Contribution of crosslinking products in the flavour enhancer processing: the new concept of Maillard peptide in sensory characteristics of Maillard reaction systems

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Revised: 13 May 2016 / Accepted: 24 May 2016 / Published online: 4 July 2016
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Abstract In this study, the flavour-enhancing properties of the Maillard reaction products (MRPs) for different systems consisted of different peptides (sunflower, SFP; corn, CP and soyabean SP) with, xylose and cysteine were investigated. Maillard systems from peptides of sunflower, corn and soyabean with xylose and cysteine were designated as PXC, MCP and MSP, respectively. The Maillard systems were prepared at pH of 7.4 using temperature of 120°C for 2 h. Results showed that all systems were significantly different in all sensory attributes. The highest scores for mouthfulness and continuity were observed for MCP with the lowest peptides distribution between 1000 and 5000 Da, known as Maillard peptide. This revealed that the MCP with the lowest Maillard peptide content had the strongest “Kokumi” effect compared to the other MRPs and demonstrated that “kokumi effect” of MRPs was contributed by not only the “Maillard peptide” defined by the molecular weight (1000–5000 Da). Results on sensory evaluation after fractionation of PXC followed by enzymatic hydrolysis showed no significant differences between

PXC, P-PXC and their hydrolysates. This observation therefore confirmed that the presence of other contributors attributed to the “Kokumi” effect rather than the Maillard peptide. It can be deduced that the unhydrolyzed crosslinking products might have contributed to the “Kokumi” effect of MRPs. The structures of four probable crosslinking compounds were proposed and the findings have provided new insights in the sensory characteristics of xylose, cysteine and sunflower peptide MRPs.

Keywords Maillard reaction · Cross linking products · Flavour enhancer · Kokumi effect · Sensory evaluation

Abbreviations

SFP	Sunflower peptide
CP	Corn peptide
SP	Soybean peptide
PXC	Sunflower peptide, xylose, cysteine Maillard system
MCP	Corn peptide, xylose, cysteine Maillard system
MSP	Soybean peptide, xylose, cysteine Maillard system
MRPs	Maillard reaction products
P-PXC	Purified sunflower Maillard reaction products
H-PXC	Sunflower Maillard reaction products hydrolysate
HP-PXC	Purified sunflower Maillard reaction products hydrolysate
MALDI-TOF/ TOF MS/MS	Matrix-assisted laser desorption/ ionization time of flight mass spectrometry
FAA	Free amino acid
TAA	Total amino acid

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MWD	Molecular weight distribution
Da	Dalton
MW	Molecular weight
m/z	Mass-to-charge ratio

Introduction

The integration of proteolytic reactions with Maillard reaction can play an important role in development of flavour in protein-rich foods. During proteolysis, pure enzymatic processes free amino acids and peptides are formed. Hydrolyzed vegetable proteins, for example, soy sauce, are widely used as savory ingredients in a variety of foods because of their “umami” taste properties (Schlichtherle-Cerny and Amadò 2002). In addition, flavour enhancers have been produced from free amino acids and peptides derived from enzymatic hydrolysis and MR. The flavour enhancers have presented different taste characteristics, including umami and Kokumi taste.

“Kokumi” is a term used throughout the flavour industry to describe taste characteristics such as continuity, mouthfulness, richness and thickness; while in contrast; the monosodium glutamate is usually characterized as having the “Umami” taste (Ueda et al. 1994). It is a distinct taste quality, or rather a taste-enhancing quality, which can be easily detected and differentiated by sensory tests by a trained panel. The compounds that provide a kokumi taste are usually tasteless in water, but enhance the taste in combination with other tastants such as umami solution and consommé soup (Hofmann and Dunkel 2009). According to Dunkel et al. (2007) who reported that although peptide identified in raw as well as thermally treated beans were tasteless molecules, they were found to induce mouthfulness, thickness, and increase the continuity of savory foods. They were coined as “kokumi” flavour compounds.

In recent years, the presence of peptides in flavour enhancers’ development promoted the full-bodied perception of flavour in the finished product, and they have been described of having the “kokumi” enhancement effect. Dunkel et al. (2007) studied the molecular and sensory characterization of γ -glutamyl peptides as key contributors to the kokumi taste of edible beans (*Phaseolus vulgaris* L.). In their findings, they found that γ -glutamyl peptides significantly decreased and remarkably enhanced mouthfulness, complexity, and long-lastingness of the taste when added to a savory matrix such as sodium chloride and monosodium glutamate solutions or chicken broth. Ogasawara et al. (2006a) studied the taste properties of Maillard reaction products prepared from 1000 to 5000 Da peptide and concluded that the Maillard peptide produced an enhanced effect on flavour, including umami, continuity

and mouthfulness in the umami solution and in consommé soup. Similarly, Ogasawara et al. (2006b) also studied the taste enhancer from the long-term ripening of miso (soybean paste) and found that the Maillard-reacted peptide was considered to be a key substance, which gives the characteristic flavour (mouthfulness and continuity) of long-ripened miso. Furthermore, the use of purified di, tri, tetra and other short peptides in different MR systems to produce various flavour and taste characteristics has been extensively studied (Chen and Ho 1999; Elmore et al. 2002; Lu et al. 2005; Münch et al. 1997; Yang et al. 2012).

During the last decade, a number of researchers defined the “Kokumi” effect enhancement as the result of Maillard peptide content (Eric et al. 2013; Ogasawara et al. 2006a, b; Song et al. 2013). “Maillard peptides” are defined as peptides of molecular weight between 1000 and 5000 Da (Ogasawara et al. 2006a). However, it has been previously reported that during Maillard reaction (MR), Maillard peptides were not the only products of peptide degradation. Cross linking products as well as low molecular weight (LMW) peptides and free amino acids were also revealed as products of peptide change during MR. In our research work, it was observed that peptides below 1000 Da could easily crosslink to form Maillard peptides with flavour enhancing capacities (Lan et al. 2010). Some researchers have previously reported that the high molecular weight (HMW) peptides, including Maillard peptides could be prepared through the crosslinking of sugar and small peptides with a certain molecular weight (MW) range (Liu et al. 2012a, b).

However, it is evidently clear that the impact of crosslinking products on “Kokumi” effect and sensory characteristics of MRPs seemed to be still unclear. Therefore, the aim of this study was to evaluate the role of crosslinking products on enhancing the “Kokumi” effect (mouthfulness and continuity taste) of MRPs systems. Additionally, the study was also aimed at identifying the crosslinking compounds formed during xylose, cysteine and sunflower peptide MR system through MALDI-TOF-TOF mass spectroscopy. It is expected that findings could provide more useful information for the production of flavour enhancers with great and pleasant taste characteristics.

Materials and methods

Chemicals

Sunflower, corn and soybean protein hydrolysates were prepared according to the methods described by Eric et al. (2013), Liu et al. (2012a, b). Alcalase 2.4 L FG and Flavourzyme 500 MG were purchased from Novo Co., Ltd. (Novozyme Nordisk, Bagsvaerd, Denmark). The other solvents/chemicals used were of analytical grade and obtained

from Shanghai Chemical Reagent Co., Ltd. (Shanghai, China).

Preparation of Maillard reaction products

The Maillard reaction products were prepared according to the method of Eric et al. (2013). The equivalent weight of 1 g peptide was weighed for SFP, CP and SP, and appropriate amounts of D-xylose (0.5 g) and L-cysteine (0.375 g) were dissolved into the beaker with distilled water to a final concentration of 10 % (w/v). The pH of the solution was adjusted to 7.4 with 2 mol/L NaOH or 2 mol/L HCl. The solution was placed in Pyrex vial (50 mL), which was then sealed with a silicon/Teflon septum. The samples were heated at 120 °C and magnetically stirred for 120 min in an oil bath placed in a fume hood. After heating, the mixtures were immediately cooled in ice water and finally named as PXC, MCP and MSP MRPs depending on the original peptide. Samples were stored at −20 °C till further use.

Gel permeation chromatography (GPC)

An aliquot (250 mg) of the dry material of the lyophilized PXC was taken up in water (10 mL) and, centrifuged at 10,000 rpm for 10 min and filtered through 0.22 µm microporous membrane. The aliquot was then, applied on the top of a water-cooled 12 mm × 80 cm glass column (Shanghai Xiamei Biochemical Science Technique Development Ltd., Inc. Shanghai, China) filled with a slurry of Sephadex G-25 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) conditioned with degassed de-ionized water. The chromatographic separation was performed using the same solvent at a flow rate of 2 mL/min for 20 h. The effluent was monitored at 220 nm by an L-7490 type RI detector (Merck, Darmstadt, Germany). Two fractions (fractions, I–II) were collected by a fraction collector, and individually freeze-dried. The MWD of residue obtained for each GPC fraction was tested. The fraction with a higher percentage of Maillard peptide (1000–5000 Da) was named P-PXC and selected for further use.

Hydrolysis of PXC and P-PXC

Sunflower peptide MRPs (PXC) and the fraction (P-PXC) collected from GPC were subjected to enzymatic hydrolysis. 10 g of PXC and P-PXC were dissolved in distilled water to final concentrations of 8 % (w/v) and the suspensions were hydrolyzed with flavourzyme at 50 °C for 2 h with pH of 6.5 and with enzyme/substrate ratio (E/S) of 2.0 LAPU/g. The hydrolysates were incubated at 95 °C for 10 min to inactivate the enzymes and the precipitate was then removed by centrifugation (Hitachi, RX II series, Japan) at 10,000 rpm for 30 min, 4 °C. The final

supernatants termed H-PXC and HP-PXC respectively were freeze dried prior to further analysis.

Determination of total and free amino acid

Amino acids were determined as previously reported by Fekkes et al. (1995). The amino acids in the sample were analysed using an Agilent liquid chromatography 1100 (Agilent Technology, Palo Alto, CA, USA) with a UV detector operated at 338 nm. The column was ODS Hypersil (250 mm × 4.6 mm). The mobile phase consisted of 20 mM sodium acetate and 1:2 (v/v) methanol–acetonitrile, with a flow rate of 1 mL/min. The column temperature was 40 °C. An appropriate pre-treatment of the sample was done before amino acid analysis. For the determination of free amino acids, an equivalent volume of trichloroacetic acid (TCA) was added to the sample to precipitate peptides and/or proteins. For the total amino acid determination, the sample was hydrolyzed at 110 °C for 24 h with 6 M hydrochloric acid in evacuated sealed tubes. A calibration curve was obtained with standard amino acid mixture (Sigma Chemical Co., St. Louis, MO, USA). The qualitative analysis was made based on retention time and peak area of standard compounds.

Estimation of molecular weight distribution

The molecular weight distribution (MWD) profiles of the samples were estimated by high-performance gel-filtration chromatography according to the method described by Huang et al. (2011). The liquid chromatography (Waters 600; Waters Co., Milford, MA, USA) equipped with 2487 UV detector was used in this experiment. The TSK gel 2000 SWXL (7.8 i.d. × 300 mm; Tosoh Co., Tokyo, Japan) column was used. The mobile phase consisted of acetonitrile/water/trifluoroacetic acid (45/55/0.1, v/v/v) was delivered at a flow rate of 0.5 mL/min. The column temperature was 30 °C. 10 mL of sample was injected into the HPLC system. The MW calibration curve was obtained from the following standards from Sigma: cytochrome C (12,500 Da), aprotinin (6500 Da), bacitracin (1450 Da), tetrapeptide GGYR (451 Da), and tripeptide GGG (189 Da). The results were obtained using UV detector (220 nm), and the data analysis was performed using gel permeation chromatography software.

Comparative taste of MRPs from different peptide

The quantitative descriptive sensory analysis was applied for evaluating the taste characteristics of the MRPs from PXC, MCP and MSP. The panel consisted of twelve well-trained panelists aged between 25 and 50 (eight females and four males). All panelists have passed screening tests

according to ISO standards (ISO 1993), and had previous experience with sensory evaluation. The sessions took place in a sensory laboratory, which complied with international standards for test rooms (ISO 2007). Prior to the quantitative sensory analysis, the panelists had thoroughly discussed aroma and taste properties of samples through three preliminary sessions, each spent 2 h, until all of them had agreed to use them as the attributes according to the objective of the present work. Six descriptors, including meat-like; umami; caramel-like; bitterness and “Kokumi” flavour consisting of mouthfulness, continuity (long-lasting taste development) were used for the descriptive analysis. The reference materials were as follows: 10 g bouillon cube (beef flavour consisting of MSG, yeast extracts, and beef extracts), dissolved in water was labelled “mouthfulness and continuity” attributes. Defatted beef brisket (0.5 kg, 2.5 cm thick, purchased from Wal-Mart supermarket) boiled in water for 2 h was labelled “meat-like”. 2.5 g of burning white sugar in 50 mL water was labelled caramel-like aroma. Monosodium glutamate (MSG), four mM, pH 5.6) was labelled umami taste. Caffeine, 1.5 mM was labelled bitter taste. The sample solution (0.5 %, w/w) was dissolved in umami solution according to the method described by Ogasawara et al. (2006a, b). Umami solution consisted of 1.0 % (w/v) monosodium glutamate (MSG) and 0.5 % (w/v) sodium chloride (NaCl). The panel was asked to compare the gustatory impact of the blank model umami solution (control) with a solution of reduced glutathione (5 mmol/L) in umami solution (both at pH 6.5). About 30 mL of sample was served in opaque disposable plastic cups at the same time. To avoid temperature differences that could influence the assessment, the samples were kept at 45 °C in steel containers until evaluation, and the containers were completely sealed in order to avoid volatiles losses. Water and breads were available to the panelists throughout the analysis as this tended to stick to the palate. The samples were coded with random three-digit numbers and randomly presented for each panel to avoid causing a so-called order effect. The intensity of the descriptive terms was rated on a horizontal 10 cm continuous line scale, anchored “none” to the left and “extreme” to the right.

Comparative taste profile analysis

The dry material of the lyophilized extracts (50 mg) of P-PXC, PXC and their respective hydrolysates (HP-PXC and H-PXC), were dissolved in exactly 4.0 mL of the model umami solution. And the pH value was adjusted to 6.5 using trace amounts of formic acid (0.1 mmol/L) or sodium hydroxide solution (1.0 mmol/L), respectively. These solutions were then presented in a dual test together with the blank model umami solution (control) to the 12

trained sensory panelists. The intensity of the descriptors’ mouthfulness, continuity and umami was rated on a scale from 0 (not detectable) to 10 (intensely perceived).

Identification of crosslinking products in PXC and HP-PXC

Mass spectra were acquired using an ultrafleXtreme smart beam II (modified Nd: YAG laser) MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany), equipped with a UV nitrogen laser (337 nm). The samples were prepared by mixing 1 µl of analyte and 1 µl of matrix solution (2,5-Dihydroxybenzoic acid; 20 mg/mL in 10 % ethanol, 1 mM NaCl). A total of 1.0 µl of mixture was deposited on MALDI spot target and dried at room temperature. All spectra were the results of signal averaging of 200 shots. The MALDI-TOF/TOF MS/MS was run in the positive refractor mode. The mass spectrometer was operated in the full-scan mode monitoring positive or negative ions. All experiments were carried out at least three times, in order to check the reproducibility. The structures of the major peaks were performed by manual interpretation of the MS/MS spectra of the ion series in the spectra. The flex analysis software (Version 3.3; Bruker Daltonics Inc., Billerica, MA, USA) was used for homology searches between manually obtained structures.

Statistical analysis

Statistical analysis was performed using Microsoft Excel 2013 (Microsoft, Redmond, WA, USA) and SPSS 19.0 (IBM, Armonk, NY USA). Analysis of variance was used to compare means with Turkey multiple range tests for post hoc analysis. $P \leq 0.05$ was considered significant. Data reported in this work were means of triplicate (MWD, amino acid distribution and sensory evaluation) experiments.

Results and discussion

Comparison of MWD of MRPs from different peptides

Results on molecular weight distribution (MWD) of MRPs from different peptides are presented in Table 1. The molecular weight distribution (MWD) of peptides could be changed during enzymatic hydrolysis, thermal treatment and Maillard reaction. Results revealed that the LMW fractions (<1000 Da) were the major fraction in CP, SP and SFP hydrolysates and account for 92.75, 78.87 and 76.31 % respectively. The fractions between 1000 and 5000 Da were higher in SP (20.40 %) followed by SFP (13.27 %), and the

least was in CP (6.87 %). The fraction MW > 5000 Da was lowest portion in all hydrolysates accounting to 0.38 % in CP, 0.73 % in SP and 10.42 % in SFP. These results are in agreement with our previous findings (Eric et al. 2013). The MW distribution of the hydrolysates, including peptides and free amino acids, can directly or indirectly affect MRPs' properties such as sensory perception and quality of the Maillard thermo reaction flavourings (Song et al. 2010). The HMW peptides (>5000 Da) and LMW peptides (<1000 Da) decreased and increased simultaneously after MR. The content of HMW and LMW peptides ranged as follows: PXC > MSP > MCP, and MCP > MSP > PXC, respectively. The decrease in HMW peptides followed by the increase in LMW peptides might be attributed to the degradation reaction. Our previous findings demonstrated that the systems with added cysteine accelerated the high molecular weight peptide degradation to form smaller peptides and amino acids (Eric et al. 2013, 2014; Huang et al. 2011). Furthermore, results showed that the peptides between 1000 and 5000 Da were lower in MCP compared to other MRPs. The peptide between 1000 and 5000 Da also known as "Maillard peptide" might be formed from the cross linking of small peptides, and they have been reported to be the main contributors of "Kokumi" effect of MRPs (Huang et al. 2011; Lan et al. 2010; Liu et al. 2012a, b).

Comparison of amino acid content of MRPs from different peptides

Results for the distributions of free and total amino acids in different MRPs are presented in Table 1. FAA from hydrolysis served as important precursors in the thermal generation of an extensive range of aroma compounds that could be associated with sensory characteristics of MRPs.

Results showed that SFP and PXC had the lowest FAA content compared with CP, MCP, SP and MSP, respectively. However, the FAA significantly increased after MR in all samples. Cysteine, aspartic and glutamic acid gradually increased after MR. It was further observed that sulfur containing amino acids (cysteine and methionine) were most abundant in CP and MCP. The higher content in sulfur containing amino acids might have played a role in flavour characteristics of MCP. Aspartic and glutamic acids were abundant in MSP and they might have contributed to the umami taste of MRPs. The hydrophobic amino acids are generally known as bitter amino acids and may therefore impart bitter-taste properties of the final products. Some authors have previously reported that among 17 analysed amino acids, 8 bitter amino acids were identified (Val, Leu, Ile, Met, Phe, Ser, Arg and His) and further reported that their content decreased significantly after MR (Lan et al. 2010). Similarly, the total amino acids (TAA) significantly decreased after MR (Table 1). This might be due to the Strecker degradation and thermal degradation of amino acid to form volatile compounds (Eric et al. 2013, 2014).

Taste characteristics of MRPs from different peptides

In order to better understand the impact of MW on the "Kokumi" effect, the taste characteristics of MRPs from different peptides (MCP, MSP and PXC) were used as shown in Table 2. An aliquot of the aqueous MRPs was dissolved in umami solution and evaluated by a trained sensory panel to study the Kokumi-enhancing activity of the MRPs. In order to accomplish this, the panelists were tasked to rate the intensity of the taste qualities with respect

Table 1 Change in molecular weight distribution, FAA and TAA

Samples	>5 kDa (%)	1–5 kDa (%)	<1 kDa (%)	FAA (mg/g)	TAA (mg/g)
<i>Hydrolysates and MRPs</i>					
CP	0.38 ± 0.03	6.87 ± 0.036	92.75 ± 0.28	4.880 ± 0.00	82.870 ± 0.03
MCP	0.29 ± 0.02	4.65 ± 0.29	95.06 ± 0.26	5.266 ± 0.00	65.049 ± 0.00
SP	0.73 ± 0.03	20.40 ± 0.75	78.87 ± 0.63	3.833 ± 0.00	83.865 ± 0.00
MSP	0.42 ± 0.05	15.67 ± 0.55	83.91 ± 0.43	4.052 ± 0.00	61.650 ± 0.00
SFP	10.42 ± 0.06	13.27 ± 0.07	76.31 ± 0.09	1.367 ± 0.01	64.325 ± 0.08
PXC	1.03 ± 0.01	20.93 ± 0.04	78.05 ± 0.03	1.560 ± 0.00	55.056 ± 0.00
<i>After gel permeation chromatography and enzymatic hydrolysis</i>					
P-PXC (F1)	3.37 ± 0.04	50.27 ± 1.40	46.36 ± 1.44	0.088 ± 0.00	1.757 ± 0.00
HP-PXC	0.20 ± 0.01	27.59 ± 0.04	72.22 ± 0.05	0.520 ± 0.00	6.677 ± 0.00
PXC	1.03 ± 0.01	20.93 ± 0.04	78.05 ± 0.03	1.560 ± 0.00	55.056 ± 0.00
H-PXC	0.04 ± 0.01	13.02 ± 0.03	86.94 ± 0.01	2.027 ± 0.00	56.466 ± 0.00
F2	0.02 ± 0.00	10.00 ± 0.15	89.98 ± 0.14	–	–

Values are mean ± standard deviation (n = 3)

Table 2 Sensory evaluation analysis of MRPs from different peptides

Samples	Attributes					
	Mouthfulness	Meaty	Umami	Continuity	Caramel	Bitterness
Control	2.78 ± 0.76 ^a	2.11 ± 0.78 ^a	4.03 ± 0.91 ^a	2.42 ± 0.87 ^a	1.78 ± 0.72 ^a	1.75 ± 0.50 ^a
MCP	8.28 ± 0.70 ^c	7.97 ± 0.70 ^c	7.72 ± 0.66 ^c	8.03 ± 0.81 ^c	2.67 ± 0.48 ^b	2.69 ± 0.58 ^b
MSP	6.39 ± 0.80 ^b	7.17 ± 0.70 ^b	8.36 ± 0.59 ^d	6.06 ± 1.24 ^b	2.42 ± 0.50 ^b	2.50 ± 0.51 ^b
PXC	6.53 ± 0.61 ^b	7.75 ± 0.55 ^c	7.06 ± 0.86 ^b	6.28 ± 1.06 ^b	2.03 ± 0.74 ^a	2.06 ± 0.63 ^a

Mean scores (listed in ascending order) for each attribute within a column with different letters are significantly different ($P \leq 0.05$) using one way ANOVA comparison test ($n = 36$; 12 panelists with 3 replication)

to mouthfulness, continuity, meaty, umami, caramel and bitterness in the different samples on a scale of 0–10, where 0 was anchored as “none” to the left and 10 as “extreme” to the right. On one hand, the addition of MRPs to umami solution significantly increased the umami taste of MRPs. MSP showed higher umami taste compared to the other samples. This might be explained by the amount of umami amino acids (glutamic and aspartic acid). MCP and PXC showed greater meaty characteristics compared to MSP, and this might be attributed to the higher content in sulfur amino acids, which might have contributed to the formation of sulfur containing volatile compounds generated during MR. It was further revealed that all samples exhibited lower bitterness and caramel-like aroma compared to the control when added to umami solution. The increased bitterness might be attributed to the high content in bitter amino acids (Lan et al. 2010), while the caramel-like aroma might be attributed to furans and pyrazine formed during MR (Van Boekel 2006). In a related study, Ueda et al. (1994) reported that the molecules attributed to mouthfulness and thickness as well as the increase in continuity of food taste perception was coined by the Japanese as “Kokumi” flavour compounds. This therefore demonstrated that the addition of MRPs to umami solution enhanced the Kokumi effect (mouthfulness and continuity). Results on sensory evaluation revealed an increase of mouthfulness and continuity of the umami solution from 2.78 to 8.28 and 2.42 to 8.03, respectively, when the MCP was added compared to control. The increase in mouthfulness and continuity was significantly ($P \leq 0.05$) different and higher than the additional of PXC and MSP samples to umami solution (Table 2).

According to a theory previously reported by other authors, the “Kokumi” effect of MRPs added to umami solution was enhanced by an increase in content of peptide between 1000 and 5000 Da commonly known as “Maillard peptide” (Liu et al. 2012a, b; Ogasawara et al. 2006a; Song et al. 2013). However, this theory is not consistent with our findings that the “Kokumi” effect was dependent on the

Maillard peptide defined by the molecular weight distribution. As shown in Table 1, MCP has the lowest percentage in Maillard peptide compared to the PXC and MSP, but it showed the highest “Kokumi” effect when added to umami solution. These findings suggest that the “kokumi” effect of MRPs was not influenced by the Maillard peptide as defined by the molecular weight distribution. From this observation, it was hypothesized that the crosslinking products formed during MR might also contribute to the enhancement of “Kokumi” effect of MRPs. To elucidate our hypothesis, PXC with the higher percentage of Maillard peptide (1000–5000 Da) was subjected to the gel filtration followed by enzymatic hydrolysis of both PXC and purified fraction (P-PXC). All samples were further sensory evaluated.

Change in MWD and amino acid composition of PXC, P-PXC and their hydrolysates

Two fractions were obtained after GPC purification (data not shown). The results of MWD revealed that the content of Maillard peptide was higher in fraction one (50.27 %) compared to 10 % in fraction two. Our objectives were to increase the content of Maillard peptide of PXC system; therefore, the fraction one was chosen and termed P-PXC. After enzymatic hydrolysis of PXC and P-PXC, their MW pattern as well as their respective hydrolysates, H-PXC and HP-PXC were analysed. The results showed that the percentage of “Maillard peptides” dropped by 37.79 and 45.11 % in H-PXC and HP-PXC respectively, with the increase of peptides below 1000 Da (10.22 and 35.81 % in H-PXC and HP-PXC respectively; Table 1). However, the content of FAA and TAA significantly increased after enzymatic hydrolysis, indicating that flavourzyme might have helped the release of amino acids from peptides, which might meaningfully contribute to the “Kokumi” effect enhancement of MRPs. FAA increased by 23.04 % in H-PXC, and by 83.08 % in HP-PXC compared to PXC and P-PXC (Table 1). The change in peptide together with

amino acid content after enzymatic hydrolysis might have impact on the enhancement of “Kokumi” effect.

Comparative sensory characteristics of PXC, P-PXC and their hydrolysates

In order to gain an insight into the “Kokumi” effect by the cross linking compounds, PXC was purified by GPC using Sephadex G-25 as the stationary phase and water as the mobile phase followed by enzymatic hydrolysis of PXC and P-PXC. Aliquots of PXC, P-PXC, H-PXC and HP-PXC were added to umami solution in their natural concentration ratios and presented to the trained sensory panelists. The control sample was made of the mixture of dextrin 8–10 in umami solution. The results in Table 3 show an increase of Kokumi intensity and umami taste compared to the control. However, the results showed no significant ($P < 0.05$) difference in all tested samples. When comparison was made for each sample with its hydrolysate, no differences were observed in taste. The Kokumi enhancement was ranged as follows: H-PXC > PXC > HP-PXC > P-PXC. These results are consistent with our hypothesis that the “kokumi” effect was not depended on Maillard peptide defined by the molecular weight distribution. Furthermore, it can be concluded that the unhydrolyzed crosslinking products might have contributed to the “Kokumi” effects of the MRPs.

Compounds identification on MALDI-TOF/TOF spectrometer

From sensory evaluation results, the structure of P-PXC and its hydrolysate (HP-PXC) were evaluated on MALDI-TOF/TOF spectrometer. Results showed that their molecular ions $[M + H]^+$ were 3126.61 and 2513.16 respectively (Fig. 1). The decrease in molecular weight of HP-PXC suggested that its spectra were derived from the

hydrolysis of P-PXC. However, it was also observed that some fragments of P-PXC spectra were unhydrolyzed, but their intensities were significantly increased in HP-PXC. The unhydrolyzed fractions were revealed as crosslinking products, indicating that they might be responsible for “Kokumi” effect of sunflower MRPs. The cross linking products as non-volatile compounds might have been formed between sugar and low molecular weight peptides or amino acid released during MR and/or from the degradation of HMW peptides.

The cross linking products revealed by four unhydrolyzed fractions with positive molecular ion $[M + H]^+$ m/z 379, 401, 445 and 656 were further identified based on their tandem mass spectrum using MALDI-TOF/TOF MS/MS spectrometer. Peptides, xylose and cysteine were important ingredients of the Maillard reaction system. Their degradations during Maillard reaction were significant to the formation of crosslinking compounds. Based on the mechanism of pentose degradation during Maillard reaction as proposed by Hofmann and Frank (2002), Yang et al. (2012), the 2-methylfuran-3,4-diol might have been formed from the dehydration and cyclization of xylose. Ammonia, 2-mercaptoacetaldehyde and hydrogen sulphide could be generated from cysteine degradation. 1H-pyrrole-2,5-diol might be resulted from the combination of the Strecker degradation product and rearrangement of 4,5-dihydroxy-2-oxopentanal (3-Deoxyxylosone) and ammonia from cysteine degradation (Fig. 2).

The fragmentation of the positive molecular ion m/z 379 was shown in Fig. 3. The molecule lost two consecutive water molecules (H_2O) to produce the fragment at m/z 361 and 343. The fragments with m/z 361 and 343 lost a $C_7H_9NO_4$ (171 Da Loss) probably derived from glutamate to produce fragments with m/z 190 and 172 respectively. The fragment m/z 190 differed with the fragment m/z 172 by only one molecule of water. The molecular ion m/z 171.7 and 189.7 were suggested to be similar compounds with m/z 190 and 172 (Fig. 3). Further fragmentation of m/z 172 showed the presence of furan and (E)-2-amino-mercaptoacrylaldehyde, which might be from degradation of xylose and cysteine. The proposed structure was $C_{14}H_{22}N_2O_8S$, with MW 378.

The proposed structure of compound with positive molecular ion m/z 401 was $C_{17}H_{24}N_2O_7S$, MW 400, as shown in Fig. 4. The fragmentation of a molecule with m/z 401 showed the loss of 42 Da (propene) giving rise to m/z 359. The fragment m/z 359 in turn lost one molecular of (Z)-3-mercaptoacrylic acid (104 Da) to generate m/z 255. The (Z)-3-mercaptoacrylic acid might probably be derived from cysteine degradation. Further fragmentation of m/z 255 showed the lost one molecule of carbon dioxide to give a compound with m/z 211.

Table 3 Taste characteristics of PXC, P-PXC and their hydrolysates

Samples	Attributes		
	Mouthfulness	Umami	Continuity
Control	2.78 ± 0.76 ^a	4.03 ± 0.91 ^a	2.42 ± 0.87 ^a
PXC	7.86 ± 0.68 ^{b,c}	7.81 ± 0.62 ^c	7.69 ± 0.62 ^{b,c}
H-PXC	8.19 ± 0.58 ^c	7.31 ± 0.71 ^b	8.06 ± 0.75 ^c
P-PXC	7.64 ± 0.80 ^b	6.97 ± 0.74 ^b	7.39 ± 0.90 ^b
HP-PXC	7.81 ± 0.75 ^{b,c}	7.25 ± 0.81 ^b	7.64 ± 0.80 ^{b,c}

Mean scores (listed in ascending order) for each attribute within a column with different letters are significantly different ($P \leq 0.05$) using one way ANOVA comparison test ($n = 36$; 12 panelists with 3 replication)

Fig. 1 Identification of the crosslink compound from sunflower MRPs. **a** Mass spectrum of cross linking compounds identified from HP-PXC. **b** Mass spectrum of cross linking compounds identified from P-PXC

Fig. 2 Proposed formation pathways of 2-methylfuran-3,4-diol (1) and 1H-pyrrole-2,5-diol (2)

The structure of the fragment with molecular ion m/z 445 was also proposed in Fig. 5. The molecule was fragmented into two ways. Firstly, the molecule with m/z 445 lost a fragment of 212 Da giving rise to m/z 233. On the other hand, the ion m/z 445 consecutively lost one molecule of carbon dioxide (44 Da) and 2,5-diamino-5-oxopentanoic acid (146 Da) giving rise to m/z 255, the loss of

2,5-diamino-5-oxopentanoic acid (146 Da) which could be the residue of glutathione degradation might suggest the presence of glutamic acid and glycine in the proposed structure. The molecular ion m/z 255 showed two sides from xylose and cysteine degradation respectively. However, the presence of 1H-pyrrole-2, 5-diol in structure might confirm the degradation of xylose during the

Fig. 3 Proposed formation pathways of the compound with m/z 379 in sunflower-xylose-cysteine system. **a, b** LC-MS/MS profiles of HP-PXC and P-PXC, respectively, **c** structures and fragmentation assignments of ions observed in LC-MS/MS of m/z 379

reaction. Hence the proposed structure was $C_{17}H_{24}N_4O_8S$ with MW 444.

The proposed structure for the fragment with positive molecular ion m/z 656 was shown in Fig. 6. The

fragmentation of this molecule showed the loss of one molecule of ammonia (17 Da) followed by loss of 173 Da ($C_6H_7NO_3S$) giving rise to m/z 466. This might be probably due to the cysteine and glutamate degradation residue. The

Fig. 4 Proposed formation pathways of the compound with m/z 401 in sunflower-xylose-cysteine system. **a, b** LC-MS/MS profiles of HP-PXC and P-PXC, respectively, **c** structures and fragmentation assignments of ions observed in LC-MS/MS of m/z 401

Fig. 5 Proposed formation pathways of the compound with m/z 445 in sunflower-xylose-cysteine system. **a, b** LC-MS/MS profiles of HP-PXC and P-PXC, respectively, **c** structures and fragmentation assignments of ions observed in LC-MS/MS of m/z 445

Fig. 6 Proposed formation pathways of the compound with m/z 656 in sunflower-xylose-cysteine system. **a, b** LC-MS/MS profiles of HP-PXC and P-PXC, respectively, **c** structures and fragmentation assignments of ions observed in LC-MS/MS of m/z 656

fragmentation of ion m/z 466 showed the lost one molecule of carbon dioxide (44 Da) followed by loss of 167 Da ($C_8H_9NO_3$) apparently a glutamate residue, generating a positive ion m/z 255. However, the fragmentation of m/z

656 to generate ion m/z 233 showed consecutively loss of 213 Da and 210 Da. Hence, the proposed structure was $C_{26}H_{33}N_5O_{11}S_2$ with MW 655.

Conclusion

Results from the taste characteristic of MRPs from the three different peptides have shown a significant difference for all sensory attributes when umami solution was added. MCP with the lowest Maillard peptide content showed the highest “Kokumi” effect compared to PXC and MSP (MCP > PXC > MSP). It was suggested that the “Kokumi” effect was not only due to the Maillard peptide defined by the molecular weight. The fractionation of PXC to P-PXC followed by their enzymatic hydrolysis showed no significant difference in all sensory attributes. The “Kokumi” effect was ranged as H-PXC > PXC > HP-PXC > P-PXC confirming the hypothesis that “Kokumi” effect was not only due to Maillard peptide defined by molecular weight. It can therefore be concluded that the unhydrolyzed crosslinking products enhanced the “Kokumi” effect of MRPs rather than the Maillard peptides. Four crosslinking product structures were proposed from MALDI-TOF/TOF MS/MS spectrometer. These findings might provide new insights in sensory characteristics of xylose, cysteine and sunflower peptide Maillard reaction products systems.

Acknowledgments The research was supported in part by the program of “Collaborative innovation center of food safety and quality control in Jiangsu Province” and National Program of China (2016YFD0400801 and 2013AA102204). It was also founded by the Priority Academic Program Development of Jiangsu Higher Education Institutions.

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