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Synthesis and sensory characteristics of kokumi γ-[Glu]n-Phe in the presence of glutamine and phenylalanine: Glutaminase from *Bacillus amyloliquefaciens* or *Aspergillus oryzae* as the catalyst

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ABSTRACT:

The transpeptidase activity of glutaminase from *Bacillus amyloliquefaciens* (GBA) *and Aspergillus oryzae* (GAO) to yield γ-[Glu]n-Phe peptides were verified for the first time. In the presence of Gln and Phe, γ-Glu-Phe and γ-Glu-γ-Glu-Phe were synthesized by GAO, and γ-Glu-Phe, γ-Glu-γ-Glu-Phe, γ-Glu-γ-Glu-γ-Glu-Phe, γ-Glu-γ-Glu-γ-Glu-γ-Glu-Phe and γ-Glu-γ-Glu-γ-Glu-γ-Glu-γ-Glu-Phe were synthesized by GBA. The K_m values for the transpeptidation catalysed by GBA and GAO were 47.88 and 153.92 mM (Phe as the acceptor), 84.89 and 236.47 mM (γ-Glu-Phe as the acceptor), indicating that GBA had a greater affinity than GAO for Phe and γ-Glu-Phe in the transpeptidation reaction. The K_m values for the transpeptidation catalysed by GBA against acceptors, Phe and γ-$[Glu]_{(1\leqq n<5)}$-Phe (47.88 ~ 206.47 mM), increased with an elevated number of γ-glutamyl residue within the acceptor. The optimal conditions for γ-[Glu]n-Phe synthesis were pH 10 and 37°C for 3 h, 300 mM Gln, 100 mM Phe, 0.05 U/mL GBA. All the γ-$[Glu]_{(1\leqq n\leqq 5)}$-Phe exhibited astringency in water and imparted a kokumi taste to commercial soy sauce and model chicken broth. The astringent threshold values (2.5~3.92 mM) were approximately three-fold of the kokumi threshold concentrations (0.78~1.53 mM). γ-[Glu]n-Phe or the post-enzymatic reaction mixture enhanced the umami intensity of commercial soy sauce and model chicken broth.

KEYWORDS: Enzymatic synthesis, γ-[Glu]n-Phe, Kokumi, Astringency, Glutaminase

INTRODUCTION

Taste of food plays an important role in food choice. As a result, research has been directed towards not only the flavoring compounds (which can impart the five basic tastes i.e. sweet, salty, sour, bitter and umami), but also the kokumi substances (which that can modify the basic tastes, mouthfulness, thickness or continuity of foods at these effective concentrations).[1-3] Recently, γ-glutamyl peptides with kokumi-imparting properties through activating the calcium-sensing receptor in human cells have attracted increasing attention.[1, 4-7] Their sequences mainly refer to γ-Glu-Val, γ-Glu-Met, γ-Glu-Glu, γ-Glu-Gln, γ-Glu-Gly, γ-Glu-Leu, γ-Glu-His, γ-Glu-Cys-Gly, γ-Glu-Val-Gly, which were usually identified from edible beans,[2] cheese[8-11] and soy sauces[12] as the flavor contributors in these food. To the best of our knowledge, the sequence like γ-Glu-γ-Glu-Amino acid, γ-Glu-γ-Glu-γ-Glu-Amino acid, γ-Glu-γ-Glu-γ-Glu-γ-Glu-Amino acid, et al., have not yet been reported as one of the kokumi compounds.

A large-scale process for production of γ-glutamyl peptides materials is critically needed to ensure commercial use of these kokumi substances and avoid the dependence on agriculture and influence of environmental and processing conditions. Biocatalytic synthesis of γ-glutamyl peptides using enzymes could be a cost-effective alternative. L-Glutaminase (L-glutamine amidohydrolase EC 3.5.1.2) is widely distributed in microorganisms including bacteria, yeast and fungi. The enzyme catalyzes mainly the hydrolysis of glutamine to glutamic acid and ammonia and also the γ-glutamyl transfer reaction.[13] The glutaminase from *Escherichia coli* [14] and *P. Aeruginosa* [15] can catalyze the hydrolysis and then the transfer of γ-glutamyl moiety. The glutaminase from *P. nitroreducens* can catalyze γ-glutamyl transfer to ethylamine or methylamine with repressed hydrolysis of glutamine.[16] The glutaminase from *Pseudomonas nitroreducens* IFO

12694 [17] and *Aspergillus oryzae* [18] were reported to possess transpeptidase activity and have been used to catalyze the synthesis of L-theanine and a large number of γ-glutamyl peptides, respectively. To our knowledge, several microbial glutaminases might have transpeptidase activity but no systematic research has been conducted to verify.

The γ-glutamyl transfer reaction is mainly catalyzed by bacterial γ-glutamyltranspeptidase (GGT, EC 2.3.2.2) and a few microbial L-glutaminases involve in the transfer of γ-glutamyl moiety from glutathione, glutamine or other γ-glutamyl compounds to other amino acids or peptides.[19-25] The feasible sequences for the synthetic γ-glutamyl peptides might be γ-Glu-amino acid, γ-Glu-γ-Glu-amino acid, γ-Glu-γ-Glu-γ-Glu-amino acid, etc. For instance, γ-Glu-γ-Glu-Tau was found as a byproduct of the γ-Glu-Tau synthesis catalysed by GGT;[23] and γ-glutamyl-glutamine and other poly-glutamylated species were identified as the byproducts of the synthesis of γ-glutamyl-peptides catalysed by GGT.[26]

Gamma–glutamylization of bitter amino acids has been proven a powerful approach to improve the taste of bitter amino acids. For instance, Phe is known as a bitter amino acid, while γ-Glu-Phe exhibited a slightly sour, salty, metallic taste, and an intense and complex brothy which was believed close to recently termed "Kokumi".[11] γ-Glu-Phe was perceived as sourness and astringency with the taste threshold concentration as 2500 μmol/L[8] for astringency and 200-500 mg/L[11] for sourness, although its kokumi taste threshold concentration has not been disclosed yet. Moreover, γ-glutamylization of Phe was accomplished by Suzuki et al. (2002) using the GGT as the catalyst with the conversion rate of Phe against γ-Glu-Phe reaching 70%.[21] Accordingly, it is of high interest to verify that γ-glutamylation of Phe (a substrate for transpeptidation of glutaminases) could attenuate bitterness perception and the products might impart kokumi properties. This study

aimed to evaluate (1) the transpeptidase activity of two glutaminases (from *Bacillus amyloliquefaciens* (GBA) and *Aspergillus oryzae* (GAO)); (2) the synthesis of the Phe-containing γ-glutamyl peptides; (3) the affinity of glutaminases for different acceptors (Phe and γ-Glu-Phe) during the synthesis of γ-glutamyl peptides; (4) the sensory characteristics of γ-[Glu]n-Phe and post-enzymatic reaction mixture catalysed by glutaminases.

MATERIALS AND METHODS

Reagents and enzymes. L-Glutaminase from *Bacillus amyloliquefaciens* was acquired from Amano Enzyme China Ltd. (Shanghai, China) (termed as "GBA" herein). L-Glutaminases from *Aspergillus oryzae* and *Aspergillus niger* were from our lab (termed as "GAO" and "GAN", respectively, herein). Commercial peptides γ-Glu-Phe, γ-Glu-γ-Glu-Phe, γ-Glu-γ-Glu-γ-Glu-Phe, γ-Glu-γ-Glu-γ-Glu-γ-Glu-Phe and γ-Glu-γ-Glu-γ-Glu-γ-Glu-γ-Glu-Phe were purchased from Peptide Biological Technology Co., LTD (Nanjing, China). Commercial amino acids Gln and Phe were purchased from CapitalBio Corporation (Shanghai, China). Gly-Gly and γ-L-glutamyl-*p*-nitroanilide (γ-G*p*NA) were obtained from Sigma-Aldrich (Shanghai, China). Solvents and chemicals including acetonitrile and formic acid, were of HPLC grade and purchased from CapitalBio Corporation (shanghai, China).

Enzymatic synthesis of γ-glutamyl peptides. Water solutions containing Gln and Phe (both at 200 mM, pH 10.0) were prepared in duplicate to which a glutaminase (GBA or GAO, final concentration, 0.05 U/mL) was added to initiate the reaction. The mixtures were subjected to a 2-h incubation at 37 ºC and then heating at 90 ºC for 10 min (to inactivate the enzyme). The resultant

reaction mixtures in capped containers were cooled in a water bath or in the air and stored in refrigerator at 4 ºC before use (termed as "post-enzymatic reaction products"). In separate experiments, the molar ratio of substrates and reaction time were altered to evaluate, their effects on the yield of γ-glutamyl peptides. The experiment was carried out in triplicate. The yield of γ-glutamyl peptides was determined using the following equation: Yield = The amount of a specific γ-glutamyl peptide / The initial amount of Phe (mM) ×100%.

Analysis of post-reaction products using UPLC–Q-TOF–MS/MS. The identification of the γ-glutamyl peptides containing phenylalanine residue at C-terminus was conducted using an ultra performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UPLC–Q-TOF-MS/MS) system. UPLC separation was performed using an Agilent 1290 series UPLC system (Agilent Technologies) with an Agilent ZORBAX RRHD SB-C18 column (2.1 mm × 50 mm, 1.8 μm; maintained at 30 ºC). The mobile phase consisted of solvents (A) water with 0.1% formic acid and (B) acetonitrile with 0.1% formic acid and eluted at a constant flow rate of 0.3 mL/min using the following gradient: A:B = 100:0; 0–5 min, A:B from 100:0 to 90:10; 5–10 min, A:B from 90:10 to 85:15; 10–12 min, A:B from 85:15 to 100:0. Injection volume was 5 μL.

MS detection for the parent and fragment ions was performed using a maXis Impact Q-TOF MS/MS system (Bruker Daltonics, Beijing, China) fitted with an electrospray ionization (ESI) probe. The source-heated electrospray ionization (H-ESI) mass spectrometer was operated in positive ion mode with capillary voltage at 4500 V, capillary temperature at 180 C and nitrogen as curtain and collision gas (0.3 bar). Mass spectra were acquired at the rate of 1 spectra/s over the mass/charge (*m/z*) range of 50-1000. Quantitative calculation of each of the identified products was conducted by comparing the peak area of the extracted ions chromatograph (EIC) with

corresponding external calibration curves.[27] Software DataAnalysis, version 4.1 was used for identification and quantification of products.

Measurement of transpeptidase activity. The transpeptidase activity of GBA, GAO and GAN was measured following a previous method with some modifications.[28] Substrates γ-GpNA and Gly-Gly and the enzyme of interest were mixed in a 50 mM borate-NaOH buffer solution (pH 10.0) before an incubation at 37 ºC for 30 min. The reaction was then terminated by adding 0.1 M HCl. One unit (U) of enzyme was defined as the amount of the enzyme that released 1 μmol of *p*-nitroaniline per min from γ-G*p*NA through the transpeptidation reaction (which was monitored at 410 nm by a UV-visible spectrophotometer (UV765, Shanghai Youke Instrument Co., Ltd., Shanghai, China)). The pH and temperature for transpeptidation of glutaminases used in this assay were altered in separate experiments to investigate pH and temperature effects.

Enzymatic kinetic analysis of GBA or GAO catalysis for the synthesis of γ-glutamyl peptides and hydrolysis of γ-[Glu]n-Phe. GBA or GAO catalyzes the cleavage of the γ-glutamyl linkage of Gln and the transfer of the γ-glutamyl moiety to water (to release glutamate via hydrolysis) or to other amino acids and peptides (to form γ-glutamyl compounds via transpeptidation). In this study, the Michaelis–Menten constant (K_m) for GBA- or GAO-catalyzed synthesis of Phe-containing γ-glutamyl peptides through transpeptidation using Gln and Phe as substrates was determined under the following conditions: GBA or GAO, 0.0025 U/mL; substrate (Phe, or γ-$[Glu]_{(n<5)}$-Phe), 5-50 mM; Donor substrate (Gln, 50 mM (fixed)); pH 10.0, 37 ºC, reaction time 30 min. In the meantime, the Michaelis–Menten constant (K_m) for the hydrolysis of γ-[Glu]n-Phe was also determined through measuring the decrease in the amount of γ-[Glu]n-Phe over the concentration range of 5-50 mM under the following conditions: pH 10.0, 37 ºC, reaction

time 30 min; GBA or GAO, 0.0025 U/mL.

Analysis of sensory characteristics of γ-glutamyl peptides

Sensory characteristics profiling. Fifteen panelists (8 males, 7 females, aged 30–40 years), who did not have taste disorders and have been trained in similar sensory experiments at regular intervals for at least 2 years, were chosen. Each of the commercial γ-[Glu]n-Phe products and each of the single samples were reconstituted in water (5 mM), commercial soy sauce (2 mM) and model chicken broth (2 mM). These γ-glutamyl peptides are an acidity in solution for the more than one carboxylic acid groups in their molecules. Therefore, the pH values of these solutions were adjusted to 6.5 using sodium hydroxide solution (1.0 mM), and using a very small amount of diluted formic acid for the slightly excessive NaOH according to published methods.[2,29] Sensory characteristics including mouthfulness, thickness, continuity, umaminess, saltiness, sourness, astringency, and bitterness were evaluated in dual test using a 5-point intensity scale (from 0, (not detectable) to 5, (strongly detectable). Kokumi is a Japanese expression for complicated sensory characteristics of food. Kokumi does not refer to an independent taste but encompasses thickness, continuity and mouthfulness with taste-enhancing characteristics.[1,2,30] Each of these descriptors was tested separately in the sensory room at 25 2 ℃ through placing an aliquot (10 mL) of each sample in the mouth, swirling in the mouth for 10 s, swallowing at once and recording corresponding scores based on the whole 25 s taste experience. The samples for evaluation were placed in glass cups (20 mL) coded with three-digit numbers, covered with lid and served in a rationalized order. Bottled water was used to rinse the mouth between tasting trials. Thickness refers to rich complexity and was expressed as the increased taste intensity evaluated 10 s after food tasting; Continuity refers to the unbroken and consistent existence to give long-lasting

sensory effects or increased aftertaste, and was measured as persistent taste intensity 25 s after food tasting; Mouthfulness describes neither taste nor aroma but an overall perception associated with food structure, texture and morphological complexity, that is, food sensory reinforcement or increment of taste sensation throughout the whole mouth (not just on the tongue).[5,7,31]

Determination of taste recognition threshold concentrations for astringency and kokumi activity. The threshold values of the γ-[Glu]n-Phe in water, commercial soy sauce and model chicken broth were measured using a three-alternative forced-choice test.[32] The effects of γ-[Glu]n-Phe concentrations were evaluated through dose-response experiments,[33] in which samples with elevated γ-[Glu]n-Phe concentrations were prepared by adding increasing amounts of peptides (from 1 to 50 mmol/L) to solutions of model chicken broth, and presented in order of increasing concentrations to the trained panel.

Evaluation of taste-enhancing effect of post-enzymatic reaction products. The post-enzymatic reaction products prepared in this study were added at a concentration of 2 mg/mL to solutions of commercial soy sauce and model chicken broth, and subjected to panel evaluation on the taste-enhancing effects. Sensory characteristics including mouthfulness, thickness, continuity and umami quality were evaluated using a 5-point intensity scale (0, not detectable; 5, strongly detectable).

Statistical analysis. All experiments and analyses were conducted in triplicate, and the data were collected and reported as "means ± standard deviation". Analysis of variance and significance of difference were performed using SPSS 16.0 statistical software (SPSS Inc., Chicago, USA). Duncan's new multiple range test was performed to determine if the difference

187 between data within 99% confidence level.

188

189 **RESULTS AND DISCUSSION**

190 **Transpeptidase activity of glutaminase.** The transpeptidase activities of GBA and GAO were
191 detected with a specific activity of 100 and 10 U/g, respectively, at the optimal pH of 10.0 and
192 optimal temperature of 37 ºC. However, GAN exhibited no transpeptidase activity. These results
193 indicate that GBA and GAO possessed transpeptidase activity and could catalyze a γ-glutamyl
194 transfer reaction to yield Phe-containing γ-glutamyl peptides in the presence of Gln and Phe.

195 **Analysis of peptide products by UPLC-QTOF-MS/MS after catalysis by glutaminase.**
196 γ-Glu-Phe and γ-Glu-γ-Glu-Phe were both detected in the two post-reaction mixtures catalyzed by
197 GBA and GAO, whilst γ-Glu-γ-Glu-γ-Glu-Phe, γ-Glu-γ-Glu-γ-Glu-γ-Glu-Phe and
198 γ-Glu-γ-Glu-γ-Glu-γ-Glu-γ-Glu-Phe were detected only in the post-reaction mixture catalyzed by
199 GBA. The parent and fragment ion m/z values, and likely peptide sequences in GBA-catalyzed
200 post-reaction mixture were listed in Table 1. The molecular ion signals of m/z 295.1295, 424.1718,
201 553.2141, 682.2568 and 811.2996 in positive ESI mode were likely ionized γ-Glu-Phe,
202 γ-Glu-γ-Glu-Phe, γ-Glu-γ-Glu-γ-Glu-Phe, γ-Glu-γ-Glu-γ-Glu-γ-Glu-Phe and
203 γ-Glu-γ-Glu-γ-Glu-γ-Glu-γ-Glu-Phe, which are a series of γ-$[Glu]_n$-Phe short peptide chains with a
204 C-terminal Phe residue. As shown in Table 1, fragmentation of γ-Glu-Phe led to the generation of
205 a series of fragment ions i.e. mainly $[M\text{-}Phe\text{-}COOH]^+$ (m/z = 84.0446, b-CO_2 type ion),
206 $[Phe\text{-}COOH]^+$ (m/z =120.0810, y-CO_2 type ion), $[Phe+H]^+$ (m/z = 166.0860, y type ion). The ion
207 fragments of γ-$[Glu]_{(1< n \leqq 5)}$-Phe were y type and b1 type ions (b-CO_2/NH_3 type ion). The number

of y type ions was the same as that for the γ-glutamyl residue in the molecule, with the m/z of y1, y2, y3, y4 and y5 being 166.0863, 295.1283, 424.1709, 553.2134 and 682.2550, respectively. The m/z values of the parent and fragment ions for the γ-Glu-Phe and γ-Glu-γ-Glu-Phe in the post-reaction mixture catalyzed by GAO were almost identical to those for GBA (data not shown). These findings revealed that Phe-containing γ-glutamyl peptides can be synthesized through a γ-glutamyl transfer reaction catalyzed by glutaminases with Gln and Phe as substrates, and synthetic γ-$[Glu]_{(n-1)}$-Phe can be used as the acceptor to form the γ-$[Glu]_n$-Phe via the γ-glutamyl transfer reaction.[23, 26] The mass spectrometer operated in positive mode employed extracted ion chromatogram (EIC) for detection and quantitation of γ-[Glu]n-Phe products (Fig. 1). The concentrations of the γ-Glu-Phe, γ-$[Glu]_2$-Phe, γ-$[Glu]_3$-Phe, γ-$[Glu]_4$-Phe and γ-$[Glu]_5$-Phe synthesized in the presence of GBA and GAO were 61.59 ± 2.54 mM and 20.31 ± 2.34 mM, 27.64 ± 1.24 mM and 3.10 ± 0.82 mM, 14.09 ± 1.02 mM and "not detected", 1.50 ± 0.34 mM and "not detected", and 0.13 ±0.04 mM and "not detected", respectively. The yield of γ-Glu-Phe was higher than that reported previously in the literature when the same type of GAO was used,[18] whereas the yield of γ-Glu-Phe catalysed by GBA was only 43% of that catalyzed by GGT.[21] In both cases of GBA and GAO, a lower yield was associated with the γ-[Glu]n-Phe product with a higher molecular weight. γ-Glu-Phe, γ-Glu-γ-Glu-Phe and γ-Glu-γ-Glu-γ-Glu-Phe in the post-reaction mixture catalyzed by GBA accounted for 97.52% of the total γ-[Glu]n-Phe, whilst accounting for 11.70 % Phe and 13.56 % Gln in the case of GAO. The differences in the yield and type of γ-[Glu]n-Phe products between GBA and GAO catalysis suggest that GAO seemed less efficient, compared to GBA, in catalyzing the transfer of γ-glutamyl moiety to a tripeptide or a higher molecular weight peptide.

Determination of the Michaelis–Menten constant (K_m) of GBA and or GAO catalysis for the synthesis of γ-[Glu]n-Phe and hydrolysis of γ-[Glu]n-Phe. A "Ping-Pong" mechanism might be involved and led to the generation of a covalently bonded γ-glutamyl acyl-enzyme intermediate involving substantial hydrogen bonding and charge interactions which could subsequently link to Phe or existing peptides as acyl-acceptor substrates to form a γ-glutamyl peptides.[34] The two glutaminases, GBA and GAO, exhibited difference in affinity for acceptors (Phe and γ-Glu-Phe), with GBA having a greater affinity than GAO for Phe and γ-Glu-Phe in the synthesis reaction (Table 2). The K_m values for GBA against acceptor, Phe or γ-$[Glu]_{(n<5)}$-Phe of GBA increased with n value i.e. the lowest K_m value for Phe (n = 0, 47.88 mM) and the highest (206.47 mM) for γ-$[Glu]_4$-Phe. Thus, it became more difficult to synthesize γ-[Glu]n-Phe when the number of γ-glutamyl residues in the acceptor increased. Moreover, the K_m values (4.81 ~ 40.75 mM) for the hydrolysis catalyzed by GBA were lower than that for the synthesis catalyzed by GBA. The changing trend of K_m values of GAO for the synthesis of γ-$[Glu]_{(n\leqq2)}$-Phe as compared to the K_m values for corresponding hydrolysis was similar to that of GBA. GAO could not catalyze the transfer of γ-glutamyl moiety to the tripeptide (γ-$[Glu]_2$-Phe) or peptides with the number of γ-glutamyl residues greater than higher than 2 in the synthesis reaction (Table 2). These findings suggest that GBA was superior to GAO in the synthesis of Phe-containing γ-glutamyl peptides, and the transfer of γ-glutamyl moiety to a γ-[Glu]n-Phe would become more difficult with an increase of number of γ-glutamyl residues in the molecule.

Sensory characteristics of individual γ-[Glu]n-Phe. γ-[Glu]n-Phe in water (pH 6.5) exhibited astringent sensation at 5 mM which was in agreement with the previous findings that these kokumi compounds are either tasteless or astringent in water (Fig. 2).[1, 4, 5, 7] When each of

the γ-[Glu]n-Phe products was added at 2 mM to commercial soy sauce, enhanced continuity and umami taste ($p<0.05$) were perceived. The increased taste intensity and long-lasting continuity, as compared to the Control, caused by the addition of γ-[Glu]n-Phe to the commercial soy sauce were further demonstrated in the inserted figure of Fig. 2. The enhancement of commercial soy sauce with a continuity taste by γ-[Glu]n-Phe was similar to that caused by peptide-associated Maillard reaction in a umami solution.[29,30] Further, the addition of γ-[Glu]n-Phe at 2 mM to a model chicken broth led to significant ($p < 0.05$) enhanced of mouthfulness, thickness and umami taste. These findings were consistent with recent reports that γ-glutamyl peptides such as γ-Glu-Val-Gly and γ-Glu-Cys-Gly, γ-Glu-Cys-β-Ala, γ-Glu-Leu, and γ-Glu-Val are kokumi flavor compounds.[3,5,7] In summary, γ-[Glu]n-Phe would exhibit varied flavor characteristics in different food. It is the first time to describe the sensory characteristics of γ-$[Glu]_{(n>1)}$-Phe in this article. γ-$[Glu]_{(n>1)}$-Phe compounds have similar sensory characteristics to that of γ-Glu-Phe, although their taste-enhancing scores were slightly lower than that of γ-Glu-Phe (Fig. 2) i.e. the taste score in water and the taste-enhancing scores in commercial soy sauce and model chicken broth decreased progressively with an increase of the number of γ-glutamyl residues. The taste effect of the γ-[Glu]n-Phe would become weaker with an increased molecular weight. Accordingly, the type of continuity, mouthfulness, thickness, and the enhancing umami taste of food taste perception were defined as "kokumi" taste, thus all the γ-[Glu]n-Phe were confirmed as kokumi-active peptides.

Threshold concentrations and the taste intensity of γ-[Glu]n-Phe. The astringent and kokumi taste threshold concentrations of the γ-[Glu]n-Phe were determined (Table 3). γ-[Glu]n-Phe in water (pH 6.5) exhibited astringent sensation with corresponding threshold concentrations being 2.5, 3.34, 3.58, 3.69 and 3.92 mM, respectively. In comparison, γ-[Glu]n-Phe

in commercial soy sauce and model chicken broth (pH 6.5) only exhibited a kokumi flavour, with no astringent sensation detected and corresponding threshold concentrations in the range of 0.78-1.42 mM. No significant difference ($p<0.05$) was detected in the threshold concentrations between the γ-[Glu]n-Phe-fortified commercial soy sauce and γ-[Glu]n-Phe-fortified model chicken broth, and the threshold concentrations increased progressively with an increased molecular weight. The kokumi threshold concentrations of γ-Glu-Phe were approximately two-fold lower than that of γ-$[Glu]_5$-Phe, which agreed with the results of their taste profile analysis (i.e. the taste effect of these γ-[Glu]n-Phe weakened progressively with an increase of molecular weight). Further, the threshold concentration of each γ-[Glu]n-Phe compound in the commercial soy sauce and model chicken broth was significantly lower ($p<0.05$) than that in water. γ-Glu-Phe had a high astringent taste in water (2.5 mM) but a significantly lower threshold value in a commercial soy sauce or model chicken broth (0.93 and 0.78 mM, respectively). Such differences between in water and in model chicken broth were in accordance with those of γ-Glu-Leu, γ-Glu-Val γ-Glu-Cys-β-Ala and γ-Glu-Cys-Gly in water and chicken broth.[3] γ-[Glu]n-Phe only exhibited a kokumi taste at a low concentration, but an astringent sensation would be perceived when its concentration was increased to almost three-fold of the kokumi threshold value.

The dose-dependent effect of γ-[Glu]n-Phe on the model chicken broth was further evaluated (Fig. 3). The highest taste-enhancing score of the umaminess, thichness and mouthfulness (the so called "kokumi" taste) intensity occurred at about 5 mM, above which these tastes sensation began to decrease with an increased peptide dose. γ-[Glu]n-Phe at a concentration over 3 mM would also induce an astringent sensation, besides different degrees of these "kokumi" sensory attributes.

296 Thus, it was possible that the presence of astringency might suppress the kokumi sensation. A
297 similar change of flavor was also reported for the kokumi-active leucyl dipeptides.[29]

298 **Optimization of the yield of γ-[Glu]n-Phe.** This set of experiments only employed GBA as
299 catalyst. As shown by the reaction time-course (Fig. 4A), the yields of γ-Glu-Phe, γ-Glu-γ-Glu-Phe,
300 γ-Glu-γ-Glu-γ-Glu-Phe, γ-Glu-γ-Glu-γ-Glu-γ-Glu-Phe and γ-Glu-γ-Glu-γ-Glu-γ-Glu-γ-Glu-Phe
301 rapidly increased to 27.02, 9.01, 4.25, 1.12 and 0.043 %, respectively, after 1 h reaction, then
302 increased at a lower rate to 32.09, 14.34, 7.76, 1.31, and 0.065 % respectively, from the 1st to 3rd h
303 before leveling off. The yield of γ-Glu-Phe, γ-Glu-γ-Glu-Phe, γ-Glu-γ-Glu-γ-Glu-Phe,
304 γ-Glu-γ-Glu-γ-Glu-γ-Glu-Phe and γ-Glu-γ-Glu-γ-Glu-γ-Glu-γ-Glu-Phe generally increased with an
305 elevated proportion of Glu, except for a valley value for all the γ-[Glu]n-Phe at Glu/Phe of 1.5 and
306 a drop-off for γ-$[Glu]_4$-Phe and γ-$[Glu]_5$-Phe at Glu/Phe of 4.0 (Fig. 4B). The optimal yield of
307 γ-[Glu]n-Phe occurred at a Glu/Phe proportion of 3.0 (Glu:Phe = 300 mM:100 mM) i.e. 49.64%
308 for γ-Glu-Phe, 25.66% for γ-Glu-γ-Glu-Phe, 19.54% for γ-Glu-γ-Glu-γ-Glu-Phe, 3.75% for
309 γ-Glu-γ-Glu-γ-Glu-γ-Glu-Phe and 0.13% for γ-Glu-γ-Glu-γ-Glu-γ-Glu-γ-Glu-Phe, with the
310 consumption of 58.40% Gln and 98.70% Phe. Therefore, the most suitable conditions for the
311 synthesis of γ-[Glu]n-Phe were likely 300 mM Gln, 100 mM Phe, 0.05 U/mL GBA at pH 10 and
312 37°C for 3 h. An excess of L-Gln in the reaction mixture would benefit the formation of
313 γ-glutamyl-enzyme intermediate and facilitate the subsequent transpeptidation reaction.

314 **Effect of the post-reaction mixture under optimal conditions on the sensory**
315 **characteristics of commercial soy sauce and model chicken broth.** Under the above-mentioned
316 optimal conditions, 98.70% Phe was consumed as an acceptor to synthesize γ-[Glu]n-Phe, the
317 bitterness was found to be reduced and even disappeared whilst kokumi taste was present.

Subsequently, the lyophilized post-enzymatic reaction mixture (300 mM Gln/100 mM Phe as the substrates) was added at a concentration of 2 mg/mL (mainly including 1.6 mM γ-Glu-Phe, 0.8 mM γ-Glu-γ-Glu-Phe, 0.6 mM γ-Glu-γ-Glu-γ-Glu-Phe) to commercial soy sauce and model chicken broth for sensory evaluation. The greatest change in score brought by adding post-enzymatic reaction mixture was found for the intensity of continuity (2.23→3.03), followed by umami taste (3.72→4.21) in the case of commercial soy sauce. Enhancement of mouthfulness (1.99→2.74), thickness (2.55→3.24) and umami taste (3.69→4.02) was perceived in model chicken broth. These results were similar to the findings associated with commercial γ-[Glu]n-Phe products, suggesting that both γ-[Glu]n-Phe and the post-enzymatic reaction mixture can be used as food additives in commercial soy sauce and model chicken broth. Glutaminases are known to be used in the fermentation process of soy sauce to improve the content of glutamate. It is possible that kokumi-active γ-glutamyl peptides would be synthesized by the glutaminase during the production of soy sauce. The findings that γ-[Glu]n-Phe exhibited only sourness and astringent sensation in water but could impart a kokumi taste at a low concentration to commercial soy sauce and model chicken broth, along with the obtained sensory information on the series kokumi γ-[Glu]n-Phe synthesized by GBA or GAO, are undoubtedly useful to the food industry.

In this study, the transpeptidase activity of GBA and GAO to yield γ-[Glu]n-Phe peptides were verified and the Phe-containing γ-glutamyl peptides were synthesized for the first time using GBA and GAO as the catalysts. The identification of γ-Glu-Phe and γ-Glu-γ-Glu-Phe in the post-reaction mixture catalyzed by GAO, and γ-Glu-Phe, γ-Glu-γ-Glu-Phe, γ-Glu-γ-Glu-γ-Glu-Phe, γ-Glu-γ-Glu-γ-Glu-γ-Glu-Phe, γ-Glu-γ-Glu-γ-Glu-γ-Glu-γ-Glu-Phe in the post-reaction mixture catalyzed by GBA, along with the lower K_m values for the hydrolysis by GBA than the K_m values

for synthesis, indicates the feasibility for industrial production of these Phe-containing γ-glutamyl peptides. GBA was more suitable for the synthesis of Phe-containing γ-glutamyl peptides than GAO based on their respective K_m values for the transpeptidation reaction. The fact that the K_m values of GBA-catalyzed transpeptidation reaction against acceptors (Phe and γ-$[Glu]_{(n>1)}$-Phe) increased with an elevated number of γ-glutamyl residues, further suggest the greater difficulty to synthesize γ-[Glu]n-Phe with high molecular weights.

ACKNOWLEDGEMENTS

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451

Table 1. The peptides identified and corresponding m/z values of parent and fragment ions in post-enzymatic reaction mixture catalyzed by glutaminase of *B. Amyloliquefaciens* (GBA)

Parent ion (m/z; $[M+H]^+$)	Proposed peptides	Fragment ions (m/z)
295.1295	γ-Glu-Phe	84.0446 $[M\text{-Phe-COOH}]^+$, 120.0810 $[\text{Phe-COOH}]^+$, 166.0860 $[\text{Phe+H}]^+$
424.1718	γ-Glu-γ-Glu-Phe	84.0446, 120.0808, 166.0861, 232.0961 $[M-\gamma\text{-glutamyl residue}-NH_3-CO_2+H]^+$, 278.1024 $[M-\gamma\text{-glutamyl residue}-NH_3+H]^+$, 295.1285 $[M-\gamma\text{-glutamyl residue+H}]^+$
553.2141	γ-Glu-γ-Glu-γ-Glu-Phe	84.0443,166.0859, 232.0964, 295.1277, 424.1692 $[M-\gamma\text{-glutamyl residue+H}]^+$,
682.2568	γ-Glu-γ-Glu-γ-Glu-γ-Glu-Phe	130.0497 $[\text{Gln-NH}_3\text{+H}]^+$, 166.0862, 232.0964, 295.1288, 424.1714, 553.2133
811.2996	γ-Glu-γ-Glu-γ-Glu-γ-Glu-γ-Glu-Phe	130.0493 , 166.0863, 232.0964, 295.1283, 424.1709, 553.2134, 682.2550

Reaction conditions: 200 mM Gln, 200 mM Phe, pH 10, 37 °C and 2 h.Data obtained by ESI-Q-TOF-MS/MS in positive mode.

452

Table 2. The Km value (mM) of GBA and GAO for the synthesis and hydrolysis of γ-[Glu]n-Phe (n=0-5)

	Synthesis		Hydrolysis	
	GBA	GAO	GBA	GAO
L-Phe	47.88±0.47	153.92±5.47	-	-
γ-Glu-Phe	84.89±1.02	236.47±21.95	24.81±1.02	79.11±5.05
γ-$[Glu]_2$-Phe	95.23±7.05	-	30.73±2.05	99.76±8.01
γ-$[Glu]_3$-Phe	126.47±10.05	-	56.42±2.05	-
γ-$[Glu]_4$-Phe	206.47±13.05	-	70.79±3.86	-
γ-$[Glu]_5$-Phe	-	-	80.75±3.11	-

453

Table 3. Taste threshold concentrations of the Phe-containing γ-glutamyl peptides

γ-[Glu]$_n$-Phe	Taste threshold concentration (m mol/L) in		
	Water [a]	Commercial soy sauce[b]	Model chicken broth[b]
γ-Glu-Phe	2.5[#]	0.89±0.064	0.78±0.052
γ-[Glu]$_2$-Phe	3.34±0.097	1.01±0.047	0.96±0.054
γ-[Glu]$_3$-Phe	3.58±0.27	1.22±0.053	1.10±0.020
γ-[Glu]$_4$-Phe	3.69±0.21	1.34±0.037	1.25±0.032
γ-[Glu]$_5$-Phe	3.92±0.12	1.42±0.093	1.33±0.026

a: astringency; b: kokumi described as continuity and enhanced umami in commercial soy sauce; mouthfulness and thickness in model chicken broth. #: a value obtained from literature[15]. The pH of all the solutions was adjusted to 6.5.

454 **Figure Captions**

455 **Fig. 1.** Extracted ion chromatogram (EIC) of the Phe-containing γ-glutamy peptides in the
456 post-enzymatic reaction mixture by GBA. A: γ-Glu-Phe, B: γ-Glu-γ-Glu-Phe, C:
457 γ-Glu-γ-Glu-γ-Glu-Phe, D: γ-Glu-γ-Glu-γ-Glu-γ-Glu-Phe, E: γ-Glu-γ-Glu-γ-Glu-γ-Glu-γ-Glu-Phe.

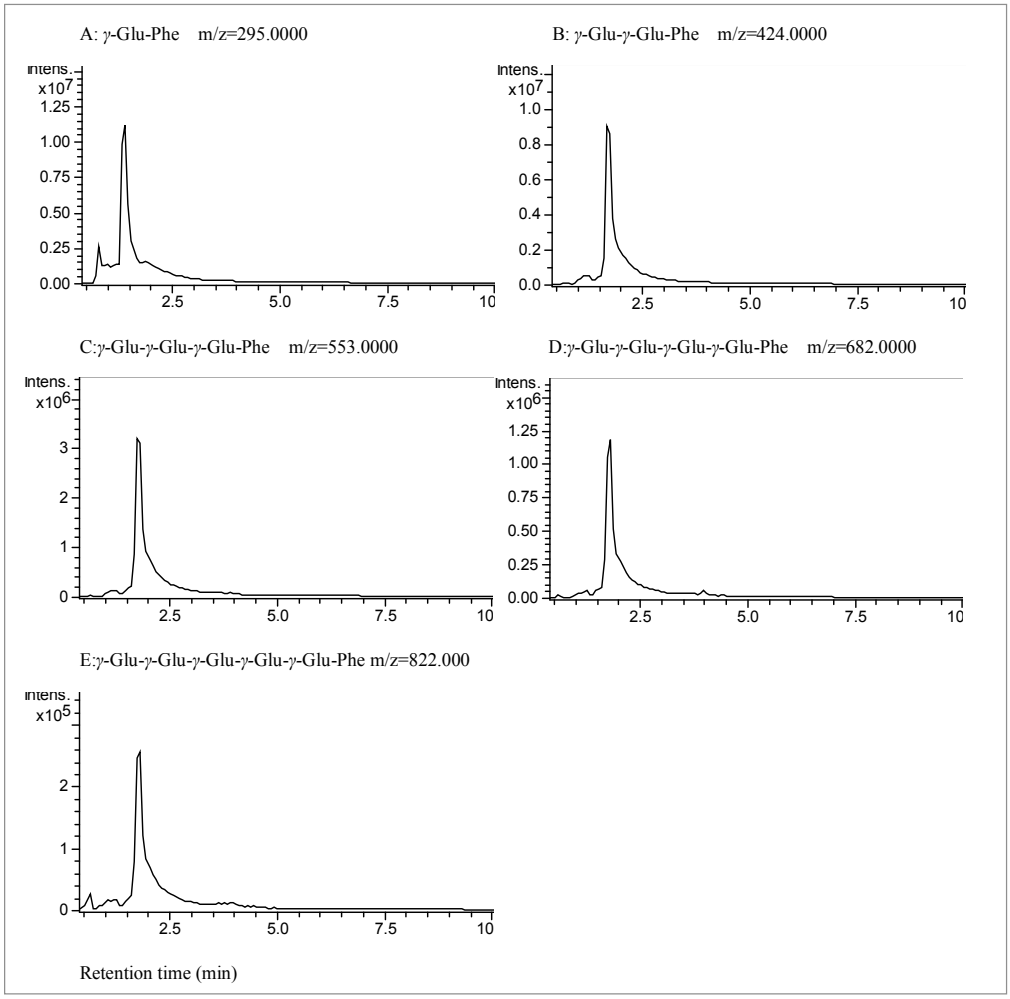
458 **Fig. 2.** Taste profile analysis of γ-[Glu]n-Phe. All the γ-[Glu]n-Phe were dissolved in water,
459 commercial soy sauce (CSS), or model chicken broth (MCB), the pH is adjusted to 6.5. Inserted
460 Figure about the intensity of aftertaste as a function of continuity time in commercial soy sauce.

461 **Fig. 3.** The dose-dependent effect of the γ-[Glu]n-Phe ("■", "■", "■", "■", "■" represented
462 γ-Glu-Phe, γ-$[Glu]_2$-Phe, γ-$[Glu]_3$-Phe, γ-$[Glu]_4$-Phe, γ-$[Glu]_5$-Phe respectively) on the umaminess,
463 thickness, mouthfulness and astringency sensations of model chicken broth.

464 **Fig. 4.** Optimization of the yield of γ-[Glu]n-Phe: (A), Effect of the reaction time on the synthesis
465 of γ-glutamylation of Phe (200 mM Phe, 200 mM Gln, 0.05 U/mL glutaminase; pH 10, and 37 °C);
466 (B), Effect of the substrate molar concentration ratio (Gln/Phe) on the γ-glutamylation of
467 phenylalanine (0.05 U/mL glutaminase; pH 10; 37 °C; 3 h).

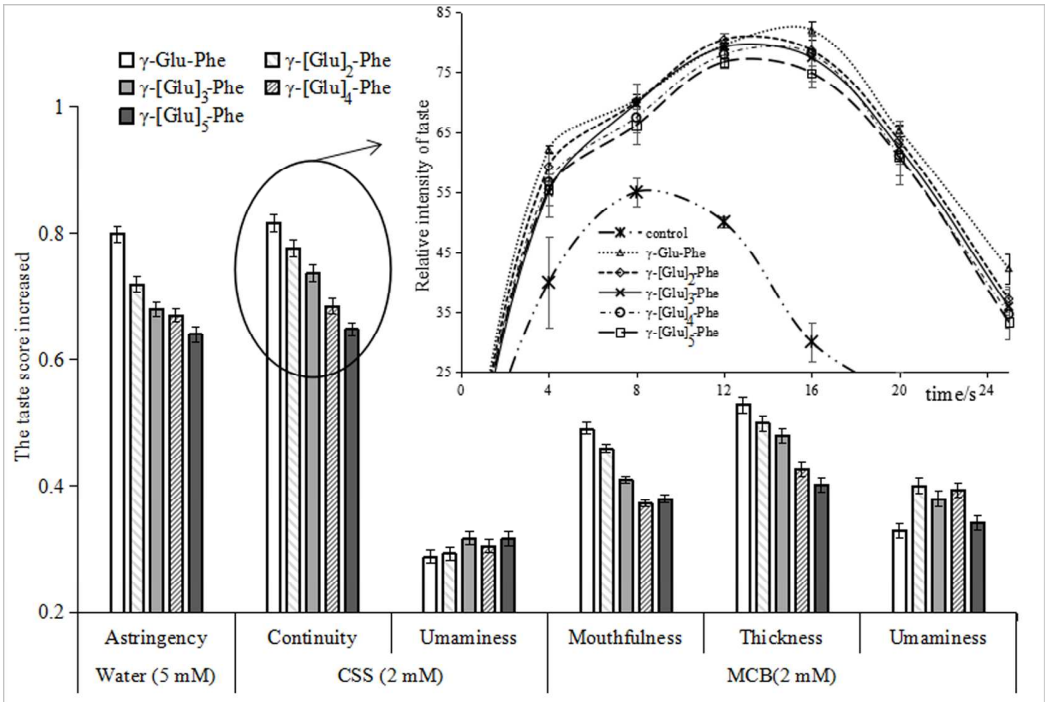
468 **Fig. 5.** Taste-modulatory activity of the post-enzymatic reaction mixture obtained under optimal
469 conditions in commercial soy sauce (CSS) and model chicken broth (MCB).

470



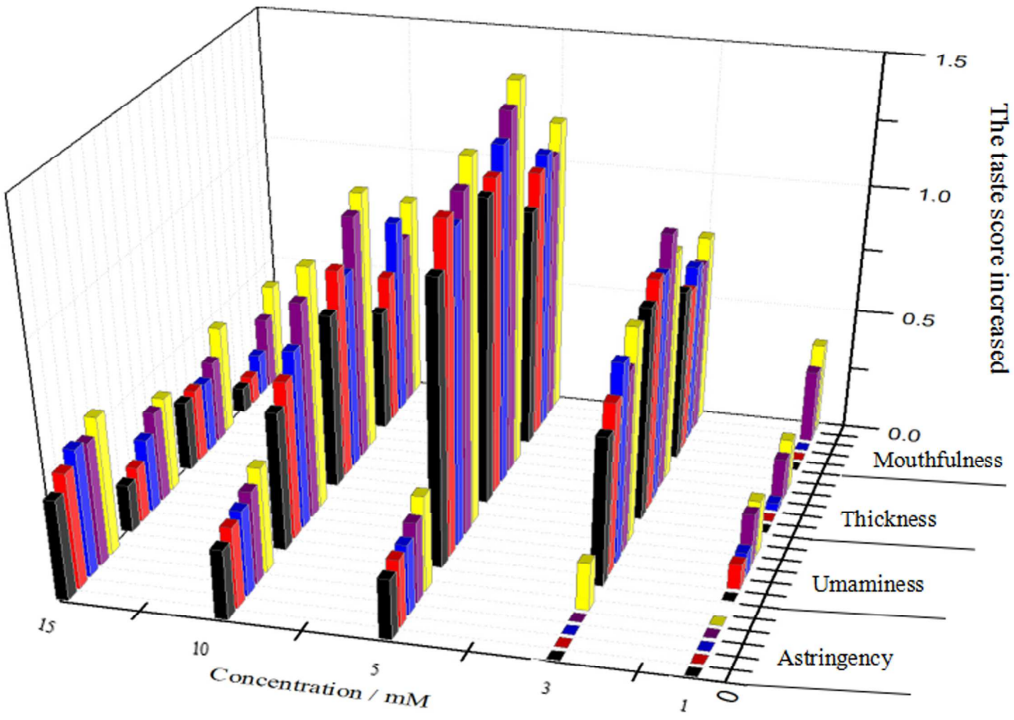
471

472 **Fig. 1.**


γ-Glu-Phe
γ-[Glu]2-Phe
γ-[Glu]3-Phe
γ-[Glu]4-Phe
γ-[Glu]5-Phe
The taste score increased
Relative intensity of taste
time/s
control
γ-Glu-Phe
γ-[Glu]2Phe
γ-[Glu]5-Phe
γ-[Glu]4-Phe
γ-[Glu]5-Phe
Astringency
Continuity
Umaminess
Mouthfulness
Thickness
Umaminess
Water (5 mM)
CSS (2 mM)
MCB(2 mM)

473

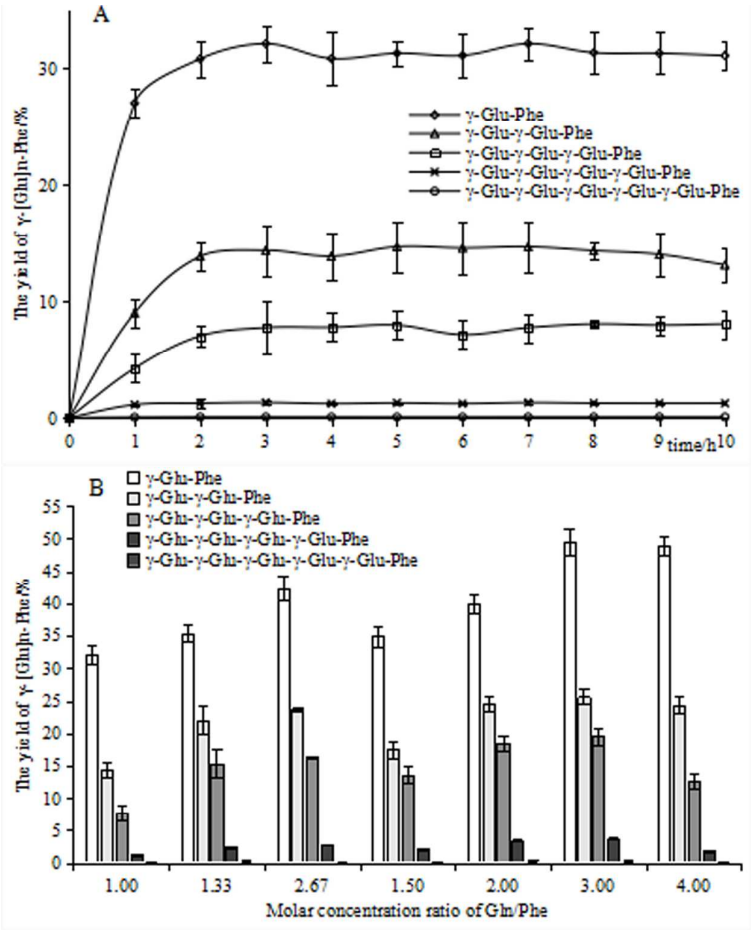
474 **Fig. 2.**


1.5
1.0
0.5
0.0
The taste score increased
Mouthfulness
Thickness
Umaminess
Astringency
15
10
5
3
1
0
Concentration / mM

475

476 **Fig. 3.**

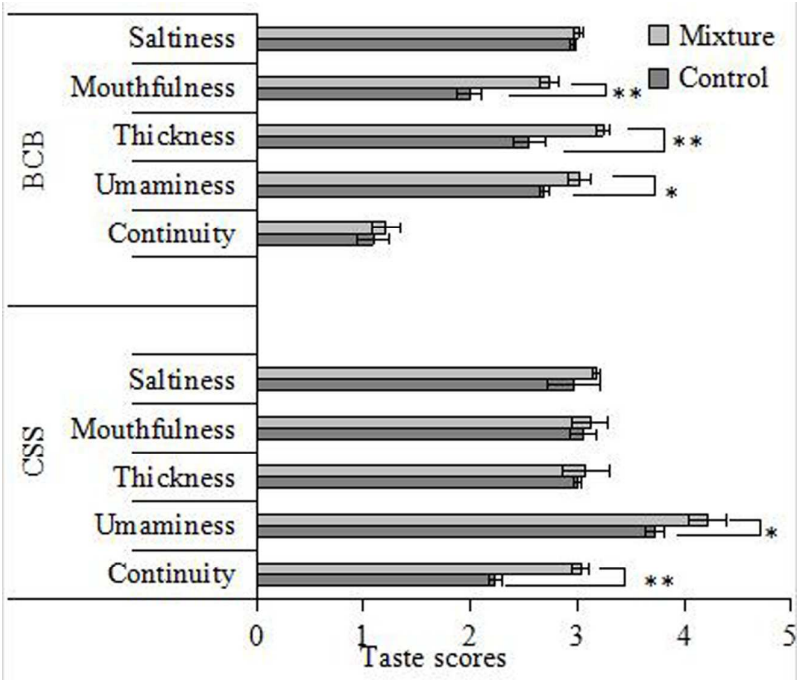
477



478

479 **Fig. 4.**

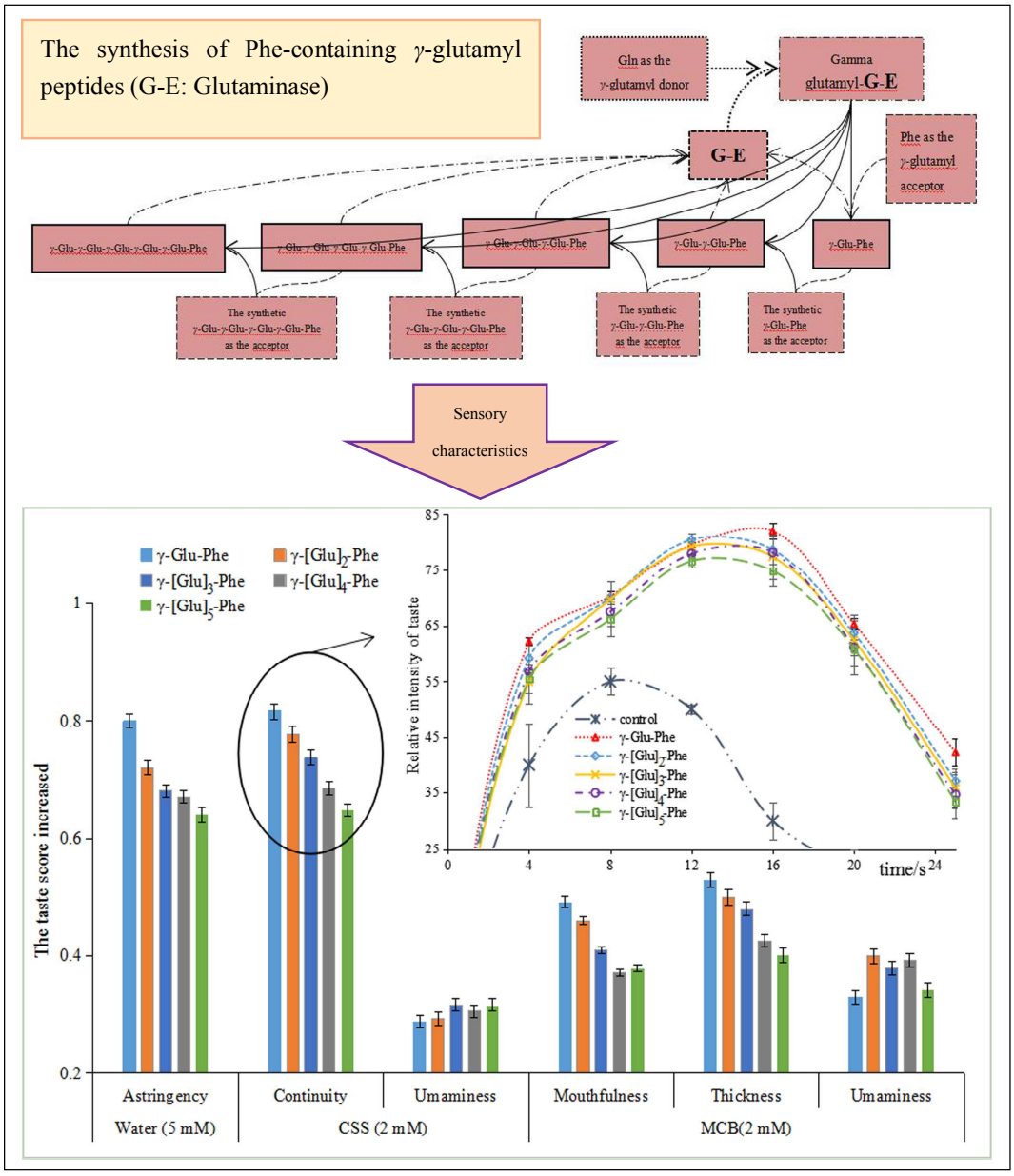
480

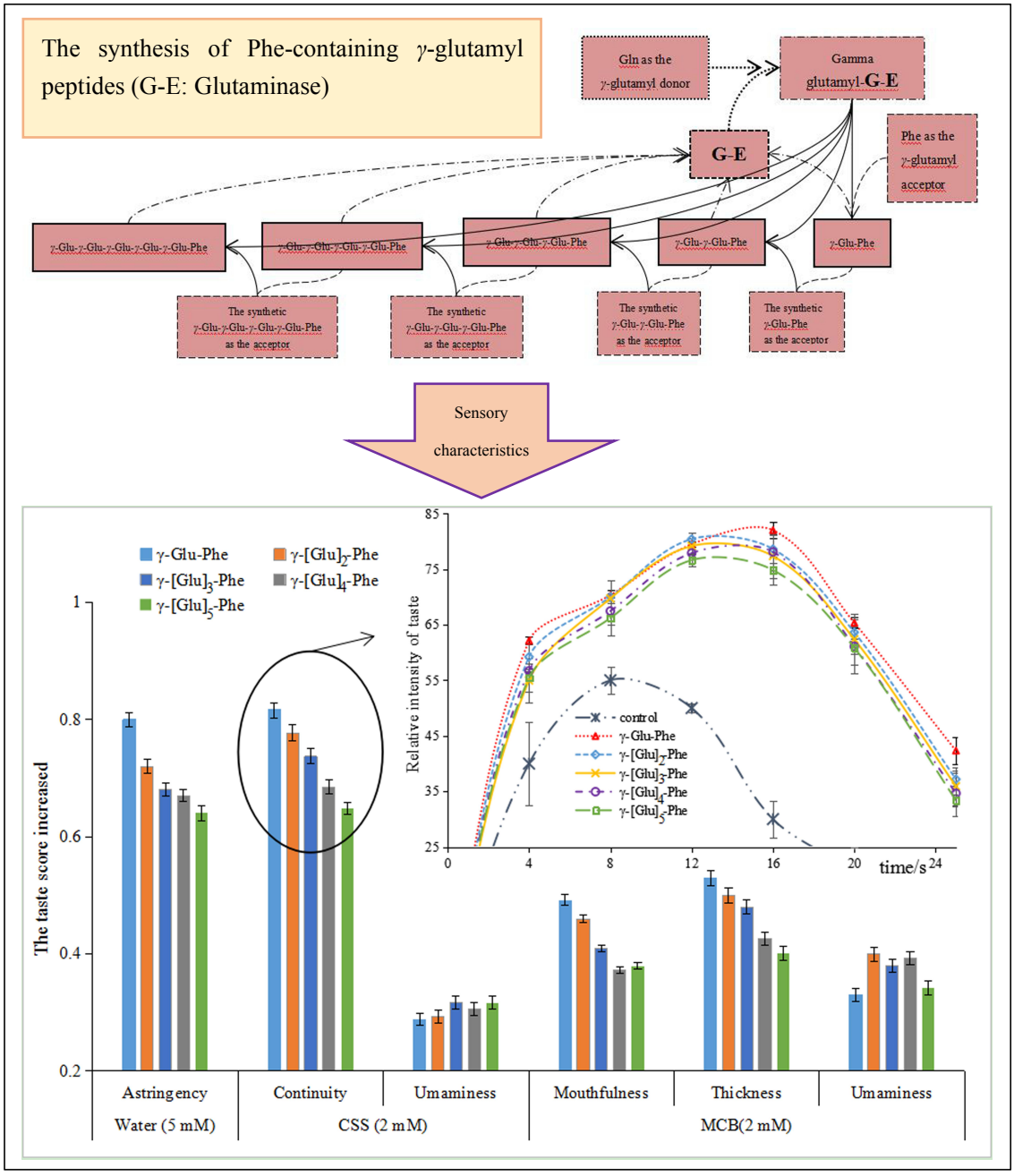


481

482 **Fig. 5.**

TOC graphic


The synthesis of Phe-containing γ-glutamyl peptides (G-E: Glutaminase)
Gln as the γ-glutamyl donor
Gamma glutamyl-G-E
G-E
Phe as the γ-glutamyl acceptor
γ-Glu-γ-Glu-γ-Glu-γ-Glu-γ-Glu-Phe
γ-Glu-γ-Glu-γ-Glu-γ-Glu-Phe
γ-Glu-γ-Glu-γ-Glu-Phe
γ-Glu-γ-Glu-Phe
γ-Glu-Phe
The synthetic γ-Glu-γ-Glu-γ-Glu-γ-Glu-Phe as the acceptor
The synthetic γ-Glu-γ-Glu-γ-Glu-Phe as the acceptor
The synthetic γ-Glu-γ-Glu-Phe as the acceptor
The synthetic γ-Glu-Phe as the acceptor
Sensory characteristics
γ-Glu-Phe
γ-[Glu]2-Phe
γ-[Glu]3-Phe
γ-[Glu]4-Phe
γ-[Glu]5-Phe
The taste score increased
Astringency
Continuity
Umaminess
Mouthfulness
Thickness
Umaminess
Water (5 mM)
CSS (2 mM)
MCB(2 mM)
Relative intensity of taste
time/s
control
γ-Glu-Phe
γ-[Glu]2-Phe
γ-[Glu]3-Phe
γ-[Glu]4-Phe
γ-[Glu]5-Phe


The synthesis of Phe-containing γ-glutamyl peptides (G-E: Glutaminase)
Gln as the γ-glutamyl donor
Gamma glutamyl-G-E
G-E
Phe as the γ-glutamyl acceptor
γ-Glu-γ-Glu-γ-Glu-γ-Glu-γ-Glu-Phe
γ-Glu-γ-Glu-γ-Glu-γ-Glu-Phe
γ-Glu-γ-Glu-γ-Glu-Phe
γ-Glu-γ-Glu-Phe
γ-Glu-Phe
The synthetic γ-Glu-γ-Glu-γ-Glu-γ-Glu-Phe as the acceptor
The synthetic γ-Glu-γ-Glu-γ-Glu-Phe as the acceptor
The synthetic γ-Glu-γ-Glu-Phe as the acceptor
The synthetic γ-Glu-Phe as the acceptor
Sensory characteristics
γ-Glu-Phe
γ-[Glu]2-Phe
γ-[Glu]3-Phe
γ-[Glu]4-Phe
γ-[Glu]5-Phe
The taste score increased
Relative intensity of taste
time/s
control
Astringency
Continuity
Umaminess
Mouthfulness
Thickness
Umaminess
Water (5 mM)
CSS (2 mM)
MCB(2 mM)