Regular Article

Structure–CaSR–Activity Relation of *Kokumi* γ-Glutamyl Peptides

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Modulation of the calcium sensing receptor (CaSR) is one of the physiological activities of γ-glutamyl peptides such as glutathione (γ-glutamylcysteinylglycine). γ-Glutamyl peptides also possess a flavoring effect, *i.e.*, sensory activity of *kokumi* substances, which modifies the five basic tastes when added to food. These activities have been shown to be positively correlated, suggesting that *kokumi* γ-glutamyl peptides are perceived through CaSRs in humans. Our research is based on the hypothesis that the discovery of highly active CaSR agonist peptides will lead to the creation of practical *kokumi* peptides. Through continuous study of the structure–CaSR–activity relation of a large number of γ-glutamyl peptides, we have determined that the structural requirements for intense CaSR activity of γ-glutamyl peptides are as follows: existence of an N-terminal γ-L-glutamyl residue; existence of a moderately sized, aliphatic, neutral substituent at the second residue in an L-configuration; and existence of a C-terminal carboxylic acid, preferably with the existence of glycine as the third constituent. By the sensory analysis of γ-glutamyl peptides selected by screening using the CaSR activity assay, γ-glutamylvalylglycine was found to be a potent *kokumi* peptide. Furthermore, norvaline-containing γ-glutamyl peptides, *i.e.*, γ-glutamylnorvalylglycine and γ-glutamylnorvaline, possessed excellent sensory activity of *kokumi* substances. A novel, practical industrial synthesis of regiospecific γ-glutamyl peptides is also required for their commercialization, which was achieved through the ring opening reaction of *N*-α-carbobenzoxy-L-glutamic anhydride and amino acids or peptides in the presence of *N*-hydroxysuccinimide.

Key words calcium sensing receptor; γ-glutamyl peptide; *kokumi* substance; glutathion; glutamic anhydride

γ-Glutamyl peptides, particularly glutathione (γ-glutamylcysteinylglycine, γ-Glu-Cys-Gly, **36**), possess various physiological activities, including activation of the calcium sensing receptor (CaSR) and sensory activity of *kokumi* substances. Figure 1 shows the structures of the γ-glutamyl peptides described in this study.

The extracellular CaSR plays a central role in extracellular calcium homeostasis in mammals.[1)] Increased blood calcium levels detected by the CaSR result in suppression of parathyroid hormone secretion, stimulation of calcitonin secretion, and induction of urinary calcium excretion to reduce the blood calcium to normal levels. It has become apparent that the CaSR is expressed not only in the parathyroid glands and kidneys but also in many other tissues, such as the liver, heart, lungs, gastrointestinal tract, lymphocytes, pancreas, and the central and peripheral nervous systems, suggesting that it is involved in a wide range of biological functions.[2)] Several types of compounds have been reported to possess CaSR agonist activity. Amino acids have been reported to bind to the large extracellular Venus flytrap domain (VFD) of CaSR, which is a structure common to all class C members of the G protein-coupled receptors (GPCRs). CaSR is strongly activated by the aromatic amino acids His, Trp, Phe, and Tyr but weakly activated by other amino acids, such as Arg, Lys, Val, or Gly. It has been suggested that all amino acids bind to the VFD binding pocket through their amino and carboxyl groups.[3,4)] γ-Glutamyl peptides have also been reported to bind to the large extracellular VFD of CaSR.[5,6)]

Previously, in a preliminary hCaSR functional assay of γ-glutamyl dipeptides and γ-glutamyl tripeptides comprising proteinogenic amino acids, we identified various γ-glutamyl peptides, including **36** and γ-glutamylvalylglycine (γ-Glu-Val-Gly, **35**), as CaSR agonists.[7)] As the agonist peptides contain an N-terminal γ-L-glutamyl residue comprising α-amino and α-carboxyl groups, as with amino acids, it is reasonable to assume that peptides containing such a unit will bind to and activate the CaSR.

Another physiological activity of γ-glutamyl peptides is sensory activity of *kokumi* substances. Ueda *et al.* investigated the flavoring effects of diluted extracts of garlic and onion, which have only minimal aroma in water, but when added to an umami solution or other types of food, they significantly enhanced the thickness, continuity, and mouthfulness of the food to which they had been added. Furthermore, they isolated and identified **36**, γ-glutamyl-*S*-allyl-L-cysteine, γ-glutamyl-*S*-allyl-L-cysteine sulfoxide, and γ-glutamyl-*trans*-*S*-propenyl-L-cysteine sulfoxide as some of the key compounds responsible for this flavoring effect.[8–10)] They proposed that substances with these properties should be referred to as *kokumi* substances. The characteristic of *kokumi* substances is that they modify the five basic tastes (especially sweet, salty, and umami) when added to basic tasting solutions or food; however, at the concentrations tested they have no taste themselves.[11)] More recently, it has been reported that many γ-glutamyl peptides such as γ-Glu-Val (**2**), γ-Glu-Leu (**5**), γ-Glu-Cys-βAla (**91**), γ-Glu-Gly (**11**), γ-Glu-Met (**9**), and **35** are found in foods, such as edible beans, Gouda cheese, and soy sauces, and they enhance the intensity of mouthfulness.[12–19)]

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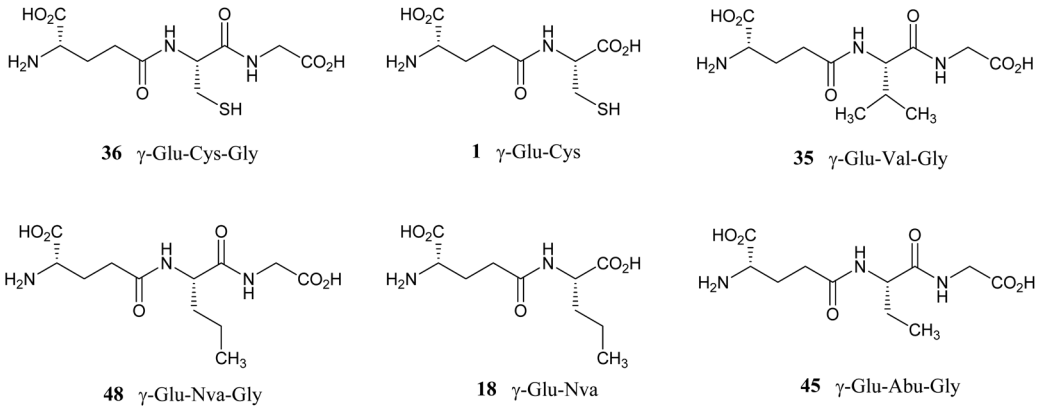

Fig. 1. Structures of γ-Glutamyl Peptides

Previously, we revealed that *kokumi* peptides such as **36** are perceived through CaSRs in humans and that the CaSR activity of previously discovered γ-glutamyl peptides comprising proteinogenic amino acids is positively correlated with the sensory activity of *kokumi* peptides.[7] A previous report also indicated that CaSR-expressing taste cells are the primary detectors of *kokumi* substances, and that the *kokumi* substance-responding taste cells might involve enhancement of basic tastes such as umami and sweet taste.[20] In other words, *kokumi* γ-glutamyl peptides can be easily identified *via* sensory evaluation of the γ-glutamyl peptides selected by primary high-throughput screening using the CaSR activity assay as an index. Thus, through further searching for active CaSR agonist peptides, the discovery of other practical *kokumi* γ-glutamyl peptides can be achieved.

In this study, to ascertain the structural requirements of γ-glutamyl peptides for activation of the CaSR, **36** was divided into three parts, N-terminus, second residue, and C-terminus, and each part was modified according to previous studies on the structure–activity relation of **36**.[21–23] Modified γ-glutamyl peptides composed of non-proteinogenic amino acids, D-amino acids, α-hydoxy acids, *etc.* were then screened using the CaSR assay as an index.

During the course of this study, **35** was filed in Flavor and Extract Manufacturers Association of the United States (FEMA) as FEMA-GRAS™ No. 4709, filed in the European Union list of flavoring substance, and approved as a food additive in Japan (GRAS: generally recognized as safe). Therefore, development of a practical industrial production method toward γ-glutamyl peptides is necessary for their commercialization. However, selective formation of the γ-glutamyl linkage occurs during their synthesis, which is a problem that must be overcome. Protected glutamic acids, such as *N*-α-carbobenzoxy-L-glutamic acid α-benzyl ester, are useful for small-scale syntheses of γ-glutamyl peptides, as shown in Experimental; however, such conventional syntheses are difficult to scale up.

The regiospecific ring opening of *N*-protected-glutamic anhydride with an amino acid or peptide is an attractive approach for the construction of γ-glutamyl peptides. There have been many reports regarding the regioselectivity of the reaction of *N*-protected-glutamic anhydride with various amines. Huang *et al.* studied the nucleophilic addition of aniline to *N*-protected-glutamic anhydride and reported that the regioselectivity of the reaction was controlled by the choice of reaction solvent. They demonstrated that the nucleophilic attack preferentially occurs at the γ-carbonyl in polar aprotic solvents such as dimethyl sulfoxide (DMSO), whereas it occurs at the α-carbonyl in nonpolar solvents such as benzene.[24,25] King and Kidd reported that the reaction of *N*-phthalyl-DL-glutamic anhydride with glycine exclusively afforded *N*-phthaloyl-γ-DL-glutamylglycine in hot acetic acid in 48% isolated yield.[26] Their methods have also been applied to the regiospecific synthesis of γ-glutamyl dipeptides.[27,28]

Quesne and Young reported that in the reaction of *N*-α-carbobenzoxy-glutamic anhydride (Cbz-Glu anhydride, **107**) with valine ethyl ester in a two phase system of ethyl acetate and aqueous potassium hydrogen carbonate, *N*-carbobenzoxy-α-glutamylvaline ethyl ester (Cbz-α-Glu-Val-OEt) was exclusively obtained in 32% yield.[29] Upon the reaction of **107** with Glu in the same solvent, Shiba and Imai obtained the α-isomer in 34% yield and the γ-isomer in 1.8% yield.[30] Manesis and Goodman reported that the α- and γ-isomers were obtained in a ratio of 2 : 3 upon the reaction of **107** with ε-*N*-*tert*-butoxycarbonyl-lysine methyl ester in tetrahydrofuran in 42% total yield.[31]

Although there have been many reports on the reaction of *N*-protected-glutamic anhydride, to the best of our knowledge, a regiospecific synthesis of γ-glutamyl peptides through the reaction of **107** and an amino acid or peptide has not yet been developed. Based on these previous reports, we have studied the reaction of **107** and an amino acid or peptide and developed a large-scale synthetic procedure of γ-glutamyl peptides, wherein the key step is a regiospecific γ-glutamyl bond formation. This was successfully achieved through the reaction of **107** and an amino acid or peptide in the presence of 0.5–1.0 eq of *N*-hydroxysuccinimide (NHS).

Results and Discussion

Determination of CaSR Agonist Activity The theory behind this experimental technique was previously described in detail[7] and is described briefly in this paper.

A two-electrode voltage clamp assay was performed using *Xenopus* oocytes microinjected with human CaSR cRNA at −70 mV. Peptides were added at various concentrations (1000, 500, 300, 100, 50, 30, 10, 3, 1, 0.3, 0.1 μM) and the minimum effective concentration at which a current could be detected was a measure of the strength of CaSR activation. The results demonstrated that many γ-glutamyl peptides display CaSR activity. No response was observed in oocytes injected with dis-

tilled water as a control. For comparison, EC_{50} values (μM) for γ-glutamyl peptides were also determined using a HEK-293 assay, which provided higher sensitivity than the oocyte assay.

Activities of γ-Glutamyl Dipeptides (γ-Glu-X) The structure–CaSR–activity relation of γ-glutamyl peptides was briefly discussed previously.[7] The CaSR activity of γ-glutamyl peptides, including previously reported results, is summarized in Tables 1–3.

Initially, we performed a CaSR agonist activity assay on a dipeptide library and found that most of the active dipeptides included a "γ-glutamyl" structure (data not shown). The presence of a γ-peptidic bond between the N-terminal glutamic acid and the second residue is the most distinct structural feature of active dipeptides. It has been suggested that a common feature of class C G-protein-coupled receptors (GPCRs) is the presence of an amino acid binding site. As γ-glutamyl dipeptides have an α-amino acid structure, it is reasonable that some γ-glutamyl dipeptides are able to activate the CaSR.

The CaSR activity of γ-glutamyl dipeptides is summarized in Table 1. Among the γ-glutamyl dipeptides composed of proteinogenic amino acids, γ-Glu-Cys (**1**) showed the most intense activity, while γ-Glu-Val (**2**) showed subsequent moderate activity. Conversely, some γ-glutamyl dipeptides, such as γ-Glu-Asp, γ-Glu-Asp, γ-Glu-Gln, γ-Glu-Glu, γ-Glu-Lys, γ-Glu-His, γ-Glu-Phe, and γ-Glu-Tyr, were inactive (data not shown). Incorporation of a large side chain or a positively or negatively charged side chain abolished the observed activity. Among the γ-glutamyl dipeptides containing non-proteinogenic amino acids, γ-Glu-Nva (Nva; norvaline) (**18**), γ-Glu-Cle (Cle; cycloleucine) (**19**), and γ-Glu-Abu (Abu; (*S*)-2-aminobutanoic acid) (**20**) showed nearly the same range of activity as **1**. This indicated that the sulfhydryl group of the cysteine residue is not essential for CaSR activity; however, the incorporation of a small-to-medium-sized neutral alkyl side chain is necessary for high activity. Incorporation of D-amino acids (*i.e.*, γ-Glu-D-Val, **29**) produced inactive dipeptides. Analogs without an α-carboxyl (*i.e.*, *N*-γ-aminobutyryl-Val) or α-amino group (*i.e.*, *N*-glutaryl-Val) were found to be inactive (data not shown). Moreover, neither α-Glu-Val (**31**) nor γ-D-Glu-Val (**30**) displayed any activity, demonstrating that the γ-L-glutamyl linkage is required for CaSR activity. Replacement of the carboxyl group at the C-terminus with an amide group

Table 1. Determination of the Calcium Sensing Receptor (CaSR) Activity of γ-Glu-X Peptides Using *Xenopus* Oocytes and HEK-293 Cells

No.	γ-Glutamyl peptides	CaSR Activity (μM)	
		Xenopus oocytes (a)	HEK-293 (b)
1	γ-Glu-Cys	3	0.16–0.46
2	γ-Glu-Val	10	1.03
3	γ-Glu-Ala	50	1.24
4	γ-Glu-Thr	50	6.97
5	γ-Glu-Leu	100	5.07
6	γ-Glu-Ile	100	9.91
7	γ-Glu-Ser	300	11
8	γ-Glu-Orn	500	70.4
9	γ-Glu-Met	500	
10	γ-Glu-Asn		159
11	γ-Glu-Gly	1000	200
12	γ-Glu-Trp		300
13	γ-Glu-Pro		409
14	γ-Glu-Cys(Me)	30	
15	γ-Glu-Tau	300	
16	γ-Glu-Cys(Me)(O)	300	
17	γ-Glu-Met(O)	1000	
18	γ-Glu-Nva		0.12
19	γ-Glu-Cle		0.15
20	γ-Glu-Abu		0.21
21	γ-Glu-Ape		0.49
22	γ-Glu-Cys(Carboxymethyl)		1.69
23	γ-Glu-Tle		3.06
24	γ-Glu-Ser(Me)		3.19
25	γ-Glu-Nle		4.41
26	γ-Glu-β-homoAla		5.53
27	γ-Glu-*allo*-Ile		8.87
28	γ-Glu-Aib		15.4
29	γ-Glu-D-Val	Inactive	
30	γ-D-Glu-Val	Inactive	
31	α-Glu-Val	Inactive	
32	γ-Glu-Val-NH_2	1000	
33	γ-Glu-Val-OMe	1000	
34	γ-Glu-Val-ol	1000	

(a) Minimum effective concentration. (b) EC_{50} values. Abbreviations: Cys(Me), *S*-(methyl)-cysteine; Tau, taurine; Cys(Me)(O), *S*-(methyl)-cysteine sulfoxide; Met(O), methionine sulfoxide; Nva, norvaline; Cle, cycloleucine; Abu, (*S*)-2-aminobutanoic acid; Ape, (*S*)-3-aminopentanoic acid; Cys(Carboxymethyl), *S*-(carboxymethyl)-cysteine; Tle, *tert*-leucine; Ser(Me), *O*-(methyl)-serine; Nle, norleucine; β-homoAla, (*S*)-3-aminobutanoic acid; Aib, α-methylalanine; Val-ol, valinol.

Table 2. Determination of the Calcium Sensing Receptor (CaSR) Activity of γ-Glu-X-Gly Peptides Using *Xenopus* Oocytes and HEK-293 Cells

No.	γ-Glutamyl peptides	CaSR Activity (μM)	
		Xenopus oocytes (a)	HEK-293 (b)
35	γ-Glu-Val-Gly	0.1	0.030–0.075
36	γ-Glu-Cys-Gly	3	0.7
37	γ-Glu-Ala-Gly	10	0.016
38	γ-Glu-Ser-Gly	10	
39	γ-Glu-Gly-Gly	30	
40	γ-Glu-Ile-Gly	100	
41	γ-Glu-Thr-Gly	300	2.8
42	γ-Glu-Leu-Gly	300	
43	γ-Glu-Pro-Gly	300	
44	γ-Glu-Cys(Me)-Gly	3	
45	γ-Glu-Abu-Gly	3	0.025
46	γ-Glu-Algly-Gly		0.011
47	γ-Glu-Ser(Me)-Gly		0.037
48	γ-Glu-Nva-Gly		0.052–0.055
49	γ-Glu-Tle-Gly		0.043–0.090
50	γ-Glu-Pen-Gly		0.16–0.23
51	γ-Glu-Aib-Gly		0.35–1.17
52	γ-Glu-Cle-Gly		0.5–1.5
53	γ-Glu-*allo*-Ile-Gly		0.63
54	γ-Glu-*allo*-Thr-Gly		0.7
55	γ-Glu-Hse-Gly		1.18
56	γ-Glu-Cys(*n*-Butyl)-Gly		4
57	γ-Glu-Nle-Gly		5
58	γ-Glu-Cys(1,2-Dicarboxyethyl)-Gly		5–10
59	γ-Glu-Cys(Allyl)-Gly	100	
60	γ-Glu-Cys(*n*-Propyl)-Gly		117

(a) Minimum effective concentration. (b) EC_{50} values. Abbreviations: Algly, α-allylglycine; Pen, penicillamine; Hse, homoserine; Cys(*n*-Butyl), *S*-(*n*-butyl)-cysteine; Cys(1,2-dicarboxyethyl), *S*-(2,3-dicarboxymethyl)-cysteine; Cys(Allyl), *S*-(allyl)-cysteine; Cys(*n*-Propyl), *S*-(*n*-propyl)-cysteine.

Table 3. Determination of the Calcium Sensing Receptor (CaSR) Activity of γ-Glu-Val-Y Peptides Using *Xenopus* Oocytes and HEK-293 Cells

No.	γ-Glutamyl peptides	CaSR Activity (μM)	
		Xenopus oocytes (a)	HEK-293 (b)
35	γ-Glu-Val-Gly	0.1	0.030–0.075
60	γ-Glu-Val-Gln	10	0.3–0.5
61	γ-Glu-Val-Cys	10	3.7
62	γ-Glu-Val-Pro	30	
63	γ-Glu-Val-Ser	30	
64	γ-Glu-Val-Phe	30	
65	γ-Glu-Val-Asn	30	
66	γ-Glu-Val-Orn	100	
67	γ-Glu-Val-His	100	
68	γ-Glu-Val-Ala	300	
69	γ-Glu-Val-Thr	1000	
70	γ-Glu-Val-Met	1000	
71	γ-Glu-Val-Asp	1000	
72	γ-Glu-Val-Arg	1000	
73	γ-Glu-Val-Lys	1000	
74	γ-Glu-Val-Glu	1000	
75	γ-Glu-Val-Val	1000	
76	γ-Glu-Val-D-Ala		1.26
77	γ-Glu-Val-D-Ser		4.27
78	γ-Glu-Ala-Leu		4.17
79	γ-Glu-Abu-Leu		2.49
80	γ-Glu-Abu-Pro		4.59
81	γ-Glu-D-Val-Gly		Inactive
82	γ-D-Glu-Val-Gly		Inactive
83	γ-D-Glu-D-Val-Gly		Inactive
84	α-Glu-Val-Gly		>10
85	α-D-Glu-Val-Gly		Inactive
86	α-Glu-D-Val-Gly		Inactive
87	α-D-Glu-D-Val-Gly		Inactive
88	β-Asp-Val-Gly		Inactive
89	β-Asp-Val-βAla		Inactive
90	γ-Glu-Val-βAla		Inactive
91	γ-Glu-Cys-βAla		5–10
92	γ-Glu-Abu-βAla		2.45
93	γ-Glu-Ala-βAla		9.96
94	γ-Glu-Val-Gly-Gly		4.91
95	γ-Glu-Val-Gly-Gln		2.03
96	γ-Glu-Val-Glycolic acid		0.05
97	γ-Glu-Abu-Glycolic acid		0.14
98	γ-Glu-Tle-Glycolic acid		0.065–0.079
99	γ-Glu-Val-L-Lactic acid		0.102
100	γ-Glu-Abu-L-Lactic acid		0.038
101	γ-Glu-Tle-L-Lactic acid		0.057

(a) Minimum effective concentration. (b) EC_{50} values. Abbreviations: βAla, β-alanine.

(γ-Glu-Val-NH_2, **32**), methoxycarbonyl group (γ-Glu-Val-OMe, **33**), or hydroxymethyl group (γ-Glu-Val-ol, **34**) yielded γ-glutamyl-amide compounds with very weak activities.

Activities of γ-Glutamyl Tripeptides (γ-Glu-X-Gly) Activities of γ-Glu-X-Gly analogs with center position modification are shown in Table 2. Tripeptides wherein the cysteine side chain was replaced by a large hydrophobic, basic, or acidic side chain were found to be inactive. Thus, γ-Glu-Arg-Gly, γ-Glu-Asn-Gly, γ-Glu-Asp-Gly, γ-Glu-Gln-Gly, γ-Glu-Glu-Gly, γ-Glu-His-Gly, γ-Glu-Lys-Gly, γ-Glu-Met-Gly, γ-Glu-Orn-Gly, γ-Glu-Phe-Gly, and γ-Glu-Tyr-Gly were inactive (data not shown). Incorporation of proteinogenic amino acids with small hydrophilic side chains or small-to-medium-sized hydrophobic side chains generated agonists. Incorporation of valine, containing a β-branched side chain, as the second residue (**35**) produced an agonist with an EC_{50} value 30 times greater than that of **36**. Moreover, intense agonists were generated due to the incorporation of non-proteinogenic L-amino acids with small-to-medium-sized hydrophobic side chains comprising one to three carbon(s) or two carbons and one sulfer or oxygen atom. Since the activity of γ-Glu-Nva-Gly (**48**) was approximately two orders of magnitude higher than that of γ-Glu-Nle-Gly (Nle; norleucine) (**57**), the steric requirements for expression of activity must be strictly controlled. However, incorporation of a D-amino acid (*i.e.*, γ-Glu-D-Val-Gly, **81**) generated inactive compounds (Table 3). These results suggest that the side chain of the second residue must have an appropriate size and orientation for activation of CaSR. In tripeptides, the cysteine thiol group is considered to play an important role, but was not found to be essential for biological activity. Thus, the CaSR agonist function of γ-glutamyl tripeptides does not depend on the functionality of the thiol group.

Activities of N-Terminal or C-Terminal Modified Analogs of γ-Glu-Val-Y Activities of **35** analogs modified at the N- or C-terminus are shown in Table 3.

Replacement of the γ-glutamyl residue with a β-aspartyl residue (**88**) or γ-D-glutamyl residue (**82**) yielded inactive tripeptides. α-Glutamyl peptide α-Glu-Val-Gly (**84**) exhibited weak activity; however, it is not clear whether the observed activity was the inherent activity of the α-glutamyl peptide or the activity of the γ-glutamyl peptide that remained after synthesis.

Almost all **35** C-terminal modified analogs comprising a proteinogenic amino acid were active, as opposed to Ile, Leu, Trp, and Tyr. However, all tripeptide C-terminal modified analogs were less active than **35**. Replacement of Gly with Gln and Cys yielded tripeptides (**60**, **61**) that were active at concentrations three orders of magnitude less than that of **35**. Incorporation of β-Ala (β-alanine) or D-amino acids, such as D-Ala and D-Ser, instead of Gly (**90**–**93**, **76**, **77**) generated inactive peptides or weak agonists. It is interesting that the replacement of Gly with an α-hydroxy acid, such as glycolic acid or L-lactic acid (**96**–**101**), afforded similar intense CaSR activities to **35**. Among the tetrapeptides, wherein another amino acid was added to the C-terminus of **35**, γ-Glu-Val-Gly-Gly (**94**) and γ-Glu-Val-Gly-Gln (**95**) exhibited weak activity.

Summary of the CaSR Activities of γ-Glutamyl Peptides With respect to the activities of several dipeptide and tripeptide analogs, the γ-glutamyl residue is mandatory for the peptide to be active, with a few exceptions. It has been indicated that the exact alignment as well as the presence of the two charged groups on the N-terminal residue are required for binding to the CaSR. A small-to-medium-sized side chain on the second residue in an L-configuration, as found in Val, Abu, Nva, α-allylglycine (Algly), *O*-(methyl)-serine (Ser(Me)), and *tert*-leucine (Tle), most likely enhanced binding to the receptor site in an active orientation. The presence of a third residue, especially one with a free carboxy terminus and no side chain (*i.e.*, glycine), significantly enhanced the activity. However, the existence of this C-terminal third residue is not essential for activity, but it is preferable. These findings were consistent with reports by Wang *et al.* and Broadhead *et al.*, in which the importance of the interaction between both N-

terminal amino acid and the C-terminal carboxyl groups of γ-glutamyl tripeptides and the active site of the CaSR was discussed.[5,6]

Figure 2 shows the putative interaction between **35** and the CaSR, where the VFD mediates **35**. The following three interactions must occur between γ-glutamyl peptides and the VFD of CaSR: γ-L-glutamyl residue with a zwitterionic binding site, which has an amino acid binding site for VFD; hydrophobic side chain of Val with a hydrophobic interaction site; and C-terminal carboxylic acid of glycine with an ionic binding site.

Brief Description of the Sensory Activity of *Kokumi* γ-Glutamyl Peptides Sensory analysis of γ-glutamyl peptides selected by screening using the CaSR activity assay was conducted. EC_{50} of **35**, **48**, and **18** in CaSR assay were 0.030–0.075, 0.052–0.055, and 0.12 μM, whereas that of **36** was 0.7 μM.

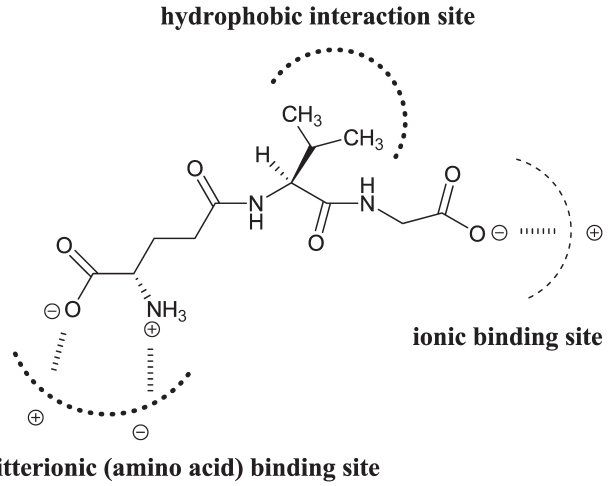

Fig. 2. Putative Interaction between γ-Glu-Val-Gly (**35**) and the CaSR

A typical profile of the sensory activity of standard *kokumi* γ-glutamyl peptide, **36**, was previously demonstrated.[7] Briefly, the peak taste intensity of basal solutions containing low concentrations of umami and salty compounds was doubled by adding 0.08% **36**. The point of subjective equivalence (PSE) of peptide **35** was determined as the concentration that was judged to produce a sensation equal to that of **36** in umami and salty taste standard solution [0.05% monosodium glutamate (MSG), 0.05% inosine monophosphate (IMP), and 0.5% sodium chloride (NaCl)] with the sensory analysis method (see Experimental). It was found that 0.01% **35** in standard solution produced the sensory activity equivalent to 0.128% **36**; therefore, we estimated that the sensory activity of *kokumi* peptide **35** is 12.8-fold greater than that of **36**.[7,32–34] Similarly, the sensory activity of Nva-containing tripeptide **48** and dipeptide **18** were approximately 200- and 100-fold greater than that of **36**, *i.e.*, 20- and 10-fold greater than that of **35**, respectively. The taste recognition threshold concentration of **48** (0.028 ppm) and **18** (0.077 ppm) were significantly lower than that of **35** (0.47 ppm) (see Experimental). Therefore, the strength of sensory activity of *kokumi* peptides **48** and **18** compared with **35** were demonstrated by two sensory analysis methods. Interestingly, although Nva is a non-proteinogenic amino acid, **48** was recently detected in human serum samples.[35]

The reason why **48** and **18** exhibited the intense sensory activity of *kokumi* γ-glutamyl peptides relative to **35** has not been fully understood yet. Quantitative analysis of the sensory activity of these peptides is currently under investigation and will be published elsewhere.

Development of a Production Procedure toward γ-Glutamyl Peptides Based on previously reported studies,[24–31] we attempted to improve upon the γ-selectivity of the reaction between Cbz-Glu anhydride (**107**) and an amino acid or peptide. Although Shiba and Imai reported that the

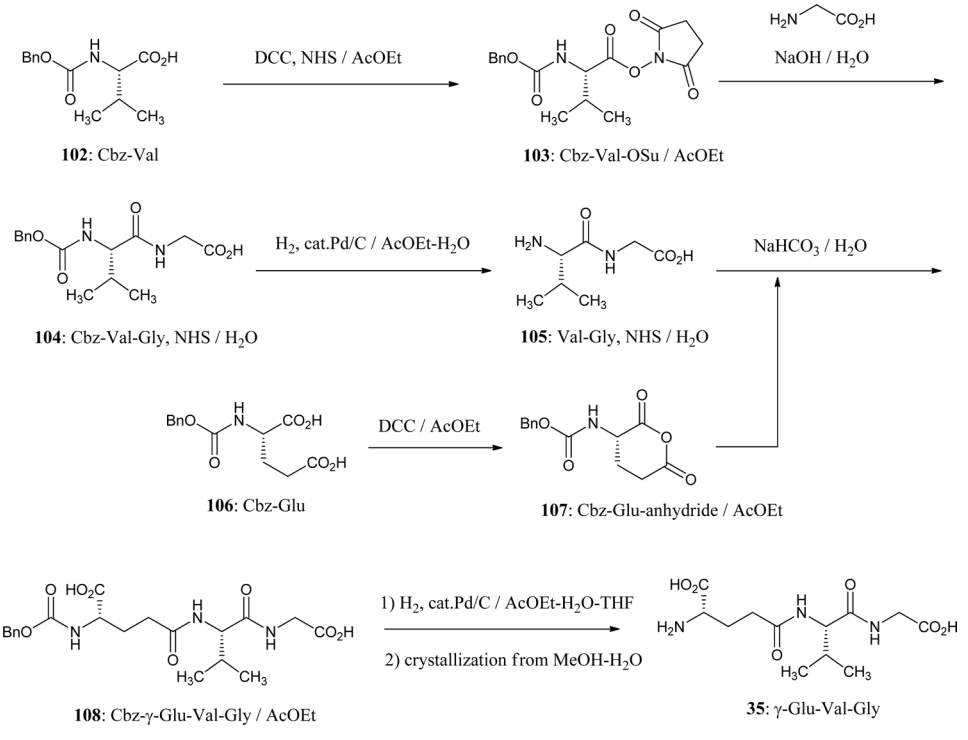

Chart 1. Large-Scale Chemical Synthesis of γ-Glu-Val-Gly (**35**)

α-isomer was predominantly obtained upon reaction of **107** and Glu,[30] we found that Cbz-γ-glutamyl peptides were predominantly obtainable by the reaction between **107** and an amino acid or peptide in the presence of 0.5–1.0 eq of NHS. For example, in the reaction between **107** and Ala in an ethyl acetate and aqueous sodium bicarbonate two layer system, the ratio of Cbz-α-Glu-Ala to Cbz-γ-Glu-Ala was 1.0 : 15.8 in the presence of 0.5 eq of NHS in 81% total yield, whereas the ratio was 1.0 : 0.7 in the absence of NHS in 68% total yield. When Val was used instead of Ala in the two layer system, the ratio of α- to γ-isomer was 1.0 : 23.7 in the presence of 1.0 eq of NHS and 1.0 : 0.7 in the absence of NHS. When reacted in a homogeneous solution such as acetone, the ratio of α- to γ-isomer was still 1.0 : 20.2 in the presence of 1.0 eq of NHS and 1.0 : 0.7 in the absence of NHS. When the dipeptide Val-Gly was used instead of Val, the ratio of α- to γ-isomer was 1.0 : 26.8 in the presence of 1.0 eq of NHS in 85% total yield after optimization of the reaction conditions.

As an example of this newly developed procedure, an outline of the synthesis of **35** on a 200 mol scale is shown in Chart 1. In this process, the NHS used in the preceding reaction was used in the subsequent reaction. Cbz-Val NHS ester (**103**) was prepared *via* condensation of Cbz-Val (**102**) and NHS with *N*,*N*′-dicyclohexylcarbodiimide (DCC). Insoluble material (*i.e.*, urea) was filtered off to give an ethyl acetate solution of **103**. An aqueous solution of the dipeptide Val-Gly (**105**) was prepared *via* the reaction of **103** and glycine in a water and ethyl acetate two layer system, followed by the deprotection of the Cbz group by hydrogenation with Pd/C in water. Cbz-Glu anhydride (**107**) was prepared by the reaction of Cbz-Glu (**106**) and DCC in ethyl acetate, followed by filtration of the generated dicyclohexylurea. To the aqueous solution of **105** containing 1 eq of each NHS used in the previous reaction and sodium bicarbonate, the solution of **107** in ethyl acetate was slowly added at room temperature to produce Cbz-α-Glu-Val-Gly and Cbz-γ-Glu-Val-Gly (**108**) in a ratio of approximately 3 : 97. Deprotection of the Cbz group *via* hydrogenation with Pd/C was performed in the water and ethyl acetate two layer system, followed by recrystallization from a mixed solvent system of water and methanol to afford **35** in approximately 54% overall yield ($\alpha/\gamma<1/99$).

The reason for this reversal of selectivity of the reaction in the presence or absence of NHS is unclear; however, the γ-selectivity can partially be explained by the steric influence of the Cbz group on both carbonyls. In glutamic anhydride modified with a large carbobenzoxy group, nucleophilic addition at the γ-position is preferred. Replacement of the intramolecular hydrogen bond existing between the hydrogen on the α-amino nitrogen and the oxygen of the α-carbonyl, which activates the electrophilicity of the α-carbonyl, with an intermolecular hydrogen bond between the hydrogen on the α-amino nitrogen and the oxygen of NHS is also a possible explanation, as discussed by Huang *et al.* regarding the role of dimethylsulfoxide in γ-selectivity.[24] Another possible explanation is activation of the less hindered γ-carboxyl group upon formation of the NHS γ-ester, which is the short-lived intermediate of the reaction of **107** and NHS. Details on this regiospecific synthesis will be reported in the near future.

Conclusion

The present study on the structure–CaSR–activity relation of a large number of γ-glutamyl peptides demonstrated the following structural requirements for the intense CaSR activity of γ-glutamyl peptides: existence of an N-terminal γ-L-glutamyl residue; existence of a moderately sized, aliphatic, neutral substituent at the second residue with an L-configuration; and existence of a C-terminal carboxylic acid, preferably with the existence of glycine as the third constituent. By the sensory analysis of γ-glutamyl peptides selected by screening using the CaSR activity assay, it was found that **35** and Nva-containing γ-glutamyl peptides, **48** and **18**, possessed excellent sensory activity of *kokumi* substances. A practical industrial synthesis of regiospecific γ-glutamyl peptides **35** was also developed for their commercialization. This was achieved through the regiospecific ring opening reaction of Cbz-Glu anhydride (**107**) and an amino acid or peptide in the presence of 0.5–1.0 eq of *N*-hydroxysuccinimide.

Experimental

Materials Chemicals for peptide synthesis were obtained from either Bachem AG (Switzerland), Watanabe Chemical Industries, Ltd. (Japan), or Kokusan Chemical Co., Ltd. (Japan). Commercially available peptide samples were purchased from Sigma-Aldrich (U.S.A.), Dojin Chemical Laboratory (Japan), Bachem AG, and Kokusan Chemical (Japan) and used as received. Dipeptide samples and tripeptides comprising proteinogenic amino acids were a contract manufactured product (Kokusan Chemical Co., Ltd. and Peptide Institute, Inc., Japan). Other peptides containing non-proteinogenic amino acids and tetrapeptides were synthesized in our laboratories using liquid phase synthesis and characterized by ^{1}H-NMR spectroscopy (Bruker AVANCE400 (400 MHz)) and electrospray ionization (ESI)-MS (Thermo Quest TSQ700).

Peptides **35**, **48**, and **18** as well as other peptides used for the sensory evaluation test were synthesized in our laboratories. Glutamine- and cysteine-containing peptides were prepared immediately before use, while other peptides were stored at −20°C after preparation. Peptides having a purity of 95% or higher were used for evaluation of the CaSR activation activity, while those having a purity of 98% or higher were used for the sensory evaluation test. However, for **1**, a purity of 80% or higher was used. When the solution in which each sample was dissolved showed an acidic or alkaline pH, the solution was adjusted to approximately neutral using a sodium hydroxide solution or hydrochloric acid solution.

Liquid Phase Synthesis of γ-L-Glutamyl-L-valylglycine (35, γ-Glu-Val-Gly) *N*-α-*tert*-Butoxycarbonyl-L-valine (Boc-Val-OH, 8.69 g, 40.0 mmol) and glycine benzyl ester hydrochloride (Gly-OBzl·HCl, 8.07 g, 40.0 mmol) were dissolved in methylene chloride (100 mL) and the solution was kept at 0°C. Triethylamine (6.13 mL, 44.0 mmol), 1-hydroxybenzotriazole (HOBt, 6.74 g, 44.0 mmol), and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (WSC·HCl, 8.44 g, 44.0 mmol) were added to the solution and the mixture was stirred overnight at room temperature. The reaction mixture was concentrated under reduced pressure and the resulting residue was dissolved in ethyl acetate (200 mL). The solution was washed with water (50 mL), 5% aqueous citric acid solution (50 mL×twice), saturated brine (50 mL), 5% aqueous sodium bicarbonate solution (50 mL×twice), and saturated brine (50 mL). The organic layer was dried over anhydrous magnesium sulfate, filtered, and the filtrate was concentrated

under reduced pressure. The residue was recrystallized from ethyl acetate/*n*-hexane to obtain Boc-Val-Gly-OBzl (13.2 g, 36.2 mmol) as white crystals.

Boc-Val-Gly-OBzl (5.47 g, 15.0 mmol) was added to a 4 N HCl/dioxane solution (40 mL) and the mixture was stirred at room temperature for 50 min. Dioxane was removed by concentration under reduced pressure, *n*-hexane (30 mL) was added to the residue, and the mixture was concentrated under reduced pressure. This procedure was repeated thrice to quantitatively obtain H-Val-Gly-OBzl·HCl.

H-Val-Gly-OBzl·HCl (15.0 mmol) and *N*-*α*-carbobenzoxy-L-glutamic acid *α*-benzyl ester (Cbz-Glu-OBzl, 5.57 g, 15.0 mmol) were dissolved in methylene chloride (50 mL) and the solution was kept at 0°C. Triethylamine (2.30 mL, 16.5 mmol), HOBt (2.53 g, 16.5 mmol), and WSC·HCl (3.16 g, 16.5 mmol) were added to the solution and the mixture was stirred at room temperature for 2 d. The reaction mixture was concentrated under reduced pressure and the resulting residue was dissolved in heated ethyl acetate (1500 mL). The solution was washed with water (200 mL), 5% aqueous citric acid solution (200 mL×twice), saturated brine (150 mL), 5% aqueous sodium bicarbonate solution (200 mL×twice), and saturated brine (150 mL). The organic layer was dried over anhydrous magnesium sulfate, filtered, and the filtrate was concentrated under reduced pressure. The deposited crystals were collected by filtration and dried under reduced pressure to obtain Cbz-Glu(Val-Gly-OBzl)-OBzl (6.51 g, 10.5 mmol) as white crystals.

Cbz-Glu(Val-Gly-OBzl)-OBzl (6.20 g, 10.03 mmol) was suspended in ethanol (200 mL), to which 10% palladium/carbon (1.50 g) was added. The reduction reaction was performed at 55°C for 5 h under a hydrogen atmosphere. During the reaction, a total of 100 mL of water was gradually added. The catalyst was removed by filtration using a Kiriyama funnel and the filtrate was concentrated under reduced pressure to half volume. The reaction mixture was further filtered through a membrane filter and the filtrate was concentrated under reduced pressure. The resulting residue was dissolved in a small volume of water and ethanol was added to deposit the crystals, which were then collected by filtration and dried under reduced pressure to obtain **35** (2.85 g, 9.40 mmol) as a white powder.

^{1}H-NMR (D_2O) δ: 0.87 (3H, d, J=6.8 Hz), 0.88 (3H, d, J=6.8 Hz), 1.99–2.09 (3H, m), 2.38–2.51 (2H, m), 3.72 (1H, t, J=6.35 Hz), 3.86 (1H, d, J=17.8 Hz), 3.90 (1H, d, J=17.8 Hz), 4.07 (1H, d, J=6.8 Hz). ESI-MS m/z: 304.1 $(M+H)^+$.

γ-L-Glutamyl-L-α-aminobutyrylglycine (45, γ-Glu-Abu-Gly) ^{1}H-NMR (D_2O) δ: 0.89 (3H, t, J=7.48 Hz), 1.63–1.69 (1H, m), 1.73–1.79 (1H, m), 2.04–2.10 (2H, m), 2.41–2.47 (2H, m), 3.74 (1H, t, J=6.36 Hz), 3.87 (1H, d, J=17.8 Hz), 3.90 (1H, d, J=17.8 Hz), 4.14 (1H, dd, J=5.66 and 8.34 Hz). ESI-MS m/z: 290.10 $(M+H)^+$.

γ-L-Glutamyl-L-*tert*-leucylglycine (49, γ-Glu-Tle-Gly) ^{1}H-NMR (D_2O) δ: 0.95 (9H, s), 2.04–2.08 (2H, m), 2.45–2.48 (2H, m), 3.73 (1H, t), 3.87–3.90 (2H, m), 4.07 (1H, s). ESI-MS m/z: 318.10 $(M+H)^+$.

γ-L-Glutamyl-L-α-aminobutyryl-glycolic Acid (97, γ-Glu-Abu-Gly Acid) In the first step of this synthesis, condensation of Boc-Abu and glycolic acid benzyl ester was carried out with WSC·HCl (1.1 equiv.) and 4-dimethylaminopyridine (0.3 equiv.).

^{1}H-NMR (D_2O) δ: 0.86 (3H, t, J=7.40 Hz), 1.60–1.74 (1H, m), 1.82–1.88 (1H, m), 2.04–2.12 (2H, m), 2.45 (2H, t, J=7.40 Hz), 3.79 (1H, t, J=6.36 Hz), 4.31–4.45 (1H, m), 4.57 (2H, s). ESI-MS m/z: 291.10 $(M+H)^+$.

Large-Scale Synthesis of γ-L-Glutamyl-L-valylglycine (35)

[Step 1]

N-Hydroxysuccinimide (NHS; 60.1 kg, 522 mol, 1.05 equiv.) and *N*,*N*′-dicyclohexylcabodiimide (DCC (107.8 kg, 522 mol, 1.05 equiv) in ethyl acetate (147 L) were added to Cbz-Val (**102**, 125 kg, 498 mol) in ethyl acetate (675 L) and the mixture was stirred under ambient conditions. After completion of the reaction, insoluble solids (dicyclohexylurea) were removed by filtration to afford Cbz-Val *N*-hydroxysuccinimide ester (**103**) in ethyl acetate. The resultant solution was added to glycine (39.2 kg, 522 mol, 1.05 equiv) in water (225 L). The mixture was stirred at 10°C while maintaining slightly basic conditions by adding an aqueous sodium hydroxide solution (25%). The water layer was then separated to afford Cbz-Val-Gly (**104**) in water. Ethyl acetate (375 L) was added to the **104** solution and the Cbz group was deprotected *via* hydrogenation in the presence of a palladium catalyst (10% Pd/C, 1.3 kg). After removing the palladium catalyst by filtration, the water phase was separated to afford a solution of Val-Glu (**105**).

[Step 2]

DCC (112.9 kg, 547 mol, 1.10 equiv.) in ethyl acetate (153 L) was added to Cbz-Glu (**106**, 146.9 kg, 522 mol, 1.05 equiv.) in ethyl acetate (514 L) at 10°C. After stirring the solution under ambient conditions overnight, the resultant solids (dicyclohexylurea) were removed by filtration to afford Cbz-Glu anhydride (**107**) in ethyl acetate. The resultant solution was added to the **105** solution obtained in step 1 in the presence of sodium bicarbonate (43.9 kg, 522 mol, 1.05 equiv.). After completion of the reaction, tetrahydrofuran (625 L) was added and the pH of the water layer was acidified with concentrated hydrochloric acid. Peptide **108** was then extracted into the organic layer (ethyl acetate and tetrahydrofuran) and washed with 10% aqueous sodium chloride solution (625 L) and 3% aqueous sodium chloride solution (625 L×twice). Water (537 L) was then added to the solution, and the Cbz group was deprotected *via* hydrogenation in the presence of a palladium catalyst (10% Pd/C, 2.1 kg). After removing the palladium catalyst, the water layer was separated and concentrated. Addition of methanol (1687 L) to the residue afforded **35** as crystals, which were collected and dried (86 kg, 283 mol, α/γ <1/99).

^{1}H-NMR (D_2O) δ: 0.88 (3H, d, J=6.8 Hz), 0.89 (3H, d, J=6.8 Hz), 1.99–2.10 (3H, m), 2.40–2.50 (2H, m), 3.72 (1H, t, J=6.4 Hz), 3.86 (1H, d, J=17.8 Hz), 3.91 (1H, d, J=17.8 Hz), 4.08 (1H, d, J=6.8 Hz). ^{13}C-NMR (D_2O) δ: 17.7, 18.7, 26.5, 31.5, 41.7, 54.2, 60.0, 173.9, 174.0, 174.5, 175.3. IR (KBr) cm^{-1}: 3321 (N–H), 3282 (N–H), 2940 (C–H), 1712 (C=O), 1654 (C(C=O)–O), 1919 (amide I band), 1541 (amide II band), 1238 (C–C(C=O)–O). ESI-MS m/z: 304.2 $(M+H)^+$. FAB-MS m/z: 304.1505 (M+H) (Calcd for $C_{12}H_{22}N_3O_6$: 304.1509). mp: 225–228°C. $[\alpha]_D^{20}$ −29 (c=1.0, H_2O).

Synthesis of γ-L-Glutamyl-L-norvalylglycine (48) γ-L-Glu-L-Nva-Gly was prepared in the same manner as γ-L-Glu-L-Val-Gly.

^{1}H-NMR (D_2O) δ: 0.81 (3H, t, J=7.4 Hz), 1.22–1.38 (2H, m), 1.55–1.73 (2H, m), 2.02–2.10 (2H, m), 2.35–2.48 (2H, m) 3.72 (1H, t, J=6.2 Hz), 3.84 (1H, d, J=17.6 Hz), 3.89 (1H, d, J=18.0 Hz), 4.20 (1H, dd, J=5.2 and 5.6 Hz). ^{13}C-NMR (D_2O)

δ: 12.7, 18.4, 26.0, 31.1, 33.0, 41.3, 53.7, 53.8, 173.5, 174.8, 175.0. IR (KBr) cm^{-1}: 3376 (N–H), 3350 (N–H), 2940 (C–H), 1679 (C=O), 1642 (amide I band), 1515 (amide II band). ESI-MS *m/z*: 304.1 $(M+H)^+$. FAB-MS *m/z*: 304.1512 (M+H) (Calcd for $C_{12}H_{22}N_3O_6$: 304.1509). mp: 191–192°C. $[\alpha]_D^{20}$ −25 to −24 (*c*=1.0, H_2O).

Synthesis of γ-L-Glutamyl-L-norvaline (18) γ-L-Glu-L-Nva was prepared in the same manner as γ-L-Glu-L-Val-Gly, with the exception that the condensation was carried out once. ^{1}H-NMR (D_2O) δ: 0.81 (3H, t, *J*=7.4 Hz), 1.22–1.35 (2H, m), 1.57–1.76 (2H, m), 2.03–2.09 (2H, m), 2.38–2.43 (2H, m) 3.74 (1H, t, *J*=6.4 Hz), 4.20 (1H, dd, *J*=5.2 and 5.2 Hz). ^{13}C-NMR (D_2O) δ: 12.7, 18.4, 26.2, 31.2, 32.6, 53.2, 53.8, 173.5, 174.6, 176.8. IR (KBr) cm^{-1}: 3310 (N–H), 2961 (C–H), 1722 (C=O), 1653 (amide I band), 1633 (C(C=O)–O), 1529 (amide II band), 1211 (C–C(C=O)–O). ESI-MS *m/z*: 247.1 $(M+H)^+$. FAB-MS *m/z*: 247.1298 (M+H) (Calcd for $C_{10}H_{19}N_2O_5$: 247.1294). mp: 197–199°C. $[\alpha]_D^{20}$ −14 to −13 (*c*=1.0, H_2O).

Preparation of cRNA The preparation of human cDNA was described previously.[7] cRNA of the hCaSR was prepared from human kidney cDNA (Clontech) using a PCR method as follows. Primer oligonucleotide DNAs (forward primer (N: 5′-ACT AAT ACG ACT CAC TAT AGG GAC CAT GGC ATT TTA TAG CTG CTG CTG G-3′) and reverse primer (C: 5′-TTA TGA ATT CAC TAC GTT TTC TGT AAC AG-3′); National Center for Biotechnology Information (NCBI) accession number NM_000388) were synthesized and PCR was performed using *PfuUltra* DNA Polymerase (Stratagene, U.S.A.) under the following conditions. After reacting at 94°C for 3 min, a cycle of reactions at 94°C for 30 s, 55°C for 30 s, and 72°C for 2 min was repeated 35 times, followed by reaction at 72°C for 7 min. The plasmid vector pBR322 (TaKaRa, Japan) was digested with the restriction enzyme EcoRV. The PCR product was ligated to the EcoRV cleavage site of pBR322 using a ligation kit (Promega, U.S.A.). hCaSR cRNA was synthesized using a cRNA preparation kit (Ambion, U.S.A.) with this sequence as a template.

Determination of CaSR Agonist Activity Using Oocytes CaSR agonist-induced currents were characterized using oocytes microinjected with hCaSR cRNA as follows.[7] *Xenopus laevis* ovarian lobes were surgically removed, defolliculated, and treated with collagenase II. Oocytes were then microinjected with 10–20 ng of hCaSR cRNA and incubated for 36–48 h at 15°C in Barth's solution. Activation of the CaSR (G_q class GPCR) expressed in oocytes leads to an increase in intercellular calcium ions, which concomitantly activates oocyte endogenous calcium-dependent chloride channels with a measurable current. The oocytes were impaled by two electrodes in a voltage clamp configuration with a Geneclamp 500 (Axon) and responses were recorded using AxoScope 9.0 recording software (Axon) at a membrane potential of −70 mV. The oocytes were challenged with a 0.1–1000 μM solution of CaSR agonists in a perfusion buffer containing 96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, and 5 mM 4-(2-hydroxyethyl)-1-piperazineethansulfonic acid (HEPES) buffer (pH 7.2). The recorded peak current was deemed to be the strength of receptor activation. Un-injected oocytes did not respond to calcium or the tested CaSR agonists.

Determination of CaSR Agonist Activity Using HEK-293 Cells hCaSR cDNA was constructed in an expression vector, pcDNA3.1, and transiently transfected into HEK-293 cells as follows.[7] The cDNA was diluted with Opti-MEM medium (Invitrogen, U.S.A.), mixed with FuGENE6 (Roche Applied Science, Switzerland), and poured onto HEK-293 cells grown at a submaximal concentration. After 24 h of culture in a 96-well plate, the cells were incubated with 5 μM Calcium-4 (Calcium-4 Assay Kit, Molecular Devices, U.S.A.) for 45–60 min and measurements were conducted using a calcium-image analyzer (FlexStation, Molecular Devices) with the associated software. Activation of CaSR expressed in HEK-293 cells leads to an increase in intercellular calcium ions, which was determined using Calcium-4, a calcium dye. This dye binds free Ca^{2+}, resulting in an increase in dye fluorescence, which is excited at 485 nm and emits at 525 nm. The concentration dependence of the fluorescence intensity was analyzed with various CaSR agonists. The assay buffer for calcium imaging contained 0.75 mM $CaCl_2$.

Evaluation of the Sensory Activity of *Kokumi* γ-Glutamyl Peptides Quantitative analyses of the sensory activity of *kokumi* peptides with standard **36**: A quantitative evaluation of the human sensory analyses was performed by a panel of 17 well trained assessors who judged the PSE between sample and reference solution of **36** in umami and salty taste standard solution. Test samples of **35** (0.01%) were mixed with umami and salty taste standard solutions (0.05% MSG, 0.05% IMP, and 0.5% NaCl). A series of **36** concentrations were chosen in logarithmically equal steps at 50% intervals (0.02, 0.03, 0.044, 0.07, 0.10, 0.15, 0.23, 0.34% (w/v)). Each test sample was paired twice with the reference **36** solution, and the assessors were required to rate the sensation produced by a test sample as either more or less intense than that of reference solution 5 s after tasting. The PSE was defined as the concentration that was judged to produce a sensation equal to that of the standard solution. The sample concentration was determined by a preliminary test. Data were analyzed by the probit method.

Determination of taste recognition threshold concentrations of *kokumi* peptides: Taste recognition threshold concentrations for sensory activity of *kokumi* peptides were determined as follows. The minimum peptide concentration at which the assessors recognize the increase of taste intensity compared with umami and salty taste standard solution without peptides was defined as the recognition threshold concentration in this study. Seven concentration sample solution were prepared for each peptide in an umami and salty taste standard solution: **35**: 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, and 5.0 ppm; **48**: 0.002, 0.005, 0.001, 0.02, 0.05, 0.1, and 0.2 ppm; and **18**: 0.005, 0.01, 0.02, 0.05, 0.1, 0.2, and 0.5 ppm. The tasting concentration level was determined in a preliminary sensory experiment. Five well trained assessors tasted these samples in these order and in the reverse order and compared them with a standard solution without peptides. The threshold value of the assessors was approximated by averaging the threshold values of individuals in three independent sessions.

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Conflict of Interest All authors were employees of Ajinomoto Co., Inc. when this study was conducted and have no further conflicts of interest to declare.

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