Algal Research 37 (2019) 145–153

Contents lists available at ScienceDirect

Algal Research

journal homepage: www.elsevier.com/locate/algal

Differential hydrolysis of proteins of four microalgae by the digestive enzymes of gilthead sea bream and Senegalese sole

A.J. Vizcaíno[a], M.I. Sáez[a], T.F. Martínez[a], F.G. Acién[b], F.J. Alarcón[a,*]

[a] Departamento de Biología y Geología, Escuela Superior de Ingeniería, Universidad de Almería, La Cañada de San Urbano, Almería, Spain
[b] Departamento de Ingeniería Química, Facultad de Ciencias Experimentales, Universidad de Almería, La Cañada de San Urbano, Almería, Spain

ARTICLE INFO

Keywords:
Electrophoresis
in vitro digestibility
Microalgae
Protein
Solea senegalensis
Sparus aurata

ABSTRACT

This study evaluates the *in vitro* protein hydrolysis of four microalgae (*Tisochrysis lutea*, *Nannochloropsis gaditana*, *Tetraselmis suecica* and *Scenedesmus almeriensis*) by intestinal proteases of gilthead sea bream (*Sparus aurata*) and Senegalese sole (*Solea senegalensis*). The hydrolysis of protein was monitored at different sampling times by electrophoretic techniques, and the quantification of the free amino acids released by proteases. Overall, *S. aurata* or *S. senegalensis* proteases hydrolysed microalgae protein in a similar way. The highest hydrolysis values (coefficient of protein degradation, CPD > 70%) were obtained for *Tisochrysis* and *Nannochloropsis* biomasses, which showed a progressive and almost complete proteolysis at the end of the *in vitro* assay. *Tetraselmis* and *Scenedesmus* protein was also hydrolysed, but SDS-PAGE revealed that two protein fractions remained virtually intact at the end of the *in vitro* assay. The final amount of free amino acids released *in vitro* by the fish digestive enzymes ranged from 9 to 25 g 100 g protein^{-1}. A linear relationship between CPD and the amount of free amino acids released was found, a fact that suggests that microalgae protein is hydrolysed efficiently by the digestive proteases of both fish species. The present study provides information about the protein availability from selected microalgae, which will aid in the initial evaluation of the microalgae as potential protein sources in feeds of two important farmed fish species.

1. Introduction

Dietary protein plays a key role in fish growth, since it represents the source of essential amino acids for protein accretion in new tissues [1,2]. In spite of the significant reduction of fishmeal in aquafeeds [3], this ingredient is still an important protein source for carnivorous fish species. However, constraints related to high prices, limited availability, and environmental concerns have promoted extensive research efforts focused on the assessment of alternative protein sources [4,5]. New protein ingredients for feeding fish should meet several requirements, the most important of which is providing high-quality protein, in terms of both quantity and bioavailability of well-balanced essential amino acids. Several microalgae species might represent an alternative ingredient of interest in aquafeeds, due to their high protein content, ranging from 30% to 55% in dry matter [6]. In this regard, in the last years numerous studies have assessed the potential of microalgae as dietary protein ingredient in practical diets for different fish species [2,4,7,8].

Nevertheless, microalgae represent a heterogeneous source of protein, owing to the specific particularities of each strain, a fact that implies that the potential bioavailability of protein for a given microalgae cannot be easily predicted. For instance, the structure and chemical composition of the cell wall vary among microalgae species [9,10] and in fact, the cell wall itself may act as a protective barrier, avoiding the release of intracellular nutrients [11] The efficiency with which fish can hydrolyse microalgae cell walls relies heavily on the carbohydrate composition (specifically, how sugars are linked to each other), as well as on the possession of the appropriate digestive enzymes. As far as we know, little research has been carried out to assess the *in vitro* hydrolysis of microalgae protein by fish digestive enzymes [12].

In vitro digestibility methods have been widely accepted as helpful procedures in order to evaluate the quality of protein ingredients for aquafeeds [2,13], in terms of both the extent of protein hydrolysis, and its further bioavailability for specific fish species [14]. These assays are faster, cheaper and less laborious than those based on *in vivo* feeding trials, but they should consider the species-specific digestive characteristics, which might determine different abilities to digest and assimilate nutrients [15,16]. When available, alternative experimental procedures are also preferred from an ethical point of view. The use of

* Corresponding author.
E-mail address: falarcon@ual.es (F.J. Alarcón).

https://doi.org/10.1016/j.algal.2018.11.018
Received 15 February 2018; Received in revised form 26 November 2018; Accepted 27 November 2018

enzyme extracts obtained from the intestine is recommended to simulate *in vitro* digestion, given that this strategy could fairly indicate the potential bioavailability of nutrients for each specific species [12,17].

The aim of this work was to assess the *in vitro* protein hydrolysis of different microalgae (*Tisochrysis lutea*, *Nannochloropsis gaditana, Tetraselmis suecica* and *Scenedesmus almeriensis*) by digestive proteases of two farmed marine fish species, gilthead sea bream and Senegalese sole. The hydrolysis of microalgae protein by digestive enzymes of the two fish species was evaluated by the sequential characterization of the protein substrates throughout the *in vitro* enzyme assay using gel electrophoresis and analysis of free amino acids.

2. Material and methods

2.1. Preparation of fish enzyme extracts

Gilthead sea bream juveniles (25 g average body weight) were obtained from a commercial nursery (Predomar SA, Almería, Spain), and they were fed a commercial diet (Skretting, crude protein: 47% DM) twice per day (9:00 and 13:00) at a rate of 3% biomass daily. Senegalese sole specimens (40 g average body weight) were provided by Centro Agua de Pino (IFAPA, Huelva, Spain) and they were fed a commercial diet (Skretting, crude protein: 55% DM) twice per day (9:00 and 17:00) at a rate of 3% of their body weight per day for 15 days. On the day of sampling, 6 h after the fish received the last feed ration, fifteen fish of each species were randomly selected, anaesthetized and sacrificed by severing their spine according to the requirements of the Council Directive 2010/63/UE. Immediately, the abdomen was opened and the whole viscera were obtained. The intestines were separated from the other organs, and all visible fat removed. For each species, three pools of five individual tissues were homogenized in distilled water at 4 °C (0.5 g mL^{-1}). Supernatants were obtained after centrifugation (12,000 rpm, 12 min, 4 °C) and stored in aliquots at −20 °C until further use. Total soluble protein in enzyme extracts was determined in triplicate using bovine serum albumin as standard [18]. Total alkaline protease activity of the enzymatic extracts was measured spectrophotometrically using 5 g L^{-1} casein in 50 mM Tris HCl (pH 9.0) as substrate [19]. One mL of casein solution was incubated with 10 μL the extracts during 30 min at 25 °C. The reaction was stopped by adding 0.5 mL of 20% (w/v) trichloroacetic acid (TCA). Blanks were prepared by adding TCA before the extracts. The reaction mixture was cooled for 15 min at −4 °C, and then centrifuged (12,000 rpm, 12 min, 4 °C). The absorbance of supernatants at 280 nm was measured spectrophotometrically (Shimadzu UV-1800, Shimadzu, Kyoto, Japan). One unit of total protease activity (UA) was defined as the amount of enzyme that released 1 μg of tyrosine per min in the reaction mixture, considering an extinction coefficient for tyrosine of 0.008 $\mu g^{-1}\, mL^{-1}\, cm^{-1}$, measured at 280 nm. Enzyme activity of each digestive extract was analysed in triplicate.

2.2. Microalgae

Freeze-dried biomass of four microalgae species (*Tisochrysis lutea,* TIS; *Nannochloropsis gaditana,* NAN; *Tetraselmis suecica,* TET; and *Scenedesmus almeriensis,* SCE) were used in this study. These strains were selected considering their high protein content, their potential to be used in aquafeeds, and the fact that they were previously assessed as feed ingredients for farmed marine fish. Microalgae were provided by Estación Experimental "Las Palmerillas" (Fundación Cajamar, Almería, Spain). Briefly, the microalgae were cultivated in a semi-industrial sized (3000 *L*) outdoor tubular photobioreactor (PBR) in continuous mode. Values of pH, temperature and dissolved oxygen were continuously monitored by using specific probes (Crison Instruments, Spain). The biomass was harvested daily by centrifugation (RINA centrifuge, Riera Nadeu SA, Spain), frozen at −18 °C, lyophilized, and finally milled (RM200 mill, Retsch, Spain) during 20 min to obtain a fine powder (< 100 μm) that was stored in the dark at −20 °C until further analysis. Each microalgae biomass was obtained from a single production batch that was homogeneously blended before using. Protein content (N × 6.25) was obtained using elemental analysis (C:H:N) with a Fisons EA 1108 analyzer (Fisons Instruments, Beverly, MA, USA), whereas amino acid profiles were analysed through a Waters HPLC system (Waters 474, Waters, Milford, MA, USA) following the method previously described by Bosch et al. [20]. Data obtained are shown in Tables 1 and 2, respectively.

Additionally, aqueous extracts (0.5 mg mL^{-1}) from each microalga were prepared, homogenized in distilled water by shaking for 30 min at room temperature and for 24 h at 4 °C. The mixtures were centrifuged for 20 min at 13000 *g* and 4 °C. Supernatants were used to determine total soluble protein in triplicate according to Bradford [18] (Table 3).

Table 1
Protein content of the microalgae evaluated in the study.

Microalgae species	Crude protein content (% dry matter)
Tisochrysis lutea (TIS)	43.6
Nannochloropsis gaditana (NAN)	44.9
Tetraselmis suecica (TET)	36.0
Scenedesmus almeriensis (SCE)	42.8

Table 2
Amino acid profile (g 100 g $protein^{-1}$) of the selected microalgae and fishmeal.

	TIS	NAN	TET	SCE	Fishmeal
Aspartic acid	8.9	7.6	9.6	8.5	9.2
Glutamic acid	11.4	10.6	11.4	10.2	13.0
Alanine	5.8	5.9	6.0	7.6	6.3
Cysteine	0.9	0.8	1.2	0.8	0.9
Glycine	5.4	5.3	6.5	5.9	5.6
Serine	3.9	3.7	4.4	3.8	3.8
Proline	3.4	7.1	3.8	3.7	4.2
Tyrosine	2.9	2.8	2.9	2.8	3.1
Arginine*	5.5	5.8	6.6	5.2	5.8
Phenylalanine*	4.8	4.5	5.9	4.7	3.9
Histidine*	1.6	1.7	1.8	1.6	2.4
Isoleucine*	4.5	4.3	4.1	3.9	4.7
Leucine*	6.8	6.9	7.0	7.3	7.6
Lysine*	4.1	4.8	3.8	4.3	6.9
Methionine*	1.57	1.3	1.3	1.2	3.0
Threonine*	4.1	4.1	4.6	4.6	4.3
Valine*	4.7	5.0	5.0	4.9	5.3
NEAA (%)	53.2	53.4	53.3	53.5	51.2
EAA (%)	46.8	46.6	46.7	46.6	48.8
Ratio EAA/NEAA	0.88	0.87	0.88	0.87	0.95

NEAA: Non-essential amino acids; EAA: essential amino acids, marked in the first column with asterisks. *Tisochrysis lutea* (TIS), *Nannochloropsis gaditana* (NAN), *Tetraselmis suecica* (TET), and *Scenedesmus almeriensis* (SCE).

2.3. In vitro protein hydrolysis assay

The *in vitro* protein hydrolysis of the microalgae biomass was carried out in 10 mL-jacketed reaction vessels connected to a circulating water bath maintained at a constant temperature of 25 °C, under

Table 3
Soluble protein content of the microalgae evaluated in the study.

	Soluble protein content (mg g dry $matter^{-1}$)
TIS	89.2 ± 12.0c
NAN	28.6 ± 3.4b
TET	24.4 ± 2.1b
SCE	8.8 ± 1.0a

Values with different lowercase letter indicate significant difference ($p < 0.05$). *Tisochrysis lutea* (TIS), *Nannochloropsis gaditana* (NAN), *Tetraselmis suecica* (TET), and *Scenedesmus almeriensis* (SCE).

continuous agitation by a magnetic stirrer. For each assay, a known amount of each microalga, providing 80 mg crude protein (8 mg mL^{-1}), was suspended in 50 mM Tris HCl buffer pH 9.0. After 15 min stirring, protein hydrolysis was initiated by the addition of a previously calculated volume (from 0.350 mL to 0.475 mL depending on the specific activity measured) of the digestive enzymatic extract, providing 200 units of total alkaline protease activity (UA) per reaction vessel. This protocol was carried out in triplicate for each microalgae species and for each of the enzymatic extracts tested. The enzyme-substrate ratio chosen for this assay has been used previously in other *in vitro* studies carried out in *Sparus aurata* [21] and *Solea senegalensis* [1]. Blank assays with microalgae in the absence of fish enzyme extracts were also carried out.

2.4. Electrophoretic analysis of protein hydrolysis

Throughout the course of the *in vitro* hydrolysis of proteins by fish digestive enzymes, the reaction mixtures were sampled at different time intervals (0, 15, 30, 45, 60, 75, and 90 min). At each time point, 50 μL samples were collected from the reaction mixture and diluted (1:1) in sample buffer (0.125 M Tris HCl, pH 6.8; 4% (w/v) SDS; 10% (v/v) β-mercaptoethanol; 20% (v/v) glycerol; 0.04% (w/v) bromophenol blue) and boiled for 5 min. After that, samples were stored at −20 °C for further analysis by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli [22], using 11% polyacrylamide and 8 × 10 × 0.075 cm gels. Electrophoresis was performed at a constant voltage of 100 V per gel for 60 min at 4 °C. The gels were stained overnight with 0.1% Coomassie brillant blue (BBC R-250) in a methanol-acetic acid solution (50:20:50). Destaining was carried out in a methanol-acetic acid-water solution (35:10:55). A protein standard including twelve proteins ranging from 6.5 kDa (aprotinin, bovine lung) to 200 kDa (myosin, porcine heart) was used (wide range molecular weight marker, S-84445, Sigma, St. Louis, USA). The relative molecular mass (Mr, in kDa) of proteins was estimated using a linear plot of log Mr. of protein standards *vs* relative mobility (Rf). Five microlitres of molecular weight marker solution prepared according to the manufacturer's instructions were loaded on each plate.

The results obtained previously by other authors when performing SDS-PAGE of similar microalgae species were used as a reference to identify the main protein fractions separated in the present study; *T. galbana* [23,24], *N. gaditana* [25,26], *Tetraselmis* sp. [27], and *Scenedesmus* sp. [28].

2.5. Calculation of the coefficient of protein degradation (CPD)

The extent of *in vitro* protein hydrolysis was expressed by a numerical value obtained considering both the decrease of optical density (expressed as percentage) for each protein band after the enzymatic hydrolysis, and the relative proportion that such band represented in the total separated protein [21]. The value obtained was called coefficient of protein degradation (CPD), and it was estimated using the following mathematical expression:

$$CPD = \sum_{i=1}^{n} \left[\frac{OD_i(t=0) - OD_i(t=90\,min)}{OD_i(t=0)} \times 100 \right] \times \frac{OD_i(t=0)}{\sum_{i=1}^{n} OD_i(t=0)}$$

where i is the major protein bands identified from 1 to n, ODi is the optical density of the protein band i and t is the time of reaction.

2.6. Quantification of free amino acids released during the in vitro assay

Analysis of free amino acids released at different sampling times (0, 15, 30, 45, 60, 75, and 90 min) was performed according to Church et al. [29]. This technique is based on the conjugation of the amino terminal group of the amino acid with o-phthaldialdehyde (OPA). In brief, 50 μL of the samples collected from the digestion mixture reactions were fixed in 50 μL of 20% TCA and centrifuged at 12,000 *g* for 15 min. Subsamples (10 μL) of the supernatant samples were added to 1 mL of OPA reagent, the solutions were incubated 5 min at room temperature, and then absorbance was read at 340 nm. The amount of free amino acids was calculated using a standard curve made with L-leucine. Blank assays were run to estimate free amino acids from enzyme extracts and microalgae suspensions, which enabled to determine the net release of amino acids attributable to enzymatic hydrolysis. Results were expressed as accumulated values of free amino acids released during the enzymatic hydrolysis (g of L-leucine equivalents 100 g protein $^{-1}$). All assays were performed in triplicate.

In addition, values of the CPD estimated as explained above were plotted against accumulated values of free amino acids released.

2.7. Statistical analysis

All experiments were repeated at least three times with three replicates. Data were expressed as mean ± SD. Comparison of means was carried out by one-way ANOVA with a 5% level of probability ($p < 0.05$) followed by a multiple comparison test. Data in percentage (%) were arcsin ($x^{1/2}$)-transformed, checked for normality (Shapiro-Wilk test) and homoscedasticity (Levene test). CDP (in percentage) and free amino acids released (in g L-leucine equivalents 100 g protein $^{-1}$) were plotted against time of digestion for different microalgae. The relationship between CDP and free amino acids released was examined using the Spearman rank correlation test, and correlations were considered significant at ($p < 0.05$). All statistical analyses were performed with Stagraphics Plus 4.0 (Rockville, Maryland, USA) software.

3. Results

The proteinograms of microalgae and fish digestive extracts are shown in Fig. 1. No protein hydrolysis was observed in microalgae samples when *in vitro* assays were carried out in the absence of fish enzyme extracts, and the protein patterns found in *S. aurata* and *S. senegalensis* enzyme extracts were negligible in comparison with microalgae proteinograms. Microalgae showed a complex protein profile made up of several fractions with a wide range of molecular weight. The time-course protein hydrolysis by the digestive proteases of *S. aurata* and *S. senegalensis* is shown in Figs. 2 and 3. Changes in optical density measured in electrophoretic gels were assessed in six protein fractions ranging from 11.7 to 56.4 kDa in TIS, seven fractions ranging from 41.0 to 85.3 kDa in NAN, eight fractions ranging from 22.9 to 58.5 kDa in TET, and six fractions ranging from 26.8 to 156.1 kDa in SCE (Figs. 2 and 3). In the case of TIS, with the exception of the 11.7 kDa protein, a noticeable hydrolysis of all the separated fractions was evidenced, especially during the initial 15 min of digestive simulation (Figs. 2A and 3A). Compared with TIS, NAN hydrolysis showed a similar trend, but in this case the optical density of all the protein fractions was decreased at the end of the *in vitro* assay (Figs. 2B and 3B). TET and SCE showed a particular pattern, with some protein fractions showing noticeable degradation, whereas 22.9 and 24.1 kDa fractions of TET (Figs. 2C and 3C), and 26.8 and 149.2 kDa fractions of SCE (Figs. 2D and 3D) remained intact after 90 min. For each specific microalgae, gilthead sea bream or Senegalese sole digestive proteases yielded similar protein hydrolysis profiles.

Changes in average CPD values during the *in vitro* assays are shown in Fig. 4. TIS and NAN yielded a similar pattern of protein hydrolysis due to both gilthead sea bream and Senegalese sole proteases. In both cases, the evolution showed an initial quick proteolysis, followed by a less marked but sustained hydrolysis until the end of the assay, reaching final CPD values over 70%. Regarding TET and SCE, the CPD values were significantly lower ($p < 0.05$) throughout the complete assay compared with those obtained for TIS and NAN.

The kinetics of free amino acids release was assessed by analysing the cumulative production of amino acids *vs* digestion time (Fig. 5).

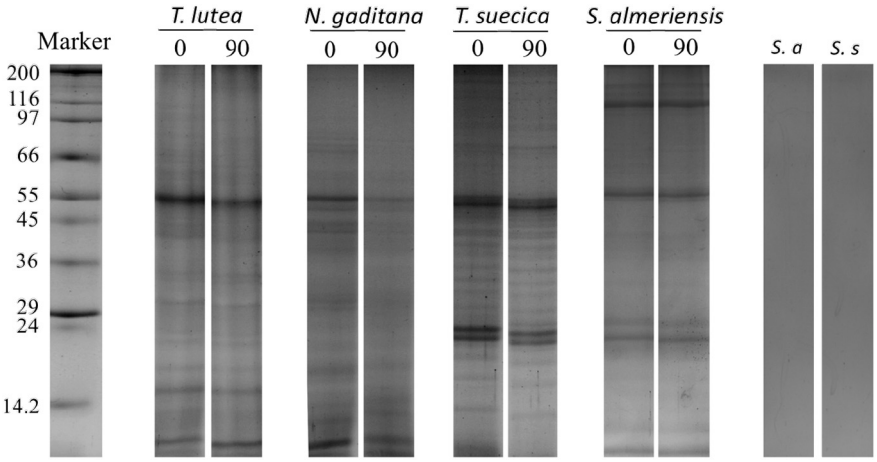

Fig. 1. SDS-PAGE of blank assays of the different microalgae carried out in absence of fish enzymes at 0 and 90 min, and protein profile of *S. aurata* (*S. a*) and *S. senegalensis* (*S. s*) enzyme extracts. *Tisochrysis lutea* (TIS), *Nannochloropsis gaditana* (NAN), *Tetraselmis suecica* (TET), and *Scenedesmus almeriensis* (SCE).

Gilthead sea bream proteases yielded two different patterns; i) the accumulation of amino acids released from TIS and SCE was progressive, whereas ii) the free amino acids released from TET and NAN levelled off at lower values, especially in the case of TET. Senegalese sole proteases generated similar amounts of free amino acids after 90 min hydrolysis for all the algae assayed, except for TET that yielded the lowest concentration. At the end of the *in vitro* assay, free amino acids released ranged from 9.5 to 26.8 g 100 g protein^{-1}. Overall, gilthead sea bream and Senegalese sole proteases released the highest cumulative values of free amino acids with TIS (25.8% and 22.4%, respectively) ($p < 0.05$). On the contrary, the lowest values were obtained for TET after hydrolysis, regardless of the fish digestive extract considered.

Finally, a clear linear relationship between the evolution of CPD and the kinetics of free amino acids released was found (Table 4), as indicated by R^2 values, which ranged from 0.833 to 0.942, and from 0.632 and 0.938 for gilthead sea bream and Senegalese sole, respectively.

4. Discussion

Simulated digestion assays for testing novel feed ingredients with species-specific digestive enzymes are highly informative, while also enabling to minimize the use of live animals. These assays can also be used as effective tools for predicting the potential protein quality prior to undertaking costly *in vivo* animal feeding trials [2]. The present study provides an initial overview of the digestive capacity of two farmed fish to hydrolyse microalgae proteins by using *in vitro* digestibility techniques. The results obtained enabled to classify the microalgae studied into two well-defined groups; a) TIS and NAN that were easily hydrolysed by the fish digestive proteases, according to the CPD values exceeding 70% after 90 min of hydrolysis, and b) TET and SCE that showed lower final CPD values.

The use of species-specific enzymatic extracts obtained from different parts of the fish digestive system is recommended to simulate *in vitro* digestion [12,17,30]. The *in vitro* simulation of fish digestion has been used in salmonids (primarily rainbow trout, *Oncorhynchus mykiss*), and other fish species, including *S. aurata* and *S. senegalensis* [17]. To date, there are some studies evaluating microalgae protein hydrolysis and nutritional quality by using commercial enzymes from mammals are available in the literature [31,32]. However, research using species-specific fish digestive enzymes is rather scarce [12].

Protein hydrolysis has been monitored by electrophoretic separation in ruminants [33], humans [34], and farmed fish species [1,35]. SDS-PAGE separation enables the identification of individual protein fractions contained in feedstuffs, as well as the assessment of the kinetic of hydrolysis under different *in vitro* simulated conditions. In our study, SDS-PAGE analysis showed, overall, that proteins of microalgae were easily hydrolysed by digestive proteases of *S. aurata* and *S. senegalensis*. A quick and almost complete hydrolysis of the main protein fractions was observed. However, TET and SCE included two protein fractions that remained undegraded at the end of the *in vitro* assay.

As for conventional feed ingredients, protein solubility is an important factor that determines the susceptibility of a given protein to be hydrolyzed enzymatically [1,12]. In the case of SCE and TET, results evidenced that the lower the content of soluble protein the lower the protein hydrolysis value. It is know that the existence of a recalcitrant cell wall in microalgae is a factor that could limit protein bioavailability in the gut of monogastric animals, including fish [12,36]. The absence of cellulase activity in fish gut can hinder the efficient utilization of intracellular nutrients [35]. Species of the genus *Scenedesmus* are characterized by a rigid and extremely resistant cell wall [36,37], a fact that might well explain the relatively low protein solubility observed in proteinograms (Figs. 2 and 3).

Taking into account that the digestive tract of the farmed fish considered in this study lack any appreciable cellulase enzyme activity, it could be of interest the rupture of the cell wall prior to including microalgae in feeds, a practical measure that could help improve the utilization of the intracellular nutrients supplied by microalgae-based diets. In the present study, the microalgal biomasses were ground into a fine powder by means of a mortar mill, in order to ensure homogeneity in all *in vitro* assays. Nevertheless, the rupture of the cell walls was not checked. For this reason, future studies aimed at linking the extent of cell-wall disruption to measures of nutrient digestibility are suggested.

The structure and spatial conformation of any given protein, as well as the amino acid composition are factors that determine its susceptibility to be hydrolysed [1,12]. Given that microalgae used in the present study display similar amino acid composition, the reduced hydrolysis observed in some specific fractions of TET and SCE might well be related to limited accessibility of proteases owing to complex structure of such protein fractions. In general, the presence of high percentage of non-polar amino acids in algal proteins compared to plant proteins [38,39] seems to be correlated with lower solubility and digestibility of microalgae [32]. Unfortunately, those aspects have not been assessed in the present study, and further studies would be required to ascertain the biochemical characteristics of microalgal proteins that determine such high resistance to hydrolysis.

The information obtained from electrophoresis gels has been used to quantify changes in optical density of protein fractions, in order to estimate a numerical quantitative index of the global hydrolysis of proteins [1,14]. In the present study, CPD values were higher than 70% in

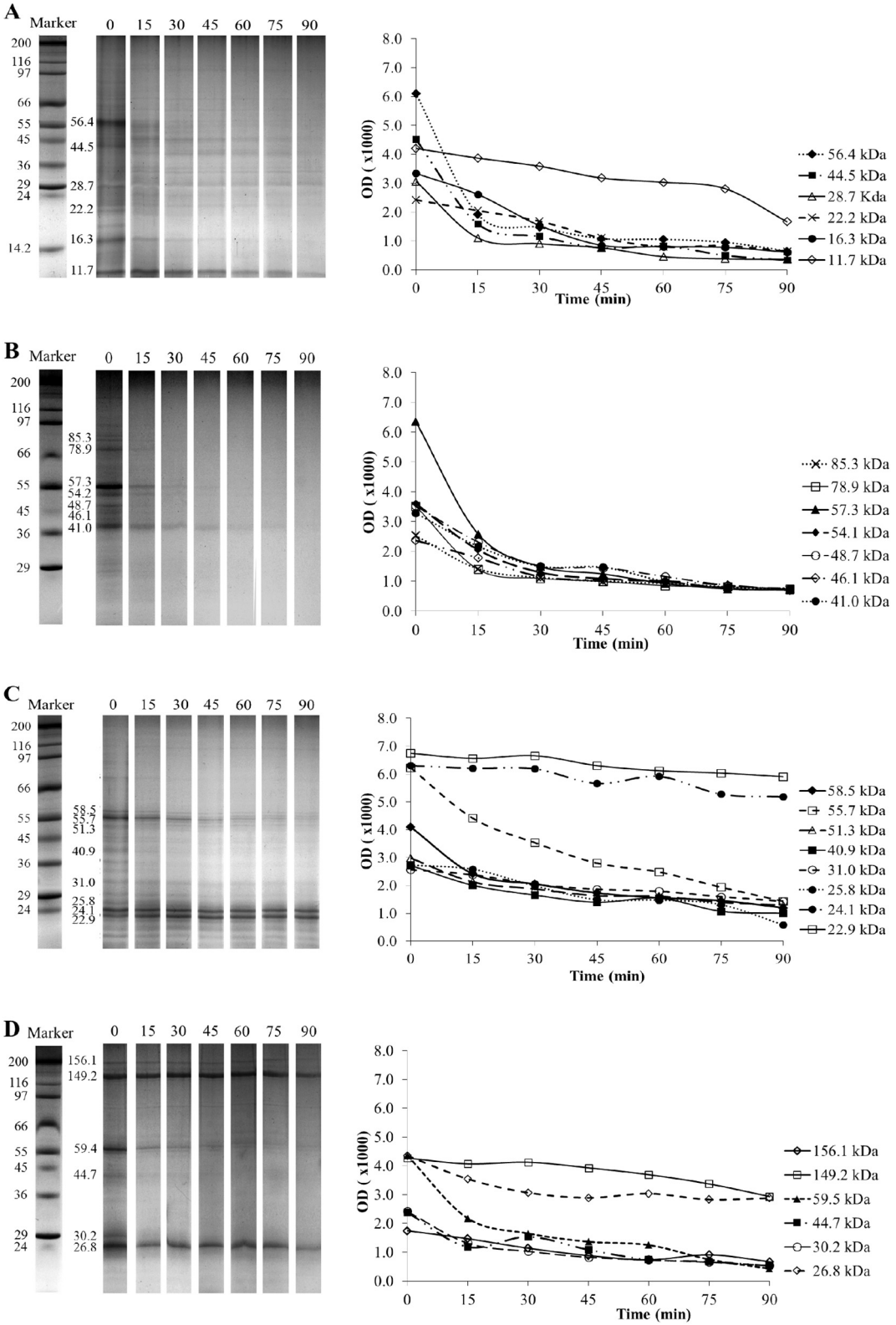

Fig. 2. Time-course of *in vitro* proteolysis of (A) *Tisochrysis lutea* (TIS), (B), *Nannochloropsis gaditana* (NAN), (C) *Tetraselmis suecica* (TET), and (D) *Scenedesmus almeriensis* (SCE) by *S. aurata* proteases. Images show SDS-PAGE hydrolysis patterns obtained at different sampling times (0, 15, 30, 45, 60, 75 and 90 min), and graphics show changes in the optical density (OD) (measured as pixels per cm^2) of the main protein fractions throughout the enzymatic *in vitro* hydrolysis. Numbers at the left of proteinograms and at the right of densitometric curves show the relative molecular weight (kDa) of the main proteins studied.

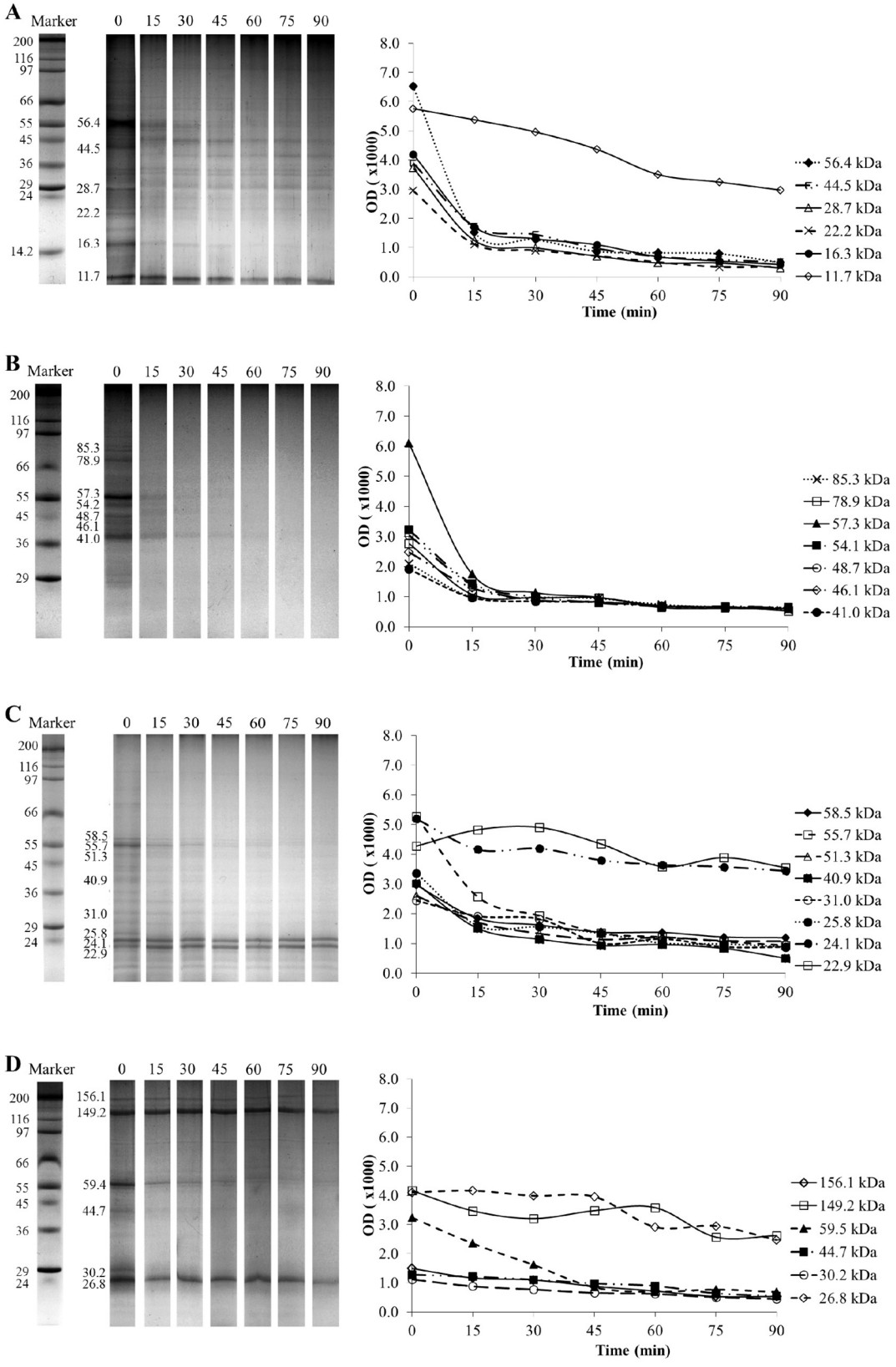

Fig. 3. Time-course of *in vitro* proteolysis of (A) *Tisochrysis lutea* (TIS), (B), *Nannochloropsis gaditana* (NAN), (C) *Tetraselmis suecica* (TET), and (D) *Scenedesmus almeriensis* (SCE) by *S. senegalensis* proteases. Details are explained in caption to Fig. 2.

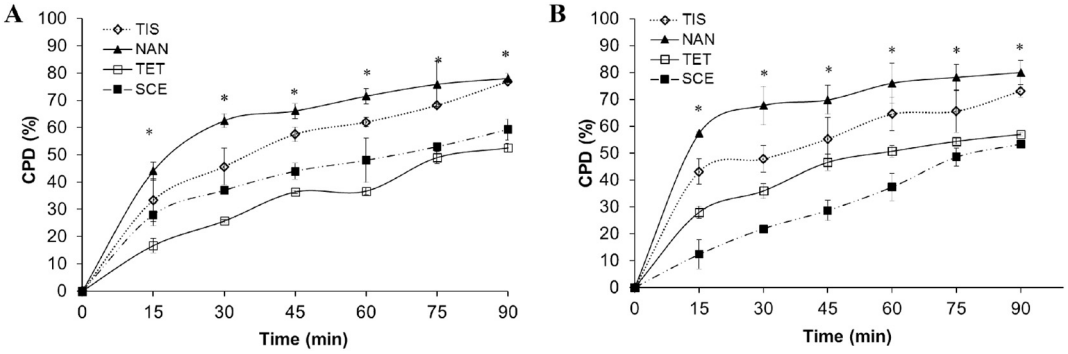

Fig. 4. Changes in coefficient of protein degradation (CPD) during *in vitro* protein hydrolysis of microalgae by *S. aurata* (A) and *S. senegalensis* (B) intestinal proteases. Asterisks denote statistically significant differences among microalgae for each sampling time ($p < 0.05$). Data are mean of triplicate determinations (n = 3). *Tisochrysis lutea* (TIS), *Nannochloropsis gaditana* (NAN), *Tetraselmis suecica* (TET), and *Scenedesmus almeriensis* (SCE).

TIS and NAN, a fact that revealed high proteolysis by *S. aurata* and *S. senegalensis* proteases. To our knowledge, there are no studies reporting the *in vitro* digestibility of *T. lutea* by digestive enzymes of farmed fish. The high protein hydrolysis observed in this microalga may be related to the fact that *T. lutea* has no distinct cell wall, and consequently, it could be expected that cells were easily hydrolyzed [40]. Regarding NAN, the high protein hydrolysis observed is in agreement with previous results reported by Tibetts et al. [12] for other *Nannochloropsis* species (*N. granulata*). In general, the observed CPD values with *S. aurata* and *S. senegalensis* proteases suggest a reasonably high bioavailability of microalgae proteins. In fact, similar values have been described for other raw materials commonly used in aquafeeds, such as soybean protein concentrate or fishmeal [1,41,42]. In the case of TET and SCE, CPD values were around 50%, which reflected that protein of these microalgae was less susceptible to the action of fish proteases under the simulated conditions used in the *in vitro* assay. In spite of this fact, TET and SCE values were higher than those reported for some plant protein sources (< 40% [43]).

The existence of microalgae showing different protein hydrolysis rates might be of practical interest in fish nutrition. One the one hand, highly hydrolysable proteins could release bioavailable amino acids easily and quickly, which might possibly act as stimulator of both the digestion and metabolism. On the other, intermediate hydrolysable proteins could end up in lower release rate of amino acids in the intestine, a fact that might prevent the saturation of amino acid membrane carriers in the microvilli of enterocytes. It should be born in mind that absorption efficiency of amino acids is influenced by the relative and total concentrations of specific amino acids in the intestine [44].

The amount of free amino acids released from proteins that might be absorbed through the intestinal epithelium is an important aspect to be considered as well [45]. The primary mechanism of intestinal absorption of digested protein is *via* enterocyte transporters, which show high specificity for free amino acids and low molecular weight peptides (di- and tri-peptides). Hence, the quantification of free amino acids released *in vitro* might be useful with the aim of estimating the bioavailability of dietary protein. In the present work, fish digestive proteases were able to release from 9% to 25% of total amino acids contained in microalgae protein after 90 min of *in vitro* hydrolysis. These values were similar to those obtained for fishmeal and plant proteins, such as soybean meal, soybean meal concentrate or pea meal [1,46,47]. The different accessibility of proteolytic enzymes to microalgal protein peptide bonds might explain the quantitative differences observed regarding the

Table 4
Relationship between partial coefficients of protein degradation (% CPD) and the amino acids released from protein (measured as the cumulative value of amino acids released at each sampling time) during the *in vitro* hydrolysis. *Tisochrysis lutea* (TIS), *Nannochloropsis gaditana* (NAN), *Tetraselmis suecica* (TET), and *Scenedesmus almeriensis* (SCE).

	Linear fitting	R^2	*p-value*
Gilthead sea bream			
TIS	y = 2.4× + 20.2	0.804	0.0063
NAN	y = 6.5× + 18.6	0.833	0.0041
TET	y = 3.9× + 2.0	0.942	0.0003
SCE	y = 2.9× + 14.7	0.848	0.0032
Senegalese sole			
TIS	y = 2.8× + 25.2	0.668	0.0247
NAN	y = 3.2× + 29.6	0.632	0.0327
TET	y = 4.8× + 5.3	0.938	0.0003
SCE	y = 3.3× − 4.4	0.832	0.0042

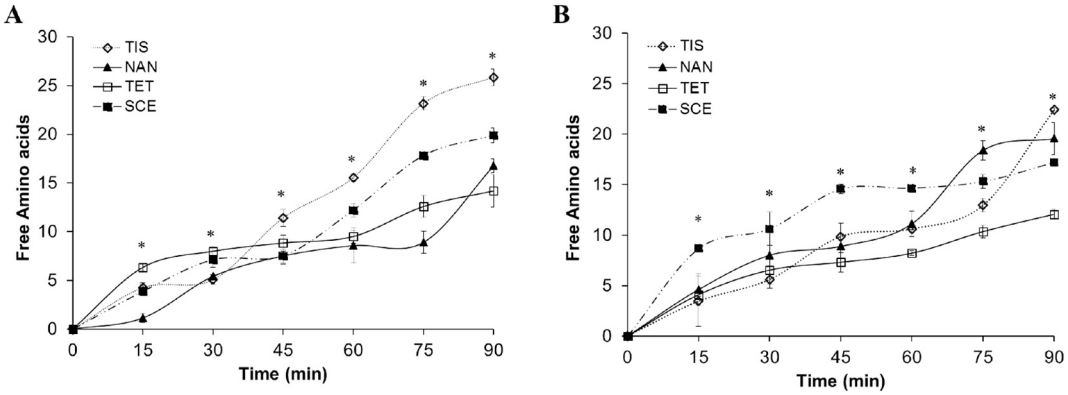

Fig. 5. Concentration of free amino acids released (g 100 g protein^{-1}) during the *in vitro* proteolysis of microalgae by *S. aurata* (A) and *S. senegalensis* (B) intestinal proteases. Asterisks symbolize statistically significant differences among microalgae for each sampling time ($p < 0.05$). *Tisochrysis lutea* (TIS), *Nannochloropsis gaditana* (NAN), *Tetraselmis suecica* (TET), and *Scenedesmus almeriensis* (SCE).

amount of amino acids released and, therefore, this factor could represent a crucial aspect for the bioavailability and further absorption of free amino acids into the enterocytes [48].

Additionally, a linear relationship between CPD and free amino acids released was found for all the microalgae evaluated. This suggests that both parameters are complementary indices that, together, enabled to measure *in vitro* the overall protein hydrolysis of microalgae. The presence of a wide range of enzymes in the digestive extracts of farmed fish species has been reported [19], including both *exo*- and *endo*-proteases. Such variety of digestive enzymes confers fish intestinal extracts a clear advantage in terms of simulation of protein breakdown under *in vitro* conditions, compared to the use of commercial enzymes. CPD values likely reflected the action of endoproteases on microalgal protein, whereas free amino acids are released from protein and small peptides by the action of exoproteases. The values of CPD and release of free amino acid obtained in the present study suggest that microalgal protein can be hydrolysed by digestive proteases of gilthead sea bream and Senegalese sole.

In conclusion, *S. aurata* and *S. senegalensis* proteases hydrolysed similarly the protein of each specific microalga, but the pattern of hydrolysis changed depending on the microalgae species considered, as revealed by differences in the coefficient of protein degradation and the rate of total free amino acids released. The highest hydrolysis values were obtained for *Tisochrysis* and *Nannochloropsis* biomasses, but in the case of *Tetraselmis* and *Scenedesmus* at least two protein fractions remained intact at the end of the *in vitro* assay. These *in vitro* assays represent a valuable preliminary tool aimed at selecting feed ingredients for farmed fish. The present study provides useful species-specific information of microalgae as potential sustainable ingredients for aquafeeds. Further research is needed to improve understanding of the factors that determine microalgae protein bioavailability, as well as to assess the *in vivo* biological performance of fish fed with diets supplemented with these microalgae.

Acknowledgements

This research was funded by the projects SABANA (grant # 727874) from the European Union's Horizon 2020 - Research and Innovation Framework Programme, MICROAQUA-UAL (TRFE-I-2018/001), and CEIJ-C05.1-CEI-MAR.

Author contributions

F.J. Alarcón conceived and designed the experiments; A.J. Vizcaíno and M.I. Sáez carried out the experiments and data acquisition; A.J. Vizcaíno and F.J. Alarcón and T.F. Martínez analysed and interpreted the data; F.J. Alarcón, A.J. Vizcaíno and T.F. Martínez drafted the manuscript; F.G. Acién obtained the funding; all authors critically revised and approved the manuscript.

Conflict of interest

The authors declare no conflict of interests.

Statement of informed consent, human/animal rights

The authors state that no conflicts, informed consent, human or animal rights are applicable.

Declaration of authors' agreement

All authors agree to the authorship and submission of the manuscript to Algal Research for peer review.

References

[1] M.A. Sáenz de Rodrigánez, E. Medina, F.J. Moyano, F.J. Alarcón, Evaluation of protein hydrolysis in raw sources by digestive proteases of Senegalese sole (*Solea senegalensis*, Kaup 1858) using a combination of an *in vitro* assay and sodium dodecyl sulphate polyacrylamide gel electrophoresis analysis of products, Aquac. Res. 42 (2011) 1639–1652.

[2] D.M. Silva, L.M.P. Valente, I. Sousa-Pinto, R. Pereira, M.A. Pires, F. Seixas, P. Rema, Evaluation of IMTA-produced seaweeds (*Gracilaria*, *Porphyra*, and *Ulva*) as dietary ingredients in Nile tilapia, *Oreochromis niloticus L.*, juveniles. Effects on growth performance and gut histology, J. Appl. Phycol. 27.4 (2014) 1671–1680.

[3] R.W. Hardy, Utilization of plant proteins in fish diets: effects of global demand and supplies of fishmeal, Aquac. Res. 41.5 (2010) 770–776.

[4] E. Tibaldi, G. Chini Zittelli, G. Parisi, M. Bruno, G. Giorgi, F. Tulli, S. Venturini, M.R. Tredici, B.M. Poli, Growth performance and quality traits of European sea bass (*D. labrax*) fed diets including increasing levels of freeze-dried *Isochrysis sp*. (T-ISO) biomass as a source of protein and n-3 long chain PUFA in partial substitution of fish derivatives, Aquaculture 440 (2015) 60–68.

[5] C. Minjarez-Osorio, S. Castillo-Alvarado, D.M.I.I.I. Gatlin, M.L. González-Félix, M. Perez-Velazquez, W. Rossi Jr., Plant protein sources in the diets of the sciaenids red drum (*Sciaenops ocellatus*) and shortfin corvina (*Cynoscion parvipinnis*): a comparative study, Aquaculture 453 (2015) 122–129.

[6] C.V. López, M.C. Cerón, F.G. Acién, C. Segovia, Y. Chisti, J.M. Fernández, Protein measurements of microalgal and cyanobacterial biomass, Bioresour. Technol. 101 (2010) 7587–7591.

[7] A.J. Vizcaíno, G. López, M.I. Sáez, J.A. Jiménez, A. Barros, L. Hidalgo, J. Camacho-Rodríguez, T.F. Martínez, M.C. Cerón-García, F.J. Alarcón, Effects of the microalga *Scenedesmus almeriensis* as fishmeal alternative in diets for gilthead sea bream, *Sparus aurata*, juveniles, Aquaculture 431 (2014) 34–43.

[8] S.F. Velasquez, M.A. Chan, R.G. Abisado, R.F.M. Traifalgar, M.M. Tayamen, G.C.F. Maliwat, J.A. Ragaza, Dietary Spirulina (*Arthrospira platensis*) replacement enhances performance of juvenile Nile tilapia (*Oreochromis niloticus*), J. Appl. Phycol. 28.2 (2015) 1023–1030.

[9] E. Teuling, P.A. Wierenga, J.W. Schrama, H. Gruppen, Comparison of protein extracts from various unicellular green sources, J. Agric. Food Chem. 65.36 (2017) 7989–8002.

[10] H.G. Gerken, B. Donohoe, E.P. Knoshaug, Enzymatic cell wall degradation of *Chlorella vulgaris* and other microalgae for biofuels production, Planta 237.1 (2013) 239–253.

[11] R. Lavecchia, G. Maffei, A. Zuorro, Evaluation of dissolved organic carbon (DOC) as a measure of cell wall degradation during enzymatic treatment of microalgae, Chem. Eng. Trans. 49 (2016) 373–378.

[12] S.M. Tibbetts, F. Yasumaru, D. Lemos, *In vitro* prediction of digestible protein content of marine microalgae (*Nannochloropsis granulata*) meals for Pacific white shrimp (*Litopenaeus vannamei*) and rainbow trout (*Oncorhynchus mykiss*), Algal Res. 21 (2017) 76–80.

[13] F.J. Alarcón, F.J. Moyano, M. Díaz, C. Fernández-Díaz, M. Yúfera, Optimization of the protein fraction of microcapsules used in feeding of marine fish larvae using *in vitro* digestibility techniques, Aquac. Nutr. 5 (1999) 107–113.

[14] F.J. Alarcón, F.J. Moyano, M. Díaz, Evaluation of different protein sources for aquafeeds by an optimised pH-stat system, J. Sci. Food Agric. 82 (2002) 697–704.

[15] M.C. Hidalgo, E. Urea, A. Sanz, Comparative study of digestive enzymes in fish with different nutritional habits: proteolytic and amylase activities, Aquaculture 170 (1999) 267–283.

[16] I. Rønnestad, M. Yúfera, B. Ueberschär, L. Ribeiro, Ø. Sæle, C. Boglione, Feeding behaviour and digestive physiology in larval fish: current knowledge, and gaps and bottlenecks in research, Rev. Aquacult. Suppl. 1 (5) (2013) S59–S98.

[17] F.J. Moyano, M.A. Sáenz de Rodrigañez, M. Díaz, A.G.J. Tacon, Application of *in vitro* digestibility methods in aquaculture: constraints and perspectives, Rev. Aquac. 6 (2014) 1–20.

[18] M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.

[19] F.J. Alarcón, M. Díaz, F.J. Moyano, E. Abellán, Characterization and functional properties of digestive proteases in two sparids; gilthead sea bream (*Sparus aurata*) and common dentex (*Dentex dentex*), Fish Physiol. Biochem. 19 (1998) 257–267.

[20] L. Bosch, A. Alegría, R. Farré. Application of the 6-aminoquinolyl-N-hydroxysccinimidyl carbamate (AQC) reagent to the RP-HPLC determination of amino acids in infant foods. J. Chromatogr. B 831,1–2(2006) 176–183.

[21] F.J. Alarcón, F.J. Moyano, M. Díaz, Use of SDS-page in the assessment of protein hydrolysis by fish digestive enzymes, Aquac. Int. 9 (2001) 255–267.

[22] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.

[23] F.G. Plumley, D.I. Kirchman, R.E. Hodson, G.W. Schmidt, Ribulose bisphosphate carboxylase from three chlorophyll *c*-containing algae, physical and immunological characterizations, Plant Physiol. 80 (1984) 685–691.

[24] P. Song, L. Li, J. Liu, Proteomic analysis in nitrogen-deprived *Isochrysis galbana* during lipid accumulation, PLoS ONE 8.12 (2013) e82188, , https://doi.org/10.1371/journal.pone.0082188.

[25] Y.K. Kim, W.I. Yoo, S.H. Lee, M.Y. Lee, Proteomic analysis of cadmium-induced protein profile alterations from marine alga *Nannochloropsis oculata*, Ecotoxicology 14 (2005) 589–596.

[26] S. Basso, D. Simionato, C. Gerotto, A. Segalla, G.M. Giacometti, T. Morosinotto, Characterization of the photosynthetic apparatus of the Eustigmatophycean *Nannochloropsis gaditana*: evidence of convergent evolution in the supramolecular

organization of photosystem I, Biochim. Biophys. Acta 1837 (2014) 306–314.
[27] A. Schwenzfeier, P.A. Wierenga, H. Gruppen, Isolation and characterization of soluble protein from the green microalgae *Tetraselmis sp*, Bioresour. Technol. 102 (2011) 9121–9127.
[28] J. Schnackenberg, R. Schulz, H. Senger, Characterization and purification of a hydrogenase from the eukaryotic green alga *Scenedesmus obliquus*, FEBS Lett. 327 (1993) 21–24.
[29] F.C. Church, H.E. Swaisgood, D.H. Porter, G. Catignani, Spectrophotometric assay using o-phthaldehyde for determination of proteolysis in milk proteins, J. Dairy Sci. 66 (1983) 1219–1227.
[30] N.F. Haard, L.E. Dimes, R.E. Arndt, F.M. Dong, Estimation of protein digestibility–IV. Digestive proteinases from the pyloric caeca of coho salmon (*Oncorhynchus kisutch*) fed diets containing soybean meal, Comp. Biochem. Physiol. B 115 (1996) 533–540.
[31] S.M. Tibbetts, J.E. Milley, S.P. Lall, Chemical composition and nutritional properties of freshwater and marine microalgal biomass cultured in photobioreactors, J. Appl. Phycol. 27 (3) (2015) 1109–1119.
[32] S.M. Tibbetts, T. MacPherson, P.J. McGinn, A.H. Fredeen, *In vitro* digestion of microalgal biomass from freshwater species isolated in Alberta, Canada for monogastric and ruminant animal feed applications, Algal Res. 19 (2016) 324–332.
[33] A.A. Sadeghi, A. Nikkhah, P. Shawrang, M.M. Shahrebabak, Protein degradation kinetics of untreated and treated soybean meal using SDS-PAGE, Anim. Feed Sci. Technol. 126 (2006) 121–133.
[34] L.H. Jonge, J.W. Speka, H. van Laarc, J. Dijkstra, Effects of pH, temperature and osmolality on the level and composition of soluble N in feedstufs for ruminants, Anim. Feed Sci. Technol. 153 (2009) 249–262.
[35] M. González-Zamorano, M.A. Navarrete Del Toro, F.L. García-Carreño, Exogenous proteinases as feed supplement for shrimp: *in vitro* evaluation, Aquac. Nutr. 19 (2013) 731–740.
[36] C. Safi, M. Charton, O. Pignolet, F. Silvestre, C. Vaca-Garcia, P.Y. Pontalier, Influence of microalgae cell wall characteristics on protein extractability and determination of nitrogen-to-protein conversion factors, J. Appl. Phycol. 25 (2013) 523–529.
[37] H. Takeda, Cell wall sugars of some *Scenedesmus* species, Phytochemistry 42 (1996) 673–675.
[38] R. Kumar, V. Choudhary, S. Mishra, I.K. Varma, B. Mattiason, Adhesives and plastics based on soy protein products, Ind. Crop. Prod. 16 (2002) 155–172.
[39] K. Borgen, Evaluation of Physicochemical Properties of Modified Algae Protein Adhesives (M.Sc. Thesis), Kansas State University, Manhattan, Kansas, 2012, p. 42.
[40] C.J. Zhu, Y.K. Lee, T.M. Chao, Effects of temperature and growth phase on lipid and biochemical composition of *Isochrysis galbana* TK1, J. Appl. Phycol. 9 (1993) 451–457.
[41] Z. Sultana, Md.S. Ahmed, Md.S. Iqball, Md.A. Hasan Chisty, Determination of in vitro protein digestibility of different feed ingredients for nilotica (*Oreochromis nilotica*), Bangladesh Res. Pub. J. 4.1 (2010) 87–94.
[42] C. Hernández, R.W. Hardy, D.G. Márquez-Martínez, P.V. Domínguez-Jimenez, B. González-Rodriguez, Evaluation of apparent digestibility coefficients of individual feed ingredients in spotted rose snapper *Lutjanus guttatus* (Steindachner, 1869), Aquac. Res. 21.6 (2015) 835–842.
[43] A.S.C. Chong, R. Hashim, A.B. Ali, Assessment of dry matter and protein digestibilities of selected raw ingredients by discus fish (*Symphysodon aequifasciata*) using *in vivo* and *in vitro* methods, Aquacult. Nut. 8 (2002) 229–238.
[44] B.H. Hirst, Dietary regulation of intestinal nutrient carriers, Proc. Nutr. Soc. 52 (02) (1993) 315–324.
[45] T. Verri, A. Romano, A. Barca, G. Kottra, H. Daniel, C. Storelli, Transport of di- and tripeptides in teleost fish intestine, Aquac. Res. 41 (2010) 641–653.
[46] G.A. Morales, F.J. Moyano, Application of an *in vitro* gastrointestinal model to evaluate nitrogen and phosphorus bioaccessibility and bioavailability in fish feed ingredients, Aquaculture 306 (2010) 244–251.
[47] G.A. Morales, M. Sáenz de Rodrigáñez, L. Marquez, M. Díaz, F.J. Moyano, Solubilisation of protein fractions induced by *Escherichia coli* phytase and its effects on *in vitro* fish digestion of plant proteins, Anim. Feed Sci. Technol. 181 (2013) 54–64.
[48] A.I. Román-Gavilanes, E. Martínez-Montaño, M.T. Viana, Comparative characterization of enzymatic digestion from fish and soybean meal from simulated digestive process of Pacific bluefin tuna, *Thunnus orientalis*, J. World Aquacult. Soc. 46.4 (2015) 409–420.