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Factors Affecting the Autolysis Rate of Crude Preparations of Proteolytic Enzymes from Bouri Fish (*Mugil cephalus*)

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ABSTRACT

Dried fish viscera prepared from Bouri fish showed a sufficiently high activity as a crude proteolytic enzyme preparation. A rapid change in the rate of hydrolysis was observed in the first 2 h. The hydrolysis reaction increased as the amount of substrate increased from 0·1 to 0·4 g in the reaction mixture. An increase in activity was observed when enzyme increased from 0·025 to 0·4 g in the reaction mixture. The rates of protein hydrolysis at pHs ranging from 2 to 9 and temperatures ranging from 20 to 70°C were determined. Hydrolysis rate was generally greatest at neutral and basic pHs and began to decrease after temperatures exceeded 60°C. Crude preparations prepared from Borboni, Bolti and Mabrouk fish were tested for proteolytic activity under the optimum conditions found with Bouri fish for comparison. All the preparations were found to be more active on casein as substrate than on gelatin. Barboni fish was the most active preparation. Fish viscera differ in activity with the fish species and age (weight of fish), while no apparent differences within the same species of fish appeared.

INTRODUCTION

The utilization of protein foods has always involved some modifications of their native state, and the protein undergoes physical and organoleptic

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changes (Akiva, 1976). The use of proteolytic enzymes is recently one of the approaches used to modify proteins through partial hydrolysis. The enzyme system utilized may be endogenous autolytic enzymes and/or commercial exogenous enzymes. Miyada & Tappel (1956) compared nine proteolytic enzyme preparations for their action on rehydrated freeze-dried beef muscle. The relative activities of more than twenty commercially available proteolytic enzymes were measured for the digestion of a washed and freeze-dried fish protein substrate prepared from haddock (Hale, 1969). Several proteolytic enzymes from plants, animals and microorganisms were studied to obtain information for use in a continuous fish protein concentrate solubilization process (Cheftel *et al.*, 1971). Autolytic activities in tissues can have a significant effect on the yield of protein in the preparation of concentrates from animal and plant tissues. Koury *et al.* (1971) observed that fish flesh and viscera contain highly active proteolytic enzymes.

The work on utilization of enzymes from dehydrated portions of the fish digestive tract was undertaken in the expectation that dried preparations of certain digestive organs might be of use commercially for protein solubilization processes or as leather dressing. Fish viscera comprise about one-sixth of waste weight obtained from fish after its preparation for processing. It would be clearly advantageous if the waste material, or a part of it, were converted into a by-product of commercial value. The present work attempts to determine the total proteolytic activity of the digestive organs of Bouri fish and the effect of a number of factors which affect the rate and extent of protein degradation; namely, pH, temperature and time, as well as substrate and enzyme concentrations. These factors are considered for use in the production of fish protein concentrate. The present work deals also with the comparison of the activity with crude enzyme preparations of viscera of other commercial fish; namely, Barboni, Bolti and Mabrouk, under the optimum conditions used with the crude enzyme prepared from viscera of Bouri fish.

MATERIALS AND METHODS

Materials

Bouri fish (*Mugil cephalus*) was used as a main source of fish viscera in this study. Two sizes were obtained from the El-Zawia fish farm at Kafr El-Sheikh. The average lengths and weights of the fish were 22·4 cm and 107 g and 26·5 cm and 220 g for small and large sizes, respectively. Commercial fishes—Bouri (*Mugil cephalus*) and Barboni (*Mullus barbatus*)—were

purchased from Alexandria market, Bolti fish (*Tilapia nilotica*) from Kafr El-Sheikh market and Mabrouk (*Cyprinus carpis*) from El-Zawia fish farm for comparison purposes.

The fish used throughout this work were obtained during the fishing season of September–December 1984. All samples were obtained fresh, immediately frozen and held at −20°C.

Methods

Preparation of crude mixture of enzymes

The crude dry preparation of viscera enzymes was made as described by Johnston (1941) with some modifications. Fish viscera were immersed in 3·5 times their weight of a solution consisting of acetone:ether (9:1 v/v). After standing at room temperature for half an hour with stirring occasionally, the mixture was filtered. The remaining solid matter was immersed again in the same solution for 30 min. The residue was squeezed and left to dry at room temperature. The dried preparations were then kept in polyethylene bags and stored in a refrigerator.

Measurement of activity

The method used was that of Johnston (1941) with some modifications. The substrate for determination of enzyme activity was composed of a 5% casein solution held at pH 8·0 with a borate buffer at a temperature of 40°C. The enzyme–substrate ratio used was 1:2·5 (w/w). The activity of the preparation was determined by following the progress of the hydrolysis of casein. The incubation mixture was treated with an equal volume of TCA (10%) after an hour of incubation, allowed to stand for 30 min and then filtered. The digestion products in the filtrates were determined by Biuret reagent and presented in terms of optical density (OD) measurements at 540 nm. To define the optimal conditions of proteolysis by using the crude enzyme preparation, rates of enzyme activity at pHs of 2–9 and temperatures of 20–70°C were determined. Controls were performed by adding the enzyme preparation to TCA before the latter was added to the substrate.

RESULTS AND DISCUSSION

Yield of crude enzyme preparation and preliminary measurement of activity

As shown in Table 1, the viscera constitute about 6–10% of the total weight of fish. Dried crude enzyme preparation represented 14–20% of the

TABLE 1
The Average Weight of Fish Viscera[a] and the Yield[b] of Crude Enzyme Preparation from Various Fishes

Fish	*Weight of fish (g) (FW)*	*Weight of viscera[a] (VW)*	$\frac{VW}{FW}$ (%)	*Yield of dried preparation (%)[b]*
Farm Bouri (small size)	107	6·3	5·9	19·8
Farm Bouri (large size)	220·8	13·3	6·0	17·8
Bouri from Alexandria market	143	11·3	7·9	15
Barboni from Alexandria market	55·5	4·0	7·2	20·8
Bolti from Kafr El-Sheikh market	328	31·8	9·7	14·6
Farm Mabrouk	446	37·4	8·4	14

[a] Fish viscera includes stomach, intestines, liver, pyloric caeca and other organs in the abdominal cavity of fish.

[b] Yield of dried preparation $= \frac{\text{Weight of dried preparation}}{\text{Weight of viscera used}} \times 100\%$.

initial weight of the viscera used. It was observed that the pyloric caeca of Bouri fish were hard in texture and not easily homogenized with the rest of the viscera, so preliminary work was done to make a crude preparation of the pyloric caeca alone (a) and to examine its activity. The crude enzyme preparation, obtained from the viscera without pyloric caeca, which remained on the filter paper, was manually divided into two parts—a coarse part easily separated with a spatula (b) and a fine powder retained on the filter paper (c) and obtained by scraping gently with a straight-edged piece of glass. Activity was measured for each part alone. As shown in Table 2, only negligible proteolytic enzyme activity was observed for the pyloric caeca of Bouri fish (a), as reacted on casein at pH 2, which indicated that the peptic activities of the pyloric caeca are very low, if not absent. Their activities at pH 8 were lower than those of parts (b) and (c) of the viscera preparation. These findings are in contrast with that of Johnston (1941) who found that the pyloric caeca of four species of mackerel were the most active portions of the fish digestive organs. The low activity of pepsin-like enzymes during preparation may be due to auto-digestion at pH values greater than 2·5, because no steps were taken to minimize auto-digestion of pepsin-type enzymes. Each of the above-mentioned preparations comprises a third of the whole preparation. The pyloric caeca preparation was discarded due to its negligible activity and the remaining two parts—(b)

TABLE 2
Enzyme Activity of Bouri Viscera Preparations at Different pH Values

Enzyme preparation	*Optical density (OD) measured at 540 nm and after 1 h of digestion*	
	pH 2	*pH 8*
Pyloric caeca (a)	0·016	0·372
Whole viscera without (a):		
Coarse preparation (b)	0·372	0·578
Fine preparation (c)	0·487	0·683

and (c)—of the viscera preparation were mixed and taken as the crude enzyme preparation in all the following experiments.

Effect of duration of hydrolysis

The duration of hydrolysis was estimated from the moment of the enzyme addition. As shown in Fig. 1, a rapid increase in the rate of recovery of proteolytic products (as indicated by the value of optical density) was generally observed to occur during the first 2 h. The rate reached its maximum after 4 h at pH 8·0 after which it slowed down. The rate of proteolytic hydrolysis is greater at pH 8·0 than pH 2·0. The rate of proteolysis after 6 h of digestion at pH 2·0 was nearly equal to that obtained after only half an hour at pH 8·0. Thus, the use of an alkaline pH to determine the proteolytic activity gives an indication of the economic usefulness of this crude enzyme preparation for the digestion of protein.

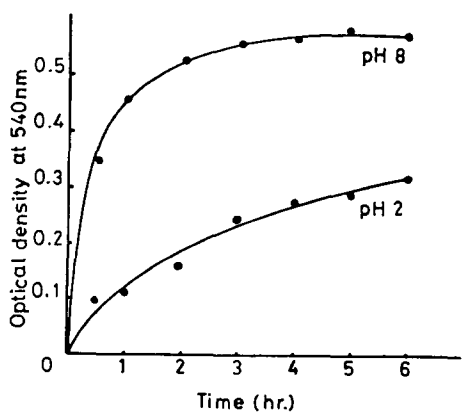

Fig. 1. Effect of duration of hydrolysis on protein hydrolysis rate by crude preparation from fish viscera. Reaction conditions: 50 mg/ml of casein, 20 mg/ml of crude preparation of enzymes, 40°C, pH 2 and 8.

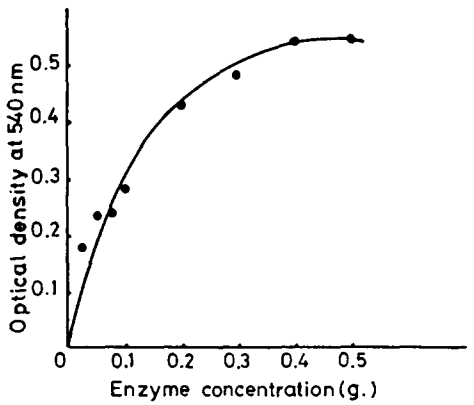

Fig. 2. Effect of enzyme concentration on protein hydrolysis rate by crude preparation from fish viscera. Reaction conditions: 50 mg/ml of casein, 40°C, pH 8, 1 h reaction time.

Effect of enzyme concentration

Figure 2 shows an expected relationship of proteolysis against enzyme concentration. Recovery in terms of optical density is greater at 0·5 g of dried preparation (OD = 0·55) than at 0·1 g (OD = 0·28). An increase in product output was obtained with an approximate twofold increase in enzyme concentration from 0·025 to 0·2 g/reaction mixture. Further increases in enzyme levels slowly increased the output. Enzyme concentrations greater than 0·4 g did not improve performance. There appears to be no apparent difference in the rates of proteolysis at the 0·4 and 0·5 g enzyme concentrations.

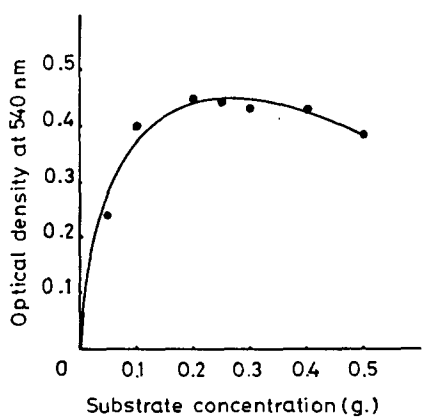

Fig. 3. Effect of substrate concentration on protein hydrolysis rate by crude preparation from fish viscera. Reaction conditions: 40 mg/ml of crude preparation of enzymes, 40°C, pH 8, 1 h reaction time.

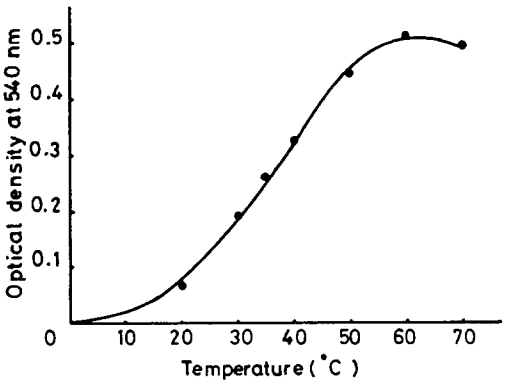

Fig. 4. Effect of temperature on protein hydrolysis rate by crude preparation from fish viscera. Reaction conditions: 20 mg/ml of casein, 40 mg/ml of crude preparation of enzyme, pH 8, 1 h reaction time.

Effect of substrate concentration

It is clear from Fig. 3 that the hydrolysis reaction increases as the substrate concentration increases. The optimum concentration of casein was 0·2 g in the reaction mixture. Protein degradation decreases as the concentration of casein increases. The protein hydrolysis by protease is generally known to proceed effectively at a low concentration of substrate (Yamashita *et al.*, 1976).

Effect of temperature and pH

The hydrolysis behaviour depends, not only on the concentration of enzyme and substrate, but also on such parameters as the temperature and

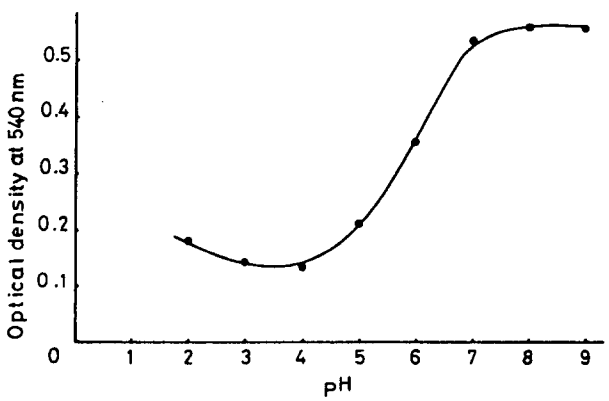

Fig. 5. Effect of pH on protein hydrolysis rate by crude preparation from fish viscera. Reaction conditions: 20 mg/ml of casein, 40 mg/ml of crude preparation of enzymes, 50°C, 1 h reaction time.

pH. The rate of proteolytic activity increased and remained elevated until the temperature exceeded 60°C; then a slight decrease appeared (Fig. 4). Similar results were obtained by Koury *et al.* (1971) in their studies on the protein autolysis in some fishes and by Archer *et al.* (1973) on the enzymatic solubilization of fish protein concentrate.

The amount of proteolytic products was greater at neutral or alkaline pH than at acidic pH (Fig. 5). No great differences were observed in the rates of activity as the pH was lowered. In this case, the activity extended into the pH 2–5 range at about half-optimal value.

Comparison of relative activities of the enzyme preparation from viscera of various species of fish

In the light of our present knowledge, and to estimate the degree of digestion attainable near optimum conditions, a 4% casein solution of 0·2 g of enzyme preparation was incubated at 50°C and pH 7 for 1 h. These conditions were chosen as the basis for comparison of the relative activities

TABLE 3
Relative Activities (In Terms of Optical Density) of the Enzyme Preparations Prepared from Viscera of Various Fish Species

Fish	*Optical density (OD) measured at 540 nm and after 1 h of digestion*		
	At reviewed conditions[a]	*At optimum measured conditions*[b]	*At optimum measured conditions*[c]
Farm Bouri (small size)	0·328	0·555	0·190
Farm Bouri (large size)	0·355	0·555	0·178
Bouri from Alexandria market	0·375	0·553	0·185
Barboni from Alexandria market	0·345	0·575	0·233
Bolti from Kafr El-Sheikh market	0·175	0·520	0·193
Farm Mabrouk	0·350	0·530	0·150
Pancreatin enzyme	0·438	0·530	0·160

[a] Digestion was carried out at 40°C and pH 8 for 30 min. Reaction mixture consisted of 5 ml of 5% casein solution + 0·1 g enzyme preparation (obtained from the literature).
[b] Digestion was carried out at 50°C and pH 7 for 1 h. Reaction mixture consisted of 5 ml of 4% casein solution + 0·2 g of enzyme preparation.
[c] Digestion was carried out at 50°C and pH 7 for 1 h. Reaction mixture consisted of 5 ml of 4% gelatin solution + 0·2 g of enzyme preparation.

of the various enzyme preparations obtained from different species of fish. Table 3 lists the optical density values of each fish proteolytic enzyme preparation, as measured under these optimum conditions of reaction and also under the conditions reviewed in the literature (Sen *et al.*, 1962; Hale, 1969; Koury *et al.*, 1971). A 10 mg concentration of protease bovine pancreatin enzyme (107 Tame units per milligram, 11 Baee units per milligram, NBCO), was used as standard enzyme. An examination of this Table shows that all enzyme preparations from fish viscera (except Bolti) are more active than pancreatin as measured under optimum conditions. The most active fish was Barboni fish (a salt-water fish), while Bolti fish represented the lower proteolytic activity. No apparent differences were found within the same species of fish differentiated according to fishing ground or age (size).

Using gelatin as substrate, Barboni also exhibited the highest activity, and Mabrouk the lowest. The enzyme activity for small farm Bouri fish was near that for Bouri from the Alexandria market (nearly the same size and weight for the two fish). Some apparent differences were observed with the large fish; its activity was somewhat low. Generally, enzyme preparations from fish viscera showed more activity when reacted on casein than gelatin.

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