Effect of dietary protein level and source on digestive proteolytic enzyme activity in juvenile Senegalese sole, Solea senegalensis *Kaup 1850*

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Abstract The effect of dietary protein level and protein source on growth and proteolytic activity of juvenile *Solea senegalensis* was studied. In Experiment 1, fish were fed on four experimental diets containing increased protein levels (36, 46, 56 and 67%). In Experiment 2, Senegalese soles were fed on five diets with partial substitution of fish meal by soybean meal, soybean protein concentrate, soybean protein isolate, wheat gluten meal or pea protein concentrate. Results prove that growth and proteolytic activity in the distal intestine of fish were affected by the quantitative increase in dietary protein. The origin of protein source used in the elaboration of experimental diets affected both the amount and composition of the alkaline proteases secreted into the intestinal lumen; however, it did not decrease animal growth. Juvenile Senegalese sole showed capability to modulate digestive protease secretion when the concentration and/or source of dietary protein were modified. Quantity and quality of dietary protein affected protein hydrolysis in Senegalese sole intestine. This study establishes that 30% fish meal protein can be replaced by soybean derivatives without affecting intestinal proteases. Replacement with wheat gluten meal or pea protein concentrate should be taken cautiously, but further research is needed to establish whether growth performance and digestive enzyme physiology of Senegalese sole are affected by plant protein-supplemented diets in a long-term trial.

Keywords Dietary protein · Digestive proteases · Plant protein sources · Protease inhibitor · Replacement · Senegalese sole · Zymogram

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Abbreviations

C	Control diet
CKK	Cholecystokinin
CL	Crude lipid
CP	Crude protein
DI	Distal intestine
FBW	Final body weight
FM	Fish meal
IBW	Initial body weight
PI	Proximal intestine
PPC	Pea protein concentrate
RIL	Relative intestinal length
SBM	Soybean meal
SGR	Specific growth rate
SPC	Soybean protein concentrate
SPI	Soybean protein isolate
TPA	Total protease activity
VSI	Viscerosomatic index
WGM	Wheat gluten meal

Introduction

Senegalese sole (*Solea senegalensis*, Kaup) is a promising flatfish species for intensive aquaculture in Southern European countries (Dinis et al. 1999; Imsland et al. 2003). However, the development of their commercial farming needs, such as (1) improved egg and larval quality, (2) to develop vaccines and therapies to reduce disease incidence and (3) improved formulation of feeds for their use in open and recirculation systems (Howell et al. 2009). Focusing on the nutrition and feeding of Senegalese sole, which seem a particularly critical key issue, several significant advances on nutritional requirements have been reported (Aragão et al. 2003; Dias et al. 2004; Rema et al. 2008; Rubio et al. 2009). Other recent findings on juvenile *S. senegalensis* proved their high ability to use plant proteins and so offer good prospects for high levels of fish meal replacement (Silva et al. 2010; Dias et al. 2010). In these in vivo trials, fish were fed on diets with different protein levels or including plant protein sources, they mainly focus on obtaining zootechnical indexes (Rema et al. 2008; Silva et al. 2010), though Dias et al. (2010) also studied the apparent digestibility of several plant protein sources in this species. However, no information regarding the influence of these experimental diets on the digestive physiology of this species has been published so far.

The replacement of fish meal by a variety of vegetable protein sources has been a trend for some years, and their successful use in the genus *Solea* provides good prospects for fish meal replacement in these flatfish species (Aragão et al. 2003; Silva et al. 2010; Bonaldo et al. 2006; Gatta et al. 2010). The presence of secondary plant compounds in aquafeeds can affect protein digestion, causing adverse physiological effects and reducing fish growth performance (Olli et al. 1994). Fish species differ in their sensitivity and response to protease inhibitors contained in plant protein sources (Garcia-Carreño et al. 1993; Moyano et al. 1999; Francis et al. 2001; Alarcón et al. 2002; Chong et al. 2002; Gatlin et al. 2007).

Getting to know the effects of these antinutritive factors on the digestive physiology of Senegalese sole may have important implications on the formulation of specific aquafeeds for this flatfish.

As the ability of fish to use the ingested nutrients depends on the presence of an adequate set of digestive enzymes, general knowledge on nutrient hydrolysis in the digestive tract is a key factor to optimize fish feeding procedures (Suárez et al. 1995). It is well accepted that the digestive enzyme profile can reflect the digestive capacity of a given fish (Smith 1980). Moreover, fish are capable of modulating their digestive enzyme pattern in response to the source, quality and concentration of dietary nutrients (Debnath et al. 2007; Santigosa et al. 2008; González-Félix et al. 2010). These responses are the basis of the metabolic adaptation of fish to dietary changes, and knowing the mechanisms involved in this process can provide useful information to improve fish growth performance and health status (Eusebio and Coloso 2002).

In the case of *S. senegalensis*, the existence of a possible modulation of digestive enzymes that could maintain or improve their growth in response to quantitative or qualitative dietary protein changes has not been studied. Because dietary protein is recognized as a key factor influencing both adequate fish nutrition and feeding costs (Watanabe 2002), and considering the lack of specific knowledge about Senegalese sole digestive physiology, the objectives of the present work were studying the effect of (1) dietary crude protein level, and (2) dietary protein source on growth performance and digestive proteolytic activity of this flatfish species.

Materials and methods

Experimental animals and feeding experiments

For Experiment 1, Senegalese sole juveniles were obtained from a reproductive stock at the IFAPA "Agua del Pino" (Cartaya, Huelva, Spain); 516 fish (mean body weight of 22.3 ± 2.5 g) were selected and randomly distributed (43 fish tank^{-1}) in 12-gray rectangular PVC tanks (77 × 56 × 22 cm deep; volume: 80 L). Four isolipidic (120 g kg^{-1} crude lipid, CL) and isocaloric (16.3 MJ kg^{-1}) diets were formulated with different crude protein (CP) levels: 358 (diet 35), 463 (diet 46), 562 (diet 56) and 672 (diet 67) g kg^{-1} CP (Tables 1, 2). Each dietary treatment was randomly assigned to triplicate tanks.

For Experiment 2, Senegalese sole juveniles were obtained from a commercial farm (STOLT SEA FARM S.A. Vigo, Spain). After 15 days of acclimation at the IFAPA "Agua del Pino" facilities, 192 fish (mean corporal weight of 21.5 ± 2.8 g) were selected and 16 fish per tank were randomly distributed in 12-gray rectangular PVC tanks (77 × 56 × 22 cm deep; volume: 80 L). Moreover, sole were individually marked with tattoo ink using an insulin syringe. Five isoproteic (550 g kg^{-1} CP), isolipidic (120 g kg^{-1} CL) and isocaloric (19.7 MJ kg^{-1}) diets were formulated replacing 30% of fish meal protein by soybean meal (SBM), soybean protein concentrate (SPC), soybean protein isolate (SPI), wheat gluten meal (WGM) or pea protein concentrate (PPC). A 100% fish meal (FM)-based diet was used as a control feed (C) (Tables 1, 2). Each dietary treatment was randomly assigned to two replicate tanks.

Feeding trials were conducted during 10 (Experiment 1) or 11 weeks (Experiment 2) in an indoor recirculating marine water system located at the IFAPA "Agua del Pino" (Cartaya, Huelva), with 0.48 L min^{-1} of water flow, constant temperature (21.0 ± 1.1°C)

Table 1 Formulation (average of three replicates) of experimental diets

Ingredient (% DM)	Experiment 1: protein level (% CP)				Experiment 2: protein source					
	36	46	56	67	C	SBM	SPC	SPI	WGM	PPC
Herring meal[a]	31.7	43.3	54.8	64.5	58.9	40.5	40.5	40.5	40.5	40.5
SBM[b]	–	–	–	–	–	29.0	–	–	–	–
SPC[c]	–	–	–	–	–	–	20.0	–	–	–
SPI[d]	–	–	–	–	–	–	–	16.1	–	–
WGM[e]	–	–	–	–	–	–	–	–	17.3	–
PPC[f]	–	–	–	–	–	–	–	–	–	28.0
Casein[g]	7.2	7.2	7.2	7.2	5.0	5.0	5.0	5.0	5.0	5.0
Mollusk meal	2.0	2.0	2.0	2.0	5.0	5.0	5.0	5.0	5.0	5.0
FM hydrolysate[h]	2.5	2.5	2.5	2.5	5.0	5.0	5.0	5.0	5.0	5.0
Fish oil[i]	4.6	2.9	2.8	1.9	2.3	3.6	4.2	3.5	3.9	3.1
Lecithin-HUFA mixture	3.9	3.9	3.9	3.9	3.0	3.0	3.0	3.0	3.0	3.0
Others	43.3	33.4	22.0	13.2	15.3	3.4	11.8	16.4	14.8	4.9
Premix[j]	4.8	4.8	4.8	4.8	5.5	5.5	5.5	5.5	5.5	5.5

[a] Scotia Garden Seafood Incorporated (Yarmouth, NS, Canada)

[b] Bunge Canada (Oakville, ON, Canada)

[c] Soycomil® and Pro-Fam®, respectively; Archer Daniels Midland (Decatur, IL, USA)

[d] Roquette UK Limited (Northants, UK)

[e] Parrheim Foods (Portage La Prairie, MB, Canada)

[f] Serva, Feinbiochemica (Heidelberg, Germany)

[g] US Biochemical, Cleveland, OH, USA

[h] Concentre proteique soluble de poisson, Sopropêche, France

[i] Stabilized with 0.06% ethoxyquin, Comeau Seafood, Saulnierville, NS, Canada

[j] PREMIX: Tibbetts et al. (2004)

and salinity (3.5 ± 1%). Oxygen was kept at 7.0 ± 0.4 mg L^{-1} with supplemental aeration. Ammonia, nitrite and nitrate were determined spectrophotometrically weekly. Feed was offered twice at day (09:00 and 15:00 h), and the feeding rate was adjusted every 2 weeks at 3% of the tank biomass. Feed losses were controlled all over the trials and were minimal. However, as mentioned by Dias et al. (2004), when necessary, remaining feed was estimated and deducted from feed intake for overall calculations. Experiments were carried out in compliance with the Guidelines of the European Union Council (86/609/EU) and the Spanish Government (RD1201/2005) for the use of animals in research under the supervision of trained scientists (category C).

Zootechnical indices

Specific growth rate (SGR) was calculated as $SGR = 100 \times (\ln W_{fin} - \ln W_{in})/t$, where W_{fin} and W_{in} are final and initial weights, respectively, and t is trial duration in days. Viscerosomatic index was calculated as VSI = (viscera weight/body weight) × 100. Relative intestinal length (RIL) as intestinal length (cm)/total length (cm).

Table 2 Chemical composition of experimental diets

Proximate composition (% DM)	Experiment 1: protein level (% CP)				Experiment 2: protein source					
	36	46	56	67	C	SBM	SPC	SPI	WGM	PPC
Dry matter	92.3	91.6	91.4	91.0	95.5	94.3	94.7	93.5	92.6	93.8
CP	35.8	46.3	56.2	67.2	54.9	55.6	55.7	54.9	55.8	55.5
CL	11.3	12.1	11.7	12.8	10.5	11.0	10.3	10.6	12.4	12.0
Ash	8.6	9.7	9.5	12.5	11.3	10.4	9.0	8.9	8.3	9.4
Carbohydrates[a]	44.3	31.9	22.6	7.5	23.3	23.0	25.0	25.6	23.5	23.1
Gross energy (MJ kg^{-1})	16.7	16.4	16.2	15.9	19.4	19.5	19.8	19.8	20.3	19.5
P/E ratio (g MJ^{-1})	21.4	28.2	34.7	42.3	28.3	28.5	28.1	27.7	27.5	28.5

Composition of raw materials and diets was analyzed following AOAC (1990) procedures: dry matter (105°C to constant weight), ash (incinerated at 550°C to constant weight) and CP ($N \times 6.25$) by the Kjeldahl method after an acid digestion (Kjeltec 2300 Auto Analyser, Tecator Höganas, Sweden), CL extracted with diethyl ether (Soxtec 1043 extraction unit, Tecator) and crude fiber by acid and basic digestion (Fibertec System M., 1020 Hot Estractor, Tecator). Energy by direct combustion in an adiabatic bomb calorimeter (Parr Model 1108 oxygen combustion bomb, IL, USA)

[a] Carbohydrates = 100 − (CP + CL + ash)

Sampling

At the end of the each experimental trial, 30 18-h fasted fish per treatment (Experiment 1) were randomly selected and killed after 2-phenoxyethanol overdose according to the requirements of Council Directive 86/609/EEC. In Experiment 2, samplings were performed 1, 3 and 6 h after manual feeding to obtain digestive tracts; three animals per diet and time were randomly selected. All fish were dissected from the esophagus to the anal area. Stomach, proximal intestine (PI) and distal intestine (DI) extracts were obtained by manual homogenization of individual samples in distilled water (1:2). Supernatants were obtained after centrifugation (12,000 rpm, 4°C, 15 min) and stored at −20°C for further enzymatic analysis.

Analysis of digestive protease activities

Total acid and alkaline protease activities in digestive extracts were measured following the procedures described by Alarcón et al. (1998). In brief, acid protease activity was determined using 5 g L^{-1} hemoglobin in 0.1 M glycine–HCl (pH 2.0), whereas alkaline protease activity was measured using 5 g L^{-1} azocasein in 50 mM Tris–HCl (pH 9.0). One milliliter of substrate was added, and enzymatic reaction was carried out for 30 min at 37°C. Reaction was stopped by incorporating 0.5 mL of TCA (20%) and then cooled for 15 min at −20°C. Samples were centrifuged, and supernatant absorbance was recorded at 280 or 366 nm (acid or alkaline activity, respectively) in a spectrophotometer (Shimadzu UV-1800, Shimadzu, Kyoto, Japan). Blanks were established by adding TCA before enzyme extract. All measures were determined in triplicate. One unit of enzyme activity (U) was defined as 1 μg of tyrosine released per minute (tyrosine extinction coefficient: 0.005 mL μg^{-1} cm^{-1}). Activity was referred to as U per mg soluble protein.

Specific trypsin- and chymotrypsin-like activities were analyzed with 0.5 mM BAPNA (Nα-benzoyl-DL-arginine 4-nitroanilidehydrochloride) and 0.2 mM SAPNA

(*N*-succinyl-Ala–Ala-Pro_phe *p*-nitroanilide) substrates, respectively, in 50 mM Tris–HCl (pH 9.0), using 96-well plates. The slope until saturation curve was recorded at 410 nm in a Tecan Sunrise spectrophotometer (Tecan, Salzburg, Austria) using Magellan V.1.12 software package (Tecan, Salzburg, Austria).

Electrophoresis analysis

To visualize the active fractions present in proximal intestinal extracts of fish fed on different amounts of dietary protein (Experiment 1) or different plant protein sources (Experiment 2), substrate–SDS–PAGE electrophoresis gels were used. Intestinal enzyme extracts were mixed with SDS sample buffer (1:1), and SDS–PAGE was performed according to Laemmli (1970) using 12% polyacrylamide (100 V per gel, 45 min, 4°C). Zymograms revealing protease active bands were made according to Garcia-Carreño et al. (1993) as modified by Alarcón et al. (1998). Thus, after electrophoresis, gels were washed and incubated in 5 g L^{-1} casein at 4°C. After 30 min, a new fresh casein solution was used and temperature was increased (37°C for 90 min). Gels were stained in methanol:acetic acid:water (40:10:50) containing 1 g L^{-1} Coomassie brilliant blue and then washed with the same solution containing no colorant. Gels images were scanned using a Universal Hood II (Bio-Rad, Segarte, Italy). Clear gel zones revealed the presence of active proteases.

Testing the protease inhibitory activity in diets of Experiment 2

Two different approaches were used to analyze the presence of potential protease inhibitors in the diets containing plant proteins: (1) the quantification of inhibition on intestinal alkaline protease activity and (2) the identification of protease inhibitory activity in zymograms.

For the preparation of inhibitory extracts, diets from Experiment 2 (C, SBM, SPC, SPC, WGM and PPC) were grounded and powder was extracted with five volumes of distilled water by shaking for 120 min at room temperature and for 22 h at 4°C. The mixture was centrifuged for 20 min at 12,000×*g* and 4°C. The supernatant was stored at 4°C until use in inhibition assays.

The potential inhibitory effect of diets on Senegalese sole alkaline proteases was evaluated according to Alarcón et al. (2001). In brief, 10 µL of diet extracts was incubated with 10 µL of a standardized sole enzymatic extract (from fish fed control diet and providing 10 U) in 0.5 mL of 50 mM Tris–HCl pH 9.0 for 1 h at 25°C. Residual protease activity was then evaluated by adding 0.5 mL of 5 g L^{-1} azocasein. A control was prepared by adding distilled water instead of aqueous diet extracts. Enzyme inhibition was assessed as a percentage of residual activity by comparison with a control assay (absence of diet extracts).

For the identification of protease inhibitory activity in zymograms, 7 µL of intestinal extract (containing 7 U) was mixed with 7 µL of the different diet extracts, being incubated for 1 h at 25°C under continuous stirring. An additional sample (E) was prepared by incubating the standardized enzymatic extract with distilled water. The mixtures were then mixed with sample buffer (1:1) containing SDS but no mercaptoethanol, and 3.5 µL of the final mixture was loaded in each cell of SDS–PAGE gels. After electrophoresis, gels were revealed as detailed above. The patterns of active fractions were compared between samples incubated or not in the presence of aqueous diet extracts.

Statistical analysis

Data are presented as mean ± standard deviation (SD) of at least triplicate determinations. Normality and homogeneity analysis were performed with Kolmogorov–Smirnov and Levene tests, respectively. One-way ANOVA was used to test differences among dietary treatments. In Experiment 2, the proteolytic activity was statistically analyzed by a two-way ANOVA test using all possible combinations between the 3 main effects: protein source, postprandial time and intestine segment. When appropriate, differences among means at $P < 0.05$ were analyzed using the Tukey's or Kruskal–Wallis tests. All statistical tests were performed using the Statgraphic Plus V.4 software package (Rockville, ML, USA).

Results

Experiment 1: protein level

After 10 weeks of feeding, Senegalese sole showed high survival (99%). SGR, viscero-somatic index (VSI) and RIL were not influenced by the level of dietary CP ($P = 0.0699$). Final body weight was significantly high in fish fed on diets containing 46 and 56% of CP (Table 3).

Total acid and alkaline proteolytic activities measured in Senegalese sole digestive extracts are shown in Fig. 1. Results show that mean values of acid protease activity progressively increase when dietary protein content raises up to 46% CP, but no significant differences among dietary treatments were found. Total alkaline protease activity was separately measured in PI and DI (Fig. 1b, c). Results obtained reflect the existence of an inverse linear relationship between total alkaline protease activity and dietary protein content (% CP) ($P = 0.029$) (total protease activity, TPA $= -0.11 \times$ % CP $+ 12.07$; correlation coefficient $= -0.375$), this correlation being significant for DI extracts ($P = 0.049$) (for DI; TPA $= -0.07 \times$ % CP $+ 6.29$; correlation coefficient: -0.503). Fish fed on 67% CP diet showed the lowest level of total proteolytic activity in both PI and DI segments, being significant only for DI when compared to the protease activity level of fish fed on 36% CP diet (Fig. 1c).

Table 3 Growth performance and zootechnical parameters of Senegalese sole fed on experimental diets

Experiment	Diets	IBW (g)	FBW (g)	SGR	VSI	RIL
1. Protein level (% CP)	36	22.1 ± 2.6	37.4 ± 8.0[a]	0.7 ± 0.1	7.4 ± 4.3	2.5 ± 0.6
	46	22.0 ± 2.9	44.1 ± 5.5[b]	1.0 ± 0.1	6.3 ± 4.4	2.4 ± 0.5
	56	22.3 ± 2.7	47.0 ± 6.4[b]	1.0 ± 0.2	6.7 ± 4.3	2.5 ± 0.3
	67	22.6 ± 2.7	37.7 ± 8.4[a]	0.7 ± 0.1	6.5 ± 5.0	2.3 ± 0.5
2. Protein source	C	21.7 ± 2.7	51.6 ± 14.2	1.1 ± 0.3	6.8 ± 1.5	2.5 ± 0.2
	SBM	21.6 ± 2.7	54.6 ± 11.7	1.2 ± 0.3	6.2 ± 1.8	2.5 ± 0.3
	SPC	21.5 ± 2.3	49.2 ± 11.7	1.1 ± 0.3	5.4 ± 3.4	2.5 ± 0.3
	SPI	21.9 ± 3.2	50.3 ± 11.4	1.1 ± 0.2	6.7 ± 2.0	2.4 ± 0.2
	WGM	21.0 ± 3.0	46.6 ± 10.8	1.1 ± 0.3	6.5 ± 1.5	2.3 ± 0.3
	PPC	21.3 ± 2.9	49.0 ± 12.6	1.1 ± 0.3	6.8 ± 2.4	2.4 ± 0.3

Values are mean ± SD. Within a column, means with different superscripts differ significantly ($P < 0.05$)

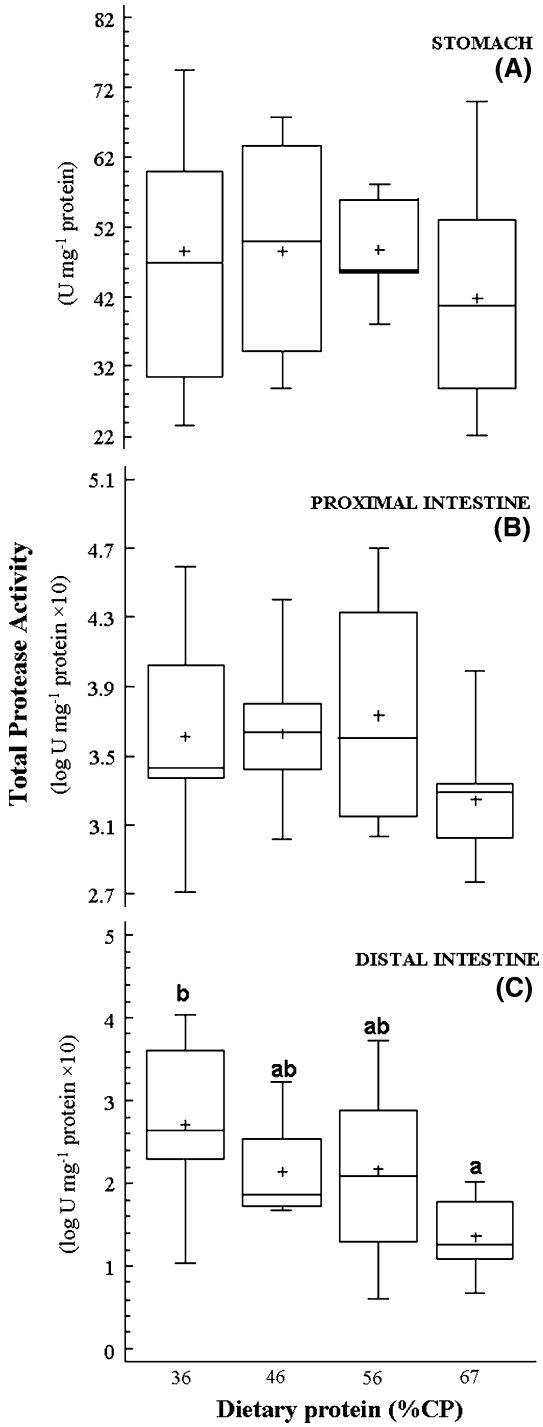

Fig. 1 *Box*-and-*whisker plot* of the values of proteolytic activity in stomach, PI and DI extracts of juvenile Senegalese sole fed on diets with different dietary protein level (Experiment 1). The *central box* covers the middle 50% of the data, and the *whiskers* extend out to the *lower* and *upper values*. The mean is represented as a *cross* and the *horizontal line drawn* through the *box* is the median. *Different letters* denotes statistically different medians with $P < 0.05$

In the zymograms of PI extracts, three groups of active bands (77, 40 and 27 kDa) with proteolytic activity were detected (Fig. 2). The use of specific protease inhibitors allowed the identification of the group of highest molecular mass (77 kDa), as trypsin-like enzymes, while the two groups with lowest molecular mass (40 and 27 kDa) showed chymotrypsin-like activity (data not shown).

Zymography analysis shows the pattern of intestinal proteases of juvenile Senegalese sole fed on diets with different levels of dietary protein (Fig. 2a). The profile of proteases found in PI extracts seems not to be influenced by dietary protein concentration, as all fish showed the same number and distribution of active fractions (Fig. 2a).

Experiment 2: protein source

During the assay, fish consumed well the six experimental diets and the recorded survival was over 95%. After 11 weeks of trial, fish doubled their initial corporal weight and no significant differences were found in FBW, SGR, RIL or VSI among treatments (Table 3).

A previous in vitro assay of inhibition was carried out to assess whether experimental diets contain protease inhibitors. Inhibition results on the activity of alkaline proteases after incubation with the aqueous solution of diets are shown in Fig. 3. Inhibitory activity was tested by (1) spectrophotometric quantification of residual activity (Fig. 3a) and (2) visualization of the inhibitory activity through zymograms (Fig. 3b). The highest mean inhibition values were obtained for the PPC diet, which reduces 52% of alkaline protease activity in a standardized sole extract (Fig. 3a). SPI and WGM only inhibit 20% of the proteolytic activity. By contrast, no inhibition was found after incubation of intestinal extracts with C, SBM and SPC aqueous extracts. Zymograms confirmed the above-mentioned results and revealed the effect of inhibitors on specific active fractions of Senegalese sole (Fig. 3b). The effect of protease inhibitors on digestive proteases was visualized by either a partial or total disappearance of one or more active bands, or by the formation of

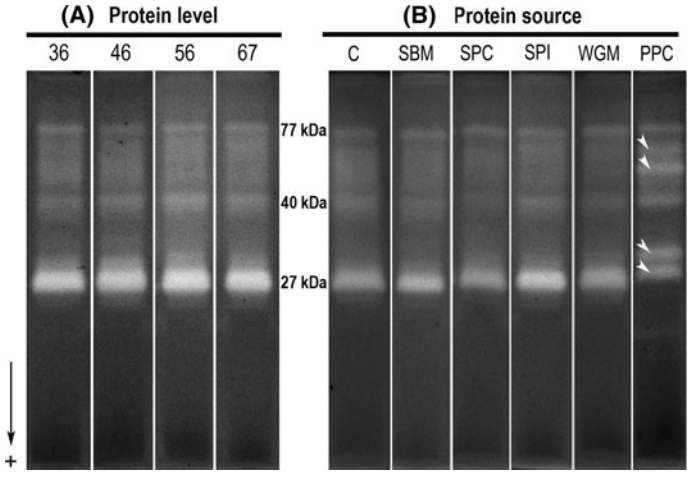

Fig. 2 Zymograms of alkaline proteolytic activity in PI extracts of Senegalese sole fed on diets with increasing protein levels (**a**) and on diet with 30% of FM replacement by different plant protein sources (**b**). All *lanes* in zymograms contain the same activity (2 units of activity per well). *White arrows* in *lane* PPC denote reversible protease–inhibitor complexes of high molecular weight

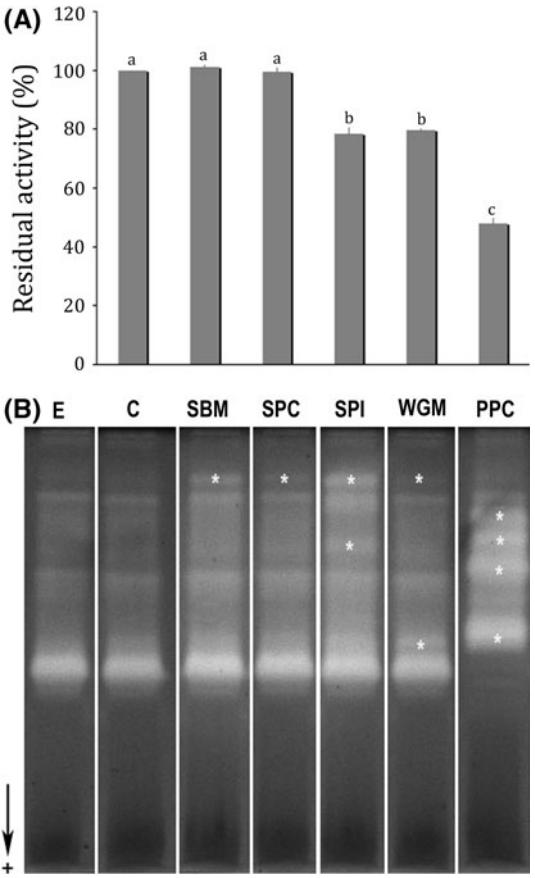

Fig. 3 Inhibition of intestinal proteases (**a**) and substrate–SDS–PAGE (**b**) obtained after incubation of Senegalese sole enzyme extract with the aqueous solution of diets from the Experiment 2. **a** The enzyme extract activity was adjusted in order to produce an increase of absorbance at 366 nm in TCA-soluble fractions of 0.5 in 30 min at 37°C. The amount of diet extract employed in the in vitro inhibitory assay was equivalent to 2 mg of feed. Results are given as residual activity (%) relative to that of a control assay made by using distilled water instead of the diet extracts. Data are mean ± SD ($n = 3$) and *different superscript letters* being significantly different ($P < 0.05$). **b** SDS–PAGE zymograms obtained after incubation of digestive extracts with aqueous solutions of diets. *Lane* E enzyme extract with distilled water. *Lanes* C, SBM, SPC, SPI, WGM and PPC are enzyme extract (E) incubated with aqueous extracts of control, SBM, SPC, SPI, WGM and PPC diets, respectively. All *lanes* in zymograms contain the same activity (2 units of activity per well). *White asterisks* denote reversible protease–inhibitor complexes

enzyme–inhibitor complexes when compared to the profile of a standardized enzyme extract pre-incubated with distilled water (Fig. 3b, lane E). PPC clearly inhibited all the active fractions of Senegalese sole intestine extracts and produced a characteristic pattern of inhibition showing four different reversible protease–inhibitor complexes (lane PPC, white asterisks). Diet based on soybean derivatives (SBM, SPC and SPI) did not affect the different active fraction of sole extract, but the appearance of two slightly active protease–inhibitor complexes, specially in the case of SPI, shows the residual presence of at least two protease inhibitors. The profile of inhibition obtained for WGM was quite close to that found for enzyme control or the control diet (lanes E and C, respectively). However, two

slight enzyme–inhibitor complexes revealed that this diet contains residual levels of protease inhibitors.

Total protease, trypsin and chymotrypsin activities were measured in PI and DI 1, 3 and 6 h after feeding (Fig. 4). Mean values generally increased after feeding, with the exception of fish fed on SPC diet, where proteolytic activities in the PI segment decreased along post-feeding time. Fish fed on the PPC diet showed lower marginal proteolytic activities at all post-feeding times than fish fed on the other experimental diets, but with no significant difference. Regarding the PI segment, the chymotrypsin activity of fish fed on SBM diet showed the highest 1/6 h ratio. The highest 1/6 h total protease, trypsin and chymotrypsin ratios were found in the DI of fish fed on SPC diet.

Proteolytic activities were significantly affected by the experimental diet, as well as by the post-feeding time, but not by the intestinal segment (Table 4). However, a significant interaction of postprandial time and intestinal segment on intestinal proteolytic activities was found ($P < 0.05$). Two-way ANOVA analysis showed that mean values of proteolytic activities were significantly lower in fish fed on PPC diet than in fish fed on other plant-supplemented diets ($P < 0.05$). An inverse tendency was observed for the trypsin/chymotrypsin ratio. Thus, this ratio significantly decreased along postprandial time, though the values of intestinal proteolytic activities showed the opposite trend ($P < 0.05$). Higher values of acid protease activity and acid protease/alkaline protease and trypsin/chymotrypsin ratios were found for PPC than those estimated in fish fed on other diets (Table 4).

Figure 2b shows representative zymograms of PI homogenates corresponding to 3 h post-feeding. Results confirm that the alkaline protease profile secreted into the intestinal lumen is modified by the experimental diets. Intestinal proteases were clearly affected in case of fish fed on PPC diet. PPC protease inhibitors were shown to be able to form four active protease–inhibitor complexes with sole proteases, thus delaying the migration of trypsin- and chymotrypsin-like fractions and altering the enzyme pattern in comparison with fish fed on the control diet (Fig. 2b, lane C). Moreover, this profile was quite similar to that obtained in the in vitro inhibitory assay (Fig. 3b, lane PPC) and in accordance with

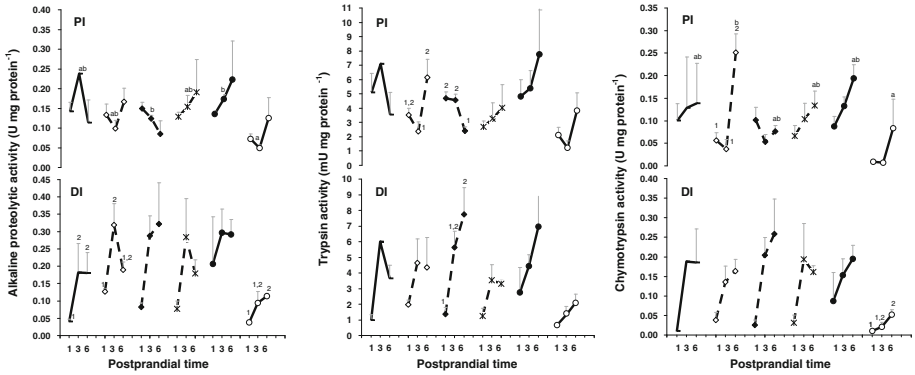

Fig. 4 Postprandial total protease activity, trypsin- and chymotrypsin-like in PI and DI of juvenile Senegalese sole fed on diets with 30% of FM protein replacement by different plant protein sources (Experiment 2). Control (*solid line*), SBM (*dash line*; *open rhombus*), SPC (*dash line*; *closed rhombus*), SPI (*dash line*; *asterisk*), WGM (*solid line*; *closed circles*) and PPC (*solid line*; *open circles*). Values are shown as mean ± SEM. Differences for each postprandial time among diets are marked with *different letters* and differences in among postprandial times for each diet with numbers ($P < 0.05$)

Table 4 Proteolytic activities[1] and trypsin/chymotrypsin and acid/alkaline protease ratios of Senegalese sole fed on diets of Experiment 2

	Total alkaline protease (U mg protein^{-1})	Trypsin (mU mg protein^{-1})	Chymotrypsin (U mg protein^{-1})	Trypsin/ chymotrypsin ratio	Acid protease (U mg protein^{-1})	Acid protease/ alkaline protease ratio
Diet[2]						
C	0.15^{ab}	4.39^{b}	0.12^{b}	0.13^{bc}	2.50^{ab}	13.95^{ab}
SBM	0.17^{b}	3.84^{b}	0.11^{b}	0.07^{ab}	2.19^{ab}	6.87^{a}
SPC	0.18^{b}	4.40^{b}	0.12^{b}	0.05^{ab}	1.99^{ab}	6.93^{a}
SPI	0.17^{b}	3.02^{ab}	0.12^{b}	0.05^{a}	0.99^{a}	3.72^{a}
WGM	0.22^{b}	6.08^{b}	0.14^{b}	0.07^{ab}	1.94^{ab}	4.57^{a}
PPC	0.08^{a}	1.88^{a}	0.03^{a}	0.15^{c}	2.87^{b}	18.31^{b}
Pooled SEM	0.02	0.77	0.02	0.02	0.40	2.53
Postprandial time (PT)[3] (h)						
1	0.11^{a}	2.66^{a}	0.05^{a}	0.14^{c}	1.99	10.45
3	0.19^{b}	4.12^{b}	0.11^{b}	0.07^{b}	2.42	9.09
6	0.18^{b}	5.02^{b}	0.16^{c}	0.05^{a}	1.83	7.63
Pooled SEM	0.01	0.54	0.02	0.02	0.28	1.79
Segment[4]						
PI	0.14	4.36	0.10	0.09	–	–
DI	0.19	3.51	0.12	0.08	–	–
Pooled SEM	0.01	0.45	0.01	0.01	–	–
Interactions						
Diet × PT	ns	ns	ns	ns	ns	ns
Diet × segment	ns	ns	ns	ns	–	–
PT × segment	<0.05	<0.05	<0.05	<0.05	–	–

[1] Values are mean of [2] 18, [3] 36 or [4] 53 replicates. Different superscript letters denote significant differences among diet combinations, postprandial time or between intestinal segments ($P < 0.05$)

ns Not significant

those obtained by the previously described spectrophotometric techniques (Table 4; Fig. 4). On the contrary, extracts of fish fed on SBM, SPC, SPI or WGM showed the same proteolytic pattern that those fed on control diet (Fig. 2b, lane C).

Discussion

The aim of this study was determining the influence of (1) the level of dietary protein (Experiment 1) as well as (2) the source of dietary protein (Experiment 2) on growth performance and proteolytic activities of juvenile Senegalese sole. The SGR values obtained in this study agree with those previously reported for Senegalese sole juveniles cultured under similar experimental conditions (Rema et al. 2008; Silva et al. 2010) and those described for other species in the Soleidae family (Gatta et al. 2010; Bonaldo et al. 2006). In the present work, SGR was not affected by changes in the dietary protein level or the protein source type used in feed formulation. However, FBW was significantly

increased in fish fed on diets containing 46 or 56% CP. These values are slightly lower than those reported by Rema et al. (2008), who defined the optimum CP level for this species at 600 g kg^{-1}, but the same trend was not observed in the 67% diet.

Taking into account the results obtained in Experiment 1 and available data from literature (Rema et al. 2008; Rodiles et al. 2007), 6 experimental diets containing 55% CP were formulated in Experiment 2, but replacing 30% protein of FM by different plant protein sources. Results obtained indicate that—despite its high dietary protein requirement—Senegalese sole is able to perform equally irrespective of the protein source origin, as previously stated by Silva et al. (2010) using a mixture of plant ingredients (SBM, corn and wheat gluten).

Fish growth depends on numerous factors and is not always correlated with dietary protein level (Kohla et al. 1992). Thus, fish digestive capacity is often studied to optimize the proportion of macronutrients in the diet (Eusebio and Coloso 2002; Debnath et al. 2007; González-Félix et al. 2010). The digestive physiology of *S. senegalensis* is characterized by a residual gastric phase, which contributes modestly to the global digestive process (Sáenz de Rodrigáñez et al. 2005, 2011), followed by an alkaline phase in a long multiple *S*-shaped intestine, where most protein digestion occurs (Yúfera and Darías 2007). In the current experiment, a clear relationship of dietary protein level on stomach proteolytic activity cannot be established. However, gastric protease activity was influenced by dietary protein source. Thus, partial replacement of FM by PPC tends to higher increase in the acid protease activity and the acid protease/alkaline protease ratio than other plant-based diets. However, there is no clear mechanism regarding this modulation. Cholecystokinin/gastrin-like peptides are well known to play a critical role in digestive physiology control in vertebrates. Johnson (2001) reported that gastrin could influence the secretion of pepsinogen, while Temler et al. (1985) stated that SBTI stimulates gastrin secretion in rats. Similarly, Santigosa et al. (2010) found changes in acid protease activity over time in *Sparus aurata* fed on SBTI-supplemented diets, suggesting that acid protease activity variations could be caused by negative feedback due to the presence of an inhibitor at intestinal level. This hypothesis could be used in the current research, as the PPC diet contains high protease inhibitors content, as shown in the zymogram of fish fed on this diet. Thus, the presence of protease inhibitors in pea seed seems to be related to their low in vitro protein hydrolysis, as reported by Sáenz de Rodrigáñez et al. (2011) for Senegalese sole. In *S. aurata,* acid pre-treatment of green pea seed meal with gastric enzymes does not improve their intestinal in vitro protein hydrolysis (Alarcón et al. 2002). Alarcón et al. (2001) describe that the inhibitory effects of pea seed meal reduce alkaline protease activity, possibly due to the presence of acid and/or heat stable protease inhibitors that are able to react with both trypsin- and chymotrypsin-like enzymes. In the present study, fish fed on PPC diet are not able to overcome protease inhibitor effects on their digestive physiology, as revealed by spectrophotometric (Table 4; Fig. 4) and electrophoretic (Figs. 2b, 3) techniques.

Increased dietary protein level tends to reduce alkaline total protease activity in PI and DI extracts of Senegalese sole ($P < 0.05$), since an inverse linear relationship was found in both intestinal segments. These observations are not in line with data reported by other authors, who describe increased intestinal protease activity when fish are fed on diets containing increased protein content (Bazaz and Keshavanath 1993; El-Saidy et al. 2000; Eusebio and Coloso 2002; Mohanta et al. 2008). For instance, Cahu and Zambonino Infante (1994) found that trypsin was unresponsive to dietary CP in early-weaned sea bass larvae. However, the present study cannot establish a clear correlation between growth and total alkaline protease activity. Similar results were found by Lopez-Lopez et al. (2005),

who found no correlation between protease activity and fish growth. The high levels of intestinal protease activity found in the DI of fish fed on a low level of dietary protein might be related to the high protein requirement of this species. This fact could be provoked by the action of cholecystokinin. CCK has been proven to play a critical role in digestive physiology control in vertebrates, and it stimulates the pancreas in some fish species (Einarsson et al. 1997; Olsson et al. 1999; Olsson and Holmgren 2001). CCK secretion is regulated by the digestive products of diets, and dietary proteins seemed to stimulate postprandial CCK secretion from the gut, as well as pancreatic trypsin secretion, thus suggesting that both factors contribute to protein digestion in Atlantic halibut larvae (*Hippoglossus hippoglossus*) (Rojas-García and Rønnestad 2002). In rats, dietary proteins seem to perform their stimulatory effect on CCK secretion via trypsin inhibition, and this has been postulated as the main connection in the feedback loop regulating the intestinal phase of pancreatic exocrine secretion (Sharara et al. 1993). Therefore, it is interesting to speculate that change in endogenous enzyme activity is probably due to changes in CCK secretion as a result of the stimulation from different dietary protein levels in the present research. However, further research is needed to prove this fact.

In Experiment 2, the total dietary protein of experimental diets was fixed. Hence, the observed variations in the level of proteolytic activities can be attributed to the partial replacement of FM by plant protein sources. These digestive variations may be an adaptive physiological response to the poor quality of plant meal and/or the presence of antinutritional factors (Francis et al. 2001, Gatlin et al. 2007). Changes in the distribution and activity of digestive enzymes have been identified as a compensation mechanism of digestion processes (Krogdahl et al. 1994; Olli et al. 1994; Haard et al. 1996; Francis et al. 2001; Sveier et al. 2001; Escaffe et al. 2007; Romarheim et al. 2007; Santigosa et al. 2008). Thus, results reported in this work showed the lowest and highest values of total alkaline protease, trypsin- and chymotrypsin-like in fish fed on PPC and WGM, respectively. As mentioned above, the lower values of alkaline protease activity in fish fed on PPC agree with the presence of a noticeable concentration of protease inhibitors. In addition, no growth-related differences were found, thus suggesting the existence of a compensation mechanism in Senegalese sole. A similar result was reported by Gouveia and Davies (1998, 2000), who found that 35% of different pea products in feeds for juvenile European Sea bass have no adverse effect on growth. Øverland et al. (2009) report the same finding in 150 g Atlantic salmons (*Salmo salar*) fed on 27% PPC. On the contrary, fry *S. salar* (2 g) fed with 78% inclusion of PPC resulted in reduced relative weight, increased trypsin activity and induced enteropathy in the DI (Penn et al. 2011). On the other hand, fish fed on WGM showed the maximum values of protease activity. This fact may be related to the moderate protein digestibility of wheat meal reported for this species (Moyano et al. 1999; Dias et al. 2010). Thus, an induction of the secretion of alkaline proteases might compensate the presence of residual protease inhibitors, as proven in the protease profile of WGM-fed fish. Similar results were reported for Krogdahl et al. (1994), Olli et al. (1994) and Santigosa et al. (2008) in trout, Atlantic salmon and seabream, respectively. Krogdahl et al. (1994) point out a possible imbalance in sulphur-containing amino acids as a result of increased protease synthesis (note that proteases contain cysteine). The additional synthesis of digestive proteases might involve increased energy cost for WGM-fed fish, and this fact seems to reflect no growth improvement. A similar tendency is reported by various authors working on other fish species (Moyano et al. 1999; Takii et al. 2001; Ostaszewska et al. 2005; Escaffe et al. 2007; Romarheim et al. 2007; Santigosa et al. 2008).

Omnivorous fish readily accept feeds containing plant feedstuffs better than other fish species (Arndt et al. 1999; Hidalgo et al. 1999). In vitro assay with *S. senegalensis*

intestinal extracts shows higher proteolysis of soybean proteins (Sáenz de Rodrigáñez et al. 2011), even as compared with *Oreochromis niloticus* or *S. aurata* enzyme extracts (Moyano et al. 1999). In the current experiment, the SBM diet leads to slightly higher mean growth values than SPC and SPI diets. This is a surprising fact, as the purification of soybean protein by aqueous extraction or alcohol should reduce the levels of secondary compounds and increase its nutritional value. However, results obtained prove that SPI diets contain higher levels of protease inhibitors than SBM or SPC diets (Fig. 3). This fact explains why different SPC or SPI batches can lead opposite growth results (Bureau et al. 1998). Accordingly, this may explain the differences found in the present study among soybean products. The inhibition produced by PPC was more unspecific and affected several proteases, as revealed by the existence of several active protease–inhibitor complexes, thus suggesting the presence of at least two different inhibitors in this diet.

The partial replacement of FM by plant protein sources affects the amount of secreted alkaline proteases in Senegalese sole juveniles, especially in the DI. Trypsin-like activity was affected 6 h after feeding mainly by SPC and WGM diets. Trypsin acts as a key enzyme in the activation cascade of other pancreatic zymogens (chymotrypsin, carboxypeptidases and elastase) during digestion (Guillaume and Choubert 2001). The absorption pattern could be modified in response to FM replacement and may imply differential availability of luminal oligopeptides and amino acids, which may lead to amino acid imbalance under these feeding conditions (Santigosa et al. 2008). These results could suggest a compensation mechanism in alkaline protease secretion (mainly trypsin), thus resulting in delayed digestion, to compensate the presence of plant protease inhibitors in experimental diets. However, delay in the action of digestive enzymes was not accompanied by decreased fish growth, as reported by Santigosa et al. (2008) for trout fed on diets with <50% of FM replacement by plant proteins. However, higher replacement levels (>75%) lead to decreased weight gain in trout and sea bream (Escaffe et al. 2007; Santigosa et al. 2008).

Zymograms revealed that dietary protein level did not affect the pattern of active proteases of *S. senegalensis*. Thereby, protein concentration on diet affected the amount but not the composition of intestinal proteases secreted into the intestinal lumen (Fig. 2a). In contrast, protein source quality affects the composition of secreted intestinal proteases. Differences among the effects of inhibitors on alkaline proteases of *S. senegalensis* were evident in zymograms (Fig. 3b). The physiological response of fish to the presence of residual protease inhibitors in SBM, SPI, SPC and WGM diets suggests the existence of some fish mechanism to compensate the presence of plant inhibitors (Fig. 2b).

To conclude, juvenile Senegalese sole show capability to modulate digestive protease secretion when the concentration or source of dietary protein is modified. Intestinal protease activities were affected by quantitative changes in dietary protein. However, the type of dietary protein source modifies both the amount and the composition of the pancreatic proteases secreted into the intestinal lumen, involving no growth reduction. This study establishes that 30% FM protein can be replaced by soybean derivatives with no impact on either the growth or intestinal proteases of this flatfish species. Replacement with PPC or WGM should be taken cautiously, but further research is needed to determine whether growth performance and digestive enzyme physiology of Senegalese sole can be affected by these protein ingredients in a long-feeding trial.

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