Oxidation reactions in model systems simulating the processing of soybeans into soymilk: role of lipase and lipoxygenase in volatile flavors formation

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ABSTRACT

In recent decades, the process of lipase (LIP) activation hydrolysis and lipoxygenase (LOX) oxidation for energy supply during soybean germination has been well understood, but the formation mechanism of volatile flavor substances in soymilk grinding has not been understood in detail. Different model systems were prepared using protein, LOX, LIP, and triacylglycerol (TAG) derived from soybeans to study the reaction process of soymilk flavor. The study revealed that the simultaneous existence of LOX, LIP, and TAG was a necessary condition for flavor formation, and the type and amount of volatile flavor substances were related to the composition of the lipid substrates. Compared with soymilk, the model system with LOX and LIP had a high degree of similarity in the composition of volatile flavors, but there were differences in quantity. This indicates that the flavor formation of soymilk is also positively influenced by other ingredients in soymilk.

Introduction

Soymilk is a traditional product derived from soybeans that are consumed as a health food and beverage and have long been an important source of protein in the oriental diet. Since the late 20th century, soymilk has become a major milk substitute for lactose-intolerant consumers in western countries. However, the undesirable beany flavor in soymilk has greatly limited its popularity and promotion.¹⁻³ Studies have shown that the beany flavor of soybean products is due to the enzymatic oxidation of unsaturated free fatty acids (FFAs) in a process that largely involves the enzyme LOX.⁴⁻⁶ LOXs are dioxygenases that catalyze the addition of O₂ to polyunsaturated FFAs which contain cis,cis-1,4-pentadiene structures. Linoleic acid (C18:2) and linolenic acid (C18:3), are examples of polyunsaturated FFAs containing such structures, and are abundant in seeds and plant tissues, respectively. The primary products are free hydroperoxy radicals, which further generate hydroxy acids and hydroperoxides. The LOX pathway plays an important role in the formation of key (C-6) flavor compounds such as hexanal which is largely associated with the undesirable flavor in soybean products. Soybean seeds contain at least three LOX isozymes (LOX-1, LOX-2, and LOX-3), accounting for about 1–2% of the soybean protein.⁷,⁸ Each isozyme differs in terms of its substrate preference, optimal pH, product formation, and stability; LOX-1 is most active with linoleic acid at pH 9 while LOX-2 and LOX-3 (pH optima 6.5) are more active with TAG than FFAs.⁹ In addition, soybean seeds contain a significant amount of TAGs (about 20% by weight composed of mainly linolenic, linoleic, oleic acid, and saturated FFAs), a small quantity of phospholipids, and a trace amount of unsaturated FFAs, which can be used as substrates in the LOX pathway.¹⁰

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Mature intact soybean seeds do not have a typical undesirable (beany) flavor. However, the processed products, such as soymilk, soybean protein, and tofu, have a remarkable beany flavor. This is because LOX and substrate precursors such as linoleic acid remain separated in the cell of the intact soybean. Hence, the enzyme remains in an inactive state and off-flavor substances are not be produced.\cite{7,11} Whether the dried soybeans are used to prepare powdered products or soaked to form slurries, many volatile flavor substances are produced during grinding.\cite{1} The grinding of soybean seeds during the processing of soybean products is a key process in the formation of volatile flavor substances. The reaction of soybean components during the grinding is complex, as water and oxygen participate in a series of reactions that determine the reaction products. At the same time, soybean LIP hydrolyses TAGs to increase the amount of polyunsaturated FFAs available for enzyme-catalyzed oxidation.\cite{12} The polyunsaturated FFAs substrates are rapidly assimilated on contact with LOX to form an abundance of flavor substances. These substrates, and the products of the LOX pathway, are believed to play an important role in the catalytic-oxidation reaction of LOX.\cite{7}

LIPs (TAG acylhydrolases EC: 3.1.1.3) are ubiquitous enzymes found in plants, animals, fungi, and bacteria and are of considerable physiological significance.\cite{13,14} The biological function of LIPs is to catalyze the hydrolysis of TAGs to yield FFAs, DAGs, monoacylglycerols (MAGs), and glycerol.\cite{15} Whether supplying energy for plant seed germination and seedling growth in vivo or causing the deterioration of food quality, LIP catalysis is the first step in lipid hydrolysis.\cite{16,17} Previous studies showed the in vitro activity of LIP was very low compared to LOX in soybean seed extracts.\cite{18} Inspection of the earlier work also suggested that the activity of LIP may be partially inhibited, indicating LIP-catalyzed formation of substrates, although much reduced, were still available to the LOX pathway.\cite{19,20} While considerable evidence has accumulated implicating soybean LOX-catalyzed LIP oxidation as the critical excitation step in the initial stage of soybean seeds growth, to date, no research has specifically examined the role of LIP in the volatile flavor of soybean products.

Previously in our laboratory, we developed a soymilk reaction system (SRS) to follow the evolution of flavor compounds during processing. The optimum incubation conditions for the formation of volatile flavor compounds in the SRS were pH 7.0°C and 40°C for a 6 mL sample incubated for 30 min.\cite{21} In this work, the SRS was used to study oxidation reactions during the processing of soybeans into soymilk. Combinations of LOX, LIP, protein, and TAG, isolated from soybeans, were incubated and their reaction products analyzed. The role of LIP and LOX in the formation of volatile flavor compounds in soymilk was discussed.

**Materials and methods**

**Materials**

Soybeans (*Glycine max (L.) Merr.*) were purchased from Neijiang Food Co., Ltd. (Neijiang, China); all preparations described below were obtained from the same batch of soybeans. Soybean oil was purchased from Shanghai Kerry Food Industry Co., Ltd. (Shanghai, China). All chemicals were AR grade unless stated. 2-Methyl-3-heptanone, methanol (anhydrous), isopropanol, n-hexane, and volatile standards (see Table 3) were HPLC grade and purchased from Sigma-Aldrich Trading Co., Ltd. (Shanghai, China). Acetone, diethyl ether (anhydrous), and 98% formic acid were purchased from Sinopharm Group Co., Ltd. (Shanghai, China). Carboxen/Polydimethylsiloxane (CAR-PDMS) solid-phase microextraction (SPME) fibers (85 μm) were purchased from ANPEL Laboratory Technologies (Shanghai) Inc. (Shanghai, China).

**Preparation of soymilk**

Soybeans (50 g) were washed and then soaked in deionized water (500 mL) for 18 h at 4°C prior to grinding (2 min) using an MJ-60BE01B blender (Midea, China). The soymilk mixture was filtered using four layers of cheesecloth to obtain the raw soymilk filtrate and an aliquot was immediately
taken for the measurement of flavor volatile substances according to the method described below. Protein and lipid contents of the soymilk were measured by the Kjeldahl method and Soxhlet extraction method, respectively.\textsuperscript{22} LIP and LOX activities were measured according to the methods described by Yeşiloğlu et al.\textsuperscript{23} and by Axelrod et al.\textsuperscript{4} respectively.

\textbf{Extraction of LOX}

Soybean LOX was extracted according to the methods described by Axelrod et al.\textsuperscript{4} and Fukushige et al.\textsuperscript{24} with some modifications. Dry soybean seeds (200 g) were milled to a fine flour using an XA-1 high-speed grinder (Yinhe, China), sieved (60-mesh), and mixed with deionized water (960 mL; 1:6 w/w). The resultant slurry was adjusted to pH 4.5 with HCl (0.5 M), stirred for 1 h at 4°C, filtered through four layers of cheesecloth, and the resultant filtrate was centrifuged at 8000 g_{av} and 4°C for 30 min. The supernatant was adjusted to pH 6.8 with 2 M NaOH, ammonium sulfate powder was added slowly to a concentration of 40\% and the mixture was stirred for 1 h at 4°C before centrifuging as described above. The supernatant was again adjusted to pH 6.8 with 2 M NaOH, ammonium sulfate was added to a final concentration of 60\% and the mixture was stirred for 1 h at 4°C before centrifuging as described above. The supernatant was removed, and the precipitate was dissolved in sodium phosphate buffer (100 mL, 0.02 M, pH 6.8). The enzyme solution was subjected to dialysis using an MD 44 dialysis bag (Solarbio, China) prior to vacuum freeze-drying. The activity of LOX was determined with the method described by Axelrod et al.\textsuperscript{4}

\textbf{Extraction of LIP}

Soybean LIP was extracted by the ammonium sulfate precipitation procedure according to the method of Yeşiloğlu et al.\textsuperscript{23} Briefly, soybean flour (50 g) was defatted by stirring with cold acetone (150 g) for 30 min in the water bath at 4°C. The extraction procedure was repeated three times using a fresh solvent. After standing at room temperature to allow the acetone to evaporate, the defatted soybean flour was suspended overnight in sodium phosphate buffer (350 g, 0.1 M, pH 7.0), and then centrifuged for 30 min at 4,000 g_{av} and 4°C. The supernatant was precipitated using solid ammonium sulfate (1 h at 4°C) to collect the 70\% salt saturation fraction followed by centrifugation at 13,000 g_{av} for 30 min. The obtained precipitate was dissolved in a small volume of sodium phosphate buffer (0.1 M, pH 7.0). The suspension was then dialyzed at 4°C for 24 h with three changes of sodium phosphate buffer (0.05 M, pH 7.0) using cellulose membrane dialysis tubing (Sigma Chemical Co., USA) prior to vacuum freeze-drying. The LIP activity was assayed with the method described by Yeşiloğlu et al.\textsuperscript{23}

\textbf{Extraction of soybean protein}

Soybean proteins were extracted according to the method reported by Huang et al.\textsuperscript{22} with minor modifications. Defatted soybean flour (120 g), prepared according to the procedure described above, was deeply degreased by chloroform-methanol extraction, and then heated at 95°C for 30 min to inactivate LOX before extracting the protein. The obtained lipid-reduced soybean flour (100 g) was suspended in deionized water (1000 g) and adjusted to pH 7.0 with 1 N NaOH. The mixture was stirred for 30 min then centrifuged at 3000 g_{av} for 15 min at room temperature. The supernatant was adjusted to pH 4.5 with 1 N HCl and centrifuged at 3000 g_{av} for 15 min. The protein precipitate was re-suspended in an appropriate volume of water and adjusted to pH 7.0 with 1 N NaOH prior to vacuum freeze-drying with a freeze drier LGJ-18 (BEIJING, China). The emulsifying activity (EA) and emulsion stability (ES) of the soybean protein were determined according to previously published methods.\textsuperscript{25,26}
**Purification of soybean oil and detection of HPLC-ELSD**

TAGs were purified from the soybean oil by silica gel column chromatography, as reported by Wang et al. [27] with minor modifications. Soybean oil (2.5 g) was dissolved in 50 mL eluent (n-hexane/anhydrous diethyl ether, 250 mL, 95:5 v/v) eluted at a flow rate of 2 mL/min. The collected solution was evaporated in a vacuum to remove the organic solvent, which was the TGA.

At the end of SRS3 and SRS4 reactions, 6 M HCl was used to terminate the reaction, anhydrous ethyl ether was used to extract the oil products, TAG reaction product, TGA, and soybean oil were, respectively, dissolved in n-hexane to prepare a 0.30 mg/mL solution. The chromatographic analyses were performed with an HPLC (Waters inc., USA). Detection was performed with an ELSD (model 2424, Waters inc., USA). The chromatographic separation of the compounds was achieved with a Lichrospher Silica gel column (2.00 mm × 250 mm) operating at ambient temperature (20°C). Mobile phase A and mobile phase B were mixtures of n-hexane/isopropanol (99:1, v/v) and n-hexane/isopropanol/acetic acid (1:1:01, v/v/v), respectively. The elution flow rate was 0.30 mL/min. Gradient elution distribution is as follows: 0 min 100% mobile phase A, 10 min 80% A, 15 min 100% A, 20 min 100% A. Column temperature air, 35°C; evaporator temperature of ELSD, 55°C; carrier gas flow rate, 1.8 L/min; injection volume, 5 μL.

**Soymilk reaction system**

The composition of the SRSs is given in Table 1. Protein solution (4.5 mL of 2% solution in deionized water) was added to a 15 mL glass tube. Soybean TAG (60 mg) was added (as required, see Table 1) and the tube vortex mixed for 1 min prior to high shear mixing for 1 min using a T10 ultra-turrax (IKA, Germany). The mixture was then transferred to a 15 mL glass headspace vial. LOX (1.0 mL) and/or LIP (0.5 mL) was added to the glass vial with a pipette according to the sequences given in Table 1. For SRS2 and SRS3, the total volume of each system was made up to 6.0 mL with distilled water as appropriate. The vial was then fitted with a septum cap. For SRS5 and SRS6, the system was preincubated at 40°C for 30 min before the addition of the second enzyme with a gas-tight syringe according to the conditions given in Table 1. Samples were immediately stirred (magnetic stir bar, 120 r/min) at 40°C for 30 min. The volatile flavor of a single component of the soymilk reaction system was also determined according to the reaction conditions of the system.

**Incubation and analytical sampling**

Separate SRS runs were made for the analysis of LOOHs and flavor volatiles. Prior to HS-SPME analysis, 2-methyl-3-heptanone (1 μL of 0.5025 mg/mL in anhydrous methanol) internal standard was added via syringe. For the analysis of flavor chemicals, an SPME fiber was inserted into the headspace of the vial at the commencement of the main incubation period (t = 0); at the end of the incubation period, the fiber was then immediately desorbed into a GC-MS system according to the procedure described below. For the analysis of LOOHs, 0.3 mL of each SRS sample solution was removed via syringe at t = 30 min for analysis according to the procedure described below.

**Table 1. Major constituents of the reaction system.**

| Sample | Protein (mg) | Triglyceride (mg) | Lipoxigenase (mL) | Lipase (mL) | Deionized water (mL) |
|--------|--------------|-------------------|-------------------|-------------|----------------------|
| SRS1   | 4.50         | —                 | 1.00              | 0.50        | —                    |
| SRS2   | 4.50         | 60.00             | 1.00              | —           | 0.50                 |
| SRS3   | 4.50         | 60.00             | —                 | 0.50        | 1.00                 |
| SRS4   | 4.50         | 60.00             | 1.00              | 0.50        | —                    |
| SRS5   | 4.50         | 60.00             | 1.00              | 0.50        | —                    |
| SRS6   | 4.50         | 60.00             | 1.00              | 0.50        | —                    |

SRS4 Enzyme solution of Lipoxigenase and Lipase was added to the test tube at the same time.
SRS5 Lipoxigenase solution was added with a syringe after the lipase hydrolyzing for 30 min.
SRS6 Lipase solution was added with a syringe after the lipoxigenase catalyzing for 30 min.
Measurement of volatile flavor substances

Volatile substances were measured by HS-SPME with gas chromatography-mass spectrometry (GC-MS) according to the method reported by Achouriet al.\textsuperscript{[28]} with some modifications. The CAR–PDMS SPME fiber (85 μm) was exposed to the HS above samples (6 mL) containing 2-methyl-3-heptanone internal standard (1 μL of 0.5025 mg/mL) in a 15 mL HS vial for 30 min at 40°C with stirring (magnetic stir bar, 120 r/min). SPME fiber was desorbed into a splitless injector at 260°C for 7 min on a combined 3800/1200 L GC-MS system (Varian inc., USA) equipped with a DB-WAX column (30 m × 0.25 mm i.d., 0.25 μm df). The GC oven temperature program was as follows: 40°C (3 min hold), increase to 100°C at 6°C/min, increase to 230°C at 10°C/min (7 min hold). Scanned data acquisitions were made in the positive ionization (EI) mode over the mass range of 33–350 m/z.

Mixed standard solutions (6 mL: hexanal, 1-hexanol, 1-octen-3-ol, 1-octen-3-one, (E,E)-2,4-decadienal, benzaldehyde, and 2-pentyl furan standards) were prepared at representative concentration levels each with the same internal standard concentration (1 μL of 0.5025 mg/mL 2-methyl-3-heptanone). The above-mentioned standard products were used to correct the saturated aldehydes, unsaturated alcohols, ketones, unsaturated aldehydes, aromatic hydrocarbons, and furans in the volatile flavor. A calibration curve was obtained from the peak area ratio of each flavor standard/internal standard and the concentration of each flavor standard; flavors were identified by comparison with their reference mass spectra (based on NIST version 2.2) and quantified from their response ratio and the least-squares fit to the calibration data set.

Measurement of lipid hydroperoxides

The ferrous oxidation-xylenol orange (FOX) assay was used to determine lipid hydroperoxides (LOOHs) in the reaction system.\textsuperscript{[29,30]} Sample solution (0.3 mL) and deionized water solvent (0.2 mL) were added to freshly prepared FOX reagent (4.0 mL), and vortex mixed briefly. The reaction was incubated in the dark for 45 min and then centrifuged at 3500 g<sub>av</sub> for 10 min. The absorbance of each sample at 560 nm was measured on a UV-2450 ultraviolet spectrophotometer (Shimadzu, Japan). LOOHs were quantified using a seven-point calibration curve obtained from the absorbance of the reactant and standard concentrations of H₂O₂ solution (0–25 μM) under the same conditions (Figure 1). The concentration of LOOHs was reported as H₂O₂ μM equivalents.

Statistical analysis

LOOHs measurement and GC-MS analyses were in triplicate. Data were reported as means ± SDs. The correlation analysis was carried out between LOOHs and flavors in the reaction system and soymilk by

![Figure 1](image-url)
SPSS version 19.0 (SPSS Inc., 2014). Principal component analysis (PCA) was used to determine the relationship of volatile flavors between the SRSs and the soymilk.

Results and discussion

Characteristics of the soymilk and the soybean derived protein, TAG and enzymes

The fat content of soymilk was 10 mg/mL. Soybean TAG was chosen as the lipid component of the model system as this represented the primary source of LOX-catalyzed lipid oxidation substrate in soybeans. The soybean protein had good EA and ES in the concentration range of 1–4%. The specific activity of the extracted LOX was $3.68 \times 10^4$ U/mg protein. The specific activity of the extracted LIP was 5.85 U/mg solid. Optimum enzyme activity in SRS was obtained at LOX and LIP concentrations of 5.68 mg/mL and 2.10 mg/mL, respectively. The volatile flavor contents were <10 μg/L in LOX, TAG, LIP, and 15 μg/L in the protein. Therefore, in the following experiments, the contents of volatile substances from LOX, LIP, and TAGs were considered negligible while the volatile substances from the soybean protein were included in the quantification. For an optimum emulsifying effect, 2% protein was selected as the emulsifier of the reaction system.

Analysis of HPLC-ELSD results

Chain length and the number of ester groups of the fatty acids are the main mechanisms for separating soybean glycerides in HPLC. Soybean glycerides with different polarities have different adsorption affinities in the stationary phase and are separated according to their retention time in the chromatographic column. Comparison between Figure 2 and the standard curve of glycerides showed that the peaks presented at 2.80, 5.10, 7.70, 8.90, and 17.10 min were TGA, FFAs, 1,3-DGA, 1,2-DGA, and MAG, respectively. All sample spectra were quantified using the area normalization. In addition to the first peak hydrocarbons in Figure 2, the reaction products of TGA in the SRS3 had the most peaks, with four significant peaks (C), which indicated that TGA was hydrolyzed by LIP to produce a series of products, such as FFAs, 1,3-DGA, and 1,2-DGA. The reaction product of TGA in the SRS4 had three significant peaks (D). The curve of soybean oil and purified soybean oil had two significant peaks (A) and one significant peak (B), respectively. In the above four samples, except for the equivalent amounts of TGA of A and B, the amount of TGA of C and D gradually decreased, which was mainly caused by the hydrolysis or oxidative consumption of TGA. Other lipid components of D were also

![Figure 2](image-url). HPLC chromatograms of components of oil and its reaction products: soybean oil(A), purified soybean oil soybean TGA (B), SRS3(C), SRS4 (D).
significantly reduced compared with those of C, which may be caused by oxidative degradation of TGA hydrolyzates by LOX or further inhibition of its hydrolysis. The spectrum of soybean oil (A) showed that its main component was TGA, which indicated the refined soybean oil used in the experiment was freshly produced and excluded from air thereby preventing the formation of FFAs by oxidative degradation. Purified soybean oil only detected TGA component (B), indicating that the purification effect was better and suitable for use in the model system.

**Soymilk reaction system (SRS)**

Typically, the temperature of fresh soymilk obtained post grinding is about 35–45°C at a pH of 6.8–7.0 and the conditions of the SRSs used in this study were within this range (pH 7.0, 30 min incubation at 40°C). Soymilk is a complex emulsifying system, and the formation of its volatile flavor is not only affected by its localized environmental conditions but also by its composition. Despite the complexity of the soymilk emulsifying system, the pathway of enzymatic oxidation of LOX is specific and constant. Therefore, although the selected soymilk model system was simpler in composition and physicochemical properties, it could highlight the research purpose and effect.

**LOOHs in the reaction system**

The concentrations of LOOHs generated in each of the reaction systems (SRS1-SRS6) are shown in Table 2. LOOHs were below the limit of detection in SRS1 and SRS3 indicating that little oxidation occurred in their system. Amounts of LOOHs produced when LOX, LIP, and TAGs were combined (SRS4) were 4.80 times higher than the equivalent system without added LIP (SRS2). This was probably because LIP hydrolyses TAGs to form the unsaturated FFAs substrates for the LOX pathway. This was consistent with the results of studies showing the increased formation of LOOHs from unsaturated FFAs compared with TAGs in the presence of LOX. When TAG was pre-incubated with LOX prior to the addition of LIP (SRS6), the production of LOOHs was 1.78 μM H₂O₂ equivalents less than that for the simultaneous addition of both LOX and LIP (SRS4). When TAG was pre-incubated with LIP prior to the addition of LOX (SRS5), the production of LOOHs increased by 4.38 μM H₂O₂ equivalents compared with the simultaneous addition of both enzymes (SRS4). This observation was probably due to differences in the relative amounts of the TAG hydrolyzates available to the LOX oxidation pathway. Presumably, TAG hydrolyzates were less abundant in the reaction system that added LOX before LIP (SRS6) and more abundant in the system that added LIP before LOX (SRS5). This indicated that LOX or its oxidation products may inhibit the hydrolysis of TAG by LIP to some extent in the reaction system.

**Volatile flavor substances of the reaction system**

The prepared soymilk had a strong beany flavor, and the amounts of its main volatile flavor substances are shown in Table 3. Undesirable flavors in soymilk are the result of a combination of many flavor substances, including hexanal, hexanol, 1-octene-3-ol, trans-2-hexenal, 1-octene-3-one, 2-pentylfuran, etc. Initial investigation of the prepared soymilk sample and SRSs showed that the composition of aldehydes, alcohols, ketones, furans, and other substances was consistent with the results of

| Content(μM) | SRS1 | SRS2 | SRS3 | SRS4 | SRS5 | SRS6 | soymilk |
|------------|------|------|------|------|------|------|---------|
|            | ND   | 2.57 ± 0.12 | ND   | 12.33 ± 0.26 | 16.71 ± 0.21 | 10.55 ± 0.11 | 22.57 ± 0.30 |

ND: Not detected
The data shown are the mean value ± SD (n = 3)
Different letters in the same row indicate significant differences at the 5% level.
*The content of lipid hydroperoxides was indicated with H₂O₂ μM equivalent.
Table 3. Volatile flavor substances of the reaction system and soy milk.

| Volatile flavor substance | SRS1 (μg/L) | SRS2 (μg/L) | SRS3 (μg/L) | SRS4 (μg/L) | SRS5 (μg/L) | SRS6 (μg/L) | soymilk (μg/L) |
|--------------------------|-------------|-------------|-------------|-------------|-------------|-------------|---------------|
| Pentanal                 | 0.55 ± 0.08 \(^a\) | 3.58 ± 0.13 \(^b\) | 0.32 ± 0.07 \(^a\) | 24.25 ± 1.88 \(^c\) | 25.24 ± 0.86 \(^c\) | 22.21 ± 1.16 \(^c\) | 12.64 ± 0.38 \(^d\) |
| 1-Penten-3-one           | ND          | ND          | ND          | 20.54 ± 2.53 \(^a\) | 29.34 ± 1.33 \(^b\) | 16.32 ± 0.67 \(^a\) | 5.20 ± 0.30 \(^c\) |
| 2,3-Pentanedione         | ND          | ND          | ND          | 0.60 ± 0.10 \(^a\) | 0.35 ± 0.10 \(^a\) | ND          | 3.63 ± 0.13 \(^b\) |
| Hexanal                  | 22.51 ± 3.86 \(^a\) | 40.28 ± 2.87 \(^b\) | 14.90 ± 1.00 \(^a\) | 400.30 ± 9.73 \(^c\) | 462.63 ± 15.96 \(^cd\) | 356.80 ± 11.96 \(^bc\) | 642.50 ± 10.16 \(^d\) |
| (E)-2-Pentenal           | ND          | ND          | ND          | 17.43 ± 1.45 \(^a\) | 22.45 ± 2.51 \(^b\) | 18.24 ± 1.75 \(^a\) | 23.15 ± 3.18 \(^b\) |
| Heptanal                 | ND          | ND          | ND          | 10.72 ± 1.20 \(^a\) | 12.59 ± 2.10 \(^a\) | 5.54 ± 0.96 \(^b\) | 8.73 ± 1.25 \(^c\) |
| (E)-2-Hexenal            | ND          | ND          | ND          | 2.07 ± 0.22 \(^a\) | 5.74 ± 1.24 \(^b\) | 6.47 ± 1.00 \(^b\) | 3.44 ± 0.97 \(^a\) |
| 2-pentyl-Furan           | 0.47 ± 0.09 \(^a\) | 1.23 ± 0.12 \(^b\) | 0.96 ± 0.08 \(^a\) | 13.19 ± 1.12 \(^c\) | 25.42 ± 1.87 \(^d\) | 6.72 ± 1.17 \(^bc\) | 1.35 ± 0.12 \(^b\) |
| 3-Octanone               | ND          | ND          | ND          | 1.55 ± 0.35 \(^a\) | 4.95 ± 0.74 \(^d\) | 2.25 ± 0.67 \(^a\) | 39.89 ± 2.79 \(^c\) |
| 1-Pentanol               | 0.28 ± 0.07 \(^a\) | 0.86 ± 0.14 \(^b\) | 0.20 ± 0.04 \(^a\) | 4.64 ± 0.72 \(^c\) | 5.09 ± 0.24 \(^c\) | 4.52 ± 0.50 \(^c\) | 15.50 ± 1.26 \(^d\) |
| Octanal                  | 0.55 ± 0.08 \(^a\) | 1.67 ± 0.17 \(^a\) | 0.91 ± 0.11 \(^a\) | 2.04 ± 0.17 \(^b\) | 4.31 ± 0.62 \(^c\) | 1.90 ± 0.11 \(^b\) | 4.19 ± 0.34 \(^c\) |
| 1-Octen-3-one            | ND          | 0.75 ± 0.10 \(^a\) | ND          | 4.90 ± 0.93 \(^a\) | 14.70 ± 0.99 \(^c\) | 3.87 ± 0.90 \(^b\) | 16.54 ± 0.78 \(^c\) |
| (E)-2-Heptenal           | ND          | 3.18 ± 0.20 \(^a\) | ND          | 61.35 ± 2.75 \(^b\) | 63.05 ± 3.75 \(^b\) | 47.73 ± 2.09 \(^c\) | 34.88 ± 3.09 \(^d\) |
| 1-Hexanol                | 0.42 ± 0.07 \(^a\) | 1.53 ± 0.12 \(^b\) | 0.98 ± 0.10 \(^ab\) | 9.18 ± 1.08 \(^c\) | 10.36 ± 1.46 \(^c\) | 21.08 ± 1.71 \(^cd\) | 308.28 ± 7.41 \(^d\) |
| Nonanal                  | 1.65 ± 0.28 \(^a\) | 1.66 ± 0.27 \(^a\) | 1.71 ± 0.13 \(^a\) | 2.87 ± 0.21 \(^c\) | 4.22 ± 0.45 \(^d\) | 0.56 ± 0.11 \(^c\) | 10.65 ± 1.51 \(^d\) |
| (E)-2-Octenal            | 0.68 ± 0.08 \(^a\) | 2.84 ± 0.16 \(^b\) | 0.90 ± 0.10 \(^a\) | 25.29 ± 1.72 \(^c\) | 45.58 ± 2.24 \(^d\) | 19.38 ± 2.48 \(^bc\) | 14.20 ± 1.27 \(^bc\) |
| 1-Octen-3-ol             | 0.69 ± 0.10 \(^a\) | 1.87 ± 0.25 \(b\) | 1.42 ± 0.24 \(^b\) | 13.59 ± 1.21 \(^c\) | 17.69 ± 2.01 \(^c\) | 15.82 ± 1.19 \(^c\) | 175.99 ± 7.65 \(^d\) |
| (E,E)-2,4-Heptadienal    | ND          | ND          | ND          | 4.38 ± 0.89 \(^a\) | 8.36 ± 1.11 \(^b\) | 5.20 ± 0.96 \(^a\) | 2.48 ± 0.45 \(^c\) |
| Benzoic acid             | ND          | ND          | ND          | 5.55 ± 0.68 \(^a\) | 5.67 ± 0.52 \(^a\) | 5.44 ± 0.85 \(^a\) | 1.48 ± 0.34 \(^b\) |
| (E)-2-Nonenal            | 0.23 ± 0.07 \(^a\) | 0.53 ± 0.10 \(^ab\) | 0.21 ± 0.05 \(^a\) | 2.13 ± 0.20 \(^c\) | 3.62 ± 0.70 \(^c\) | 0.80 ± 0.16 \(^ab\) | 4.89 ± 0.52 \(^d\) |
| (E)-2-Decenal            | ND          | ND          | ND          | 4.10 ± 0.11 \(^a\) | 4.58 ± 0.39 \(^a\) | 1.09 ± 0.14 \(^c\) | 4.03 ± 0.18 \(^a\) |
| (E,E)-2,4-Nonadienal     | ND          | ND          | ND          | 1.18 ± 0.38 \(^a\) | 1.39 ± 0.48 \(^a\) | 1.07 ± 0.19 \(^a\) | 0.54 ± 0.05 \(^b\) |
| (E,E)-2,4-Decadienal     | ND          | ND          | ND          | 10.16 ± 1.15 \(^b\) | 18.53 ± 1.66 \(^b\) | 5.25 ± 0.55 \(^b\) | 0.26 ± 0.05 \(^d\) |
| Total amount (μg/L)      | 28.03 ± 3.74 \(^a\) | 62.35 ± 2.20 \(^b\) | 22.51 ± 0.83 \(^a\) | 645.70 ± 11.89 \(^c\) | 796.66 ± 23.89 \(^cd\) | 505.27 ± 9.75 \(^bc\) | 1414.99 ± 14.96 \(^d\) |

ND: Not detected

The data shown are the mean value ± SD (n = 3)

Different letters in the same row indicate significant differences at the 5% level.
A range of volatile flavor substances (23), representative of soymilk, were selected for comparative analysis. Table 3 shows the composition of volatile flavor substances in the SRSs and the prepared soymilk. Amounts of volatile flavor substances were very low or not detected in SRSs where LOX, TAG, and TAG hydrolyzates were not present simultaneously (SRS1, SRS3). Low concentrations of volatile flavors were produced by LOX-catalyzed oxidation of TAG (SRS2). This may be due to the combined action of a small amount of LOX-2 and LOX-3 isoenzymes contained in the extracted LOX on TGAs. When LIP and LOX were simultaneously added (SRS4) the types and amounts of flavor substances were greatly increased and the total amount of flavor substances was 10.35 times greater than that generated without LIP (SRS2). Presumably, this was due to the increased availability of unsaturated FFAs from the LIP-catalyzed hydrolysis of TAG. The differences in the amounts of volatile flavor substances produced by SRS4, SRS5, and SRS6 were related to the addition sequence of LIP and LOX, which could be explained by the differences in substrate compositions available for LOX-catalyzed oxidation. Compared with the prepared soymilk, the amounts of flavor substances produced by SRS4 were lower, but the main types of flavor substances were identical and related to the typical flavor of soymilk. The lower amount of flavor substances in SRS4 compared with the soymilk could be due to the absence of unsaturated FFAs and factors promoting enzymatic oxidation in the model system. To examine the similarities of volatile flavors between the soymilk and the model system, PCA was performed based on the concentration of volatile flavors detected by the GC-MS, which is listed in Table 3. According to the PCA analysis, 95.15% and 4.80% of the variance were explained by the first principal component (PC1) and second principal component (PC2), respectively. As over 99% of the PCA variance was covered by the first two principal components (PC1 and PC2), the flavor profiles of the soymilk and the model system were considered to be represented by the two components. The SRS4, SRS5, SRS6, and soymilk were concentrated in the adjacent area (Figure 3), which indicated that the volatile flavors of the SRS4-6 were similar to that of the soymilk. But for PC1, SRS4, SRS5, SRS6, and soymilk were highly similar; for PC2, they were basically similar. These indicated that the SRS4-6 produce the main characteristic flavor of soymilk, which was not exactly the same due to the influence of other ingredients in soymilk. LOOHs are the primary products of LOX-catalyzed oxidation of unsaturated FFAs which can be converted into volatile flavor substances.\[35,36\] A Pearson's correlation coefficient was calculated to determine the relationship between the values of LOOHs (Table 2) and the corresponding total volatile flavor substances (Table 3) generated in the SRSs. The results showed that there was a 'very strong' positive correlation between the total amount of flavor substances and

![Figure 3. Principal component analysis plots of PC1 and PC2 of soymilk and SRS4-6.](image-url)
LOOHs produced in the SRSs ($r = .98; \ N = 5; \ p = .002$) which was in agreement with the results of a previous study.\(^{[37]}\)

**Conclusion**

In the reaction system comprising soybean protein (emulsifier) and TAG, incubation with LIP (SRS3) produced relatively small amounts of volatile substances while incubation with LOX (SRS2) catalyzed the oxidation of TAGs to a small extent and a small amount of volatile flavor substances were generated. When LIP and LOX acted together on TAG, the degree of oxidation and the volatile flavors produced were greatly increased. The order of addition of each enzyme to SRS indicated that LOX or its oxidation products could inhibit LIP hydrolysis of TAG to some extent. The profiles of the volatile flavor substances produced by the SRS and the prepared soymilk were very similar, but they differed by their relative amounts. It can be inferred that TAGs in soybean seeds were hydrolyzed by LIP in the process of grinding soymilk, although the formation of volatile flavor substances of soymilk may also be positively or negatively affected by other components present in the matrix. In future studies, the role of other components in raw soymilk on volatile flavor formation will be explored.

**Disclosure statement**

The authors declare no conflict of interest.

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