IN VITRO SIMULATION OF PROTEIN DIGESTIVE BIOACCESSIBILITY IN SENEGALESE SOLE USING THE RESPONSE SURFACE METHODOLOGY

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Introduction

Considering the digestive system as a bioreactor may give an insight on the relative influence of different factors affecting its functionality, helping to optimize it. The present work uses a novel approach to develop a mathematical model explaining the effect of three main factors - temperature, total reaction time, and enzyme:substrate ratio - in the hydrolysis of proteins by intestinal enzymes in the Senegalese sole (*Solea senegalensis*). We combined a factorial design, based on the Response Surface Methodology, and *in vitro* digestibility assays adapted to the physiology and culture conditions of this species. This fast, non-invasive, and low-cost methodology can be used as a tool to maximize the biological response under variable conditions and may orientate on-farm feeding practices.

Materials and Methods

Adult Senegalese sole (N=33; 274 ± 106 g) were sampled at different postprandial moments. Enzyme extracts used for the *in vitro* assays were prepared by homogenization of the intestine followed by centrifugation, filtering, and freeze-drying the extract. The *in vitro* assays were carried out in a bioreactor consisting of two chambers separated by a semi-permeable membrane of 3,500 kDa. The upper part of the chamber contained the substrate (fishmeal, 75.4% crude protein) and the enzyme extract dissolved in phosphate buffer (pH 7.0), while the lower part contained only buffer. The amino acids released by the hydrolysis passed across the membrane, and they were recovered at different moments to be measured. The bioreactor was maintained inside a thermal chamber.

The effect of three key factors on the hydrolysis of protein by intestinal enzymes of the Senegalese sole intestine was assessed, taking into account the farming conditions and practices in southern Europe for this species, for: 1) Temperature (T), from 16 to 26°C, based on the culturing maximum and minimum seawater temperatures. 2) Total reaction time (t), from 4 to 8 h, estimated according to feeding frequencies for medium size individuals, as well as to data on gut transit rates (Dias et al., 2010)"ISSN" : "00448486", "abstract" : "A study was conducted with Senegalese sole (Solea senegalensis. 3) Enzyme:substrate ratio (S), estimated for an 100 g model fish, of 100g considering the protease activity in the fish used in this experiment and the average protein intake per meal when using commercial feeds. The range (0.48-0.84 U. $mg.^{-1}$ protein) was achieved maintaining a fixed amount of enzyme and changing the amount of protein in the assays (from 400 to 700 mg).

A 3-level Box-Behnken factorial design, and an orthogonal least-squares calculation (Minitab® 17 software), were used to obtain empirical equations describing the effect of these factors on the response variable: the relative efficiency of the hydrolysis expressed as the percentage of the amino acids to the initial protein used in each assay (mg.aa per 100mg of protein). The general form of the polynomial equations is

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2$$

where Y is the response variable, X_1, X_2, and X_3 are the independent variables, and βs are the regression coefficients for intercept, linear, quadratic, and interaction terms.

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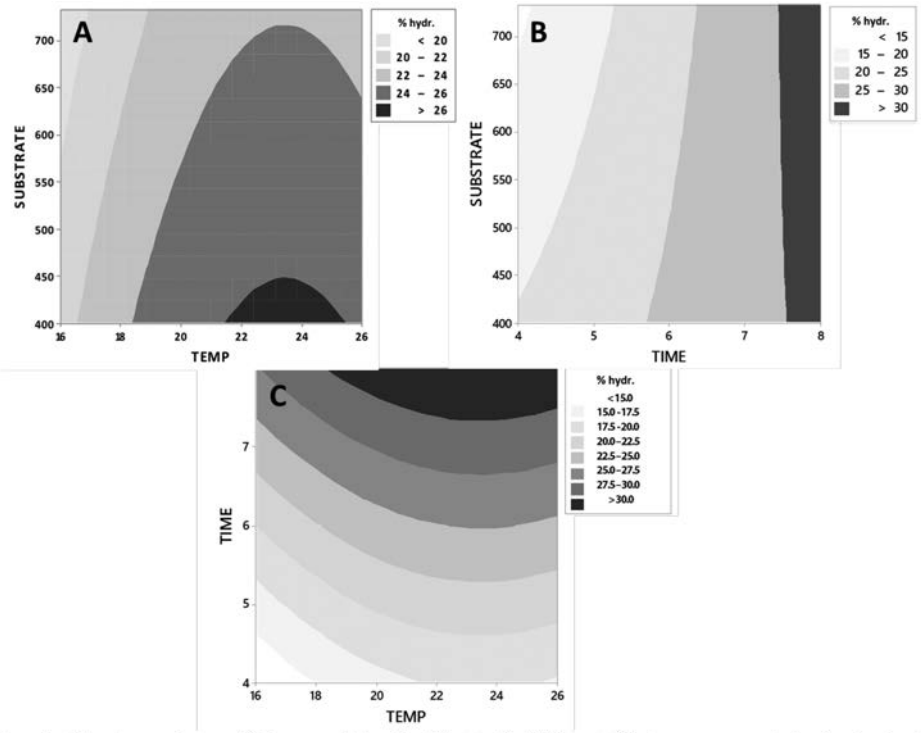

Fig. 1. Contour plots of the combined effect of different factors on protein hydrolysis. Substrate, reaction time, and temperature were fixed at 567 mg, 6 h, and 21 °C, respectively, in plots A, B, and C.

Results

Results varied thoroughly depending on the values of the considered factors: from 12.9 (16 °C, 4 h, 566.5mg of substrate) to 36.5mg per100 mg of protein (26°C, 8 h, 566.5mg of substrate) released as a result of the enzyme hydrolysis. Although the fitting of the initial regression models was acceptable ($R^2 = 0.845$), it contained a number of non-significant terms that influenced the significance of the lack-of-fit test ($P = 0.026$). Hence, a more simplified regression model was generated using the process of backward elimination of some of the non-significant coefficients:

$$\% \text{ hydrolysis} = -23.2 + 4.27\,T + 0.39\,t - 0.0420\,S - 0.0910\,T^2 + 0.00576\,T*S$$

This model maintained a high R^2 (0.816) and the significance of the lack of fit test was $P > 0.05$. It was deduced that the efficiency of protein hydrolysis by intestinal enzymes was linear and positively correlated to incubation time, showing temperature a more complex effect that combines both linear and quadratic terms and results in a non-linear response characterized by a maximum around 23°C. The amount of available substrate showed an inverse, not significant correlation to the efficiency of the hydrolysis (Fig. 1).

Discussion and Conclusion

It is clear that the operation of a real gut is much more complex than this model and that, in the living fish, a number of adaptive responses may modify to a great extent some of the obtained results. However, this approach provides a detailed knowledge on the influence of different factors in the evaluated response. As an example, results obtained here suggest that for the Senegalese sole, the efficiency of protein hydrolysis decreases notably above an optimum temperature, and that adapting feeding frequencies to optimize gut retention time may have a significant effect on such hydrolysis.

References

Dias, J., Yúfera, M., Valente, L.M.P., Rema, P., 2010. Feed transit and apparent protein, phosphorus and energy digestibility of practical feed ingredients by Senegalese sole (*Solea senegalensis*). Aquaculture 302, 94–99.

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