African Journal of Biotechnology Vol. 9(40), pp. 6740-6747, 4 October, 2010
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB10.767
ISSN 1684–5315 © 2010 Academic Journals

Full Length Research Paper

A comparative study on physicochemical properties of Chinese-type soy sauces prepared using pure *koji* and mixed *kojis*

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Accepted 2 September, 2010

Proximate indices, water soluble peptides distribution, free amino acids (FAAs) and aroma active compounds in soy sauces prepared using *Aspergillus oryzae* koji (SSAO) and mixed *kojis* (SSAON, *A. oryzae koji*: *Aspergillus niger koji* = 3:1, w/w) were determined. Results showed that SSAON resulted in 6.14, 5.97, 10.40, 71.59 and 9.62% of increases in contents of formaldehyde nitrogen, total FAAs, glutamic acid, peptides (≤1 kDa) and reducing sugar when compared with those in SSAO, whereas there were no significant differences in contents of aroma active compounds except for 2-methoxy-4-vinylphenol between these two kinds of soy sauces. The higher activities of acid protease, glucoamylase present in the mixed *kojis,* were the main reason for the increases of formaldehyde nitrogen, total FAAs, glutamic acid, peptides (≤1 kDa) and reducing sugar in SSAON. Moreover, results from sensory evaluation clarified that the taste of soy sauce prepared using mixture of *A. oryzae koji* and *A. niger koji* was obviously improved. Based on the results from the present study, soy sauce preparation using mixed *kojis* is an effective approach to improve its taste.

Key words: Chinese-type soy sauce, *Aspergillus oryzae*, *Aspergillus niger*, free amino acid, aroma active compound.

INTRODUCTION

Soy sauce, which originated in China over 2500 years ago, is widely used as a seasoning or condiment in eastern Asia and its popularity in the Western part of the world is growing due to its unique taste and aroma (Petra and Peter, 2007; Gao et al., 2009). Chinese-type soy sauce is produced by fermentation of steamed soybean and raw wheat flour with *Aspergillus oryzae* (solid state fermentation or *koji* fermentation process). Then the resulting *koji* is fermented in 20 to 25% brine to yield moromi (brine fermentation or moromi fermentation process). Finally, the ripened moromi is pressed to yield soy sauce.

Although the taste compounds in soy sauce are complex, it has been widely accepted that free amino acids (FAAs) and water soluble peptides in soy sauce are essential taste compounds and provide tactile effects (Kirimura et al., 1969; Solms, 1969; Lioe et al., 2004; Lioe et al., 2007; Ogasawara et al., 2006). However, one of the challenges, often encountered in Chinese-type soy sauce fermentation process, is the low yield of FAAs, particularly glutamic acid, which is generally ascribed to the low acid protease activity secreted by *A. oryzae* during *koji* fermentation (Kitano et al., 2002). Among the proteases secreted by *A. oryzae*, neutral and alkaline proteases, the endoprotease involved in elevating utilization rate of protein during soy sauce fermentation, are the dominant proteases, whereas acid protease, the exoprotease involved in FAAs release from protein and peptides during soy sauce fermentation, is inadequate (Coenen et al., 1998; Svendsen and Degan, 1998; Kitano et al., 2002; Lin, 2005; Lu et al., 2009). Efforts to improve the profile of proteases of *A. oryzae* by strain modification

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Abbreviations: FAAs, Free amino acids; **SSAO,** soy sauces prepared using *Aspergillus oryzae* koji; **SSAON,** soy sauces prepared using *Aspergillus oryzae* koji mixed with *Aspergillus niger koji*; **QDA,** quantitative descriptive analysis; **GC-MS,** gas chromatofraphy–mass spectrophotometer.

has been lasting for decades, whereas there is no comercial *A. oryzae* with balanced proteases available in China market till now (Christensen et al., 1988; Lu et al., 2009). Degeneration and safety of modified *A. oryzae* are main reasons for this phenomenon (Pariza and Foster, 1983; Pariza and Johnson, 2001). Thus, how to increase acid protease activity in *A. oryzae koji* by safe and reliable approach is of significance for soy sauce production.

Aspergillus niger, a fungus affirmed as GRAS by the FDA in 1994, is widely used as a cell factory in different biotechnological production processes (Zofia et al., 2006). In *A. niger*, acid protease was verified the predominating extracellular proteases and its maximal proteolytic activity in *koji* reached to 6662 U/g (Lin, 2005). However, pure *A. niger koji*, which often presents obvious "wet mushroom-like" flavor, used for Chinese-type soy sauce preparation would affect its typical flavor (Ma and Kong, 2004). Thus, mixing *A. oryzae koji* with *A. niger koji* at appropriate ratio might be one promising approach to improve the proteases profile in *koji* without affecting the typical flavor of Chinese-type soy sauce and the optimal ratio, 3:1 (w/w), of *A. oryze koji* and *A. niger koji* which was established in our laboratory.

The objectives of this study were in twofold. The first was to investigate the detailed differences in physicochemical properties between soy sauces prepared using *A. oryzae* koji (SSAO) and mixed *kojis* (SSAON, *A. oryzae koji* : *Aspergillus niger koji* = 3:1, w/w). The second was to investigate the feasibility of soy sauce preparation using mixed *kojis* by quantitative descriptive analysis (QDA).

MATERIALS AND METHODS

Materials and chemicals

Soybean, wheat starch and edible salt were purchased from Guanghui Agricultural Products Co., Ltd. (Linkou, China), Runfon Flour Co., Ltd. (Guangzhou, China) and Zhongshan Salt Industrial Co., Ltd. (Zhongshan, China), respectively. *A. oryzae* 3.042 and *A. niger* 3.350 were used in SSAO and SSAON preparation. Other chemicals used in the present study were of the highest commercial grade and obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

Soy sauces preparation

SSAO and SSAON were prepared in 20 L stainless steel fermentors. The main production processes were as follows: (1) soybean was washed thrice and soaked thoroughly with tap water, (2) the soaked soybean was steamed for 15 min at 121°C to obtain moderately denatured soybean, (3) *A. oryzae* 3.042 or *A. niger* 3.350 was inoculated into the steamed soybean to make *koji*, the resulting *koji* was sampled to determine enzyme activities and water content, (4) the *A. oryzae* 3.042 *koji* or the mixed *kojis* (*A. oryzae* 3.042 *koji* : *A. niger* 3.350 *koji* = 3 : 1, w/w) was mixed with 20% (w/w) brine to yield moromi, (5) the fermentation liquids were taken on the 150th day, all liquids were filtered through filter papers, then kept in polyethylene vials and stored in a refrigerator at -20°C until analysis.

Determination of activities of neutral and acid proteases

Activities of neutral and acid proteases in *kojis* were determined by the following procedures: 2% (w/w) casein dissolved in sodium phosphate buffer (pH 7.2) or sodium lactate buffer (pH 3.0) was used as substrate of neutral or acid protease. Before proteolysis reaction, 5 g of pulverized *koji* (pulverization for 30 s) was dissolved in approximate 100 ml distilled water to extract protease, then the filtrate (Whatman No.1) was diluted with neutral or acidic buffer to 1000 or 500 ml, respectively. One millilitre (1 ml) of substrate was incubated with 1 ml diluted protease solution for 10 min at 40°C. After 10 min of reaction, 2 ml of 5% trichloroacetic acid was added and the tubes were incubated for 20 min to settle unhydrolysed protein. After removing unhydrolysed protein by filtration (Whatman No. 1), the supernatant (1 ml) was added to 5 ml of 0.4 M sodium bicarbonate followed by 1 ml of 0.4 M Folin-Ciocalteu phenol Reagent (1:2 diluted in distilled water). Absorbance of the supernatant was measured at 660 nm after 20 min of reaction. One unit of protease (U) is defined as the amount of enzyme which yields the colour equivalent to 1 µg of tyrosine in 1 ml of reaction solution per minute at 40°C.

Determination of α-amylase and glucoamylase activities

α-Amylase and glucoamylase activities were determined by the following procedures: α-amylase activity was routinely determined by the iodine-binding assay. The substrate containing 20 ml soluble starch solution (20 g/l) and 5 ml sodium phosphate buffer (pH 6.0) was preheated at 60°C for 5 min in a thermostatic water bath. One milliliter (1 ml) α-amylase solution, which was extracted from pulverized *koji* (pulverization for 30 s) and diluted with distilled water to proper concentration, was introduced into the substrate. After 5 min of reaction at 60°C, 1 ml reactant and 5 ml I_2-KI solution (I_2: 88 mg/L, KI: 40 g/l) were mixed and absorbance of the mixed solution was determined spectrophotometrically at 660 nm. α-Amylase activity was calculated by comparing the absorbance with the absorbance-α-amylase activity table in China standard of light industry (QB/T1803–1999). The glucoamylase activity was determined using 20 g/l starch solution as substrate in 0.1 M sodium acetate buffer (pH 4.6) at 40°C. The reducing sugars formed were quantified according to China standard of light industry (QB/T1803–1999) using I_2 oxidation method in alkaline solution. One unit of glucoamylase activity is defined as the amount of enzyme that releases 1 mg of glucose per hour.

Proximate analysis

Water contents of samples were determined after drying the samples to constant weight at 105°C. Contents of total nitrogen (AOAC No: 992.23, 1995), total sugar (AOAC No: 925.35, 2000) and reducing sugar (AOAC No: 923.09, 1995) in samples were measured according to corresponding AOAC methods. Formaldehyde nitrogen and total titratable acid were measured by titration method (Jiang et al., 2007). Twenty milliliter diluted samples were mixed with 60 ml H_2O and titrated to pH 9.6 with 0.05 M NaOH before 10 ml formalin solution (37%) was added. The consumed volume was recorded to determine total titratable acids of samples. The samples were finally titrated to pH 9.2 with 0.05 M NaOH.

Determination of molecular weight distribution of water soluble peptides

The molecular weight distribution of water soluble peptides in samples was determined by gel permeation chromatography on a Superdex peptide 10/300 GL column (Amersham Biosciences

Corp., NJ, USA) with UV detection at 214 nm. Elution was carried out by using an isocratic procedure with 0.02 M sodium phosphate buffer (pH 7.2) containing 0.25 M NaCl and at a flow rate of 0.5 ml/min. The column was calibrated with Globin III (MW 2512 Da), Globin II (MW 6214 Da), Globin I (MW 8519 Da), Globin I + III (MW 10700 Da) and Globin I + II (MW 14404 Da).

Determination of FAAs

Prior to FAAs analysis, samples were filtered through filter papers (0.45 µm), then the filtrates were used to determine FAAs composition using the method of Hughes and Frutiger (1990) with high performance liquid chromatography (Waters Ltd., MA, USA) and PICO.TAG amino acid analysis column. The detection wavelength was 254 nm, temperature was set at 38°C and the flow rate was 1 ml/min. The concentrations of amino acid in samples were calculated by calibrating with standard amino acids (Amino acid standard solution, type H, Sigma AAS18, MO, USA).

Aroma active compounds collection by SPME

A SPME fiber coated with 75 µm carboxen/polydimethylsiloxane (Supelco, PA, USA) was selected to collect volatile compounds for its high sensitivity and good selectivity to polar and non-polar compounds (Lee et al., 2006). Before sampling, the fiber was preconditioned for 1 h and 30 min at 275 and 250°C, respectively, in the gas chromatography (GC) injector port to eliminate possible residues on the coated fiber. Prior to analysis, samples (100 ml) were added with 10 µl methanolic solution of 2-methyl-3-heptanone as an internal standard at final concentration of 100 ng/g. Ten milliliter sample saturated with NaCl was sealed in a dedicated bottle, preheated at 45°C and stirred by a magnetic stirring bar with a speed of 200 r/min. The adsorption time was 40 min and the concentrates were desorbed at 230°C in the injection port of gas chromatograph (Finnigan Trace GC-2000, TX, USA) by holding in the splitless mode for 3 min. The SPME fiber was cleaned by keeping it in the GC injection port for additional 5 min.

GC and GC-MS analysis

Gas chromatofraphy–mass spectrophotometer (GC-MS) analysis was carried out using Finnigan TRACE GC-2000 GS-MS (Finnigan, TX, USA), equipped with a DB-5MS column (30 m length × 0.25 mm i.d × 0.25 µm film thickness, J&W Scientific, CA, USA). Volatile compounds adsorbed on the fibers were transferred into the GC injector with a splitless mode with an injection purge-off time of 3 min and injection temperature of 230°C. The initial temperature of GC oven was held for 2 min at 50°C, then raised up to 80°C (held for 5 min) with a speed of 5°C /min, and finally, increased to 230°C (held for 10 min) at a speed of 7°C /min. Ultra high purity helium was used as carrier gas at a constant speed of 1.0 ml/min. Mass spectrometer conditions were as follows: MSD capillary direct-interface temperature was 250°C; Ionization energy was 70 eV; Mass range was 35 – 335 a.m.u; Electron-multiplier voltage was 1800 V.

Compound identification and quantification

Only aroma active compounds (Baek and Kim, 2004; Lee et al., 2006; Petra and Peter, 2007) were tentatively identified by comparing their mass spectral data with those of a NIST library (including Wiley and Mainlib). Compounds were reported on the basis of their similarity (>750). Quantitative analysis was based on the ratio between peak area of a particular compound and that of internal standard (2-methyl-3-heptanone) in sample.

Sensory evaluation

Quantitative descriptive analysis (QDA) was performed according to sensory evaluation standard of Chinese-type soy sauce (China standard: GB18186–2000). The attributes of aroma, taste and appearance of SSAO and SSAON were evaluated with an internal panel of nine members (six men and three women, aged 23 – 45 years). Commercially available Chinese-type soy sauce (Meiweixian Flavoring Foods Co., Ltd., Zhongshan, China) was used by the trained panelists as reference sample at the beginning of sensory evaluation. The three attributes of samples were evaluated using a four-point linear scale from 0 (no similarity) to 3 (high similarity). All samples were coded with random three-digit numbers and served to the panelists in a randomized complete block design. Results of the three attributes of samples were plotted in a spider web diagram.

Statistical analysis

All the determinations were conducted in triplicate. The results were subjected to one-way ANOVA. Duncan's new multiple range test was performed to determine the significant difference between sample means within 95% confidence interval using SPSS 11.5 software (SPSS Inc., IL, USA).

RESULTS AND DISCUSSION

Enzyme activities and water content

Enzyme activities and water contents of *A. oryzae koji*, *A. niger koji* and their mixed *kojis* are shown in Table 1. Significant ($p < 0.05$) differences in activities of neutral protease, acid protease and glucoamylase were observed among these three kinds of *kojis*. Compared with *A. oryzae koji*, mixed *kojis* resulted in 190.07 and 61.86% increases in activities of acid protease and glucoamylase, respectively, whereas brought about a significant decrease of 26.33% in the neutral protease activity. The results were in accordance with the report by Lin (2005). However, there were no significant differences in α-amylase activity and water contents between mixed *kojis* and *A. oryzae koji*. All these results showed that enzyme profile of *A. oryzae koji* mixed with a certain proportion of *A. niger koji* could be significantly improved.

Proximate analysis

Contents of formaldehyde nitrogen, total nitrogen, total titratable acid, total sugar and reducing sugar in SSAO and SSAON are shown in Table 2. Notably, contents of formaldehyde nitrogen, total nitrogen and reducing sugar increased by 6.14, 3.14 and 9.62%, respectively, would significantly improve the taste of SSAON, because formaldehyde nitrogen and reducing sugar are generally regarded as important taste compounds in soy sauce (Chou and Ling, 1998; Kim and Lee, 2008). Reasons for

Table 1. Enzyme activities and water contents of different *kojis**.

Parameter	*A. oryzae* koji	*A. niger* koji	Mixed *kojis* (*A. oryzae* : *A. niger* = 3 : 1, w/w)
Neutral protease (U/g)	1291.67 ± 120.03 [a]	271.33 ± 33.13 [c]	951.56 ± 69.01 [b]
Acid protease (U/g)	287.67 ± 30.01 [c]	1928.00 ± 151.01 [a]	834.44 ± 71.00 [b]
α-amylase (U/g)	239.20 ± 23.83 [a]	186.60 ± 17.80 [b]	221.67 ± 21.81 [ab]
Glucoamylase (U/g)	1840.23 ± 124.66 [c]	5255.48 ± 403.95 [a]	2978.65 ± 217.76 [b]
Water content (g/kg)	288.09 ± 10.80 [b]	329.58 ± 16.48 [a]	301.86 ± 12.61 [b]

* Each value is expressed as mean ± standard deviation (n = 3).
[a–c] Different letters in the same row indicate significant differences ($p < 0.05$).

Table 2. Chemical properties of SSAO and SSAON*.

Parameter	SSAO [a]	SSAON [a]
Formaldehyde nitrogen (g/l)	9.12 ± 0.32	9.68 ± 0.37
Total nitrogen (g/kg)	16.59 ± 0.57	16.64 ± 0.62
Total titratable acid (g/l)	16.51 ± 0.68	17.43 ± 0.73
Reducing sugar (g/l)	36.28 ± 1.40	39.77 ± 1.48
Total sugar (g/l)	52.96 ± 2.25	53.42 ± 2.19

* Each value is expressed as mean ± standard deviation (n = 3).
[a] Values in each row indicate insignificant differences ($p > 0.05$).

the increases of formaldehyde nitrogen, total nitrogen and reducing sugar in SSAON were due to the higher activities of acid protease and glucoamylase in mixed *kojis* (Table 1), which can catalyze protein, peptides and starch into amino acids and glucose (Lin, 2005). Otherwise, the increase of formaldehye nitrogen in SSAON might be responsible for the 5.57% increase of total titratable acid in it. Only slight difference in content of total sugar between SSAO and SSAON was observed. The results indicated that soy sauce preparation using mixed *kojis* is an effective approach to improve its taste.

Molecular weight distribution of water soluble peptides

Molecular weight distribution of water soluble peptides in SSAO and SSAON is shown in Table 3. Significant ($p < 0.05$) differences in peptides with molecular weight of 5 – 10 kDa and less than 1 kDa between SSAO and SSAON were observed. Peptides with molecular weight of 5 – 10 kDa and less than 1 kDa in SSAON decreased by 47.33% and increased by 71.59% when compared with those in SSAO. More low-molecular-weight peptides (≤1 kDa) present in SSAON indicated higher activity of acid protease and peptidase, which are responsible for the formation of low-molecular-weight peptides and FAAs (Kitano et al., 2002), which existed in its corresponding moromi during fermentation. Furthermore, it should be noted that peptides with molecular weight of 5 10 kDa, namely “taste enhancer peptides” (Ogasawara et al., 2006), were dominant in both kinds of soy sauces.

FAAs analysis

FAAs are regarded as important contributor to the unique taste of soy sauce (Lioe et al., 2004; Lioe et al., 2007). Therefore, investigation on FAAs in SSAO and SSAON was conducted and the results are shown in Table 4. According to the taste characteristics of FAAs described by Kato et al. (1989) and Schoenberger et al. (2002), FAAs were grouped as umami (aspartic acid and glutamic acid), sweet (alanine, glycine, serine, threonine, proline and lysine), bitter (arginine, histidine, isoleucine, leucine, methionine, phenylalanine, tryptophan, tyrosine and valine) and tasteless (cysteine).

There were no significant differences in the contents of FAAs except for glutamic acid and arginine between both kinds of soy sauces. As for most FAAs in both kinds of soy sauces, their contents were higher than their threshold values except for threonine and proline (Table 4), which indicated that FAAs were taste active compounds in both kinds of soy sauces. Notably, contents of total FAAs and glutamic acid, a key taste compound in soy sauce, in SSAON increased by 5.97 and 10.40% when compared with those in SSAO, respectively. The higher activities of acid protease and glutaminase in SSAON secreted by *A. niger* were responsible for the increases of total FAAs and glutamic acid. It is generally regarded that acid protease and glutaminase can release amino acids and glutamic acid from

Table 3. Molecular weight distribution of water soluble peptides in SSAO and SSAON*.

Molecular weight distribution	Relative content (%)	
	SSAO	SSAON
≥ 10 kDa	2.78 ± 0.25 [a]	2.57 ± 0.19 [a]
5–10 kDa	11.24 ± 0.70 [a]	5.92 ± 0.42 [b]
1–5 kDa	79.82 ± 2.01 [a]	80.94 ± 2.34 [a]
≤ 1 kDa	6.16 ± 0.37 [b]	10.57 ± 0.52 [a]

* Each value is expressed as mean ± standard deviation (n = 3).
[a–b] Different letters in the same row indicate significant differences ($p < 0.05$).

Table 4. Contents of FAAs in SSAO and SSAON*.

FFAs	Contents of FAAs (g/L)		Taste attributes and threshold values (g/L) in water	
	SSAO	SSAON		
Aspartic acid	1.10 ± 0.06[a]	1.08 ± 0.05[a]	Umami	1.00 [A]
Glutamic acid	4.09 ± 0.18[b]	4.65 ± 0.21[a]	Umami	0.30 [A]
Content of umami FAAs	5.19	5.73		
Serine	1.90 ± 0.09 [a]	1.99 ± 0.11 [a]	Sweet	1.50 [A]
Glycine	1.50 ± 0.05 [a]	1.43 ± 0.07 [a]	Sweet	1.30 [A]
Threonine	2.55 ± 0.16 [a]	2.56 ± 0.14 [a]	Sweet	2.60 [A]
Alanine	2.89 ± 0.19 [a]	2.74 ± 0.17 [a]	Sweet	0.60 [A]
Proline	2.74 ± 0.19 [a]	3.05 ± 0.20 [a]	Sweet/bitter	3.00 [A]
Lysine	3.19 ± 0.25 [a]	3.33 ± 0.23 [a]	Sweet/bitter	0.50 [A]
Content of sweet FAAs	14.77	15.10		
Histidine	2.12 ± 0.11 [a]	2.22 ± 0.14 [a]	Bitter	0.20 [A]
Arginine	4.29 ± 0.20[b]	4.85 ± 0.23[a]	Bitter	0.50 [A]
Isoleucine	3.92 ± 0.23 [a]	4.02 ± 0.26 [a]	Bitter	0.90 [A]
Leucine	6.36 ± 0.35 [a]	6.59 ± 0.41 [a]	Bitter	1.90 [A]
Methionine	1.29 ± 0.04 [a]	1.34 ± 0.06 [a]	Bitter	0.30 [A]
Phenylalanine	3.53 ± 0.22 [a]	3.91 ± 0.26 [a]	Bitter	0.90 [A]
Tyrosine	1.20 ± 0.05 [a]	1.38 ± 0.07 [a]	Bitter/sweet	0.91 [B]
Valine	4.01 ± 0.29 [a]	4.21 ± 0.33 [a]	Bitter	0.40 [A]
Tryptophan	0.90 ± 0.04 [a]	1.06 ± 0.05 [a]	Bitter	0.82 [B]
Content of bitter FAAs	27.62	29.58		
Cysteine	0.15 ± 0.02 [a]	0.17 ± 0.02 [a]	Tasteless	ND [AB]
Content of all FAAs	47.73	50.58		

* Each value is expressed as mean ± standard deviation (n = 3).
[a–b] Different letters in the same row indicate significant differences ($p < 0.05$).
[A] Kato et al. (1989).
[B] Schoenberger et al. (2002).

protein, peptides and glutamine, respectively (Veki, 1997). Meanwhile, the increase of total FAAs in SSAON was in line with the increase of formaldehyde nitrogen in SSAON (Table 2). The results further clarified that soy sauce prepared using mixed *kojis* could increase the content of total FAAs, especially that of glutamic acid and possibly improve its taste.

Aroma active compounds analysis

To clarify the differences in aroma compounds between SSAO and SSAON, aroma compounds in them were identified and quantitated, only aroma active compounds reported by Baek and Kim (2004), Lee et al., (2006) and Petra and Peter (2007) were summarized in Table 5.

Table 5. Aroma active compounds and their contents in SSAO and SSAON*.

RT	Compounds	Odour description	Relative contents	
			SSAO	SSAON
2.11	Ethanol	Alcoholic/solvent-like [B]	0.981 ± 0.178 [a]	0.975 ± 0.0163 [a]
2.69	Acetic acid	Sour [C]	0.385 ± 0.107 [a]	0.410 ± 0.113 [a]
2.79	Butanol, 3-methyl-	Malty [C]	0.039 ± 0.010 [a]	0.038 ± 0.007 [a]
2.90	Butanal, 3-methyl-	Malty [C]	0.031 ± 0.008 [a]	0.034 ± 0.011 [a]
3.16	Butanal, 2-methyl-	Malty [C]	0.012 ± 0.004 [a]	0.015 ± 0.006 [a]
5.44	Butanoic acid, 3-methyl-	Sweaty/ cheese-like [C]	0.022 ± 0.004 [a]	0.024 ± 0.005 [a]
5.77	Butanoic acid, 2-methyl-	Sweaty/cheese-like [C]	0.086 ± 0.015 [a]	0.090 ± 0.016 [a]
7.15	Propanal, 3-(methylthio)-	Cooked potato [C]	0.030 ± 0.008 [a]	0.025 ± 0.006 [a]
8.80	Benzaldehyde	Bitter [A]	0.104 ± 0.023 [a]	0.096 ± 0.019 [a]
9.01	1-Propanol,3-(methylthio)-	Cooked potato [C]	0.111 ± 0.020 [a]	0.121 ± 0.025 [a]
9.36	1-Octen-3-ol	Mushroom-like [C]	0.596 ± 0.113 [a]	0.688 ± 0.135 [a]
11.38	Hexanol, 2-ethyl-	Rosy/sweet [B]	0.230 ± 0.039 [a]	0.209 ± 0.031 [a]
12.15	Benzeneacetaldehyde	Hoeny-like [C]	0.274 ± 0.051 [a]	0.250 ± 0.043 [a]
14.34	Phenol, 2-methoxy-	Burnt [C]	0.023 ± 0.006 [a]	0.019 ± 0.004 [a]
15.52	Benzeneethanol	Flowery [C]	0.044 ± 0.013 [a]	0.052 ± 0.017 [a]
16.59	Benzoic acid	Urine-like [B]	0.027 ± 0.005 [a]	0.032 ± 0.008 [a]
16.69	Phenol, 2,6-dimethoxy-	Smoky/wood [B]	0.007 ± 0.003 [a]	0.010 ± 0.004 [a]
16.75	Ethanone, 1-(1H-pyrrol-2-yl)-	Roasty/popcorn-like [C]	0.018 ± 0.005 [a]	0.012 ± 0.004 [a]
18.10	4-Hydroxy-2,5-methyl-3(2H) furanone	Caramel-like [C]	0.008 ± 0.003 [a]	0.011 ± 0.004 [a]
18.18	2-methyl-3-hydroxy-4H-pyran-4-one	Caramel-like [C]	0.008 ± 0.004 [a]	0.007 ± 0.003 [a]
18.34	Benzeneacetic acid	Hot chocolate /sweet [C]	0.012 ± 0.004 [a]	0.015 ± 0.005 [a]
21.55	2-Methoxy-4-vinylphenol	Spicy [C]	0.298 ± 0.040 [b]	0.479 ± 0.075 [a]
Total content			3.346	3.612

* Each value is expressed as mean ± standard deviation (n = 3).
RT: Retention time.
[a–b] Different letters in the same row indicate significant differences ($p < 0.05$).
[A] Baek and Kim (2004); [B] Lee and Kim (2006); [C] Petra and Peter (2007).

Totally, 22 aroma active compounds including 5 acids, 5 alcohols, 4 aldehydes, 1 furanone, 3 phenols, 1 pyranone, 1 pyrrol and 2 sulfur-containing compounds were identified in SSAO and SSAON.

ANOVA showed that there were no significant ($p > 0.05$) differences in contents of most aroma active compounds except for 2-methoxy-4-vinylphenol between these two kinds of soy sauces. Notably, fermentation of mixed *kojis* caused 60.74, 15.44 and 9.01% increases in contents of 2-methoxy-4-vinylphenol, 1-octen-3-ol, 1-propanol, 3-(methylthio)- and 33.33, 9.13 and 8.76% decreases in contents of ethanone, 1-(1H-pyrrol-2-yl)-, hexanol, 2-ethyl- and benzeneacetaldehyde for SSAON when compared with those in SSAO. Among the aroma active compounds (Table 5), 1-octen-3-ol, one of the major components in Japanese-type soy sauce, is regarded as the degraded products of unsaturated fatty acid, such as linoleic and linolenic acids (Lee et al., 2006). 2-methoxy-4-vinylphenol, which might arise from wheat fraction by *Candida* (*Torulopsis*) yeasts fermentation, is a very strong contributor to the good aroma of Japanese-type soy sauce (Nunomura and Sasaki, 1992). The differences in contents of 1-octen-3-ol and 2-methoxy-4-vinylphenol might cause the aroma difference between SSAO and SSAON due to their high contents. Furthermore, total content of the 22 aroma active compounds in SSAOS increased by 7.95% when compared with that in SSAO, which was a potential reason to give rise to aroma discrepancy between these two kinds of soy sauces.

Sensory evaluation

The sensory profiles of SSAO and SSAON based on QDA test by nine panelists are shown in Figure 1. The sensory score of taste in SSAON was obviously higher than that in SSAO, the higher contents of formaldehyde nitrogen, reducing sugar, low-molecular-weight peptides (≤1 kDa) and FAAs, especially glutamic acid, in SSAON were responsible for the taste difference between SSAO and SSAON (Tables 2 - 4). Although differences in contents of 2-methoxy-4-vinylphenol, 1-octen-3-ol, 1-propanol, 3-(methylthio)-, ethanone, 1-(1H-pyrrol-2-yl)-, hexanol, 2-ethyl-, benzeneacetaldehyde and all the 22 aroma active compounds between SSAO and SSAON were observed

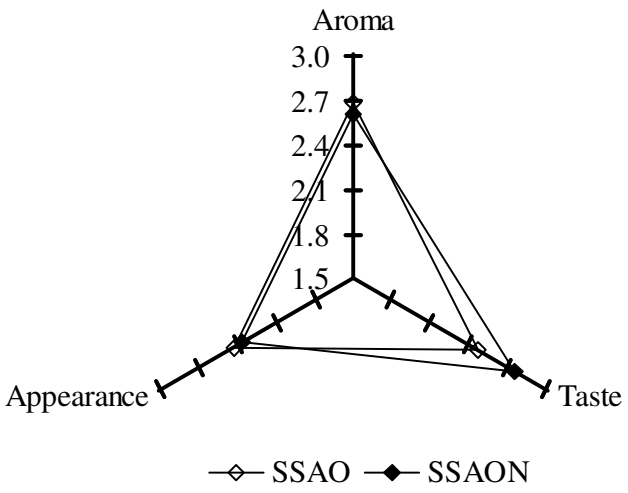

Figure 1. Sensory evaluation of SSAO and SSAON.

(Table 5), the difference in sensory score of aroma between them was almost negligible. Aroma formation in soy sauce depends on not only effect of a few aroma compounds, but synergistic effect of all the aroma compounds and complicated matrix of soy sauce. Thus the possible aroma discrepancy between SSAO and SSAON caused by the differences in contents of aroma compounds was significantly weakened by the complicated matrices in them. Furthermore, no obvious difference in sensory score of appearance between SSAO and SSAON was observed according to result of QDA. The results indicated that there was obvious difference in taste, but no significant differences in aroma and appearance between SSAO and SSAON.

Conclusion

A comparative study on physicochemical properties including proximate indices, water soluble peptides distribution, FAAs, aroma active compounds and sensory evaluation of SSAO and SSAON was conducted. Results showed that contents of formaldehyde nitrogen, total FAAs, glutamic acid, peptides (≤ 1 kDa) and reducing sugar in SSAON markedly increased when compared with those in SSAO, whereas there were no significant differences in contents of aroma active compounds except for 2-methoxy-4-vinylphenol between these two kinds of soy sauces. Different activities of acid protease, glucoamylase in pure *A. oryzae koji* and mixed *kojis* caused the differences in physicochemical properties for both kinds of soy sauces. Sensory evaluation further clarified that the taste of SSAON was obviously improved when compared with that of SSAO. Based on the current results, soy sauce preparation using mixed *kojis* (*A. oryzae koji*: *A. niger koji* = 3:1, w/w) is an effective approach to improve its taste by increasing taste compounds in it.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the National Science-Technology Supporting Project for 11th Five-Year Plan (Nos. 2007BAK36B03 and 2008BAI63B06) and the Key Technology R&D Program of Guangdong Province (Nos. 2007A010900001, 2008A010900001 and 2009A010700004) for their financial support.

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