A quick method for the assessment of activity and inhibition of fish amylases

I. FERNÁNDEZ, M. DÍAZ, T. MARTÍNEZ & F.J. MOYANO

Dpto. Biología Aplicada, Escuela Politécnica Superior, Universidad de Almería, La Cañada de San Urbano, Spain

Abstract

A sensitive and quick method was developed to determine the presence of α-amylase in the gut of aquatic organisms, as well as its sensitivity to inhibitors. The assay is based on the utilization of Petri dishes filled with starch–agarose gel as a substrate for the enzyme solution, which is placed in small wells punched in the surface. Circular zones produced by the action of amylase remain colourless after staining with lugol. Pure commercial porcine amylase was used to fit the better conditions for developing the assay (1 g L^{-1} starch in the gels, 4 h of incubation). The diameter of the cleared zones were related to the activity of enzyme and the method detected linearly amylase activity in a range of 2–20 U $well^{-1}$, so it was used to reveal the presence of amylase in digestive extracts obtained from different sparid fish. The method was also used to evaluate the effect produced by a specific inhibitor on fish amylases, showing a linear response when the ratio inhibitor:enzyme (in units) changed from 20:1 to 2:1. Comparison of the cleared zones produced by amylases of sparid fish in the presence or absence of inhibitor, revealed differences in their sensitivity to inhibition, which ranged from 15 to 50% of total activity. The assay is proposed for a preliminary evaluation of possible inhibitors contained in feedstuffs used in fish feeding.

KEY WORDS: α-amylase, fish, inhibition, starch–agarose gel

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Correspondence: F.J. Moyano, Dpto. Biología Aplicada, Escuela Politécnica Superior, Universidad de Almería, La Cañada de San Urbano, 04120 Almería, Spain. E-mail: fjmoyano@ualm.es

Introduction

After the early works of Vonk (1937), a great number of papers have been focused to the study of amylase activity in fresh water and marine fish (Keddis 1957; Fish 1960; Ushiyama *et al*. 1965; Ugwumba 1993; Hidalgo *et al*. 1999). The secretion of amylase seems to be greatly determined by the species and the feeding habit. This enzyme is produced in significant amounts in different parts of the digestive tract in herbivorous fish, being less abundant in carnivorous fish, where it is produced only in the pancreas (Nagayama & Saito 1968). A number of chemical methods are routinely used to determine activity of amylase in animal and plant extracts. Some of the more usual are those of Bernfeld (1955) and Somogy–Nelson, described by Robyt & Whelan (1968). Nevertheless, some of those methods are expensive and time consuming. Methods of radial diffusion in agar gels are commonly used in immunological applications for the rapid determination of specific protein levels in large number of samples. Those methods may offer a useful and time-saving alternative to the spectrophotometric or titrimetric assays.

The aim of the present paper was to propose a simple and quick method for both qualitative and quantitative assessment of amylase activity in non-purified extracts obtained from aquacultured species. It is based on the utilization of a starch–agarose gel as a substrate for the enzyme, with the activity further revealed using lugol. The method may be useful for a rapid evaluation of the presence of amylase in fish digestives and for the evaluation of the effect produced by inhibitors present in plant ingredients. Such an information may be useful in the preliminary tests of raw materials used in the elaboration of fish feeds.

Materials and methods

Preparation of the starch–agar plates

A melted agar (Panreac, Barcelona, Spain) solution (100 g L^{-1} in distilled water), was mixed with different amounts of soluble starch (Sigma, St. Louis, USA). After mixing, 10 mL were quickly poured into sterile 8.5 cm diameter Petri dishes. Once the gel was solidified, nine wells (4 mm diameter, 3 mm

height) were punched at intervals that would accommodate the expected size of the zones. Punching was performed using a hollow metal cork borer. Fresh plates were used in all cases.

The radial amylase diffusion assay (RADA)

A standard solution of porcine amylase (Sigma, A6255) was prepared in distilled water (4100 U μL^{-1}) and diluted as required for the preliminary assays. After filling the wells with 5 μL of the amylase solution, the Petri dishes were sealed with parafilm and incubated at 37 °C. A staining solution prepared with lugol (1:10 v/v) was poured into the plates to cover the agar. After a few minutes, once the unstained zones were well appreciated, the solution was poured off and the dishes were rinsed with distilled water. The diameter of such zones was estimated on a horizontal illuminated screen to the nearest 0.1 mm using a digital gauge. The uncoloured zones were stable for various weeks when stored at 4 °C. The influence of different factors on the development of diffusion areas was studied, in order to establish the better conditions for future assays. The influence of substrate concentration on the agar plates was evaluated by using different relative concentrations of starch in the gels, ranging from 0.5 to 8 g L^{-1}. The effect of enzyme activity included in the wells was assayed by changing the amount of an enzyme solution (12 U μL^{-1}) and completion with distilled water to a final volume of 5 μL. The effect of time in the development of cleared zones was evaluated performing assays at different incubation times from 1 to 12 h. In all cases, the diameter of the halo was correlated to the changing values of substrate concentration, enzyme activity in the wells or reaction time, in order to establish both the type of correlation and the more suitable conditions to develop further assays.

Use of the RADA to assess amylase activity in fish extracts

Wild fish of different species, ranging from 85 to 260 g, were captured in the Mediterranean coast; bogue (*Boops boops*), seabream (*Sparus aurata*), saupe (*Sarpa salpa*), common dentex (*Dentex dentex*), pandora (*Pagellus erytrhinus*), red sea bream (*P. bogaraveo*), annular bream (*Diplodus annularis*) and couch's sea bream (*S. pagrus*). Intestines of five fish of each species were dissected, rinsed with distilled water, pooled and after mechanically homogenized in cold (4 °C) phosphate buffer (10 mM, pH 8.0) using a mechanical homogenizer (Heidolph, Kelheim, Germany). Samples were sonicated during 2 s (Microson, Misonix, Farmingdale, NY, USA) to ensure complete cell rupture. Crude extracts were centrifuged (4 °C, 10 000 ***g***, 15 min) and supernatants were stored at –20 °C until enzyme assays. Amylase activity in extracts was assayed following the previously cited Somogy–Nelson method using soluble starch as substrate. One unit of enzyme activity was defined as the amount of enzyme releasing 1 μg maltose min^{-1}. Amylase activity in extracts was revealed as described previously, including an equivalent amount of activity in the wells (Table 1).

Table 1 Amylase activity measured in digestive extracts obtained from different sparid fish. Data are means of three replicate measurements made on pooled samples of five fish

Species	Amylase activity ± SD (U mL^{-1})
Diplodus annularis	29 157 ± 100
Pagellus bogaraveo	26 169 ± 150
Sparus aurata	25 128 ± 250
Boops boops	20 640 ± 200
Dentex dentex	7157 ± 22
Sarpa salpa	7143 ± 46
Pagellus erythrinus	5151 ± 25
Sparus pagrus	2484 ± 20

Use of the RADA to assess amylase inhibition

In a second series of experiments, the RADA was used to evaluate both the effect of increasing amounts of triticale amylase inhibitor (Sigma, A1520) on amylase activity and differences in sensitivity to such inhibitor presented by different fish species. In the first case, inhibitor solutions were mixed to highly active extracts obtained from tilapia (*Oreochromis niloticus*) obtained from the Institute of Oceanography in Alexandria (Egypt) to give ratios ranging from 20:1 to 2:1 expressed in units. One inhibitor unit is defined as the quantity of inhibitor required to reduce the activity of two units of amylase by 50%, that is, one unit of inhibitor inhibits one unit of enzyme under specified assay conditions (O'Donnell & McGeeney 1976). In the second series of experiments, once the more suitable ratio enzyme:inhibitor was established, the aforementioned extracts obtained from the different sparid fish were assayed. The diameters of cleared zones obtained in plates treated with the inhibitor were compared to those obtained in the assay performed using only crude extracts. Inhibition was expressed in percentage, being calculated as follows:

$$[(\text{diameter without inhibitor} - \text{diameter with inhibitor}) / \text{diameter without inhibitor}] \times 100$$

Statistical methods

One-way ANOVA followed by Duncan's multiple range test were used to compare values. Simple regression analysis used

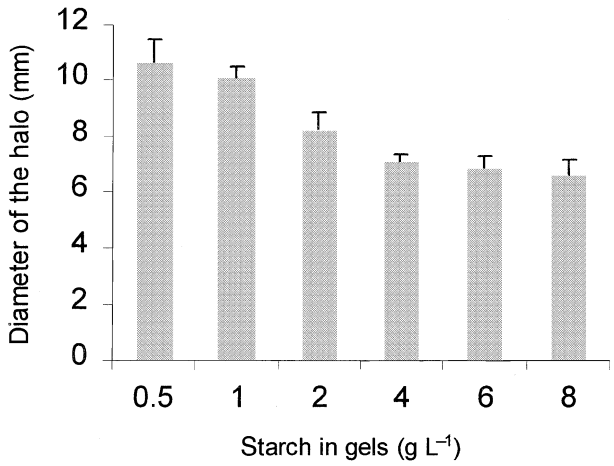

Figure 1 Influence of substrate concentration on the diameter of the cleared zones produced in the Petri dishes after addition of commercial amylase. Measurements were made in triplicate.

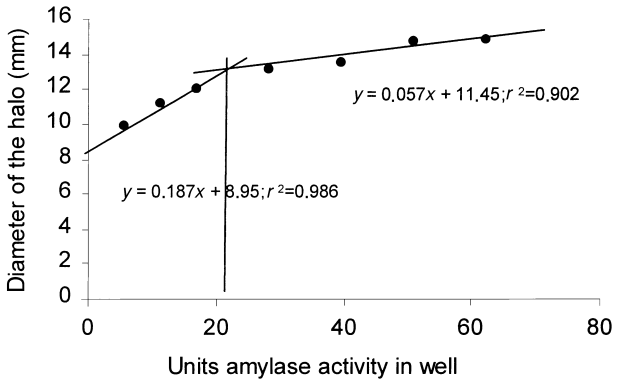

Figure 2 Influence of the amount of amylase activity included on the wells in the diameter of the cleared zones produced in the Petri dishes. Measurements were made in triplicate.

when evaluating the effect of substrate or enzyme concentration were performed using the statistical module of the spreadsheet Excel© (Microsoft, USA).

Results

The influence of substrate concentration in the halo produced in Petri dishes for a given amount of enzyme activity is shown in Fig. 1. From 0.5 to 4 g L^{-1} an almost linear reduction in the diameter of the areas was observed, but no significant differences were detected when concentration of starch was increased over this later concentration. The use of gels including a very low amount of starch (0.5 g L^{-1}) showed some inconveniences (not uniform staining, difficulties in obtaining homogeneous wells), so a concentration of 1 g L^{-1} was selected. The influence of the concentration of enzyme included in the well on the diffusion area could be represented as a two-slope line, which is shown in Fig. 2. Greater changes in the halo diameter corresponded to activities ranging from 0 to 20 U $well^{-1}$. When the concentration of enzyme exceeded this amount, changes in the halo were much less evident, so the optimum activity was noted by the breakpoint in the general trend. The influence of the incubation time on the assay is shown in Fig. 3. The diameter of the cleared zone progressively increased with time during the first four hours of incubation. In contrast, no significant changes were observed when incubation was maintained for a further 8 h. This result was confirmed when assays were performed using three different enzyme concentrations: 5, 10 and 20 U $well^{-1}$.

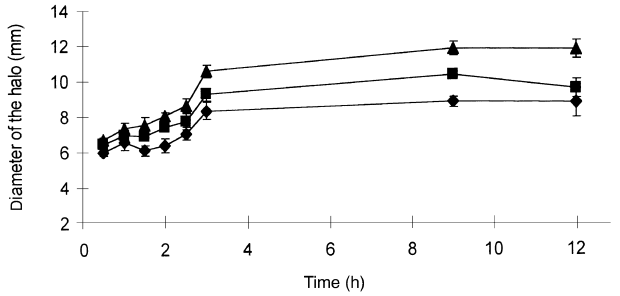

Figure 3 Influence of the incubation time on the diameter of the cleared zones produced in the Petri dishes by three given amounts of amylase activity (◆; ■; ▲: 3, 6 and 9 U $well^{-1}$, respectively). Measurements were made in triplicate.

Once the better conditions for developing the assays were selected (1 g L^{-1} starch in the gels, less than 20 U $well^{-1}$, 4 h of incubation), the RADA was used to evaluate amylase activity in extracts obtained from different fish species (Fig. 4a). The diameter of the clear zones produced ranged from 9 to 14 mm.

The effect of an increasing concentration of inhibitor on the activity of amylase was tested using the highly active extract obtained from tilapia and it is represented in Fig. 5. Changes in the ratio inhibitor:enzyme units from 20:1 to 2:1 resulted in nearly a 20% reduction in the diameter of the cleared zones. The progressive reduction in the diameter of such areas was fitted to a polynomial function with $r^2 = 0.95$ at $P < 0.05$.

Inhibition produced by the amylase inhibitor on the different fish extracts was tested in triplicate including equivalent activities in the wells. Results obtained are shown

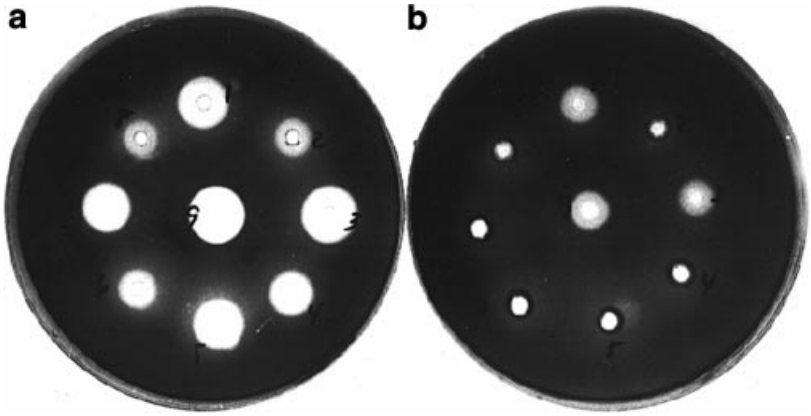

Figure 4 (a) Amylase activity present in extracts obtained from different fish species revealed using the RADA. (b) Inhibition of such activity by a specific amylase inhibitor (Sigma, A1520). The assayed species were (reading clockwise); *Sparus aurata*, *Sarpa salpa*, *Diplodus annularis*, *Pagrus pagrus*, *Dentex dentex*, *Pagellus erytrhinus* and *Boops boops*. The central well contained commercial amylase as a reference.

in Figs 4(b) and 6. A 15% inhibition was measured for the amylase activity of *S. aurata* but in contrast, more than 50% was measured for amylase activity of *S. pagrus* and *P. erythrinus*.

Discussion

The industry of fish feeds has been growing quickly during the last few years, together with a noticeable increase in total production and a diversification in diet formulations, this to cover the demand generated by the culture of new species (Tacon 1994). Because of their low cost and local availability, the use of vegetable feedstuffs is increasing everywhere. Nevertheless, one of the main obstacles for their use is their content in digestive enzyme inhibitors, mainly for protease and amylase (Tacon 1997). A quick and easy assessment of the presence of such antinutritional compounds should be helpful for the feed industry allowing a better selection of ingredients. A method of radial diffusion in agar gels has been successfully used in the determination of protease inhibitors by Jongsma *et al.* (1993) being suitable for analysing hundreds of samples in only a few days. In the present study, an equivalent method suitable to test the inhibition in fish amylases is proposed.

The method is based in the utilisation of crude intestinal extracts showing amylase activity. Different factors affecting diffusion of amylase in the starch gel, thus influencing the formation of cleared zones, were evaluated. It was observed that the better conditions for diffusion in gels were: 1.5 g L^{-1} starch, 5 units of activity in 3 μL extract and less than 4 h incubation at 37 °C. Under such conditions cleared zones up 10 mm in diameter are produced and after revealed with lugol. Once the better conditions for revealing amylase activity in crude extracts were established, assays performed using fish extracts revealed the suitability of the method in the evaluation of differences in activity existing in different fish species. The selected species showed quite different feeding habits, this being reflected by variations in amylase activity present in the extracts, which ranged from 2.5 to

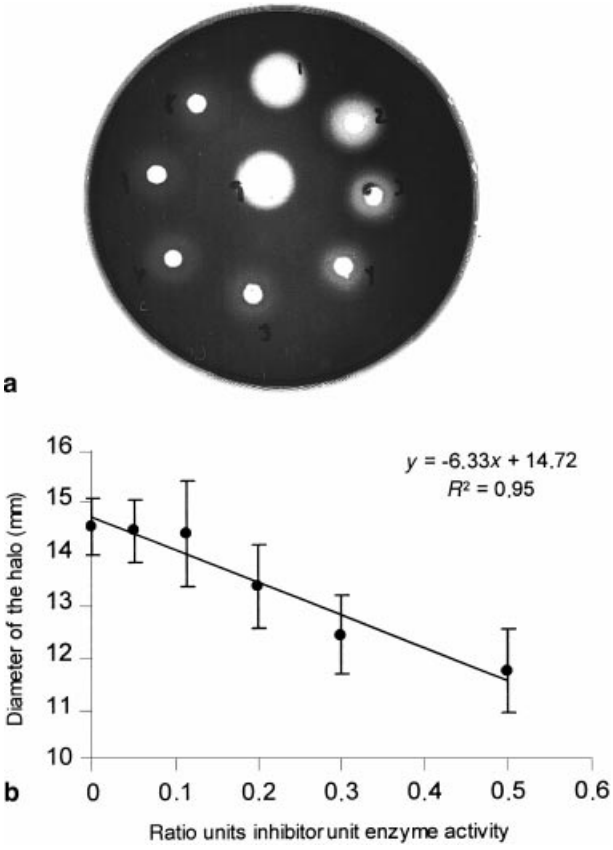

Figure 5 (a) Changes in the diameter of the cleared zones produced by amylase present in extracts obtained from tilapia when mixed with variable ratios inhibitor:enzyme. Reading clockwise, the ratios ranged from 20:1 to 2:1. (b) Graphical plotting of the results ($n = 3$).

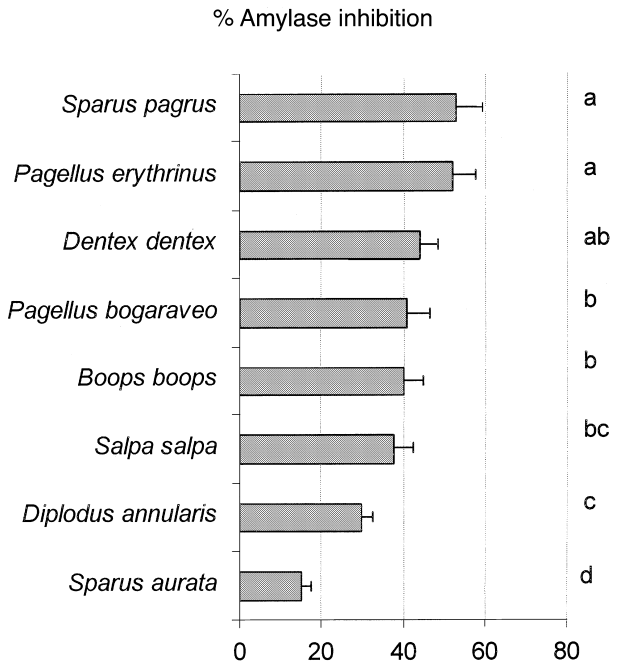

Figure 6 Inhibition of amylase activity present in extracts obtained from different fish species measured using the RADA. Measurements were made in triplicate. Values not sharing the same superscript are significantly different with $P < 0.05$.

29.2 U μL^{-1} (Table 1). It is noteworthy that, in spite of including the same units of amylase activity in each well in the plate, the diameter of the halo generated was not similar for all the species. This may be owing to differences in the size of the amylase molecules, which may affect their diffusion in the agarose-starch gel. Such differences existing in the amylase equipment of closely related fish species have been assessed using electrophoretic techniques (Fernández *et al*., in preparation) and were also confirmed by the great variations found in sensitivity to the specific inhibitor, which resulted in a reduction of activity from 15 to 50%. This confirms the need of a previous evaluation of plant ingredients to be used in feeds for each species.

As a resume, a cheap, rapid and sensitive method for the assessment of amylase inhibition in fish is described. The quick development of the assay makes possible to carry out a great number of tests in one day. The assay is applicable to a wide range of species and may be used in the selection of plant ingredients to be used in the elaboration of feeds, preventing the utilisation of those contents, which in amylase inhibitor could negatively affect digestion of carbohydrates.

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