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Effect of non-enzymatic browning products on the activity of gastric proteases from the rainbow trout *Oncorhynchus mykiss*

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Abstract

The products resulting from the non-enzymatic browning in processed diets are known to exert negative effects on the digestive enzymes of vertebrates. In addition, browning heavy products (BHP's) are known to co-precipitate with proteins depending on the pH and ionic strength of the medium and on the isoelectric point of the protein. As the manufacture of aquafeeds as well as aquafeed ingredients frequently implies heating processes, the effects of BHP's from a well-known model mixture (glucose + glycine) on the gastric proteases of the rainbow trout *Oncorhynchus mykiss* were investigated in the present work. The results support that BHP's interact with gastric proteases most probably to form a co-precipitate, thus potentially reducing the

protease activity in the fish stomach. The maximal loss of activity found in the experiments was above 20%. The interaction is pH and ionic strength-dependent and relatively stable against mechanical perturbation. In the range of BHP concentration 500-1000 µg/mL, the interaction was dose-dependent at pH 3.5 with a maximum above 20%, and apparently independent of the dose at pH 4.0. Above an ionic strength of 100 mM due to monovalent ions (NaCl), the intensity of the interaction is reduced. On the other hand, divalent cations such as Ca^{2+} and Mg^{2+} in the range 30-80 mM disturb the interaction between BHP's and gastric proteases even at ionic strengths below 100 mM, thus alleviating the inactivating effect of BHP's.

Keywords: Non-enzymatic browning, gastric proteases, gastric pH, ionic strength, divalent cations, rainbow trout

1. Introduction

The global aquaculture industry depends on a suitable formulation and processing of aquafeeds both from the economic and the nutritional points of view. Currently, a great amount of aquafeeds is produced by extrusion-cooking, a process involving thermal and mechanical stress affecting the physical and chemical properties of the diet (Singh et al., 2007). In general, heating of a mix of nutrients determines an impairment of the nutritional value tightly connected to a change in colour known as "non-enzymatic browning" (Corzo-Martínez et al., 2012). The non-enzymatic browning is the result of a complex net of chemical reactions called Maillard reaction which begins with the formation of covalent links between amino groups and either reducing glucides or products from lipid oxidation (Hidalgo et al., 1999; Martins et al., 2001). As a consequence, lysine, tryptophan and, to a lesser extent, arginine residues in proteins are particularly destroyed (Becker and Yu, 2013). The resulting set of new molecules are usually named "Maillard reaction products" or MRP's. This series of reactions ends up with a kind of brown-coloured, heavy products called melanoidins. Neither the structures of model melanoidins nor those of dietary melanoidins have been completely disclosed to date, but they are known to be brown in colour and strong absorbers in the UV spectrum (Kim and Lee, 2009), poly-carboxylic (Fang and Schmidt-Rohr, 2009), mostly anionic and with a high (although variable) molecular weight (Lee et al., 1987).

But extrusion is not the only heating process involved in the production of aquafeeds. For example, a number of protein raw materials suffer one or several drying steps before been used as ingredients: fish meal (Samuelsen, 2015), soybean meal (Dozier and Hess, 2011; Refstie, 2007), corn gluten meal (Rausch and Belyea, 2006), blood meal (Waibel et al., 1977) or distilled dry grains with solubles (DDGS) (Liu, 2011) among others. Fermented fish silages are ingredients making a potentially high contribution to the content of browning products when included in the formulation of animal diets, since sugarcane molasses are usually added during the fermentation of silages (van Wyck and Heydenrich, 1985). Interestingly, molasses are known to be rich in recalcitrant melanoidins (Chandra et al., 2008), and diets including fermented silages often show a reduction in protein digestibility (Borghesi et al., 2008; Hernández et al., 2013; Mach et al., 2010) and, sometimes, a conspicuous excretion of browning products in feces (Borghesi et al., 2008).

When the degree of browning is quantified according to the CIE (Commission Internationale de l´Eclairage) *L* a* b** colour system (Pathare et al., 2013), the lightness (*L**) and redness (*a**) coordinates are correlated to the digestibility of nitrogen and/or energy of the dietary mixture (Cabrita et al., 2011; Cozannet et al., 2010; Morken et al., 2012). Other authors have reported negative effects of glucose-lysine browning products (less than 6-8 kDa in molecular weight) on the digestibility of dietary proteins *in vivo* in rats (Öste and Sjödin, 1984).

One of the potential reasons for the negative relationship between the intensity of the non-enzymatic browning and feed digestibility is the partial destruction of lysine, arginine and tryptophan residues which are targets for trypsin, chymotrypsin and pepsin (Kageyama, 2002; Marcuschi et al., 2010; Zhao et al., 2011). Another potential factor can be the negative effect of Maillard-reaction products on the activities of trypsin, demonstrated both with model and dietary browning heavy products (BHP's) (Hirano et al., 1994 1996, 1996b), together with the negative effect of browning products (without reference to their molecular weights) on intestinal carbohydrases (Chung et al., 2012). On the other hand, the interaction between gastric proteases and BHP's has been poorly studied, but the precipitation of complexes between BHP's derived from a mixture of glucose and glycine and commercial pepsin at pH values characteristic of mammal stomachs was early reported by Horikoshi and Gomyo (1976). Therefore, a negative effect of BHP's on the activity of vertebrate gastric proteases in an acidic environment should not completely be ruled out. Based on this hypothesis, the main objective of the present work is to test for the effect of BHP's from a heated solution of glucose and glycine on the gastric protease activity from the rainbow trout *Oncorhynchus mykiss*, and in relation to the biochemical environment of fish digestive tract.

2. Material and Methods

The preparation of high molecular weight browning products was based in Hirano et al. (1994). Mainly, a solution (25 mL) with glucose 0.9 M, glycine 1.0 M and sodium hydrogen-carbonate 0.1 M was heated at 103°C for 24 h. in a closed screw-cap tube. The heated mixture was let to cool to room temperature, acidified

with HCl to pH 2.5 and stored at 7° C overnight. The so obtained mixture was deep-brown in color and viscous. It was mixed 1:1 with distilled water to reduce the viscosity, aliquoted and centrifuged at 17000 g for 15 min at 4°C. Supernatants were discarded and the pellets were manually homogenized with distilled water acidified to pH 2.5 with HCl. The homogenates were centrifuged at 3700 g for 10 min. at 4°C, the supernatants discarded and the pellets homogenized with acidified distilled water; this step was repeated three times. The pellets so obtained were dissolved in NaOH 10 mM and the whole volume (aprox. 27 mL) was dialyzed (Spectrapore No. 6, MWCO 10 kDa) against water (4.0 L) for 120 h. at 7°Cdialysis water being changed daily. The dialysate (final pH = 5.1) was acidified to pH 2.5 with HCl and stored at 7°C for 48 h. Finally, it was centrifuged at 4800 g for 30 min at 4°C, the supernatant discarded and the pellets freeze-dried for 24 h prior to be stored at -20°C until use.

Rainbow trout *(Oncorhynchus mykiss)* were obtained from "Wenüy" fish farm and stocked at Catholic University of Temuco hatchery in Araucanía region (Chile). The specimens were maintained in 500 L fiberglass tanks connected to a flow-through water system at 15°C and natural photoperiod. Fish were fed a maintenance ration (1% body weight, Nutra Parr 30, Skretting, Chile: 48% protein, 19% lipid) once a day. Prior to the sampling, fish used in the experiments (291.2±9.2 g) were starved for three days, anaesthetized with benzocaine (0.15 mL/L), and euthanized by cranial puncture to the brain. The abdominal cavity of euthanized fish were cut open and the stomach immediately dissected and stored at -80° C until the preparation of the gastric extracts. All experiments were conducted in compliance with the Chilean legal requirements for the experimentation on live animals. A collection of fifteen gastric extracts were prepared for the study. Random samples of at least 8 individuals from this collection of extracts were used for each experiment. The gastric extracts were prepared by chopping the stomachs in small pieces while thawing, mixing with chilled distilled water (pH ~5.6) in proportion 1:4 (w/w, tissue:water), and homogenizing in ice with an IKA Ultraturrax T25 Digital homogenizer. The homogenates were centrifuged at 15800 g and 4°C for 15 min, and the supernatans aliquoted and stored at -20°C until use. The soluble protein of each extract was determined following the method of Bradford (1976) based on the binding of proteins to the Comassie G250 dye; bovine serum albumin (BSA) was used as a reference protein to calibrate the Bradford assays.

All the experiments further described followed the same three-step protocol: a) gastric extracts (300 μg/mL of soluble protein) were pre-incubated for 1 hour at 15°C (within the thermal range for growth in juvenile *Oncorhynchus mykiss* (Austreng et al., 1987)) in a 50 mM formic/formate buffer (except in the experiment 6, in which the buffer was 50 mM formic/formate + 50 mM acetic/acetate) under the pH and ionic strength conditions required in the different assays of each experiment, b) pre-incubated solutions were centrifuged at 15800 g and 15°C, c); finally, the supernantant was assayed for the activity of total acid proteases as described by Anson (1938). In addition, all the experiments followed the same general experimental design, i. e., a repeated-measures design in which a set of individual gastric extracts was subject to the different levels of the factor or factors under investigation.

The activity assays were performed by pipetting 0.01 mL of the pre-incubation supernatant into 1 mL buffer glycine 100 mM at pH 2.5 and containing bovine hemoglobin 0.5%, and incubated at 25°C for 25-30 min. The reaction solution was stopped with 0.5 mL of TCA 20%, cooled at 7°C for 15 min and centrifuged (15800 g, 20°C, 15 min) and the supernantant was read for absorbance at 280 nm. Two activity assays plus one blank assay (in which the TCA was added before the gastric extract) were performed for each individual in each experiment. The specific activity was calculated from the increment in absorbance from the blank assay and assuming an extinction coefficient for TCA-soluble, Tyr-containing peptides of 0.008 mL $(\mu g\ Tyr)^{-1}\ cm^{-1}$. The unit of activity (U) was defined as 1 $(\mu g\ Tyr)\ min^{-1}$ $(mL\ of\ extract)^{-1}$, and the unit of specific activity as 1 U $(\mu g\ of\ extract\ soluble\ protein)^{-1}$. The results of the different experiments were expressed as percent losses of activity from a reference condition, the resulting value being not necessarily in the range [0, 1]. Following this basic protocol, six specific experimental designs were implemented that are described below:

2.1. Combined effects of pH and BHP's on the activity of gastric proteases

Two experiments were performed on 11 individuals. In the first experiment, the combined effects of different concentrations of BHP (0, 500, 666 and 1000 μg/mL) and pH (3.5 and 4.0) on gastric extract (300 μg/mL soluble protein.) maintained at constant temperature and ionic strength (15ºC and 33.5 mM) were assayed.

Protease activity measured after a 1-hour pre-incubation in the absence of BHP at 15ºC and pH 3.5 or 4.0 was used as a reference.

Because a minor part (1:100) of the BHP's present in the pre-incubation step were transferred to the activity assays together with the gastric enzymes, a second experiment was designed to ascertain that the negative effects of BHP's were mainly due to interactions taking place during the pre-incubation time. In this experiment, a similarly diluted gastric extract (300 µg/mL prot. sol.) was assayed (without pre-incubation) for total acid-protease activity in the presence of 10 µg/mL BHP (just the concentration of BHP in the incubation step after a pre-incubation with 1000 µg/mL BHP). For each individual (n=11), the protease activity in the absence of BHP and at 25ºC was defined as the 100% reference activity.

2.2. Combined effects of ionic strength and BHP's on the activity of gastric proteases

Designed to test the possible effect of ionic strength (IS) on the interaction between BHP's and gastric proteases from rainbow trout. In this experiment, the gastric extracts were always pre-incubated at pH 3.5 but using seven different ionic environments:

i) IS 33.5 mM (adding NaCl) without BHP's
ii) IS 250 mM (adding $CaCl_2$) without BHP's
iii) IS 33.5 mM (adding NaCl) with 1000 µg/mL BHP's
iv) IS 100 mM (adding NaCl) with 1000 µg/mL BHP's
v) IS 250 mM (adding NaCl) with 1000 µg/mL BHP's
vi) IS 100 mM (adding $CaCl_2$) with 1000 µg/mL BHP's
vii) IS 250 mM (adding $CaCl_2$) with 1000 µg/mL BHP's

For each individual, the activity after pre-incubating a diluted gastric extract (to 300 µg/mL prot. sol.) without BHP's at pH 3.5, 15ºC and IS 33.5 mM (adding NaCl) was defined as the 100% reference value (n=10).

2.3. Effect of Ca^{2+} vs. Mg^{2+} on the interaction between BHP's and gastric proteases

Since Mg^{2+} is as important as Ca^{2+} in the ionic composition of chyme of the rainbow trout during digestion (Bucking and Wood, 2011), an experiment was designed to differentiate the effects of these two divalent cations on the interaction between HBP's and gastric proteases. For each individual, the activity after pre-incubating a diluted gastric extract (to 300 µg/mL prot. sol.) without BHP's at pH 3.5, 15ºC and IS 33.5 mM (adding NaCl) was defined as the 100% reference value (n=13). In this experiment, the total concentration of divalent cations was set at 50 mM which is a figure within the range reported by Bucking and Wood (2011) for the prost-prandial gastric fluid of rainbow trout fed a commercial diet in freshwater. Three combinations of divalent cations were tested: i) 50 mM Ca^{2+} (from $CaCl_2$), ii) 25 mM Ca^{2+} + 25 mM Mg^{2+} (from $CaCl_2$ and $MgCl_2$ respectively), iii) 50 mM Mg^{2+} (from $MgCl_2$). In all cases, the total IS was fixed at 250 mM adding NaCl.

2.4. Stability of the interaction between BHP's and gastric proteases in the activity assay medium

To assess the reversibility of the effect of BHP's on the activity of gastric proteases, two experiments were designed in which the solution obtained after the pre-incubation step was centrifuged or directly assayed without centrifugation to measure the protease activity. By skipping the centrifugation step, all the protease molecules at the end of the pre-incubation period (those in solution and also those in the form of precipitated particles) are introduced in the assay medium, in these conditions:

i) If the loss of protease activity without centrifugation is significantly lower than the value obtained with a centrifugation step, the precipitates can be interpreted as partly reversible.
ii) If the loss of protease activity without centrifugation is not significantly lower than the value obtained with a centrifugation step, the precipitates including the gastric proteases can be interpreted as irreversible.

In the first experiment (Figure 3A), two factors were tested: BHP's (0 vs. 1000 µg/mL) and centrifugation after the pre-incubation step (centrifugation vs. no centrifugation). In the absence of centrifugation the precipitate was homogenized by gently tapping the Eppendorf immediately before the transference to the protease assay medium. This transference implied a change in the pH from 3.5 to 2.5. For each individual, the activity after a 1-hour of pre-incubation without BHP's and with centrifugation was considered as the 100% reference value (n=8).

A second experiment with the same individuals was designed to test for the specific effect of the change in pH (after the transference to the activity assay medium) on the stability of the interaction between the BHP's and gastric proteases,:

i) the pre-incubation was always performed in the presence of 1000 µg/mL BHP and pH 3.5, whereas the centrifugation step was the factor tested (presence vs. absence),
ii) the incubation was always assayed at pH 3.5, therefore *there is not a change in pH* after the transference of digestive enzymes from the pre-incubation medium to the activity assay medium.

For each individual, the activity after 1-hour of pre-incubation with BHP's and without centrifugation was considered as the 100% reference value (n=8). In this second experiment, the inactivation rates of gastric extracts pre-incubated with 1000 µg/mL of BHP's at pH 3.5, centrifuged and assayed at pH 3.5 were calculated with respect to gastric extracts pre-incubated with BHP's at pH 3.5, *not centrifuged*, and also assayed at pH 3.5 (first bar in Figure 3B),

$$100 \times \frac{(Act.pH\ 3.5;\ Preinc.\ with\ \ HBP,\ \ with\ cent.,\ pH\ 3.5) - (Act.pH\ 3.5;\ \ Preinc.\ with\ HBP,\ \ without\ cent.,\ pH\ 3.5)}{(Act.\ at\ pH\ 3.5;\ \ Preinc.\ with\ \ HBP,\ \ without\ cent.,\ pH\ 3.5)}$$

The average inactivation resulting from the above calculation is compared with the equivalent inactivation value when the activity assay was performed at pH 2.5 (calculated from the first experiment data, second bar in Figure 3B),

$$100 \times \frac{(Act.pH\ 2.5;\ Preinc.\ with\ \ HBP,\ \ with\ cent.,\ \ pH\ 3.5) - (Act.pH\ 2.5;\ \ Preinc.\ with\ HBP,\ \ without\ cent.,\ \ pH\ 3.5)}{(Act.\ \ at\ pH\ 2.5;\ \ Preinc.\ with\ \ HBP,\ \ without\ cent.,\ \ pH\ 3.5)}$$

2.5. Effects of mechanical perturbation on the interaction between BHP's and gastric proteases

Since the chyme is mixed by peristaltic contractions during the gastric digestion in fish (Olsson and Holmgren, 2001), an experiment was designed to assess the effect of mechanical perturbation on the intensity of the inactivation of gastric proteases by BHP. For each individual (n=8), the activity after pre-incubating a diluted gastric extract with 1000 µg/mL of BHP's at pH 3.5, 15ºC and under still conditions was defined as the 100% reference value. The perturbation assay consisted in inserting the eppendorfs into a plastic plate which was inside a water bath at 15ºC and, at the same time, attached to an orbital shaker (Selecta Rotaterm) set at 150 rpm.

2.6. Effect of the pre-incubation pH on the interaction between BHP's and gastric proteases

An additional experiment was designed to test for the effect of the pre-incubation pH in a wide rage range between 3.0 and 5.0. A combination of two monoprotic weak acids was used in the buffer solution: formic/formate 50 mM + acetic/acetate 50 mM, with IS fixed at 100 mM (adding NaCl). In this experiment, each individual gastric extract (final concentration of protein soluble of 300 µg/mL) was pre-incubated at 5 pH values, 3.0, 3.5, 4.0, 4.5, and 5.0, with or without BHP's (0 vs. 1000 µg/mL). A repeated-measures 5×2 factorial design was implemented. For each assay performed in the presence of BHP's, inactivation rates were calculated as usual (Figure 5A), i.e. considering the activity after pre-incubating the gastric extract under the same pH but without BHP's as the 100% reference activity (n=11).

In this experiment, not only inactivation rates but also the effect of pre-incubation pH on the stability of gastric proteases was assessed. For each individual, enzymatic stabilities at different pH values were estimated by normalizing the activities obtained in the absence of HBP's with respect to the maximal activity found in the pH range [3.0, 5.0] also without HBP's. Therefore, enzymatic stabilities were expressed as

percentages within the interval [0, 100]. The average stabilities for each pH were calculated for the whole sample (Figure 5B).

2.7. Statistics

Experimental data were analyzed with repeated-measures Anova or, in the case of those experiments with only two experimental conditions (experimental design 5), with a dependent-samples t-test. Because enzymatic stabilities (experimental design 6) are strictly within the interval [0, 100], they were transformed with the arcsin(sqrt(Stability/100)) function prior to the analysis. When necessary, a Bonferroni multiple comparison test was applied to locate significant differences among means. The significance level was set at 0.05. Average data were expressed as means±SEM throughout the text and in figures.

3. Results

In all the experiments, whenever the gastric extracts were pre-incubated with BHP>0, a conspicuous biphasic solution with a transparent yellowish phase at the top of the tube and a turbid, brown phase towards the bottom was produced after the pre-incubation time.

3.1. Combined effects of pH and BHP's on the activity of gastric proteases

In the first experiment, the activity of gastric proteases (reference average activities: 1.326±0.088 and 1.308±0.090 U/μg prot. sol. for pre-incubation pH 3.5 and 4.0 respectively) was significantly modified during the pre-incubation step by the presence of BHP ($p<0.001$) and the value of the environmental pH ($p<0.001$). The interaction BHP×pH was also significant ($p<0.001$). At pH 3.5, the increase in BHP from 500 to 1000 μg/mL, produced a reduction in activity from 5 to 22%, whereas at pH 4.0, the presence of BHP in the same range of concentrations reduced the activity about 11% irrespective of the concentration (Figure 1A). This difference explains the significant interaction. In the second experiment (reference average activity: 1.133±0.088 U/μg prot. sol.), the activity of gastric protease was not significantly inactivated by the presence

of BHP during the incubation step (n=11, average loss of activity = 1.12±0.57%, p=0.080), supporting that the effects of BHP's were mostly due to the interactions taking place during the pre-incubation time (Figure 1B).

3.2. Combined effects of ionic strength and BHP's on the activity of gastric proteases

A clearly significant effect of IS on the inactivation of gastric proteases (reference average activity: 0.726±0.039 U/µg prot. sol.) by BHP's was obtained (n=10, p=0.001) (Figure 2A). When the IS was set by adding only monovalent ions (NaCl) the maximal inactivation rate, approx. 28%, was maintained until IS 100 mM, but reduced to 20.5±2.4% when IS was incremented till 250 mM. Interestingly, the presence of divalent cations ($CaCl_2$) reduced the inactivation rates to a larger extent at the same IS values, i.e., the inactivation only reached a 17.1±2.1% at IS 100 mM when this value of IS was get by adding $CaCl_2$ to the pre-incubation buffer. It can be concluded that the ionic strength and the presence of calcium cations are important factors determining the intensity of the interaction between HBP's and gastric proteases of the rainbow trout.

3.3. Effect of Ca^{2+} vs. Mg^{2+} on the interaction between BHP's and gastric proteases

There was a significantly higher inactivation of acid proteases (reference average activity: 0.808±0.041 U/µg prot. sol.) by BHPs in the presence of Mg^{2+} than in the presence of Ca^{2+} (13.6±2.8% vs 10.0±2.8%; n=13, p=0.001) (Figure 2B). This difference pointed to a stronger interaction between HBP's and Ca^{2+} than between HBP's and Mg^{2+}.

3.4. Stability of the interaction between BHP's and gastric proteases in the activity assay medium

In the first experiment, when the gastric extracts (reference average activity at pH 2.5=1.131±0.077 U/µg prot. sol.) were pre-incubated with 1000 µg/mL BHP and centrifuged before being assayed for the protease activity, the inactivation rate reached the usual value above 20% (n=8, average inactivation = 24.9±2.1%), but the inactivation rate in the presence of BHP's was clearly reduced when the pre-incubated mixture was not centrifuged (Figure 3A) (n=8, p<0.001).

In the second experiment (reference average activity at pH 3.5= 0.893±0.049 U/μg prot. sol.), the average inactivation (calculated for the presence of BHP's, *with centrifugation* and activity assay at pH 3.5 with respect to a situation with BHP's, *but without centrifugation* and also with the activity assay at pH 3.5) amounted to 14.1±1.4% (first bar in Figure 3B). When the equivalent inactivation rate is calculated for the first experiment, it reached 21.8±2.3% (second bar in Figure 3B). This difference was statistically significant (n=8, p=0.012), pointing to the effect of the pH change (after the transference of the pre-incubated gastric extract) on the reversibility of the interaction between BHP's and gastric proteases.

3.5. Effects of mechanical perturbation on the interaction between BHP's and gastric proteases

No significant effect of the mechanical perturbation on the interaction between BHP's and gastric proteases (reference average activity: 0.601±0.051 U/μg prot. sol.) was obtained (n=8, average inactivation: -0.8±2.8%, p=0.773, see Figure 4).

3.6. Effect of the pre-incubation pH on the interaction between BHP's and gastric proteases

A significant effect of the pre-incubation pH on the interaction between BHP's and gastric proteases was found (n=11, p<0.001) (Figure 5A). The average loss of activity ranged between 6% and 21% for the pH interval [3.0, 5.0], and the maximal value was found at pH 3.5 (20.8%±1.0). In relation to the enzymatic stabilities (reference average activity for the optimum pH 3.5: 0.671±0.030 U/μg prot. sol), a significant effect of pH was obtained (n=11, p<0.001) (Figure 5B): the stability of the protease activity was maintained nearly constant in the pH interval [3.0-4.0] and dropped beyond pH 4.0.

4. Discussion

The two-step protocol (pre-incubation + assaying for residual activity) used in the present investigation has proven to be useful when testing for anti-nutritional effects on the activity of fish digestive enzymes (Morales

et al., 2011). In that way, the results offered valuable information on the type of interaction between BHP's and fish gastric proteases. It is probably a sort of electrostatic binding since it is affected by the pH, the ionic strength, the presence of divalent cations and even affected by the type of divalent cation. As previously reported for commercial pepsin (Horikoshi and Gomyo, 1976), the interaction probably implies the formation of non-covalent precipitating complexes, and it can be reversed by decreasing the pH to 2.5, the isoelectric point reported for glucose-glycine melanoidins. To the best of our knowledge, there is a lack of data about the content of melanoidins in animal feestuffs, but the content of melanoidins in a Western-type human diet has been recently estimated to be about 7.9 g a day for solid foods (Pastoriza and Rufián-Henares, 2014, Table 3). If the total ingestion of solid foods is probably less than 1000 g per day, it is prudent to estimate a concentration of BHP's above 0.8% for the solid fraction of the diet for Western people. Assuming a similar concentration of BHP's in aquafeeds, and that gastric contents reach a moisture about 50% in juvenile rainbow trout (Kristiansen and Rankin, 2001), the concentration of BHP's in the gastric fluid would be about 8.0 mg/mL. As a conservative approach, the BHP concentrations used in the present work are 8-16 times lower.

The most probable mechanism for the herein studied interaction is electrostatic. In the first place, model melanoidins are known to contain numerous carboxylic groups (Fang and Schmidt-Rohr, 2009) and model and real melanoidins have proven to be mainly anionic in nature (Lee et al., 1987). Secondly, the isoelectric points (IP) of fish pepsins are in the range 4.0-6.5 (Zhao et al., 2011) and that of glucose-glycine BHP's IP is about 2.5. In the pH range 3.5-4.0, in which stomachs of juvenile trout spent a noticeable part of the digestion time (Bucking and Wood, 2009; Sugiura et al., 2006), the net charge of BHP's is expected to be negative, whereas that of fish pepsins will be positive, making the formation of charge-based complexes possible. In addition, the maximal rate of inactivation recorded in the present work, 22-28%, is in good agreement with the measured values of precipitation of commercial pepsin when mixing this protein with glucose-glycine melanoidins at pH 2.0, (Horikoshi and Gomyo, 1976). In the case of commercial pepsin, the precipitation rate is zero above pH 2.5, however, the maximal loss of activity for gastric proteases of the rainbow trout in our experiment was obtained at pH 3.5, which can be soundly explained considering the comparatively higher isoelectric point (4.0-6.5) of fish pepsins (Zhao et al., 2011).

The effects of model BHP's derived from a mixture of amino acids and reducing sugars on the activity of digestive enzymes have been investigated in the case of intestinal proteases, mainly trypsin of mammal origin (Hirano et al., 1994, 1996, 1996b; Ibarz et al., 2009). Glucose-glycine melanoidins were reported to co-precipitate with commercial pepsin (probably from mammal origin) in the pH range 1.5-2.5 with an optimum at pH 2.0 (Horikoshi and Gomyo, 1976) but, to the best of our knowledge, no data exists about the potential effects on the activity of fish gastric proteases or, in general, on vertebrate gastric proteases. Results obtained in the present work point to a potentially significant inactivation of fish gastric proteases by a precipitation mechanism mediated by BHPs, taking place within the physiological range of pH values in fish stomachs. This precipitation phenomenon is most probably reversible as indicated in Figure 3A. When the pre-incubation and the incubation steps were both performed at the pH value in which the precipitates forms (pH 3.5, i.e., at the pH value in which the precipitates are known to be stable), the effect of the centrifugation (step intended to remove the precipitates) in the loss of activity is significantly reduced, so that a higher stability of the precipitates in the incubation medium when the pH is 3.5 can be interpreted, in other words, the precipitated proteases hardly returned to the aqueous phase in the incubation period. It can be expected that as long as the gastric pH gets closer to the isoelectric point of the HBP's (pH 2.5), the protease inactivation weakens.

According to experiment 5, gastric motility is expected to exert a minor effect on the interaction between BHP's and gastric proteases with respect to the changes in pH and ionic composition of the chyme. A concentration of 33.5 mM of Ca^{2+} (IS=100 mM) is high enough to reduce the interaction between BHP-proteases at pH 3.5. Despite de scarcity of data on the ionic composition of gastric chyme in digesting fish, Bucking and Wood (2009, 2011) reported a concentration of Ca^{2+} + Mg^{2+} about 60 mM, and an IS above 240 mM, in the gastric fluid phase of the rainbow trout fed a commercial diet, just when the pH begins to drop below 4.0 (approx. from the 10^{th} hour of digestion). Under such an ionic environment, it is not expected to find a full inactivating effect of BHP's. Nevertheless, the ionic composition of chyme fluid phase is most probably diet and species-specific and further research is needed to provide a general picture of the topic. The

buffering capacity of the diet (Lawlor et al., 2005) can be considered as another relevant factor for the interaction HBP-protease through its indirect effect on the acidification capacity of the stomach.

In spite of the little effect of heated mixtures of glucose-lysine and glucose-methionine on the solubility of Ca^{2+} and Mg^{2+} ions under intestinal pH and IS conditions (6.8-7.0 and 165 mM respectively) (Delgado-Andrade et al., 2004), in the present work, the alleviating effect of divalent cations on the interaction between BHP's and fish gastric proteases can be interpreted as a competition process for the negatively charged moieties in the glucose-glycine BHP molecules. This is in keeping with the results published by Horikoshi and Gomyo (1976) about the coagulation of glucose-glycine melanoidins in the presence of metallic ions in the pH range of fish stomachs.

Interestingly, the concentration of Ca^{2+} and Mg^{2+} in the gastric chime of the trout during digestion may be influenced not only by the chemical environment, but also by the ability of the stomach to produce hydrochloric acid. For example, the acidification of the gastric lumen leads to the solubilisation of calcium salts in humans and birds (Guinotte et al., 1999; Kopic and Geibel, 2013), which make Ca^{2+} ions available to co-precipitate with HBP´s whenever the pH is above 3.5. On the other hand, in mammals, Ca^{2+} ions can also bind to Ca-sensing-receptors (CaSR) in the gastric epithelium which in turn stimulates the production of gastric acid in humans (Kopic and Geibel, 2013). There is also some evidence of the stimulation of the production of gastric acid trough the action of CaSR in fish (Márquez and Fuentes, 2014). Therefore, it is possible to hypothesize that Ca^{2+} ions can promote its own solubilisation from the salts and fish bones present in fishmeal used in aquafeeds, and also its potential precipitation with dietary HBP's, thus alleviating their inactivating effect on fish gastric enzymes. As a consequence, the resulting concentrations of Ca^{2+} and Mg^{2+} in the gastric fluid during digestion can be interpreted not only as a balance among solubilisation, secretion and absorption processes (Bucking and Wood, 2011), but also precipitation.

To summarize, the negative effect of BHP's on the gastric protease activity of the rainbow trout is most probably due to an electrostatic interaction leading to the precipitation of the digestive enzymes. Since the inactivating effect can be considered to be moderate to high (depending on pH, IS, and ionic conditions), it

can be hypothesized to partially contribute to the usual decrease in protein digestibility of heat-processed aquafeeds.

5. Aknowledgements

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Figure captions

Fig. 1. Combined effects of pH and BHP's on the activity of gastric proteases. **a** Average loss of activity after pre-incubating gastric extracts in the presence of BHP's and expressed with respect to the assays pre-incubated without BHP's and at same pH (3.5 or 4.0). **b** Average loss of activity without pre-incubation and adding 10 μg/mL of BHP's in the activity assay (pH 2.5) expressed with respect to the assays without BHP's. Different letters on top of the columns indicate significant differences at the level 0.05. n.s.: not significant different from zero. Bars: standard error of the mean.

Fig. 2. Combined effects of the ionic composition of the medium and BHP's on the activity of gastric proteases. **a** Average loss of activity after pre-incubating gastric extracts at different IS values get with NaCl or $CaCl_2$ at pH 3.5 expressed with respect to the assays pre-incubated without BHP's, pH 3.5 and IS 33.5 mM (NaCl). **b** Average loss of activity after pre-incubating gastric extracts at 250 mM in IS with $CaCl_2$, $MgCl_2$ or a 1:1 mixture of both salts, with respect to the assays pre-incubated without BHP's, pH 3.5 and IS 33.5 mM (NaCl). Different letters on top of the columns indicate significant differences at the level 0.05. Bars: standard error of the mean.

Fig. 3. Stability of the interaction between BHP's and gastric proteases in the activity assay medium. **a** Average loss of activity after pre-incubating gastric extracts at pH 3.5 expressed with respect to the assays pre-incubated at the same pH but without BHP's and with centrifugation before the activity assay. **b** Average loss of activity after pre-incubating gastric extracts with 1000 μg/mL at pH 3.5 and performing the subsequent activity assay at pH 2.5 or 3.5, and expressed with respect to the assays pre-incubated at pH 3.5 without BHP's and with centrifugation and incubated at the same pH (2.5 or 3.5). Different letters on top of the columns indicate significant differences at the level 0.05. Bars: standard error of the mean.

Fig. 4. Effect of mechanical perturbation on the interaction between BHP's and gastric proteases. Average loss of activity after pre-incubating gastric extracts with 1000 μg/mL at pH 3.5 and under orbital shaking at 150 rpm is expressed with respect to the assays pre-incubated with 1000 μg/mL at pH 3.5 and without shaking. n.s.: not significant different from zero. Bars: standard error of the mean.

Fig. 5. Effect of the pH during the pre-incubation on the interaction between BHP's and gastric proteases. **a** Average loss of activity after pre-incubating gastric extracts at different pH values with 1000 μg/mL BHP's expressed with respect to the assays pre-incubated at the same pH but without BHP's. **b** Average stability of gastric proteases after pre-incubating gastric extracts at different pH values without BHP's, and expressed as a percent residual activity with respect to the maximum value from the same individual. Different letters on top of the columns indicate significant differences at the level 0.05. Bars: standard error of the mean.

Figure 1

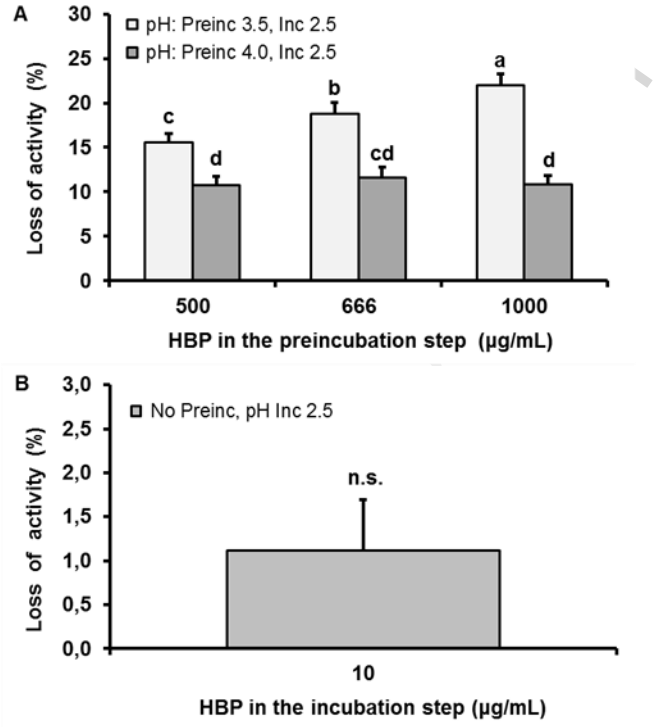


Figure 2

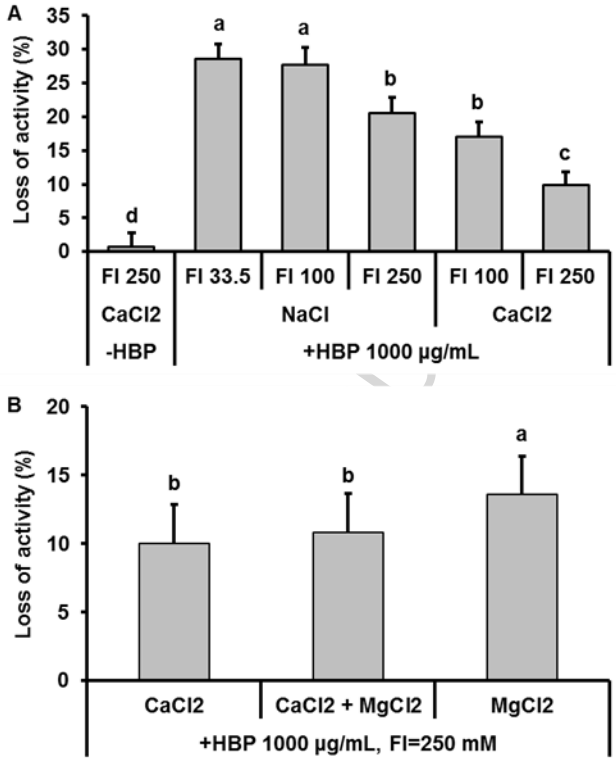


Figure 3

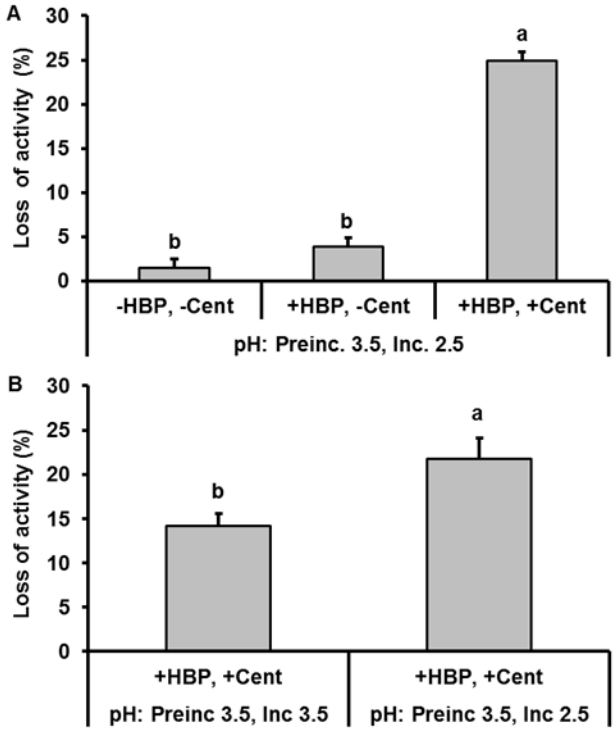


Figure 4

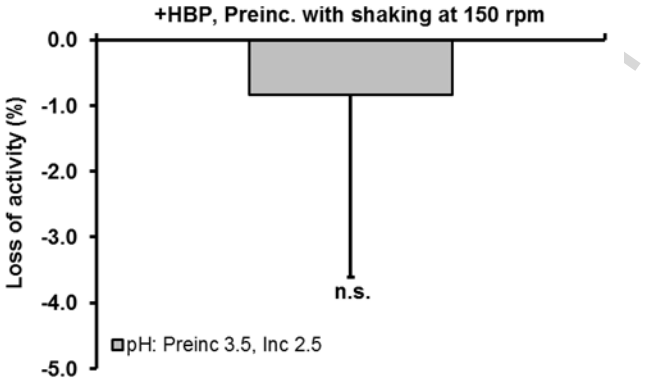
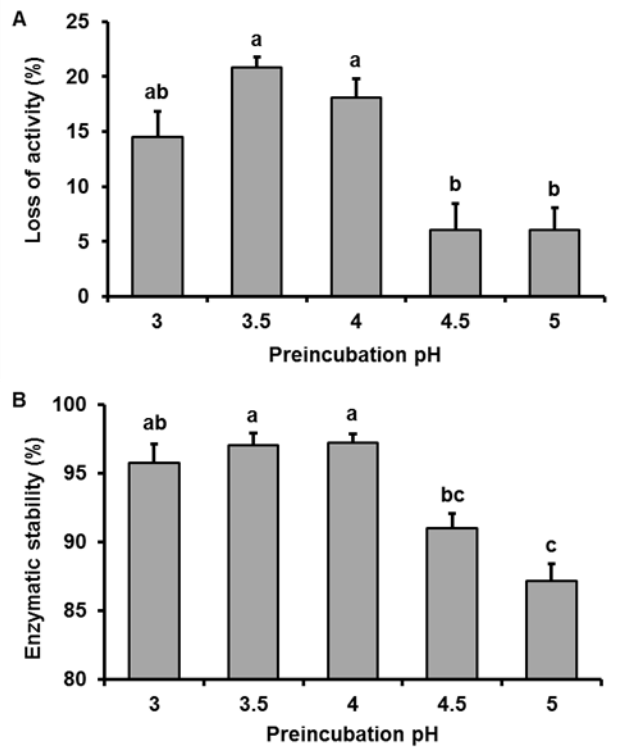


Figure 5



Statement of relevance

Aquafeeds are subject to heating processes producing the non-enzymatic browning of the diet. The effects of browning heavy products on digestive enzymes of commercial fish species are poorly researched. This work shed some light on this point and call researchers' attention to a nearly skipped over topic, being of the interest of aquaculture nutritionists and aquafeed producers

Highlights

Browning heavy products from a glucose-glycine model reduce the activity of gastric proteases of the rainbow trout

The activity reduction is dependent on the pH, ionic strength and the presence of divalent cations in the medium

The mechanism of the activity reduction is most probably based on the co-precipitation of gastric proteases and browning products