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Effect of Storage Conditions on Carbon-Centered Radicals in Soy Protein Products

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Using electron paramagnetic resonance (EPR) spectroscopy, the levels of carbon-centered radicals in retail samples of isolated soy protein (ISP), soy protein concentrate (SPC), and powdered soy milk were estimated to contain from 6.12×10^{14} to 1.98×10^{15} spins/g of soy product. Roasted soy nuts contained about 5.70×10^{15} spins/g. The peak to peak line width of the carbon-centered radicals from soy nuts was about 10 gauss, whereas ISP samples with a similar peak height had a peak to peak line width of about 8 gauss. Retail snack bars containing ISP, SPC, and/or roasted soy nuts with a total protein content of either 13, 21, or 29% contained 5.32×10^{14}, 6.67×10^{14}, and 5.74×10^{14} spins/g of snack bar, respectively. Levels of carbon-centered radicals in newly prepared samples of ISP were much lower than levels in the retail soy protein products and levels previously reported for commercial ISP and laboratory ISP samples. The levels of radicals in ISP samples increased over a 12−25 week period of storage in the dark at 22 °C and exposed to air from about 8.00×10^{13} spins/g immediately after preparation to 9.95×10^{14} spins/g of ISP. Storing the ISP samples under nitrogen at 22 °C greatly reduced the increase in radical content, whereas storing the ISP in 99.9% oxygen at 40 °C accelerated the formation of stable carbon-centered radicals. ISP samples hydrated at either 22 or 92 °C, rapidly frozen, and dried lost about 92% of the trapped radicals. The level of carbon-centered radicals in these same ISP samples immediately began to increase during subsequent storage exposed to the air and gradually returned to similar levels obtained before they were hydrated.

KEYWORDS: Soy protein; soy nuts; carbon-centered free radicals; electron paramagnetic resonance spectroscopy; inert atmosphere

INTRODUCTION

Boatright and others (*1*) demonstrated that the carbon-centered free radical content of commercial and laboratory isolated soy proteins (ISP) and powered drink mixes (made from ISP) ranged from 2.96×10^{14} to 4.10×10^{15} spins/g. The higher radical contents were found in the powered drink mixes. There are three characteristics that should be considered in the identification of the type of radical by its electron paramagnetic resonance (EPR) spectrum; *g* value, the shape of the spectrum, and the power saturation of the paramagnetic species. This is particularly true when one is distinguishing between nitrogen-centered and carbon-centered radicals because their *g* values are so close. Published *g* values for nitrogen-centered radicals range from 2.0022 to 2.0065 (*2*) and from 2.0041 to 2.0054 for carbon-centered radicals (*3*−*8*). Nitrogen-centered radicals exhibit a triplet pattern (*4*,*6*,*9*), whereas carbon-centered radicals typically exhibit a single broad peak with power saturation levels of 2−8 mW (*10*). Microwave power saturation for nitrogen-centered radicals has been reported to be greater than 10 mW (*11*) and 20 mW (*12*) and approximately 62 mW (*13*). The observed *g* value, line shape, and power saturation level (4 mW) of the signal from soy protein products is indicative of carbon-centered radicals (*1*).

Jonsson and others (*14*) estimated that the one-electron oxidation potentials for the α-carbon-centered radicals of glycine anhydride, L-alanine anhydride, and DL-alanine anhydride were 1.19, 1.00, and 0.99 V versus NHE, respectively. Amino acid anhydrides were used as model compounds for peptides. These carbon-centered radicals have oxidation potentials similar to those of the alkyl peroxyl radicals and likely contribute to numerous reactions once the protein is hydrated, including the generation of hydroxyl radials from the reaction with molecular oxygen.

Carbon-radical generating systems (e.g., azo-compounds) have been used extensively to initiate oxidative degradation of various proteins, lipids (unsaturated fatty acids and cholesterol), and lipoproteins as well as DNA strand scission and cell death (*15*−*23*). This type of oxidative damage is thought to play a pivotal role in aging and in a number of degenerative diseases (*24*−*26*). High-protein diets, in general, have been associated with several types of cancer (*27*−*29*).

The current investigation was undertaken to determine, when carbon-centered radicals are being formed in ISP, if selective treatments during processing or storage of the ISP can inhibit the

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formation of these radicals and if the radical content that is diminished when the protein is hydrated will return upon subsequent drying and storage in an atmosphere containing oxygen.

MATERIALS AND METHODS

Chemicals. ISP, soy protein concentrate, powdered soy milk, roasted soy nuts, snack bars containing ISP, and whey protein drink mixes were obtained from local markets. Snack bars were freeze-dried before EPR analyses.

Preparation of Isolated Soy Proteins. Defatted soybean (white) flakes were provided by the Archer Daniels Midland Co. The defatted soybean flakes were ground for 15 s in a retail coffee grinder prior to use. Laboratory ISP was prepared by dispersing hexane-defatted soybean flour in water (1 part flour to 10 parts water) at the designated temperature (either 22 or 50 °C), followed by additions of 1 N sodium hydroxide, as needed, until a pH of 8.5 was achieved and maintained for 30 min (*30*). The supernatant resulting from centrifugation at 1500*g* for 10 min was adjusted to a pH of 4.6 and centrifuged at 1500*g* for 10 min. The precipitated proteins were washed once with water, and the resulting protein isolate was suspended in water at about a 1:10 ratio of solids to water. The pH was then adjusted to pH 7 with 1 N NaOH. The resulting ISP was rapidly frozen by pouring the slurry into a −40 °C stainless steel pan, held at −40 °C overnight, and then freeze-dried.

To evaluate the effect of hydrating ISP, 0.6 g of ISP was placed into a 50 mL glass tube with 20 mL of water (either 22 or 92 °C), votexed for 30 s, and then stirred for 30 min. After 30 min of stirring, each tube was lowered into liquid nitrogen, where it was held for 5 min. Each tube was then transferred to a −80 °C freezer overnight and freeze-dried.

EPR Spectroscopy. EPR spectroscopy was performed at 20 °C on a Bruker EMX EPR spectrometer with the following parameters: microwave frequency, 9.42 GHz; receiver gain, 2×10^4; modulation amplitude, 5.0 G; modulation frequency, 100 kHz; microwave power, 1 mW; time constant, 327.68 ms; conversion time, 183.84 ms; field sweep, 800 gauss. Soy protein samples were packed into a 4 mm quartz sample tube (Wilmad, Buena, NJ) to a density of 0.03 ± 0.002 g/cm of tube length. The *g* value axis was calibrated relative to crystalline 1,1-diphenyl-2-picrylhydrazine (DPPH) using Bruker WINEPR System software. The mean *g* value from five scans of DPPH was 2.00381 ± 0.00002 and that from five scans of ISP, 2.00517 ± 0.00013. Soy protein spin concentrations were estimated using a standard curve of powdered K_3CrO_8 in K_3NbO_8 prepared according to the method of Cage and others (*31*) diluted with powdered KCl. The Cr(V) spin concentrations were calculated from the ESR signal of Fremy's salt (dipotassium nitrosodisulfonate) solutions at −196 °C after double integration. Spin concentration within the EPR cavity (calculated from the standard curve) was divided by the weight of sample in the cavity to obtain the "spins/g" of sample.

RESULTS AND DISCUSSION

Retail samples of ISP, soy protein concentrate (SPC), and powdered soy milk (**Table 1**) all had levels of carbon-centered radicals ($g = 2.005$) from about 1 to 4 times as high as the levels previously reported for ISP samples obtained directly from the processor (*1*). All ISP, SPC, and powdered soy milk samples examined contained carbon-centered free radicals from 3.5 to 12 times higher than whey proteins (**Table 1**; **Figure 1**). The carbon-centered radical contents of whey protein samples examined were similar to those reported for egg albumin and sodium caseinate (*1*). Roasted soy nuts contained about 5.70×10^{15} carbon-centered radicals/g. The peak-to-peak line width of the carbon-centered radicals from soy nuts was about 10 gauss, whereas ISP samples with a similar peak height had a peak width of about 8 gauss. Kosuge and others (*32*) and Uyeta and others (*33*) reported on the formation of mutagenic substances formed from the pyrolysis of proteins. Uchiyama and Uchiyama (*34*) demonstrated that heating soy protein above 75 °C for 1 h elevated the level of free radicals at $g = 2.0037-2.0045$.

Table 1. Peak Areas after Double Integration of Primary EPR Signal (Symetrical at $g = 2.005$) in Laboratory-Isolated Soy Protein Samples and Various Protein Products Obtained from Local Markets

product	peak area × 10^5
protein products from local markets	
"Now" ISP	15.28
"Bulk Foods" ISP	14.44
'Life Extension" soy protein concentrate	5.24[a]
'The SausageMakers" soy protein concentrate	18.34[b]
"Now" powdered soy milk	16.65
roasted soy nuts	53.86[c]
"ON - 100% Whey Protein"	1.51
'GNC - 100% Whey Protein"	1.58
29% protein bar (Kraft South Beach Living, peanut butter)	4.87
21% protein bar (Luna S'mores)	5.90
13% protein bar (SoyJoy Apple)	4.58
laboratory ISP, all analyzed within 1 day of being prepared	
lab ISP extracted at 22 °C, pH 9	0.48
lab ISP extracted at 50 °C, pH 8	1.54
lab ISP extracted at 50 °C, pH 9	0.99
lab ISP extracted at 50 °C, pH 9, held for 30 min before freezing	1.47
lab ISP extracted at 50 °C, pH 8.5	0.78

[a] Estimated spin concentration (spins per gram of soy protein) = 6.12×10^{14}. [b] Estimated spin concentration (spins per gram of soy protein) = 1.98×10^{15}. [c] Estimated spin concentration (spins per gram of soy protein) = 5.70×10^{15}.

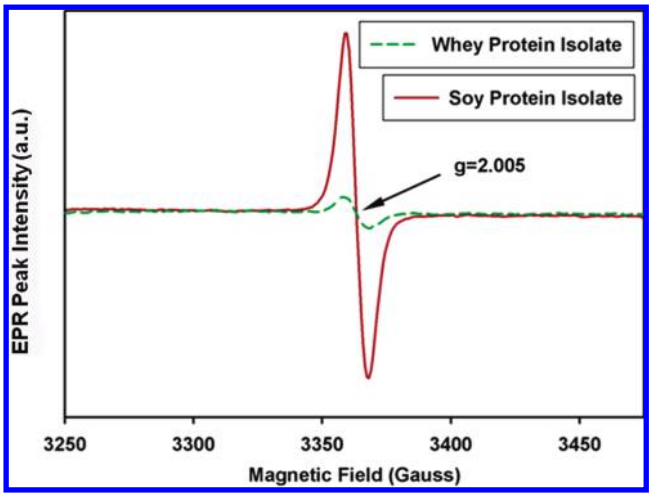

Figure 1. EPR spectra from a whey protein isolate (GNC) and a soy protein isolate (Now). Peak areas are provided in **Table 1**. Both were rcorded in the solid state at 1 mW power.

The broader EPR peak in soy nuts resulting from pyrolysis indicates that the higher temperature process is more random than the stable-radical forming process that occurs over time at room temperature.

Retail snack bars containing ISP, SPC, and/or roasted soy nuts with a total protein content of either 13, 21, or 29% contained 5.32×10^{14}, 6.67×10^{14}, and 5.74×10^{14} spins/g of snack bar, respectively (**Table 1**). These are levels similar to those reported in some ISP samples (at about 85% protein). This type of food product consumed as-is without further processing would result in ingestion of higher levels of radicals. Boatright and others (*1*) demonstrated that about 80% of the carbon-centered free radicals in ISP reacted to produce nonradical species after being hydrated and stirred for 30 min.

Because the level of free radicals in proteins was reported to be elevated by exposing the protein to a mixture of

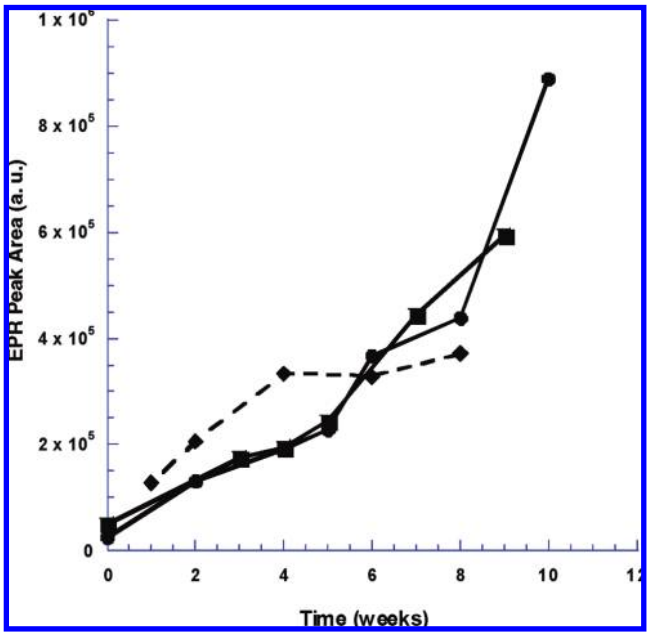

Figure 2. EPR peak area (after double integration) over time for the level of carbon-centered radicals in three different laboratory ISP samples exposed to air at 23 °C (◆, ■, ●).

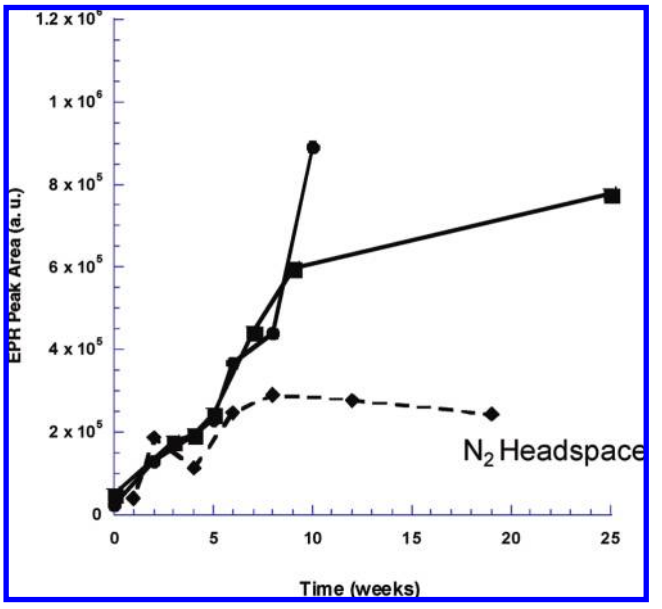

Figure 3. EPR peak area over time for the carbon-centered radicals in two laboratory ISP samples exposed to air at 22 °C (●, ■) and another ISP sample stored under nitrogen (◆).

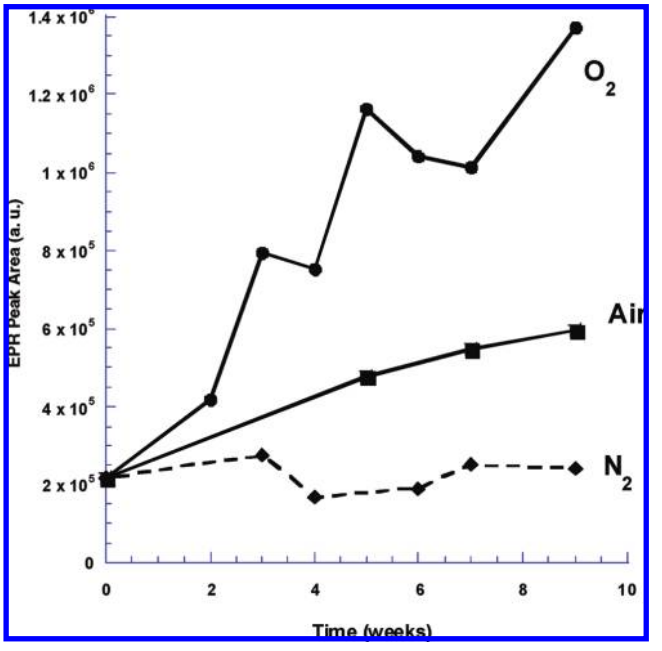

Figure 4. EPR peak area over time for the carbon-centered radicals in a single batch of laboratory ISP with one portion exposed to air at 23 °C (■), another portion stored under nitrogen at 23 °C (◆), and a third portion stored under 99.9% oxygen at 40 °C (●).

lipoxygenase/linoleic acid (*3*), ISP process variations that might affect the ability of naturally occurring lipoxygenase to contribute to the carbon-centered radicals of ISP were investigated. Laboratory ISP samples were processed at either 22 or 50 °C, using extraction pH at either 8 or 9 and varying the time the protein slurry was held before freezing at either 0 or 30 min. Regardless of the treatment, all laboratory ISP samples analyzed within 48 h of being dried had carbon-centered free radical contents at about $^{1}/_{10}$ to $^{1}/_{30}$ the level of previously analyzed laboratory ISP and commercial ISP samples (**Table 1**) (*1*).

Huang and others (*3*) employed high levels of lipoxygenase/substrate at the optimal pH for enzymatic activity (pH 9), and the reaction mixture was exposed to flash freezing to help trap the radicals. Also, because there were no quantitative data provided, no comparisons of the levels of radicals trapped in commercial ISP samples can be made. Whereas these findings indicate that some protein oxidation can occur as a result of oxidizing lipids during ISP processing, there appears to be another mechanism responsible for the elevated levels in "dry" commercial ISP products.

After laboratory ISP samples had been stored in the dark at 22 °C in sealed 500 mL jars (with a large air headspace), it was observed that the level of free radicals increased by as much as 35-fold during the first 9 weeks of storage (**Figure 2**). Storing the ISP under nitrogen inhibited the increase in radicals over time (**Figure 3**). The jar containing the ISP stored under nitrogen was opened periodically to remove enough samples for EPR analysis, which allowed some oxygen to contact the sample.

The effect of oxygen on the free radicals in ISP was further investigated by preparing a single batch of ISP and dividing it into three portions, with one portion stored exposed to air at 23 °C, one protein stored under nitrogen at 23 °C, and a portion stored under 99.9% oxygen at 40 °C (**Figure 4**). A temperature of 40 °C was chosen to accelerate the oxidation process without significantly altering the protein structure. The level of carbon-centered radicals in the ISP stored under nitrogen remained nearly constant, whereas that of the sample exposed to air gradually increased and the sample exposed to oxygen at 40 °C demonstrated a much greater increase in radical content. We repeated this experiment with a single batch of ISP divided between 99.9% oxygen, air, and nitrogen but with all portions stored at 23 °C (**Figure 5**). A similar trend was observed, indicating the accelerating effect of a higher oxygen content.

These results clearly demonstrate that in the presence of oxygen, the rate of radical formation is accelerated. The reaction mechanism involving oxygen may follow well-known pathways, including the formation of superoxide radical anions (subsequent pathways involve the relatively high content of iron in soy proteins, leading to hydroperoxide and hydroxyl radicals) and the reaction of oxygen with carbon radicals leading to hydroperoxides, alkoxy radicals,

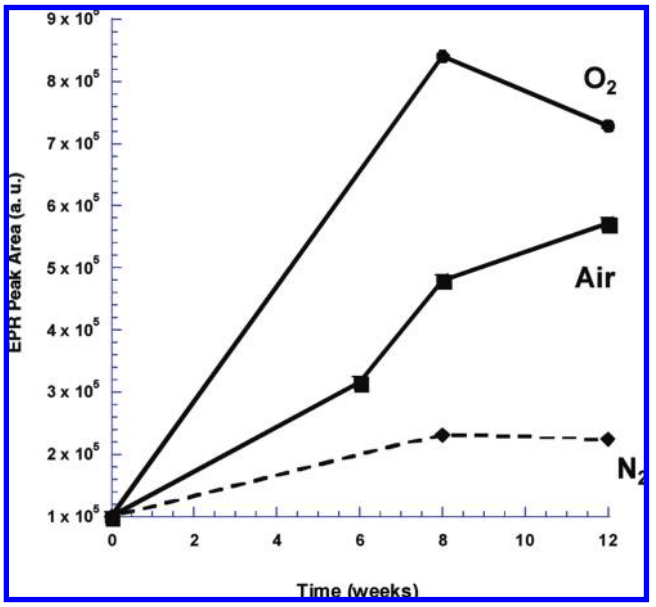

Figure 5. EPR peak area over time for the carbon-centered radicals in a single batch of laboratory ISP with all portions held at 23 °C. One portion was stored in air (■), another portion was stored under nitrogen (◆), and a third portion was stored under 99.9% oxygen (●).

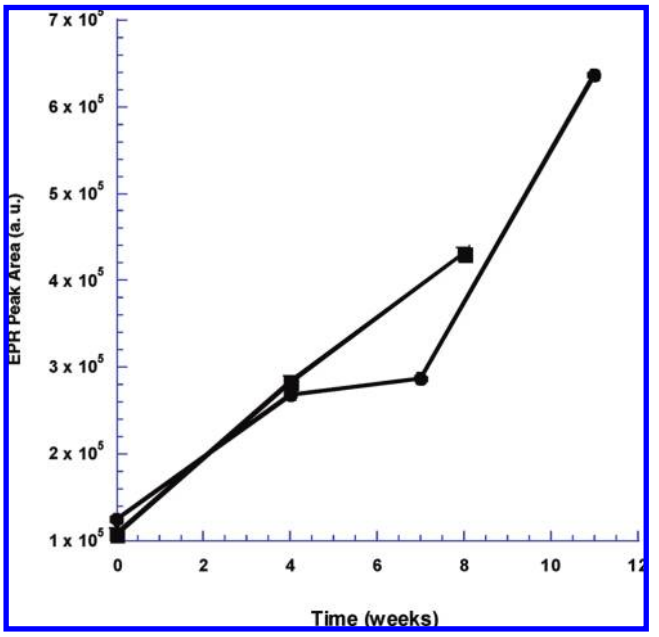

Figure 6. EPR peak area over time for the carbon-centered radicals in ISP after being hydrated at either 22 °C (●) or 92 °C (■), stirred for 30 min, and freeze-dried followed by storage in air at 22 °C.

carbon−carbon bond cleavage, and the subsequent propagation of free radical reactions (*35*, *36*). Because oxygen readily reacts with carbon radicals, this second mechanism raises the question: how then can the carbon-centered radical content of soy protein be so stable and rise to such high levels? The answer to this question is beyond the scope of the current investigation; however, because the levels of carbon-centered radicals are clearly being elevated to such high levels, there must be some stearic hindrance or other means to stabilize the carbon radicals lodged deep within the proteins. Soy proteins appear to be particularly suited to accomplish this. Most reports of oxygen reacting with carbon radicals in proteins occur in an aqueous environment (*37*). The structure of the protein that promotes the trapping of carbon-centered radicals in "dry" soy proteins may be altered when the protein is hydrated.

Boatright and others (*1*) noted that when ISP samples were hydrated, stirred for 30 min, and then rapidly frozen and dried, the level of free radical in the protein was reduced by about 80%. The effect of hydration leading to an alteration of the protein conformation and a decrease in trapped radicals was previously reported for myosin (*38*) and for powdered soybean axes and cotyledons (*39*). We repeated this experiment to see if, after the initial loss of trapped radicals from the soy protein, the free radicals would gradually increase again during storage exposed to the air. We also hydrated a sample of ISP at 92 °C to determine if thermally denaturing soy protein affected it ability to trap radicals (**Figure 6**). The ISP sample used initially exhibited an EPR peak area after double integration of 14.44×10^5 before being hydrated. Thus, the hydration process resulted in losses of approximately 91 and 92.5% of the trapped radicals by the 22 and 92 °C treatments, respectively. The level of carbon-centered radicals in these same ISP samples immediately began to increase during subsequent storage exposed to the air at a rate of increase similar to that observed in laboratory ISP samples during the same period of time. The 92 °C treatment appeared to have no significant effect on the ability of radicals to be formed during subsequent storage compared to the 22 °C treatment. Thus, soy proteins in food products with low water activity, whether or not they were processed in an aqueous environment, can lead to unintentional consumption of high levels of potentially harmful free radicals.

The relatively high levels of carbon-centered free radical in soy protein products appear to be formed in the dry protein during storage exposed to oxygen. Hydrating soy proteins allows the majority of the trapped radicals to react and form nonradical species; however, during subsequent storage of the dried protein, the elevated levels of free radicals can return. Further study is required to determine why soy proteins are more prone to form and trap carbon-centered radicals compared to other source proteins and what reactions are catalyzed by carbon-centered radicals when soy proteins are hydrated.

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