

Assessment of added ingredient effect on interaction of simulated beef flavour and soy protein isolate by gas chromatography, spectroscopy and descriptive sensory analysis

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Abstract

The influence of ingredients such as glucosamine, sucrose, ascorbic acid, and/or polyethylene glycol, on the release of beefy aroma components of simulated beef flavour (SBF) in the presence of soy protein isolate (SPI), were investigated. Conformational changes of SPI protein structure induced by the added ingredients were also detected. Addition of ascorbic acid alone or with polyethylene glycol resulted in reduction of disulfide bonds, increase in surface hydrophobicity and increase in unordered structure of SPI. The SPI–SBF mixtures containing ascorbic acid alone or with polyethylene glycol showed increased GC peak areas of indicator peaks, which were associated with an increase in the perceived beef characteristic attributes in descriptive analysis, as expressed by enhancement of roasted note and diminishing of soymilk-like and cereal notes. These results provide the basis for further research to elucidate strategies to maximize perception of beefy aroma in soy based products.

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1. Introduction

Soy protein has gained increasing attention as a promising protein source with various health benefits in addition to functional properties. Due to the problematic retention of indigenous off-flavour arising from the action of lipoxygenases present in soybean, much research has been conducted to investigate the interaction of soy protein with model compounds. In contrast, research is required but lacking to elucidate the binding and release of intentionally added, desirable flavour components by soy protein, which is important in formulating soy-based products with simulated meat flavours for the vegetarian market (Malcolmson & McDaniel, 1987).

Several studies have investigated the interaction of flavour compounds with soy proteins in model systems (Beyeler & Solms, 1974; Damodaran & Kinsella, 1981a, 1981b; Gremler, 1974; Li, Grün, & Fernando, 2000; Thissen, 1982; Wilson, 1985; Zhou & Cadwallader, 2006). Comparison of the reported literature reveals some conflicting data in terms of nature and types of interaction between flavour compounds and soy proteins such as hydrophobic interaction, hydrogen bonding, dipole forces or van der Waals forces, which may be attributed to differences in methods and experimental conditions for analysis, as well as origin and degree of denaturation of the soy protein starting material (O'Keefe, Wilson, Resurreccion, & Murphy, 1991). Furthermore, most of the studies conducted to date have used model systems of single ingredients or selected volatile model compounds such as series of aldehydes, ketones, alcohols, or alkanes, while flavour ingredients in the real food system usually contain various subclasses of compounds. Depending on the nature and the strength of

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the binding, each aroma compound could be released at a different rate from SPI, which could have an impact on flavour suppression or alteration of flavour profiles in the final food products due to changes in the odour balance.

For the development of soy products with acceptable flavour quality it is imperative to elucidate the nature of the interactions of soy proteins with the mixture of compounds present in the actual flavour ingredients. Recently, we have conducted research to elucidate the impact of SPI on aroma characteristics of a commercially produced simulated beef flavour (SBF) ingredient (Moon, Cliff, & Li-Chan, 2006; Moon & Li-Chan, accepted for publication). By using headspace solid phase microextraction coupled with gas chromatography (GC), GC–mass spectrometry (GC–MS) and GC–olfactometry (GC–O) analysis, the odour-active components were recognized, and several high aroma impact compounds from the volatile compounds in SBF were identified (Moon et al., 2006). Descriptive analysis (DA) and GC analysis were conducted to assess the aroma characteristics of SBF in mixtures with SPI, and volatile compounds in SBF that were related to a “beefy” note were tentatively identified. Addition of SPI resulted in decreasing of “beefy”, “roasted”, and “yeasty” aroma notes in the samples (Moon & Li-Chan, accepted for publication). Several GC indicator peaks were selected to represent the beefy aroma notes based on their odour-active properties as well as the correlation between the indicator peak areas of SBF–SPI mixtures and beefy attribute intensity by DA. However, the nature of the SBF–SPI interactions resulting in the changes in aroma attributes was not explored.

Therefore, the objectives of this study were to further examine the interactions between SPI and SBF as influenced by food ingredients or additives that may affect SPI conformation. Specifically, glucosamine, sucrose, ascorbic acid, and polyethylene glycol were selected based on their potential to alter hydrogen bonding, hydrophobic interactions or disulfide bonds in SPI, in order to explore the effect of conformational changes of SPI on its interactions with flavour compounds, particularly with regard to beefy attribute related aroma compounds in SBF. The interactions between SPI and flavour compounds as influenced by the additional ingredients were investigated from the flavour “releasing” point of view, focusing on the “availability of flavour compounds from the bulk food into the gas phase for sensory perception” (McGorin, 1996). The results from this study may provide a foundation for better understanding of the interactions between SPI and SBF.

2. Materials and methods

2.1. Materials

Simulated beef flavour (SBF; vegetarian beef type flavour F96x49 containing maltodextrin, autolyzed yeast extract, natural flavours, onion powder and silicon dioxide)

was a commercially available product from Mastertaste (Arlington Heights, IL). Soy protein isolate (SPI; lot # 02060631-532) was a commercially available product from the Solae Company (St. Louis, MO), containing $89.3 \pm 0.1\%$ protein as analyzed by the nitrogen combustion method using a LECO FP-428 (LECO Corporation, Joseph, MI). Sucrose, ascorbic acid (min. 99%), glucosamine (min. 99%), and polyethylene glycol (average Mw. 8000) were purchased from Sigma Chemical Co. (St. Louis, MO) and spectral-grade methanol was from Fisher Scientific (Fairlawn, NJ). The fluorescent probe, 6-propionyl-2-(dimethylamino) naphthalene (PRODAN), and Ellman’s reagent, 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB) were from Sigma Chemical Co. (St. Louis, MO). The solid phase assembly holder, 50/30 μm stableflex divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS), 15 mL capacity GC sample vials and polypropylene hole cap with PTFE/silicone septa were purchased from Supelco (Sigma–Aldrich Canada, Oakville, ON).

2.2. Differential scanning calorimetry (DSC)

The thermal behaviour of SPI was investigated by a multi-cell differential scanning calorimeter thermal analyzer (model 4207 MC-DSC, Calorimetry Science Corp). Three concentrations of SPI (0.2%, 5%, and 10%) were prepared in pH 7.5 phosphate buffer (3.88 mM NaH_2PO_4 , 15.5 mM Na_2HPO_4). Approximately 0.05 g of each sample was placed in Hastelloy C ampoules, which were sealed then weighed accurately. A sealed empty cell was used as a reference. For DSC analysis, each sample was held at 25 °C and equilibration time was set at 600 (10 min). Heating scans were conducted from 25 °C to 180 °C at a rate of 1 °C/min for each sample.

2.3. Gas chromatography

For headspace solid phase microextraction (HS-SPME), 100 mg of each ingredient i.e. glucosamine, sucrose, polyethylene glycol, or ascorbic acid, was first mixed with 250 mg SPI in a 15 mL capacity GC sampling vial with a magnetic stirring bar. Following addition of 5 g buffer (0.05 M Tris–HCl buffer pH 7.4), the vial was incubated at either room temperature (RT; 23 °C) or 60 °C for 20 min. To investigate the effects of a mixture of polyethylene glycol and ascorbic acid, the sample was first incubated with 100 mg of ascorbic acid for 20 min at RT or 60 °C, and then 100 mg of polyethylene glycol was added and the mixture was incubated for another 20 min at the same temperature before adsorption. SBF (500 mg) was then added to each vial containing the incubated SPI and ingredients, and the vial was tightly capped with a polypropylene hole cap with a PTFE/silicone septum. As a control, a sample containing SPI and SBF was prepared without any additional ingredients and analyzed under the same condition. A sample with only SBF (no SPI) was also ana-

lyzed. Stirring with a magnetic stirring bar was consistently applied.

To extract headspace volatile compounds, adsorption was performed with 50/30 μm DVB/CAR/PDMS SPME fibre exposed to the headspace above the sample solution for 60 min at 60 °C in a thermostat controlled water bath (± 2 °C). Analysis of the samples adsorbed at RT was also conducted to observe changes in volatile components induced by adding ingredients to SPI in the absence of heat. Analysis of volatile components was performed by gas chromatography – flame ionization detector as previously described (Moon & Li-Chan, 2004). The effect of each ingredient at each temperature condition was analyzed in triplicate with three independently prepared samples and the results were presented as the mean of the triplicate values. In addition, SBF alone without either SPI or ingredients as well as SBF with SPI but no ingredients were also analyzed in triplicate; the average coefficients of variation for individual peak areas of these triplicates were 10.1% and 10.6%, respectively. The area of each individual peak in each sample was expressed as relative peak area (%) compared to the peak area of SBF (100%).

2.4. Sulfhydryl and disulfide content

Samples of SPI with or without added ingredient in pH 8.0 buffer (85 mM Tris, 100 mM glycine, 4 mM EDTA, 10 mg/mL SDS) were prepared as described for GC analysis. Ingredient blank samples without any SPI were also prepared. The content of total sulfhydryl groups (SH) was determined with Ellman's reagent (DTNB) by the method of Beveridge, Toma, and Nakai (1974) as modified by Chung, Lei, and Li-Chan (2005). Total sulfhydryl and disulfide content (SH + SS) was measured by the method of Thannhauser, Konishi, and Scheraga (1984), with incubation in the dark as suggested by Damodaran (1985). The content of disulfide (SS) groups was determined by subtracting total SH content from (SH + SS) content. Results were expressed as the mean of duplicate analyses.

2.5. Surface hydrophobicity

SPI stock solution (10 mL of 1% w/v SPI in 0.05 M Tris buffer, pH 7.4, with 0.02% sodium azide) was mixed with the ingredient (40 mg of glucosamine, sucrose, polyethylene glycol, or ascorbic acid). Ingredient blank samples were prepared using buffer without any SPI. The samples were heated at 60 °C for 20 min, with additional heating at 60 °C for 20 min after adding polyethylene glycol for the sample mixture of ascorbic acid and polyethylene glycol. After cooling down the samples in cold water for 15 min, serial dilution was performed to obtain five SPI concentrations ranging from 0.010% to 0.0025%.

Surface hydrophobicity of SPI samples was determined by the hydrophobic fluorescent probe method using PRO-DAN as described by Alizadeh and Li-Chan (2000). Relative fluorescence intensity (RFI) of samples and blanks was

measured by a Shimadzu RF-540 (Shimadzu Corp., Kyoto, Japan) spectrofluorometer. The initial slope (S_0) of net RFI versus SPI concentration was calculated by linear regression analysis and reported as surface hydrophobicity of the sample.

2.6. FT-Raman spectroscopy

Samples containing SPI with or without ingredient were prepared as described above for “gas chromatography” analysis and then freeze-dried for FT-Raman spectroscopy, due to the poor signal to noise ratio of the spectra of the aqueous samples observed in preliminary experiments. The freeze-dried samples and original (not freeze-dried) SPI powder as well as ingredients were placed in NMR tubes and FT-Raman spectral data were collected using a Nexus 670 FT-Raman spectrometer equipped with CaF_2 beam splitter, Ge detector and OMNIC version 6.0a software (Thermo Nicolet Corp., Madison, WI). The analyses for each sample were performed under the following conditions: laser power, 0.5 W; scan number, 512; spectral resolution, 4 cm^{-1} . The spectrum of polystyrene standard was used to adjust sample position for maximal signal intensity to verify the system before analyzing samples. Spectral data processing including baseline correction, subtraction of ingredient spectra and normalization to the intensity of phenylalanine peak at 1003 cm^{-1} was performed using GRAMS/AI Version 7.02 (Thermo Galactic, Galactic Industries Corp., Salem, NH). Assignments of the bands in the Raman spectra to the specific vibrational modes of amino acid chain or polypeptide backbone were based on the literature (Li-Chan, 1996). Secondary structure analysis based on the amide I region using the Raman Spectral Analysis Package (RSAP) program (version 2.1) of Przybycien and Bailey (1988) was conducted with ingredient subtracted Raman spectral data. FT-Raman spectroscopic analyses were conducted in duplicate; coefficients of variation of peak intensities for duplicate spectra were 1% or less.

2.7. Sensory evaluation

Descriptive analysis was performed using five panelists from the previous study (Moon & Li-Chan, [accepted for publication](#)) and two additional panelists recruited from graduate students in the Food Science program at UBC and from staff at a food company producing meat substitute products. A 20 min-training session was held to remind the panelists of the five sensory attributes, i.e., roasted, beefy, yeasty, soymilk-like, and cereal, as previously established (Moon & Li-Chan, [accepted for publication](#)). Three different samples (SBF only, SBF with SPI, and SBF with ascorbic acid treated SPI) were used for the training session.

For the descriptive sensory analysis, the following samples were prepared: 250 mg SPI in 5 g distilled deionized (dd-) water with 150 mg SBF (SPIF), 250 mg ascorbic

acid-treated SPI in 5 g dd-water with 150 mg SBF (AF), and 250 mg ascorbic acid and polyethylene glycol-treated SPI in 5 g dd-water with 150 mg SBF (APF). In comparison to the previous study (Moon & Li-Chan, [accepted for publication](#)) in which SPI and SBF were prepared in batch scale and then aliquoted into individual vials, in the present study SBF was added into each individual vial for sensory evaluation, in order to minimize possible loss of volatiles during the aliquoting process. The three samples (coded with 3-digit random numbers) and a reference (250 mg SPI in 5 g dd-water with 150 mg SBF) were covered with aluminum foil and held at 60 °C for 20 min before serving. The intensity of each attribute in a sample was rated on an unstructured 15 cm line scale anchored at 1.3 cm from each end and labeled with “Slight” on the left, “Moderate” in the center, and “Intense” on the right. The reference (R) was identified as “moderate” in the scale. Data were quantified as distance from the origin in centimeter.

2.8. Statistical analysis

General linear model of analysis of variance (ANOVA) was performed using Minitab software (version 13.30, Minitab Inc. PA), and Fisher's least significant difference (LSD) test was performed to compare samples at the 95% confidence level. For FT-Raman spectral data, the mean values from duplicate analyses were reported, and significant differences between samples were determined using lower and upper 95% confidence limits calculated based on the assumption of 5% coefficient of variation for the FT-Raman analysis (Badii & Howell, 2003).

3. Results and discussion

3.1. Differential scanning calorimetry

The thermograms of SPI at three different concentrations did not show any peaks in the 25–180 °C temperature range investigated (data not shown). Generally, native proteins show endothermic changes associated with the rupture of hydrogen bonds or exothermic changes upon weakening of hydrophobic interactions and aggregation of proteins by thermally induced denaturation (Li-Chan & Ma, 2002). The absence of endothermic or exothermic peaks in the thermograms of the SPI used in this study, suggests that the proteins were already considerably denatured, most likely during the processing of SPI. Arrese, Sorgentini, Wagner, and Añón (1991) also reported that 7 out of 19 commercial SPI they examined did not show any peaks in DSC analysis.

3.2. Gas chromatography

The relative % areas of individual peaks in each sample after incubation at room temperature (RT, 23 °C) or 60 °C are presented in [Tables 1 and 2](#), using HS-SPME adsorp-

tion at room temperature and at 60 °C, respectively. The relative % areas were calculated based on the area of each peak relative to the corresponding peak in SBF alone, which was designated as 100%, and the tables only show peaks whose areas were significantly ($p \leq 0.05$) positively correlated with beefy note in sensory evaluation in the previous study (Moon & Li-Chan, [accepted for publication](#)). Some of these peaks represented odour-active compounds with detection frequency $\geq 50\%$ by GC-olfactometry, but many peaks were not odour-active and were not identified (“unknown”). Nevertheless, it could be noted that the areas of most peaks were reduced by addition of SPI to SBF, and partial recovery of the peak areas was achieved to varying extents by first incubating SPI with the ingredients, prior to mixing with SBF. Bold numbers in [Tables 1 and 2](#) indicate significant difference ($p \leq 0.05$) in GC peak area in the presence of the added ingredients compared to the sample containing SBF with only SPI (abbreviated in the tables as “SPIF”).

The ability of SPI to suppress release of beefy related volatile compounds in the headspace, as indicated by the GC peak areas, was noticeably reduced in SPI containing glucosamine under the conditions of room temperature incubation and room temperature adsorption, as shown in [Table 1](#). However, the effect of glucosamine disappeared under the condition of 60 °C incubation or 60 °C adsorption. The observed result may be attributed to the changes in hydrogen bonding interactions of the SPI affected by the hydrogen in the amino group of glucosamine. The energy of hydrogen bonds was reported as 8–40 kJ/mol and is readily weakened by heating, while hydrophobic interaction is enhanced by heating (Cheftel, Cuq, & Lorient, 1985). Ionic interactions between SPI and glucosamine were not considered to contribute significantly in this study where Tris buffer at pH 7.4 was used, since these forces would only be expected to play an important role in the pH range between 4.6 and pH 6.9, i.e. between the *pI* of SPI and *pKa* of glucosamine where SPI would be negatively charged and glucosamine would be positively charged.

Moreover, addition of ascorbic acid alone or with polyethylene glycol showed recovery of some peaks almost to the level found in SBF alone ([Tables 1 and 2](#)). A number of these peaks were reported in our previous study (Moon & Li-Chan, [accepted for publication](#)) to be highly positively related to the beefy note from SBF ($p \leq 0.001$), e.g. the unidentified peaks with retention time of 29.971, 30.729, 31.881 and 33.306 min in [Table 1](#), and 31.317 min in [Table 2](#), and delta-3-carene and beta-cubebene with retention times of 12.132 and 30.377 min in [Table 2](#). In addition, 3-methyl-furan, delta-3-carene, decanal, an unidentified compound and beta-cubebene eluting with retention time at 1.734, 12.132, 22.151, 25.794, and 30.377 min, respectively, were shown to be associated with high detection frequency (greater than 50%) in GC-O, and may therefore be expected to have an impact on perceived aroma profile.

Table 1
Effect of added ingredient in SBF–SPI mixtures^a incubated at room temperature (RT, 23 °C) or 60 °C on the peak areas^b of volatile compounds captured by HS-SPME under the adsorption condition at RT

IP# ^c	Retention time ^d	SBF		RT incubation–RT adsorption						60 °C incubation –RT adsorption						Peak characteristics		
				Relative % area						Relative % area						Peak identification	DF ^e (%)	Beefy ^f
		Area	%	SPIF	GF	SF	PF	AF	APF	SPIF	GF	SF	PF	AF	APF			
IP1	1.734	11512	100	71	94	68	60	88	48	42	42	40	32	55	56	3-Methyl furan	50	0.710*
	13.271	2514	100	19	15	0	0	0	0	17	0	0	0	0	0	Limonene		0.713**
IP13	28.288	13837	100	42	72	50	50	63	64	50	45	42	34	57	57	delta-Elementene	50	0.931***
	29.122	933	100	129	387	159	104	58	133	0	0	0	0	0	0	Unknown		0.713**
	29.971	15016	100	48	73	57	58	71	74	49	45	42	35	59	59	Unknown		0.928***
	30.729	1897	100	0	57	56	42	75	77	22	39	19	21	71	67	Unknown		0.929***
	31.271	4513	100	57	89	65	74	288	184	66	64	55	43	110	78	Unknown		0.638*
	31.530	2735	100	24	83	29	50	70	66	49	53	0	26	87	78	Unknown		0.750**
	31.881	113227	100	49	78	58	61	63	76	54	52	46	41	67	68	Unknown		0.924***
	32.231	3743	100	53	79	59	61	70	77	61	52	53	38	73	75	Unknown		0.879***
	33.166	3793	100	50	75	54	61	68	72	58	46	50	37	67	70	Unknown		0.826**
	33.306	6149	100	50	79	58	63	73	83	56	55	49	45	71	72	Unknown		0.894***
	34.691	828	100	0	52	0	0	50	106	0	0	0	0	0	0	Unknown		0.881***
	35.365	1501	100	57	80	0	0	26	57	0	0	0	0	0	29	Unknown		0.823**
	35.759	417	100	0	105	0	0	98	220	0	0	0	0	85	90	Calamenene	75	0.883***
	35.887	1624	100	0	40	32	215	58	393	81	181	77	147	82	296	Unknown		0.685*
	36.322	5318	100	46	81	55	60	68	82	51	48	44	43	66	67	Unknown		0.822**

^a SPIF: SBF in the presence of SPI; GF, SF, PF, AF, and APF: SBF with SPI containing glucosamine, sucrose, polyethylene glycol, ascorbic acid, and ascorbic acid with polyethylene glycol, respectively.

^b Peak areas are the average values from three replicate GC analyses. Relative % area of SBF–SPI mixtures are based on the peak area for SBF alone, designated as 100%. Bold numbers indicate significant ($p \leq 0.05$) difference by Fisher's LSD test in GC peak area of SBF–SPI mixtures with added ingredient compared to SBF with only SPI (SPIF).

^c Indicator peak number described in the previous study (Moon & Li-Chan, accepted for publication).

^d Retention time in minutes on GC chromatogram in the present study.

^e Detection frequency (%) of eight panelists who perceived the aroma compound in GC–olfactometry (Moon et al., 2006).

^f Pearson correlation coefficient for beefy notes in descriptive sensory analysis (Moon & Li-Chan, accepted for publication).

Table 2
Effect of added ingredient in SBF–SPI mixtures^a incubated at room temperature (RT, 23 °C) or 60 °C on the peak areas^b of volatile compounds captured by HS-SPME under the adsorption condition at 60 °C

IP # ^c	Retention time ^d	SBF		RT incubation–60 °C adsorption						60 °C incubation–60 °C adsorption						Peak characteristics		
				Relative % area						Relative % area						Peak identification	DF ^e (%)	Beefy ^f
		Area	%	SPIF	GF	SF	PF	AF	APF	SPIF	GF	SF	PF	AF	APF			
IP1	1.734	6953	100	106	91	113	94	163	158	80	83	85	78	131	146	3-Methyl furan	50	0.710*
IP2	7.893	5537	100	122	83	141	89	83	74	77	62	75	65	59	63	2-Acetylfuran	75	0.658*
IP3	12.132	3360	100	66	58	67	54	62	49	20	31	32	17	43	45	delta-3-Carene	88	0.872***
	13.271	1929	100	24	0	30	42	0	0	18	0	0	0	0	20	Limonene		0.713**
	13.915	1352	100	33	34	39	31	240	174	0	82	25	0	133	148	Unknown		0.648*
	15.014	6302	100	84	63	62	27	89	73	67	73	68	67	60	67	Unknown		0.773**
	15.877	1761	100	54	51	88	80	87	47	64	50	53	52	48	51	2-Ethyl-3,6-dimethylpyrazine	88	0.601*
IP4	16.247	2543	100	49	23	68	75	82	49	45	35	36	32	43	49	Unknown	100	0.852***
IP5	16.953	1935	100	20	0	49	39	38	0	20	18	0	0	18	18	Unknown	100	0.827**
IP8	19.917	2881	100	0	23	35	28	62	0	46	0	53	0	0	37	2,3-Diethyl-5-methylpyrazine	50	0.590*
	20.608	3328	100	47	44	61	59	99	81	49	46	45	48	75	71	4-Terpeneol		0.678*
IP9	22.151	3951	100	57	52	59	58	77	64	54	55	50	47	64	75	Decanal	75	0.715**
IP10	24.232	468	100	0	0	0	0	0	0	0	0	0	0	0	0	2-Isoamyl-6-methylpyrazine	50	0.599*
IP12	25.794	8033	100	41	47	46	51	50	40	38	42	45	50	71	43	Unknown	75	0.794**
	26.319	3792	100	19	18	40	41	38	34	0	0	17	0	16	19	Unknown		0.684*
	26.954	2156	100	0	19	21	99	63	19	24	24	0	0	0	43	Unknown		0.752**
	27.841	3710	100	16	35	38	56	22	95	40	22	35	21	0	0	Unknown		0.887***
	28.288	19545	100	48	36	55	55	62	42	50	42	47	42	46	46	delta-Elementene	50	0.931***
	28.830	8259	100	44	41	53	61	35	28	43	41	47	50	25	26	Unknown		0.678*
	29.122	7556	100	131	130	130	128	158	109	116	149	117	151	115	123	Unknown		0.713**
	29.971	25200	100	46	39	52	53	62	54	51	45	49	44	50	53	Unknown		0.928***
	30.220	1260	100	0	0	0	0	0	0	0	0	0	0	0	0	Unknown		0.617*
	30.377	3031	100	16	14	49	50	62	60	40	17	15	19	36	36	beta-Cubebene	63	0.850***
IP14	30.615	1639	100	0	0	0	0	0	0	0	0	0	0	0	0	Unknown		0.645*
	30.729	9478	100	34	29	42	39	46	42	40	37	40	35	37	43	Unknown		0.929***
	30.950	721	100	0	0	0	0	0	0	0	0	0	0	0	0	Unknown		0.868***
	31.271	1849	100	88	0	106	102	0	0	34	0	46	41	0	0	Unknown		0.638*
	31.317	11730	100	54	71	60	66	158	131	69	77	67	57	94	104	Unknown		0.914***
	31.530	4843	100	44	50	39	56	98	76	50	74	72	82	74	73	Unknown		0.750**
	31.881	262026	100	50	43	57	57	55	50	55	47	54	49	48	53	Unknown		0.924***
	32.231	7117	100	41	25	43	44	53	47	42	38	40	33	54	48	Unknown		0.879***
	32.679	4244	100	42	29	48	44	30	30	42	39	40	36	40	48	Unknown		0.863***
	33.166	6537	100	43	38	44	45	56	53	51	45	45	38	56	57	Unknown		0.826**
	33.306	24572	100	50	45	57	57	55	51	54	48	53	49	48	52	Unknown		0.894***
	33.620	4026	100	9	13	12	25	25	14	29	9	11	0	24	34	Unknown		0.779**
	33.813	4478	100	0	0	0	0	0	0	0	0	0	0	0	0	Unknown		0.689*
	34.211	7156	100	31	17	31	49	26	30	32	26	33	37	15	24	Unknown		0.775**
	34.346	11082	100	30	26	31	35	31	28	36	32	35	32	22	26	Unknown		0.787**
	34.481	2104	100	0	0	0	0	0	0	0	0	62	47	0	0	Unknown		0.600*
	34.691	9569	100	40	34	44	46	45	43	43	38	29	26	39	41	Unknown		0.881***
	34.871	4778	100	33	9	11	10	11	9	20	18	18	19	8	0	Unknown		0.781**

lower than the range of values reported in the literature, and may be related to the observed turbidity of the samples, suggesting potential inaccessibility of interior SH groups to Ellman's reagent due to SS bonds that would limit unfolding and exposure of interior groups, or due to other potential covalent cross-linkages such as lysinoalanine formation upon alkali or heat treatment during commercial processing of soy (Wu, Hettiarachchy, & Kalapathy, 1999). In comparison, Boatright and Hettiarachchy (1995) reported total SH and total (SH + SS) contents of SPI of 8.3 and 52.9 $\mu\text{mole/g}$ protein, respectively, compared to 10.1 and 43.9 $\mu\text{mole/g}$ protein, respectively, for SPI in the presence of the antioxidant Tenox 22. Total SH contents of 0.29 and 5.4 $\mu\text{mole/g}$ protein were observed for SPI in buffer at pH 8 and pH 3, respectively, and these values were decreased with high pressure treatment between 400 and 600 MPa (Puppo et al., 2004).

Table 3 shows that no or only small differences in the total SH content were observed by addition of glucosamine, sucrose, and polyethylene glycol to SPI, while considerable difference resulted in the presence of ascorbic acid, or ascorbic acid with polyethylene glycol. Furthermore, a significant decrease of disulfide content was observed in samples containing ascorbic acid and ascorbic acid with polyethylene glycol. Since ascorbic acid is a potent reducing agent, it can be postulated that its addition to SPI may cause reduction of disulfide linkages leading to possible changes in the conformation of SPI. The decreases of total (SH + SS) contents in SPI containing ascorbic acid or ascorbic acid with polyethylene glycol could suggest possible involvement of some thiol groups in other bonds upon reduction of the disulfide bonds by ascorbic acid.

3.4. Surface hydrophobicity

The surface hydrophobicity S_0 of the samples as determined by the neutral fluorescent probe PRODAN is shown in Table 3. The S_0 of SPI was not affected by addition of glucosamine or sucrose, but was significantly increased by addition of polyethylene glycol or ascorbic acid. Mild denaturing treatment by polyethylene glycol may lead to increasing surface hydrophobicity on SPI. Polyethylene glycol is highly soluble in water but includes a repeating hydrophobic region ($-\text{CH}_2-\text{CH}_2-$) per monomer unit, which could evoke relocation of the hydrophobic side chains of non-polar amino acid residues in SPI. The denaturing effect by polyethylene glycol seemed to be enhanced by reduction of disulfide bond by ascorbic acid, resulting in considerable increase of surface hydrophobicity in SPI treated with both ascorbic acid and polyethylene glycol. It can be hypothesized that addition of ascorbic acid increased the surface hydrophobicity through structural changes in SPI accompanying the reduction of disulfide bonds to sulfhydryl groups, enabling buried amino acid residues with non-polar side chains to become exposed to the surface of the protein molecule. The increase in S_0 was amplified in the sample treated by the combination

of ascorbic acid and polyethylene glycol implying additive or complementary effects of ascorbic acid and polyethylene glycol in exposing surface hydrophobic groups in SPI.

3.5. FT-Raman spectroscopy

Fig. 1 shows a typical FT-Raman spectrum of SPI (freeze-dried powder from 5% w/w of SPI solution in 0.05 M Tris-HCl buffer pH 7.4), with tentative assignment of selected major bands based on the literature (Li-Chan, 1996). Effects of additional ingredients on the FT-Raman spectrum of SPI are also depicted in Fig. 1, and the normalized intensities of the selected bands for SPI in the absence or presence of the additional ingredients are shown in Table 4.

The SS-stretching band near $510\text{--}550\text{ cm}^{-1}$ clearly indicated an effect of addition of ingredients on the disulfide bonds, as shown in Table 4. Significant increase in intensity at 540 cm^{-1} was shown in the sample with added sucrose, while significantly decreased intensity at 537 and 539 cm^{-1} was observed in samples containing ascorbic acid and ascorbic acid with polyethylene glycol, respectively. These changes may be explained by possible formation or

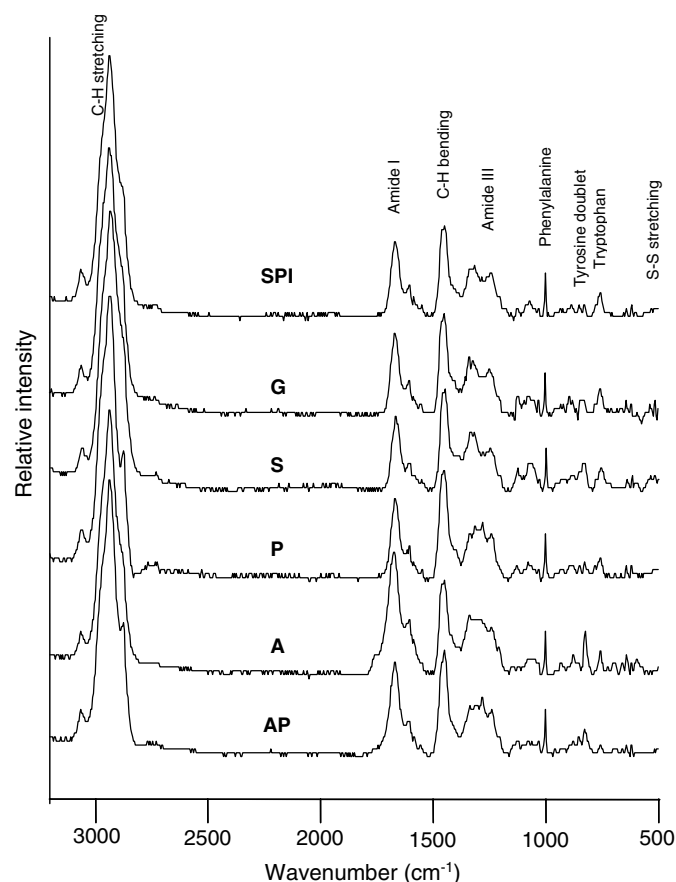


Fig. 1. FT-Raman spectra of freeze-dried SPI and SPI treated with various ingredients (baseline corrected, ingredient spectrum subtracted, and normalized to the intensity of phenylalanine peak at 1003 cm^{-1}). G: glucosamine; S: sucrose; P: polyethylene glycol; A: ascorbic acid; AP: ascorbic acid with polyethylene glycol.

Table 4

Normalized intensity values at selected regions of the FT-Raman spectra of SPI and SPI treated with various ingredients^a

Band assignment	Mean of normalized peak intensity ^b [wavenumber (cm ⁻¹)]					
	SPI	G	S	P	A	AP
SS of cystine	0.55 b	–	–	–	–	–
(<i>gauche-gauche-trans</i>)	–	0.60 b	0.64 c	0.52 b	0.48a	0.48a
(<i>trans-gauche-trans</i>)	[522]	[537]	[540]	[541]	[537]	[539]
Tryptophan indole ring	0.74 b	0.80 b	0.76 b	0.74 b	0.76 b	0.58a
	[760]	[759]	[759]	[759]	[759]	[759]
Tyrosine doublet ratio	0.97 b	0.99 b	0.89 b	0.98 b	0.60a	0.85a
	[850/830]	[850/830]	[850/830]	[850/830]	[850/830]	[850/830]
Aliphatic residues	1.62 a	1.71 a	1.76 a	1.80 b	1.64 a	1.81 b
C–H bending	[1448]	[1450]	[1450]	[1449]	[1449]	[1449]
Aliphatic residues	3.79 a	3.79 a	3.99 a	4.10 a	3.75 a	4.06 a
C–H stretching	[2934]	[2935]	[2932]	[2933]	[2934]	[2932]

^a G: glucosamine; S: sucrose; P: polyethylene glycol; A: ascorbic acid; AP: ascorbic acid with polyethylene glycol.^b Mean values are shown from duplicate analyses. Samples with different lower case letters (a–c) within a row are significantly ($p \leq 0.05$) different. Numbers in bold letter are significantly different ($p \leq 0.05$) from SPI.

stabilization of disulfide bonds in SPI by addition of sucrose, in contrast to disulfide bond breakage in samples containing ascorbic acid or ascorbic acid with polyethylene glycol, probably due to reduction by ascorbic acid, which is in good agreement with the results of SH and SS contents determined by colorimetric analysis. In addition, the Raman wavenumber shift of the SS-stretching band from 522 cm⁻¹ in SPI to 537–540 cm⁻¹ by addition of ingredients to SPI, suggests changes in disulfide bonds from *gauche-gauche-trans* to *trans-gauche-trans* conformation when the ingredient was added to SPI. No peaks were found in the region of 2550–2558 cm⁻¹ assigned to S–H stretching vibrations, probably due to the low concentration of sulfhydryl groups in these samples as shown by analysis with Ellman's reagent.

Comparison of the tryptophan band near 760 cm⁻¹ in each sample spectrum shows a significant decrease in peak intensity in the sample containing ascorbic acid with polyethylene glycol compared to SPI alone (Table 4), indicating decrease of “buriedness” or hydrophobic microenvironment of the tryptophan indole ring (Li-Chan, 1996). Further changes in aromatic amino acid residues may be noted in the decreased intensity ratio of the tyrosine doublet bands at 850 and 830 cm⁻¹ for SPI in the presence of ascorbic acid or ascorbic acid with polyethylene glycol compared to SPI alone (Table 4). A decrease in the tyrosine doublet intensity ratio indicates intermolecular interactions of the tyrosine residues as a strong hydrogen donor or increased buriedness around the residues (Howell & Li-Chan, 1996). The observed decrease in the ratio of the doublet in the samples containing ascorbic acid alone or with polyethylene glycol suggests the possibility of changes in SPI characteristics through the interaction between tyrosine residues and dehydroascorbic acid, the oxidized form of ascorbic acid, or amino acids on SPI containing potential hydrogen bond acceptor in their side chains such as aspartic acid, glutamic acid, asparagine, and glutamine, which are reported to be the most abundant amino acids in SPI (Krishnan, 2005).

The microenvironment of aliphatic amino acid residues was examined by Raman bands at 1450 cm⁻¹ (C–H₂ bending), 1465 cm⁻¹ (C–H bending) or 2935 cm⁻¹ (C–H stretching). Although there were no significant differences in the intensities at 2935 cm⁻¹, a significant increase in the intensity at 1450 cm⁻¹ was observed in samples containing polyethylene glycol and ascorbic acid with polyethylene glycol, which may be attributed to decreased interior hydrophobic interactions due to increasing polarity of the environment around hydrocarbon chains, which implies unfolding of proteins in those samples.

Among several distinct vibrational modes of the -CO-NH- amide or peptide bond, the amide I and III bands are the most useful to determine the secondary structure of proteins (Li-Chan, 1996). Based on least squares analysis of the amide I band, the SPI in this study contained 14.4% α -helix, 55.2% β -sheet, 19.0% β -turns, and 11.5% unordered structures (Table 5). The secondary structure composition was not changed by freeze-drying, except for a slightly increased proportion of β -sheet.

In comparison, Rampon, Robert, Nicolas, and Dufour (1999) investigated the mid-infrared spectra of commercial SPI and reported relative contribution of α -helices and random segments (1658 cm⁻¹), intermolecular β -sheets (1622 cm⁻¹), and β -turns (1673 cm⁻¹) in the SPI to be 28.4%, 49.7%, and 20.9%, respectively. Using infrared spectroscopy, Abbott et al. (1996) reported 25% α -helix, 33% β -sheet, 31% turns, and 12% unordered structures for unheated glycinin in aqueous buffer and also noted that glycinin in solution had the same secondary structure as glycinin in hydrated solids. The propensity of secondary structure in purified β -conglycinin was reported as 13.2% α -helix, 37.9% β -sheet, 24.7% β -turn and 23.0% unordered structures (Hou & Chang, 2004).

The observed secondary structure composition of SPI in the present study (Table 5) shows higher β -sheet and lower α -helix than the reported values in the literature for glycinin and β -conglycinin but similar values to those reported for commercial SPI. This result may be related to the

Table 5
Composition of secondary structure in SPI and SPI treated with various ingredients^a

	% Secondary structure ^b						
	NOFD	SPI	G	S	P	A	AP
Total α -helix	14.4 a	13.6 a	13.1 a	15.0 ab	16.5 bc	18.9 d	17.5 cd
Total β -sheet	55.2 ab	57.2 c	59.6 d	57.2 c	55.1 a	56.8 bc	56.9 c
β -reverse turn	19.0 cd	18.6 cd	20.0 d	19.8 d	17.0 c	1.4 a	7.6 b
Unordered structure	11.5 b	10.6 b	7.3 a	8.0 a	11.4 b	22.9 d	18.0 c

^a NOFD: original SPI (not freeze-dried); SPI: freeze-dried SPI without any ingredient; G: glucosamine; S: sucrose; P: polyethylene glycol; A: ascorbic acid; AP: ascorbic acid with polyethylene glycol.

^b Mean values are shown from duplicate analyses. Samples with different lower case letters (a–d) within a row are significantly ($p \leq 0.05$) different.

results from DSC which indicated denaturation of the SPI used in this study. Increase in the β -sheet content at the expense of existing α -helices has been reported to be associated with denaturation and/or aggregation of proteins (Puppo et al., 2004; Herald & Smith, 1992).

Sucrose seemed to have the least effect on changing the secondary structure of SPI, with the only difference being a decrease in unordered structure. When SPI was treated with glucosamine, the proportion of β -sheet increased while total unordered structure decreased. The decrease of unordered structure by addition of glucosamine and sucrose might be explained by the reported role of sugars and polyols in the stabilization of the structure of protein molecules (Gekko, Li, & Makino, 1999). Various concentrations of glycerol and sorbitol enhanced thermal stability of soy protein, β -conglycinin and glycinin in aqueous solution. Addition of polyols was assumed to strengthen intramolecular hydrophobic interaction due to the preferential solvent interaction with the protein molecules. According to the mechanism described by Gekko and Timasheff (1981), polyols such as glucosamine and sucrose in this study are fundamentally hydrophilic compounds, which can occupy a part of the solvation sheath around the protein with concomitant stabilization of solvent structure. Polyols were reported to show a stabilization effect on hydrophobic interaction of protein molecules more effectively compared to peptide–peptide hydrogen bonds (Gekko, 1981).

Addition of polyethylene glycol caused an increase in the proportion of α -helix and a decrease in β -sheet compared to SPI. The greatest change in terms of propensity of secondary structure of SPI was detected by addition of ascorbic acid, observed in both the amide I ($1650\text{--}1685\text{ cm}^{-1}$) and amide III ($1235\text{--}1305\text{ cm}^{-1}$) bands (Fig. 1). Table 5 shows that the α -helix and unordered structure were significantly increased by more than 5% and 12%, respectively, while β -reverse turn was drastically decreased by about 17% when SPI was treated with ascorbic acid. Similar changes occurred in SPI treated by ascorbic acid with polyethylene glycol, but to a lesser extent. Furthermore, the fraction of unordered structure was significantly increased in the samples containing ascorbic acid or ascorbic acid with polyethylene glycol, and considerable decrease was observed in samples containing sucrose or glucosamine, while no significant difference was shown in

samples containing polyethylene glycol. Increased unordered structure in the samples containing ascorbic acid alone or with polyethylene glycol might be related to their increased surface hydrophobicities facilitated by disulfide breakage as indicated by determination of S_0 , total SH and SS contents.

3.6. Sensory evaluation

According to the results from GC analysis, the areas of beefy attribute related peaks increased by addition of ascorbic acid alone or with polyethylene glycol. Furthermore, some of these peaks were associated with high detection frequency in GC–O analysis, which might imply improvement of aroma perception of SBF in mixtures of SPI and SBF. Therefore, descriptive sensory analysis was conducted to explore the effects of ascorbic acid alone or ascorbic acid with polyethylene glycol added to SPI on the five aroma attributes of SPI–SBF mixtures.

The comparison of the five attributes for the three samples, (i.e. SPI without any ingredient added (SPIF), SPI containing ascorbic acid (AF) and SPI containing ascorbic acid with polyethylene glycol (APF)) is illustrated in Fig. 2. The sample of SBF with SPI treated by ascorbic acid with polyethylene glycol showed considerable changes in the sensory scores of all the attributes, with a significantly higher score for roasted attribute and significantly lower scores for soymilk-like and cereal attributes compared to SPI without any ingredient. A higher score for beefy attribute and lower score for yeasty attribute were also noted for SPI containing ascorbic acid with polyethylene glycol, but these differences were not statistically significant ($p > 0.05$). The sample of SBF with SPI treated with ascorbic acid alone showed a similar trend to the sample containing both ascorbic acid and polyethylene glycol, but the scores were not statistically significantly different from the control mixture of SBF and SPI.

It could be postulated that adding ascorbic acid alone or with polyethylene glycol to SPI prior to interacting with SBF resulted in reduction of disulfide bonds, increased surface hydrophobicity and increased unordered structure in SPI. These protein structural changes resulted in an increased release of flavour compounds involving the beefy characteristic, as measured by the increase in peak areas of indicator peaks by GC analysis. The lack of a significant

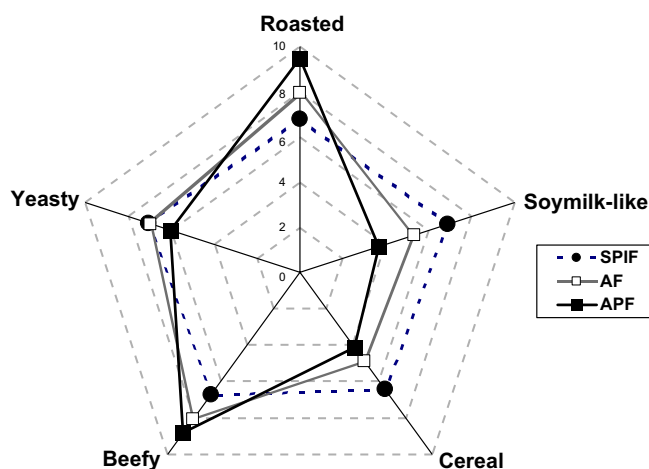


Fig. 2. Cobweb diagram of the sensory scores from the descriptive analysis of simulated beef flavour in the presence of soy protein isolate (SPIF), SPI containing ascorbic acid (AF) and SPI containing ascorbic acid with polyethylene glycol (APF) ($n=14$; 7 panelists with two replications).

difference in beefy note *per se* in descriptive analysis may likely be attributed to the complexity of “beef” aroma, which cannot be constructed easily, and to the possible deformation of beefy profile due to the unbalanced increase among beefy related aroma compounds. Nevertheless, the beefy characteristic in the sample treated with ascorbic acid and polyethylene glycol was expressed by enhancement of roasted note and alleviation of soymilk-like and cereal notes.

4. Conclusions

The results of this study clearly showed that interaction of SPI with odour-active compounds in SBF was affected by addition of different ingredients. GC analysis showed higher peak areas of individual beefy attribute related odour-active compounds in the mixture of SBF and SPI containing ascorbic acid alone or ascorbic acid with polyethylene glycol, compared to SBF–SPI without any additional ingredients. The increase in GC indicator peak areas by addition of ascorbic acid with polyethylene glycol to SPI prior to mixing with SBF was accompanied by an enhancement of roasted note and diminished soymilk-like and cereal notes by descriptive sensory analysis of the SBF–SPI mixture containing ascorbic acid and polyethylene glycol. According to the results of sulfhydryl and disulfide content, surface hydrophobicity, and FT-Raman spectroscopy, it can be postulated that while glucosamine and sucrose had little effect on protein structure of SPI, the addition of ascorbic acid especially in conjunction with polyethylene glycol triggered conformational changes in SPI structure mainly through disulfide bond reduction, resulting in increased surface hydrophobicity and unordered structure on SPI, which in turn affected improvement of SBF perception in the SBF–SPI mixture during descriptive sensory analysis. The present results pave the way for

further research to elucidate strategies maximizing perception of beefy aroma in soy products.

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