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Characterisation of Digestive Enzymes and *In Vitro* Screening of Feed Ingredients for the New Hybrid Grouper, *Epinephelus coioides* (Hamilton 1822) × *Epinephelus lanceolatus* (Bloch 1790)

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Abstract

The new hybrid grouper [*Epinephelus coioides* (Hamilton 1822) × *Epinephelus lanceolatus* (Bloch 1790)] has been recently hybridised by fertilising the egg of orange-spotted grouper with sperm of giant grouper. Information on nutritional requirements for this hybrid is still limited. Characterisation of the main digestive enzymes (including pepsin, trypsin, chymotrypsin and amylase) as well as *in vitro* screening of feed ingredients suitable for the hybrid were therefore investigated in this study. The body weight of the hybrid was positively correlated with gastrointestinal weight (r = 0.8952, P < 0.001, n = 30). The characteristic observation suggested that optimal conditions for studying digestive enzyme activities in hybrid grouper were: pH 1 and 4 at 40 ^{o}C for pepsin, pH 10 at 40 ^{o}C for trypsin, pH 10 at 35 to 45 ^{o}C for chymotrypsin and pH 8 at 50 ^{o}C for amylase. *In vitro* screening of 16 feed ingredients using digestive enzymes extracted from the intestine indicated that wheat gluten, soybean meal, squid liver meal and fish meal were good sources of protein while appropriate carbohydrate sources were broken rice, rice bran, wheat bran, wheat flour and corn meal. These findings can be used for understanding the nutritional requirement via the enzymatic analysis, whereas *in vitro* digestibility could provide information on the appropriate feed ingredients that can be used for the hybrid feed formulation.

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Introduction

The new hybrid grouper [*Epinephelus coioides* (Hamilton 1822) × *E. lanceolatus* (Bloch 1790)] has been successfully hybridised by fertilising eggs of orange-spotted grouper (*E. coioides*, Serranidae) with sperm of giant grouper (*E. lanceolatus*, Serranidae) (Kiriyakit et al. 2011). Both species have desirable characteristics due to tolerance to crowding (Ahmad et al. 2000) and rapid growth (Sadovy et al. 2003), respectively. Therefore, hybridisation between the two species may result in fast growth, which is an important trait for improving aquaculture. However, the lack of information on nutritional requirements is a major problem for growth performance of the new hybrid fish. Development of artificial diet with optimised feed ingredients is therefore essential.

Fish growth is positively correlated with an increase of gastrointestinal volume for digestion and absorption of nutrients (Engrola et al. 2007; Thongprajukaew et al. 2011; Thongprajukaew and Kovitvadhi 2013). Therefore, digestive enzymes found along the alimentary tracts appear to have an important role for understanding nutritional status in animals (Thongprajukaew et al. 2011; Albentosa et al. 2012). Recent studies have reported the associations among digestive protease activity, protein digestibility, feed conversion efficiency, weight and specific growth rate of fish (Rungruangsak-Torrissen et al. 2002). Moreover, carbohydrate-digesting enzyme, α-amylase, has been applied to study fish feeding habit, growth and carbohydrate digestibility (Thongprajukaew et al. 2011).

Digestive enzyme activities contribute to the digestibility of feed in animals. *In vitro* digestibility techniques, based on digestive enzyme extracts, have been widely used for screening feeds before testing *in vivo*. This technique is less-time consuming, more economical and simpler than conventional growth trials (Supannapong et al. 2008; Hamdan et al. 2009). Moreover, changes in digestibility are closely related with protein quality that affects enzymatic digestion of feed such as disulphide bond formation, *D*-aspartic acid (Rungruangsak-Torrissen et al. 2002) and protein subunit degradation (Ebrahimi et al. 2009), as well as gelatinisation, water solubility, crystallinity, amylose content and starch diameter (Kaur et al. 2010) for carbohydrate. Therefore, studies on digestive enzyme characterisation and *in vitro* digestibility of feed ingredients may provide a suitable feed formulation for the new hybrid fish.

Materials and Methods

Chemicals

Acetic acid, Folin-Ciocalteu reagent and sodium chloride were purchased from Merck (KGaA, Germany). Sodium hydroxide and hydrochloric acid were acquired from J.T. Baker (Malaysia) and VWR BDH Prolabo (UK), respectively. These chemicals were purchased from Sigma-Aldrich Co., including *DL*-alanine (USA), *N*-α-benzoyl-*L*-arginine-*p*-nitroanilide (Japan), casein (New Zealand), chloramphenicol (Singapore), dinitrosalicylic acid (India), maltose (Japan), *p*-nitroanilide (China), soluble starch (USA), *N*-succinyl-ala-ala-pro-phe-*p*-nitroanilide (USA), trichloroacetic acid (Germany), trinitrobenzene sulphonic acid (USA) and *L*-tyrosine

(Germany). Luteinising hormone-releasing hormone analogue and metoclopamide were purchased from Sanofi-Aventis Co., (Germany) and Nida Phama Inc., (Thailand), respectively. All chemicals were of analytical grade.

Preparation and hybridisation of grouper

Adult orange-spotted female grouper and male giant grouper were reared in a floating sea cage (5 m width×5 m length×2.5 m height) at Samed Island, Sub-station of Rayong Coastal Fisheries Research and Development Center, Department of Fisheries, Thailand. The technique to produce hybrid grouper larvae were modified from Peatpisut and Bart (2010) and Kiriyakit et al. (2011). Briefly, mature orange-spotted female grouper (6.50 kg, oocytes diameter > 450 µm) was selected in order to induce ripe eggs. Two injections of luteinising hormone-releasing hormone analogue (LHRHa) and metoclopamide were administered intramuscularly behind the dorsal fin. The initial LHRHa (10 $\mu g \cdot kg^{-1}$) and metoclopamide (2 $mg \cdot kg^{-1}$) were administered and then followed by second doses (20 $\mu g \cdot kg^{-1}$ and 2 $mg \cdot kg^{-1}$, respectively) at 24 h after the first injection. After ovulation. (approximately 10-12 h after the second injection), eggs were stripped.

Hybridisation by fertilising stripped eggs with the cryopreserved sperm was performed according to Kiriyakit et al. (2011). The frozen sperms of giant grouper were placed on the eggs (approximately 9.0×10^5 spermatozoa per egg) and gently mixed for 3 min. Filtered seawater (salinity 32 ppt) was poured into the bowl to activate the fertilisation process.. After 15 min, the eggs were rinsed and transferred into a hatching tank (1,000 L-round black plastic tank). Water temperature was maintained at 30 ºC by using an aquarium heater and water salinity was maintained at 32 ppt.

Fish husbandry and sampling

Newly hatched 1 day old hybrid grouper (1 DAH) were stocked (10 $individual \cdot L^{-1}$) in a 12 m^3-round cement rearing tank . The feeding techniques for newly hatched larvae were slightly modified from Sutthinon et al. (2012). Briefly, during 3-10 DAHs, hybrid grouper larvae were fed with phytoplanktons, *Nannochloropsis* sp., *Tetraselmis* sp. and *Isochrysis* sp. at a total density of 1.0×10^6 $cells \cdot mL^{-1}$ once daily (09.00 h), and screened rotifers, *Brachionus rotundiformis* (80-90 µm lorica length) at 20 $individual \cdot mL^{-1}$ twice daily (09.00 and 15.00 h). During 10-16 DAHs, enriched rotifers were added at 10-15 $individual \cdot mL^{-1}$ without screening. From 17-35 DAHs, the larval hybrid groupers were fed with enriched Artemia nauplii (5-10 $individual \cdot mL^{-1}$) and commercial marine pellet feeds (>55% crude protein). Subsequently, the larvae were fed with adult Artemia (10-20 $individual \cdot mL^{-1}$) and pellet feeds. At 42 DAH, the fish were transferred and reared in 1.5 m^3-round cement tank. They were maintained in flow-through seawater (30-32 ppt) system at 28-30 ^oC, 12-h light/12-h dark, and daily fed to satiation with commercial marine pellet feed (42% crude protein). Approximately 80% of the water was replaced weekly to maintain optimal water quality. The 90 DAH hybrid groupers (9.56±0.09 cm total length and 15.95±0.47 g body weight, Fig. 1), which is a marketable stage for intensive rearing, were starved for 24 h prior to sampling. The fish were killed by chilling in ice and then the gastrointestinal tracts were carefully collected for use in digestive enzyme extraction.

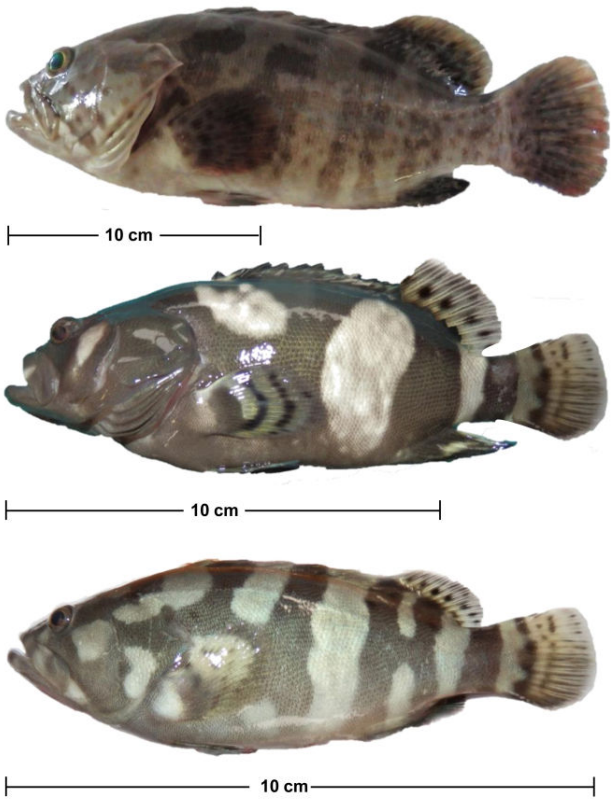

Fig. 1. Comparative images showing general morphology of 6 months old orange-spotted grouper (top), 5 months old giant grouper (middle) and 3 months old hybrid grouper *Epinephelus coioides* × *E. lanceolatus* used in this study (bottom). Scale bars are 10 cm.

Digestive enzyme extraction

Digestive enzymes were extracted according to the method of Rungruangsak and Utne (1981) with slight modification. Briefly, the stomach and intestine (including pyloric caeca) were homogenised in 10 mM and 1 mM HCl (1:2 *w/v*), respectively, using a micro-homogeniser (THP-220; Omni International, Kennesaw GA, USA). Centrifugation of the homogenate was carried out at 15,000×*g* for 30 min at 4 ^{o}C. The supernatant was carefully collected after removing the lipid layer and then kept in small portions at –80 ^{o}C until analysis.

Characterisation of digestive enzymes

The characteristics of digestive enzymes including pepsin (from stomach extracts), trypsin, chymotrypsin and amylase (from intestine and pyloric caeca extracts) were performed at pH 1 or 2-12. The profiles were studied at ambient temperature using: KCl-HCl buffer for pH 1-2, citrate-phosphate buffer for pH 3-5, phosphate buffer for pH 6-8, $NaHCO_3$-Na_2CO_3 buffer for pH 9-10, Na_2HPO_4-NaOH for pH 11, and KCl-NaOH for pH 12. For temperature characteristics (25-80 ^{o}C), the profiles of each enzyme were determined at optimal pH. The relative activities of digestive enzymes were calculated as percent conversion of 1 μmol substrate per min.

The assay of digestive enzymes was performed as described below: Pepsin activity (EC 3.4.23.1) was assayed according to the method described in Rungruangsak and Utne (1981) with slight modification from Rick (1974). Briefly, 200 µL of 1% casein was mixed with an equal volume of crude enzyme extract, incubated for 10 min and stopped by adding 1 mL of 5% trichloroacetic acid. The mixture was centrifuged at 5000×*g* at room temperature for 20 min. One mL of 0.5 M NaOH was added to 0.5 mL of the supernatant, followed by 0.3 mL of Folin-Ciocalteu reagent (four-fold dilution). Subsequently, the mixture was measured spectrophotometrically at 720 nm and compared with *L*-tyrosine standard curve.

Trypsin (EC 3.4.21.4) and chymotrypsin (EC 3.4.21.1) activities were assayed based on the method of Rungruangsak-Torrissen et al. (2006) with slight modification from Erlanger et al. (1961) and del Mar et al. (1979), using 1.25 mM *N*-α-benzoyl-*L*-arginine-*p*-nitroanilide (BAPNA) and 0.1 mM *N*-succinyl-ala-ala-pro-phe-*p*-nitroanilide (SAPNA) as substrates, respectively. The assay was performed by mixing 700 µL of substrate with 100 µL of digestive enzyme extracts, incubating for 10 min, and then stopping the reaction by adding 800 µL of 30% acetic acid. The activities of both enzymes were determined by measuring the absorbance at 410 nm and compared with *p*-nitroanilide standard curve.

Amylase activity (EC 3.2.1.1) was determined according to Areekijseree et al. (2004) with slight modification from Bernfeld (1951). The reaction mixture containing 25 µL of 5% soluble starch, 62.5 µL of buffer, 37.5 µL of 20 mM NaCl and 125 µL of crude enzyme extract was incubated for 15 min. Subsequently, the liberated maltose was stained by adding 250 µL of 1% dinitrosalicylic acid, boiled at 100 ^{o}C for 5 min, cooled to room temperature and mixed with 2.5 mL of distilled water. The amylase activity was measured spectrophotometrically at 540 nm and compared with maltose standard curve.

In vitro digestibility of feed ingredients

Feed ingredient preparation

All feed ingredients were provided by Coastal Aquatic Feed Research Institute, Chonburi, Thailand. Feed ingredients used as sources of protein (including chicken meal, fermented soybean meal, fish meal, meat and bone meal, shrimp head meal, soybean meal, squid liver meal and wheat gluten) and carbohydrate (including alpha starch, broken rice, corn flour, corn meal, rice bran, tapioca, wheat bran and wheat flour) were ground, sieved and dried using a freeze dryer (Delta 2-24 LSC, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) for 48 h. All samples were packed in polyethylene bags and then kept in desiccators for later analysis of *in vitro* digestibility.

In vitro digestibility of protein and carbohydrate

The intestine (including pyloric caeca) extracts were dialysed overnight against the extraction buffer before being used in this study.

In vitro digestibilities of protein and carbohydrate were performed under weak alkaline condition based on activities of trypsin and amylase, respectively, according to the methods described by Thongprajukaew et al. (2011) with slight modification from Rungruangsak-Torrissen et al. (2002). The reaction mixtures containing 5 mg of feed ingredients, 10 mL of 50 mM phosphate buffer pH 8, 50 µL of 0.5% chloramphenicol and 125 µL of dialysed enzyme, were incubated at 25 °C for 24 h. The enzyme: substrate (E: S) ratio used in the assays was 1: 40.

In vitro protein digestibility was determined by measuring the increase of liberated reactive amino groups of cleaved peptides. The reaction mixture containing 200 µL of digested solution, 2 mL of 50 mM phosphate buffer pH 8 and 1 mL of 0.1% trinitrobenzene sulphonic acid (TNBS) was incubated in the dark at 60 °C for 1 h and then 1 mL of 1 M HCl added. The absorbance was measured at 420 nm and compared with *DL*-alanine standard curve. For carbohydrate digestibility, the increase in reducing sugar was analysed by mixing 1 mL of digested solution with 500 µL of 1% dinitrosalicylic acid, heating in boiling water for 5 min, cooling to room temperature, measuring absorbance at 540 nm and comparing with maltose standard curve. Protein and carbohydrate digestibilities were calculated and expressed as µmol *DL*-alanine equivalent g^{-1} and µmol maltose g^{-1}, respectively.

Statistical analysis

Data analyses were performed using SPSS Version 14 (SPSS Inc., Chicago, USA). All data were expressed as mean±SEM in triplicate observations. Pearson correlation coefficients (r) between weights of body and gastrointestinal tract were calculated. Significant differences between means were ranked using Duncan's multiple range test at 95% significance level.

Results and Discussion

Morphometric relationship between weights of body and gastrointestinal tract

Gastrointestinal changes are directly associated with food process assimilation (Segner et al. 1993). The highly positive correlations between weights of body and gastrointestinal tract (Fig. 2; $r = 0.8952$, $P < 0.001$, $n = 30$) were observed and contributed 7.33 ± 0.09% of their body weight. The finding is similar to the positive relationship between the two parameters ($r = 0.8214$, $P < 0.0001$, $n = 362$) found in juvenile Siamese fighting fish, *Betta splendens* (Thongprajukaew et al. 2011). The basic information could be used for calculating the required amount of tissue when gastrointestinal tract is used for digestive enzyme extraction.

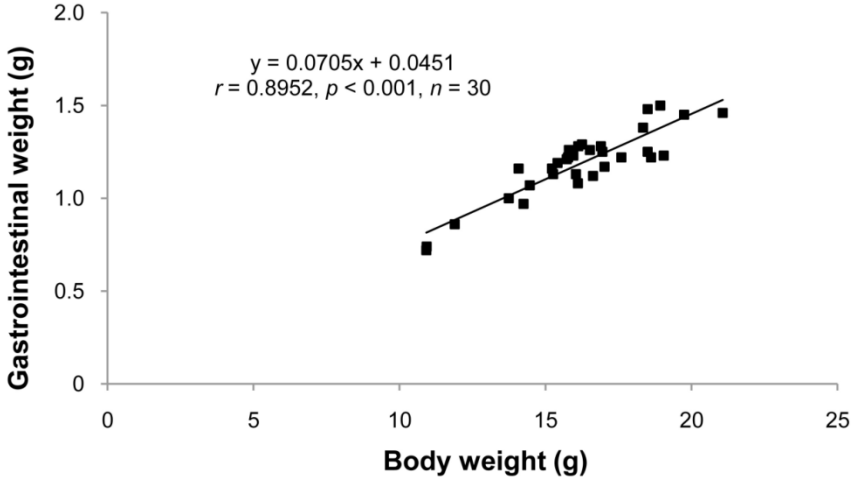

Fig. 2. Morphometric relationship between weights of body and gastrointestinal tract.

Characterisation of digestive enzymes

Characterisation of pepsin

Pepsin is released from chief cells in the stomach for breaking down large-chain polypeptides. Feng et al. (2008a and b) found two and three forms of pepsinogens A and C transcripts, respectively, expressed during developmental stages of orange-spotted grouper. This prominence indicates the stomach functionality as well as marks the shift from intracellular to extracellular digestion (Salze et al. 2012). The main isoform of hybrid grouper was found in pH 1 (Fig. 3a) at 40 ^{o}C (Fig. 3b) whereas the other isoform exhibited at pH 4 (Fig. 3a) with optimal temperature at 40 and 50 ^{o}C (Fig. 3b). The activity of all isoforms was relatively high in the temperature range of 25 to 55 ^{o}C and decreased dramatically at 60 ^{o}C. The few pepsin isoforms with broad temperature range and also close to the water temperature in natural habitat indicates the crucial role for digestion of various dietary proteins from foods. However, substrate–gel electrophoresis of acid proteases (Díaz-López et al. 1998) in this hybrid should be classified in the future. The presence of pepsins in the hybrid is in agreement with, and has similar characteristics as, that observed in many fish species, such as pH 3.0-3.5 at 45 ^{o}C in pectoral rattail, *Coryphaenoides pectoralis* (Gilbert 1892) (Klomklao et al. 2007), and pH 2.5-3.5 at 35-40 ^{o}C in European eel, *Anguilla anguilla* (Linnaeus 1758) (Wu et al. 2009).

Characterisation of trypsin

Trypsin in hybrid grouper showed at least two isoforms at pH 5 and 10 (Fig. 3c). The relative activity of acidic isoform increased with temperature and exhibited the highest activity at 80 ^{o}C (Fig. 3d). This isoform might be important for digesting protein at the stomach-intestine border or digesting reverse-digested foods from intestine to stomach. Benjakul and Morrissey (1997) pointed that acidic condition can cause changes in charge distribution and conformation of enzyme which affects substrate binding capacity.

However, many studies in fish have reported the pH stability of trypsin at pH 5 (Lu et al. 2008; Elhadj-Ali et al. 2009). Marcuschi et al. (2010) hyphothesised that trypsin activity might be stabilised by substrate at high temperature. This finding is in agreement with maximal activity found in pyloric caeca of Amazonian tambaqui, *Colossoma macropomum* (Cuvier 1816) (Marcuschi et al. 2010). However, protein denaturation occurs at high temperature. Therefore, the thermophilic isoform might suggest the lower activity in gastrointestinal tract of fish, which is close to water temperature.

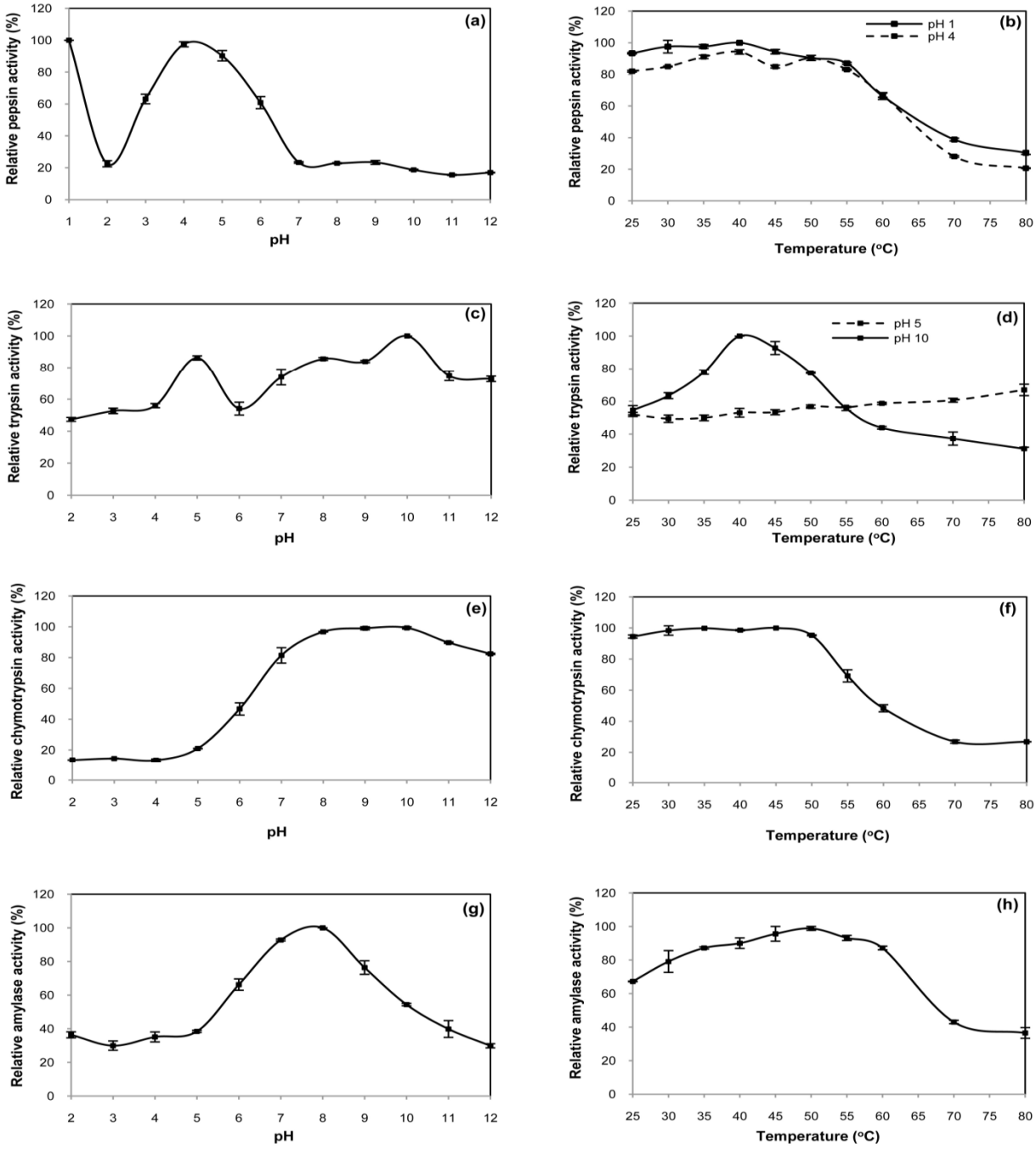

Fig. 3. Relative activity (%) of the main digestive enzymes in hybrid grouper. The pH characteristics at ambient temperature of pepsin (a), trypsin (c), chymotrypsin (e) and amylase (g) and the temperature characteristics at chosen optimal pHs of the same enzymes (b, d, f and h, respectively). Each data point represents the mean of triplicate analyses.

The alkaline isoform of trypsin gave the highest activity at 40 ^{o}C, a slight change at 45 ^{o}C and then dramatically decreased above 50 ^{o}C (Fig. 3d). This isoform covered the pH and temperature stability of most trypsin isoforms reported in fishes (Bougatef 2013) and may therefore be a major enzyme for controlling the protein digestion in the hybrid. Similar conditions have been reported, such as pH 10.5 at 40 ^{o}C in grey triggerfish, *Balistes capriscus* Gmelin 1789 (Jellouli et al. 2009) and pH 10 at 30-35 ^{o}C in Siamese fighting fish (Thongprajukaew et al. 2010a). Liu et al. (2012) reported the highest activity of trypsin detected in pyloric caeca of orange-spotted grouper, followed by anterior, middle and hind sections of intestine, respectively. This enzyme exhibited the optimal condition at pH 8-10 at 50 ^{o}C whereas stability occurred at 50 ^{o}C in the pH range of 6-8. Therefore, stabilisation of trypsin in hybrid grouper during enzymatic assay by lowering alkalinity could be possible.

Characterisation of chymotrypsin

The relative activity of chymotrypsin was more than 95% in pH range of 8-10 (Fig. 3e). This enzyme had higher activity in alkaline than in acidic conditions. The highest activity at pH 10 exhibited a broad range temperature of 25-50 ^{o}C whereas a dramatic decrease in activity was observed above 55 ^{o}C (Fig. 3f). However, the temperature of approximately 25-30 ^{o}C, which is close to the natural habitat, may be more suitable for digesting food protein along the alimentary tract of the hybrid grouper. This optimal condition is similar to that reported in many fish species, such as pH 10 at 40 ^{o}C in Siamese fighting fish (Thongprajukaew et al. 2010a) and pH 9 at 40 ^{o}C in guppy, *Poecilia reticulata* Peters 1859 (Thongprajukaew and Kovitvadhi 2013). This condition also covers and is in agreement with pH and temperature stability studies in cuttlefish, *Sepia officinalis* Linnaeus 1758 (Balti et al. 2012) and vermiculated sailfin catfish, *Pterygoplichthys disjunctivus* (Weber 1991) (Villalba-Villalba et al. 2013). Furthermore, the optimal condition of the chymotrypsin from hybrid grouper exhibited some potential application where low processing temperature is required.

Characterisation of amylase

The activity of amylase was relatively low in acidic and strong alkaline conditions as shown in Fig. 3g. The highest activity was found at pH 8 (Fig. 3g) with optimal temperature at 50 ^{o}C (Fig. 3h) whereas more than 85% relative activity was exhibited in the temperature range of 35 to 60 ^{o}C. This optimal pH is close to the neutral condition measured from minced gastrointestinal tract of the hybrid grouper (pH 6.68±0.04) and also similar to findings for the pancreas and intestines of many fishes (Chakrabarti et al. 1995). Relative activity in a wide range of temperature may represent an ecological advantage for adaptation. The suitable condition for assaying amylase in hybrid is similar to that observed in seabream, *Sparus aurata* (Linnaeus 1758) (Munilla-Moran and Saborido-Rey 1996), Pacific bluefin tuna, *Thunnus orientalis* (Temminck & Schlegel 1844) (de la Parra et al. 2007) and Siamese fighting fish (Thongprajukaew et al. 2010b). However, sex and age of fish may play a major role in changing the amylase isoforms during the lifespan (Thongprajukaew et al. 2010b).

In vitro digestibility of feed ingredients

Protein digestibility is a primary indicator for determining feed protein digestion and the digested products obtained after proteolytic digestion primarily depend on feed protein quality such as disulphide bond formation, *D*-aspartic acid and protein subunit degradation (Rungruangsak-Torrissen et al. 2002; Ebrahimi et al. 2009). The highest digestibility was observed after incubating digestive enzymes in the presence of wheat gluten, followed by soybean meal, squid liver meal and fish meal, respectively (Fig. 4a). These feed ingredients are generally used in both aquatic and terrestrial animals. Linear regression based on data from earlier reports in various fish species exhibited the apparent protein digestibility in range of 99.60-102.00% (5 fish species), 40.70-99.40% (32 fish species), 87.00-94.20% (2 fish species) and 56.00-99.00% (32 fish species) for wheat gluten, soybean meal, squid meal and fish meal, respectively (Sales 2008). For fermented soybean meal, the protein digestibility was similar to that found in chicken meal but higher than for shrimp head meal and meat and bone meal. Therefore, based on protein digestibility, the pellet feed for successive growth of the hybrid grouper should contain the selected feed ingredients from this study.

Carnivorous fish require substantial protein for growth and development whereas dietary carbohydrate is also important as it is necessary for improving protein utilisation (Thongprajukaew et al. 2011). However, limited use of carbohydrate due to insufficient carbohydrate-digesting enzymes has been reported in carnivorous fish. This leads to improved utilisation efficiency of carbohydrate by supplementation of carbohydrate-digesting enzymes or modification of starch by different methods (Mohapatra et al. 2002; Thongprajukaew et al. 2011). In hybrid grouper, the highest maltose liberation was obtained after digesting broken rice with crude enzymes (Fig. 4b). This product was significantly lower in rice bran, wheat bran, wheat flour and corn meal. However, the digestibility of theses feed ingredients is influenced by the developmental stage and sex of fish (Thongprajukaew et al. 2010a, b; Thongprajukaew and Kovitvadhi 2013).

Therefore, comparisons in digestibility should be avoided due to the differences in various factors that affect the digestion capacity within the same species as well as between species. For other sources of feed ingredients, the lowest carbohydrate digestion was observed for tapioca and alpha starch. The differences in digestibility values were indirectly contributed by carbohydrate contents, but proportionally affected by starch physicochemical properties such as degree of gelatinisation, water solubility, crystallinity, amylose content and starch diameter (Kaur et al. 2010). Therefore, pre-treatment of feed ingredients for improving physicochemical properties of carbohydrate to enhance digestive enzyme hydrolysis should be of interest.

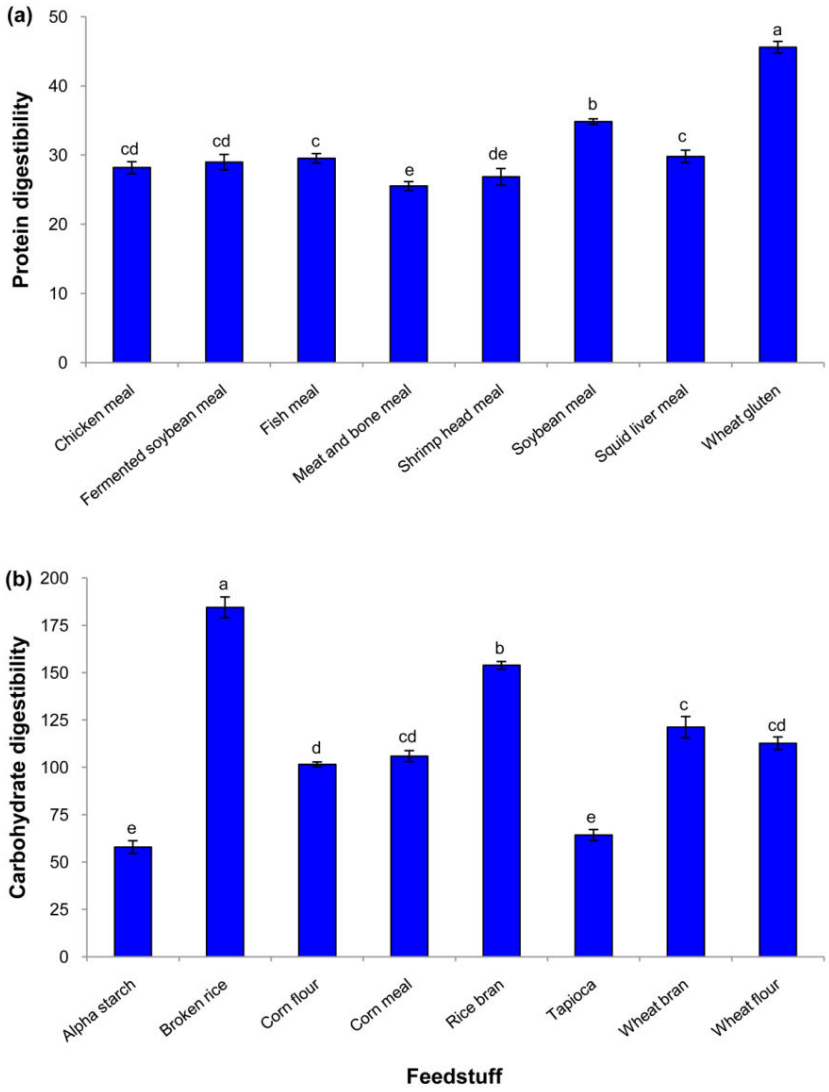

Fig. 4. *In vitro* digestibilities of tested feed ingredients using digestive enzyme extracts from intestine of hybrid grouper. Digestibilities of protein (a, µmol *DL*-alanine equivalent g^{-1}) and carbohydrate (b, µmol maltose g^{-1}) were calculated from triplicate observations. Data with different superscripts are significantly different ($P < 0.05$).

Conclusions

Body weight of hybrid grouper was closely related to weight of the gastrointestinal tract. This species showed carnivorous feeding habit, as indicated by having many isoforms of protein-digesting enzymes. The characteristic observation suggests that the suitable conditions for studying digestive enzymes in the hybrid were: pH 1 and 4 at 40 ^{o}C for pepsin, pH 10 at 40 ^{o}C for trypsin, pH 10 at 35-45 ^{o}C for chymotrypsin and pH 8 at 50 ^{o}C for amylase. The major enzymes showed high relative activity in a wide range of temperature which may represent an ecological advantage for fish adaptation. The *in vitro* digestibility based on digestive enzyme from the intestine suggests the appropriate feed ingredient sources. The ingredients, including wheat gluten, soybean meal, squid liver meal and fish meal, should be used as protein source in the future pellet diets whereas broken rice, rice bran, wheat bran, wheat flour and corn meal were among common carbohydrate sources.

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