JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Tastes and Structures of Bitter Peptide, Asparagine-Alanine-Leucine-Proline-Glutamate, and Its Synthetic Analogues

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Asn-Ala-Leu-Pro-Glu (NALPE) is a strong bitter peptide with a minimum response threshold (MRT) of 0.074 mM. To elucidate the relationship of spatial structure and bitterness on peptides, NALPE and its analogues, NALPW, NALPS, NALPL, NALPP, NALPD, and NALPR, were synthesized and sensorially evaluated. Structural analysis using computer simulation for each peptide revealed that the presence of a polar group and hydrophobic bitter amino acids, the composition of hydrophobic regions, the spatial orientation of the polar group and hydrophobic regions, and the proximity between polar groups and hydrophobic regions faced within the same plane space may be the major determinants for the taste type and intensity of peptide bitterness.

KEYWORDS: Bitterness; bitter peptide; NALPE; three-dimensional structures; computer simulation

INTRODUCTION

Bitter peptides are frequently generated during enzymatic processes that produce functional, bioactive protein hydrolysates or during the aging process of fermented products such as cheese, soybean protein, and wine. Isolation of bitter peptides from food protein hydrolysate has been widely studied for a long time, especially for the identification of bitter peptides in casein. For example, BPI (Gly-Pro-Phe-Pro-Val-Ile), BPII (Phe-Phe-Val-Ala-Pro-Phe-Pro-Glu-Val-Phe-Gly-Lys), and BPIII (Phe-Ala-Leu-Pro-Gln-Tyr-Leu-Lys) are well-known bitter peptides purified from casein hydrolysate (*1*). Fujimaki et al. (*2*) and Kukman et al. (*3*) isolated bitter peptides from the hydrolysate of whole soybean protein. Recently, we isolated a total of 28 peptides from the bitter hydrolysates of ^{11}S glycinin, one subunit of soybean, and proglycinin, one subunit of ^{11}S glycinin using gel permeation HPLC and three series of C_{18} RP-HPLC (*4, 5*).

According to Ney's Q-rule (*6*), peptides with an average hydrophobicity value >1400 cal/mol are bitter, and those with an average hydrophobicity value <1300 cal/mol are not bitter. Most of the bitter peptides from casein hydrolysate are known as having strong hydrophobicities. However, most of the bitter peptides isolated from soybean glycinin have a hydrophobicity much less than 1400 cal/mol, although many hydrophobic amino acid residues were contained in almost every analyzed peptide (~40%). These peptides possessed a relatively large amount of glutamine, glycine, asparagine, glutamate, and proline (*5*). Although the Q-rule generally is accepted by researchers, the accuracy of the Q-rule is sometimes thought to be limited because steric parameters and the spatial structure, which are not reflected in the average hydrophobicity, are important for the intensity of bitter taste.

The relationship between the bitterness potency and the chemical structure of peptides has been studied extensively (*4, 5, 7–12*). These studies reported that hydrophobicity, primary sequence, spatial structure, peptide length, and bulkiness of the molecule are important for bitter taste perception. Ishibashi et al. (*8*) and Tamura et al. (*13*) suggested that bifunctional units, namely, a bulky basic or hydrophobic group as the stimulating unit and a hydrophobic group as the binding unit, are necessary participants in the mechanism of bitter taste perception of peptides (*8, 12*). The adjacency of these two sites in the steric conformation of peptides is essential (*9*), and the steric distance between the two sites was estimated as 4.1 Å (*8*) with a pocket size of 15 Å (*11*). On the basis of these studies, they proposed bitter receptor models. Recently, taste receptors were identified, and taste signaling in the cell was explained by molecular biological approaches (*14–20*). Bitter receptors, T2Rs, consist of more than 30 different G protein coupled receptor (GPCR) family members that function as heteromeric

Table 1. Hydrophobicities and Bitterness Comparison of Synthetic Peptides

synthetic peptide	concn (mg/mL)	hydrophobicity (cal/mol)	bitter intensity[a]
SDNF	0.0625	550	0
SAEFG	0.1868	540	1
EQGGEQG	0.1380	0	0
NALPE	0.1000	980	3

[a] Bitter intensities were rated as follows: not bitter (0), slightly (1), distinctly (2), moderately (3), very (4), or extremely (5). These ratings were evenly distributed on a line, and each degree represents a quinine-HCl concentration of 1.6, 2.4, 3.2, 4.0, 4.8, and 5.6 × 10^{-5} M, respectively.

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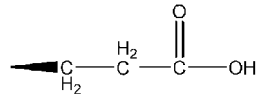
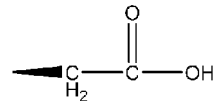
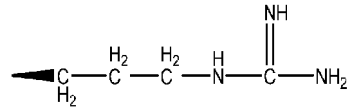
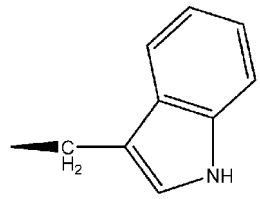
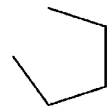
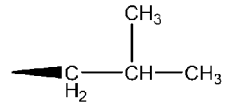
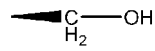
10.1021/jf7036664 CCC: $40.75
Published on Web 06/25/2008

Table 2. Characteristics and Chemical Structures of C Terminus of Synthetic NALP Analogue Peptides

Synthetic peptide	Characteristic of C-terminal amino acid	C-terminal R-group	M.W. (Da)	Hydrophobicity (cal/mol)	Taste description
NALPE	Separated bitter peptide		542	980	Distinctly bitter
NALPD	Acidic		528	980	sour/ astringent
NALPR	Basic		569	1130	Bitter/ astringent
NALPW	Bulky		599	1660	bitter/ tickling
NALPP	Proline		510	1500	sour/ bitter
NALPL	Hydrophobic		526	1340	Strongly bitter
NALPS	Hydrophilic		500	920	Slightly bitter

receptors to accommodate the great chemical diversity of bitter tastes (*21, 22*). Generally, two steps are involved in sensing the taste of a molecule (*23*). In the first step, the molecule must move to the receptor site, which is related to its solubility and hydrophobicity. In the second step, the molecule may interact with a receptor, depending on the reaction group within that molecule and its stereoalignment. If the molecule and receptor site interact, signal transduction will begin (*24*). In this process, taste ligands must conform to the special three-dimensional structures to interact with the taste receptors. The structures of taste receptors have not been identified, but it is clear that the taste ligands must assume specific three-dimensional structures when interacting with the taste receptor. However, structural information for individual bitter peptides rarely was available with a few exceptions until now.

In this study, therefore, to explore the structural information for bitter peptides purified from ^{11}S soybean glycinin, we synthesized various analogue peptides of a model bitter peptide with modification at the C-terminus and evaluated the tastes of the analogue peptides. We then explained the relationship between peptide structure and bitterness employing computer simulation. This study indicates the significance that the spatial structure of a peptide has in bitter taste perception.

MATERIALS AND METHODS

Peptide Synthesis. The peptides, Asn-Ala-Leu-Pro-Glu (NALPE), Ser-Asp-Asn-Phe (SDNF), Ser-Ala-Glu-Phe-Gly (SAEFG), and Glu-Gln-Gly-Gly-Glu-Gln-Gly (EQGGEQG), and the analogues, Asn-Ala-Leu-Pro-Glu-Asp (NALPD), Asn-Ala-Leu-Pro-Arg (NALPR), Asn-Ala-Leu-Pro-Thr (NALPW), Asn-Ala-Leu-Pro-Pro (NALPP), Asn-Ala-Leu-Pro-Leu (NALPL), and Asn-Ala-Leu-Pro-Ser (NALPS) were chemically synthesized and chromatographically purified, and their molecular weight was confirmed by mass spectrometry, which was obtained from Sawady Technology, Co. (Tokyo, Japan). The synthesized peptides were then injected individually into an analytical TSK ODS 80 column (4.6 mm × 150 mm, 5 μm, Tosho, Tokyo, Japan) as part of a Waters Millenium PDA HPLC system. Solution A was 0.1% HCl in 1% EtOH, and solution B was 0.1% HCl in 90% EtOH. A linear gradient of 0−50% solvent B at a flow rate of 0.7 mL/min was developed over 45 min. An accelerated gradient from 50 to 80% solvent B was completed in another 5 min. The mobile phase finally returned to 100% solvent A for 10 min. The separation profile was monitored at 214 nm. In this purification system, the mobile phase, consisting of HPLC grade water containing aqueous ethanol and hydrochloric acid, was treated according to the method of Lee and Warthesen (*25*) to use the eluent for the subsequent sensory evaluation. After lyophilization and reconstitution with water, the aliquots could be used for sensory evaluation without interference from solvents or problems of potentially hazardous solvents.

Table 3. Threshold Values and Structural Similarity of Synthetic NALPE Analogue Peptides

synthetic peptide	sensorial description	bitterness MRT (mM)	rmsd (nM)	size (Å^3)
NALPE	distinctly bitter	0.074		549.30
NALPL	strongly bitter	0.149	0.27	525.29
NALPW	bitter/tickling	0.105	0.36	520.36
NALPS	slightly bitter	0.250	2.02	506.62
NALPR	bitter/astringent	0.420	2.30	573.09
NALPP	sour/bitter		1.60	542.72
NALPD	sour/astringent		1.92	595.16

Concentrations of Peptides. The concentrations of synthesized peptides were measured by a modified TNBS method (*26*). The absorption coefficient, ϵ, of asparagine at the N-terminus of peptides was 22 000.

Hydrophobicity. The hydrophobicity of peptides was calculated according to Bigelow and Channon's method (*27*) on the basis of the change in free energy, Δf, for the transfer of 1 mol of amino acid from ethanol to water, which was proposed by Tanford in 1962 (*28*).

Sensory Evaluation. The concentrations of synthetic peptides, SDNF, SAEFG, EQGGEQG, and NALPE, were normalized with a similar concentration. For sensory evaluation of bitter peptides, 150 μL samples were spotted directly on the rear of the extended tongue since the sample volume was very small (*29, 30*). A sensory panel composed of four volunteers from Korea University compared the bitterness of each sample to a diluted quinine-HCl solution by a line-scaling test. Before evaluating the bitterness, the panelists were trained with standard quinine-HCl solutions at several different concentrations near a threshold quinine-HCl concentration of 2.4×10^{-5} M. The panelists rinsed their mouths thoroughly with water before testing and kept the samples in their mouths for 30 s. A rating sheet was provided to evaluate the bitterness of the samples. The intensity of bitterness was rated as follows: not bitter (0), slightly (1), distinctly (2), moderately (3), very (4), and extremely (5). These ratings were evenly distributed on a line, and each degree represented a quinine-HCl concentration of 1.6, 2.4, 3.2, 4.0, 4.8, and 5.6×10^{-5} M, respectively.

For taste descriptions of synthetic NALPE analogue peptide, samples also were prepared at 0.05 mg/mL. Panels composed of six volunteers from Korea University were trained for sweet, bitter, sour, and salty tastes with sugar, quinine-HCl, acetic acid, and sodium chloride solutions, respectively, each near the threshold concentration.

To determine the minimum response threshold for bitterness of the synthetic NALPE analogue peptides, the three-drop method of Weiffenbach et al. (*30*) with modifications was applied against four sensorial panels. Sample solutions were serially diluted to the following concentrations in doubly distilled water: 0.03125, 0.0625, 0.125, 0.25, and 1 mM. Sample solutions were dispensed as single 150 μL drops from a dropper. For each trial, a series of three drops was placed on the rear of the extended tongue. One drop contained taste fluid; the other two drops were doubly distilled water. Panelists then reported as to which drop tasted different than the other two, guessing when uncertain. The geometric mean of the recognized concentrations was calculated, which was defined as the best estimate threshold value (BET). The geometric mean of BETs was calculated as the minimal response threshold value (MRT).

Computer Simulation of Three-Dimensional Structures of Synthetic Peptides. Spatial structures of the synthetic peptides were analyzed using a computer simulation by the Discovery program, Insight II (Accelrys Software, Inc., San Diego, CA) and ChemBio3D Ultra 11.0 (CambridgeSoft Co.). The internal bonding energies of the peptide structures were stabilized and minimized in a vacuum system at 398 K. Finally, the peptide structures were simulated by minimizing the energies of the peptides within a 30 Å^3 water box at 298 K. The sizes of the peptides were calculated in units of cubic angstroms. The similarity of peptide structures was estimated as the root-mean-square deviation (rmsd). The rmsd was calculated as the average distance between α-carbons in the backbones of superimposed proteins. NALPE was used as the backbone structure.

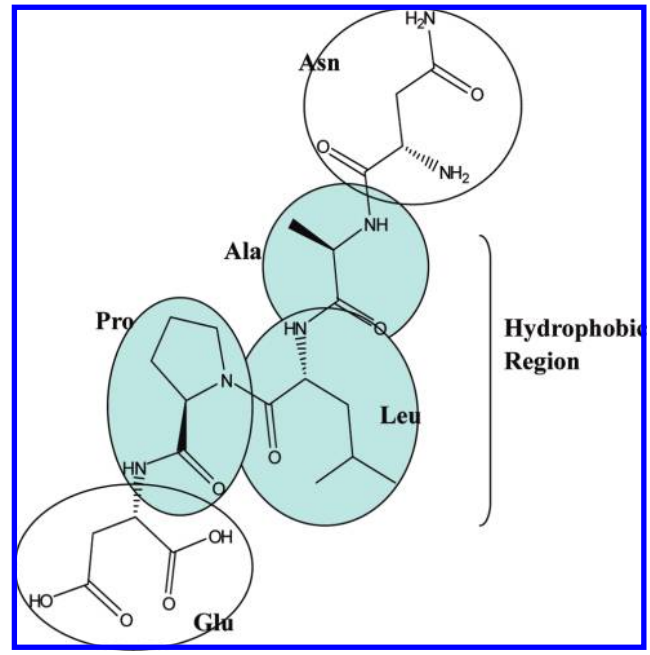


Figure 1. Chemical structures of NALPE, a bitter peptide isolated from soybean ^{11}S glycinin.

RESULTS

Effect of Hydrophobicity on Bitterness of Peptides. In previous studies (*4, 5*), numerous predicted bitter peptides were purified from several bitter fractions of soybean ^{11}S glycinin and proglycinin hydrolysates. To elucidate the limitation of hydrophobicity on the bitterness of pepetides, several bitter peptides with a hydrophobicity <1400 cal/mol were selected and chemically synthesized: Ser-Asp-Apn-Phe (SDNF), Ser-Ala-Glu-Phe-Gly (SAEFG), Glu-Gln-Gly-Gly-Glu-Gln-Gly (EQGGEQG), and Asn-Ala-Leu-Pro-Glu (NALPE). As shown in **Table 1**, although SDNF, SAEFG, and EQGGEQG were peptides purified from bitter fractions of soybean hydrolysates, they showed a "slightly bitter" characterization corresponding to a 2.4 mM quinine-HCl equivalent for SAEFG or "not bitter" for SDNF and EQGGEQG. However, NALPE with a hydrophobicity <1400 cal/mol was evaluated as "very bitter," corresponding to a 4.0 mM quinine-HCl equivalent. Even if all peptides with a hydrophobicity <1400 cal/mol did not display bitterness, some peptides with a hydrophobicity <1400 cal/mol showed bitterness. On the basis of these results, it is apparent that the hydrophobicity of a peptide is not a main factor for peptide bitterness.

Characteristics of Synthetic NALPE Analogue Peptides. Among synthetic peptides, NALPE was confirmed as a bitter peptide and was therefore further studied as the model bitter peptide. To investigate the structural characteristics of peptides that elicit bitterness, the structure of NALPE was modified with various amino acids at the C-terminus of the peptide. The modified amino acids at the C-terminus of the peptide were categorized by the respective properties of the residual side chain: hydrophobic (Leu, L), hydrophilic (Ser, S), basic (Arg, R), acidic (Asp, D), folding (Pro, P), and bulky (Trp, W). The characteristics and residual structure of C-terminal amino acids, molecular weights, hydrophobicities, and taste descriptions of the synthetic NALPE analogue peptides are shown in **Table 2**.

The molecular weights of the synthetic NALPE analogue peptides ranged from 500 to 600 Da. The hydrophobicities of synthetic peptides with bulky and folding residues were 1340−1660 cal/mol (*5*). To determine changes in taste that

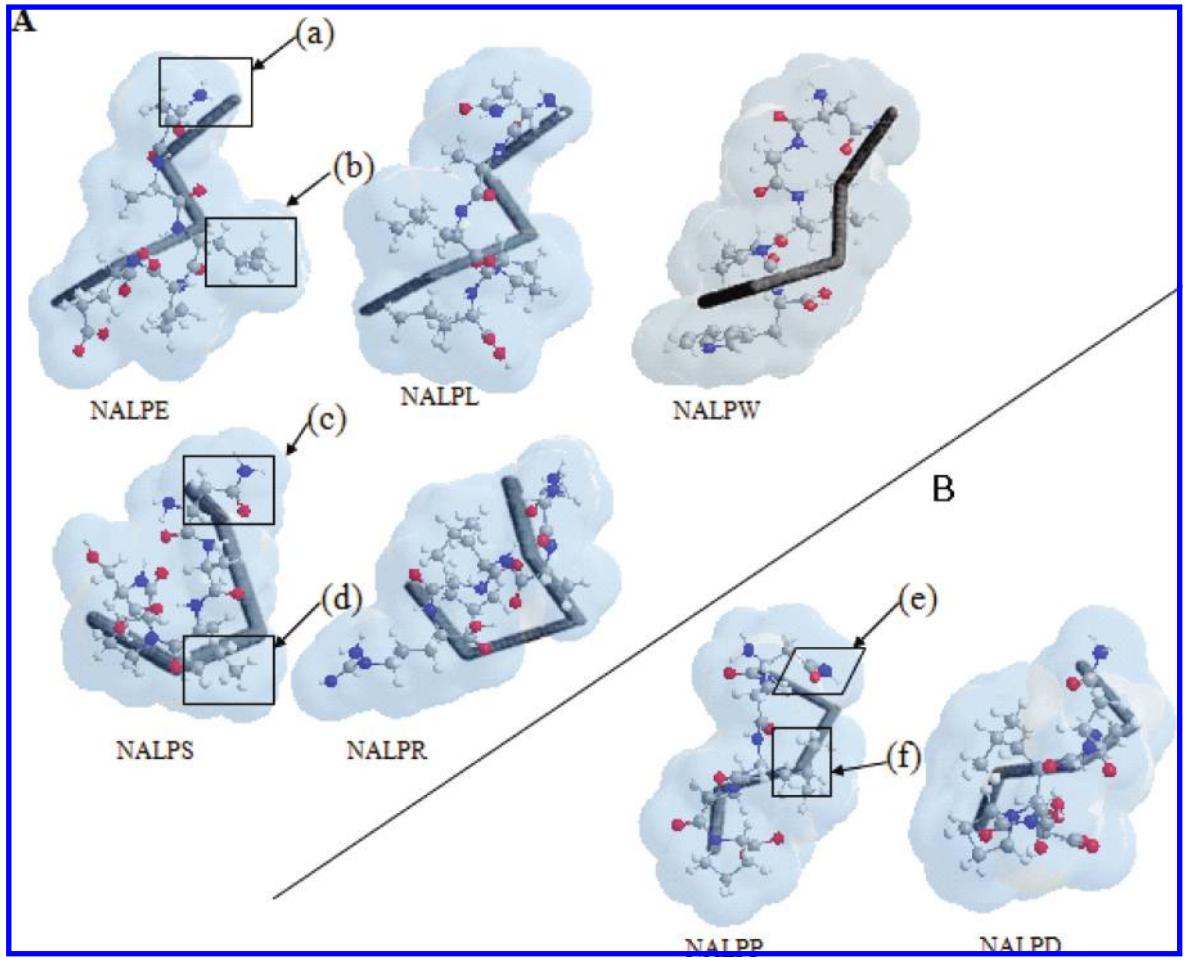


Figure 2. Computer-simulated structure for analogue peptides showing one of the minimum energy structures: (**A**) peptides having a bitter taste and (**B**) peptides having a nonbitter taste.

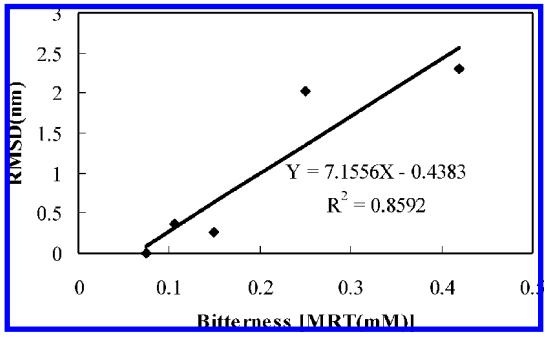


Figure 3. Relationship between bitterness and structural similarity of peptides.

resulted from changing the primary structure of the peptide, a sensorial descriptive test was conducted for each synthetic peptide. The synthetic analogues of NALPE were categorized as various tastes, such as sour, astringent, tickling, or bitter. As compared to the very bitter taste of NALPE with a glutamic acid residue, NALPD, with a similar acidic side chain (aspartic acid) in the C-terminal position, registered as sour and astringent in taste. NALPL and NALPW, with bulky and hydrophobic side chains at the C-terminus, were strongly bitter. Meanwhile, NALPS and NALPR, with hydrophilic and basic residues, were slightly bitter. In this study, we did not determine any relationship between the taste and the primary structure, size, or hydrophobicity of the peptides but only that peptides with a high hydrophobicity mostly exhibit bitter properties, which suggests that the presence of hydrophobic amino acids within molecules needed for peptide bitterness, even the total hydrophobicity of the molecule, was not a main factor.

Bitter Intensity of Synthetic NALPE Analogue Peptides. To evaluate the intensity of bitterness for the synthetic analogues of NALPE, we used a modified Weiffenbach's three-drop method (*30*) due to the limited sample amounts and bitterness properties. Because the bitterness of peptides was generally sustained for a rather long time, solutions of bitter peptides were prepared near the bitterness threshold concentration for the sensory panels. NALPE, NALPW, NALPL, NALPS, and NALPR, which were categorized as bitter peptides in the sensorial descriptive test, were selected for the sensory evaluation tests. The MRT values for the bitter analogues of NALPE were measured and are given in **Table 3**. The MRT value of NALPE was 0.074 mM, which is comparable to the MRT value, 0.05 mM, of the BPIa (Arg-Gly-Pro-Pro-Phe-Ile-Val) peptide, a well-known bitter peptide purified from casein hydrolysates (*31*). The MRT values of NALPL and NALPW, with hydrophobic and bulky amino acid residues, were 0.105 and 0.149 mM, respectively, which are comparable to that of NALPE. Meanwhile, the MRT values were higher for NALPS with a hydrophilic amino acid and for NALPR with a basic amino acid, 0.25 and 0.42 mM, respectively, indicating weak bitterness.

Spatial Structure of Synthetic NALPE Analogue Peptides. To explore the relationship between the properties of the C-terminal residues of synthetic NALPE analogue peptides and the changes in taste, including the intensity of bitterness, we attempted to analyze the spatial three-dimensional structures of

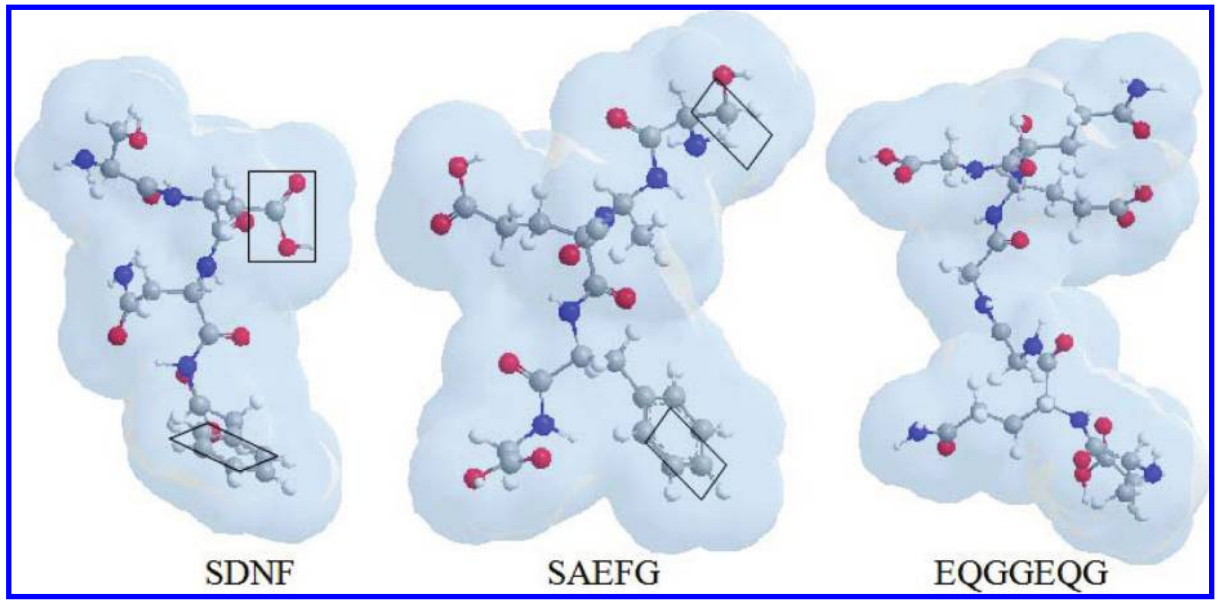


Figure 4. Computer-simulated structures for SDNF, SAEFG, and EQGGEQG showing one of the minimum energy structures.

the synthetic analogue peptides. **Figure 1** shows the chemical structure of NALPE. NALPE was composed of an asparagine residue at the N-terminal position and hydrophobic region of alanine, leucine, and proline at the center of the peptide.

The pentapeptides, composed of five amino acids, were too small to analyze as fixed structures in solution. Because the internal bonding energy of the pentapeptides was not large enough to sustain a certain fixed structure, the molecular structure of pentapeptides could be affected by an external environmental energy and might exist as flexible forms in solution. Therefore, the computer-simulated spatial structures of synthetic NALPE analogue peptides were analyzed under identical external energy conditions and compared (**Figure 2**). The structures of NALPE and analogues were grouped into three types according to physicochemical properties. The peptides within each group, those exhibiting sourness (NALPP and NALPD), peptides with weak bitterness (NALPS and NALPR), and peptides with strong bitterness (NALPE, NALPL, and NALPW), had similar structures. These categories were based on the orientation and proximity between C═O groups at N-terminal amino acids and hydrophobic regions, which might affect the taste of the peptides.

The similarity of peptide structures was estimated as the measure of the average distance between the backbones of superimposed peptides and NALPE (rmsd). Because the rmsd is the distance between the backbones of superimposed proteins, a lower rmsd value indicates closer structural similarity. NALPW and NALPL had the closest structural similarity to NALPE and also had a similar bitterness to NALPE in sensorial evaluation. NALPD and NALPP, which were the least similar to NALPE structurally, had a nonbitter taste (**Table 3**).

The relationship between intensity of bitterness (MRT value) and structural similarity with NALPE (rmsd) was analyzed by regression analysis as shown in **Figure 3**. Interestingly, the rmsd results coincide well with the intensity of bitterness ($R^2 = 0.859$ and $p = 0.023$), implying that the structure of peptide is important for eliciting bitterness.

DISCUSSION

To estimate the taste based on the structure, a general and common structure of sweet compounds was already proposed (*32*). In the case of bitter compounds, there are few structural models to explain bitterness because the peptide structures vary too much to determine a general relationship between bitterness and structure. However, the general major factors that affect the bitterness of peptides are summarized as follows: hydrophobic amino acids, hydrophobicity of total molecules, and characterization and numbers of side chains (*32–34*). In addition, on the basis of data already reported, it was generally said about the structural characteristics of bitter compounds that there is always a polar function and a hydrophobic group within the molecule, the former probably affecting taste quality and the latter affecting taste intensity.

In this study, NALPE, a bitter peptide purified from the hydrolysate of soybean ^{11}S glycinin, has a small molecular weight (542 Da) and a hydrophobicity of 980 cal/mol, which is less than the 1400 cal/mol proposed by Ney's Q-rule (*6*). In addition, it does not have a basic amino acid at the N-terminus or a hydrophobic amino acid at the C-terminus, which were reported to be required for bitterness (*12, 35*). Despite these differences, NALPE had a strong bitterness as compared to BPIa peptide from casein hydrolysate (*31*).

This might be explained by the presence of hydrophobic amino acids, leucine and proline, which are frequently found in known bitter peptides (*7, 9, 36, 37*). However, the analogue peptides, which contain the same leucine and proline, have different tastes. In addition, the hydrophobic side chains, molecular weights, and hydrophobicities of the synthetic analogues of NALPE did not explain the bitterness. Indeed, changing only one amino acid within the peptides resulted in drastic changes in taste, which could be explained by the spatial structure of the peptides.

Among these bitter peptides from ^{11}S glycinin and proglycinin hydrolysates, many peptides with an asparagine (Asn) residue at its N-terminal amino acid were found: Asn-Leu-Gln-Gly (NLQG), Asn-Ala-Leu-Glu-Pro-Asp-His-Arg-Val-Glu (NALEPDHRVE), Asn-Ala-Leu-Pro-Glu (NALPE), Asn-Asn-Glu-Asp-Thr (NNEDT), and Asn-Phe-Asn-Asn-Gln-Leu-Asp-Gln-Thr-Pro-Arg (NFNNQLDQTPR) from ^{11}S glycinin hydrolysate and Asn-Ala-Leu-Lys-Pro-Asp (NALKPD) from proglycinin hydrolysates (*4, 5*). The common structural characteristic of these peptides is the presence of Asn at the N-terminus and the following hydrophobic amino acid. The residual property of Asn is polar but uncharged. Therefore, the presence of a polar group

in the N-terminus and hydrophobic region within these peptides were assumed to be a basic structural core for bitter peptides. This assumption is in accordance with previous structural characteristics of bitter compounds that there is always a polar function and a hydrophobic group within the molecule.

Then, the spatial configuration of pentapeptides produced from the modification of amino acids at the C-terminus may determine the taste type and intensity of bitterness of the peptides. As shown in **Figure 2A(a−d)**, the Asn residues and hydrophobic regions faced each other at the same plane space in the case of bitter peptides. However, two units in the sour peptides crossed each other at differently oriented plane spaces (**Figure 2B(e,f)**). This observation might explain the difference of taste type on peptides. On the other hand, the distances between Asn residues and hydrophobic regions on the peptides with a strong bitterness (**Figure 2A(a,b)**) were closer than those with a weak bitterness (**Figure 2A(c,d)**), which might be induced by hydrophobic moieties of the sequential amino acids (-ALP-) and the characteristics of C-terminal amino acids. This observation may elucidate the intensity of peptide bitterness. Saroli proposed that the bitter compound denatonium chloride interacts with a bitter receptor by two polar groups and by two hydrophobic interactions in a structure−activity relationship study (*38*). Kubo also proposed that bitterness seems to be due to a balance between the bitter unit and the hydrophobic portions of molecules (*34*). His study based on rabdosia diterpenoids indicated that a bitter unit consists of a proton donor DH group and a proton acceptor A group. The bitter compounds could enter the molecular structure of the receptor site with the bitter unit oriented into the aqueous phase by hydrogen bonding and the hydrophobic portion aligned into the lipid phase by dispersion forces. Kim and Li-Chan demonstrated that bulky hydrophobic amino acids at the C-terminus and bulky basic amino acids at the N-terminus were highly correlated to bitterness in the study of bitterness prediction for 48 dipeptides and 12 pentapeptides (*39*). Our present result seems to fit with these previous theories that bitterness is due to a balance between the bitter unit (polarity) and the hydrophobic portions (lipophilicity) of molecules. Using the present results, it also is possible to explain the bitterness of SDNF, SAEFG, and EQGGEQG (**Figure 4**). SDNF and SAEFG have a polar group in the N-terminus and hydrophobic region within molecules, while EQGGEQG did not have this basic structure for bitterness. Therefore, EQGGEQG was not bitter. In addition, the polar group in the N-terminus and the hydrophobic region in the spatial structure of SAEFG eliciting distinct bitterness faced each other but were far from each other. In the case of SDNF, although the type of taste was not sensorially determined, its spatial structure showed the possibility of tastes different from bitterness.

The present study was designed to explore as to whether the type of taste and intensity of bitterness on bitter peptides isolated from soybean are related to the spatial configuration produced from the modification of the amino acid at the C-terminus. In conclusion, we can suggest that the presence of a polar amino acid and hydrophobic amino acid, the composition of hydrophobic regions, the spatial orientation of the polar group and hydrophobic regions, and the proximity between polar groups and hydrophobic regions faced within the same plane space may be the major determinants for taste type and intensity of peptide bitterness.

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Received for review December 17, 2007. Revised manuscript received April 27, 2008. Accepted May 10, 2008. This work was partially supported by a grant from the Ministry of Health and Welfare, Republic of Korea (AO5O376).

JF7036664