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Original Article

Tuna viscera hydrolysate products prepared by different enzyme preparations improve the feed intake and growth of Asian seabass, *Lates calcarifer*, fed total fishmeal replacement diets

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Abstract

Protein hydrolysate products prepared using different enzymes at different degrees of hydrolysis (DH) may have different qualities when applied in a fish diet. In this study, hydrolysis enzymes, including endoproteinase from *Bacillus licheniformis* (A), endoproteinase and exopeptidase from *Aspergillus oryzae* (F), and endoproteinase from *B. amyloliquefaciens* (N) were used to prepare tuna viscera hydrolysate products (TVHPs) at low (1) and high (2) DH (40 and 60%, respectively) for supplementation in fishmeal free diets for Asian seabass. The TVHPs were tested in a feeding trial composed of ten diets: A1- and F1-TVHP (50g/kg), F2- and A2-TVHP (25 g/kg) of diet, and N-hydrolysed TVHP (either low or high DH) at 25, 50, and 75 g/kg of diet. A control poultry by-product based diet and a fishmeal reference diet were included. Feed intake of fish fed the TVHP supplemented diets improved by 25.4-41.0% which significantly increased growth performance compared to the control groups ($P<0.05$). Growth response was better with diets containing 60% DH-TVHP suggesting that using a TVHP with a higher DH makes it possible to reduce the inclusion rate while still obtaining a performance benefit. In conclusion, the inclusion levels of TVHPs at 25-75 g/kg of diet could be suitable as a feeding stimulant in fishmeal free diets for this carnivorous fish.

Keywords: tuna viscera, degree of hydrolysis, inclusion level, feeding stimulant, Asian seabass

1. Introduction

The low palatability of zero-fishmeal diets or diets with high inclusion levels of alternative plant and animal protein ingredients have resulted in poor feed consumption by malabar grouper, *Epinephelus malabricus* (Wang *et al*., 2008), poor performance in Asian seabass, *Lates calcarifer* (Glencross *et al*., 2011), and reduced feed intake and specific growth rate in Japanese seabass, *Lateolabrax japonicus* (Hu *et al*., 2013). To overcome these problems, several studies have added feeding stimulants such as amino acids, nucleotides, betaine, and organic acids to these types of feeds to improve their palatability to promote increased feed intake and enhance growth rates (Barnard, 2006; Kasumyan & Døving, 2003; Xue & Cui, 2001). Natural substances such as fish protein hydrolysates containing high levels of free amino acids and peptides have recently become candidate feeding attractants or stimulants for aquaculture diets, while also being alternative sources of protein. Refstie *et al*. (2004)

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found that fish protein hydrolysate as a fishmeal replacer at 5-15% of the diet was also an efficient feeding stimulant that improved feed intake, growth, nutrient retention, and nutrient digestibility in early seawater-stage Atlantic salmon (*Salmo salar*). In our earlier study in Asian seabass, palatability of a poultry by-product based diet was improved with supplementation of tuna viscera hydrolysate at 10, 20, 30, and 40 g/kg (Chotikachinda *et al*., 2013) which was probably a result of the high percentage of small peptides in the hydrolysates. Several studies have shown that amino acids and low molecular weight peptides are effective in stimulating feed intake to bring about growth improvements in different fish species (Erteken & Nezaki, 2002; Hidaka *et al*., 2000; Ostaszewska *et al*., 2013).

Different proteases with different site-specificity have an effect on hydrolysate characteristics. Foh et al. (2010) obtained tilapia hydrolysate composed of low molecular weight peptides of <3,000 Da when hydrolysed by Alcalase while those produced by Flavourzyme and Neutrase were much larger (>8,000 Da). Silver carp muscle hydrolysates produced by both Alcalase and Flavourzyme hydrolysis contained peptides with a molecular weight <5,000 Da; however, more than 60% of the peptides found in the hydrolysates using Alcalase were <1,000 Da (Dong *et al*., 2008). Furthermore, differences in peptide composition of hydrolysates also depend on the degrees of hydrolysis (DH) and the concentration of enzymes used in the production. For example, You *et al*. (2009) reported that DH and protease influenced the molecular weight of peptide and amino acid residues of loach (*Misgurnus anguillicaudatus*) protein hydrolysates.

Therefore, the present study examined the effects on the palatability of diets, growth performance, and feed utilization of Asian seabass by supplementing fishmeal free diets with tuna viscera hydrolysate products (TVHPs) that were produced using different enzymes.

2. Materials and Methods

The study was carried out in three consecutive procedures: (1) preparation of tuna viscera hydrolysate using different enzymes at two DH levels, (2) determination of suitable levels of TVHP as an effective feeding stimulant in a 56-day feeding trial, and (3) a feeding preference test for the determination of effective TVHPs as feeding attractants/stimulants.

2.1 Preparation of tuna viscera hydrolysates

2.1.1 Tuna viscera

Viscera of skipjack tuna (*Katsuwonus pelamis*) including spleen, stomach, intestine, bile sac, liver, and pancreas were obtained from Chotiwat Manufacturing Co., Ltd., Songkhla, Thailand. The viscera were minced and packed in plastic bags (0.3-0.4 kg per unit) and stored at -20 ˚C until use.

2.1.2 Enzymes

The enzymes used to prepare the protein hydrolysates were endoprotease from *Bacillus licheniformis*, (activity 2.4 AU/g) (A), endoprotease and exopeptidase from *Aspergillus oryzae* (activity 500 LAPU/g) (F), and endoprotease from *B. amyloliquefaciens* (activity 0.8 AU-N/g) (N) by Novozymes, Bagavaerd, Denmark that were purchased from a local distributor.

2.1.3 Hydrolysis of tuna viscera

Tuna viscera were hydrolysed to obtain the low (1) and high (2) DH levels (40 and 60%, respectively) according to Chotikachinda *et al*. (2013). The frozen minced tuna viscera samples were thawed by rinsing the storage bags with tap water. Each hydrolysis was performed in 250 mL Erlenmeyer flasks containing 30 g of minced tuna viscera mixed with distilled water at a ratio of 1:2 (w/v) and homogenized for approximately 1 min using an overhead mixer (Braun MR 400 HC, Kronberg, Germany). The pH of the mixtures was adjusted to 8.0 for enzyme A and 7.0 for enzymes F and N for hydrolysis using 1 N NaOH. The samples were pre-incubated in shaking water baths (GFL 1083, Burgwedel, Germany) at 50 °C with constant agitation (100 rpm) for 10 min. To create hydrolysates with low and high DH, two enzyme/substrate ratios were utilized with each enzyme. The concentrations of the enzymes added to the hydrolysis mixtures were as follows: enzyme A at 0.5% (w/w) and 1.64% (w/w) to A1- and A2-TVHP, enzyme F at 0.5% (w/w) and 1.32% (w/w) to F1 and F2-TVHP, and enzyme N at 0.5% (w/w) and 2.37 % (w/w) to N1 and N2-TVHP, respectively. All enzyme-substrate mixtures were held at 50 ºC for 1 h followed by enzyme activity termination by heating at 90 °C for 15 min. The hydrolysed mixtures were centrifuged (Beckman Avanti™, CA, USA) at 7400×*g* at 4 °C for 20 min to remove insoluble materials. The supernatant was concentrated overnight at 60 °C in an oven (Memmert, Schwabach, Germany) and stored at -20 °C. All six hydrolysates, A1-TVHP, A2-TVHP, F1-TVHP, F2-TVHP, N1-TVHP, and N2-TVHP, were characterized for chemical composition, peptide size, free amino acid, and total amino acid content and later used in the diets for the feeding trial and preference test.

2.2 Feeding trial

2.2.1 Diet preparation

The fishmeal-free diets used for the feeding trial and the feeding preference test were formulated with poultry by-product meal (PBM) and soybean meal (SBM) as the primary protein sources to contain 450-470 g/kg protein and 110-130 g/kg lipid. Twelve diets were prepared consisting of the control diet, fishmeal reference diet, and ten experimental diets of A1-TVHP (50 g/kg), A2-TVHP (25 g/kg), F1-TVHP (50 g/kg), F2-TVHP (25 g/kg), N1-TVHP (25, 50, and 75 g/kg), and N2-TVHP (25, 50, and 75 g/kg) (Table 1). The diets were prepared using a laboratory Hobart mixer and pelleter (Hobart, Ohio, USA). The proximate and total amino acid compositions of the diets were analyzed as described in 2.2.4 and 2.2.5.

Table 1. Free and total amino acid content (g/kg) of tuna viscera hydrolysate products produced using different enzymes and degree of hydrolysis

Amino acid composition [1]	Free amino acid[2]						Total amino acid[2]					
	A1-TVHP (L)[4]	A2-TVHP (H)[4]	F1-TVHP (L)	F2-TVHP (H)	N1-TVHP (L)	N2-TVHP (H)	A1-TVHP (L)	A2-TVHP (H)	F1-TVHP (L)	F2-TVHP (H)	N1-TVHP (L)	N2-TVHP (H)
Essential amino acid												
Arginine/Glycine (Arg/Gly)[3]	71±4 ^{a}	80±1 ^{abc}	74± 6 ^{ab}	87±1 ^{bc}	89±4 ^{c}	86± 4 ^{bc}	232±10	233±23	213±10	231±8	280±20	240±26
Histidine (His)	13±0	14±4	14±0	18±4	17±1	15±3	30±4	32±8	25±2	33±1	39±3	33±4
Isoleucine (Ile)	35±2	38±3	37±3	45±1	44±1	43±8	72±5	77±7	65±5	79±7	84±4	77±6
Leucine (Leu)	59±3	64±6	64±4	76±2	75±1	71±7	114±2	124±12	108±8	127±10	133±7	121±9
Lysine (Lys)	59±7 ^{a}	67±3 ^{ab}	67±3 ^{ab}	81±3 ^{b}	67±2 ^{ab}	79±7 ^{b}	135±7	118±11	116±16	131±15	132±10	136±15
Methionine (Met)	21±0 ^{a}	25±3 ^{ab}	24±0 ^{ab}	26±2 ^{ab}	31±2 ^{b}	27±2 ^{ab}	39±2	31±3	34±2	40±4	43±5	40±3
Phenylalanine (Phe)	33±1	35±3	34±3	42±2	39±1	39±7	64±4	64±1	53±9	70±3	72±3	68±5
Threonine (Thr)	31±2	34±3	33±6	42±0	40±1	40±5	73±0	78±10	67±9	83±8	88±8	80±5
Tryptophan (Trp)	30±1 ^{ab}	31±2 ^{ab}	32±4 ^{ab}	40±1 ^{b}	22±1 ^{a}	28±5 ^{a}	41±7^{a}	56±4 ^{ab}	49±7 ^{ab}	60±4 ^{b}	40±1^{a}	42±1 ^{ab}
Valine (Val)	43±2	47±0	44±6	54±1	54±1	53±3	86±4	90±7	78±8	92±10	99±5	92±7
Total essential amino acid	395	435	423	511	478	481	886	903	808	946	1010	929
Non-essential amino acid												
Alanine (Ala)	46±3	51±1	47±8	58±0	58±1	57±3	87±1 ^{ab}	96±5 ^{ab}	74±6 ^{a}	105±14^{ab}	112±9^{b}	104±7 ^{ab}
Asparagine (Asn)	11±1	12±4	10±2	13±0	12±0	11±2	1±0	1±0	0±0	0±0	1±0	2±0
Aspartic acid (Asp)	50±2 ^{a}	55±2 ^{ab}	51±6 ^{a}	63±7 ^{ab}	65±1 ^{ab}	72±2 ^{b}	141±2	154±11	133±14	158±2	163±10	159±13
Glutamic acid (Glu)	57±3 ^{a}	62±5 ^{ab}	58±3 ^{a}	74±1 ^{c}	75±0 ^{c}	68±0 ^{bc}	192±9	207±20	179±17	209±23	231±19	211±20
Glutamine (Gln)	0±0 ^{a}	0±0 ^{a}	0±0 ^{a}	1±0 ^{ab}	2±0 ^{b}	0±0 ^{a}	0±0^{a}	0±0 ^{a}	0±0 ^{a}	1±0^{b}	0±0 ^{a}	0±0 ^{a}
Serine (Ser)	28±2	31±2	30±6	35±0	27±1	35±6	35±7	74±0	28±3	79±2	68±4	75±8
Total non-essential amino acid	192	211	196	244	239	243	456	532	414	552	575	551
Total amino acid	423	466	453	546	505	516	921	977	836	1025	1078	1004

[1] Grams of amino acid per kg of hydrolysate.

[2] Means±standard deviation are as dry matter basis (n = 2). Means with different superscripts are significantly different (P<0.05).

[3] Arginine and glycine are reported together because their peaks merge.

[4] (L) = low degree of hydrolysis, (H) = high degree of hydrolysis

2.2.2 Fish

Juvenile seabass were obtained from the National Institute of Coastal Aquaculture, Department of Fisheries, Thailand and reared at the Department of Aquatic Science, Faculty of Natural Resources, Prince of Songkla University, Thailand until reaching the required sizes of 2.60 g and 4.43 g for the feeding trial and the preference test, respectively.

2.2.3 Feeding and response measurement

Thirty-six 100-liter aquaria were used and stocked with 12 fish/aquarium. The fish were fed twice daily at 10% of body weight from Day 1 to Day 7 and then to apparent satiation from Day 8 until the end of the trial. Feed intake was determined daily and corrected for uneaten diet which was removed, dried, and reweighed. Fish weight was recorded individually at the beginning and termination of the feeding trial. At the end of the 56-day feeding, the survival rate, weight gain (WG), thermal unit growth coefficient, specific growth rate (SGR), feed conversion ratio (FCR), and protein efficiency ratio (PER) were determined.

2.3 Preference test

The test was conducted in three replicated 66-liter aquaria, each of which was divided into two unequal compartments using a plastic divider. Two feeding trays were placed in the smaller front part. Nine fish per tank were initially placed in the back compartment for one day for adjustment prior to the test. A paired preference test was conducted by introducing the control diet and one of the test diets (at 5% body weight) into trays 1 and 2, respectively. The divider was then slowly raised up from the bottom. Each group of fish was allowed to consume either of the two feeds for 5 minutes. Uneaten feed was removed and dried to a constant weight at 105 °C and reweighed to calculate the actual consumed amount of each diet. The relative diet preference value (%) was measured by quantitative comparisons of eaten amounts and calculated as follows:

[(Test diet consumed (g) – Control diet consumed (g)) x 100]/Control diet consumed (g)

2.4 Analysis of hydrolysate products and experimental diets

2.4.1 DH

The DH levels of the TVHPs were determined using the trinitrobenzenesulfonic acid method (Alder-Nissen, 1979).

2.4.2 Molecular weight distribution of peptides

Molecular weights of the peptide fractions were separated on a fast protein liquid chromatography (FPLC) column (Superdex^{TM} peptide 10/300 GL, Amersham Biosciences, Uppsala, Sweden) and compared with distributions of the standard compounds: cytochrome c (M_w=12,384), Aprotinin (M_w=6512) and Vitamin B12 (M_w=1355) (Sigma Chemical, MO, USA). The areas of each fraction were given in percentage relative to the total area (Šližyte et al., 2005).

2.4.3 Free amino acid and total amino acid content

Freeze-dried hydrolysates were precipitated for protein and the supernatant was collected for analysis of free amino acid content. The total amino acid compositions of the hydrolysates were determined after digesting the samples in 6M hydrochloric acid at 105 ºC for 22 h. Both free and total amino acid content of the hydrolysates and diets were determined using reversed phase high-performance liquid chromatography (HPLC) by pre-column fluorescence derivatization with *o*-phthaldialdehyde (SIL-9A Auto Injector, LC-9A Liquid Chromatograph, RF-530 Fluorescence HPLC Monitor [Shimadzu Corporation, Kyoto, Japan]) and Nova-Pak C18 cartridge (Waters Corporation, Milford, MA, USA) following the method of Flynn (1988).

2.4.4 Composition of experimental diets and hydrolysates

The proximate compositions of the hydrolysates and diets were determined in triplicate following the standard method of Association of Official Analytical Chemists (AOAC, 1990).

2.5 Statistical analysis

The data were subjected to analysis of variance followed by Tukey's HSD test. The paired t-test was employed for the feeding preference results between the test diets against the control diet. Differences were regarded as significant when $P<0.05$.

3. Results

3.1 Characterization of tuna viscera hydrolysates and experimental diets

On a dry matter basis, the hydrolysates contained 826.4-878.3 g/kg protein, 33.4-44.3 g/kg lipid, and 85.2-107.1 g/kg ash (Table 1). The nitrogen solubility of the TVHP was high (93.85-97.52%) but not significantly different among the samples ($P>0.05$). The peptide compositions of the different hydrolysates had predomimantly small peptides of <500 Da (Table 1); however, A2-TVHP, F1-TVHP, and F2-TVHP also contained a high amount (81-95%) of free amino acids (MW<200 Da).

Most of the free amino acid content of the TVHPs was not significantly different between the samples, but some differences were noted for arginine/glycine, lysine, methionine, tryptophan, aspartic acid, glutamic acid, and glutamine (Table 2). A2- and F2-TVHP had higher arginine, methionine, and glutamic acid content than the A1- and F1-TVHP. All hydrolysates with high DH (A2-, F2-, and N2-TVHP) had higher lysine, tryptophan, and aspartic acid content than the lower DH hydrolysates (A1-, F1-, and N1-TVHP). The total amino acid composition of the TVHPs was not significantly different among the samples except for tryptophan, alanine, and glutamine ($P<0.05$) (Table 2).

Aspartic acid, glutamic acid, arginine/glycine, leucine, and lysine were high in all hydrolysate samples.

The proximate compositions and amino acid compositions of the TVHP supplemented diets are shown in Tables 3 and 4, respectively. The levels of essential amino acids in the diets were in a close range but there were distinctive differences in the levels of glutamic acid and glycine. Glutamic acid was high in diets 4 through 9 and glycine in diets 5 through 9.

Table 2. Proximate composition (dry matter basis), degree of hydrolysis, ammonia nitrogen, nitrogen solubility index, protein recovery and molecular weight distribution of tuna viscera hydrolysate products (TVHPs).

Composition [1]	A1-TVHP [2]	A2-TVHP [3]	F1-TVHP [4]	F2-TVHP [5]	N1-TVHP [6]	N2-TVHP [7]
Protein (g/ kg)	857.2±26.4^{ab}	878.3±16.8 ^{b}	826.4±15.3 ^{a}	837.1±11.7 ^{ab}	842.4±17.1 ^{ab}	854.1±20.9 ^{ab}
Lipid (g/kg)	44.3±1.8^{b}	44.0±1.2^{b}	39.9±1.0^{b}	33.4±0.8^{a}	42.4±0.6^{b}	41.5±1.8^{b}
Ash (g/kg)	103.5±4.5^{b}	107.1±3.3^{b}	85.2±7.6^{a}	99.6±1.9^{ab}	91.5±9.2^{ab}	92.8±4.5^{ab}
Degree of hydrolysis (%)	41.7±1.2^{a}	62.6±2.3^{b}	43.7±1.4^{a}	64.5±2.3^{b}	43.0±1.8^{a}	63.8±2.7^{b}
Nitrogen solubility index (%)	93.9±2.4	97.5±1.9	94.0±1.6	96.2±3.7	93.9±3.0	94.8±0.8
Molecular weight [8]						
>2500 Da	0.2±0.0	0.3±0.2	2.3±3.1	0.6±0.1	0.9±0.1	0.7±0.9
500 to 2500 Da	9.8±2.4	0.0±0.0	0.2±0.2	1.5±2.1	7.9±1.5	3.8±6.5
200 to 500 Da	38.0±12.6^{b}	13.3±0.5^{ab}	16.1±2.4^{ab}	3.1±4.3^{a}	26.7±0.9^{ab}	22.0±10.3^{ab}
<200 Da	52.0±10.2^{a}	85.4±0.3^{bc}	81.4±0.4^{bc}	94.9±6.5^{c}	64.6±0.7^{ab}	70.9±10.5^{abc}

[1] Values are presented as mean±standard deviation (n=3). Means with different superscripts are significantly different (P<0.05).
[2] TVHP hydrolyzed using endoprotease from *Bacillus licheniformis* at low degree of hydrolysis
[3] TVHP hydrolyzed using endoprotease from *Bacillus licheniformis* at high degree of hydrolysis
[4] TVHP hydrolyzed using endoprotease and exopeptidase from *Aspergillus oryzae* at low degree of hydrolysis
[5] TVHP hydrolyzed using endoprotease and exopeptidase from *Aspergillus oryzae* at high degree of hydrolysis
[6] TVHP hydrolyzed using endoprotease from *B. amyloliquefaciens* at low degree of hydrolysis
[7] TVHP hydrolyzed using endoprotease from *B. amyloliquefaciens* at high degree of hydrolysis
[8] Values are the mean± standard deviation of the percentage of peak area (n=2). Means with different superscripts are significantly different (P<0.05).

3.2 Feeding trial

Fish showed a better feed acceptance during the first 7 days when receiving diets formulated with hydrolysates (Figure 1). The total feed intake at the end of the trial was significantly higher than those of the PBM control diet (P<0.05) (Table 5). When compared with the fishmeal reference diet, which had a feed intake higher than the control diet by 25.47%, none of the hydrolysate treatments were significantly different from the reference group (P>0.05). Feed intake improved by 25.40%, 36.76%, 41.00%, and 33.01%, in fish fed diet 2 (A1-50), diet 3 (A2-25), diet 4 (F1-50), and diet 5 (F2-25), respectively. Fish fed diets containing N1-TVHP showed increased feed intake by 21.82%-36.84% compared with the control, while the fish with the N2-TVHP treatments improved by 29.12%-32.05%. However, there were no significant differences among enzyme treatments at all inclusion levels.

Significant differences in fish growth were observed between the groups fed the control diet and most treatments fed the TVHP supplemented diets (P<0.05), except treatments 2 (A1-50) and 6 (N1-25). Similarly, the percentage weight gain and SGR of all TVHP supplemented groups, except diet 6 (N1-25), were significantly higher than the control group (Table 5).

The final weights, WG, and SGR of fish fed diets with 25 g/kg of the higher DH-TVHP (F2- and N2-TVHP) were similar to those fed diets containing 50 g/kg of the lower DH-TVHP (F1- and N1-TVHP). The FCR ranged from 1.1 to 1.3 which was better in the groups fed diet 5 (F2-25) and the reference diet, and inferior in those fed the control diet and diet 6 (N1-25) (Table 5). The PER was not significantly different among the dietary treatments (P>0.05). The survival rate was 100% for all treatments.

3.3 Preference test

The preference test results showed that all test diets were preferred over the control diet (P<0.05) (Table 6). Diets 7 (N1-25), 10 (N2-50), 11 (N2-75), and 12 (Reference) were the preferred diets having relative preference values above 65% as compared with only 18.4% for the control diet. However, only diet 11 with the preference value of 71.8% was better than the reference diet.

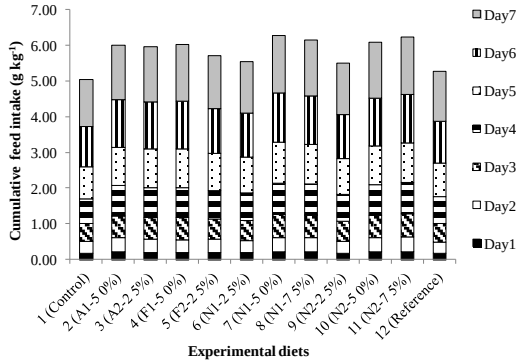

Figure 1. Cumulative feed intake (g fish^{-1}) of fish feds of different experimental diets (g fish^{-1}) during the first 7 days.

Table 3. Composition of the experimental diets

Ingredients	Experimental diets (g/kg)											
	1 (Control)	2 (A1-50)	3 (A2-25)	4 (F1-50)	5 (F2-25)	6 (N1-25)	7 (N1-50)	8 (N1-75)	9 (N2-25)	10 (N2-50)	11 (N2-75)	12 (Reference)
Fishmeal	-	-	-	-	-	-	-	-	-	-	-	129.0
PBM[1]	542.0	483.0	512.0	484.0	513.0	512.0	482.0	452.0	513.0	484.0	455.0	542.0
Soybean meal	180.0	180.0	180.0	180.0	180.0	180.0	180.0	180.0	180.0	180.0	180.0	0.0
Wheat flour	158.6	162.4	160.9	161.5	160.1	160.9	163.2	165.5	160.1	161.5	162.9	216.0
Soybean oil	40.0	45.2	42.7	45.1	42.5	42.7	45.4	48.1	42.5	45.1	47.7	34.2
Premix [2,3,4]	66.0	66.0	66.0	66.0	66.0	66.0	66.0	66.0	66.0	66.0	66.0	66.0
Lysine HCL	5.2	5.2	5.2	5.2	5.2	5.2	5.2	5.2	5.2	5.2	5.2	5.2
DL-Methionine	8.2	8.2	8.2	8.2	8.2	8.2	8.2	8.2	8.2	8.2	8.2	7.6
A1-TVHP [5]	-	50.0	-	-	-	-	-	-	-	-	-	-
A2-TVHP [6]	-	0.0	25.0	-	-	-	-	-	-	-	-	-
F1-TVHP [7]	-	-	-	50.0	-	-	-	-	-	-	-	-
F2-TVHP [8]	-	-	-	-	25.0	-	-	-	-	-	-	-
N1-TVHP [9]	-	-	-	-	-	25.0	50.0	75.0	-	-	-	-
N2-TVHP [10]	-	-	-	-	-	-	-	-	25.0	50.0	75.0	-
Protein	459.6	459.8	454.7	457.7	461.4	462.8	461.9	460.8	459.6	456.5	456.8	466.8
Lipid	112.8	111.8	111.8	109.2	109.5	118.8	108.7	109.4	107.8	109.9	108.1	115.1
Ash	119.2	122.6	112.7	117.9	128.2	123.0	120.5	120.7	120.7	120.2	119.1	126.2

[1] PBM = Poultry by product meal
[2] Premix contains 20 g/kg CMC
[3] Vitamin premix (mg/kg diet): vitamin AD_3 (500 IU of A and 100 IU of D_3/mg) 8, α-tocopherol acetate 100, menadione 50, thiamin HCl 60, riboflavin 100, Ca-pantothenate 100, pyridoxine HCl 40, niacin 10, folic acid 15, biotin 6, vitamin B_{12} 0.1, inositol 2,000, vitamin C 1000
[4] Mineral premix (g/kg diet): $CaHPO_4$ 8 , $NaH_2PO_4.2H_2O$ 15 , KH_2PO_4 10, KCl 5
[5] TVHP hydrolyzed using endoprotease from *Bacillus licheniformis* at low degree of hydrolysis
[6] TVHP hydrolyzed using endoprotease from *Bacillus licheniformis* at high degree of hydrolysis
[7] TVHP hydrolyzed using endoprotease and exopeptidase from *Aspergillus oryzae* at low degree of hydrolysis
[8] TVHP hydrolyzed using endoprotease and exopeptidase from *Aspergillus oryzae* at high degree of hydrolysis
[9] TVHP hydrolyzed using endoprotease from *B. amyloliquefaciens* at low degree of hydrolysis
[10] TVHP hydrolyzed using endoprotease from *B. amyloliquefaciens* at high degree of hydrolysis

Table 4. Total amino acid composition (g/kg) of experimental diets

Amino acid composition[1]	Experimental diet[2]											
	1 (Control)	2 (A1-50)	3 (A2-25)	4 (F1-50)	5 (F2-25)	6 (N1-25)	7 (N1-50)	8 (N1-75)	9 (N2-25)	10 (N2-50)	11 (N2-75)	12 (Reference)
Essential amino acid (EAA)												
Arginine (Arg)[3]	17 (37)	19 (41)	18 (46)	19 (41)	18 (38)	18 (35)	19 (39)	20 (42)	18 (37)	19 (44)	20 (40)	17 (35)
Histidine (His)	12 (26)	11 (24)	11 (28)	10 (22)	10 (21)	12 (24)	13 (27)	12 (26)	11 (23)	10 (23)	12 (24)	10 (21)
Isoleucine (Ile)	26 (57)	23 (50)	23 (59)	25 (54)	26 (55)	27 (53)	28 (58)	27 (57)	26 (54)	23 (53)	24 (49)	23 (48)
Leucine (Leu)	46 (100)	41 (89)	40 (103)	45 (98)	45 (96)	47 (92)	49 (102)	47 (100)	46 (96)	40 (93)	42 (86)	42 (88)
Lysine (Lys)	46 (100)	46 (100)	39 (100)	46 (100)	47 (100)	51 (100)	48 (100)	47 (100)	48 (100)	43 (100)	49 (100)	48 (100)
Methionine (Met)	17 (37)	16 (35)	14 (36)	17 (37)	17 (36)	18 (35)	18 (38)	18 (38)	18 (38)	14 (33)	15 (31)	18 (38)
Phenylalanine (Phe)	29 (63)	26 (57)	25 (64)	28 (61)	28 (60)	30 (59)	31 (62)	29 (62)	29 (60)	25 (58)	26 (53)	26 (54)
Threonine (Thr)	24 (52)	21 (46)	20 (51)	23 (50)	24 (51)	32 (63)	26 (53)	25 (53)	25 (52)	21 (49)	22 (45)	22 (46)
Tryptophan (Trp)	18 (39)	16 (35)	24 (62)	20 (43)	19 (40)	17 (33)	19 (36)	17 (36)	17 (35)	18 (42)	21 (43)	15 (31)
Valine (Val)	30 (65)	26 (57)	26 (67)	29 (63)	29 (62)	31 (61)	32 (66)	31 (66)	30 (63)	26 (60)	28 (57)	28 (58)
ΣEAA	265	245	240	262	263	283	283	273	268	239	259	246
Non-essential amino acid (NEAA)												
Alanine (Ala)	31	27	27	30	31	31	33	32	31	27	28	30
Asparagine (Asn)	1	0	0	0	0	0	1	1	0	0	1	0
Aspartic acid (Asp)	50	47	47	53	50	56	58	56	52	45	50	47
Glutamic acid (Glu)	83	81	79	89	92	118	97	93	95	83	82	80
Glutamine (Gln)	0	0	0	0	0	0	0	0	0	0	0	0
Glycine (Gly)[3]	64	72	64	77	80	85	88	82	84	69	60	78
Serine (Ser)	28	27	26	30	29	31	33	31	30	26	28	27

[1] Gram of amino acid per kg of diet and the values in parentheses are the percentage of amino acid content relative to the lysine content of each dietary treatment.

[2] Means are as-fed basis (n = 2) and there are no significant difference ($P>0.05$).

[3] The content of Arg and Gly calculated from the analysis of Arg/Gly value. Difficulty in separating analytical peaks of arginine and glycine necessitated that the quantity of each amino acid be calculated from the analysis of the arginine/glycine values using a reference amino acids profile (Dozier III and Hess, 2011).

Table 5. weights, weight gain (WG), thermal unit growth coefficient (TGC), specific growth rate (SGR), feed intake (FI), feed conversion ratio (FCR) and protein efficiency ratio (PER) of Asian seabass fed diets containing different levels of tuna viscera hydrolysates for 56 days.

Variable	Experimental Diets											
	1 (control)	2 (A1-50)	3 (A2-25)	4 (F1-25)	5 (F2-25)	6 (N1-25)	7 (N1-50)	8 (N1-75)	9 (N2-25)	10 (N2-50)	11 (N2-75)	12 (Reference)
IBW [1]	2.61±0.01	2.60± 0.00	2.60± 0.01	2.60±0.00	2.61±0.01	2.61±0.01	2.60±0.02	2.60±0.00	2.60±0.00	2.60±0.00	2.60±0.01	2.60± 0.00
FBW [2]	17.5±0.7 [a]	22.5±1.1 [ab]	24.4±0.5 [b]	25.6±1.5 [b]	25.1±3.1 [b]	21.0±4.6 [ab]	24.1±1.7 [b]	25.1±4.3 [b]	23.3±1.0 [b]	22.9±1.7 [b]	23.3±1.6 [b]	24.4±2.5 [b]
WG 3	570.9±27.7[a]	764.4±40.3[ab]	838.1±16.9[b]	883.1±59.0[b]	861.0±117.2 [b]	705.4±178.9[ab]	827.7±61.1[b]	866.2±163.5[b]	796.8±39.1[b]	779.4±66.4 [b]	795.8±60.2 [b]	839.6±96.1[b]
TGC [4]	3.52±0.16 [a]	4.70±0.25[ab]	5.15±0.12 [b]	5.43±0.36 [b]	5.31±0.73 [b]	4.35±1.09 [ab]	5.09±0.39 [b]	5.33±1.01 [b]	4.90±0.24 [b]	4.79±0.41 [b]	4.90±0.37 [b]	5.16±0.59 [b]
SGR [5]	3.4±0.1 [a]	3.9±0.1 [b]	4.0±0.0 [b]	4.1±0.1 [b]	4.0±0.2 [b]	3.7±0.4 [ab]	4.0±0.1 [b]	4.0±0.3 [b]	3.9±0.1 [b]	3.9±0.1 [b]	3.9±0.1 [b]	4.0±0.2 [b]
FI [6]	18.7±0.5 [a]	23.5±0.8 [b]	25.6±0.4 [b]	26.4±1.2 [b]	24.9±1.7 [b]	22.8±3.2 [b]	24.7±1.2 [b]	25.6±2.8 [b]	24.2±1.1 [b]	24.7±1.9 [b]	24.7±0.7 [b]	23.5±2.3 [b]
FCR [7]	1.3±0.0[b]	1.2±0.0 [ab]	1.2±0.0 [ab]	1.2±0.0 [ab]	1.1±0.1 [a]	1.3±0.1 [b]	1.2±0.0 [ab]	1.2±0.1 [ab]	1.2±0.0 [ab]	1.2±0.0 [ab]	1.2±0.1 [ab]	1.1±0.0 [a]
PER [8]	1.7±0.0	1.8±0.0	1.9±0.0	1.9±0.0	2.0±0.1	1.7±0.2	1.9±0.1	1.9±0.2	1.9±0.0	1.8±0.0	1.8±0.1	2.0±0.0

Values are presented as mean±standard deviation (n=3). Means in each column with different superscripts are significantly different (P<0.05).
[1] Initial body weight (g)
[2] Final body weight (g)
[3] WG (%) = [$(W_f - W_i) / W_i$] * 100
[4] TGC = [$(W_f^{1/3} - W_i^{1/3}) / (T \times \Delta t)$] *1000
[5] SGR (% day^{-1}) = [$(ln\ W_f - ln\ W_i) / t$] *100
[6] Feed intake (g/fish)
[7] FCR = Total as-fed feed intake (g) / Total wet weight gain (g)
[8] PER = Average weight gain (g) / Protein intake (g)

Table 6. Mean feed intake of test and control diets within each dietary treatment in the preference test

	2 (A1-50)	3 (A2-25)	4 (F1-50)	5 (F2-25)	6 (N1-25)	7 (N1-50)	8 (N1-75)	9 (N2-25)	10 (N2-50)	11 (N2-75)	12 (Reference)
Test diet[1]	1.84±0.13	1.82±0.34	1.89±0.10	2.05±0.13	1.83±0.18	1.82±0.35	1.99±0.16	1.70±0.40	1.67±0.34	2.08±0.06	1.81±0.30
Control diet[1]	1.54±0.16	1.25±0.28	1.52±0.19	1.59±0.21	1.52±0.20	1.05±0.58	1.51±0.18	1.38±0.38	0.93±0.33	1.20±0.06	1.35±0.44
P-value [2]	0.03	0.04	0.04	0.01	0.02	0.03	0.02	0.03	0.01	0.00	0.00
Relative preference Value (%) [3]	18.36±3.62[a]	46.28±5.95[a]	31.04±9.41[a]	29.72±11.11[a]	20.99±4.13[a]	65.15±9.77[b]	32.14±5.78[a]	23.97±5.60[a]	66.17±14.73[b]	73.39±2.28[b]	71.84±0.11[b]

[1] Feed intake = given feed – uneaten feed, values are means ± SD of feed intake of three replicates and presented in gram of diet per tank (9 fish per tank).
[2] Paired samples T-test between test and control diet. There are significantly different when P<0.05.
[3] Relative diet preference value (%) = (gram of test diet consumed – gram of control diet consumed)/ gram of control diet consumed *100 and means with different superscripts in the same column are significantly different (P<0.05).

4. Discussion

All prepared tuna viscera hydrolysates had a high protein content which was consistent with the selection of animal tissues as raw materials. The high solubility of all finished hydrolysates, particularly of those at high DH, could be explained by the release of soluble low molecular weight peptides and free amino acids and the removal of undigested substances by centrifugation and partial removal of lipids after hydrolysis (Benjakul & Morrissey, 1997). The high DH-TVHPs had greater peptide content below 200 Da, which was similar to other studies in that peptide size decreased with increasing DH (Benjakul & Morrissey, 1997; Kristinsson & Rasco, 2000). Moreover, the use of different enzymes with different substrate specificities yielded hydrolysates with different peptide and amino acid compositions. The F1-TVHP and F2-TVHP showed a high portion of <200 Da peptides which was probably due to a mixture of endoprotease and exopeptidase in the enzyme F that could hydrolyze protein into smaller fragments than the enzyme A and N which consisted of only endoproteases. Choi *et al*. (2009) found that the peptide distribution of croaker hydrolysates varied between 100 and 4000 Da and the hydrolysate that was produced using Flavourzyme (endoprotease and exopeptidase) had more peptides with smaller molecular size compared to the Protamex (endoprotease) treated hydrolysate.

The contents of free amino acids Ala, Asp, Glu, Gly/Arg, Leu, and Lys were high in all hydrolysate samples. The results were similar to those reported for fermented viscera from skipjack tuna (*Katsuwonus pelamis*) (Lee *et al*., 2004), Persian sturgeon (*Acipenser persicus*) viscera hydrolysate (Ovissipour *et al*., 2009), and Indian carp (*Catla catla*) viscera hydrolysates (Bhaskar *et al*., 2008). In the present study, the high level of these free amino acids appeared to enhance the effectiveness of the hydrolysates as good attractants and stimulants in diets with total fishmeal replacement, particularly for treatments A2 and F2, which had higher levels of Arg/Gly, Asp, and Glu than the treatments of A1 and F1. It is known that alanine, glycine, proline, valine, tryptophan, tyrosine, phenylalanine, lysine, and histidine are major components of effective feeding stimulants for many fish species (Leal *et al*., 2010). In addition, neutral amino acids containing few or two carbon atoms and having unbranched and uncharged side chains are highly stimulatory for the gustatory system of fish (Caprio, 1978). However, taste preferences of fish are highly species-specific and the effectiveness of substances acting as gustatory stimuli depends on the species of the fish (Kasumyan & Døving, 2003).

The overall results indicated that dietary supplementation with TVHPs promoted palatability of diets which are commonly associated with increased feed intake and growth performance of fish if the diet is nutritionally complete. Kolkovski *et al*. (2000) also found that krill (*Euphausia superba*) hydrolysate improved diet acceptance and feed intake, and growth performance in larvae and juveniles of yellow perch (*P. flavescens*), walleye (*Stizostedion vitreum*), and lake whitefish (*Coregonus clupeaformis*). The influence of feed intake on the specific growth rate was observed for the TVHP-supplemented diets while the PER was not significantly different among dietary treatments. The higher final weights and weight gains observed in all of the supplemented groups, except diets 2 (A1-50) and 6 (N1-25) compared with fish fed the control diet, were due to better feed consumption and possibly a better essential amino acid balance of the former TVHP groups. The responses also differed among dietary treatments as a result of both DH and inclusion levels of TVHPs produced by the three different enzymes. It was observed that the diets containing 60% DH-TVHPs were more effective than the 40% DH-TVHPs. This implied that the lower DH-TVHP at the 25 g/kg inclusion rate was not enough to improve the palatability of the diet. It may be because higher DH-TVHPs contained higher concentrations of effective free amino acids and small peptides that induced the active feeding activity, promoted increased feed intake, and subsequently the good growth in Asian seabass. The results showed that the method used in preparation of enzyme hydrolyzed protein hydrolysates in the present study was effective in liberating a high level of free amino acids and small peptide products in the range of 200-500 Da. Zheng *et al*. (2012) showed that fish fed diets containing an ultra-filtered fraction (<1000 Da) of fish hydrolysate resulted in growth improvement and feed utilization efficiency when fed to Japanese flounder (*Paralichthys olivaceus*) in comparison to those with non-ultra-filtered hydrolysate. In addition, Velez *et al*. (2007) found that the majority of potent substances in evoking olfactory activity from homogenate of ragworm macerate were small molecular weight compounds of <500 Da with glycine, praline, and aspartic acid as the most abundant amino acids. In the present study, TVHP-supplemented diets contained free amino acids between 10^{-4}-10^{-2} M that might very well play an important role in the gustatory response, which was also observed by Kohbara *et al*. (2000). They studied gustatory and olfactory sensitivity to extracts of jack mackerel muscle in young yellowtail (*S. quinqueradiata*) by recording electrical responses from the palatal taste nerve and the olfactory bulb and reported that the ultra-filtrate (molecular weight cut-off at 10,000 Da) of the extract stimulated both chemosensory systems. Their thresholds in the olfactory bulb response were around 10^{-5} and 10^{-8} M of the original concentration and those in the gustatory nerve response were 10^{-4} and 10^{-2} M.

Feeding stimulants are useful when introducing fish to new feeds or switching between different feed formulations, particularly for fishmeal-free feeds. Xue *et al*. (2004) showed that feeding stimulants significantly affected feed intake of juvenile and adult gibel carp (*Carassius gibelio*) after a 3-day adaptation. In our study, there was no adaptation period because a clear response could be better evaluated when the TVHPs were introduced. The results indicated that the TVHPs successfully enhanced palatability of the fishmeal-free diets as confirmed by fish accepting the supplemented diets from the first day of feeding without a transitional period. Similarly, summer flounder larvae accepted squid hydrolysate-based larval diets during the first 3 days which led to an improvement in SGR and survival rate (Lian *et al*., 2008). In addition, we observed in the preference test that the frequency of visits and feed intake were higher for the supplemented test diets than those of the control diet. This study demonstrated that fish could discriminate and choose between different diet compositions using both olfactory and gustatory cues to identify chemicals, locate food, and respond

to nutrients with respect to feeding behavior and consumption, as also reported by Volkoff and Peter (2006) and Derby and Sorensen (2008). However, swimming behavior is not easy to observe for the fast swimming Asian seabass. It is best to assess the feeding responses of seabass in the consummatory phase, which allows the fish to take food into the mouth, followed by separation of palatable from unpalatable particles by the fish, and culminating in the ingestion of food items or the expulsion of those that are unpalatable (Lamb, 2001). The preferred diets in the preference test were in line with the feeding trial results. Overall responses indicated that Asian seabass were able to discriminate and choose the diets of different compositions using both olfactory and gustatory chemosensory cues, responding to preferred components and nutrients in their feed, which led to the initiation of feeding (Derby & Sorensen, 2008; Volkoff & Peter, 2006). Therefore, the preference test may be an appropriate preliminary method to select feeding attractants, which is not time consuming and can be practically applied for potential single and multiple taste and flavor-enhancing ingredients formulated into diets.

5. Conclusions

This study demonstrated that hydrolysates produced from tuna viscera by-products of the canning industry, by using suitable enzymes and adjusting the degree of hydrolysis, contained a large amount of small peptides and free amino acids which were effective feeding stimulants for carnivorous Asian seabass. Therefore, not only are these hydrolysates value added by-products of the seafood processing industry, but also a promising solution for palatability improvement and enhancement of fishmeal free diets which are important for culturing carnivorous fish species. Based on the growth performance and potential cost effectiveness in producing various hydrolysates in this study, the TVHPs produced using endoproteinase and exopeptidase (F) would be the most beneficial TVHPs as feeding stimulants in Asian seabass feeds. However, the main consideration, when considering the cost effectiveness of hydrolysis is the quantity of enzymes used to produce the TVHP at the desired DH.

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