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In vivo and *in vitro* protein digestibility in juvenile bagrid catfish *Mystus nemurus* (Cuvier and Valenciennes 1840) fed soybean meal-based diets

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Abstract

In vivo and *in vitro* protein digestibility of 0% to 60% soybean meal protein (SBM) substitution from fishmeal protein was conducted for bagrid catfish *Mystus nemurus* juveniles. Seven experimental diets containing 35% protein and 15% lipid were fed to bagrid catfish to determine the *in vivo* protein digestibility. *In vitro* methods were determined using pH stat, pH shift, spectrophotometric assay and sodium dodecyle sulphate polyacrylamide gel electrophoresis (SDS-PAGE) with different enzyme mixtures (crude intestinal extract of bagrid catfish, Lazo 1-enzyme, Hsu 3-enzyme, Saterlee 4-enzyme). *In vivo* and *in vitro* protein digestibility decreased with increased SBM protein substitution with highest tolerance level of 10% (*in vivo*). pH stat and pH drop methods showed the highest degree of hydrolysis (DH) and relative protein digestibility (RPD) using Saterlee 4-enzyme system. However, pH stat method showed highest correlations (r^2 = 0.9263) with the *in vivo* results using crude intestinal enzyme extract compared to other enzyme systems. The highest correlation of the *in vitro* methods using crude intestinal enzyme extracts of bagrid catfish was determined using spectrophotometric assay (r^2 = 0.9284) followed by pH stat (r^2 = 0.9263), SDS-PAGE (r^2 = 0.8348) and pH drop (r^2 = 0.6777). All the *in vitro* methods tested are suitable to rapidly determine protein digestibility for bagrid catfish except for pH drop.

Keywords: digestive proteases, pH shift, pH stat, soybean meal, SDS-PAGE, spectrophotometric assay

Introduction

Bagrid catfish is a commercially important fish in fisheries, aquaculture and ornamental industry in Southeast Asia particularly Malaysia, Thailand, Indonesia (Leesa-Nga, Siraj, Daud, Sodsuk, Tan & Sodsuk 2000), Vietnam (Widjaja, Abdulamir, Saari, Bakar & Ishak 2009) and Laos (Somboon 2006). The high economic value of bagrid catfish is due to its excellent taste (Rahmah, Kato, Yamamoto, Takii, Murata & Senoo 2014) and high content of protein, vitamin E (Mesomya, Cuptapun, Jittanoonta, Hengsawadi, Boonvisut, Huttayanon & Sriwatana 2002) and polyunsaturated fatty acids (Mesomya *et al.* 2002; Widjaja *et al.* 2009). Although various attempts have been conducted to culture this valuable fish to meet the increasing demand, insufficient information is available for the culture of bagrid catfish. Feeding is commonly done using trash fish or commercial feeds formulated specifically for African catfish and tilapia *Oreochromis* sp. (Eguia, Kamarudin & Santiago 2000). Nutrient requirements of farmed bagrid catfish have not been fully determined, despite several growth studies conducted to determine the optimum protein requirement (Eguia *et al.* 2000; Ng, Soon & Hashim 2001) and carbohydrate utilization (Hamid, Mahayat & Hashim 2011).

In the aquaculture feed industry, protein is the most expensive nutrient as its cost accounts for more than 50% of the total aquaculture feed production (El-Sayed 2006). The high demand of fishmeal usage as protein source in aquaculture feed

industry has resulted in increased price due to the limited supply (Olsen & Hasan 2012) which eventually increasing the total cost of aquaculture production. Therefore, replacing fishmeal protein with cheaper and more accessible alternative sources (especially plant proteins) has become highly essential (Cabral, Fernandes, Campos, Castro-Cunha, Oliveira, Cunha & Valente 2013). Soybean meal is one of the promising candidates for fishmeal protein replacement due to its high protein and favourable amino acids profile, stable supply and reasonable price (Fuertes, Celada, Carral, Sáez-Royuela & González-Rodríguez 2012). However, the limitation of soybean meal inclusion in fish diets is primarily due to the presence of antinutritional factors, which affects the bioavailability of amino acids (Francis, Makkar & Becker 2001). Soybean meal also lacks some of the essential amino acids such as methionine (Pillay & Kutty 2005), cystine (Tibaldi & Tulli 1999) and lysine (Bai, Wang & Shin 2005). Therefore, the ability of cultured fish to digest and absorb protein in feed ingredients needs proper assessment which is made possible through digestibility studies.

The conventional *in vivo* method used to measure apparent protein digestibility in feed involves feeding trials, which are tedious, time-consuming, expensive, requires close monitoring and care of the experimental fish. The difficulties of faecal matter collection from fish tanks and leaching of nutrients from feeds or faeces complicate the estimation of apparent protein digestibility (Belal 2005). However, more reliable *in vitro* protein digestibility methods have been conducted to assess the quality of protein through stimulated conditions such as the pH stat (Dimes & Haard 1994; Ezquerra, Garcia-Carreño & Carrilo 1998; Tibbetts, Verreth & Lall 2011), pH drop (Dimes & Haard 1994; Ezquerra *et al.* 1998), spectrophotometric assay (Chong, Hashim & Ali 2002a; Maitra, Ramachandran & Ray 2007) and SDS-PAGE (Alarcon, Garcia-Carreño & Navarrete del Toro 2001; Lemos, Navarrete del Toro, Córdova-Murueta & Garcia-Carreño 2004; Maitra *et al.* 2007). The *in vitro* methods use either a single or multiple mixtures of commercial enzymes like proteases, trypsin, chymotrypsin and peptidase originating from mammals or bacteria (Hsu, Vavak, Satterlee & Miller 1977; Saterlee, Marshall & Tennyson 1979). Several reports have shown that *in vitro* enzymatic digestion using proteases isolated from digestive system of the experimental fish itself delivers a better correlation with the *in vivo* data (Dimes & Haard 1994). The *in vitro* technique is also useful for determining the protein quality of potential feedstuffs to be included in the diet for that particular fish species. This information is important to formulate nutritionally efficient, least cost feeds, while minimizing the excessive nutrient wastage with potential detrimental effects on the environment.

The objective of this study was to determine the *in vivo* and *in vitro* (pH stat, pH drop, spectrophotometric assay and SDS-PAGE) protein digestibility of experimental diets with increased level of soybean meal protein replacement from fishmeal protein. The results from the *in vitro* methods were then correlated with the *in vivo* data to determine the reliability of the *in vitro* methods to measure protein digestibility for bagrid catfish.

Materials and methods

Experimental diets

Seven iso-nitrogenous and iso-caloric (20 kJg^{-1}) experimental diets were formulated to contain 35% protein and 15% lipid with 0%, 10%, 20%, 30%, 40%, 50% and 60% protein replacement from fishmeal with soybean meal (Table 1). Protein sources were contributed from Danish fishmeal (FM), soybean meal (SBM) and squid meal (SM) (SM included at 3% level as attractant). Lipids were incorporated into the diets at a ratio of 1:1 of animal (FM, SM and fish oil) to plant (SBM and corn oil) source. Chromium oxide was added at 1% in all the experimental diets to measure the *in vivo* apparent protein digestibility.

Ingredients were mixed in a feed mixer (Tyrone, Model TR 202, L.J. Stuart and Company, Sydney, Australia) and passed through a pelletizer (Model MH 237, Miao Hsien Ltd., Taichung, Taiwan) to produce 0.25 cm diameter pellets. The pellets were dried in an oven at 60°C for 24 h. The dried pellets were kept in airtight containers and stored at −20°C prior to feeding trials. Proximate analysis was performed on the feed ingredients and experimental diets to determine the contents of moisture, crude protein, crude lipid, fibre and ash according to AOAC (1997) as presented in Table 1.

Fish and culture facility

Bagrid catfish juveniles were purchased from Enggor Aquaculture Development Centre, Perak,

Table 1 Formulation and proximate composition of experimental diets

Dry basis ingredients (g kg^{-1})	Diet 1 0	Diet 2 10	Diet 3 20	Diet 4 30	Diet 5 40	Diet 6 50	Diet 7 60
Fishmeal*	469	422	375	328	281	234	188
Soybean meal†	0	73	145	217	290	362	435
Squid meal‡	30	30	30	30	30	30	30
Corn α-starch	272	249	225	202	178	155	131
Fish oil	19	24	29	34	39	44	49
Corn oil	75	73	71	69	67	65	63
Cellulose	65	60	55	50	45	40	35
Vitamin mix§	20	20	20	20	20	20	20
Mineral mix**	20	20	20	20	20	20	20
Chromium oxide	10	10	10	10	10	10	10
CMC (binder)	20	20	20	20	20	20	20
Proximate composition (g kg^{-1})							
Moisture	35.6	46.5	39.2	34.7	31.6	36.6	33.2
Crude protein	355.4	355.1	354.4	357.5	355.6	358.2	357.0
Crude lipid	150.4	146.7	145.4	144.2	142.0	141.3	144.5
Fibre	66.4	66.3	68.2	65.0	68.3	65.7	69.3
Ash	98.9	98.1	98.2	94.9	97.2	96.2	92.5
NFE††	328.9	333.8	333.9	338.5	336.9	338.6	336.6
Gross energy (kJ g^{-1})‡‡	20.0	19.9	19.8	19.9	19.8	19.8	19.9

*Danish fishmeal supplied by Sri Purta Trading, Alor Setar, Kedah; containing 718 g kg^{-1} protein and 107.5 g kg^{-1} lipid.
†Supplied by Sri Purta Trading, Alor Setar, Kedah; containing 464.6 g kg^{-1} protein and 28.8 g kg^{-1} lipid.
‡Supplied by Sri Purta Trading, Alor Setar, Kedah; containing 448.6 g kg^{-1} protein and 183.9 g kg^{-1} lipid.
§Vitamin mix kg^{-1} (Rovimix 6288, Roche Vitamin Ltd., Basel, Swizerland): Vit. A 50 million i.u.; Vit. D3 10 million i.u.; Vit. E 130 g; Vit. B1 10 g; Vit. B2 25 g; Vit. B6 16 g; Vit. B12 100 mg; Biotin 500 mg; Pantothenic acid 56 g Folic acid 8 g; Niacin 200 g; Anticake 20 g; Antioxidant 200 mg; Vit. K3 10 g and Vit. C 35 g.
**Mineral mix kg^{-1} Calcium phosphate (monobasic) 397.65 g; Calcium lactate 327 g; Ferrous sulphate 25 g; Magesium sulphate 137 g; Potassium chloride 50 g; Sodium chloride 60 g; Potassium iodide 150 mg; Copper sulphate 780 mg; Manganese oxide 800 mg; Cobalt carbonate 100 mg; Zinc oxide 1.5 g and Sodium selenite 20 mg.
††NFE (Nitrogen Free Extract) = 1000 - (protein + lipid + fibre + ash).
‡‡Gross Energy calculated based on protein*23.4 kJ g^{-1}, lipid *39.8 kJ g^{-1}, NFE*17.2 kJ g^{-1}.
CMC = Carboxymethyl Cellulose.

Malaysia and transported to the aquaculture facility of Universiti Sains Malaysia in aerated polyethylene bags. Fish were acclimatized to laboratory conditions for 1 week prior to experiment in a rectangular fibreglass tank, and fed on a commercial tilapia diet (32% protein and 5% lipid) from Cargill (M) Sdn. Bhd., Prai, Malaysia, diet used to wean the fish at Enggor. After the conditioning period, 120 fish with mean initial weight of 12.0 ± 1.0 g were stocked in round fibreglass tanks in triplicates, filled with 230 L of dechlorinated tap water, in a flow-through system at 0.6 L min^{-1}. To supply continuous dechlorinated tap water, tap water was first held in a separate 5 tonnes fibreglass tank and treated with sodium thiosulphate before channelling it into the experimental tanks. The tanks were supplied with continuous aeration through an air compressor. Water temperature was measured at 28°C throughout the study, while natural photoperiod of 12 h L:12 h D was adopted. The fish were fed the assigned experimental diets to satiation twice daily at 09:00 and 17:00 hours for 30 days. Uneaten feed was syphoned from the tanks 2 h after feeding. The tanks were cleaned once weekly to maintain water quality and avoid stress to the fish.

Determination of *in vivo* protein digestibility of experimental diets

After 30 days of feeding, sufficient faeces were carefully collected from the respective tanks, 2 h after feeding in the morning, daily. Collection of faeces was conducted using a small and fine scoop net with necessary precaution to minimize leaching of nutrient. The faeces were pooled into 2 mL microtubes, centrifuged (4 000 rpm, 15 min) and

freeze dried prior to analysis. Apparent protein digestibility (APD) was determine using the methods of Furukawa and Tsukahara (1966) and calculated as: APD (%) = 100 – 100 [(% *I* diet)/(% *I* faeces) × (% *N* faeces)/(% *N* diet), where *I* is the inert marker (chromium oxide) and *N* is the nutrient (protein).

Determination of *in vitro* protein digestibility of experimental diets

Protein suspension mixtures for the experimental diets were prepared according to (Saterlee *et al.* 1979). Experimental diets were finely ground and weighed to prepare a protein suspension mixture of 6.25 mg protein in 50 mL of distilled water. The protein suspension mixtures were vortexed separately and supernatant were obtained for each experimental feed by centrifuging the mixture at 10 000 rpm. Protein concentration of the supernatants was determined using Bradford (1976) method with Bovine Serum Albumin (BSA) as standard.

Four types of enzyme systems were used to determine the protein digestibility of the experimental diets. All commercial enzymes were freshly prepared prior to analysis.

- Crude intestinal enzyme extract of bagrid catfish. Upon crude intestinal enzyme extraction, fish were fasted for 24 h and then fed as usual at 09:00 hours. At five post-feeding hours (based on preliminary data of the highest protease activity in the intestine after feeding), the fish were sampled and immediately submerged in ice-cold water to sedate them. The intestines were then removed, weighed and homogenized in cold distilled water at a ratio of tissues: water of 1:2 (w/v) using Ultra Turrax T25 homogenizer. The homogenate samples were then centrifuged at 10 000 rpm for 15 min at 4°C. Supernatant obtained were frozen at −20°C prior to analysis. Protein concentration of the crude intestinal enzyme extract was determined by Bradford (1976) using Bovine Serum Albumin (BSA) as standard.
- Lazo 1-enzyme (Lazo, Romaire & Reigh 1998) consisting 1.5 mg mL^{-1} trypsin (Sigma®, St. Louis, MO, USA type IX, bovine pancreas)
- Hsu 3-enzyme (Hsu *et al.* 1977) consisting 1.6 mg mL^{-1} trypsin (Sigma® type IX, bovine pancreas), 3.1 mg mL^{-1} chymotrypsin (Sigma® type II, bovine pancreas), 1.3 mg mL^{-1} peptidase (Fluka®, St. Louis, MO, USA, *Rhyzopus oryzae*)
- Saterlee 4-enzyme (Saterlee *et al.* 1979) consisting 1.6 mg mL^{-1} trypsin (Sigma® type IX, bovine pancreas), 3.1 mg mL^{-1} chymotrypsin (Sigma® type II, bovine pancreas), 1.3 mg mL^{-1} peptidase (Fluka®, *Rhyzopus oryzae*), 7.95 mg mL^{-1} protease (Sigma® type 1, bovine pancreas)

In vitro protein digestibility of the experimental diets was performed using four methods of analysis; pH stat, pH drop, spectrophotometric assay and SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis). In the pH stat and pH drop methods, four different types of enzyme systems were used. For inhibition assay using spectrophotometric assay and SDS-PAGE methods, only the crude intestinal enzyme extract of bagrid was used.

pH stat

Fifty mL of protein suspension mixtures of feed ingredients were individually pippetted into a test tube, each placed in a water bath at 37°C. The pH of the protein suspension mixture was adjusted to 8.0 using 0.01 M HCl or NaOH. The reaction was initiated by the addition of 5 mL of the enzyme system mixture (pH adjusted to 8.0 using 0.01 M HCl or NaOH) into the test tube (containing the protein suspension mixture) and allowed to continue for 60 min. Subsequently, the enzyme-protein mixture was titrated using 0.01 M NaOH and the amount of 0.01 M NaOH required to maintain the reaction mixture at pH 8.0 for 60 min was recorded. All analysis was done in triplicates. Degree of hydrolysis (DH) was calculated based on Cordova-Murueta and Garcia-Carreño (2002) as: $DH\% = [B \times N_B \times 1.4 \times (S\%/100)]/8 \times 100$, where, B is the amount (mL) of standard alkali (0.01 N NaOH) consumed to maintain the reaction mixture at pH 8.0, N_B is the normality of the titrant and S is the protein content in the reaction mixture expressed as %.

pH drop

Fifty mL of the protein suspension mixture was pippetted into a test tube each placed in a water bath at 37°C. The pH of the protein suspension mixture was adjusted to 8.0 using 0.01 M HCl or

NaOH. For crude intestinal enzyme extract of bagrid catfish, Lazo 1-enzyme and Hsu 3-enzyme system, the reaction was initiated by the addition of 5 mL of the enzyme system mixture (pH adjusted to 8.0 using 0.01 M HCl or NaOH) into the test tube (containing the protein suspension mixture). The drop of pH was measured and recorded at 1 min intervals for 10 min.

For Saterlee 4-enzyme system, pH drop method was carried out by adding 5 mL of 1.6 mg mL^{-1} trypsin, 3.1 mg mL^{-1} chymotrypsin and 1.3 mg mL^{-1} peptidase mixture (pH adjusted to 8.0 using 0.01 M HCl or NaOH) into a test tube in a water bath at 37°C containing 50 mL of protein suspension mixture for 10 minutes. Subsequently, 1 mL of 7.95 mg mL^{-1} commercial protease was added and the enzyme-protein mixture was transferred into a water bath at 55°C for 9 min. The enzyme-protein mixture was then transferred back into the 37°C water bath for 1 min and the pH was recorded on the 20th min at the end of the whole procedure. Casein was used as control and all analysis were carried out in triplicates. Relative protein digestibility was calculated based on Lazo, Romaire and Reigh (1998) as: Relative Protein Digestibility (RPD) = [(− ΔpH feed ingredient)/(− ΔpH casein)] × 100.

Spectrophotometric assay

Assessment of the inhibitory effects of the protein suspension mixtures on the crude intestinal enzyme extract of bagrid catfish was conducted according to Kunitz (1947) as modified by Walter (1984) using casein as a substrate at 26°C.

SDS-page

Assessment of the inhibitory effects of protein suspension mixtures on the crude intestinal enzyme extract of bagrid catfish using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970). Crude intestinal enzyme extract was incubated with the respective protein suspension mixtures at 26°C for 60 min at a ratio of 1:1 (v/v). The mixture was then added with the sample buffer (1 M Tris-HCl pH 6.8, glycerol, SDS, bromophenol blue) at a ratio of 2:1 (enzyme-inhibitor mixture: sample buffer). Equivalent mixtures of 30 μg protein concentration were loaded in separate wells using 12% polyacrylamide on an 8 × 10 × 0.075 cm gel (Laemmli 1970). Six μL of the Precision Plus Protein™ Standard Unstained molecular weight marker (10 to 250 kD, Bio-Rad, CA, USA) were loaded in each gel. Electrophoresis was carried out at 4 to 8°C with 120 V in electrophoresis buffer (Tris-glycine-SDS) using Mini Protean III® (Bio-Rad). Zymogram was prepared as described in Chong, Hashim, Lee and Ali (2002c) and analysed using the UN-SCAN-IT gel Version 6.1 according to the molecular weights. Percentage of inhibition of the protease bands was calculated as: (Intensity of protease bands for control-Intensity of protease bands for diet)/Intensity of protease bands for control.

The apparent protein digestibility of the experimental diets from the *in vivo* method was correlated with the *in vitro* data to compare the reliability of the *in vitro* methods to determine protein digestibility for bagrid catfish.

Statistical analysis

All data were analysed statistically by one–way analysis of variance (ANOVA) followed by Duncan Multiple Range Test using SPSS software, version 11.5 (SPSS, Chicago, IL, USA) to assess the differences in mean values.

Results

Generally, the *in vivo* apparent protein digestibility (APD) of bagrid catfish decreased with increasing SBM protein substitution from 0% to 60%. The APD for Diets 1 (90.27 ± 0.38%) and 2 (87.56 ± 0.15%) did not vary significantly ($P > 0.05$) but were significantly ($P < 0.001$) higher than Diets 3 to 7 (80.46 ± 1.57, 79.57 ± 1.56, 78.06 ± 3.35, 78.23 ± 6.98 and 75.81 ± 3.31% respectively). No significant ($P > 0.05$) reduction in APD was recorded between Diets 3 to 7.

In vitro protein digestibility of the experimental diets using pH stat and pH drop methods shows a decreasing degree of hydrolysis (DH) and relative protein digestibility (RPD) with increased SBM for all enzyme systems tested, similar to the trend observed in the *in vivo* data. For both the pH stat and pH drop methods, the values for DH and RPD were the highest using Saterlee 4-enzyme system (0.62% to 0.71% and 62.72% to 74.22%), followed by crude intestinal enzyme extract of bagrid catfish (0.34% to 0.45% and 55.70% to 68.34%), Hsu 3-enzyme system (0.38% to 0.51% and 30.78% to 53.09%) and Lazo single enzyme

Table 2 *In vivo* and *in vitro* protein digestibility of experimental diets

Diets	*In vivo* protein digestibility (%)	*In vitro* protein digestibility							
		pH stat (DH%)				pH drop (RPD%)			
		Crude intestinal enzyme extract	Lazo 1-enzyme system	Hsu 3-enzyme system	Saterlee 4-enzyme system	Crude intestinal enzyme extract	Lazo 1-enzyme system	Hsu 3-enzyme system	Saterlee 4-enzyme system
Diet 1	90.27 ± 0.38^{A}	0.45 ± 0.01^{Ac}	0.32 ± 0.01^{Ad}	0.51 ± 0.00^{Ab}	0.71 ± 0.01^{Aa}	68.34 ± 4.24^{Ab}	28.74 ± 1.84^{Ad}	53.09 ± 2.82^{Ac}	74.22 ± 2.94^{Aa}
Diet 2	87.56 ± 0.15^{A}	0.43 ± 0.01^{Bc}	0.27 ± 0.01^{Bd}	0.50 ± 0.00^{Bb}	0.67 ± 0.00^{Ba}	67.32 ± 2.58^{Aa}	28.58 ± 4.93^{Ac}	52.42 ± 2.83^{Ab}	73.01 ± 5.97^{Aa}
Diet 3	80.46 ± 1.57^{B}	0.40 ± 0.01^{Cc}	0.26 ± 0.01^{Cd}	0.46 ± 0.00^{Cb}	0.63 ± 0.01^{Ca}	66.07 ± 4.18^{ABa}	28.03 ± 3.02^{ABc}	52.20 ± 2.97^{Ab}	72.07 ± 6.58^{Aa}
Diet 4	79.57 ± 1.56^{B}	0.39 ± 0.00^{Cc}	0.22 ± 0.00^{Dd}	0.45 ± 0.01^{Db}	0.63 ± 0.01^{Ca}	65.01 ± 1.37^{ABa}	27.51 ± 2.36^{ABa}	42.76 ± 0.33^{Ba}	70.06 ± 4.47^{ABa}
Diet 5	78.06 ± 3.35^{B}	0.37 ± 0.01^{Dc}	0.23 ± 0.01^{Dd}	0.42 ± 0.00^{Eb}	0.63 ± 0.01^{Ca}	61.49 ± 1.31^{BCb}	27.23 ± 1.33^{ABd}	41.77 ± 3.98^{Bc}	67.22 ± 2.11^{ABa}
Diet 6	78.23 ± 6.98^{B}	0.36 ± 0.00^{Ec}	0.20 ± 0.01^{Ed}	0.41 ± 0.01^{Fb}	0.62 ± 0.00^{Ca}	57.77 ± 3.38^{CDa}	25.95 ± 3.28^{ABc}	34.53 ± 0.57^{Cb}	63.01 ± 4.10^{Ba}
Diet 7	75.81 ± 3.31^{B}	0.34 ± 0.00^{Fc}	0.18 ± 0.01^{Fd}	0.38 ± 0.00 ^{Gb}	0.62 ± 0.01^{Ca}	55.70 ± 2.85^{Db}	22.63 ± 2.26^{Bd}	30.78 ± 2.31^{Cc}	62.72 ± 3.30^{Ba}

Results are mean ± SD of triplicate samples.

A, B Values in the same column assigned as different superscript letters are significantly different ($P < 0.05$).

a, b Values in the same row assigned as different superscript letters are significantly different ($P < 0.05$).

system (0.38% to 0.51% and 22.63% to 28.74%), respectively, as shown in Table 2.

Figure 1 shows the percentage of inhibition of the experimental diets on the crude intestinal enzyme extract using the spectrophotometric assay. From the results, inhibition value was the lowest in Diet 1 (13.68 ± 0.26%) and increased as the protein from FM were replaced with SBM from 10% to 60% in Diets 2 to 7 (15.88 ± 0.56, 22.25 ± 0.52, 25.32 ± 0.33, 27.45 ± 0.90, 30.15 ± 0.92 and 33.95 ± 0.80% respectively). The percentage of inhibition between all the experimental diets were significantly ($P < 0.000$) different.

Analysed zymogram of the effects of the experimental diets on protease activity is presented in Fig. 2. Eight protease bands with molecular weights of 8.4, 13.0, 20.0, 26.8, 35.3, 39.7, 46.6 and 56.9 kDa were detected. The zymogram shows a gradual reduction in intensity and increased in percentage of inhibition of protease bands for Diets 2 to 7 (6.82%, 9.91%, 13.41%, 16.58%, 20.38% and 28.72% respectively) when compared with Diet 1 (0%).

pH stat method showed better correlation with the *in vivo* method compared to the pH drop method. The sequence of correlation between the enzyme systems for pH stat method was in the following decreasing order; crude intestinal enzyme extract of bagrid catfish ($r^2 = 0.9263$) > Saterlee 4-enzyme ($r^2 = 0.9242$) > Hsu 3-enzyme ($r^2 = 0.9144$) > Lazo 1-enzyme system ($r^2 = 0.8659$). On the other hand, the order of correlation among the enzyme systems for the pH drop method was; Saterlee 4-enzyme ($r^2 = 0.6951$) > crude intestinal enzyme extract of bagrid catfish ($r^2 = 0.6777$) > Hsu 3-enzyme ($r^2 = 0.6670$) > Lazo 1-enzyme system ($r^2 = 0.5386$). Spectrophotometric assay and SDS-PAGE method using crude intestinal enzyme extract of bagrid catfish revealed correlations values of 0.9284 and 0.8348 respectively.

Discussion

Various studies have been conducted to substitute protein from fishmeal (FM) with soybean meal (SBM) to compensate the shortage supply of FM as well as the increased demand and price. However, SBM has varying acceptability in different species including bagrid catfish. In the present study, low tolerance of SBM may be related to the presence of antinutritional factors (Francis *et al.* 2001) which have more intense negative

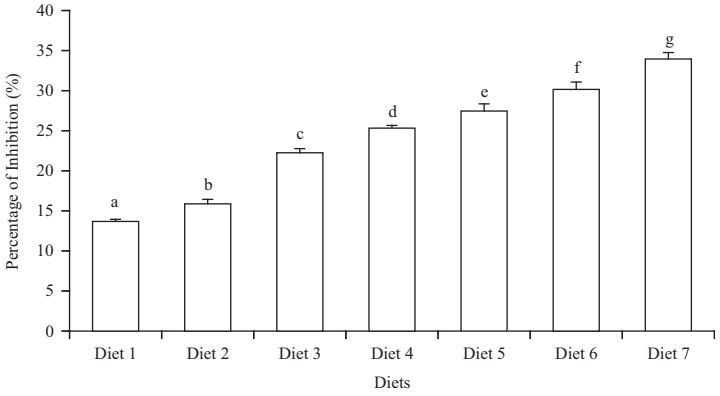

Figure 1 Inhibition of experimental diets on the crude intestinal enzyme extract of bagrid catfish via spectrophotometric assay. Results are mean±SD of triplicate samples. Values assigned as different superscript letters are significantly different.

effects on fish compared to terrestrial animals (Peres, Lim & Klesius 2003). Although SBM inclusion level is acceptable only up to 10% in the present study, the APD (75.8%) was fairly high even at 60% SBM inclusion. The APD of SBM for bagrid catfish could be improved by heat treatment (Peres *et al.* 2003) with careful regulation of heat to prevent degradation of essential amino acids (Sorensen, Ljokjel, Storebakken, Shearer & Skrede 2002).

Amino acids deficiency in SBM such as methionine (Pillay & Kutty 2005), cystine (Tibaldi & Tulli 1999) and lysine (Bai *et al.* 2005) may also be responsible for the reduced APD noted in bagrid catfish. Deficient amino acids in SBM could be improved by synthetic amino acids supplementation in the diets (Bai *et al.* 2005) or combination of different protein sources (Hansen, Rosenlund, Karlsen, Koppe & Hemre 2007).

In vivo digestibility method requires feeding trials using many fish samples and laborious analysis, tedious collection of faeces, which disintegrates easily into the water causing nutrient leaching and overestimation of the APD (Belal 2005). Various collection methods were employed to collect samples of faeces including stripping (Glencross, Hawkins, Evans, Rutherford, Dods, Maas, McCafferty & Sipsas 2006), anal dissection (Peres *et al.* 2003) and anal suction (Percival, Lee & Carter 2001). However, these methods may underestimate the APD by including the undigested feed from the anal cavity. Contamination of urinal material and mucus from the fish may also affect APD values (Hajen, Beames, Higgs & Dosanjh 1993a). Higher mortality and loss of scales have also been reported (Hajen, Higgs, Beames & Dosanjh 1993b). Although the most accurate method to measure protein digestibility is using the *in vivo* method, its disadvantages have forced the development of a more convenient, reliable and cost-effective *in vitro* methods.

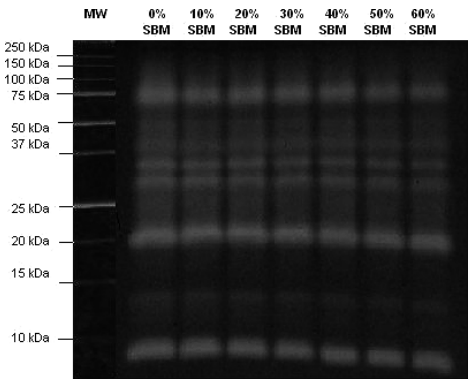

Figure 2 Inhibition of experimental diets on the crude intestinal enzyme extract of bagrid catfish via SDS-PAGE zymogram.

The pH stat (Garcia-Carreño, Navarrette del Toro & Ezquerra 1997; Ezquerra *et al.* 1998) and pH drop (Lazo *et al.* 1998; Chong, Hashim & Ali 2002b) methods are among the most studied *in vitro* methods. In the present study, the results of the pH stat correlated better with the *in vivo* data compared to the pH drop as also reported by Ezquerra *et al.* (1998). Procedures for pH stat and pH drop methods involve mixing of enzymes and proteins under continuous agitation at constant pH (pH 8.0), involving the hydrolysis of protein to produce small chains of amino acids and releasing protons (H^+) as evidenced by decreasing pH. The pH stat is advantageous due to its stability of pH throughout the digestion process and the rate of protein digestion can be determined rapidly by directly measuring the percentage of hydrolysed peptide bonds of the protein (DH) (Dimes & Haard

1994). El-Sayed, Nmartinez and Moyano (2000) stated that the pH stat method can differentiate between highly digestible and poorly digestible proteins provided that pure protein extracts are used. The pH drop is less accurate compared to the pH stat method due to the inconsistent pH throughout the digestion processes (Dimes & Haard 1994).

Assessment of *in vitro* protein digestibility assessment of feed ingredients is better determined using the enzymes from the experimental species itself compared to commercial enzymes from mammals, bacteria or other sources (Dimes & Haard 1994; Bassompierre, Larsen, Zimmermann & McLean 1998). This is because protease inhibitors have been reported to be species-specific (Dimes & Haard 1994; Garcia-Carreño *et al.* 1997) depending on the anatomical and biochemical activities (Glass, Macdonald, Moran and Stark (1989). Some of the commercial enzymes used may not be naturally present in bagrid catfish. For instance, trypsin is a major protease present in crustaceans (Lazo *et al.* 1998) and showed a better correlation when Lazo 1-enzyme system (trypsin) was used. However, bagrid catfish showed the lowest correlation with Lazo 1-enzyme system probably due to the lower trypsin activity in the intestine and the existence of other important serine proteases (unpublished data).

Spectrophotometric assay and SDS-PAGE have been used to identify the inhibitory effects of feedstuffs or diets on protease activity of fish (Lemos *et al.* 2004). In the present study, the correlation of the inhibitory effects using both the spectrophotometric assay and SDS-PAGE demonstrate negative response of protease activity of bagrid catfish with increased SBM in the experimental diets. Spectrophotometric assay showed higher correlation with the *in vivo* method compared to the SDS-PAGE. However, SDS-PAGE zymogram provided more information on the inhibition of protease bands by the experimental diets according to the molecular weights compared to other *in vitro* methods (Alarcon *et al.* 2001; Chong *et al.* 2002b; Lemos *et al.* 2004).

Conclusion

Bagrid catfish can tolerate up to 10% SBM protein replacement determined through the *in vivo* method. pH stat, spectrophotometric assay and SDS-PAGE *in vitro* methods could be used to determine protein digestibility in bagrid catfish. However, it should not be relied as the sole method and still requires the *in vivo* method to justify its results. Hence, further improvement of the *in vitro* methods should consider the digestive physiology of the fish to closely resemble the *in vivo* conditions.

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