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Evaluation of different protein sources for aquafeeds by an optimised pH-stat system

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Abstract: This paper presents the results of some experiments oriented to optimise and standardise the measurement of protein hydrolysis by fish proteases using the pH-stat technique. Various factors affecting the degree of hydrolysis (DH) were considered (autohydrolysis of the protein sources, type of enzymes utilised, substrate/enzyme ratio and effect of inhibitors). The crude extracts obtained from fish digestive tissues showed their suitability for the determination of protein hydrolysis, rendering better results than those obtained using mixtures of commercial enzymes. DH values for a given protein were greatly affected by the substrate/enzyme ratio, since small modifications in protein concentration resulted in significant variations in DH. Protease inhibitors present in various plant protein sources produced a reduction in DH values.

Keywords: aquafeed; digestibility; enzyme; fish; hydrolysis; inhibitor; pH-stat; protease; protein; *Sparus aurata*

INTRODUCTION

The determination of the digestibility of the major nutrients is one of the main steps in the evaluation of their bioavailability for a given species. This is the reason why studies aimed at the measurement of digestibility of protein and energy are key to the formulation of adequate feeds for any species, being routinely performed in a great number of research centres all over the world. In the last few years, interest in the feasibility of developing *in vitro* digestibility tests has been renewed for a number of reasons, most of them related to both the high cost of analyses and the social actions concerned with animal welfare. Some of the advantages supporting the use of such methodologies are *cost-effectiveness, easy performance* and *ready availability of results*.[1,2] In this context the evaluation of a great number of ingredients employed in aquafeed formulation may benefit from the recent advances in various methodologies applied to the *in vitro* measurement of digestibility in feeds for aquatic animals.[3]

One the most interesting *in vitro* techniques is the measurement of protein hydrolysis using the pH-stat system, developed by Pedersen and Eggum,[4] who adapted the method previously assayed in Carlsberg laboratories. This method has proved its accuracy in predicting digestibility in both human foods and feeds for various terrestrial animals.[5–9] Dimes and Haard[10] suggested its utilisation in the evaluation of feeds for salmonids, providing the good correlation the authors found with digestibility measurements in those fish. More recently, the technique has been used for the optimisation of the protein fraction of microcapsules employed in feeding of marine fish larvae.[11] However, the use of the pH-stat technique in the measurement of protein hydrolysis by fish digestive enzymes requires a previous standardisation of the method. Several experiments were designed to determine the best conditions for future application of such methodology in fish nutritional studies. Studies were performed using digestive extracts of seabream (*Sparus aurata* L), an omnivorous marine fish, whose culture is rapidly expanding along the Mediterranean coasts.

EXPERIMENTAL

Preparation of active extracts

Live specimens of juvenile seabream (*S aurata*) ranging from 35 to 45 g in weight were provided by a local fish farmer (Predomar, Almería, Spain). Each fish had been fed a commercial diet (45% protein) three times a day to reach a total amount of feed representing 3.5% of its body weight. After killing specimens, previously starved for 6 h, by submersion in cold water (4 °C), the digestive tract was removed and cleaned. A portion of the anterior intestine containing pyloric caeca was homogenised (100 g l^{-1}) in cold distilled water. Supernatants obtained after centrifugation (16 000 × *g* for 30 min at 4 °C) were adjusted to pH 8.0 and stored at −20 °C until enzyme analysis. The concentration of soluble protein in enzyme extracts was determined by the Bradford method

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using bovine serum albumin (1 mg ml^{-1}) as a standard.[12]

Determination of protease activity in fish digestive extracts

Alkaline protease activity of pyloric caecal extracts was measured using casein, as previously done by Walter.[13] In brief, 20 µl of the enzyme preparation was mixed with 0.5 ml of 0.1 mol l^{-1} Tris-HCl buffer at pH 8.0 at 25 °C. Reaction was initiated by the addition of 0.5 ml of casein and stopped 30 min later by adding 0.5 ml of 20% trichloroacetic acid (TCA). After 10 min at 4 °C the reaction mixture was centrifuged at 16 500 × *g* for 5 min and the absorbance at 280 nm was recorded. For the control, TCA was added before the substrate. One unit of enzyme activity was defined as 1 µg tyrosine released min^{-1} using the extinction coefficient for tyrosine of 0.005 ml µg^{-1} cm^{-1}. All measurements were carried out in triplicate.

Protein solutions

Substrate solutions were prepared by manual homogenisation of different protein sources in distilled water. The amounts were calculated to give a final concentration of 8 g protein l^{-1}. The origin and proximate composition of the protein sources used in the assays are given in Table 1. Moisture, protein, fat, ash and total dietary fibre were determined according to AOAC methods.[14] Nitrogen was determined by the Kjeldahl method, and the nitrogen content was converted to protein by multiplying by 6.25.

Determination of degree of hydrolysis (DH) of proteins utilised in feeding of seabream using pH-stat

DH was determined by pH-stat titration following the method described by Dimes and Haard.[10] A 10 ml suspension of each protein source was adjusted to pH 8.0 with 0.1 mol l^{-1} NaOH and equilibrated to 37 °C for 10 min in a jacketed reaction vessel, prior to the addition of 0.2 ml of seabream digestive extract that provided an enzyme activity of 250 units. Such relative proportion between protease activity and feed protein was first established taking into account the potential intake and total enzyme activity determined for seabream of similar size.[15] The mixture was maintained under continuous agitation by a magnetic stirrer, purged with nitrogen gas to prevent incorporation of atmospheric CO_2 into the assay mixture, and at constant temperature (37 °C) using a thermal bath. The course of the reaction was automatically recorded for 60 min and plotted using a 718 Stat Titrino (Methrom Ltd, Switzerland). Protein hydrolysis was calculated from the hydrolysis equivalent (h) of the volume of standard alkali (0.1 mol l^{-1} NaOH) required to maintain the pH of the reaction mixture at 8.0:

$$h = [B \times (1/\alpha) \times N_b]/[M \times (S/100)]$$

where B (ml) is the volume of NaOH consumed during 60 min of enzymatic digestion,

$$\alpha = 10^{pH-pK}/(1 + 10^{pH-pK})$$

N_b is the normality of the titrant, M (g) is the mass of the reaction mixture and S (%) is the protein concentration in the reaction mixture. The degree of

	Protein	*Lipid*	*Ash*	*Fibre*	*Moisture*
Animal proteins					
Casein[a]	900	—	—	—	—
Squid meal[b]	748	88	34	ND	81
Fish meal 1[c]	624	83	174	99	59
Fish meal 2[d]	606	93	203	—	81
Fish meal 3[e]	700	—	—	—	—
Blood meal[f]	870	15	20	—	70
Meat and bone meal[c]	641	103	254	24	77
Plant proteins					
Soybean meal (thermally treated)[c]	499	26	64	139	105
Lupin meal[c]	429	153	35	202	91
Corn gluten meal[g]	600	60	—	50	120
Soybean meal[b]	245	12	—	—	96
Bean meal[b]	255	20	75	—	98
Pea meal[b]	220	26	55	—	80

[a] Casein Hammerstein. Source: ICN Biomedicals, Madrid, Spain.
[b] Source: local supplier.
[c] Source: Grupo de Investigación en Acuicultura, Las Palmas de Gran Canaria, Spain.
[d] Source: Piensos La Foca, Almería, Spain.
[e] Papain-hydrolysed fish meal. Source: local supplier.
[f] Source: Aprocat SA, Palencia, Spain.
[g] Source: Campoebro Industrial SA, Zaragoza, Spain.
ND, not detected.

Table 1. Nutrient composition of protein sources utilised in assays (g kg^{-1} dry weight)

protein hydrolysis (DH) was calculated from h as follows:

$$DH(\%) = (h/h_{tot}) \times 100$$

where h_{tot} is the total number of peptide bonds in the protein expressed as meq g^{-1} protein. For defined proteins the number of peptide bonds was estimated from the average molecular weight of amino acid residues, which was calculated from the amino acid composition. If the amino acid composition is unknown, an average value of 8.0 meq g^{-1} protein can be assumed. Each determination was performed in triplicate and various experiments were designed in order to determine how different factors could affect the measurement of DH.

Experiment 1. Effect of autohydrolysis of protein sources in absence of fish enzymes
Basal values of protein hydrolysis were measured in different feedstuffs or compound feeds using the methodology described above. Fish digestive extracts were replaced by an equal volume of distilled water. DH values were expressed as a percentage of those obtained when fish enzymes were utilised.

Experiment 2. Effect of type of enzyme utilised in analysis
DH measurements were carried out by incubating different feedstuffs or compound feeds in the presence of either crude fish digestive extracts or a mixture of three commercial enzymes as detailed by Hsu *et al*:[16] 1.6 g l^{-1} porcine trypsin (EC 3.4.21.4, Sigma T-8003), 3.1 g l^{-1} bovine chymotrypsin (EC 3.4.21.1, Sigma C-7762) and 1.3 g l^{-1} porcine amino peptidase (Sigma P-7500). The mixture was adjusted to reach an activity equivalent to that of crude extracts (250 units of protease activity).

Experiment 3. Effect of acid pre-digestion of sample proteins
An acid pre-treatment of protein samples was carried out as described by Saunders *et al*[17] to simulate stomach digestion. Protein solutions were incubated at 37 °C for 60 min in 0.1 mol l^{-1} HCl (pH 2.0) + 1000 units of acid protease (porcine pepsin, EC 3.4.23.1, Sigma P-7012). Then the pH of the solution was adjusted to 8.0 with 1 mol l^{-1} NaOH and the rest of the pH-stat analysis was performed as usual. DH values were compared with those obtained without acid pre-treatment.

Experiment 4. Effect of substrate concentration
Changes in DH values obtained when varying the protein substrate/enzyme (S/E) ratio, ranging from 50 to 1500 μg protein substrate $unit^{-1}$ protease activity, were assayed using lupin meal and fish meal. This assay was done by progressively increasing the amount of protein substrate (g of meal) in the reaction mixture while keeping the volume of enzyme preparation constant (0.2 ml to provide 250 units of alkaline protease activity).

Experiment 5. Effect of protease inhibitors present in protein sources
Taking into account the results obtained in the previous experiment, DH values were measured in mixtures made using casein and different plant proteins combined in different proportions (9:1; 8:2; 7:3; 6:4 and 5:5) but maintaining a fixed S/E ratio of 320 μg protein $unit^{-1}$ protease activity. This design allowed the separation of the inhibitory effects produced by an increasing concentration of hydrolysis products from those due to specific inhibitors present in the assayed feedstuffs (soybean meal, lupin meal and corn gluten meal).

Statistical methods
All determinations were carried out in triplicate. Before one-way ANOVA, percentages were changed to arcsin($x^{1/2}$). Differences at $P < 0.05$ were analysed by the Tukey test. The Statistix 4.0 package (Analytical Software, Pheonix, Arizona, USA) was used. The fitting of data for plotting curves in inhibition assays was performed using the statistical module of the Excel 5.0 spreadsheet (Microsoft, Atlanta, Georgia, USA).

RESULTS
Experiment 1. Effect of autohydrolysis of protein sources in absence of fish enzymes
The degree of hydrolysis measured in protein samples in the absence of enzyme extracts is detailed in Table 2. This autohydrolysis accounted for nearly 30% of DH values measured in some proteins (fish meal, meat and bone meal and soybean meals) after enzymatic digestion. The effect was less in the other protein sources studied, and almost negligible in the case of hydrolysed fish meal, blood meal and casein.

Experiment 2. Effect of type of enzyme utilised in analysis
The degree of hydrolysis measured for different animal and plant proteins using either a three-enzyme system or crude fish digestive extracts is detailed in Table 2. In most cases the values obtained using crude extracts were significantly higher than those measured using commercially prepared enzymes, although the standard deviation in measurements also increased. DH values obtained using both types of enzymes were significantly correlated ($r = 0.875$; $P < 0.05$).

Experiment 3. Effect of acid pre-digestion of sample proteins
DH values measured for different animal and plant proteins and commercial fish feeds, including or not an acid pre-digestion of samples, are detailed in Table 3. The results obtained allowed the classification of the proteins into two groups: (a) those in which DH values were not significantly affected by an acid pre-digestion

	Baseline				
	Absolute	*%*	*Three-enzyme*	*Crude extract*	Δ
Casein	0.10±0.02	0.6	11.39±0.63	16.98±0.15	*
Fish meal 1	1.39±0.22	25.1	5.46±0.12	5.53±0.56	NS
Fish meal 2	1.67±0.32	29.8	4.68±0.04	5.61±0.45	NS
Fish meal 3	0.59±0.06	5.4	6.21±0.03	10.89±0.25	*
Meat and bone meal	1.96±0.09	36.4	2.83±0.37	5.38±0.69	*
Blood meal	0.18±0.04	3.9	1.52±0.13	4.61±0.23	*
Soybean meal (thermally treated)	1.35±0.08	24.3	4.72±0.17	5.56±0.36	NS
Soybean meal	1.70±0.07	35.5	—	4.79±0.13	
Lupin meal	0.48±0.12	6.9	4.30±0.12	6.97±0.30	*
Corn gluten meal	1.09±0.26	18.9	2.27±0.27	5.76±0.39	*

Table 2. Values of autohydrolysis and degree of hydrolysis (% DH) obtained after enzymatic digestion of different protein sources. Data are mean of triplicate measurements ± SD

* $P < 0.05$.
NS, not significant.

(squid meal, lupin meal and green pea meal); and (b) those which were much better hydrolysed after such treatment, where DH values increased by 67–131% (corn gluten meal, meat and bone meal, fish meal, soybean meal and blood meal).

Experiment 4. Effect of substrate concentration

Changes in DH values of lupin meal and fish meal obtained when varying the S/E ratio are presented in Fig 1. Small variations in the relative concentration of protein within the range 50–500 μg protein unit^{-1} protease activity resulted in two- to fourfold increases in DH values, especially when lupin meal was tested. A further increase in the relative concentration of substrate protein did not result in noticeable DH changes.

Experiment 5. Effect of protease inhibitors present in protein sources

Variations in DH values obtained when total S/E was held constant but when the relative proportion of three different plant proteins to total protein was changed are shown in Fig 2. A progressive decrease in DH was observed in all cases as their relative proportion in the assay mixture was increased. Reductions in DH accounted for 30–60% of the values measured when casein was used as the only substrate.

DISCUSSION

The pH-stat technique has proved to be highly valuable in the prediction of protein digestibility in foods.[18–20] Recently, it has also been used to assess the digestibility of different proteins for marine fish[21] and crustaceans.[22,23] In spite of the good results that have been obtained when correlating digestibility values of protein determined *in vivo* and *in vitro* for salmonids, a number of features not considered in such studies should be assessed for an improved use of the technique in fish nutrition.

The early pH-based methods measured the drop in pH during the enzymatic reaction. However, some substances present in the protein may influence the pH decline in this assay, thus affecting the *in vitro* estimation. The pH-stat method has improved this limitation by keeping the pH constant during the enzymatic digestion. Nevertheless, some authors suggest that pH-stat is not a valid method for the measurement of protein digestibility, because differences in the buffering capacity of proteins could interfere with the measurement of protein hydrolysis.[24,25] In this sense the determination of baselines can be used as an indicator of such buffering capacity.[26,27] The baseline consumption of alkali was highly variable and, in some samples, considerable, amounting to up to 35% of the total uptake of alkali during the enzymatic digestion period. Results showed

	Alkaline	*Acid + alkaline*	*Difference*	*%*
Fish meal 1	5.53	10.10±0.40	*	82.6
Squid meal	14.25	14.65±0.25	NS	2.8
Ovalbumin	2.91	1.88±0.03	NS	−35.4
Meat and bone meal	5.38	9.47±0.60	*	76.0
Blood meal	4.61	10.67±0.78	*	131.5
Soybean meal (thermally treated)	5.56	10.55±0.37	*	89.7
Lupin meal	6.97	7.29±0.09	NS	4.6
Corn gluten meal	5.76	9.67±0.45	*	67.9
Green pea meal	8.37	8.41±0.06	NS	0.5

Table 3. Effect of previous enzymatic acid digestion on final values of hydrolysis (DH). The increase in DH is also expressed as a percentage. Data are mean of triplicate measurements ± SD

* $P < 0.05$.
NS, not significant.

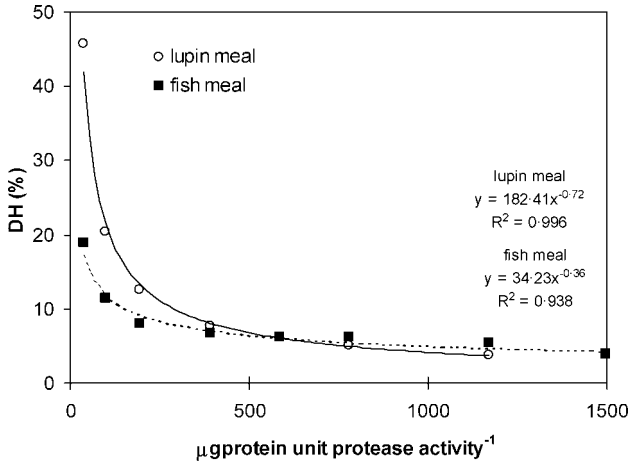

Figure 1. DH of lupin meal and fish meal proteins measured under different substrate/enzyme ratios.

that higher autohydrolysis values do not correspond with higher DH values in the presence of enzymes. Surprisingly, fish meal has a higher baseline uptake of alkali in comparison with enzymatic hydrolysed fish meal (Table 2). In contrast, papain hydrolysed fish meal yielded twice the DH value of fish meals 1 and 2. The uptake of alkali measured in assays without mammal or fish enzymes does not adequately predict the autohydrolysis in the presence of enzymes.[4] For instance, endogenous enzymes in samples that may contribute to the autohydrolysis might be degraded by the added proteolytic enzymes. This might partially explain the lower autohydrolysis value of fish meal 3. The exogenous enzyme employed in the elaboration of the hydrolysate could inactivate endogenous enzymes. For production of hydrolysates, after the desired DH was achieved, the enzymes were inactivated, and a lower baseline confirms complete inactivation.[27] In studies relating *in vitro* hydrolysis to digestibility, it seems reasonable to use total values comprising the autohydrolysis and hydrolysis produced by intestinal proteases. However, Pedersen and Eggum[4] found that

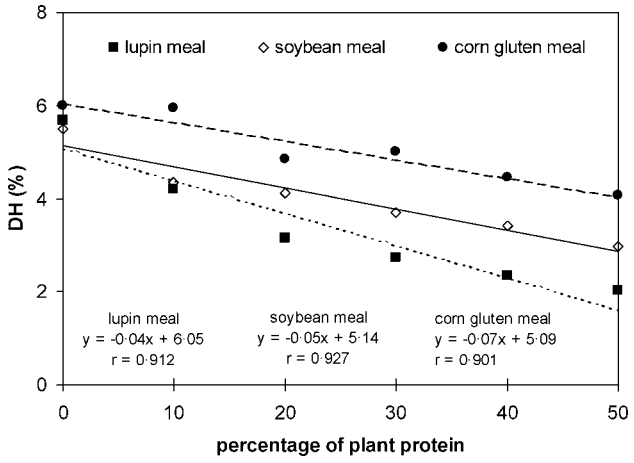

Figure 2. Changes in DH of protein solution obtained when amount (in % of total protein) of different plant protein sources is increased.

corrections of autohydrolysis did not result in a more accurate estimation of *in vitro* digestibility. Nevertheless, when weighing the ability of fish enzymes to hydrolyse a given protein, such basal values must be taken into account in total measurements.

DH quantifies the number of peptide bonds hydrolysed in a given protein, irrespective of the site of hydrolysis. Hydrolysis by endo- or exo-proteases was not differentiated by this method. The differences in DH values between the fish meals studied could be related to the localisation of the site of hydrolysis. Proteins within fish meal 3 have been pre-hydrolysed by papain and transformed into oligopeptides, whereas proteins within fish meals 1 and 2 were non-hydrolysed. The presence of oligopeptides in the reaction mixture provides a suitable substrate for endo- and/or exo-proteases, but particularly exo-proteases (amino- and/or carboxypeptidases). Under such conditions the action of exo-proteases is independent of the occurrence of specific amino acids within peptides. In the presence of exo-proteases the higher concentration of oligopeptides (fish meal 3) in the reaction mixture leads to a greater number of total peptide bonds being broken down when compared with the same non-pre-hydrolysed proteins. This effect occurs when the enzymatic mixture includes exo-proteases (both the three-enzyme and the crude fish enzyme systems), but it is more evident when the enzyme preparation includes several types of exo-proteases, as seems to be the case of fish digestive extracts.

Assays with pH-stat routinely use commercial proteases developed for assay of human protein digestibility.[16,28] Nevertheless, such enzymes are obtained from mammals, and several of their functional characteristics are greatly different from those of fish proteases.[29] Mammal enzymes are inappropriate for examining protein digestibility in fish owing to differences in digestive physiology between phyla. For example, characterisation of fish enzymes reveals differences with respect to properties such as catalytic activity at low reaction temperature, pH and temperature activity and stability and inhibitor sensitivity when compared with mammalian enzymes.[30] *In vivo* hydrolysis of food proteins is the result of the proteolytic action of a battery of proteases, endo- and exo-proteases. *In vitro* hydrolysis by a wide range of proteases could better reflect the *in vivo* hydrolytic process. Commercial multi-enzyme assays involve three or four proteases, while crude fish digestive extracts include several proteases such as trypsin, chymotrypsin, carboxypeptidases and aminopeptidases.[15] *In vitro* evaluation of protein digestibility requires the use of appropriate enzymes in the digestion reactor. In the present study it was demonstrated that crude extracts obtained from the seabream digestive system produced higher DH values than those measured using commercial enzymes. Since noticeable differences in substrate specificity or sensitivity to inhibitors have been found in proteases of different fish species,[31] the use of species-specific

extracts is a better alternative for the development of a pH-stat system in fish.[10,32,33]

Considering that *in vitro* assays tend to reproduce processes occurring in the digestive tract, some authors have tried to simulate stomach and intestine digestion using pepsin and one or more alkaline proteases.[17,34] The effect of such an acid pre-digestion was evaluated in this study using a combination of HCl and pepsin. Results indicated that in most cases an acid pre-treatment of samples performed under the described conditions increased final DH values. This resulted in more homogeneous values for all samples when compared with those obtained using only the alkaline phase (eg 4.61 for blood meal and 5.56 for soybean meal changed into 10.67 and 10.55 respectively). This suggests that an excessive hydrolysis could take place and that a less drastic acid treatment (shorter time, higher pH) could better simulate stomach digestion in fish. This should be in agreement with modifications in the methodology suggested for the avoidance of excess hydrolysis.[35] An improved methodology including the use of enzymatic extracts from fish stomach and intestine is currently being assayed.[36]

Standardisation of DH determinations also comprises the assessment of relative S/E concentrations utilised in the assays. Quite different ratios have been used by different authors, ranging from 5–10[26,37] to 40 and even 100 g kg^{-1}.[27,38] All these authors employed commercial enzymes. Consequently, a somewhat constant activity should be expected in their experiments. Nevertheless, if crude extracts showing a variable activity were employed, it would be more useful to express an S/E ratio standard in mg substrate unit^{-1} activity (or the inverse). In this study it was demonstrated that DH changes greatly if this ratio is modified. DH values of lupin meal and fish meal proteins measured at a ratio of 50 μg protein unit^{-1} protease activity (45 and 20% respectively) were reduced to only 5% when the ratio exceeded 500 μg protein unit^{-1} protease activity (Fig 1). Possible reasons accounting for the decrease in reaction rates are loss of enzyme activity, substrate exhaustion and end-product inhibition. Loss of enzyme activity was not recorded at the pH and hydrolysis time assayed.[15] Substrate concentrations were such that substrate exhaustion was also unlikely. Such a decrease in reaction rate may be due to end-product inhibition.[39] One of the limitations of employing closed assays is related to the potential for inhibition of enzyme activity by reaction products and indigestible food residues, because their accumulation is likely to affect the efficiency and rate of hydrolysis of the substrate.[40]

Since there were greater differences between DH measured in both protein sources using low enzyme concentrations, it can be suggested that better accuracy of measurements could be obtained under such conditions. Although this conclusion presupposes a much better digestibility of lupin meal protein than that of fish meal, it is not backed by data on protein digestion obtained for such protein sources by Robaina *et al.*[41] Such a high hydrolysis of lupin meal protein measured when a low substrate concentration was used (more than twice that of fish meal) may be explained by the convergence of different factors. *First*, there may be differences in solubility and/or buffering capacity of proteins. Even though comparative assays employed the same amount of fish and lupin protein (Kjeldahl × 6.25), protein solubility differs between samples. In this sense, Carbonaro *et al*[42] found that legume proteins vary their protein solubility profile, showing maximum values of solubility at pH above 7.0 and below 2.0. *Second*, the percentage of amino acids susceptible to cleavage by alkaline proteases (trypsin- or chymotrypsin-like) is greater than 25% in lupin protein but less than 22% in fish meal protein. The quantitative level of these amino acids in a given protein could determine the extent of enzymatic hydrolysis. *Third*, the susceptibility of peptide bonds to proteases depends on their accessibility and flexibility.[43] The accessibility and localisation of amino acid residues (lysine, arginine, phenylalanine, tryptophan and tyrosine) specific to protease action would be of great importance because of the restricted specificity of the main proteases trypsin and chymotrypsin.

It follows that if DH values are used as indicators of protein digestibility, conditions of the assay should resemble more closely what occurs *in vivo*. A 50 g seabream is able to produce approximately 2000 units of alkaline protease activity immediately after a meal;[15] if it receives (in each meal) an amount of feed representing nearly 4% of its weight and containing 50% protein, the expected final ratio S/E reached in the gut should be close to 500 μg protein unit^{-1} protease activity. This value should be situated in the part of the curve where variations in DH are mainly influenced by the quality and not the quantity of protein.

The observed reduction in DH when the concentration of substrate in the medium is increased suggests the existence of some type of inhibition affecting measurements. Such inhibition should be due to either enzyme saturation by the final products of protein hydrolysis or to the presence of specific protease inhibitors present only in some protein sources. In the first case, DH values measured for different proteins at the same S/E should be similar, but they should be clearly modified if protein hydrolysis is affected by protease inhibitors. The second hypothesis was evaluated using a constant amount of total protein (constant S/E ratio) but changing the relative proportion of plant protein from 0 to 100%. Specific inhibition was evidenced by a progressive decrease in DH as the relative proportion of the protein source containing the inhibitor increased (Fig 2). This effect was detected when lupin, soybean and corn gluten meals were assayed, which is in agreement with the demonstrated existence of protease inhibitors in such protein sources.[44]

To date, most researchers concerned with the

development of *in vitro* digestibility techniques have found difficulties in correlating their results with those obtained *in vivo*. Furuya[45] found that predictability of ileal digestibility in pigs depends on the type of food and particle size, being low for cereal grains. As a rule, best results seem to be obtained for compound feeds, probably because errors in the determination of the different ingredients are mutually compensated.[46] In the case of fish, additional difficulties are related to the scarcity of data on protein digestibility in fish other than salmonids. A better predictability of *in vivo* digestibility of protein using DH is expected when more data become available.

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