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Evaluation of β-cyclodextrin masking effect on the bitterness of angelwing clam *(Pholas orientalis)* hydrolysate

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Abstract

Angelwing clam (*Pholas orientalis*) hydrolysate was prepared by hydrolysis using bromelain. The hydrolysate named as bromelain hydrolysate (BH) was then treated with β-cyclodextrin in the ratio of 1:0.8 (v/w) by physical mixing and kneading methods producing the physical mixed hydrolysate (PMH) and kneaded method hydrolysate (KMH), respectively. The masking effect of β-cyclodextrin on bitterness was evaluated based on sensory analysis, amino acid analysis and determination of flavor compound by gas chromatography- mass spectrometry (GC-MS) and field emission scanning electron microscope (FESEM). Sensory analysis showed that KMH has least bitter taste compared to BH. Amino acids analysis showed that hydrophobic amino acids content that contributed to the bitter taste were lower in KMH and PMH compared to BH. GC-MS analysis also showed that benzothiazole compounds were present in KMH. The absence of benzene, 1-phenyl-4-2-(2-cyano-2-phenylethyl) in KMH and PMH indicated that phenylalanine in BH had been masked by β-cyclodextrin. FESEM showed that the new solid phase formed by kneading method has a crystal structure which was completely different from the original morphology of BH and β-cyclodextrin. Therefore, the bitterness in BH had successfully been masked by β-cyclodextrin, thus indicates its potential to be used as food ingredient..

Introduction

Protein hydrolysate is used for various purposes such as food ingredient, fertilizer, protein supplement, beverage stabilizer and flavor enhancer (Nazeer *et al*., 2013). Angelwing clam (*Pholas orientalis*) is a marine bivalve species exhibiting a sweet taste, delicious flavor and soft texture (Tizon *et al*., 2012). This makes it suitable to be used as flavor enhancer or other food ingredient. However, most protein hydrolysate is mainly used for animal nutrition due to its bitter taste and fishy odor which limit its acceptability for human consumption (Thiansilakul *et al*., 2007). Thus, the production of protein hydrolysate with the non-bitter taste is important in order to pave the way for full utilization of the marine protein hydrolysate.

The hydrophobicity of a peptide is the key factor to bitterness in protein hydrolysate. It has been shown that blocking the hydrophobic side chains and α-amino groups of bitter amino acids effectively reduced bitterness (Tamura *et al*., 1989). Taste masking can be defined as covering, obscuring and disguising the taste of protein hydrolysate by the addition of a certain substances, without changing the component of the protein hydrolysates (Hamman and Calton, 2002). However, other studies stated that debittering of protein hydrolysate by masking or selective separation will give some changes on many properties of globular protein such as essential amino acids, viscosity, solubility, hydration and stability of nutraceutical antioxidant (Hou *et al*.,2013; Hippel and Schleich, 1969). Cyclodextrin is one of the debittering agents and often involves in masking the bitter taste of hydrolysate (Saha and Hayashi, 2001). The hydrophobic cavity of cyclodextrin can form inclusion complex with the hydrophobic amino acids or peptides thus masking the bitter taste of protein hydrolysate (Linde *et al*., 2009).

β-cyclodextrin has a unique shape consisting of a large and small openings of ring-shaped molecule that is exposed to the secondary and primary hydroxyl groups (Del Valle, 2003). The ring-shaped molecule has the ability to act as molecular container by entrapping the quest molecules in their internal cavity (Urban *et al*., 2012). Since the interior cavity is less hydrophilic than the aqueous environment it is able to host other hydrophobic molecules within it and therefore stop them from being perceived by the senses of taste and odor (Imelda, 2007).

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β-cyclodextrin could be used in a small amount to reduce bitter taste. Such sensation of bitter taste is reduced by 90% when 5% β-cyclodextrin was added into the hydrolysate (Linde *et al*., 2010). Besides, β-cyclodextrin has also been shown to reduce bitterness and enhance the stability of whey protein hydrolysate (Yang *et al*., 2012). Hence, β-cyclodextrin is recommended for masking bitter taste of new functional food products (Linde *et al*., 2010). The objective of this study was to determine the effect of physical mixing and kneading methods in reducing bitterness of angelwing clam hydrolysate.

Materials and Methods

Materials

Angelwing clam was purchased from Pantai Remis, Selangor, Malaysia. The clam was placed in ice and transported to the laboratory. Upon arrival, the clam was washed and the flesh was separated manually and then stored at -20°C until used for experimental work. Bromelain (1.5 AU/g) was obtained from Novozymes Sdn Bhd, Malaysia and stored at 4°C until further used.

Preparation of angelwing clam hydrolysates

Angelwing clam hydrolysate was prepared according to the method by Normah and Nur Fazlika (2013). Five hundred grams angelwing clam flesh was mixed with 531.33 ml of distilled water and then minced in a blender. The mixture was transferred into a 1 L beaker which was then placed in a water bath. The water bath was set at 45°C and the pH of the mixture was adjusted to 6. Once the temperature and pH were constant, bromelain (enzyme substrate ratio 3%) was added and the hydrolysis was performed for two hours. The mixture was continuously stirred at 200 rpm using a stirring propeller. The pH was kept constant throughout the hydrolysis by the addition of 1 N NaOH. At the end of the hydrolysis, the reaction was terminated by heating at 90ºC for 15 minutes in a water bath. The mixture was then centrifuged (Hettich ZENTRIFUGEN, UNIVERSAL 320R) at 10000 rpm at 4ºC for 20 minutes. The supernatant was collected and freeze dried using SANYO-Biomedical freeze dryer (Alpha 1-4, Martin Christ). The resulting hydrolysate was named as bromelain hydrolysate (BH).

Taste masking of hydrolysate by physical mixing

For taste masking effect, hydrolysate was prepared according to the procedure mentioned earlier, however, β-cyclodextrin was added into the supernatant at the ratio of 1:0.8 (v/w) at 38.5±1°C. The mixture was consistently agitated at 150 rpm for 12 minutes by using an incubator shaker (Innova 4080 INCUBATOR SHAKER, United States). The product was then freeze dried using a SANYO-Biomedical freeze dryer (Alpha 1-4, Martin Christ) (Hou *et al*., 2013).

Taste masking of hydrolysate by kneading method

The procedure for physical method was repeated. However, after agitation, the sample was placed in a mortar and ground for 45 minutes. The product was freeze dried.

Quantitative descriptive analysis (QDA)

To determine the intensity of bitter taste, QDA was conducted according to Nilsang *et al*. (2005). Samples were evaluated by fifteen panelists who have been trained for three weeks using caffeine as a reference solution. Six different concentrations of caffeine solutions (0 to 1000 ppm, intervals of 200 ppm) were served to the panelists. Each panelist was required to range these concentrations according to the least to most bitter. Concentration with the least bitter intensity was identified at the end of the training session and used for the evaluation of the hydrolysate samples. During the evaluation session, panelists were seated in an individual booth. They were provided with 0.04% (w/v) citrus water and warm water. Intensity of bitterness was identified and marked on a given 15 cm line scale anchored from 'none' to 'very bitter'. After each tasting, they were asked to rinse their mouth with citrus water and twice with warm water.

Amino acid compositions

Amino acid compositions were analysed by AccQ Tag HPLC (Waters, Milford, MA, USA) equipped with Water 1525 Binary HPLC Pump and Waters 2475 Multi λ Fluorescence detector and 2414 Refractive Index Detector. Column used was Waters AccQ- Tag column (3.9 × 150 mm). The column was thermostated at 37°C; the flow rate was 1.0mL/min and the injection volume was 5 µL. Mobile phase A was 0.3M sodium acetate containing 5% acetonitrile while mobile phase B comprised of acetonitrile and methanol. Prior to analysis, sample was hydrolysed in 6N HCl at 110°C for 24 hours. Breeze 2 HPLC System software was used to measure the amino acid compositions.

Determination of flavor compounds by Gas Chromatography- Mass Spectrometry (GC-MS)

GC-MS analysis was performed using Agilent GC-MS (Santa Clara, CA, USA) to identify the

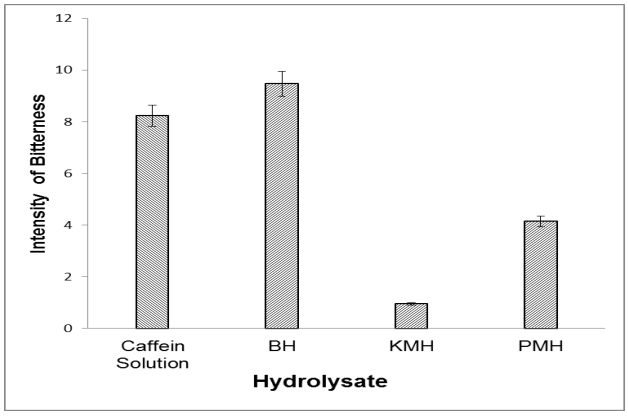

Figure 1. Bitterness intensity of caffeine reference solution, bromelain hydrolysate (BH), hydrolysate produced by physical mixing (PMH) and hydrolysate produced by kneading method (KMH). Different letters on the bars denote significant difference ($p < 0.05$)

flavor compounds that contribute to the bitter flavor in hydrolysates. Twenty five mg sample was placed in each test tube containing 2 ml sodium methoxide (Ranjith *et al*., 2007). The mixture was then shaken vigorously. After 20 minutes, 1 ml hexane was added and the upper layer formed was pipetted and transfered to a sample vial. The qualitative results were obtained from the GC-MS library. The temperature of the column (50ºC) was then programmed at 25ºC min-1 to 200ºC after injection and maintained at that temperature for 5 min. Split injection was conducted with a split ratio of 10:1, the flow-rate was 1.0 mL min-1. Helium was used as the carrier gas and the injector temperature was 250ºC. The MS detection conditions were as follows: interface temperature, 230ºC; ionization mode, EI+; electron energy, 70 eV; full scan acquisition mode; mass range, 33-450 amu.

Morphology of BH - β-cyclodextrin complexes

Particle size and structure of the hydrolysates were evaluated by a FESEM, SUPRATM40VP FESEM (Carl Zeiss SMT AG, Germany). The sample was pasted to FESEM stubs and examined at 100-1000 kV.

Statistical analysis

The data obtained was analysed using the Analysis of Variance (ANOVA) to determine significance at 5% level. Duncan Multiple Range Test (DMRT) was used to identify differences between means. Statistical analysis was performed using the Statistical Package for Social Science (IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY, 2011).

Results and Discussion

Quantitative Descriptive Analysis

Quantitative Descriptive Analysis (QDA) was carried out by fifteen trained panelists and the result is presented in Figure 1. Bitterness intensity of the hydrolysates was in the sequence of BH > PMH > KMH which was 9.47, 3.14 and 0.95, respectively. This result revealed that the intensity of bitterness of hydrolysates treated by the physical mixing and kneading method were significantly ($p<0.05$) lower than the caffeine reference solution. The intensity of bitterness for kneaded hydrolysate was below 1 which denotes negligible bitterness. Peptide sizes contribute to bitter taste (Geisenhoff, 2009). Low molecular weight peptides consist mainly of hydrophobic amino acids (Jeffery *et al*., 2002). Hydrophobic side chains of amino acids are buried in the interior part of protein by hydrophobic interaction (Imelda, 2007). During hydrolysis, protein was degraded thus exposing the hydrophobic amino acids. During consumption, the amino acids come in contact with bitter taste bud and generate the bitter taste (Pederson, 1994). This study was in line with amino acids composition where the amount of hydrophobic amino acids was reduced in PMH and KMH compared to BH.

Treatment of the hydrolysate after the hydrolysis played a key role in masking bitter taste in which the physical mixing or kneading methods could influence the complex formation. It was found that kneading method effectively masked the bitter taste compared to physical mixing. Kneading method was applied in two steps; the slurry followed by kneading step. In the slurry step, β-cyclodextrin form complexes with the hydrolysates while during kneading, complexation was completed by increasing the duration of contact time between the hydrolysate and β-cyclodextrin (Allen, 1998). In the physical method, the hydrolysates only undergo the slurry steps. As a result, the panelist still perceived bitterness in PMH sample. This was supported by FESEM observation where BH molecules remain unattached to β-cyclodextrin in the physical mixed hydrolysate (PMH) samples.

Amino acid compositions

Previous studies suggested that hydrophobic amino acids such as glycine, alanine, valine, leucine, isoleucine, phenylalanine, proline, tryptophan and methionine gave bitter taste in peptide (Imelda, 2007). The reduction in hydrophobic amino acids such as alanine (Ala), proline (Pro), cysteine (Cys), valine (Val), methionine (Met) and phenylalanine (Phe) in KMH and PMH indicated the formation of

Table 1. Total amino acids composition (g 100 g^{-1}) of bromelain hydrolysate (BH), kneaded method hydrolysate (KMH) and physical mixed hydrolysate (PMH)

Amino acid	BH	KMH	PMH
Aspartic acid	5.83^{a}±0.15	5.94^{a}±0.04	5.58^{b}±0.04
Serine	1.47^{a}±0.33	1.08^{a}±0.05	1.24^{a}±0.10
Glutamic acid	1.32^{b}±0.08	1.73^{a}±0.00	1.01^{c}±0.00
Glycine	2.64^{a}±0.27	2.07^{a}±0.04	2.64^{a}±0.27
Histidine	1.92^{a}±0.09	2.01^{a}±0.00	1.96^{a}±0.04
Arginine	2.45^{a}±0.08	2.01^{b}±0.00	2.42^{a}±0.01
Threonine	4.12^{a}±0.08	4.00^{b}±0.00	4.16^{a}±0.03
Alanine	1.63^{a}±0.05	0.81^{b}±0.00	1.69^{a}±0.02
Proline	3.97^{a}±0.05	1.51^{b}±0.01	3.64^{a}±0.44
Cysteine	1.82^{a}±0.02	1.55^{b}±0.02	1.81^{a}±0.01
Tyrosine	2.35^{a}±0.08	0.78^{c}±0.01	1.04^{b}±0.04
Valine	3.09^{a}±0.04	1.49^{c}±0.00	3.04^{b}±0.00
Methionine	8.69^{a}±0.03	5.19^{b}±0.06	8.37^{a}±0.26
Lysine	4.89^{a}±0.04	4.24^{b}±0.04	3.12^{c}±0.09
Isoleucine	2.36^{b}±0.03	2.62^{a}±0.01	2.11^{c}±0.01
Leucine	3.86^{a}±0.02	2.81^{b}±0.00	2.45^{c}±0.01
Phenylalanine	4.36^{a}±0.16	0.90^{c}±0.00	1.64^{b}±0.05
Total amino acid	56.77^{a}	40.74^{b}	48.84^{c}

Values are expressed as means ± standard deviation from triplicate determinations. Different letters within rows indicate significant difference at $p < 0.05$

complexes between amino acids and β-cyclodextrin, thus reduces the bitter taste.

KMH contains relatively less amount of Phe, Met and Val. This observation was in line with QDA where KMH was identified to have lower degree of bitterness. The reduction of the amino acids in PMH and KMH also indicated that these amino acids formed complexes with β-cyclodextrin. This is supported by previous study where the interaction between β-cyclodextrin and Phe increased especially when the Phe residue was positioned at the N- and C-terminus of the peptides (Caso *et al*., 2015).

Alanine content was lower in KMH compared to PMH and BH. Alanine gave a pleasant sweet taste in peptide and also involved in the formation of complexes with β-cyclodextrin (Ekka and Roy, 2013). Both KMH and PMH had lesser amount of tyrosine than BH (Table 1). This showed that tyrosine might have formed complexes with β-cyclodextrin. This was agreed by previous study in which the affinity of tyrosine for the β-cyclodextrin significantly increases when the residue is located in the middle of amino acids sequence (Caso *et al*., 2015). Moreover, tyrosine is suited for interaction with β-cyclodextrin and form stable complexation with β-cyclodextrin (Caso *et al*., 2015).

PMH has less amount of lysine compared to BH and KMH. Lesser lysine was detected in PMH probably due to oxidation. BH contains higher amount of lysine most probably because BH was not

Table 2. Volatile compounds identified in bromelain hydrolysate (BH), kneaded method hydrolysate (KMH) and physical mixed hydrolysate (PMH)

	Area (%)		
Compound	BH	KMH	PMH
Hydrocarbon			
Benzene,1-phenyl-4-2-(2-cyano-2-phenylethyl)	0.85	nd	nd
cyclohexasiloxane,dodecamethyl	3.08	3.94	3.45
cycloheptasolixane, -1-tetradecamethyl	1.39	1.7	1.37
5-octadecen	0.16	0.39	0.12
1-Tridecene	0.21	0.15	0.15
Benzothiazole	nd	0.61	nd
Acid			
Azelaic	0.11	nd	nd
Methyl myristate	0.14	nd	nd
Palmitic	1.12	5.72	nd
Margaric	0.19	nd	0.08
oleic	5.81	1.25	3.07
stearic	3.07	nd	0.91
linoleic	2.25	nd	nd
pipecolic acid	5.80	nd	nd
Nitrogen containing compound			
Aniline	4.06	nd	4.14
decanamide	5.45	1.8	nd
oleamide	1.07	5.54	2.28
octadecanamide	2.53	0.87	1.9
Phenethylamine	0.19	nd	nd
hexadecanamide	nd	nd	2.12
4-nitrosoveratrole	nd	0.96	0.85
Alcohol			
4-Dodecen-1-ol	1.07	nd	nd
Phenolic			
1,2-Benzenediol, 3,5-bis(1,1-dimethylethyl)	0.13	nd	nd
4-(2-Amino ethyl)bezene-1,2-diol	1.52	nd	nd

nd: not detected

subjected to further treatment which are the kneading method and physical mixing. This treatment might cause some parts of the hydrolysate that are not interacted with β-cyclodextrin tend to oxidize. Lysine was the target of oxidative reaction because it is a nucleophilic amino acid and therefore vulnerable to modification by lipid peroxidation (Uchida, 2003). Lysine is also susceptible to be attacked by free radicals (Watson and Preedy, 2013). Therefore, reduced amount of lysine by oxidation suggested poor complex formation between BH and β-cyclodextrin in PMH samples compared to KMH.

Determination of flavor compounds by Gas chromatography- mass spectrometry (GC-MS)

Identification of volatile compound in food product is important because food flavor depends on the composition of volatile compound. Twenty five

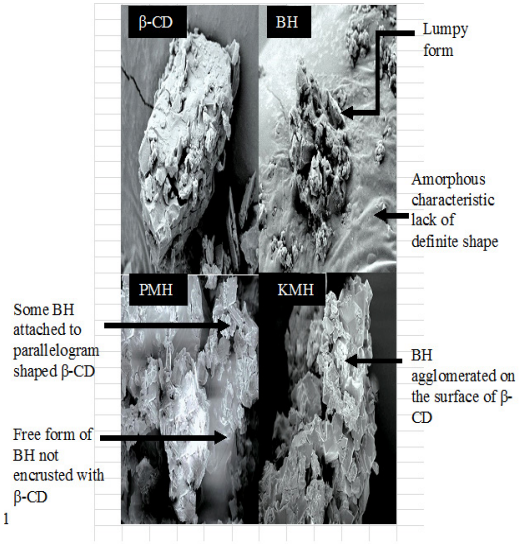

Figure 2. Field emission scanning electron microscope (FESEM) of β-cyclodextrin (β-CD), bromelain hydrolysate (BH), physical mixed hydrolysate (PMH) and kneaded method hydrolysate (KMH) at 800x magnification

different compounds were detected (Table 2). BH contained twenty two volatile compounds; KMH and PMH each contains eleven compounds. Hydrocarbon such as cyclohexasiloxane, cycloheptasiloxane, 5-octadecene and tridecene are commonly present in hydrolysate (Nor Qhairul Izzreen and Vijaya Ratnam, 2011). Phenylalanine is the amino acid precursor of amine (2-phenylethyl) that is present in benzene,1-phenyl-4-2-(2-cyano-2-phenylethyl) (Belitz *et al*., 2009). Phenylalanine which is a hydrophobic amino acid elucidates bitter taste and could form inclusion complex with β-cyclodextrin (Sompornpisut *et al*., 2002; Wong *et al*., 2008). Benzene,1-phenyl-4-2-(2-cyano-2-phenylethyl) was only detected in BH. The absence of benzene,1-phenyl-4-2-(2-cyano-2-phenylethyl) in KMH and PMH probably due to phenylalanine that might has been masked by β-cyclodextrin. Therefore, benzene,1-phenyl-4-2-(2-cyano-2-phenylethyl) was not detected.

Benzothiazoles found in KMH probably had been synthesized by the reaction of aromatic aldehydes mediated by β-cyclodextrin (Katla *et al*., 2015). Therefore, the benzothiazoles compounds presence in KMH is evidence that BH had form complex with β-cyclodextrin in kneading method. 4-(2-Amino ethyl) benzene-1, 2-diol also known as dopamine was present in BH, however, PMH and KMH do not contain any dopamine. Phenylalanine and tyrosine are precursors of dopamine (Lou, 1994). The reduced amount of phenylalanine and tyrosine in KMH and PMH could be due to the formation of complex between BH and β-cyclodextrin. As a result, dopamine is not detected in KMH and PMH, thus reducing the bitterness of KMH and PMH.

As shown in Table 2, BH contains unsaturated free fatty acids; linoleic and oleic acid, however, PMH and KMH samples do not contain any linoleic acid. In the study, both linoleic and oleic acid presence in BH might be due to lipid oxidation occurring during the hydrolysis. These are supported by previous study where the presence of double bond in these unsaturated fatty acids caused the compounds to be readily undergoing oxidative reactions (Rong, 2007).

Morphology of BH - β-cyclodextrin complexes

The structural analysis of the bromelain hydrolysate/β-cyclodextrin complex was carried out through field emission scanning electron microscope (FESEM, SUPRATM 40VP) with voltage acceleration of 15 kV. In order to record FESEM micrographs, the samples were fixed on aluminum stubs and vacuum coated with a fine layer of gold. The morphologies and particle sizes of β-cyclodextrin, BH, PMH and KMH are presented in Figure 2. An apparent difference in the microstructure of BH, β-cyclodextrin and the modified hydrolysate (KMH and PMH) could be observed. β-cyclodextrin appears in regular parallelogram-shaped crystals while BH was lumpy with amorphous character that is lack of definite shape and was unorganized (Figure 2). It has been stated that β-cyclodextrin had a regular parallelogram-shaped crystals (Jianbin *et al*., 2002). However, KMH and PMH appeared to form different sizes of agglomerates with β-cyclodextrin (Dima *et al*., 2014).

For KMH, it is clearly showed that β-cyclodextrin was encrusted with BH. Furthermore, the loading degree of β-cyclodextrin with BH was better by kneading method compared to physical mixing. For PMH, it was clearly showed that there were free forms of BH which are not encrusted with β-cyclodextrin. Similar results were reported in the preparation of fish oil/β-cyclodextrin complex (Choi *et al*., 2010). BH and β-cyclodextrin are difficult to differentiate in KMH sample. However, in PMH, the BH formed agglomerates on the surface of β-cyclodextrin. The FESEM image of the PMH confirmed the presence of crystalline hydrolysate but BH particles was observed either partially mixed or adhered loosely on the surface of β-cyclodextrin. In contrary, a drastic change in the morphology of BH and β-cyclodextrin were observed in KMH complex and had lost their original shape in KMH. Thus, this study reveals that kneading method is a better way for forming complex between bromelain hydrolysate and β-cyclodextrin rather than physical mixing.

Conclusion

Bitterness of BH was successfully masked by β-cyclodextrin by using two different methods; the kneading and physical mixing. Sensory analysis showed that PMH was less bitter than BH while bitterness of KMH was negligible. Hydrophobic amino acids such as Phe, Met, Val and Cys had been reduced in the modified hydrolysates probably due to complexation between BH with β-cyclodextrin. From GC-MS results, the presence of benzothiazole compounds in KMH indicated that BH had formed complex with β-cyclodextrin. Besides, the absence of benzene, 1-phenyl-4-2-(2-cyano-2-phenylethyl) in KMH and PMH indicated that phenylalanine might had been masked by β-cyclodextrin. Evidence for FESEM analysis which demonstrated the presence of a completely different crystal structure in KMH suggested that a new solid phase was formed. Therefore, these studies suggested that the absence of bitter taste in KMH was probably due to the masking effect by the complexes formed with β-cyclodextrin.

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