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Quantitative structure-activity relationship study of bitter di- and tri-peptides including relationship with angiotensin I-converting enzyme inhibitory activity

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Abstract: Bitterness represents a major challenge in industrial application of food protein hydrolysates or bioactive peptides and is a major factor that controls the flavor of formulated therapeutic products. The aim of this work was to apply quantitative structure-activity relationship modeling as a tool to determine the type and position of amino acids that contribute to bitterness of di- and tri-peptides. Datasets of bitter di- and tri-peptides were constructed using values from available literature, followed by modeling using partial least square (PLS) regression based on the three *z*-scores of 20 coded amino acids. Prediction models were validated using cross-validation and permutation tests. Results showed that a single-component model could explain 52 and 50% of the Y variance (bitterness threshold) of bitter di- and tri-peptides, respectively. Using PLS regression coefficients, it was determined that hydrophobic amino acids at the carboxyl-terminus and bulky amino acid residues adjacent to the carboxyl terminal are the major determinants of the intensity of bitterness of di- and tri-peptides. However, there was no significant ($p > 0.05$) correlation between bitterness of di- and tri-peptides and their angiotensin I-converting enzyme-inhibitory properties.

Keywords: bitter peptides; partial least square regression; QSAR; angiotensin converting enzyme; amino acid descriptors

INTRODUCTION

Proteins are hydrolyzed by proteases to improve their chemical, functional and biological properties [1], which enhance the production of desirable functional protein hydrolysates for the food and nutrition industries. However, the formation of off-flavor bitterness during hydrolysis has been one of the major limitations in the practical use of protein hydrolysates [2–4]. Several bitter peptides have been characterized from protein hydrolysates of soybean and milk hydrolysates, especially cheese products. It is now believed that off-flavor bitterness can be attributed mainly to hydrophobic-containing peptides released by the action of proteases. However, to the best of our knowledge, there is scanty information about the positional arrangements of amino acids on the primary structure that influences the bitter taste of peptides.

The importance of hydrophobic amino acid residues in bitter peptides was well characterized using average hydrophobicity (Q value), which according to the hypothesis of Ney [5] means that a peptide is almost certainly bitter when its Q value exceeds 1400 cal/mol regardless of its primary sequence, provided its molecular weight is less than 6000 D [6]. Matoba and Hata [7] also supported this hypothesis stating that the amino acids in a peptide chain independently contribute to bitterness regardless of the amino acid sequence and configuration. Based on Ney's Q value hypothesis, controlled protein hydrolysis is used to avoid the formation of bitter peptides, and technologies are developed to remove, eliminate or mask the bitterness of peptides [3,8]. However, there are exceptions and limitations in Ney's hypothesis. For example, lysine and proline have very high Q values despite being found in nonbitter peptides [9] while bitter peptides that contain hydrophilic amino acids have been reported [10]. Moreover, steric parameters have not been reflected in the Q values but are known to be important for the intensity of bitter taste [11,12]. Furthermore, the importance of the primary structure of peptides has been implied in works that used synthetic peptides in addition to hydrophobicity. It was reported that in order to increase the bitterness of peptides, the hydrophobic amino acids need to be located at the *C*-terminal and, conversely, the basic amino acid should be located at the *N*-terminal [13,14].

Since the off-flavor bitterness represents a technical problem that has not been adequately solved by the food industry, research on structure and activity relationship of bitter peptides could improve our understanding of the structural requirements of bitter peptides and thus guide technology development for improving the tastes of protein hydrolysates [2,4,15]. Quantitative structure-activity relationship (QSAR) of bitter peptides was previously performed using whole structural parameters of peptides [11]. However, knowledge of the structure-activity relationships of

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bitter peptides remains limited and requires further elucidation. The presence of bioactive peptides in protein hydrolysates and fermented products has received great attention, among which angiotensin converting enzyme (ACE)-inhibitory peptides are the most extensively documented. The importance of hydrophobic amino acids in ACE-inhibitory peptides was previously reported [16,17]. Quantitative structure-activity relationship (QSAR) study of ACE-inhibitory peptides also suggested that bulky, hydrophobic and acidic amino acids are important for enhancing ACE-inhibitory potency of di- and tri-peptides [18]; the results suggest structural similarities with bitter peptides. Bitterness could have a significant limiting effect on the formulation and consumer acceptability of functional foods that contain ACE-inhibitory peptides. The primary objective of this study, therefore, was to provide the structural information that relates arrangement of amino acids on the peptide chain to bitterness, using the well-known *3-z* physicochemical descriptors and sensory evaluation scores for published peptides. A secondary objective was to review possible correlations between bitterness and ACE-inhibitory potency of the di- and tri-peptides.

MATERIALS AND METHODS

Peptide Dataset

Data on the primary structure of bitter di- and tri-peptides as well as their bitterness scores were collected from previously published reports. The reports showed that bitterness of peptides was organoleptically determined via panel evaluation and presented as threshold values (TV) (Tables 1 and 2). ACE-inhibitory activities, expressed as IC_{50} value (peptide concentration that reduces ACE activity by 50%), was from our previously collected ACE-inhibitory dataset or was predicted using the previously established ACE-inhibitory peptide models if literature values were not available [18].

Analysis of Peptide Datasets

The characterization of each individual amino acid by the *3-z* score, namely, z_1, z_2, *and* z_3 scores, were calculated by principal component analysis (PCA) from a matrix

Table 1 Bitterness and ACE-inhibitory activity of di-peptides

Peptide sequence	ACE-inhibitory activity [log (IC_{50})][a]	Bitterness [log (TV)][b]	Peptide sequence	ACE-inhibitory activity [log (IC_{50})][a]	Bitterness [log (TV)][b]
FF	3.03*	0.08	PR	0.61	0.48
LL	2.45*	0.40	RR	1.38*	0.90
LG	3.94	1.30	RG	3.08	0.90
LV	2.10*	0.78	GR	3.51	2.00
LI	2.43*	0.60	GV	3.66	0.65
VL	2.19*	0.78	VI	2.16*	0.78
IL	1.74	0.18	VD	1.44*	0.48
FL	1.20	0.08	VE	1.35*	0.78
FG	3.57	0.78	IV	2.07*	1.10
YY	2.36*	0.36	DV	1.34*	0.18
FV	2.31*	0.78	EV	1.44*	0.18
FL	1.20	0.18	PP	2.07*	0.65
FI	2.64*	0.18	PK	1.32*	0.78
FP	2.50	0.18	AD	1.17*	0.78
IF	2.97	0.18	VD	1.44*	1.11
YF	2.78*	−0.10	LD	1.70*	0.78
IF	2.97	0.18	LE	1.61*	0.48
GR	3.51	1.88	RG	3.08	1.00
VI	2.16*	0.78	IV	2.07*	1.10
RP	1.85	−0.10	RF	2.17	0.36
KF	1.76	0.65	VY	1.37	0.48
RF	2.17	0.36	RP	1.85	−0.1
GP	2.60	0.78	KP	1.51	0.48
GF	2.70	0.08	LF	2.40	0.08
YG	3.18	0.48	GF	2.70	0.08
AF	1.78	1.1	VF	1.44	0.48
GY	2.20	0.48		—	—

[a] Values with an asterisk were predicted using the ACE di-peptide QSAR model of Wu *et al.* [18]. Values without asterisk are from published literature.
[b] Published literature values.

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Table 2 Bitterness and ACE-inhibitory activity of tri-peptides

Peptide sequence	Predicted ACE-inhibitory [log (IC_{50})][a]	Bitterness [log (TV)][b]	Peptide sequence	Predicted ACE-inhibitory [log (IC_{50})][a]	Bitterness [log (TV)][b]
V V V	1.63	0.65	R R R	1.77	0.60
R P G	3.09	−0.10	P P P	1.86	0.30
G R P	0.48	−0.10	F F F	1.20	−0.70
L L L	1.35	0.08	R G P	1.73	1.11
L G G	2.49	1.88	P G R	2.67	1.40
G L G	2.45	1.00	G G V	1.99	1.58
G G L	1.63	1.00	G V V	1.82	0.65
L L G	2.33	0.70	P P G	3.18	0.98
L G L	1.52	0.70	P G G	3.14	0.65
G L L	1.47	0.18	P G P	1.82	0.98
F G G	2.79	0.65	G P G	2.65	1.30
G F G	2.53	0.48	G G P	1.28	0.98
G G F	1.10	0.18	P G I	2.23	0.36
F F G	2.71	0.34	K P K	2.63	0.48
F G F	1.29	0.08	A D A	2.07	1.11
G F F	1.02	−0.22	L D L	1.42	−0.10
G G G	2.61	−0.70	G E G	2.28	0.18
Y G G	3.07	0.36	L E L	1.19	−0.40
G Y G	2.33	1.28	R G P	1.73	1.11
G G Y	1.35	0.18	P I P	1.69	0.15
Y Y G	2.79	−0.22	F P F	1.32	−0.40
Y G Y	1.82	−0.10	V I F	0.78	0.11
G Y Y	1.07	−0.40	K P F	1.51	−0.40
Y Y Y	1.54	−0.70	R P F	1.59	0.18
F I V	2.04	0.18	P P F	1.68	0.36
F P P	1.50	0.65	V Y P	0.82	0.48
F P K	2.45	0.48	Y P F	1.60	−0.52
P F P	1.74	−0.40		—	—

[a] ACE-inhibitory activity values were predicted using the ACE tri-peptide QSAR model of Wu *et al.* [18].
[b] Published literature values.

consisting of 29 physicochemical variables [19]. These three resulting principal components, so called principal properties, are linear combinations of the primary data and were tentatively interpreted to represent largely lipophilicity (z_1), steric properties (z_2) or side chain bulk/molecular size, and electronic properties (z_3), respectively. As a common practice in QSAR analysis, all the threshold values were log-transformed prior to modeling. The amino acid at the amino-terminus was designated as n_1, and its properties were described as n_1z_1, n_1z_2 and n_1z_3; the amino acid adjacent to the amino-terminus was designated as n_2, and its properties were described as n_2z_1, n_2z_2, n_2z_3, and so on.

Partial least square (PLS) regression analysis between amino acid descriptors (predictors, X) and log-transformed TV (dependent, Y) was carried out using SIMCA-P version 10 (Umetrics Inc., Umeå, Sweden). All variables were centered and scaled to unit variance prior to the analyses to ensure that all variables would have an equal participation in the model. In SIMCA-P, the number of significant PLS components is chosen automatically by using various rules based on a statistic called Q^2. Q^2 is the cross-validation correlation coefficient, calculated from predicted residual sum of squares (PRESS), referred to as the model's predictive ability in SIMCA. Another important parameter in PLS analysis is the multiple correlation coefficient (R^2), which provides estimates of the model fit. The optimal model is made where a reasonable balance between the model's fit and predictive ability is achieved [20,21] followed by validation with response permutation [22]. Correlations between ACE-inhibitory activities and TV of peptides were determined using Microsoft Excel.

RESULTS

Relationships between Bitterness and Peptide Primary Structure

QSAR analysis of bitter di-peptides in Table 1 resulted in a single-component model that could explain 52.2% (i.e. multiple correlation coefficient, R^2) of the Y variance with the predictive ability (cross-validation correlation coefficient, Q^2) of 43.4% (Figure 1(a)); similarly, QSAR analysis of bitter tri-peptides in Table 2 generated a

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single-component model that could explain 50.0% of the Y variance with the predictive ability of 42.6% (Figure 1(b)).

The predictive power of these two models was validated by response permutation, where the response data vectors (log TV) were each randomly reordered and permuted a number of times, but with unperturbed X data; then a QSAR model was computed and used for refitting the model, respectively [22]. SIMCA-P displays the plot of the correlation coefficient between the original Y and the permuted Y *versus* the cumulative R^2 and Q^2, and draws the regression line. The intercept (R^2 and Q^2 when correlation coefficient is zero) is a measure of the over fit [21]. Twenty times permutation and cross-validation rounds computed the resulting intercepts of R^2 to be 0.0082 and 0.0457 for di-peptide and tri-peptide sets, respectively. The intercept values for Q^2 are −0.124 and −0.113 for di-peptide and tri-peptide sets, respectively. It was suggested that the desirable intercept limits for R^2 should be less than 0.3 and less than 0.05 for Q^2 in order to obtain a valid model [23]. The intercept values in our models are lower than these limits; therefore, the models developed in this work are valid. The root-mean-square error of estimation, which describes the fitting error of the model were 0.3111 and 0.4044, respectively, for di-peptide and tri-peptide datasets.

The importance of a given X-variable for Y is proportional to its distance from the origin in the loading space (zero) and corresponds to the PLS regression coefficients [24]. The expected amino acid properties in each position are evaluated according to their importance to the Y variable; high coefficient values indicate that the *z* property is more relevant to the corresponding amino acid position when compared to low coefficient values. For di-peptides the *z1* at position 2 (*n2z1*) is more important than that of position 1 (*n1z1*), while *z2* is more important for the *N*-terminus than for the *C*-terminal; *z3* was relevant only to the *C*-terminal amino acid (Figure 2(a)). In Figure 2(b), all

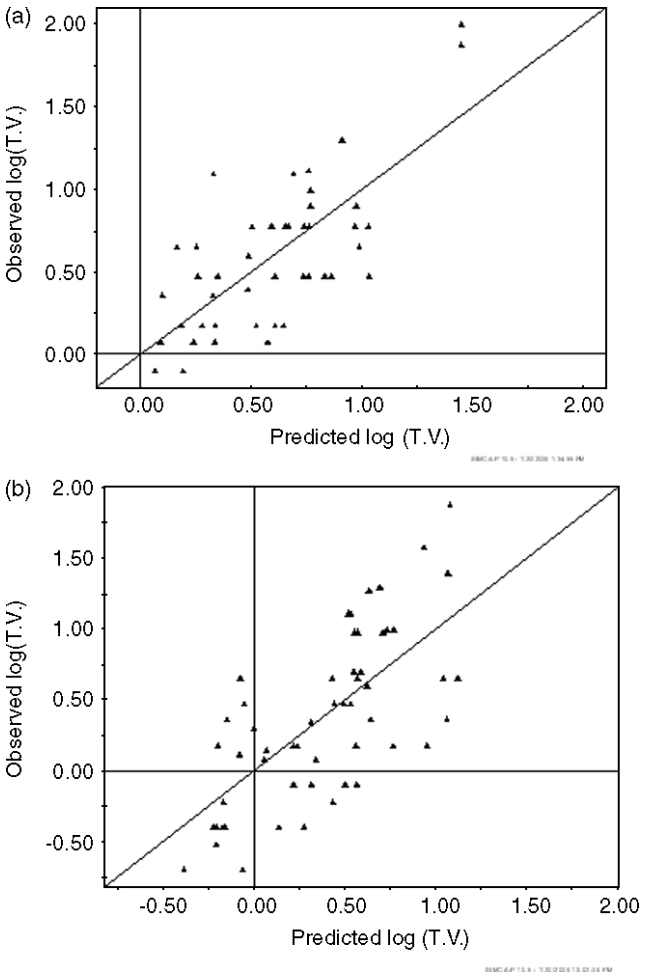

Figure 1 The relationships between observed *versus* calculated values of bitterness threshold values (log TV) for di-peptides (a) and tri-peptides (b).

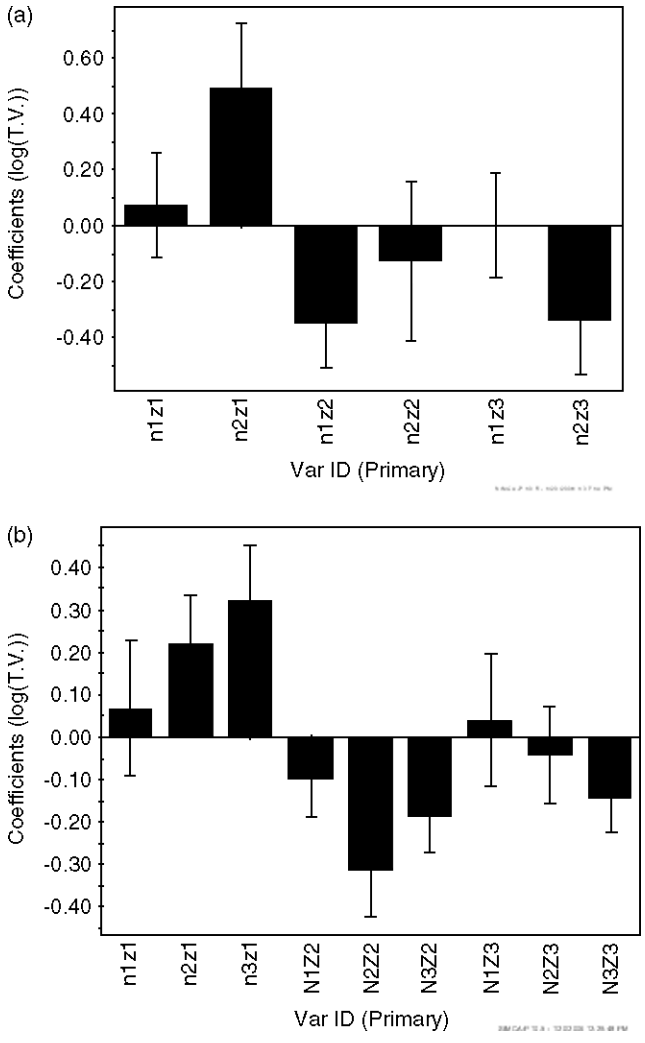

Figure 2 PLS regression coefficients for (a) di-peptides and (b) tri-peptides. The bars indicate 95% confidence intervals based on jack-knifing. The importance of a given x-axis variable is proportional to its distance (coefficient value) from the origin (zero).

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z1 are positively related to the bitterness threshold values and the relevance increases as we move from the *N*-terminal (*n1*) to the *C*-terminal (*n3*). In contrast the *z2* property is negatively related to bitterness and the contribution to the middle amino acid (*n2*) is greater than the *N*- or *C*-terminal amino acids (Figure 2(b)). The third property (*z3*) had a small positive coefficient for *n1* but negative coefficients for *n2* and *n3*; overall contributions of *z3* were substantially less than the contributions of *z1* and *z2* to the bitterness of tri-peptides.

Relationships between Bitterness and ACE-Inhibitory Activity of Peptides

Because of the high occurrence of hydrophobic amino acid residues in ACE-inhibitory peptides, there is the possibility of bitterness if we apply the Q rules [5]. To the best of our knowledge, the taste of ACE-inhibitory peptides has not been studied and the correlation between these two activities has not been reported either. ACE-inhibitory activities were either cited from our previous paper or predicted according to our established models [18] if the activity was not available in the literature (Tables 1 and 2). In case of peptides with multiple reported ACE-inhibitory activity values, the average value was used for correlation analysis. Our results showed that the bitterness of peptides had no significant ($p > 0.05$) correlation with the ACE-inhibitory activity (Figure 3) with R^2 value of 0.32.

DISCUSSION

Our understanding of the structural requirements of bitter peptides has been improved mainly through systematic synthesis of peptide analogues of isolated or known bitter peptides. However, the relationship between the bitterness intensity and type of amino acid or arrangement of amino acid on the peptide chain has not been fully characterized. QSAR is a basic tool that can be used to search for information relating chemical structure to biological and other activities [24]. The biological activity (BA) within a set of compounds is related to the structural variation of the compounds, i.e. BA can be modeled as a function of molecular structure [25]. The importance and utility of QSAR as a predictive and modeling tool for BA of proteins and peptides have been previously reported [20,26]. Early studies on the QSAR of bitter peptides were limited by applying overall peptidic structural properties without considering the positional effects of individual amino acids [11,27]. Previous studies on QSAR of bitter di-peptides examined the feasibility of application of new physicochemical descriptors of amino acids in QSAR analysis rather than the QSAR of bitter peptides [14,16,28–33]. Our previous research on QSAR of ACE-inhibitory di- and tri-peptides using the *3-z* descriptors has generated models that enabled *de novo* prediction of new and potent ACE-inhibitory peptides [18].

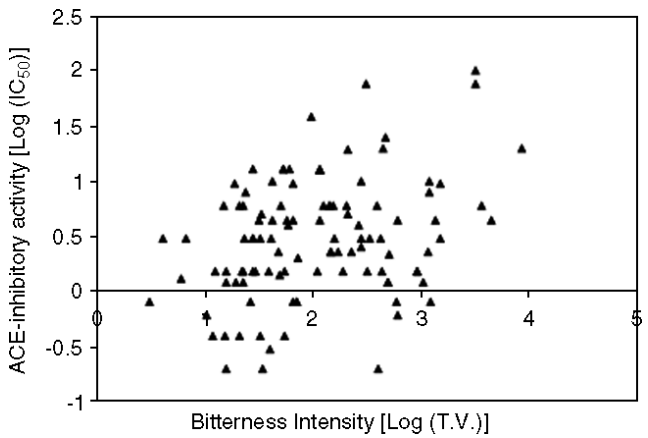

Figure 3 Correlations between angiotensin converting enzyme-inhibitory activity (log IC_{50}) and bitterness intensity (log T.V) of di- and tri-peptides.

According to Hellberg *et al.* [19], *z1* represents largely hydrophobicity of amino acids, which we have shown in this work to be positively related with the bitterness for both di- and tri-peptides; therefore, amino acids with high hydrophobicity values will contribute more to bitterness than those with low values. Our results are in good agreement with previous reported results [3–5]; our results also showed that hydrophobic amino acids (high *z1* values) at the carboxyl-terminus (*n2* for di-peptides and *n3* for tri-peptides) are more important than the other positions in terms of relative contribution to bitterness intensity. The contribution of hydrophobic amino acids to the bitterness is well accepted; however, the positional effect of hydrophobic amino acids has not been fully elucidated. An earlier work proposed that bitterness is attributable to hydrophobic amino acids irrespective of amino acid sequences [7], though no statistical analysis was performed. Similarly, the widely accepted Q rule did not take into account the positional effect of amino acids [5]. However, another report [34] suggested that the hydrophobic *C*-terminal sequence had an important effect on the bitterness. Other groups also reported the significance of the positional effect of hydrophobic amino acid residues when the residue was located at the *C*-terminus of peptides [14,35,36].

A previous study had suggested a role for the steric parameters in the intensity of bitterness [11], but the authors were unable to clarify the positional influence of the individual amino acid residue because they used whole molecular descriptors, such as the total length along the zigzag peptide backbone chain of the molecule. In this study, we have clearly showed that the steric property (*z2*) of amino acids in bitter peptides has significant influence on the intensity of bitterness as shown in Figure 2; more specifically, for di-peptides (Figure 2(a)) the *N*-terminus (*n1z2*) is more important

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than *C*-terminus (*n2z2*). For tri-peptides, the middle amino acid residue is more important than both *C*- and *N*-terminal amino acid residues. According to our results, the presence of bulky amino acid residues such as Trp, Arg and Tyr etc., adjacent to a hydrophobic amino acid at the *C*-terminus increases intensity of bitterness. Electronic property (*z3*) of the *C*-terminus was mostly negatively correlated with the bitterness intensity of di- and tri-peptides, except at the *N*-terminus (Figure 2). The *z3* of *N*-terminus position was positively correlated with bitterness but the contribution could be negligible because of the relatively small coefficient values. Therefore, the presence of positively charged amino acids (with the smallest *z3* values) at the *N*-terminus did not affect the intensity of bitterness, which is different from the bifunctional unit theory [37], and an earlier report [38] that emphasized an important role for basic amino acids in determining bitterness of peptides.

The lack of a significant correlation between ACE-inhibitory activity and bitterness of di- and tri-peptides is understandable because the detailed structural requirements of bitter peptides obtained in this work (hydrophobic amino acids at *C*-terminus with aromatic amino acid at the adjacent position) are different from those reported by Wu *et al.* [18] for ACE-inhibitory peptides (bulky aromatic amino acids at *C*-terminus with hydrophobic or positively charged amino acid at the adjacent position).

In conclusion, our results showed that elucidation of structural requirements of bitter peptides through QSAR study would be helpful in designing new technologies and methods for eliminating or reducing the bitter taste of hydrolyzed protein products. Even though we did not find any significant correlation between peptide bitterness and ACE-inhibitory activity, the presence of bulky amino acids and hydrophobic amino acid in ACE-inhibitory peptides could contribute some level of bitterness to formulated products. Knowledge of the type and position of amino acids that contribute to bitterness could provide a basis for elimination of certain residues from food proteins or rearrangement of residues on the primary structure using genetic engineering techniques. The results can also enhance the production of less bitter protein hydrolysates through appropriate choice of enzymes that cleave the bonds between the amino acid residues that we have shown to be important determinants of peptide bitterness. However, it should be noted that the current work was limited to di- and tri-peptides and the results may not be extrapolated to peptides with higher numbers of amino acid residues. Finally, in order to further demonstrate the illustrated relationships between peptide primary structure and bitterness, it will be necessary to perform the appropriate experiments using synthesized peptides.

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