Food Sci Biotechnol (2022) 31:767–785
https://doi.org/10.1007/s10068-022-01097-w

RESEARCH ARTICLE

Evaluation of taste active peptides and amino acids from anchovy proteins in fish sauce by in silico approach

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Received: 28 October 2021 / Revised: 14 April 2022 / Accepted: 2 May 2022 / Published online: 19 May 2022

Abstract Enzymatic activity and microbial fermentation play a prominent role in the bioconversion of complex muscle tissue into smaller units of peptides and amino acids, possibly contribute to sensory properties. Thus, this study screens and evaluate anchovy proteins with taste-active peptides and amino acids by the reaction of multiple enzymes using an in silico approach. Information about sensory components was provided based on an in silico analysis using tools available in the BIOPEP-UWM database. Proteins from anchovy, namely myosin heavy chain 6 alpha, myosin light chain 1, cytochrome B, and NADH ubiquinone oxidoreductase, were subjected to in silico digestion with the combination of 23 enzymes. This led to the release of taste-active peptides and amino acids, including umami, sweet, salty, sour, and bitter sensory properties. The combination of multiple enzymes released a more significant number of taste-active peptides and amino acids for both myosins compared to other proteins.

Keywords Fermentation · Enzyme · Proteolysis · Bioinformatics · Flavor

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Introduction

Fish sauce is a condiment with a unique aroma and flavor produced as the product of fish and salt fermentation. It is often used as a flavoring additive in cooking. Usually, fish species of *Stolephorus* sp., *Sardinella* sp. and *Engraulis japonicus* were used as the primary raw material (Ray and Montet, 2015). During the long period of the fermentation process, fish muscle or protein tissues were slowly broken down into smaller peptides and amino acids units due to enzymatic activity mainly from its digestive tract. This proteolysis is then furthered by the activity of halotolerant microorganisms, mainly lactic acid producing bacteria through a biological process known as fermentation. The continuous release of peptides and amino acids during fermentation is believed to play the role of taste-active component in the development of fish sauce overall taste. Previous research has found that fish sauce contains a high concentration of nitrogen-based compounds, including sensory peptides (Phewpan et al., 2019) and essential amino acids such as lycine, leucine, valine, isoleucine, threonine, valine, and phenylalanine (Ahmad et al., 2019).

Myofibrillar proteins are proteins forming myofibril, which covers approximately 66% and up to 77% of the total protein in fish. These proteins consist of actin and myosin that could also be observed as actomyosin in Dewi (2002)

in the study of dried salted anchovy (*Stolephorus* sp.). Since myosin is a complex protein molecule, this protein consists of both myosin heavy chain and light chain. Other than that, proteins such as tropomyosin, troponin T, troponin C, and actin are also found in myofibrillar protein, especially in anchovy, as found in a protein identification study by Herrero et al. (2000). In another study on anchovy myofibrillar proteins (*Engraulis japonicus*) from the fish sauce with enzymes, the identified proteins from SDS-PAGE analysis also showed the presence of myosin heavy chain, actin, tropomyosin, and troponin T (Choi et al., 2004).

In anchovies, major endogenous proteolytic enzymes include trypsin-like proteinase, pepsin, chymotrypsin, elastase, and aminopeptidase (Siringan et al., 2006), with trypsin, chymotrypsin, and pepsin are usually found in the viscera of fish as its primary digestive juices. Enzymes, especially cathepsins, peptidases, transaminases, amino acid decarboxylases, glutamic dehydrogenases are all common in fish muscle tissue, where trypsin, chymotrypsin and cathepsin are highly involved in protein breakdown during the fermentation process. Among these enzymes, chymotrypsin was found as the highest contribution for breaking down of anchovy muscle in fish sauce (Choi et al., 2004; Fernandes, 2016). As for exogenous enzyme such as oligopeptidase and aminopeptidase that were mainly produced by microbial community, these enzymes were found to contribute to the production of sensory components during fermentation process (Faisal et al., 2015; Law and Haandrikman, 1997). Thus, variation of enzymes during fermentation period will be producing their own unique sensory components to any fermented products.

Several chemical components in food have been reported to be responsible for its sensory properties, including salts, sugars, nucleotides, peptides, and free amino acids, which these components can have synergistic or antagonistic effect to elevate the taste in foods or mask the taste from other sensory components (Chan and Cheung, 2010). For instance, mixture of L-glutamic acid and 5' nucleotide at 1:1 produced 7 times umami strength in comparison with the taste strength of glutamate itself, Na^+ and Cl^- substantially elevate overall umami intensity of mushrooms and dried bonito (Wang et al., 2020). One of the earliest documented peptides with sensory properties was from Arai et al. (1973), where Glu-Asp and Glu-Glu peptides from proteinase-modified soybean protein were observed to have a brothy taste. According to Park et al. (2002), the peptide sequence of Val-Pro in Vietnamese fish sauce *Nuoc-mam* showed sweet taste activity, peptide Asp-Glu with umami taste and Gly-Phe showed bitter taste properties.

Amino acids, in general possess primary taste properties, which aspartic acid and glutamic acid play a direct role in contributing to sour taste. These amino acids in the derived form of salt are characterized as umami, savory, and meaty with a chicken broth-like taste. Meanwhile, L-amino acids with hydrophobic side chains, particularly leucine, isoleucine, tyrosine, and valine, are attributed to bitter taste (Temussi, 2012). On the other hand, the D-form of amino acids, such as proline, alanine, lysine, glycine, serine, and threonine, is usually attributed to a sweet taste. As for peptides, it has been shown to also portray sensory attributes apart from a variety of biological activity and functional properties (Khositanon et al., 2018; Lacou et al., 2016). Sensory active peptides have been documented from multiple studies, these including dipeptide Val-Pro as a sweet peptide, Asp-Glu as a sour peptide, Tyr-Pro-Orn as a bitter peptide, and Asp-Met-Pro as an umami peptide (Park et al., 2002). Another older study also showed fragments of 'delicious peptide' including Lys-Glys-Asp-Glu-Glu-Ser-Leu-Ala as sour and umami peptide, Lys-Gly •HCL with salty and umami peptide, and the role of Lys-Gly •HCL with both salty and umami taste (Tamura et al., 1989).

The use of in silico analysis is a recent, useful and rapid technique for predicting the release of peptides from a known protein sequence. BIOPEP-UWM is a database of biologically active peptides, sensory peptides, and amino acids documented from various resource materials and proteomic studies. BIOPEP-UWM also has the feature to perform simulation of enzymatic hydrolysis, which can be done by applying the desired protein sequence into the system, followed by determining specific enzyme to predict the release of the peptides and amino acids. Applying in silico tools will reduce the cost and time requirement for theoretical estimation of potential bioactivities after specific enzymatic hydrolysis. BIOPEP-UWM has been successfully used to investigate proteolysis and its biological activity of proteins from tomato seeds and multiple food products (Kartal et al., 2020). There is generally a good agreement between the in silico prediction and the in vitro bioactive effects of the peptides (Nongonierma and FitzGerald, 2016).

Previous sensory studies on fish sauce samples only limited to taste active components in *Nuoc-mam*, salt-taste enhancing components in *Nam-pla*, kokumi peptide in *Pla-ra*, *Yu-lu*, *Shottsuru*, *Ishiru* and *Garum* (Kuroda et al., 2012; Miyamura et al, 2015; Park et al., 2002; Schindler et al., 2011), while there is more variety of fish sauces to discover. Since most of fish sauces especially in Asia were mainly made from anchovy (Lopetcharat et al., 2007), there is a need to explore more potential taste-active peptides and amino acids from anchovy proteins which covers five basic human tastes. In this article, in silico analysis is applied to screen and to perform in silico digestion in order to discover potential taste-active peptides and amino acids. Thus, the implementation of this study could be taken as reference guide for any study of the same interest and to provide a theoretical basis in designing

in vitro and in vivo studies to explore taste-active components from anchovy-based proteins, especially in fish sauce products.

Materials and methods

Materials

The sequence of proteins applied in the study was obtained from the UniProtKB database at https://www.uniprot.org. The proteins were selected based on 'anchovy' keyword search with '*Engraulis* sp.' and '*Coila* sp.' as filter species and 'muscle' as a filter for protein name and tissue. Search results listed 70 anchovy proteins. Four protein sequences were selected based on previous studies (Choi et al., 2004; Dewi, 2002) and consideration on preferring high molecular weight proteins with long amino acid sequences. Details of analyzed proteins are summarized in Table 1.

Screening of potential taste active peptides and amino acids

The potential of all sensory peptides and amino acids were screened in BIOPEP-UWM http://www.uwm.edu.pl/biochemia/index.php/en/biopep. The application of 'sensory peptides and amino acids' shown on the BIOPEP-UWM homepage is selected and continued with the 'analysis' application button. Then the 'profile of sensory activity' feature is selected, and the sequence obtained from UniProt is applied in the 'for your sequence'. The screening analysis outcome could be obtained by pressing the 'report' button. A parameter as an indicator of strength is implemented to evaluate peptides and amino acids influences on sensory characteristics in a protein. The calculation was based on the following equation:

$$A = a\backslash N,$$

where 'A' is a frequency of bioactive fragments/sensory active fragments occurrence in a protein sequence, as for 'a' is the number of peptides or amino acids with taste activity, meanwhile 'N' is the number of amino acid residues in a protein.

In silico digestion and prediction of taste active peptides and amino acids

Protein sequences selected were subjected to proteolysis simulation feature in BIOPEP-UWM (Minkiewicz et al., 2019). For this purpose, 'sensory peptides and amino acids' are chosen, with the current database of 493 (as accessed on June 10th, 2021) and continued with 'analysis'. Then, the enzyme(s) action' tool of the BIOPEP-UWM database was used to predict the theoretical peptide sequences and amino acids resulting from enzymatic proteolysis. The selection of enzymes was based on evidence and previous reports of enzymes found in anchovy (Choi et al., 2004; Fernandes, 2016; Siringan et al., 2006). The sequence is then applied in 'for your sequence'. Enzymes used were trypsin (EC 3.4.21.4), chymotrypsin (EC 3.4.21.1), pepsin (EC 3.4.23.1), cathepsin (EC 3.4.21.20), and oligopeptidase B (EC 3.4.21.83). The simulated digestion was implemented with 23 enzyme actions comprising single-acting and combined enzymes of two and three combinations. To view the analysis outcome, 'view report with results' is selected. The calculation to indicate taste strength was based on the following equation:

$$A_E = d\backslash N,$$

where 'A_E' is the frequency of release of fragments with given activity from the selected enzymes, meanwhile, 'd' is the number of amino acids and peptides with taste activity released by single enzyme or enzyme combinations, and 'N' is the number of amino acid residues in a protein.

Table 1 Information on selected proteins for in silico studies

Protein code	Protein	Uniprot entry identifier	Uniprot entry name	Scientific name	Total amino acid residues	Molecular weight (Da)
1	Cardiac muscle myosin heavy chain 6 alpha	D6BT34	9TELE	*Engraulis eurystole* (Silver anchovy)	232	25,988
2	Myosin light chain 1	Q9IB21	ENGJA	*Engraulis japonicus* (Japanese anchovy)	195	21,592
3	Cytochrome B	Q8M227	ENGEN	*Engraulis encrasicolus* (European anchovy)	380	42,394
4	NADH-Ubiquinone Oxidoreductase Chain 5	A0A0U2A1V5	9TELE	*Coilia brachygnathus* (Yangtse grenadier anchovy)	611	67,591

Results and discussions

Screening analysis

In fish sauce, the sensory components were generated as the results of proteolysis and further refined via bacterial fermentation during the long period of the fermentation process (Montero, 2017). In this study, screening analyses were intended to display all possible taste active peptides and amino acids present in a fish sauce based on protein sequences selected in reference to the current database (as assessed in June 2021). Table 2 shows the outcome of screening analysis in this study.

According to the screening results shown in Table 2, there were, in general, a total of 10 possible sensory properties found in the proteins selected with different intensities displayed as A values. These sensory properties include umami taste, umami enhancing, bitter, bitter suppressing, sweet, sweetness suppressing, sour, salty, salty enhancing, and astringent taste. Protein 2 showed the highest of all six proteins analyzed with the A of 0.307, followed by 0.2672 from protein 1. However, protein 2 was observed with fair umami enhancing intensity as the third highest with 0.0103 after protein 1 with A value of 0.0043 compared to other proteins. Based on Table 2, both protein 2 and protein 1 were screened with a total of 65 and 61 umami sensory contributing amino acids and peptides. The umami-active components screened include free amino acids of D (Aspartic Acid), E (Glutamic Acid), dipeptides, and tripeptides, where most of it contains either glutamic acid or aspartic acid. Both glutamic acid and aspartic acid have been identified as umami active amino acids, which have been documented responsible for eliciting umami taste in several foods (Kaneko et al., 2011; Su et al., 2012). In another study of sea-urchin involving omission test for taste study, omission of glutamic acid from the synthetic extract yielded reduced umami property and enhanced the sweetness (Fuke and Konosu, 1991). In addition, it is evidenced that some of the dipeptides shown in Table 2, such as Glu-Asp, have been documented in Zhang et al. (2016) as umami active dipeptides in fish.

As for the bitter sensory properties screened, protein 3 showed the highest intensity for bitterness with the A of 0.8711, as shown in Table 2. Protein 3 has the highest number of amino acids and peptides with bitter properties with a total of 329, including R (arginine), P (proline), F (phenylalanine), V (valine), L (leucine), K (lysine). The presence of these amino acids in a peptide sequence will be influencing the peptide to be perceived as a bitter peptide, such as Pro-Ala, Pro-Arg, Lys-Phe, and Leu-Glu. According to Chan and Cheung (2010), L configurated amino acids such as lysine and proline have a predominant taste of both sweet and bitter at a detection threshold of 50 and 300 mg/100 mL respectively. Meanwhile, arginine, phenylalanine, valine, and leucine come with a predominant taste of bitter with threshold ranging from 40 to 90 mg/100 mL. According to Nishimura and Kato (2009), hydrophobic amino acids such as L-Phe, L-Tyr, L-Trp, L-Leu, L-Val, and L-Ile produced bitter taste, which almost all peptides with these amino acids also produced the same bitter taste. For the bitter suppressing, protein 4 has the highest in total, followed by protein 1; however, in terms of strength or intensity, both protein 2 and protein 1 were observed the highest compared to the other proteins screened, with A value of 0.1538 and 0.1422 respectively. Conversely, bitter active amino acids screened in this study were also found as bitter suppressing amino acids, such as arginine and lysine. This might be the synergistic interaction between components, such as the combination of arginine and aspartic acid, in enhancing the salty flavor of sodium chloride, masking the sour and bitter taste. Other than that, in a soy sauce study, the presence of bitter amino acids at sublevel threshold enhanced umami taste in soy sauce, which might also result in masking of bitter taste (Lioe et al., 2005). According to Tokita and Boughter (2012), synergism of glutamate with sodium salts and umami active peptides such as α-Glu-Asp could suppress bitter taste.

Sweetness sensory properties have been observed highest in strength for protein 2 and protein 1 with A of 0.4256 and 0.3190 (Table 2). However, the total number of amino acids and peptides with corresponding sensory properties was higher in protein 4, with 163, compared to protein 2 with 83. These components with sweet sensory properties include V (valine), G (glycine), P (proline), A (alanine), and K (lysine). The activity of alanine, glycine, and proline possessing or imparting a sweet taste has been proven in multiple studies in several food samples (Lioe et al., 2005; Toelstede and Hoffman, 2009). The arrangement or configuration of amino acids has a significant influence on its taste-active properties, where commonly, most of the hydrophobic L-amino acids display bitterness. Meanwhile, D-amino acids tend to produce a sweet taste. For instance, proline and lysine have been described as both sweet and bitter (Chan and Cheung, 2010), L-configurated phenylalanine, valine, leucine, and tryptophan are bitter. However, these amino acids in D-configuration impart a sweet taste (Nishimura and Kato, 2009). This might explain the activity of the same amino acids sharing more than one or different taste properties in this study. According to Table 2, protein 1 showed the highest sweetness suppressing strength with an A value of 0.0086, followed by 0.0051 from protein 2. A total of two sweetness suppressing components were identified from protein 1 and five from protein 2. As shown in Table 2, sweet suppressing peptides are dipeptides Glu-Asp and Glu-Glu, which had been identified as umami peptides, in the interaction between taste components in food, enhancement, and masking of one taste properties do occur, umami taste perception

Table 2 BIOPEP-UWM screening analysis for peptide and amino acid with sensory properties

Protein	Possible taste active components					
	Umami\Umami enhancing	Bitter\Bitter suppressing	Sweet\Sweet suppressing	Sour	Salty\Salty enhancing	Astringent
Cardiac muscle myosin heavy chain 6 alpha	D(14), E(16), ED, DE(2), EE(2), VG(2), VGG, VD, DES, ES(2), AE(2), DA, DG, EL(2), EA, EK(2), KG, ET, TE, DEL, EY, AD (4), DL **[23,61]** **{A 0.2672}** \ PE **[1,1]** **{A 0.0043}**	R(9), P(5), F(9), V(13), L(19), K(18) PR, RF,KP, FG(3), GF, YG, YY, FI, AF, IF, YF, KF, VY, VL, VI, LE(3), VD, GE(2), AD(4), LD, ELL, LL(3), LG(2), GL(2), II, GI, LV, LI, DA, DL, EL, EI, EY, ADE, VG(2), DG, DL, DV, EE(2), EA, KG(3), VA, PA, FY, DY, YL(2), IA, IE, IN, IQ(2), PY **[57, 142]** **{A 0.6250}** \ R(9), K(18), ED, EE(2), ES(2), DES **[6,33]** **{A 0.1422}**	V(13), G(18), P(5), A(17), AA, GGA, ADE **[7,56]** **{A 0.3190}** \ ED, EE(2) **[2,2]** **{A 0.0086}**	D(14), E(16), K(18),DE(2), ED, VD, DV, ADE, DES **[9, 55]** **{A 0.2371}**	ED, DE(2), EE(2), DES **[4,6]** **{A 0.0862}** \ D(14),RV(3, VR **[3,18]** **{A 0.0172}**	K(18) **[1,18]** **{A 0.0776}**
Myosin light chain 1	D(14), E(21), ED(4), DE, EE, VE, VD, EDE, EV, AE(2), DA(2), EL, EG(2), EA, EK(3), ET, EDF, EK(3), ET, EDF, AD, VE **[22,65]** **{A 0.3077}** \ PE(2) **[1,2]** **{A 0.0103}**	R(6), P(17), F(9), V(11), L(14), K(19), PP, KP(3), PK(3), GF, FL, FI, AF, VF, LF, VL, VI, VE, IV, VD, GE, AD, LD, LG(3), GL, LI, IL, DA(2), DL, EG(2), K(19), EI, EF, DL, EE, EV, ID, EA, APK, VA, PA(5), DY, RL, IA, IE, IN, SL **[47,147]** **{A 0.6667}** \ R(6), K(19), ED(4), EE **[4,30]** **{A 0.1538}**	K(19), V(11), G(10), P(17), A(21), AA(4), EV **[7,83]** **{A 0.4256}** \ ED(4), EE **[2,5]** **{A 0.0051}**	D(14), E(21), K(19),DE, ED(4), VE, VD, EV, SPE **[9,63]** **{A 0.3231}**	ED(4), DE, EE, EDE, SPE **[5,8]** **{A 0.1128}** \ D(14), RA, RV **[3,16]** **{A 0.0103}**	K(19) **[1,19]** **{A 0.0974}**

Table 2 (continued)

Protein	Possible taste active components					
	Umami\Umami enhancing	Bitter\Bitter suppressing	Sweet\Sweet suppressing	Sour	Salty\Salty enhancing	Astringent
Cytochrome B	D(11), E(6), VG(2), VV(3), VE, VD(2), DA(2), EL, ET(2), TE, AD(2), VE, DL(2) **[13,36]** **{A 0.0895}** \ PE **[1,1]** **{A 0.0026}**	R(8), P(21), F(30), V(28), L(56), K(8), W(13) [7,164], PP, RG(2), FF(3), FFF, RP, RF, KP, FG, GF(2), GGF, YG(2), GY, YY, YYG, FV(2), FL(6), FI(2), FP, FPF, AF(4), LF(7), IF, YF(3), PF(2), DLL, GV(3), GGV, GVV, VL(5), VI(3), VE, LE, VD(2), AD(2), LL(11), LG(4), GL(4), II(3), IG(3), GI, LLL(2), LLLL, LGG, LLG(3), LGL, LV(6), LI, IL(6), DA(2), DL(2), EL, SLA, VV(3), VG(2), WL(2), FW(2), WW, IW, PL(2), DV(2), VA(3), LA(6), PA(3), GLY, LY(3), YL, IA(5), IP, PY(2), SL(4) **[77,329]** **{A 0.8711}** \ R(8), K(8) **[2,16]** **{A 0.0421}**	K(8), V(28), G(26), P(21), A(34) **[5,117]** **{A 0.3079}** \ -	D(11), E(6), K(8), VE, VD(2),DV(2) **[6,30]** **{A 0.0789}**	D(14), DD, EE **[3,16]** **{A 0.0289}** \ D(11), AR, RG(2), RS, RP **[5,16]** **{A 0.0132}**	K(8) **[1,8]** **{A 0.0211}**

Table 2 (continued)

Protein	Possible taste active components					
	Umami\Umami enhancing	Bitter\Bitter suppressing	Sweet\Sweet suppressing	Sour	Salty\Salty enhancing	Astringent
NADH-Ubiquinone Oxidoreductase Chain 5	D(13), E(13), ED(2), DE, VG(2), VV, EV, ES, DA, EL, EG, EA, EK, KG, AD **[15,41]** **{A 0.0704}** \ -	R(9), P(28), F(41), V(23), L(87), K(23), W(14), PP,FF(5), PR, RF(2), PK(3), GR, GP(2), FG(2), GF, GFF, YG, GY, YY, FV(2), FL(9), FI(2), FP, AF(5), LF(4), IF (6), YF, PF(3), KF, VIF, YP, GV, VL, VI(5), V, LE(2), AD, LL(8), LG(5), GL(9), II(4), IG(5), GI(3), LGL (2), GGL, LV(2), LI(13), IL(5), DA(2), EG(2), EL, EI, EF, SLA, VV, VG(2), WL, WF, WW, PL(2), EV, EA, KG, FLL(3), VA(3), LA(12), PA(3), DY, LY, YL(2), RL(3), IA(4), IE, IN(4), IQ, PY, SL(8) **[79,414]** **{A 0.6890}** \ R(9), K(23), ED(2), ES **[4,35]** **{A 0.0573}**	K(23), V(23), G(32), P(28), A(52), AA(4), EV **[7,163]** **{A 0.2688}**	E(13), K(23), DE, ED(2), EV **[5,40]** **{A 0.0867}**	ED(2), DE **[2,3]** **{A 0.0262}** \ RV(2) **[1,2]** **{A 0.0033}**	K(23) **[1,23]** **{A 0.0376}**

especially coming from peptides was found to suppress bitterness via human taste receptor, where several interactions between umami components also have been found to impart bitterness and sourness (Briand and Salles, 2016).

Next, both protein 2 and protein 1 again showed the highest strength in terms of possible sour sensory properties with an A value of 0.3231 and 0.2371 as screened by the BIOPEP-UWM analysis shown in Table 2. These two proteins also showed a comparatively high total of sour components consisting of both amino acids and peptides, which a total of 63 components from protein 2 and 55 components from protein 1. Each amino acids and peptides of these sour active components include D (aspartic acid), E (glutamic acid), K (lysine), and peptides consisting of either aspartic acid, glutamic acid, or both. These amino acids have been known for their umami-active capability. However, these amino acids also elicit a sour taste since both have a predominant taste of sour as referring to L-amino acids taste properties in Linden and Lorient (1999). Both aspartic acid and glutamic acid in their dissociated form are sour stimuli. However, when in the form of sodium salt, these amino acids are described as umami, savory, meaty, or chicken broth taste (Nishimura and Kato, 2009). According to Kirimura et al. (1997), dipeptides containing glutamic acid and/or aspartic acid elicit a sour taste in water.

As for salty taste properties, it has been shown protein 2 possibly has the strongest salty sensory properties compared to the other proteins with A of 0.1128 then followed by 0.0862 from protein 1. These two proteins have the total salty components of eight from protein 2 and six components from protein 1. Salty active components screened by BIOPEP-UWM only comprise dipeptides and tripeptides, mostly including umami active dipeptides such as Glu-Asp, Asp-Glu, and Glu-Glu. According to literature, salty taste is elicited mainly by Na^+ with some other cations such as Ca^{2+}, K^+, and NH^{+4}, where it has also been reported that peptides do stimulate salty sensation, especially dipeptides hydrochlorides but not free amino acids (Briand and Salles, 2016; Nishimura and Kato, 2009). According to Table 2, these peptides sharing both salty taste and umami taste might result from synergistic interactions with other components to bring out a variety of tastes from individual components. Salty enhancing sensory properties have been observed highest on protein 1 with A value of 0.0172 and total number components of 18, followed by protein 3 with A 0.0132 and 16 total components of corresponding sensory properties. In a related study, Schindler et al. (2011) had documented a series of arginyl dipeptides, including Arg-Pro, Arg-Ala, Arg-Gly, and Val-Arg, as salty taste enhancing components in fish hydrolysates and fermented fish sauces. As the last sensory properties identified in the BIOPEP-UWM screening analysis, Astringent was found the strongest in protein 2 with A value of 0.0974 and a total number of components of 19 astringent amino acids, followed by protein 1 with A 0.0776 and a total number of components of 18 astringent amino acids.

In silico digestion analysis: protein 1

Table 3 shows all in silico enzyme actions on protein 1. In this simulated digestion, other than the enzyme chymotrypsin, the application of one enzyme towards protein 1 has been found not to produce an umami-active component at all. Among all two enzyme combinations applied, the combination of trypsin-chymotrypsin produced the highest A_E of 0.0083 compared to 0.0042 from other combinations. Meanwhile, for three enzymes, all combinations with trypsin-chymotrypsin managed to produce the highest A_E as well as the combination of chymotrypsin, cathepsin, and oligopeptidase. According to Palmer and Bonner (2007), chymotrypsin and trypsin are serine proteases that are considered to give out their function with a common mechanism and optimum activity at pH 8. These endopeptidases break down peptide bonds in the middle polypeptide chains, which chymotrypsin with a wide hydrophobic active site will bind and cleave carbonyl sides of phenylalanine, tryptophan, and tyrosine chains. Meanwhile, trypsin has specificity in cleaving basic side chains of amino residues, including lysine or arginine (Terra and Ferreira, 2005).

All enzymes and combined enzymes were observed to yield bitter active components, which these biter components were comprised of both amino acids and peptides rather than peptides as the sole active components for umami taste. In single enzyme application, the highest number of bitter active components were produced by enzyme and oligopeptidase with A_E of 0.0083, meanwhile in two enzyme applications, both combinations of trypsin-chymotrypsin and chymotrypsin-oligopeptidase produced the highest strength of bitter active components with A_E of 0.1250 as well as three enzyme applications with trypsin-chymotrypsin and chymotrypsin, cathepsin and oligopeptidase combination. In terms of bitter suppressing attribute, for single enzyme application, it has been observed that only enzyme trypsin and oligopeptidase managed to yield bitter suppressing components from protein 1, with A_E of 0.0083 for both enzymes. As for two and three enzyme applications, the combination consisting of trypsin-chymotrypsin and chymotrypsin-oligopeptidase scored the highest A_E with a value of 0.0292 followed by 0.0250 by other combinations.

As shown in Table 3, components from protein 1 with sweet, sour, and astringent activity have a common relationship with singular and combined enzymes applied. According to the outcome obtained, a single enzyme application did not produce any taste components at all. The combination of enzyme trypsin-chymotrypsin and chymotrypsin-oligopeptidase yielded the highest number of sweet, sour,

Table 3 Potential sensory attributes from Cardiac muscle myosin heavy chain 6 alpha after in silico digestion

Enzyme	Sensory Attribute				
	Umami	Bitter \ Bitter suppressing	Sweet	Sour	Astringent
Trypsin	–	R(2) **[1,2] {A_E 0.0083}** \ R(2) **[1,2] {A_E 0.0083}**	–	–	–
Chymotrypsin	EY **[1,1] {A_E 0.0042}**	F(2), L(7), GF, IF, KF, GL, EY, DY, PY **[9,16] {A_E 0.0667}**	–	–	–
Cathepsin	–	F(2), L(7), DY, GF, IF, KF, GL **[7,14] {A_E 0.0583}**	–	–	–
Pepsin	–	L(3), GF **[2,4] {A_E 0.0167}**	–	–	–
Oligopeptidase	–	R(2) **[1,2] {A_E 0.0083}** \ R(2) **[1,2] {A_E 0.0083}**	–	–	–
Trypsin + Chymotrypsin	EK, EY **[2,2] {A_E 0.0083}**	R(2), F(5), L(10), K(5), PR, GF, IF, GL(2), EY, DY, PY **[11,30] {A_E 0.1250}** \ R(2), K(5) **[2,7] {A_E 0.0292}**	K(5) **[1,5] {A_E 0.0208}**	K(5) **[1,5] {A_E 0.0208}**	K(5) **[1,5] {A_E 0.0208}**
Trypsin + Cathepsin	EK **[1,1] {A_E 0.0042}**	R(2), F(5), L(10), K(4), PR, GF, IF, GL(2), DY [9,27] \ R(2), K(4) **[2,6] {A_E 0.1125}** \ R(2), K(4) **[2,6] {A_E 0.0250}**	K(4) **[1,4] {A_E 0.0167}**	K(4) **[1,4] {A_E 0.0167}**	K(4) **[1,4] {A_E 0.0167}**
Trypsin + Pepsin	EK **[1,1] {A_E 0.0042}**	R(2), F(3), L(6), K(2), GF, GL **[6,15] {A_E 0.0625}** \ R(2), K(2) **[2,4] {A_E 0.0167}**	K(2) **[1,2] {A_E 0.0083}**	K(2) **[1,2] {A_E 0.0083}**	K(2) **[1,2] {A_E 0.0083}**
Trypsin + Oligopeptidase	–	R(2) **[1,2] {A_E 0.0083}** \ R(2) **[1,2] {A_E 0.0083}**	–	–	–
Chymotrypsin + Cathepsin	EY **[1,1] {A_E 0.0042}**	F(2), L(7), GF, IF, KF, GL, EY, DY, PY **[9,16] {A_E 0.0667}**	–	–	–
Chymotrypsin + Pepsin	EY **[1,1] {A_E 0.0042}**	F(2), L(7), GF, IF, KF, GL, EY, DY, PY **[9,16] {A_E 0.0667}**	–	–	–
Chymotrypsin + Oligopeptidase	EK **[1,1] {A_E 0.0042}**	R(2), F(5), L(10), K(5), PR, GF, IF, GL(2), EY, DY, PY **[11,30] {A_E 0.1250}** \ K(5) **[1,5] {A_E 0.0292}**	K(5) **[1,5] {A_E 0.0208}**	K(5) **[1,5] {A_E 0.0208}**	K(5) **[1,5] {A_E 0.0208}**
Cathepsin + Pepsin	–	F(2), L(7), GF, IF, KF, GL, DY **[7,14] {A_E 0.0583}**	–	–	–
Cathepsin + Oligopeptidase	EK **[1,1] {A_E 0.0042}**	R(2), F(5), L(10), K(4), PR, GF, IF, GL(2), DY **[9,27] {A_E 0.1125}** \ R(2), K(4) **[2,6] {A_E 0.0250}**	K(4) **[1,4] {A_E 0.0167}**	K(4) **[1,4] {A_E 0.0167}**	K(4) **[1,4] {A_E 0.0167}**
Pepsin + Oligopeptidase	EK **[1,1] {A_E 0.0042}**	R(2), F(3), L(6), K(2), GF, GL **[6,15] {A_E 0.0625}** \ R(2), K(2) **[2,4] {A_E 0.0167}**	K(2) **[1,2] {A_E 0.0083}**	K(2) **[1,2] {A_E 0.0083}**	K(2) **[1,2] {A_E 0.0083}**
Trypsin + Chymotrypsin + Cathepsin	EY, EK **[2,2] {A_E 0.0083}**	R(2), F(4), L(10), K(5), PR, GF, IF, GL(2), EY, DY, PY **[11,29] {A_E 0.1250}** \ R(2), K(5) **[2,7] {A_E 0.0292}**	K(5) **[1,5] {A_E 0.0208}**	K(5) **[1,5] {A_E 0.0208}**	K(5) **[1,5] {A_E 0.0208}**

Table 3 (continued)

Enzyme	Sensory Attribute				
	Umami	Bitter \ Bitter suppressing	Sweet	Sour	Astringent
Trypsin + Chymotrypsin + Pepsin	EK, EY **[2,2] {A_E 0.0083}**	R(2), F(5), L(10), K(5), PR, GF, IF, GL(2), EY, DY, PY **[11,30] {A_E 0.1250}** \ R(2), K(5) **[2,7] {A_E 0.0292}**	K(5) **[1,5] {A_E 0.0208}**	K(5) **[1,5] {A_E 0.0208}**	K(5) **[1,5] {A_E 0.0208}**
Trypsin + Chymotrypsin + Oligopeptidase	EK, EY **[2,2] {A_E 0.0083}**	R(2), F(5), L(10), K(5), PR, GF, IF, GL(2), EY, DY, PY **[11,30] {A_E 0.1250}** \ R(2), K(5) **[2,7] {A_E 0.0292}**	K(5) **[1,5] {A_E 0.0208}**	K(5) **[1,5] {A_E 0.0208}**	K(5) **[1,5] {A_E 0.0208}**
Trypsin + Cathepsin + Pepsin	EK **[1,1] {A_E 0.0042}**	R(2), F(5), L(10), K(4), PR, GF, IF, GL(2), DY **[9,27] {A_E 0.1125}** \ R(2), K(4) **[2,6] {A_E 0.0250}**	K(4) **[1,4] {A_E 0.0167}**	K(4) **[1,4] {A_E 0.0167}**	K(4) **[1,4] {A_E 0.0167}**
Trypsin + Cathepsin + Oligopeptidase	EK **[1,1] {A_E 0.0042}**	R(2), F(5), L(10), K(4), PR, GF, IF, GL(2), DY **[9,27] {A_E 0.1125}** \ R(2), K(4) **[2,6] {A_E 0.0250}**	K(4) **[1,4] {A_E 0.0167}**	K(4) **[1,4] {A_E 0.0167}**	K(4) **[1,4] {A_E 0.0167}**
Chymotrypsin + Cathepsin + Pepsin	EY **[1,1] {A_E 0.0042}**	F(2), L(7), GF, IF, KF, GL, EY, DY, PY **[9,16] {A_E 0.0667}**	–	–	–
Chymotrypsin + Cathepsin + Oligopeptidase	EK, EY **[2,2] {A_E 0.0083}**	R(2), F(5), L(10), K(5), PR, GF, IF, GL(2), DY, PY **[10,29] {A_E 0.1250}** \ R(2), K(5) **[2,7] {A_E 0.0292}**	K(5) **[1,5] {A_E 0.0208}**	K(5) **[1,5] {A_E 0.0208}**	K(5) **[1,5] {A_E 0.0208}**
Cathepsin + Pepsin + Oligopeptidase	EK **[1,1] {A_E 0.0042}**	R(2) F(5), L(10), K(4), DY, PR, GF, IF, GL(2) **[9,27] {A_E 0.1125}** \ K(4) **[1,4] {A_E 0.0250}**	K(4) **[1,4] {A_E 0.0167}**	K(4) **[1,4] {A_E 0.0167}**	K(4) **[1,4] {A_E 0.0167}**

and astringent-active components with five in total with A_E of 0.0208 then followed by a total number of four with A_E 0.0167 from the other enzyme combinations. As for three enzyme applications, the same highest A_E can be observed from combinations including trypsin-chymotrypsin and chymotrypsin-oligopeptidase.

In silico digestion analysis: protein 2

As shown in Table 4, the application of single enzyme towards protein 2 did not produce umami-active components at all. As for double enzyme combination, trypsin-chymotrypsin and chymotrypsin-oligopeptidase produced the strongest umami components with A_E of 0.0099, which can also be seen as the highest value throughout all enzyme combinations. In three enzyme applications, all combinations including trypsin-chymotrypsin and chymotrypsin-oligopeptidase, scored the highest A_E, followed by 0.0049 by the other three enzyme combinations excluding chymotrypsin, cathepsin, and pepsin combination.

In using a single enzyme for digestion to analyze bitter active components, pepsin produced the lowest of all common A_E of 0.0197 from other enzymes. In two and three enzyme applications, combinations including trypsin-chymotrypsin and chymotrypsin-oligopeptidase scored the highest A_E of 0.0985 and followed by 0.0837 as the second-highest by other enzyme combinations. On the other hand, bitter suppressing components have been observed to be produced by only trypsin and oligopeptidase in a single enzyme application with an A_E of 0.0197. Meanwhile, for two and three enzyme usages can be seen highest with the value of A_E 0.0542 from enzyme combinations comprising of trypsin-chymotrypsin and chymotrypsin-oligopeptidase. According to Morty and Burleigh (2013), oligopeptidase B hydrolyzes peptide bonds of a small peptide and low molecular weight peptide with cleaving specificity on the carboxy side basic amino acid which preference are more on Arg rather than Lys (basic amino acid residues), which the cleaving also includes glycine, phenylalanine, leucine, threonine and proline.

Table 4 Potential sensory attributes from Myosin light chain 1 after in silico digestion

Enzyme	Sensory Attribute				
	Umami	Bitter \ Bitter suppressing	Sweet	Sour	Astringent
Trypsin	-	R, K(3) **[2,4] {A_E 0.0197}** \ R, K(3) **[2,4] {A_E 0.0197}**	K(3) **[1,3] {A_E 0.0148}**	K(3) **[1,3] {A_E 0.0148}**	K(3) **[1,3] {A_E 0.0148}**
Chymotrypsin	–	F, L(2), IL **[3,4] {A_E 0.0197}**	–	–	–
Cathepsin	–	F, L(2), IL **[3,4] {A_E 0.0197}**	–	–	–
Pepsin	–	F, L, IL **[3,3] {A_E 0.0148}**	–	–	–
Oligopeptidase	–	R, K(3) **[2,4] {A_E 0.0197}** \ R, K(3) **[2,4] {A_E 0.0197}**	K(3) **[1,3] {A_E 0.0148}**	K(3) **[1,3] {A_E 0.0148}**	K(3) **[1,3] {A_E 0.0148}**
Trypsin + Chymotrypsin	EK(2) **[1,2] {A_E 0.0099}**	R(5), F, L(5), K(6), VF, IL, APK **[7,20] {A_E 0.0985}** \ R(5), K(6) **[2,11] {A_E 0.0542}**	K(6) **[1,6] {A_E 0.0296}**	K(6) **[1,6] {A_E 0.0296}**	K(6) **[1,6] {A_E 0.0296}**
Trypsin + Cathepsin	EK **[1,1] {A_E 0.0049}**	R(5), F, L(5), K(3), VF, IL, APK **[7,17] {A_E 0.0837}** \ R(5), K(3) **[2,8] {A_E 0.0394}**	K(3) **[1,3] {A_E 0.0148}**	K(3) **[1,3] {A_E 0.0148}**	K(3) **[1,3] {A_E 0.0148}**
Trypsin + Pepsin	–	R(4), F, L(4), K(3), VF, IL **[6,14] {A_E 0.0690}** \ R(4), K(3) **[2,7] {A_E 0.0345}**	K(3) **[1,3] {A_E 0.0148}**	K(3) **[1,3] {A_E 0.0148}**	K(3) **[1,3] {A_E 0.0148}**
Trypsin + Oligopeptidase	–	R, K(3) **[2,4] {A_E 0.0197}** \ R, K(3) **[2,4] {A_E 0.0197}**	K(3) **[1,3] {A_E 0.0148}**	K(3) **[1,3] {A_E0.0148}**	K(3) **[1,3] {A_E 0.0148}**
Chymotrypsin + Cathepsin	–	F, L(2), IL **[3,4] {A_E 0.0197}**	–	–	–
Chymotrypsin + Pepsin	–	F, L(2), IL **[3,4] {A_E 0.0197}**	–	–	–
Chymotrypsin + Oligopeptidase	EK(2) **[1,2] {A_E 0.0099}**	R(5), L(5), K(6), VF, IL, APK **[6,19] {A_E 0.0985}** \ R(5), K(6) **[2,11] {A_E 0.0542}**	K(6) **[1,6] {A_E 0.0296}**	K(6) **[1,6] {A_E 0.0296}**	K(6) **[1,6] {A_E 0.0296}**
Cathepsin + Pepsin	–	F, L(2), IL **[3,4] {A_E 0.0197}**	–	–	–
Cathepsin + Oligopeptidase	EK **[1,1] {A_E 0.0049}**	R(5), K(3), F, L(5), VF, IL, APK **[7,17] {A_E 0.0837}** \ R(5), K(3) **[2,8] {A_E 0.0394}**	K(3) **[1,3] {A_E 0.0148}**	K(3) **[1,3] {A_E 0.0148}**	K(3) **[1,3] {A_E 0.0148}**

Table 4 (continued)

Enzyme	Sensory Attribute				
	Umami	Bitter \ Bitter suppressing	Sweet	Sour	Astringent
Pepsin + Oligopeptidase	–	R(4), F, L(4), K(3), VF, IL [6,14] **{A_E 0.0690}** \ R(4), K(3) **[2,7] {A_E 0.0345}**	K(3) **[1,3] {A_E 0.0148}**	K(3) **[1,3] {A_E 0.0148}**	K(3) **[1,3] {A_E 0.0148}**
Trypsin + Chymotrypsin + Cathepsin	EK(2) **[1,2] {A_E 0.0099}**	R(5), F, L(5), K(6), VF, IL, APK **[7,20] {A_E 0.0985}** \ R(5), K(6) **[2,11] {A_E 0.0542}**	K(6) **[1,6] {A_E 0.0296}**	K(6) **[1,6] {A_E 0.0296}**	K(6) **[1,6] {A_E 0.0296}**
Trypsin + Chymotrypsin + Pepsin	EK(2) **[1,2] {A_E 0.0099}**	R(5), F, L(5), K(6), VF, IL, APK **[7,20]** \ R(5), K(6) **[2,11]**	K(6) **[1,6] {A_E 0.0296}**	K(6) **[1,6] {A_E 0.0296}**	K(6) **[1,6] {A_E 0.0296}**
Trypsin + Chymotrypsin + Oligopeptidase	EK(2) **[1,2] {A_E 0.0099}**	R(5), F, L(5), K(6), VF, IL, APK **[7,20] {A_E 0.0985}** \ R(5), K(6) **[2,11] {A_E0.052}**	K(6) **[1,6] {A_E 0.0296}**	K(6) **[1,6] {A_E 0.0296}**	K(6) **[1,6] {A_E 0.0296}**
Trypsin + Cathepsin + Pepsin	EK **[1,1] {A_E 0.0049}**	R(5), F, L(5), K(3), VF IL, APK **[7,17] {A_E 0.0837}** \ R(5), K(3) **[2,8] {A_E 0.0394}**	K(3) **[1,3] {A_E 0.0148}**	K(3) **[1,3] {A_E 0.0148}**	K(3) **[1,3] {A_E 0.0148}**
Trypsin + Cathepsin + Oligopeptidase	EK **[1,1] {A_E 0.0049}**	R(5), F, L(5), K(3), VF, IL, APK **[7,17] {A_E 0.0837}** \ R(5), K(3) **[2,8] {A_E 0.0394}**	K(3) **[1,3] {A_E 0.0148}**	K(3) **[1,3] {A_E 0.0148}**	K(3) **[1,3] {A_E 0.0148}**
Chymotrypsin + Cathepsin + Pepsin	–	F, L(2), IL **[3,4] {A_E 0.0197}**	–	–	–
Chymotrypsin + Cathepsin + Oligopeptidase	EK(2) **[1,2] {A_E 0.0099}**	R(5), L(5), K(6), VF, IL, APK **[6,17] {A_E 0.0985}** \ R(5), K(6) **[2,11] {A_E 0.0542}**	K(6) **[1,6] {A_E 0.0296}**	K(6) **[1,6] {A_E 0.0296}**	K(6) **[1,6] {A_E 0.0296}**
Cathepsin + Pepsin + Oligopeptidase	EK **[1,1] {A_E 0.0049}**	R(5), L(5), K(3), VF, IL, APK **[6,16] {A_E 0.0837}** \ R(5), K(3) **[2,8] {A_E 0.0394}**	K(3) **[1,3] {A_E 0.0148}**	K(3) **[1,3] {A_E 0.0148}**	K(3) **[1,3] {A_E 0.0148}**

Single enzyme usage in producing sweet, sour, and astringent components in protein 2 can only be seen in the application of trypsin and oligopeptidase, with both given A_E values of 0.0148. In a combined enzymes application, combinations comprising enzymes trypsin-chymotrypsin and chymotrypsin-cathepsin are able to produce the highest A_E of 0.0296 rather than 0.0148 from other combinations except for chymotrypsin-cathepsin and chymotrypsin-pepsin.

In silico digestion analysis: protein 3

Table 5 shows a series of enzyme actions towards protein 3. According to the result obtained, all five single enzymes

Table 5 Potential sensory attributes from Cytochrome B after in silico digestion

Enzyme	Sensory Attribute				
	Umami	Bitter \ Bitter suppressing	Sweet	Sour	Astringent
Trypsin	–	K **[1,1] {A_E 0.0025}** \ K **[1,1] {A_E 0.0025}**	K **[1,1] {A_E 0.0025}**	K **[1,1] {A_E 0.0025}**	K **[1,1] {A_E 0.0025}**
Chymotrypsin	–	F(16), L(23), W(4), GF, GGF, PF(2),VL(2), GL, IW, PL(2), PY **[11,54] {A_E 0.1371}**	–	–	–
Cathepsin	–	F(14), L(19), GF, PF(2), VL(2), GL, PL, PY **[8,41] {A_E 0.1041}**	–	–	–
Pepsin	–	F(10), L(16), GF, YF, PF, VL, GL **[7,31] {A_E 0.0787}**	–	–	–
Oligopeptidase	–	K **[1,1] {A_E 0.0025}** \ K **[1,1] {A_E 0.0025}**	K **[1,1] {A_E 0.0025}**	K **[1,1] {A_E 0.0025}**	K **[1,1] {A_E 0.0025}**
Trypsin + Chymotrypsin	DL **[1,1] {A_E 0.0025}**	R(3), F(17), L(24), K(5), W(4), GF, GGF, PF(2), VL(2), GL(2), DL, IW, PL(2), PY **[14,66] {A_E 0.1701}** \ R(3), K(5) **[2,8] {A_E 0.0203}**	K(5) **[1,5] {A_E 0.0127}**	K(5) **[1,5] {A_E 0.0127}**	K(5) **[1,5] {A_E 0.0127}**
Trypsin + Cathepsin	DL **[1,1] {A_E 0.0025}**	R(3), F(15), L(20), K(3), GF, PF(2), VL(2),GL(2), PL, DL, PY **[11,51] {A_E 0.1320}** \ R(3), K(3) **[2,6] {A_E 0.0152}**	K(3) **[1,3] {A_E 0.0076}**	K(3) **[1,3] {A_E 0.0076}**	K(3) **[1,3] {A_E 0.0076}**
Trypsin + Pepsin	DL **[1,1] {A_E 0.0025}**	R(3), F(11), L(17), K(2) GF, YF, PF, VL GL(2), DL **[10,40] {A_E 0.1041}** \ R(3), K(2) **[2,5] {A_E 0.0127}**	K(2) **[1,2] {A_E 0.0051}**	K(2) **[1,2] {A_E 0.0051}**	K(2) **[1,2] {A_E 0.0051}**
Trypsin + Oligopeptidase	–	K **[1,1]{A_E 0.0025}** \ K **[1,1] {A_E 0.0025}**	K **[1,1] {A_E 0.0025}**	K **[1,1] {A_E 0.0025}**	K **[1,1] {A_E 0.0025}**
Chymotrypsin + Cathepsin	–	F(16), L(23), W(4), GF, GGF, PF(2), VL(2), GL, IW, PL(2), PY **[11,54] {A_E 0.1371}**	–	–	–
Chymotrypsin + Pepsin	–	F(16), L(23), W(4), GF, GGF, PF(2), VL(2), GL, IW, PL(2), PY **[11,54] {A_E 0.1371}**	–	–	–
Chymotrypsin + Oligopeptidase	DL **[1,1] {A_E 0.0025}**	R(3), F(17), L(24), K(5), GF, GGF, PF(2), VL(2), GL(2), DL, IW, PL(2), PY **[13,62] {A_E 0.1701}** \ R(3), K(5) **[2,8] {A_E 0.0203}**	K(5) **[1,5] {A_E 0.0127}**	K(5) **[1,5] {A_E 0.0127}**	K(5) **[1,5] {A_E 0.0127}**
Cathepsin + Pepsin	–	F(14), L(19), GF, PF(2), VL(2), GL, PL, PY **[8,41] {A_E 0.1041}**	–	–	–
Cathepsin + Oligopeptidase	DL **[1,1] {A_E 0.0025}**	R(3), F(15), L(20), K(3), GF, PF(2), VL(2), GL(2), DL, PY **[10,48] {A_E 0.1320}** \ R(3), K(3) **[2,6] {A_E 0.0152}**	K(3) **[1,3] {A_E 0.0076}**	K(3) **[1,3] {A_E 0.0076}**	K(3) **[1,3] {A_E 0.0076}**

Table 5 (continued)

Enzyme	Sensory Attribute				
	Umami	Bitter \ Bitter suppressing	Sweet	Sour	Astringent
Pepsin + Oligopeptidase	DL **[1,1] {A_E 0.0025}**	R(3), F(11), L(17), K(2), GF, YF, PF, VL, GL(2), DL **[10,40] {A_E 0.1041}** \ R(3), K(2) **[2,5] {A_E 0.0127}**	K(2) **[1,2] {A_E 0.0051}**	K(2) **[1,2] {A_E 0.0051}**	K(2) **[1,2] {A_E 0.0051}**
Trypsin + Chymotrypsin + Cathepsin	DL **[1,1] {A_E 0.0025}**	R(3), F(17), L(24), K(5), W(4), GF, GGF, PF(2), VL(2), GL(2), IW, PL(2), DL, PY **[14,66] {A_E 0.1701}** \ R(3), K(5) **[2,8] {A_E 0.0203}**	K(5) **[1,5] {A_E 0.0127}**	K(5) **[1,5] {A_E 0.0127}**	K(5) **[1,5] {A_E 0.0127}**
Trypsin + Chymotrypsin + Pepsin	DL **[1,1] {A_E 0.0025}**	R(3), F(17), L(24), K(5), W(4), GF, GGF, PF(2), VL(2), GL(2), DL, IW PL(2), PY **[14,66] {A_E 0.1701}** \ R(3), K(5) **[2,8] {A_E 0.0203}**	K(5) **[1,5] {A_E 0.0127}**	K(5) **[1,5] {A_E 0.0127}**	K(5) **[1,5] {A_E 0.0127}**
Trypsin + Chymotrypsin + Oligopeptidase	DL **[1,1] {A_E 0.0025}**	R(3), F(17), L(24), W(4), K(5), GF, GGF, PF(2), VL(2), GL(2), DL, IW, PL(2), PY **[14,66] {A_E 0.1701}** \ R(3), K(5) **[2,8] {A_E 0.0203}**	K(5) **[1,5] {A_E 0.0127}**	K(5) **[1,5] {A_E 0.0127}**	K(5) **[1,5] {A_E 0.0127}**
Trypsin + Cathepsin + Pepsin	–	R(3), F(15), L(20), K(3), GF, PF(2), VL(2), GL(2), PY **[9,49] {A_E 0.1320}** \ R(3), K(3) **[2,6] {A_E 0.0152}**	K(3) **[1,3] {A_E 0.0076}**	K(3) **[1,3] {A_E 0.0076}**	K(3) **[1,3] {A_E 0.0076}**
Trypsin + Cathepsin + Oligopeptidase	DL **[1,1] {A_E 0.0025}**	R(3), F(15), L(20), K(3), GF, PF(2), VL(2), GL(2), DL, PY **[10,50] {A_E 0.1320}** \ R(3), K(3) **[2,6] {A_E 0.0152}**	K(3) **[1,3] {A_E 0.0076}**	K(3) **[1,3] {A_E 0.0076}**	K(3) **[1,3] {A_E 0.0076}**
Chymotrypsin + Cathepsin + Pepsin	–	F(16), L(23), W(4), GF, GGF, PF(2), VL(2), GL, IW, PL(2), PY **[11,54] {A_E 0.1371}**	–	–	–
Chymotrypsin + Cathepsin + Oligopeptidase	DL **[1,1] {A_E 0.0025}**	R(3), F(17), L(24), K(5), W(4), GF, GGF, PF(2), VL(2), GL(2), DL, IW, PL(2), DL, PY **[15,67] {A_E 0.1701}** \ R(3), K(5) **[2,8] {A_E 0.0203}**	K(5) **[1,5] {A_E 0.0127}**	K(5) **[1,5] {A_E 0.0127}**	K(5) **[1,5] {A_E 0.0127}**
Cathepsin + Pepsin + Oligopeptidase	DL **[1,1] {A_E 0.0025}**	R(3), F(15), L(20), K(3), GF, PF(2), VL(2), GL(2), DL, PL, PY **[11,51] {A_E 0.1320}** \ R(3), K(3) **[2,6] {A_E 0.00152}**	K(3) **[1,3] {A_E 0.0076}**	K(3) **[1,3] {A_E 0.0076}**	K(3) **[1,3] {A_E 0.0076}**

did not produce umami-active components towards the selected protein, which only combined enzymes, either two or three enzyme applications, managed to release umami active dipeptide with A_E of 0.0025. Among the combined enzymes applied, trypsin-oligopeptidase, chymotrypsin-cathepsin, chymotrypsin-pepsin, and cathepsin-pepsin did not produce umami-active component at all.

Then, bitter active components were released from all five single enzymes, which were found highest from enzyme chymotrypsin with A_E 0.1371 and followed by 0.1041 by enzyme cathepsin. The combination of trypsin-chymotrypsin was observed to attain the highest in releasing bitter active components with an A_E value of 0.1701 either in two or three enzyme usage for protein 3. Meanwhile, only trypsin

and oligopeptidase yet again have the capability to release bitter suppressing components with A_E 0.0025. Looking at two and three enzyme applications, the highest A_E 0.0203 came from combinations of enzymes with trypsin-chymotrypsin and chymotrypsin-oligopeptidase.

In the release of sweet, sour, and astringent components from protein 3, it can be seen on the action from enzyme trypsin and oligopeptidase, with both sharing the same strength of releasing these taste active components with A_E of 0.0025. As can be seen from the table, the release of sweet, sour, and astringent components were highest with an A_E of 0.0127 from the combination of enzymes with trypsin-chymotrypsin and chymotrypsin-oligopeptidase, followed by 0.0076 and 0.0051 from other enzyme combinations.

In silico digestion analysis: protein 4

According to Table 6, single enzyme application towards protein 4 has been observed limited in releasing only one umami active component when compared to the combination of two and three enzymes. In a single enzyme action, chymotrypsin and cathepsin each release one umami dipeptide with A_E 0.0016 while other enzyme usage did not. As for combined enzymes, the highest A_E obtained for both two and three enzyme combinations were 0.0032 with two of the same umami active dipeptides followed by a 0.0016 A_E value. In referring to Table 6, two umami dipeptides can be released from protein 4 by using enzyme combination comprising trypsin-chymotrypsin, chymotrypsin-oligopeptidase, and cathepsin-oligopeptidase.

As for the release of bitter active components from protein 4, every five single enzyme applications managed to release bitter components with different release capabilities. According to Table 6, both trypsin and oligopeptidase only managed in releasing bitter amino acids with A_E of 0.0047. Meanwhile, the other enzymes produced both bitter peptides and amino acids. The highest number of bitter components with an A_E value of 0.1074 was released by the enzyme chymotrypsin. In the application of two and three enzymes, the highest A_E value obtained from these enzyme combinations was 0.1422, which were found on combinations with trypsin-chymotrypsin and chymotrypsin-oligopeptidase. On the other hand, only trypsin and oligopeptidase could release bitter suppressing components in the application of a single enzyme comprising two kinds of amino acids with A_E of 0.0047, and both share the same bitter suppressing and bitter components. As for two and three enzyme applications, combinations with trypsin-chymotrypsin and chymotrypsin-oligopeptidase produced the highest A_E of 0.0205 and followed by 0.0108 by other enzyme combinations.

For the remaining sensory attributes of sweet, sour, and astringent assessed, it is observed that only trypsin and oligopeptidase are able to release these taste active components with an A_E value of 0.0032 in the application of a single enzyme. Looking at two and three usages of enzymes, combinations capable of producing the highest A_E of 0.0142 from protein 4 are the enzyme combinations including trypsin-chymotrypsin and chymotrypsin-oligopeptidase, then followed by 0.0126 as the second-highest A_E. According to Alvarez et al. (2012), sequencing and gene encoding of oligopeptidase B has shown its similarities with prolyl oligopeptidase, grouping it along with the S9A family, which prolyl oligopeptidase specifically cleaves peptide bonds at the C-terminus of proline. At the same time, oligopeptidase B also portrayed carboxypeptidase-like activity, which is highly specific on basic amino acids with the requirement of two basic residues at C-terminus of the substrate.

In short, this work highlighted the application of the in silico approach, specifically BIOPEP-UWM to list out all taste active peptides and amino acids from a protein sequence. This study also showed the release of taste-active components from anchovy proteins with multiple enzyme treatments in simulating the breakdown of proteins during fermentation. Among the proteins taken for screening, protein 1 and protein 2 had shown a meaningful potential sensory profile since both proteins have almost double the total umami active components and significantly produced higher potential sweet and salty enhancing components than protein 3 and protein 4. The application of enzyme action in this in silico study showed better results of releasing a higher number of taste active components using combined enzymes instead of using a single enzyme. Among the 23 total enzyme treatments applied, the combination of trypsin-chymotrypsin and chymotrypsin-oligopeptidase towards hydrolyzing the selected proteins had shown almost consistent and good results in releasing higher number of taste active components compared to other enzyme combinations applied. Since oligopeptidase is an exogenous enzyme, this in silico analysis managed to show the significance of microbial community into producing taste active components during fermentation. Theoretical knowledge found in this study could be applied to determine enzyme combinations for further in vitro and in vivo research towards production of taste active components from anchovy proteins, which would be another measure in improving the taste quality of the fish sauce.

Table 6 Potential sensory attributes from NADH Ubiquinone Oxidoreductase chain 5 after in silico digestion

Enzyme	Sensory Attribute				
	Umami	Bitter \ Bitter suppressing	Sweet	Sour	Astringent
Trypsin	–	R, K(2) **[2,3] {A_E 0.0047}** \ R, K(2) **[2,3] {A_E 0.0047}**	K(2) **[1,2] {A_E 0.0032}**	K(2) **[1,2] {A_E 0.0032}**	K(2) **[1,2] {A_E 0.0032}**
Chymotrypsin	EL **[1,1] {A_E 0.0016}**	F(15), L(25), W(3), GY, AF(2), IF(3), PF, KF, VL, GL(4), GGL, IL(2), EL, EF,PL(2), DY, IN, SL(3) **[18,68] {A_E 0.1074}**	–	–	–
Cathepsin	EL **[1,1] {A_E 0.0016}**	F(11), L(23), GY, AF(2), IF(3), PF, KF, VL, GL(4), GGL, IL(2), EL, EF, PL(2), DY, SL(3) **[16,58] {A_E 0.0916}**	–	–	–
Pepsin	–	F(9), L(17), AF, IF(3), KF, VL, GL(3), IL(2), EF, PL, SL **[11,40] {A_E 0.0632}**	–	–	–
Oligopeptidase	–	R, K(2) **[2,3] {A_E 0.0047}** \ R, K(2) **[2,3] {A_E 0.0047}**	K(2) **[1,2] {A_E 0.0032}**	K(2) **[1,2] {A_E 0.0032}**	K(2) **[1,2] {A_E 0.0032}**
Trypsin + Chymotrypsin	EL, EK **[2,2] {A_E 0.0032}**	R(4), L(31), K(9), W(3), GR, GY, AF(2), IF(3), PF, VIF, VL, GL(4), GGL, IL(2), ELEF, PL(2), DY, IN, SL(3) **[19,72] {A_E 0.1422}** \ R(4), K(9) **[2,13] {A_E 0.0205}**	K(9) **[1,9] {A_E 0.0142}**	K(9) **[1,9] {A_E 0.0142}**	K(9) **[1,9] {A_E 0.0142}**
Trypsin + Cathepsin	EL, EK **[2,2] {A_E 0.0032}**	R(2), F(13), L(29), K(8), GR, GY, AF(2), IF(3), PF, VIF, VL, GL(4), GGL, IL(2) EL, EF, PL(2), DY, SL(3) **[19,77] {A_E 0.1216}** \ R(2), K(8) **[2,10] {A_E 0.0158}**	K(8) **[1,8] {A_E 0.0126}**	K(8) **[1,8] {A_E 0.0126}**	K(8) **[1,8] {A_E 0.0126}**
Trypsin + Pepsin	–	R(2), F(11), L(23), K(8), AF, IF(3), VIF, VL, GL(3), IL(2), EF, PL, YL, SL **[14,59] {A_E 0.0932}** \ R(2), K(8) **[2,10] {A_E 0.0158}**	K(8) **[1,8] {A_E 0.0126}**	K(8) **[1,8] {A_E 0.0126}**	K(8) **[1,8] {A_E 0.0126}**
Trypsin + Oligopeptidase	–	R,K(2) **[2,3] {A_E 0.0047}** \ R, K(2) **[2,3] {A_E 0.0047}**	K(2) **[1,2] {A_E 0.0032}**	K(2) **[1,2] {A_E 0.0032}**	K(2) **[1,2] {A_E 0.0032}**
Chymotrypsin + Cathepsin	EL **[1,1] {A_E 0.0016}**	F(15), L(25), GY, AF(2), IF(3), PF, KF, VL, GL(4), GGL, IL(2), EL, EF, PL(2), W(3), DY, IN, SL(3) **[18,68] {A_E 0.1074}**	–	–	–
Chymotrypsin + Pepsin	EL **[1,1] {A_E 0.0016}**	F(15), L(25), W(3), GY, AF(2), IF(3), PF, KF, VL, GL(4), GGL, IL(2), EL, EF, PL(2), DY, IN, SL(3) **[18,66] {A_E 0.1074}**	–	–	–
Chymotrypsin + Oligopeptidase	EL, EK **[2,2] {A_E 0.0032}**	R(4), F(17), L(31), K(9), GR, GY, AF(2), IF(3), PF, VIF, VL, GL(4), GGL, IL(2), EL, EF, PL(2), W(3), DY, IN, SL(3) [21,90] \ R(4), K(9) **[2,13] {A_E 0.1422}** \ R(4), K(9) **[2,13] {A_E 0.0205}**	K(9) **[1,9] {A_E 0.0142}**	K(9) **[1,9] {A_E 0.0142}**	K(9) **[1,9] {A_E 0.0142}**
Cathepsin + Pepsin	EL **[1,1] {A_E 0.0016}**	F(11), L(23), GY, AF(2), IF(3), PF, KF, VL, GL(4), GGL, IL(2), EL, EF, PL(2), DY, SL(3) **[16,58] {A_E 0.0916}**	–	–	–

Table 6 (continued)

Enzyme	Sensory Attribute				
	Umami	Bitter \ Bitter suppressing	Sweet	Sour	Astringent
Cathepsin + Oligopeptidase	EL, EK **[2,2] {A_E 0.0032}**	R(2), F(13), L(29), K(8), GR, GY, AF(2), IF(3), PF, VIF, VL, GL(4), GGL, IL(2), EL, EF, PL(2), DY, SL(3) **[19,77] {A_E 0.1216}** \ R(2), K(8) **[2,10] {A_E 0.0158}**	K(8) **[1,8] {A_E 0.0126}**	K(8) **[1,8] {A_E 0.0126}**	K(8) **[1,8] {A_E 0.0126}**
Pepsin + Oligopeptidase	EK **[1,1] {A_E 0.0016}**	R(2),F(11), L(23), K(8), AF, IF(3), VIF, VL, GL(3), IL(2), EF, PL, YL, SL **[14,59] {A_E 0.0932}** \ R(2), K(8) **[2,10] {A_E 0.0158}**	K(8) **[1,8] {A_E 0.0126}**	K(8) **[1,8] {A_E 0.0126}**	K(8) **[1,8] {A_E 0.0126}**
Trypsin + Chymotrypsin + Cathepsin	EL, EK **[2,2] {A_E 0.0032}**	R(4), F(17), L(31), K(9), W(3), GR, GY, AF(2), IF(3), PF, VIF, VL, GL(4), GGL, IL(2), EL, EF, PL(2), DY, IN, SL(3) **[21,90] {A_E 0.1422}** \ R(4), K(9) **[2,13] {A_E 0.0205}**	K(9) **[1,9] {A_E 0.0142}**	K(9) **[1,9] {A_E 0.0142}**	K(9) **[1,9] {A_E 0.0142}**
Trypsin + Chymotrypsin + Pepsin	EL, EK **[2,2] {A_E 0.0032}**	R(4), F(17), L(31), K(9), W(3),GR, GY, AF(2), IF(3), PF, VIF, VL, GL(4), GGL, IL(2), EL, EF, PL(2), DY, IN, SL(3) **[21,90] {A_E 0.1422}** \ R(4), K(9) **[2,13] {A_E 0.0205}**	K(9) **[1,9] {A_E 0.0142}**	K(9) **[1,9] {A_E 0.0142}**	K(9) **[1,9] {A_E 0.0142}**
Trypsin + Chymotrypsin + Oligopeptidase	EL, EK **[2,2] {A_E 0.0032}**	R(4), F(17), L(31), K(9), W(3), GR, GY, AF(2), IF(3), PF, VIF, VL, GL(4), EF, PL(2), DY, IN, SL(3) **[18,86] {A_E 0.1422}** \ R(4), K(9) **[2,13] {A_E 0.0205}**	K(9) **[1,9] {A_E 0.0142}**	K(9) **[1,9] {A_E 0.0142}**	K(9) **[1,9] {A_E 0.0142}**
Trypsin + Cathepsin + Pepsin	EL, EK **[2,2] {A_E 0.0032}**	R(2), F(13), L(29), K(8), GR, GY, AF(2), IF(3), PF, VIF, VL, GL(4), GGL, IL(2), EL, EF, PL(2), DY, SL(3) **[19,77] {A_E 0.1216}** \ R(2), K(8) **[2,10] {A_E 0.0158}**	K(8) **[1,8] {A_E 0.0126}**	K(8) **[1,8] {A_E 0.0126}**	K(8) **[1,8] {A_E 0.0126}**
Trypsin + Cathepsin + Oligopeptidase	EL, EK **[2,2] {A_E 0.0032}**	R(2), F(13), L(29), K(8), GR, GY, AF(2), IF(3) PF, VIF, VL, GL(4), GGL, IL(2), EL, EF, PL(2), DY, SL(3) **[19,77] {A_E 0.1216}** \ R(2), K(8) **[2,10] {A_E 0.0158}**	K(8) **[1,8] {A_E 0.0126}**	K(8) **[1,8] {A_E 0.0126}**	K(8) **[1,8] {A_E 0.0126}**
Chymotrypsin + Cathepsin + Pepsin	EL **[1,1] {A_E 0.0016}**	F(15), L(25), W(3), GY, AF(2), IF(3), PF, KF, VL, GL(4), GGL, IL(2), EL, EF, PL(2), DY, IN, SL(3) **[18,68] {A_E 0.1074}**	–	–	–
Chymotrypsin + Cathepsin + Oligopeptidase	EL, EK **[2,2] {A_E 0.0032}**	R(4), F(17), L(31), K(9), W(3), GR, GY, AF(2), IF(3), PF, VIF, VL, GL(4), GGL, IL(2), EL, EF, PL(2), DY, IN, SL(3) **[21,90] {A_E 0.1422}** \ R(4), K(9) **[2,13] {A_E 0.0205}**	K(9) **[1,9] {A_E 0.0142}**	K(9) **[1,9] {A_E 0.0142}**	K(9) **[1,9] {A_E 0.0142}**

Table 6 (continued)

Enzyme	Sensory Attribute				
	Umami	Bitter \ Bitter suppressing	Sweet	Sour	Astringent
Cathepsin + Pepsin + Oligopeptidase	EL, EK **[2,2] {A_E 0.0032}**	R(2), F(13), L(29), K(8), GR, GY, AF(2), IF(3), PF, VIF, VL, GL(4), GGL, IL(2), EL, DY, SL(3) **[17,74] {A_E 0.1216}** \ R(2), K(8) **[2,10] {A_E 0.0158}**	K(8) **[1,8] {A_E 0.0126}**	K(8) **[1,8] {A_E 0.0126}**	K(8) **[1,8] {A_E 0.0126}**

Acknowledgements The work was supported by the Fundamental Research Grant Scheme (FRGS/1/2018/WAB09/UMT/03/2). The author appreciated everyone involved in the process of article writing.

Declarations

Conflict of interest All authors have declared no conflict of interest in this study.

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