of protein, and damage the structure of protein which leads to the change of its functional properties. It is speculated that the "activation" of water molecules in the microwave field could generate free radicals, which can lead to the changes in the structure of the protein. When the water activity is high, the dehydration reaction between C–C is not easy to occur to reduce the generation of free radicals.

The correlation between free radicals generation and protein oxidation

In order to study the regularity of oxidation of rice protein and free radical formation under microwave irradiation, the samples were processed with a constant power (80 W g^{-1}) and the electron spin resonance and related oxidation indexes were detected. The content of carbonyl and sulfhydryl was used as an important index for the characterization of protein oxidation, compared with the corresponding free radical signal strength. The results showed that (Fig. 5), after microwave heating, the signal changes slowly, heating to 80 °C, the carbonyl content increased significantly, the sulfhydryl group in the heating process showed a slow downward trend. The growth rate of the free radical signal intensity increased gradually, when the temperature is heated to 100 °C, the free radical content is increased by 35% when compared to the 90 °C. So we can conclude that, in the process of microwave heating, pyrolysis behaviors would be occurred at first and the intramolecular or intermolecular disulfide bonds and fewer free radicals were generated, and later with the increase of the sample temperature, part of the secondary and tertiary structure of protein were destroyed and a large number of free radicals were generated. So we inferred that the generation of C centered radical is mainly focused on microwave heating to 80 °C.

Other researches also proved that the changes of the interior secondary and tertiary protein structure were caused by dipole rapid rotation (2.450×10^9 s^{-1}) and orderly arrangement in microwave field with the electric field alternating and polarization of molecules or ions, or electromagnetic field.[26] Our

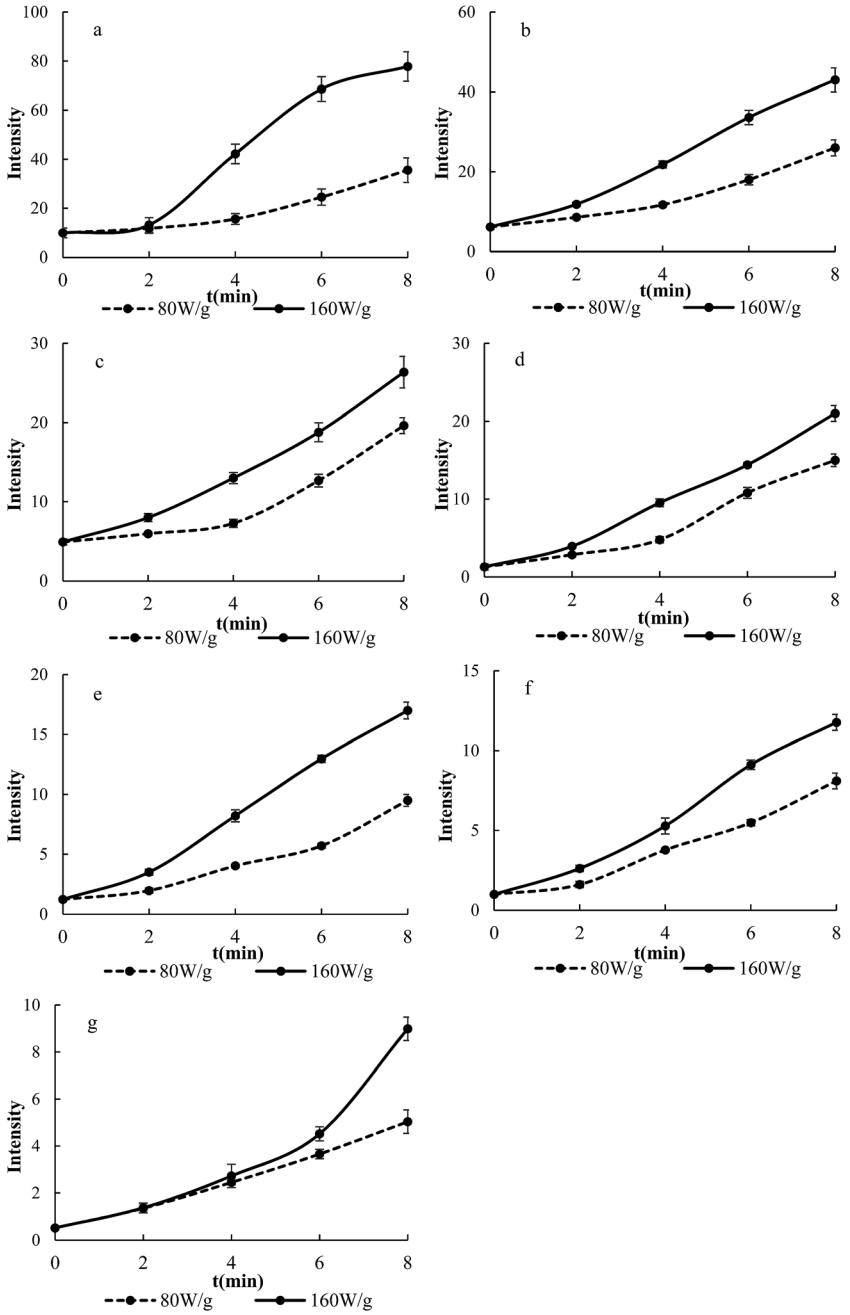

Fig. 4 Intensity of rice protein radicals with water activity 0.54–0.98 after treatment for 0–8 min: (a) rice protein (Aw = 0.42) irradiated under 80 W g^{-1} and 160 W g^{-1}; (b) rice protein with Aw = 0.54; (c) rice protein with Aw = 0.69; (d) rice protein with Aw = 0.78; (e) rice protein with Aw = 0.84; (f) rice protein with Aw = 0.94; (g) rice protein with Aw = 0.98.

results were consistent with the study conducted by Li Y., which revealed the relationship between protein oxidation and free radical changes. It was showed that the protein carbonyl content, surface hydrophobicity and turbidity of myofibrillar proteins were increased, while total sulfhydryl groups were decreased by increasing concentration of hydroxyl radicals.[27]

Attenuation of rice protein radicals during storage

We studied the effect of different water activities on the protein radicals, which had been generated by microwave heating, during subsequent storage. As the water activities of food systems are mostly higher than 0.8, rice proteins with water activities of 0.84, 0.94 and 0.98 were selected. The microwave power was 160 W g^{-1} and the electron paramagnetic resonance was detected after heating for 6 min.

After processing at 160 W g^{-1} for 6 min, the radical signal decayed rapidly within the first hour (Fig. 6). The signal dropped to about 85% of the intensity obtained immediately after the microwave heating. It is hypothesised that after microwave treatment, some short-lived, high-spin free radicals are

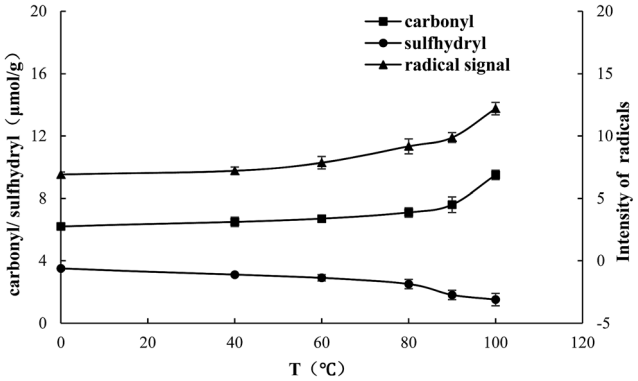

Fig. 5 Carbonyl, sulfhydryl contents and radicals of rice protein under microwave radiation.

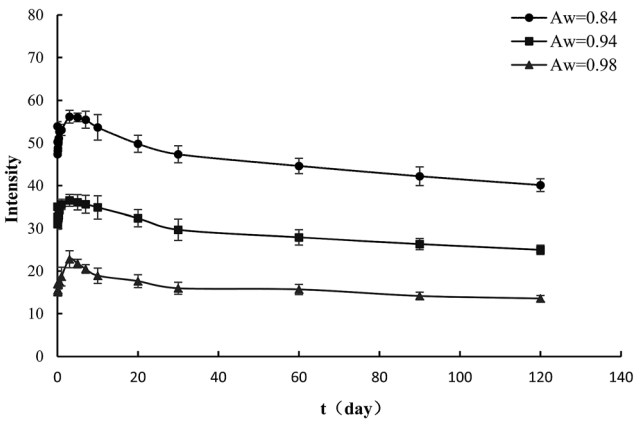

Fig. 6 Changes in the signal intensity of rice protein radicals after treatment for 6 min with microwave radiation at 160 W g^{-1}.

produced. These radicals are unstable, highly reactive and can react with each other quickly – thus, the free radicals quench one another, causing the observed decrease in the radical signal. After storing for 5 d, the radical signal gradually increased with respect to the original level, which could have been due to some effect in the samples that stabilised the free radicals. The radical signal intensity decreased slowly but steadily with storage time after 5 d. This change was mainly due to the large number of stable free radicals in the system, resulting from the dipole–dipole interactions of the stable free radicals, causing the signals to broaden and gradually decline. The intensity of the radicals stabilised after 60 d and showed little change thereafter.

Growth of rice protein free radicals under microwave irradiation and conventional heating

To compare the effect of microwave heating and conventional heating on the generation of free radicals, the microwave synthesiser was set to maintain the same temperature and heating time but vary the microwave power from 0 to 200 W to ensure a temperature and heating time in conformance with conventional heating. Then, samples at different heating

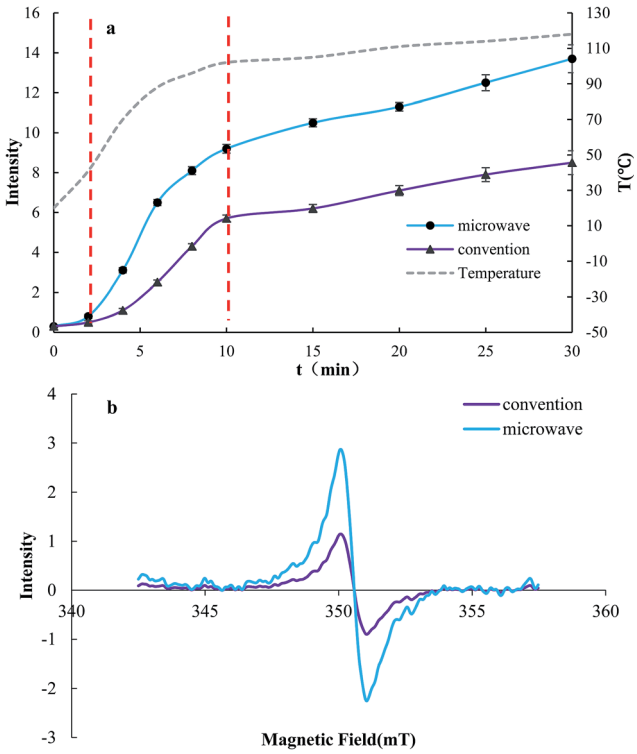

Fig. 7 (a) Growth of protein radicals under microwave irradiation and conventional heating; (b) EPR spectrum of rice protein after oven heating and microwave heating.

times were measured with EPR, and the radical growth curve of protein samples with Aw = 0.84 in the two heating modes was explored. The thermal and electromagnetic effects on the free radicals generated were studied during microwave irradiation.

The signal results of the free radicals (Fig. 7) indicated that the heat absorbed by the sample was mostly converted into internal energy within the first 2 min of heating, and was not sufficient to excite the water molecules to attack the protein molecules and generate free radicals; therefore, there were no obvious changes in the number of free radicals in either of the two heating modes. When heating for 2–10 min, the free radicals increased rapidly, and the growth rate of free radicals in microwave heating was greater than that of conventional heating. Presumably, the targeted dielectric effects of electromagnetic fields can directly act on polar groups in the water molecules or protein molecules to impart kinetic and internal energy under microwave radiation, which can further provide bond dissociation energy for the formation process of free radicals. Conventional heating provides energy by heat transfer; there is a single energy source, and the energy is limited, thus inducing a lower growth rate of free radicals. The growth of free radicals slowed after heating for 10 min. A possible explanation is that the water molecules escaped from the interior of the system after heating for 10 min, thus reducing the content of water molecules available to stimulate proteins to produce free radicals, so the free radical growth became slower.

Conclusions

Radicals in rice protein materials induced by microwave treatment were measured by electron paramagnetic resonance (EPR). The results showed that the protein radicals were carbon-centred and stable during a period of time, moreover, the protein itself contained a high number of radicals, and water had a certain ability to quench the radicals. The protein radicals were affected by water activity and parameters of microwave heating.

Acknowledgements

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