Journal of Applied Phycology (2021) 33:967–977
https://doi.org/10.1007/s10811-020-02337-4

Bromelain enhances digestibility of Spirulina-based fish feed

Samskrathi A. Sharma[1] · Siddharthan Surveswaran[1] · James Arulraj[2] · Krishnakumar Velayudhannair[1]

Received: 10 July 2020 / Revised and accepted: 11 November 2020 / Published online: 7 January 2021

Abstract

Microalgae like Spirulina (*Arthrospira platensis*) are protein rich and can be alternative protein sources to fishmeal and soybean meal in fish feed formulation. The present study aims to improve the protein bioavailability of Spirulina by cost-effective protein extraction followed by protease supplementation in fish feed, using in vitro studies. Different extraction procedures such as microwave-assisted, high pressure, and temperature-mediated extraction, boiling and an isoelectric precipitation were employed to study the protein yield from Spirulina powder, and this was compared with the conventional soybean meal and fishmeal conditioning during feed manufacture. Bromelain is a potent protease that has not been widely used as a feed additive with Spirulina. To study the comparative efficiency of bromelain and other proteases like papain and trypsin on Spirulina and conventional feed substrates, a protease assay was performed at different temperatures and enzyme concentrations. The digestibility of these substrates was also studied in vitro, using gut extracts from the fingerlings of Mozambique tilapia (*Oreochromis mossambicus*). Unlike an in vivo feeding trial, a novel method was used to study the effect of protease supplementation on the inherent digestibility of the gut with an in vitro method. Bromelain showed the highest activity on all the substrates at both the temperatures. Bromelain supplementation improved the in vitro digestibility of the Spirulina that were subjected to protein extraction, more than the un-extracted one. The results of the present in vitro study suggest that Spirulina could serve as an alternative protein source, and bromelain-based supplementation could improve the digestibility of Spirulina-based fish diets.

Keywords *Arthrospira platensis* · In vitro digestibility · Microalgae · *Oreochromis mossambicus* · Papain · Trypsin

Introduction

The aquaculture industry is one of the major contributors to the total food production and artificial feeds need to be formulated to provide adequate amounts of nutrients, especially proteins (Kim et al. 2012), to the candidate species. Although this is species-specific, feed accounts for about 40–60% of the total operational costs in the aquaculture industry (Bais 2018), due to the inclusion of expensive protein sources such as fishmeal and soybean meal (Tacon 1997). Being expensive, it becomes important that all the protein present in the feed is not wasted or excreted without assimilation into the fish body, especially in juvenile fishes, where the utilization of dietary protein is even lower, due to the lower levels of proteolytic enzymes (Kolkovski 2001).

Spirulina (*Arthrospira platensis*), a filamentous cyanobacterium, is rich in minerals and antioxidant peptides and has among the highest recorded protein amounts (Bleakley and Hayes 2017). The present cost-intensive culturing and variability in the composition of microalgae bring up the costs up to 30% of the total cost in aquaculture feed formulation (Borowitzka 1997). Although various non-conventional methods of protein extraction from microalgae like the use of pulsed electric fields, high-voltage electrostatic fields, high field electric discharges, ultrasound, and high pressure, microwave-assisted, sub- and supercritical fluid extraction are available, most of them are expensive, involve longer treatment times, or may even show variability (Barba et al. 2015). This is further accompanied with poor protein digestibility of Spirulina due to the cell wall (Bleakley and Hayes 2017); this can be reduced by pre-treating the Spirulina and improving the bioavailability by protease supplementation.

Feed ingested by fish undergoes break down and is then exposed to various proteases, carbohydrases, and lipases in the digestive tract of fish. Various studies have been conducted on supplementation of fish diets with phytases, carbohydrases, proteases, etc. (Castillo and Gatlin 2015; Yigit et al.

✉ Krishnakumar Velayudhannair
krishnakumar.v@christuniversity.in

[1] Department of Life Sciences, CHRIST (Deemed to be University), Bangalore Central Campus, Hosur Road, Bengaluru, Karnataka 560029, India

[2] Department of Chemistry, CHRIST (Deemed to be University), Bangalore Central Campus, Hosur Road, Bengaluru, Karnataka 560029, India

2016; Sharma et al. 2019). All these are in vivo studies, involving the supplementation of diets with varying concentrations of enzymes in order to improve protein digestibility, maximize the growth, and improve the biochemistry, intestinal health, or haematological profiles. It is well established that the proteolytic enzymes bromelain and papain, present in pineapple (*Ananas comosus*) and papaya (*Carica papaya*) respectively, have a number of aquaculture applications as in vivo feed supplements (Patil and Singh 2014; Choi et al. 2016; Rachmawati and Samidjan 2018; Rostika et al. 2018; Subandiyono et al. 2018; Tewari et al. 2018; Yuangsoi et al. 2018; Sharma et al. 2019) in addition to their therapeutic and commercial uses (Amri and Mamboya 2012; Pavan et al. 2012). Papain has been used in the extraction of proteins from Spirulina, but bromelain has not been tried for this purpose (Sun et al. 2016; Fan et al. 2018).

It has been found that simulation of the physico-chemical conditions in the digestive tract of a fish during in vitro digestion tests is on par with in vivo results. A study involving in vitro digestibility of feedstuff using pyloric caecal crude enzyme extract has been reported as a valuable tool to evaluate the growth performance (Rungruangsak-Torrissen et al. 2002). In vitro studies provide a cheaper and faster alternative to in vivo feeding trials to study the effects of enzyme supplements in the feed. Various in vitro methods like apparent digestibility, degree of hydrolysis, pH-stat, and pH-drop have been employed in studies of shrimp (Ezquerra et al. 1998) and fish (Grabner 1985; Carter et al. 1999; Rungruangsak-Torrissen et al. 2002).

This study aims to assess the effect of various common proteolytic enzymes over traditional protein feed substrates in aquaculture—soybean meal and dry fish meal—and also aims to understand if Spirulina (protein extracted using various cost-effective methods) can be used as a replacement to these traditional substrates. Many studies have been conducted on the in vivo supplementation of enzymes in feed, without an explanation as to why this particular enzyme concentration was used. This study aims to assess the effect of exogenous protease supplementation in presence of the gut in vitro using a modified form of a protease assay (Cupp-Enyard 2008). The study involves a substrate-enzyme reaction followed by the estimation of the free tyrosine equivalents released. The free amino acid release can be an indicator of digestion of the provided protein and further, easier assimilation in the fish body.

Materials and methods

Protein isolation process and estimation

Various protein-rich substrates were used to check the efficiency of processes normally used to prepare feeds in the aquaculture industry. Usually protein-rich substrates are not subjected to any kind of pre-treatment to increase protein bioavailability, but after feed preparation, during conditioning, the feed mixture is subjected to thermal steaming in conditioning chambers for 30 s or 2–3 min when the feed is steam pelleted or extrusion pelleted, respectively (Hardy and Barrows 2003). In the present study, these conditions were incorporated into the preparation of soybean meal (SBM) and fishmeal (FSM), and different extraction processes were also tried for Spirulina (*A. platensis*). Substrates used for the assay include 4% protein solutions of SBM, FSM, and Spirulina. The SBM (50.25% crude protein; DMart Premia, Bengaluru, India) and FSM (solefish) (60.09% crude protein; H. S. Dry Fish, Bengaluru, India) were purchased from a local market in Bengaluru, India. Spirulina was obtained as a dry powder (62.1% crude protein; 2 M Biotech, Ramanathapuram, India).

The FSM and SBM powders were homogenized with water and heated at 120 °C for 2 min. The Spirulina samples were solubilized with 1 M NaOH solution amounting to 1/10 the total volume of solution preparation, for 15 min. These samples were subjected to protein extraction methods—by boiling at 120 °C for 2 min (boiled (BSP)), by isoelectric precipitation (ISP) (Parimi et al. 2015), by minimal grinding in distilled water (un-extracted (USP)), by autoclaving at 15 psi pressure for 20 min (autoclaved (ASP)), and by microwave-assisted extraction (MSP) for 3 min. The 'SP' in these acronymns refer to Spirulina. The Spirulina solutions were neutralized with 1 M HCl, so that these solutions had a pH of 7 ± 0.75. The SBM and FSM were also adjusted to the same pH. These solutions were then centrifuged at 12320×*g* for 15 min and the supernatants were used as the substrate (see Fig. S1 for flowchart). These were subjected to protein estimation (Lowry et al. 1951) and protein yield and protein recovery were calculated as follows:

$$\text{Protein yield}\left(\text{g g}^{-1}\text{substrate}\right) = \frac{\text{Amount of protein obtained after extraction (g)}}{\text{Amount of substrate used for extraction (g)}}$$

$$\text{Protein recovery}\,(\%) = \frac{\text{Amount of protein obtained after extraction (g)}}{\text{Amount of protein present in the substrate (g)}} \times 100$$

Substrate preparation for in vitro protein digestibility assay

Substrates used for all the in vitro assays described below were prepared using the same methods as in the previous section, but instead of 4% protein solutions, 1% protein solutions were made. The substrates include bovine serum albumin (BSA) (standard) (HiMedia, India), MSP (based on results of 'Protein isolation process and estimation'), SBM, and FSM. The previously described procedures were used to prepare 1% protein solutions of the same.

Preparation of enzymes

The enzymes used were stem bromelain (SRL, Mumbai, India), papain (SRL, Mumbai, India), and bovine trypsin (HiMedia, Mumbai, India). The bromelain and papain stock solutions were prepared at a concentration of 200 mg mL^{-1} in 0.1 M sodium acetate-acetic acid buffer (pH 5.5) (Scott et al. 1987). The trypsin stock solution of 200 mg mL^{-1} was prepared in 0.1 M sodium carbonate-bicarbonate buffer (pH 8) (Rick 1974). All assay dilutions have been described in terms of the percentage of the stock (200 mg mL^{-1}) for easy comparison with gut extract.

Maintenance of fish and preparation of gut extract

Fingerlings of the Mozambique tilapia, *Oreochromis mossambicus*, weighing 7.68 ± 0.17 g and measuring 9.15 ± 01.96 cm were purchased from the Fisheries Research and Information Centre, Hebbal, Bengaluru, India. The fingerlings were stocked in 25 litre plastic tubs at a stocking density of 1 fish L^{-1}, in replicates of three. The fish were acclimatized to the laboratory conditions for 10 days. The physiochemical parameters such as dissolved oxygen (4.59 ± 0.46 mg L^{-1}), pH (6.83 ± 0.29), temperature (25 ± 1 °C), and total hardness (221.67 ± 2.89 mg L^{-1}) were kept optimum for the fish, and they were fed ad libitum with basal diet (Taiyo, Chennai, India) (for composition, refer Online Resource 1).

For the preparation of the gut extract, a live fingerling of *O. mossambicus* was sacrificed 24 h post-feeding, (described as the state of highest gut protease activity) (Anukoolprasert et al. 2019). The fish was euthanized in clove oil (0.4 mL L^{-1} water) (Fernandes et al. 2017), and the entire gut was homogenized with ice cold 0.1 M phosphate buffer (pH 7) (1:3 (w/v)) and centrifuged at 12320× *g* for 5 min and the supernatant was used as the 'gut extract' for the further steps. This was done using three replicates.

Colorimetric protease activity assay

Effect of temperature

The proteolytic activity of the above enzymes and gut extract on the selected substrates was assayed using a modified method of Cupp-Enyard method (2008), with FSM, SBM, BSA, and MSP as protein substrates (see Fig. S1 for flowchart detailing of the substrate preparation and Fig. S3 for colorimetric assay flowchart). These substrates were prepared as described in the 'Protein isolation process and estimation' section. Briefly, 200 μL of 1% protein substrate solution and 40 μL of stock enzyme solution (bromelain, papain, trypsin, and gut extract separately) was added to each of the test tubes in triplicate and was incubated at two temperatures, 25 °C and 37 °C for 10 min. The enzyme-substrate reaction was stopped by vigorous shaking with 200 μL of 0.1 M trichloroacetic acid solution. This was centrifuged at 12320× *g* for 5 min and to 40 μL of the supernatant, 150 μL of 0.5 M sodium carbonate solution followed by 25 μL of 0.5 M Folin-Ciocalteau reagent was added and incubated for 30 min at the respective temperatures. The absorbance values were recorded at 660 nm using iMark Microplate Reader (BioRad Laboratories, India) and the number of tyrosine equivalents released was calculated from the standard graph of L-tyrosine. This value was used as a measure of the enzyme efficiency by calculating the activity in terms of μmole of tyrosine equivalents min^{-1} mL^{-1}. One unit of protease activity is the amount of enzyme solution that can release 1 μmole of tyrosine equivalents per minute per milliliter of the substrate under standard conditions.

Reference substrates

BSA, FSM, SBM, and MSP were subjected to various concentrations (20, 40, 60, 80, 100% of the stock) of the candidate enzymes (bromelain, papain, and trypsin) and gut extract by the modified Cupp-Enyard protease assay at 25 °C only as described above (based on results of the 'Effect of temperature' section).

In vitro supplementation study

To study the effect of various protease supplements on the in vitro digestibility of feed substrates, BSA, FSM, SBM, and Spirulina (MSP, ASP, ISP, and USP), a supplementation study was carried out. The modified Cupp-Enyard protease assay was performed at 25 °C, with 40 μL of gut extract along with 10 μL of varying amounts of the supplement, bromelain (0.2-1 mg bromelain mg^{-1} substrate protein) to 200 μL substrate (see Fig. S4 for flowchart). As a comparison of the inherent digestibility with the supplementation study, gut extract with no supplement was also tested. This was done to understand the amount of Spirulina that can be digested by the fish gut, and how bromelain can improve the same.

Efficacy of bromelain and gut extract on different MSP concentrations

The effect of different substrate concentrations (%) (w/v) of MSP with fixed volume of gut extract and varying amounts of bromelain was assayed by the modified Cupp-Enyard protease assay at 25 °C. The gut extract and bromelain were taken at a fixed volume (40 μL), while the MSP concentrations were varied from 1-10% (w/v).

Statistical analyses

The samples from the protein isolation process and all the colorimetric protease assays were subjected to various

statistical analyses with a significance level of $P = 0.05$ (α = 5%). One-way and two-way ANOVA were performed as appropriate, using GraphPad Prism 8.4.2 software for Windows (Muzyaka et al. 2020). The values are represented as mean ± range, calculated using three independent biological replicates ($n = 3$). All results are presented as scatter plots or bar graphs, with error bars indicating range.

Results

Protein isolation process and estimation

In the present study, the microalga Spirulina was subjected to different methods of extraction (USP, BSP, ASP, ISP, MSP) and the protein recovery percentage and protein yield (g $g^{-1}$of substrate) were estimated and presented in Table 1, along with other substrates, FSM and SBM. The protein recovery percentage was of the order: FSM > ISP > MSP > SBM = BSP > ASP > USP. Results of the one way ANOVA revealed that the protein recovery percentage showed statistically significant variations between different substrates ($F_{(6,14)} = 255.2$, $P < 0.0001$). In the present study, various extraction processes were tried to obtain maximum protein from the different substrates like boiling; microwave-assisted, isoelectric-precipitated, and autoclave-extracted Spirulina; and heat conditioning method for FSM and SBM. The protein yield was seen to be in the order: FSM > ISP > MSP > BSP > ASP > SBM > USP. Results of the one-way ANOVA revealed that the protein yield percentage showed statistically significant variations between different substrates ($F_{(6,14)} = 298.2$, $P < 0.0001$). From these results, it is evident that ISP method of processing is suitable for Spirulina to get more protein recovery and yield, but due to minimal downstream processing steps, MSP (second highest yield and recovery) was chosen over ISP.

Table 1 Protein extraction efficiency after the substrates were subjected to different kinds of protein extraction methods

Substrate		Protein recovery (%)	Protein yield (g g^{-1} of substrate)
Spirulina	MSP	26.65 ± 1.01^{c}	0.184 ± 0.006^{c}
	USP	20.67 ± 1.01^{e}	0.128 ± 0.006^{g}
	ISP	37.71 ± 0.23^{b}	0.234 ± 0.001^{b}
	ASP	23.35 ± 0.40^{d}	0.145 ± 0.003^{e}
	BSP	25.23 ± 0.84^{c}	0.156 ± 0.005^{d}
FSM		39.71 ± 0.64^{a}	0.240 ± 0.004^{a}
SBM		25.87 ± 1.00^{c}	0.130 ± 0.005^{f}

Results in the same column with different superscripted letters are significantly different ($P < 0.05$)

Colorimetric proteolytic activity assay

Effect of temperature

In the present study, the effect of temperature (at 25 °C and 37 °C) on the protease activity of bromelain, papain, trypsin, and gut extract was documented (Fig. 1). It was observed that bromelain showed the highest activity on all the substrates at both the temperatures, while FSM was the most hydrolyzed by proteases. Among the four enzymes treated on BSA at different temperatures, bromelain registered maximum activity at both the temperatures. Two-way ANOVA revealed that BSA showed statistically significant variations between different enzymes ($F_{(3,14)} = 101.5$, $P < 0.0001$). No statistically significant variations were observed between different temperatures ($F_{(1,14)} = 0.0277$, $P = 0.87$). The order of activity of different enzymes on BSA at 25 °C was bromelain > papain > trypsin = gut extract and at 37 °C was bromelain > papain = trypsin > gut extract (Fig. 1a).

On FSM, bromelain showed the highest activity among all the enzymes. Two-way ANOVA revealed that FSM showed statistically significant variations between different enzymes ($F_{(3,16)} = 19.68$, $P < 0.0001$). There were statistically significant variations between different temperatures as well ($F_{(1,16)} = 8.510$, $P = 0.01$). The activity of bromelain, papain, and trypsin showed no significant variations at both the temperatures, but the activity of gut extract was significantly higher at 25 °C. The order of activity of different enzymes on FSM at 25 °C and 37 °C was bromelain = gut extract > papain = trypsin (Fig. 1b).

Bromelain registered the maximum activity on MSP at both the temperatures. Two-way ANOVA revealed that MSP showed statistically significant variations between different enzymes ($F_{(3,16)} = 19.68$, $P < 0.0001$). There were statistically significant variations between different temperatures as well ($F_{(1,16)} = 8.510$, $P = 0.01$). The activity of bromelain, papain, and trypsin showed no significant difference at both the temperature, but the activity of the gut extract was significantly higher at 37 °C. The order of activity was found to be bromelain > trypsin = papain = gut extract and bromelain > gut extract = trypsin = papain at 25 °C and 37 °C, respectively (Fig. 1c).

On SBM, bromelain registered maximum activity at both the temperatures. Two-way ANOVA revealed that SBM showed statistically significant variations between different enzymes ($F_{(3,16)} = 14.80$, $P < 0.0001$). There were statistically significant variations between different temperatures ($F_{(1,16)} = 23.85$, $P = 0.0002$). The activity of trypsin and gut extract showed no significant variation at both the temperatures, but the activity of bromelain and papain was significantly higher at 37 °C. The order of activity was bromelain > gut extract = trypsin = papain, at both the temperatures (Fig. 1d).

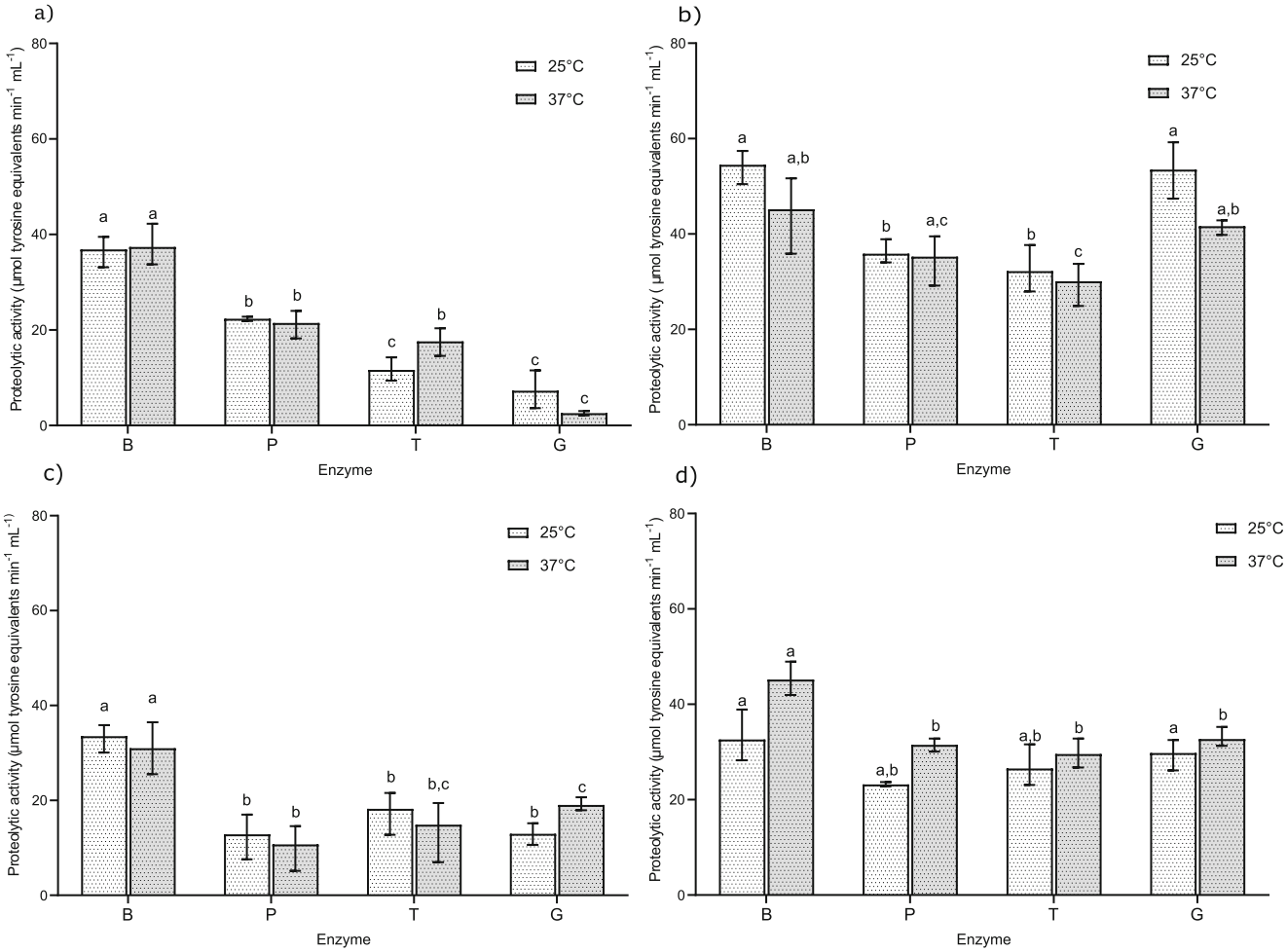

Fig. 1 Effect of temperatures on the proteolytic activity of different enzymes on selected substrates (**a**) BSA (**b**) FSM (**c**) MSP (**d**) SBM (where B, bromelain; P, papain; T, trypsin; and G, gut extract) (results at the same temperature sharing the same superscripted letters are not significantly different ($P > 0.05$))

In summary, all the substrates showed statistically significant variations between different enzymes. Between different temperatures, FSM, MSP, and MSP showed statistically significant variations. The activity of bromelain showed no significant variations at both the temperatures for BSA, MSP, and FSM, while the activity was significantly higher at 37 °C for SBM. The activity of bromelain and gut extract was highest on FSM, while the lowest activity of bromelain was on SBM, and that of gut on BSA. The order of activity of B at 25 °C was FSM > BSA > MSP > SBM and at 37 °C was FSM > SBM > BSA > MSP. The order of activity of the gut extract at 25 °C was FSM > SBM > MSP > BSA, while at 37 °C it was of the order FSM > SBM > MSP > BSA.

Reference substrates

The effect of various concentrations (20, 40, 60, 80, 100% of the stock) of the candidate enzymes on different substrates such as BSA, FSM, SBM, and MSP at 25 °C was evaluated (Fig. 2). Among the different concentrations of the different enzymes considered, the highest proteolytic activity on BSA was obtained with 100% bromelain). Two-way ANOVA revealed that BSA showed statistically significant variations between different enzyme concentrations ($F_{(4,40)} = 28.08$, $P < 0.0001$). There were statistically significant variations between different enzymes ($F_{(3,40)} = 107.4$, $P < 0.0001$) as well. The order of activity at their best activities was found to be bromelain > papain > gut extract = trypsin (Fig. 2a).

When FSM was considered, the highest proteolytic activity was seen with bromelain at 100%. Two-way ANOVA revealed that FSM showed statistically significant variations between different enzyme concentrations ($F_{(4,39)} = 6.495$, $P = 0.0004$). There were statistically significant variations between different enzymes ($F_{(3,39)} = 7.539$, $P = 0.0004$) as well. The order of activity at their best activities was found to be bromelain > gut extract = papain = trypsin (Fig. 2b).

On MSP, the highest proteolytic activity was seen with bromelain at 100%, although the variations between the

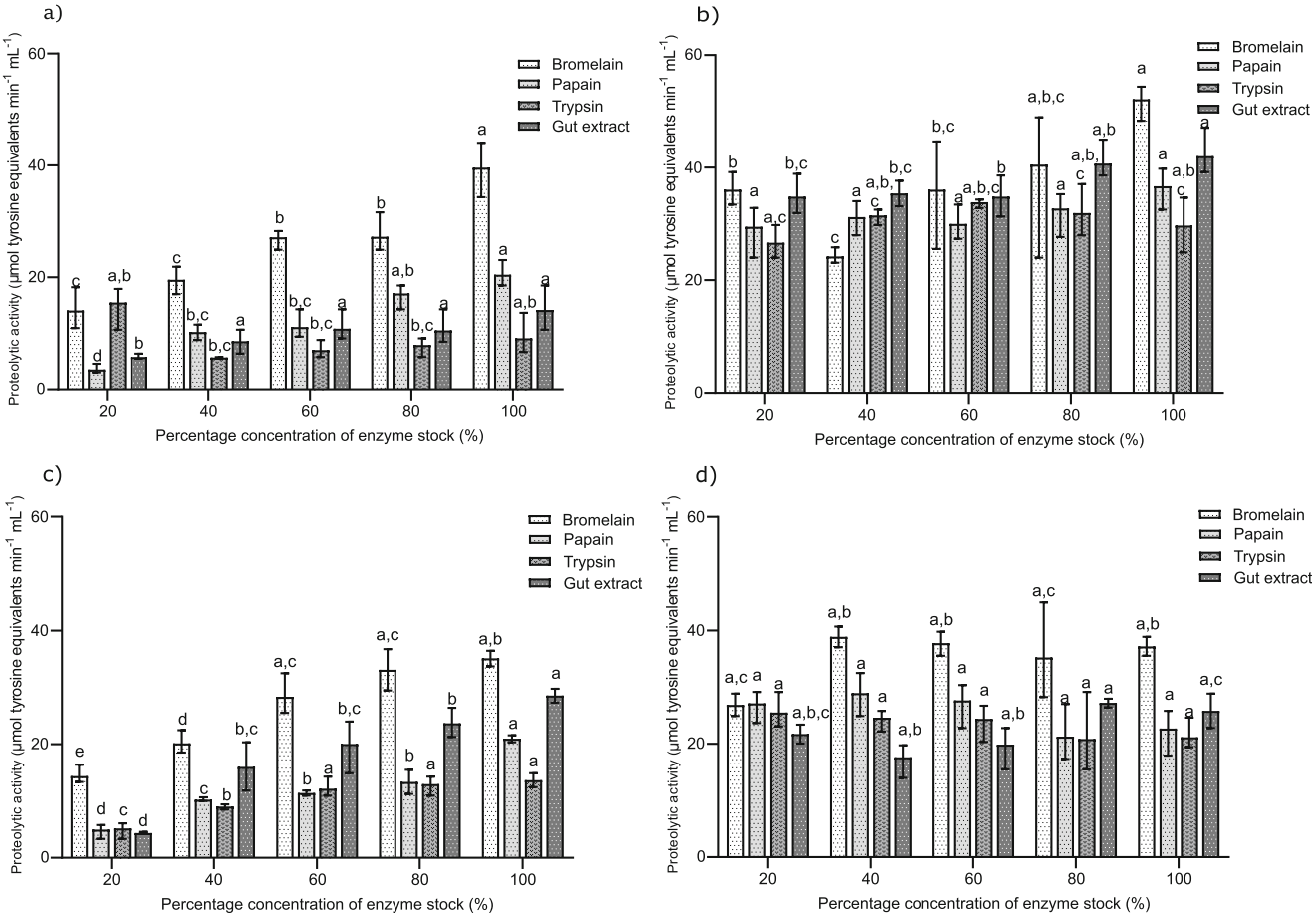

Fig. 2 Activity of selected proteolytic enzymes on different substrates (**a**) BSA (**b**) FSM (**c**) MSP (**d**) SBM. Results of the same enzyme sharing the same superscripted letters (at different concentrations) are not significantly different ($P > 0.05$)

60% and 80%, and 80% and 100% bromelain showed no significant variations. Two-way ANOVA revealed that MSP showed statistically significant variations between different enzyme concentrations ($F_{(4,34)} = 78.34$, $P < 0.0001$). There were statistically significant variations between different enzymes ($F_{(3,34)} = 112.0$, $P < 0.0001$) as well. The order of activity at their best activities was found to be bromelain > gut extract > papain > trypsin (Fig. 2c).

The highest proteolytic activity on SBM was seen with bromelain at 40%, after which no significant variations were seen. Two-way ANOVA revealed that SBM showed no statistically significant variations between different enzyme concentrations ($F_{(4,35)} = 0.5107$, $P = 0.7282$). There were statistically significant variations between different enzymes ($F_{(3,35)} = 24.00$, $P < 0.0001$) as well. The order of activity at their best activities was found to be bromelain > gut extract = papain = trypsin (Fig. 2d).

In summary, all the substrates showed statistically significant variations between different enzyme concentrations, except SBM, and, between different enzymes, all substrates showed statistically significant variations. Bromelain showed the highest activity among all the enzymes considered. Hence, it was considered as a supplement for the in vitro supplementation study.

In vitro supplementation study

The result of the supplementation of the gut with different amounts of bromelain on all the substrates considered (FSM, SBM, BSA, ISP, MSP, USP) was documented. It was seen that the ASP and BSP samples had high content of free amino acids owing to the harsh extraction conditions; thus, they were not used for the supplementation study (personal observation). The order of highest activities was seen to be ISP > FSM > BSA = MSP > USP > SBM. The inherent gut capacities to digest the substrates are in the order: FSM > MSP = ISP = SBM = BSA > USP. Two-way ANOVA revealed that the different amounts of bromelain supplement added showed statistically significant variations between different enzyme concentrations ($F_{(5,60)} = 7.306$, $P < 0.0001$). There were statistically significant variations between different substrates considered for the supplementation study ($F_{(5,60)} = 108.0$, $P < 0.0001$) as well. It can be seen that on supplementation with 1 mg bromelain mg^{-1} substrate protein, ISP shows an

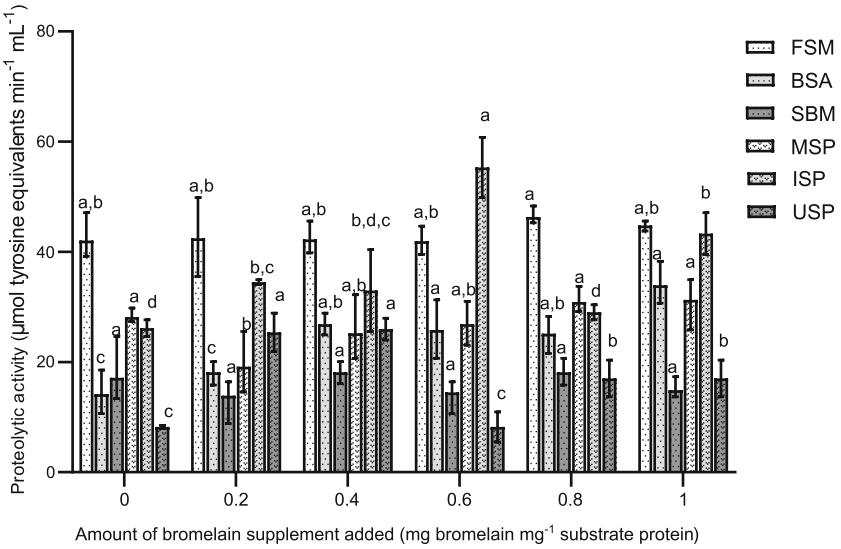

Fig. 3 *In vitro* supplementation of the gut extract with different amounts of bromelain. Results of the same substrates sharing the same superscripted letters (at different mg bromelain mg^{-1} substrate protein) are not significantly different ($P > 0.05$)

extremely significant variation ($P < 0.0001$) and at 0.8 mg bromelain mg^{-1} substrate protein, MSP significant variation ($P = 0.007$) when compared to USP (Fig. 3).

Efficacy of bromelain and gut extract on different MSP concentrations

The efficacy of bromelain and gut extract on different concentrations of MSP was documented. The highest activity of bromelain and gut extract was seen at 7% MSP after which there was no significant increase. The activity of bromelain on MSP was significantly higher than gut extract at all MSP concentrations ($P < 0.05$) (Fig. 4). Two-way ANOVA revealed that the different concentrations of Spirulina showed statistically significant variations between different enzyme concentrations ($F_{(9,40)} = 68.72$, $P < 0.0001$). There were statistically significant variations between different enzymes considered for the supplementation study ($F_{(1,40)} = 695.7$, $P < 0.0001$) as well.

Discussion

The digestibility of protein (of raw and unprocessed form) of microalgae is poor and hence it is vital to standardize a suitable algal protein extraction method in order to improve their bioavailability (Barba et al. 2015). Various procedures are optimized to extract protein from various microalgae and are species-specific (Barba et al. 2015; Parimi et al. 2015). In the present study, different cost-effective extraction procedures were tried out for Spirulina, and isoelectric precipitation and microwave-assisted extraction were found to be the optimal

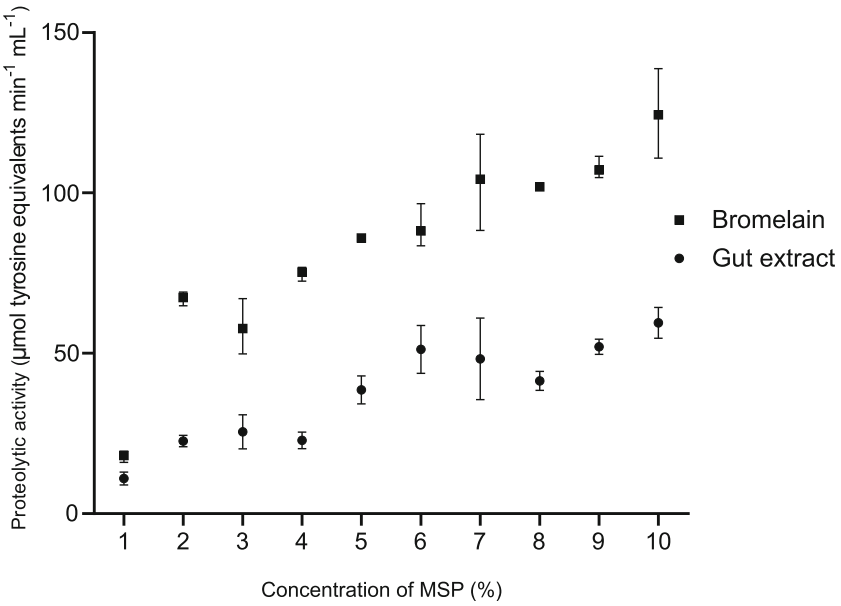

Fig. 4 Proteolytic activity of bromelain and gut extract when subjected to different concentrations of MSP

procedures. They showed higher available protein than the unextracted one, as the extraction process helped to release the protein from the cell wall. Pre-treatments such as mechanical, enzymatic, thermal, and chemical treatments or cell lysis have been reported to degrade the microalgal cell wall, in order to improve the accessibility of the intra-cellular components (Parimi et al. 2015). Although the results of the extraction indicated that ISP was the most protease-hydrolyzable form of Spirulina, it showed the presence of residual acid (from the extraction), making it unsafe as a fish food ingredient (personal observation). Since the process of extraction of Spirulina proteins for aquaculture must be cost-friendly with minimal downstream processing steps, MSP was considered as the optimum extraction procedure even though ISP showed a higher yield.

Most studies on the algal proteins are crude protein–based, using the estimation of total nitrogen, but the presence of nitrogenous non-protein constituents like nucleic acids, amines, and glucosamides, and cell wall materials which overestimate the crude protein content is not reliable (Becker 2007). The same study reported that the content of non-protein nitrogen amounts to 11.5% in Spirulina and 6% in *Dunaliella*. The pH stat method is accurate only if pure protein samples are used; if there is fat content, the release of fatty acids interferes with the pH shift caused by peptide hydrolysis (Grabner 1985). The pH-drop method for estimating protein digestibility in salmonid diets tends to overestimate samples with low apparent protein digestibility (Ezquerra et al. 1998). For these reasons, a protease assay was preferred to study protein digestibility of various enzymes. A few studies have used protease assays to study protein digestibility (Diken et al. 2016; Abdel-Warith et al. 2020).

The effect of temperature (25 °C and 37 °C) was studied in order to account for the differences in activity as most commercial enzymes have an optimum at 37 °C, and if they are to be used as feed supplements, then their activity should be comparable at lower water temperatures as fish have low body temperatures (Encarnação 2016). Various studies have performed the in vitro digestibility at various temperatures like 25, 27, 23, 37, and 15 °C as per the physiological conditions of the candidate species (Ezquerra et al. 1998; Rungruangsak-Torrissen et al. 2002). For most substrates, there were no significant variations in the enzyme activities at both the temperatures. Interestingly, in our study, the activity of the gut extract on few substrates was better at 25 °C than at 37 °C, which appears to be in conflict with the general enzyme catalytic principles. But, in a study involving hybrid juvenile tilapia (*O. niloticus* × *O. aureus*), similar results were reported, where the amylase and lipase had higher activity at 15 °C than at 20 °C, which could be attributed to the existence of isoenzymes (Jun-Sheng et al. 2006). In a study involving acetylcholinesterase from rainbow trout, it was seen that the affinities of enzyme to substrate vary with temperature and approach maximal values at temperatures corresponding to those at which the fish were acclimatized (Baldwin and Hochachka 1970). The results of the present study can also be attributed to the same, as the fishes were acclimatized at 25 °C. The results of various in vitro digestibility studies reveal that FSM was found to be more digestible than SBM (Chong et al. 2002; Valverde-Chavarría et al. 2016), while others consider SBM (Bai et al. 2016) and Spirulina (Montoya-Martínez et al. 2018) to be more digestible than FSM. In the present study, it was seen that all the enzymes showed the highest activity on FSM when compared to the other substrates. Thus, it was seen that the different concentrations of different enzymes showed varying level of interactions. A study showed that α helices and β sheets in the substrate increased and decreased the in vitro digestibility of proteinaceous substrates, respectively (Bai et al. 2016). This study showed that the digestibility (and the number of α helices) of soybean meal was better than that of fishmeal, which differs from the present study results where fishmeal showed higher digestibility.

The differences in the activities of the different proteases can be attributed to the specificity of the enzymes (Fig. 5). Bovine trypsin can make a proteolytic cleavage C-terminal to a Lys or Arg residue, as long as they are not next to a Pro (Olsen et al. 2004). Papain on the other hand cuts next to a Lys or Arg, when they are not next to Val (Sigma-Aldrich 2020b). In addition to this, the Lys or Arg must be flanked by a hydrophobic amino acid like Ala, Tyr, Trp, Val, Leu, Ile, or Phe. Bromelain makes a cleavage next to Lys, Tyr, or Ala, irrespective of the flanking sequences (Sigma-Aldrich 2020a). If it is assumed that all amino acids are equally distributed, then the probabilities of obtaining the specific sequence required for proteolytic cleavage by bromelain, papain, and trypsin can be calculated as 15, 3.33, and 9.5%, respectively (see Online Resource 1 for additional information). Thus, the activity of bromelain would be the highest among the other enzymes as the probability of finding the required cleavage sequence is the highest, and this agreed with the results of the present study. The differences in activity could also be attributed to the differences in enzyme purity of the commercial products and the differences in specific activity of the enzymes (see Online Resource 1 for specific activity of the enzymes used). The pH of optimum activity of the enzymes could also cause a difference in the activities. The optimum pH of bromelain and papain is 5.4–5.8 (Scott et al. 1987) and trypsin is 7–8 (Rick 1974) while all the reactions were carried out at pH 7 ± 0.75. This altered pH (other than optimum pHs) could also influence the activity of the enzymes. Since, the results of the present study revealed that the activity of bromelain was the highest on all the substrates, it was considered as the supplement for the next part of the study.

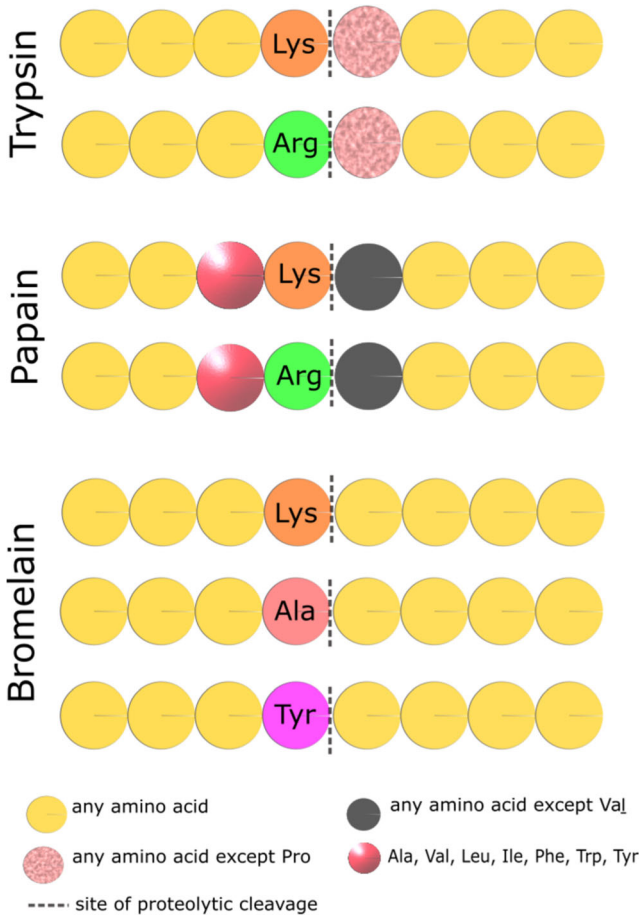

Fig. 5 Amino acid sequence specifications for proteolytic cleavage by trypsin, papain, and bromelain (drawn using information from Sigma-Aldrich (2020a, b) and Olsen et al. (2004)

In vitro digestibility studies have been found to show results that are at par with the in vivo studies (Grabner 1985). Although, the in vitro studies can be used as a precursor, it is better if they are followed up with in vivo studies. Various studies have been conducted with various substrates to check the effect of proteinaceous ingredients in vivo, with the help of feeding trials. A study on *Clarias gariepinus* revealed that full fat soybean meal could be used as a fishmeal replacement only up to 41% and after that, high contents of anti-nutrition factors (ANFs) like protease inhibitors, lectins, phytic acid, saponins, phytoestrogens, antivitamins, and allergens inhibit the digestive enzymes (Francis et al. 2001; Abdel-Warith et al. 2020). It must be taken into consideration that incorporation of microalgae like *Chlorella* sp., *Spirulina* sp., and *Schizochytrium* sp. in fish diets has shown inhibitory effects to gut proteases thereby lowering the protein digestibility of these ingredients (Diken et al. 2016). An in vivo study involving *Cyprinus carpio* revealed that *A. platensis* replaced fishmeal with no significant change in growth, while Spirulina as the sole diet showed better protein retention (Nandeesha et al. 1998). An in vivo study supplemented Spirulina in the diets of *Oncorhynchus mykiss* and they observed no differences in growth performance, but the supplementation enhanced the antioxidant profile of the gut (Sheikhzadeh et al. 2019). Another in vivo study revealed that 30% *A. platensis* inclusion is deemed the optimal level of dietary replacement for increased growth performance, improved feed utilization efficiency, and enhanced overall health status of Nile tilapia juveniles (Velasquez et al. 2016).

Supplementation studies have shown improved fish health and growth when bromelain has been supplemented in various in vivo feeding trials on *C. carpio* (Sharma et al. 2019), *O. niloticus* (Yuangsoi et al. 2018), and *Puntius javanicus* (Subandiyono et al. 2018). The present supplementation study is novel as it performs a supplementation study on the gut of *O. mossambicus* with bromelain, in vitro without the need of a feeding trial. This supplementation study is novel as it provides a method to assay protease supplementation, making it a cost-effective and a faster method to assess the same before a feeding trial is performed. However, an in vivo study should follow, in order to validate these results. The study of the efficacy of bromelain and gut extract on different concentrations of MSP is useful to estimate the amount of bromelain that can efficiently improve the protein digestibility of the gut, in order to accordingly incorporate different amounts of bromelain for different amounts of Spirulina. It also gives us information about how differently the gut and bromelain fare in their digestibility of Spirulina.

In conclusion, Spirulina (*A. platensis*) can serve as an alternative protein source to fishmeal and soybean meal and its bioavailability can be improved with cost-effective extraction procedures followed by bromelain supplementation. Considering the importance of cost-effectiveness in the aquaculture industry, MSP was considered as the optimum extraction procedure even though ISP showed a higher yield, as the former had minimal downstream processing steps. The results of the in vitro supplementation study revealed that the activity of supplemented gut extract was higher than the inherent gut capacity to digest the protein-rich substrates. For MSP, the best amount of bromelain supplement was 0.8 mg bromelain mg^{-1} substrate protein. Thus, this study is a proof of concept that bromelain can be used as a protease supplement to enhance the digestibility and bioavailability of Spirulina in fish feeds for *O. mossambicus*. Further work on in vivo systems could reinforce the efficacy of bromelain in enhancing fish feed, livestock feed, and/or human nutrition.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10811-020-02337-4.

Acknowledgements The authors are grateful to Mr. C Mugundhan of Spiking™ products (2M Biotech), Ramanathapuram, Tamilnadu, India, for providing the Spirulina powder. We thank all the staff of the Department of Life Sciences, CHRIST (Deemed to be University), Bangalore Central Campus, Bengaluru, Karnataka, for their help and all the facilities to carry out this research. We thank the two anonymous reviewers for their valuable comments on improving the manuscript.

Authors' contributions SAS and SS conceived the project. SAS designed and performed the experiments, and analyzed the data. SS, JA, and KV helped with experimental design and troubleshooting. SAS and KV performed the statistical analysis. SAS wrote the paper, and all authors commented and contributed to the previous versions. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

Abdel-Warith AA, Younis EM, Al-Asgah NA, Mahboob S (2020) Effect of replacing fish meal by full fat soybean meal on growth performance, feed utilization and gastrointestinal enzymes in diets for African catfish *Clarias gariepinus*. Braz J Biol 80:535–543

Amri E, Mamboya F (2012) Papain, a plant enzyme of biological importance: a review. Am J Biochem Biotechnol 8:99–104

Anukoolprasert T, Srinuansom K, Rukdontri T, Nonkhukhetkhong S, Petkam R (2019) Postprandial *in vitro* protease-specific activity of Nile tilapia (*Oreochromis niloticus* L.) digestive organs. Pak J Nutr 18:125–133

Bai M, Qin G, Sun Z, Long G (2016) Relationship between molecular structure characteristics of feed proteins and protein *in vitro* digestibility and solubility. Asian Australas J Anim Sci 29:1159–1165

Bais B (2018) Fish scenario in India with emphasis on Indian major carps. Int J Avian Wildl Biol 3:409–411

Baldwin J, Hochachka PW (1970) Functional significance of isoenzymes in thermal acclimatization. Acetylcholinesterase from trout brain. Biochem J 116:883–887

Barba FJ, Grimi N, Vorobiev E (2015) New approaches for the use of non-conventional cell disruption technologies to extract potential food additives and nutraceuticals from microalgae. Food Eng Rev 7:45–62

Becker EW (2007) Micro-algae as a source of protein. Biotechnol Adv 25:207–210

Bleakley S, Hayes M (2017) Algal proteins: extraction, application, and challenges concerning production. Foods 6:33

Borowitzka MA (1997) Microalgae for aquaculture: opportunities and constraints. J Appl Phycol 9:393–401

Carter CG, Bransden MP, van Barneveld RJ, Clarke SM (1999) Alternative methods for nutrition research on the southern bluefin tuna, *Thunnus maccoyii: in vitro* digestibility. Aquaculture 179:57–70

Castillo S, Gatlin DM (2015) Dietary supplementation of exogenous carbohydrase enzymes in fish nutrition: a review. Aquaculture 435:286–292

Choi WM, Lam CL, Mo WY, Wong MH (2016) Upgrading food wastes by means of bromelain and papain to enhance growth and immunity of grass carp (*Ctenopharyngodon idella*). Environ Sci Pollut Res 23:7186–7194

Chong ACS, Hashim R, Ali AB (2002) Assessment of dry matter and protein digestibilities of selected raw ingredients by discus fish (*Symphysodon aequifasciata*) using *in vivo* and *in vitro* methods. Aquac Nutr 8:229–238

Cupp-Enyard C (2008) Sigma's non-specific protease activity assay - casein as a substrate. JOVE- J Vis Exp. https://doi.org/10.3791/899

Diken G, Demir O, Naz M (2016) The potential inhibitory effects of microalgae and macroalgae on protease activities of *Argyrosomus regius* (Pisces, Scanidae) larvae using *in vitro* assays. J Int Sci Publ 4:473–483

Encarnação P (2016) Functional feed additives in aquaculture feeds. In: Nates S (ed) Aquafeed formulation. Academic Press, Amsterdam, pp 217–237

Ezquerra JM, García-Carreño FL, Carrillo O (1998) *In vitro* digestibility of dietary protein sources for white shrimp (*Penaeus vannamei*). Aquaculture 163:123–136

Fan X, Cui Y, Zhang R, Zhang X (2018) Purification and identification of anti-obesity peptides derived from *Spirulina platensis*. J Funct Foods 47:350–360

Fernandes IM, Bastos YF, Barreto DS et al (2017) The efficacy of clove oil as an anaesthetic and in euthanasia procedure for small-sized tropical fishes. Braz J Biol 77:444–450

Francis G, Makkar HPS, Becker K (2001) Antinutritional factors present in plant-derived alternate fish feed ingredients and their effects in fish. Aquaculture 199:197–227

Grabner M (1985) An *in vitro* method for measuring protein digestibility of fish feed components. Aquaculture 48:97–110

Hardy RW, Barrows FT (2003) Diet Formulation and manufacture. In: Halver JE, Hardy RW (eds) Fish nutrition, 3rd edn. Academic Press, New York, pp 505–600

Jun-sheng L, Jian-lin L, Ting-ting W (2006) Ontogeny of protease, amylase and lipase in the alimentary tract of hybrid Juvenile tilapia (*Oreochromis niloticus* × *Oreochromis aureus*). Fish Physiol Biochem 32:295–303

Kim K-D, Lim SG, Kang YJ, Kim KW, Son MH (2012) Effects of dietary protein and lipid levels on growth and body composition of juvenile far eastern catfish *Silurus asotus*. Asian Australas J Anim Sci 25:369–374

Kolkovski S (2001) Digestive enzymes in fish larvae and juveniles—implications and applications to formulated diets. Aquaculture 200:181–201

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with Folin phenol reagent. J Biol Chem 193:265–275

Montoya-Martínez C, Nolasco-Soria H, Vega-Villasante F, Carrillo-Farnés O, Álvarez-González A, Civera-Cerecedo R (2018) *In vitro* protein digestibility of animal, vegetal and microbial feed ingredients for *Macrobrachium tenellum*. Lat Am J Aquat Res 46:495–501

Muzyaka O, Musienko V, Osmak A, Plischenko A, Semiletko Y (2020) GraphPad Prism. Version 8.4.2. San Diego, California, USA. www.graphpad.com. Accessed 27 Apr 2020

Nandeesha MC, Gangadhar B, Varghese TJ, Keshavanath P (1998) Effect of feeding *Spirulina platensis* on the growth, proximate composition and organoleptic quality of common carp, *Cyprinus carpio* L. Aquac Res 29:305–312

Olsen JV, Ong S-E, Mann M (2004) Trypsin cleaves exclusively C-terminal to arginine and lysine residues. Mol Cell Proteomics 3:608–614

Parimi NS, Singh M, Kastner J, Das KC, Forsberg LS, Azadi P (2015) Optimization of protein extraction from *Spirulina platensis* to generate a potential co-product and a biofuel feedstock with reduced nitrogen content. Front Energy Res 3. https://doi.org/10.3389/fenrg.2015.00030

Patil DW, Singh H (2014) Effect of papain supplemented diet on growth and survival of post -larvae of *Macrobrachium rosenbergii*. Int J Fish Aquat Stud 1:176–179

Pavan R, Jain S, Shraddha KA (2012) Properties and therapeutic application of bromelain: a review. Biotechnol Res Int 2012:976203

Rachmawati D, Samidjan I (2018) the effects of papain enzyme supplement in feed on protein digestibility, growth and survival rate in Sangkuriang catfish (*Clarias* sp). Omni-Akuatika 14:91–99

Rick W (1974) Trypsin. Methods Enzym Anal 2:1013–1024

Rostika R, Nurhayati A, Buwono I, Rizal A, Dewanti LP, Maulana T (2018) Papain and bromelain crude enzyme extract in commercial feed, effectiveness toward pisciculture production of striped catfish (*Pangasianodon hypophthalmus*) in aquaculture facility. AACL Bioflux 11:1598–1604

Rungruangsak-Torrissen K, Rustad A, Sunde J, Eiane SA, Jensen HB, Opstvedt J, Nygård E, Samuelsen TA, Mundheim H, Luzzana U, Venturini G (2002) *In vitro* digestibility based on fish crude enzyme extract for prediction of feed quality in growth trials. J Sci Food Agric 82:644–654

Scott M, Johnsen C, Phillips KA (1987) The pH optima for papain and bromelain treatment of red cells. Vox Sang 52:223–227

Sharma SA, Krishnakumar V, Arulraj J (2019) Impact of *Ananas comosus* extract supplementation on the growth and biochemical profile of *Cyprinus carpio* fingerlings. Trends Fish Res 8:69–77

Sheikhzadeh N, Mousavi S, Oushani AK, Firouzamandi M, Mardani K (2019) *Spirulina platensis* in rainbow trout (*Oncorhynchus mykiss*) feed: effects on growth, fillet composition, and tissue antioxidant mechanisms. Aquac Int 27:1613–1623

Sigma-Aldrich (2020a) Bromelain. https://www.sigmaaldrich.com/life-science/biochemicals/biochemical-products.html?TablePage=16410479. Accessed 8 Jul 2020

Sigma-Aldrich (2020b) Papain. https://www.sigmaaldrich.com/life-science/metabolomics/enzyme-explorer/analytical-enzymes/papain.html. Accessed 8 Jul 2020

Subandiyono, Hastuti S, Nugroho RA (2018) Feed utilization efficiency and growth of Java barb (*Puntius javanicus*) fed on dietary pineapple extract. AACL Bioflux 11:309–318

Sun Y, Chang R, Li Q, Li B (2016) Isolation and characterization of an antibacterial peptide from protein hydrolysates of *Spirulina platensis*. Eur Food Res Technol 242:685–692

Tacon A (1997) Feeding tomorrow's fish: keys for sustainability. In: Tacon A, Bascuro B (eds) Feeding tomorrow's fish. Cahiers Options Méditerranéennes, No. 22. CIHEAM, Zaragosa, pp 11–33

Tewari G, Ram R, Singh A (2018) Effect of plant base digestive enzyme 'Papain'on growth, survival and behavioural response of *Cyprinus carpio*. Int J Fish Aquat Stud 6:210–214

Valverde-Chavarría S, Álvarez-González CA, Brais-Medina M, Calvo-Elizondo E, Ulloa-Rojas JB (2016) *In vitro* digestibility and proteases inhibitory effect of several feedstuffs for *Parachromis dovii* juveniles and *P. dovii* hybrid larvae. Fish Physiol Biochem 42:1767–1776

Velasquez SF, Chan MA, Abisado RG, Traifalgar RFM, Tayamen MM, Maliwat GCF, Ragaza JA (2016) Dietary Spirulina (*Arthrospira platensis*) replacement enhances performance of juvenile Nile tilapia (*Oreochromis niloticus*). J Appl Phycol 28:1023–1030

Yigit N, Koca S, Didinen BI, Diler I (2016) Effect of protease and phytase supplementation on growth performance and nutrient digestibility of rainbow trout (*Oncorhynchus mykiss* , Walbaum) fed soybean meal-based diets. J Appl Anim Res 46:29–32

Yuangsoi B, Klahan R, Charoenwattanasak S, Shi-Mei L (2018) Effects of supplementation of pineapple waste extract in diet of Nile Tilapia (*Oreochromis niloticus*) on growth, feed utilization and nitrogen excretion. J Appl Aquac 30:227–237