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Studies on digestive enzymes in fish: Characterization and practical applications

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SUMMARY - Due to the special features of the aquatic environment, fish nutritionists are well aware of the difficulties and errors obtained when determinations of digestibility are carried out *in vivo*. Nevertheless, digestibility measurements for different feed ingredients, especially in the case of proteins, remain the nucleus of most of the experiments aiming at evaluating raw materials. At present, digestibility trials performed *in vivo* may be complemented, and in some cases preceded or substituted, by several biochemical techniques offering a wide range of practical applications. A three-step approach is proposed as a suitable means to obtain valuable results. Such an approach begins with the appraisal of specific activities of selected digestive enzymes, followed by the characterization of such activities, and finally by the utilisation of all this information in order to simulate *in vitro* the digestive process taking place in fish or in aquatic organisms.

Key words: Digestive enzymes, fish, *in vitro* digestibility.

RESUME - *"Etudes sur les enzymes digestives chez les poissons : Caractérisation et applications pratiques." L'étude de la digestibilité des différents ingrédients d'un aliment, spécialement dans le cas de la protéine, constitue le centre de la majorité des expériences visant à évaluer les matières premières. Par ailleurs, il est bien connu des chercheurs en alimentation des poissons, que toutes les difficultés et erreurs dérivées de l'expérimentation "in vivo" avec ces animaux sont dues surtout à la particularité de leur milieu aquatique. Néanmoins, avec plusieurs techniques biochimiques on peut obtenir une information ayant une vaste gamme d'applications pratiques. On a proposé une approche en trois étapes comme une bonne voie pour obtenir des résultats d'intérêt. Cette approche commence par l'estimation des activités spécifiques de certaines enzymes digestives, suivie par la caractérisation de ces activités et finalement l'utilisation de toute cette information pour simuler "in vitro" le processus digestif qui a lieu chez le poisson ou tout autre organisme aquatique.*

Mots-clés : enzymes digestives, poisson, digestibilité *in vitro*.

INTRODUCTION

As the aquaculture industry grows, the need for specialized feeds designed for particular production situations is increasing. To date, nutritionists and feed manufacturers have concentrated their efforts on determining which of the wide variety of feedstuffs available to the feed industry may be used to produce lower cost aquaculture feeds. On the other hand, the successful mass rearing of fish and shrimp larvae has a large economic importance in marine aquaculture. Consequently, a great part of current research in fish nutrition and feeding is devoted to the development of artificial diets for larvae of the more common cultivated finfish. In both cases, a detailed

knowledge concerning the digestive physiology of aquatic animals is a prerequisite when formulating feeds; it follows therefore that studies aimed at determining the main features of the digestive system of different species, as well as evaluating the possible effect of antinutritive compounds present in many feeds are needed. In addition, some workers recommend that research efforts be focused on the development of simple *in vitro* digestion techniques for the rapid estimation of nutrient digestibility (Tacon, 1995).

The present paper summarizes some of the results obtained in the study of proteases, enzymes with a major importance in digestibility studies because of their involvement in the process of protein degradation, although they can also be applied to a large extent to the study of carbohydrate and lipid digestion.

THE STUDY OF DIGESTIVE ENZYME ACTIVITIES

The utilization of unespecific (casein, haemoglobin) or very specific synthetic substrates, like tosyl-arginin-methyl ester (TAME) or benzoyl-tosyl-ethyl ester (BTEE) allows to carry out quick and easy determinations of specific activities for both acid and alkaline proteases. The appraisal of specific activities is oriented to the determination of both the total amount and the temporary evolution of such activities. Obviously, total protease activity is calculated taking into account not only the specific activity of the enzymes, but also the total production of such enzymes, which in turn depends upon factors such as fish size, water temperature and feeding regime.

Data obtained from different authors have been compiled by Alarcón et al. (1995) and show that great differences exists in specific activities of alkaline proteases for several fish species (Figure 1). Such data may be useful in assessing the ability of a given species to digest proteins, since the measurement of specific activities gives an idea of how powerful are the "tools" the fish has to digest its food. A similar result can also be obtained when studying carbohydrases or lipases.

The determination of changes in enzyme activity during larval development may be useful in assessing the optimal moment for weaning as well as dependence of larvae on exogenous sources of enzymes (i.e. live food). The study of the evolution of protease activity during the first weeks of larval life gives valuable information to determine if the larva is well equipped to digest artificial feeds and allows for comparisons between species (Figure 2). This information is of a great importance for the development of artificial feeds for marine fish larvae (Moyano et al., 1996). In this sense, the selection of ingredients is critical, requiring a more detailed characterization of specific enzyme activities. For this purpose, the use of several techniques, mainly based on the use of specific substrates and inhibitors combined with the use of polyacrylamide gel electrophoresis (SDS-PAGE; Figure 3) completes the information concerning the type of enzymes present in a given organism (García-Carreño and Haard; 1993). This may be useful in assessing the most suitable composition of dietary proteins, taking into account that the selective breakdown of protein molecules is carried out by attachment of chymotrypsin or trypsin to different amino acids (tyrosine/phenylalanine or lysine/arginine, respectively). Thus, knowing the predominance of one of these enzymes in the digestive system of a fish larvae, it would be possible to select proteins rich in particular amino acids so as to enhance its digestibility

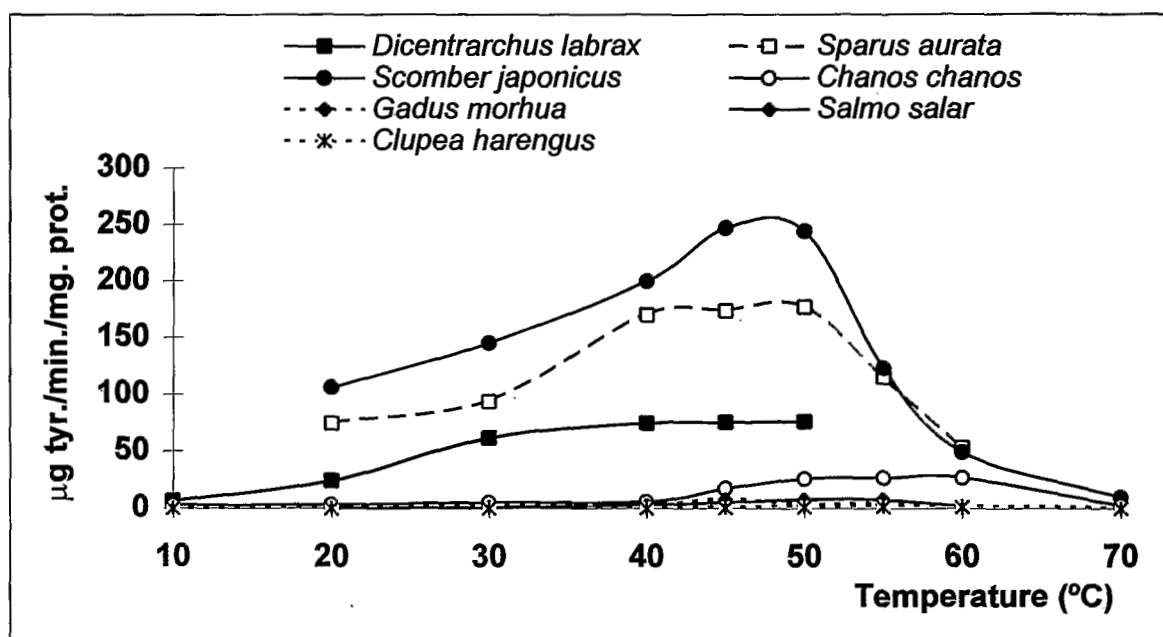


Fig. 1. Changes in alkaline protease activities in relation to temperature determined in different fish species (Alarcón et al., 1995)

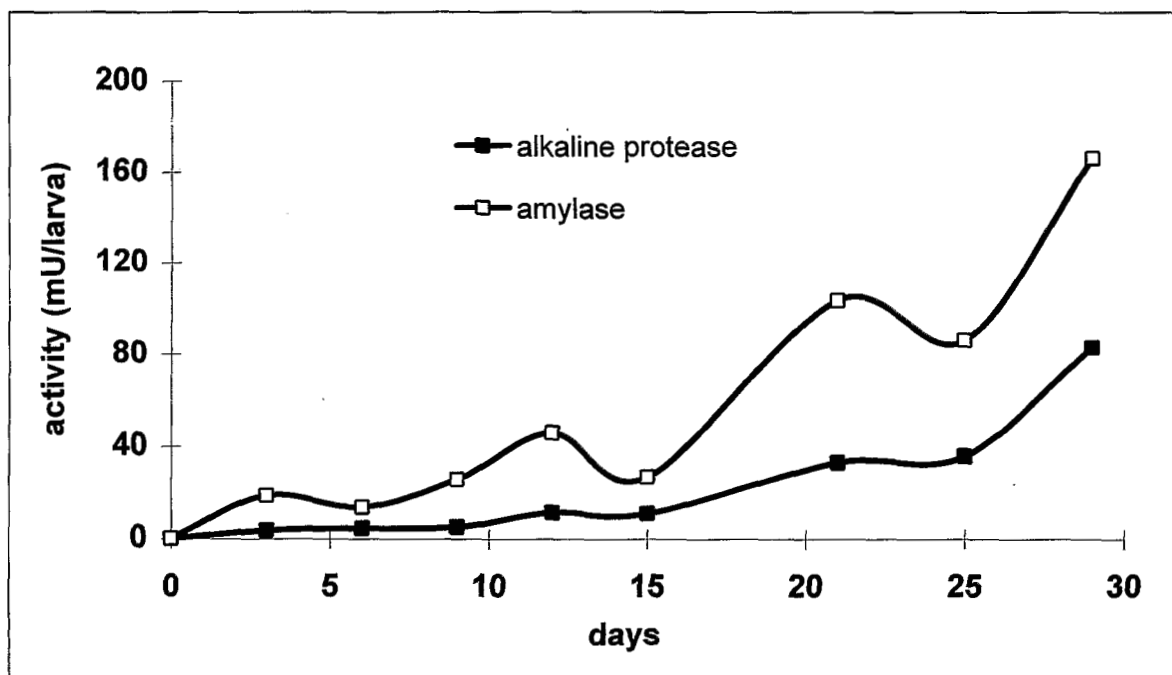


Fig. 2. Digestive enzyme activities in larval seabream during the first month of development (Moyano et al., 1996).

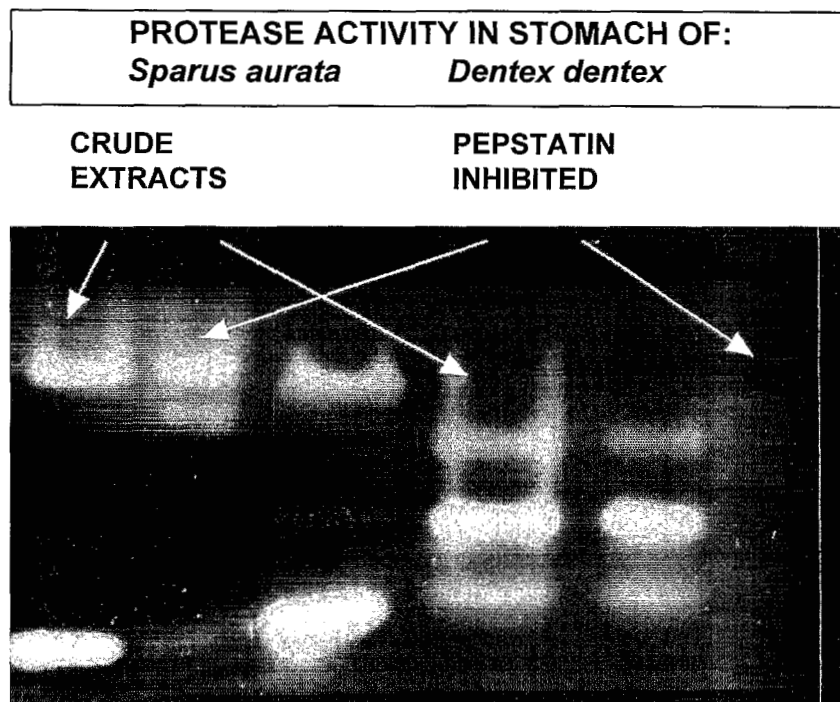


Fig. 3. Zymograms performed on the stomach extracts of seabream (*Sparus aurata*) and *Dentex dentex*. Specific inhibition of aspartic proteases by pepstatin is evidenced by disappearance of bands.

In addition, zymograms performed by SDS-PAGE are powerful tools to evaluate the relative importance of exogenous sources of enzymes (live food) in the digestive process during larval stages. For example, the application of such techniques to the extracts obtained from seabream larvae fed on rotifers and *Artemia* nauplii have been used to show if alkaline proteases existing in the rotifer were quantitatively important in relation to the total digestive proteases of seabream larvae, these being revealed as clear bands in zymograms of such extract (these being particularly evident considering that the molecular masses of these proteases have been estimated to be about 800.000 kDa; several times higher than those of fish larvae). However, bands corresponding to rotifer proteases have not been detected in zymograms of larvae fed on such organism (Díaz et al, in press). A similar result could be claimed for *Artemia*, since, as in the case of rotifers, its alkaline proteases are quite different to those existing in larvae and they did not appear in significant amounts in the digestive extracts of fish.

"IN VITRO" DIGESTIBILITY STUDIES

The simulation of the digestive process *in vitro* is widely utilized in the evaluation of feeds for terrestrial animals, and may also be applied in nutritional studies on aquatic animals. We suggest a combined approach based on the measurement of the degree of

hydrolysis (DH) for a given protein using a pH-stat, followed by a detailed study of the products obtained during such digestion that may provide useful information to characterize the type of protein digestion.

The determination of the DH for a protein using the pH-stat is based on the continuous titration of a reaction mixture composed by an extract obtained from fish gut (or hepatopancreas in the case of shrimp) and a substrate (a single protein or a diet) (Dimes and Haard, 1994). It is noteworthy that better results are obtained when using crude extracts obtained from the digestive tract of fish rather than using combinations of commercially purified enzymes, as it was the case for the three-enzyme system tested by Hsu et al (1977). This mixture is maintained under continuous agitation by a magnetic stirrer and at a constant temperature using thermal bath. The breakdown of protein chains and release of free amino acids to the medium results in a pH decrease; the later being neutralized by the continuous addition of small volumes of alkali. A plot over the course of this reaction gives a representation of the degree of hydrolysis for such a protein (Figure 4). The determination of the DH is useful, especially from a comparative point of view, in assessing the susceptibility of a given protein to be degraded by fish proteases and allows the establishment of correlations with data obtained *in vivo*. A further improvement of the technique is obtained by a two-step sequence including an acid predigestion; this enhancing total hydrolysis and resulting in a better correlation to data obtained in experiments performed using live fish.

Additional information concerning the process of protein digestion can be obtained by sampling the reaction mixture at different intervals and carrying out an SDS-PAGE; the disappearance of bands corresponding to some of the main proteins existing in a feedstuff and the appearance of new bands related to peptides is a good indicator of a potentially highly digestible feedstuff. In order to quantify the course of this process, gels can be analysed by densitometry and profiles corresponding to different intervals of molecular masses may be plotted as in Figure 5. Using this method it is possible to ascertain the size and number of peptides produced as a result of the digestive process.

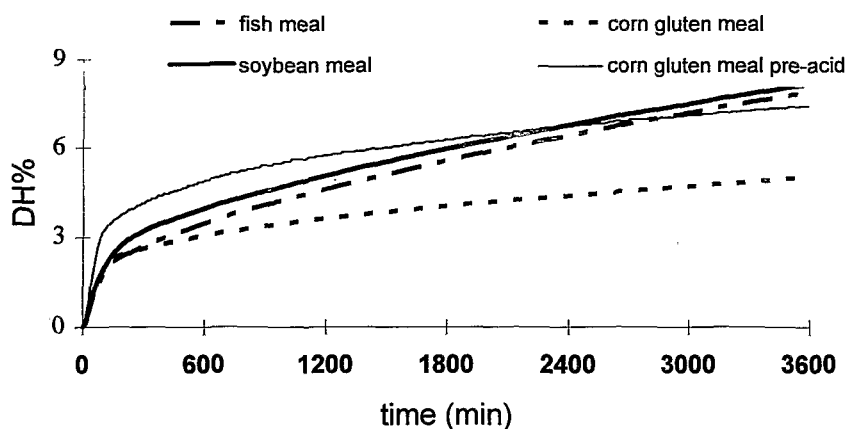


Fig. 4. Degree of hydrolysis obtained by pH-stat in several raw materials. Two different plots were obtained for gluten meal depending on the application or not of an acid predigestion.

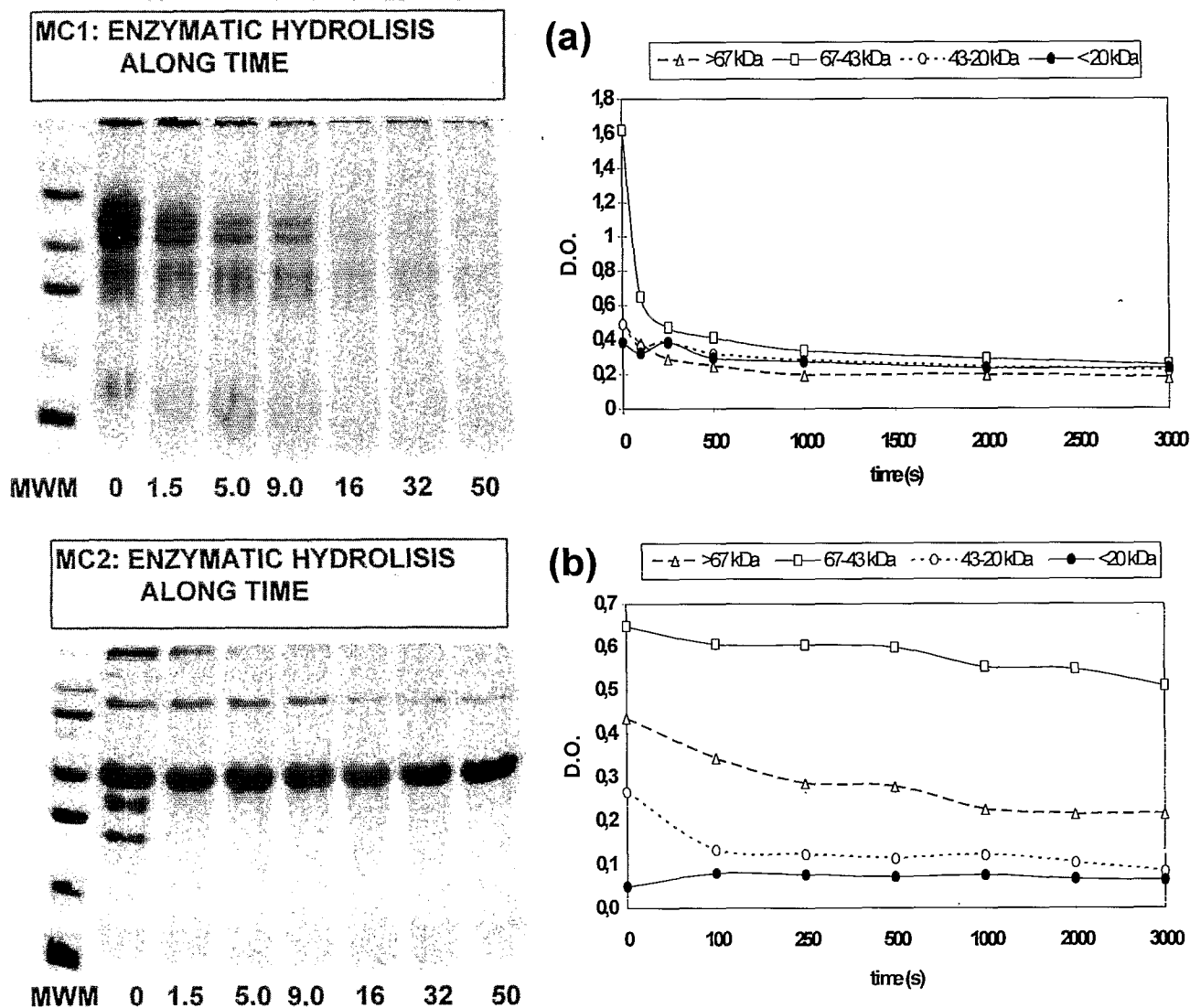


Fig. 5. SDS-PAGE profile of peptides obtained at different time intervals (min) during the digestion of two commercial microcapsules using shrimp digestive enzymes. The adjacent figures show the changes in the proportions of peptides belonging to different ranges of molecular weight along the course of digestion.

When a protein remains undigested (as in the case of Figure 5b), there are two possible explanations; the amino acid sequence of the protein is not easily attacked by the proteases or there is a certain inhibitory effect due to the protein itself or to other components of the feedstuff. The study of such inhibitory effects leads to two different concepts that may have important practical applications in the formulation of feeds for aquatic organisms. The first concerns the **rate of undigestibility**, which can be defined as the amount of a substance (expressed in mg or μg) that inhibits an unit of protease

activity, and it is calculated considering the estequiometry in the reaction enzyme-inhibitor. Since inhibitors are present in variable amounts in many feedstuffs, such levels may not be enough to completely block the activity of digestive enzymes present in an organism. Thus, it is possible to find an interval where inhibition gradually increases as the amount of feed is augmented (Figure 6).

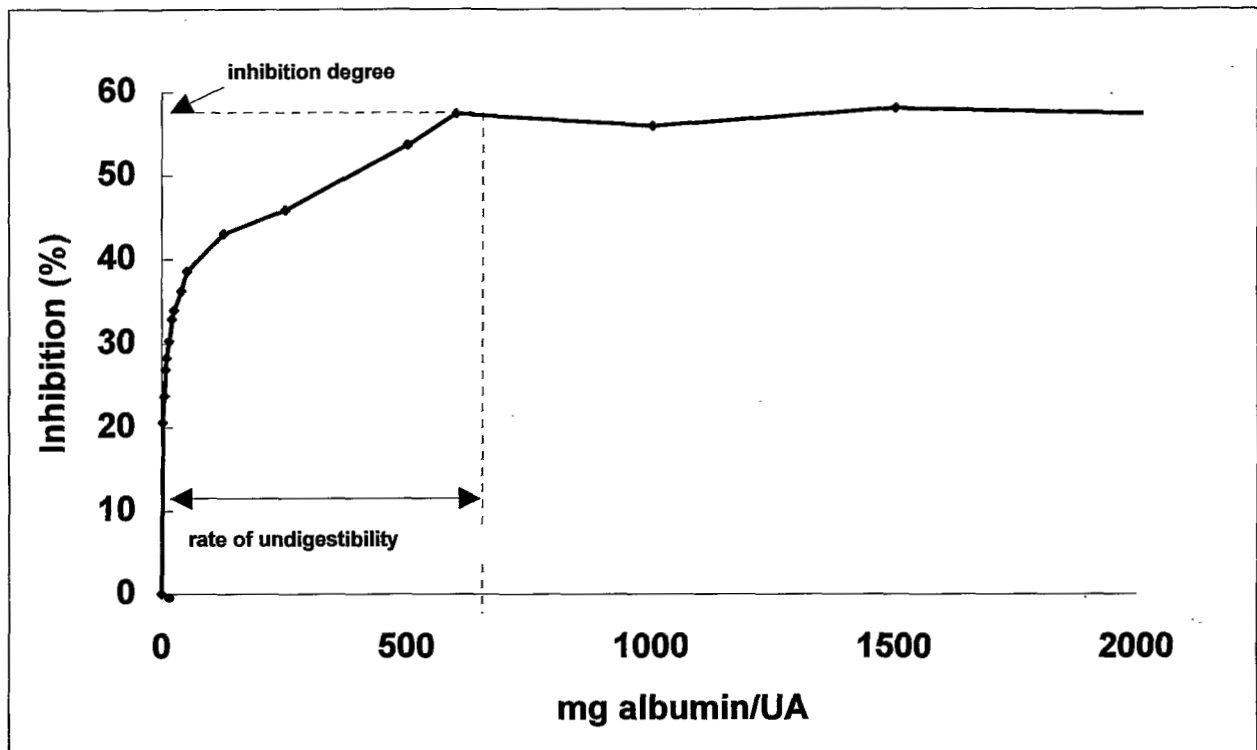


Fig. 6. Inhibition curve obtained when shrimp proteases were mixed with different proportions of albumin (UA: unit of activity).

The maximum level of inhibition is represented by an inflection point followed by a plateau and is usually termed the **inhibition degree**. The level of inhibition determined by a feed ingredient on a crude extract rarely reaches 100% because extracts obtained from fish gut generally contain several different proteases with a different susceptibility to the effect of inhibitors.

The practical applications of this information can be utilized when formulating fish feeds since once the undigestibility rate of the main feed proteins is known for a species, a different feeding pattern can be utilized in order to avoid the maximum level of inhibition for its proteases (e.g.; more frequent and smaller meals a day). On the other hand, it is possible to determine maximum allowable levels for a dietary ingredient,

simply by knowing the ratio between the amount of feed ingested in a meal and total enzyme production, as well as the undigestibility rate for the main ingredients.

CONCLUSIONS

On the basis of the above mentioned data, it is possible to make the following conclusions:

- It is possible to utilize several common biochemical techniques for the study of digestive enzymes of aquatic animals, so as to obtain information of practical value.
- Such information allows to establish the suitability of a given protein to be degraded by digestive enzymes of a particular fish species, as well as the presence and level of activity of potential inhibitors existing in feedstuffs that may affect their digestibility
- It is possible to develop an easy and inexpensive method to perform comparative digestibility studies for fish feeds.

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