Solubilization of a Fish Protein Concentrate with Proteolytic Enzymes

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Rates of proteolytic breakdown of a fish protein concentrate (FPC) were derived from its increase in solubility during enzyme digestion. The activities of three concentrations each of bromelain, Pronase, ficin, and activated ficin were compared. Pronase, at 50 °C, had twice the activity of ficin (40 °C) or bromelain (50 °C). However, ficin previously activated by cysteine and used at 40 °C was as active as Pronase at 50 °C. The maximum solubility achieved in 7 h was less than 70%, of which about 80% occurred in the first 2 h. The soluble fractions were bitter.

Fish protein concentrate (FPC) is an excellent source of protein for man and animals (Stillings, 1974; Scrimshaw, 1974; Young and Schrimshaw, 1974), but its potential as a food additive is limited because of poor functional properties, among them, lack of solubility (Spinelli et al., 1972; Archer et al., 1973a,b, 1974; Cheftel et al., 1971). Several approaches have been used in attempts to solubilize FPC, including acid, alkaline, and proteolytic enzymatic hydrolyses (Tannenbaum et al., 1970; Anglemier and Petropakis, 1974; Cobb and Hyder, 1972; Cheftel et al., 1971; Archer et al., 1973a,b, 1974). This paper presents data on rates of solubilization of a solvent-extracted menhaden FPC with the proteolytic enzymes, bromelain, Pronase, ficin, and activated ficin.

EXPERIMENTAL SECTION

Materials and Methods. Weighed amounts of crude ficin (Sigma), pineapple stem bromelain (Calbiochem), and Pronase (B grade, Calbiochem) were dispersed in distilled water, held at room temperature for 30 min, and then centrifuged at 4 °C and 12000*g* for 10 min. The supernatants were kept on ice. For activation studies, ficin was dissolved in 4.5 ml of a 0.025 M solution of cysteine·HCl which had been neutralized to pH 7 with 1 N NaOH (Sigma). The supernatant of this solution was incubated for 3–5 min at the temperature of the reaction (40 °C) in order to obtain the desired activation prior to use (Whitaker, 1957).

The sample of Gulf menhaden FPC had been prepared by College Park Fishery Products Technology Laboratory, National Marine Fisheries Service, in 1970 and had been stored in a refrigerator. The same sample was used previously in this laboratory for studies on its lipid composition (Medwadowski et al., 1971).

Reaction mixtures were prepared as follows. FPC (0.5 g) was suspended in 4.5 ml of distilled water in a 50-ml beaker, held for 5–10 min in a boiling water bath, cooled, and maintained at the temperature of reaction (40 °C for ficin and 50 °C for bromelain and Pronase) for 10 min. These temperatures are within the optimal temperature ranges for these enzymes (Hale, 1969). Then 0.5 ml of the enzyme solution was added to give a concentration of FPC in the reaction mixture of 10% (w/v). According to Cheftel et al. (1971) this concentration is adequate to give maximum velocity in a Pronase–FPC system. The beakers were held in a horizontal shaker with fixed plastic spatulas to provide continuous agitation.

At the end of the desired time, the enzyme was inactivated by heating the reaction mixture over an open flame to boiling and then held in a boiling water bath for 5 min. The beaker was covered with a watch glass to avoid excessive evaporation. Separate reaction mixtures were used each time hydrolysis measurements were taken. The pH of the mixtures was usually not controlled, but when the ficin had been activated by cysteine, the FPC had been suspended in 4.5 ml of a neutralized (pH 7.0) 0.025 M solution of cysteine·HCl.

Each of the inactivated reaction mixtures was centrifuged at 12000*g* for 10 min. The supernatants were collected, the pellets resuspended in 5 ml of deionized water, and recentrifuged. The combined supernatants were adjusted to 25 ml. Two methods were used to measure the extent of hydrolysis: (1) the solutions were treated with ninhydrin (Hirs, 1967); (2) the amount of solubilized material was measured by drying 2 ml of the stock solution to constant weight at 105 °C. Where cysteine had been added, a suitable correction was applied. Soluble material was then expressed as the percentage of the starting material. Initial rates were determined as described by Whitaker (1972, p 169).

RESULTS AND DISCUSSION

Effect of Enzyme Concentration on the Rate of Hydrolysis. The effects of enzyme concentration on the rates of hydrolysis for each of the proteases are shown in Figures 1–4. Three enzyme concentrations were used; expressed as ratios of enzyme to substrate, the relative concentrations were 1:25, 1:100, and 1:400 (enzyme:FPC). In the case of ficin (ficin:FPC, 1:25), the initial pH of 6.6 dropped to pH 5.9 after 7 h of hydrolysis.

Although solubility is not a precise measure of peptide bond splitting, the data correlate well with peptide bond splitting as measured by ninhydrin procedures (not shown). The correlation coefficients obtained with these two parameters, compared by the least-squares method for the three enzymes, were 0.98 for ficin (1:25), 0.98 for bromelain (1:100), and 0.96 for Pronase (1:100).

Figures 1–3 show that Pronase at 50 °C was more effective than bromelain at 50 °C or nonactivated ficin at 40 °C in agreement with Hale (1969) and Cheftel et al. (1971), and Figure 4 indicates that activated ficin at 40 °C is almost as effective as Pronase at 50 °C.

The broken lines in the figures represent the initial velocities for each of the enzyme concentrations. The slopes of these lines are the initial rate constants (V_0) and the data are summarized in the captions to the figures.

Enzyme Concentration–Velocity Relationship. The V_0 data plotted against enzyme concentration (Figure 5) show a reasonably uniform relationship, indicating that the estimated initial rate constants are in the right order and also confirming the observation of Cheftel et al. (1971) that a concentration of FPC of the order of 10% (w/v) is sufficient to saturate the enzymes at a ratio of 1:400 (enzyme:substrate).

The fact that the curves in Figure 5 tend to plateau

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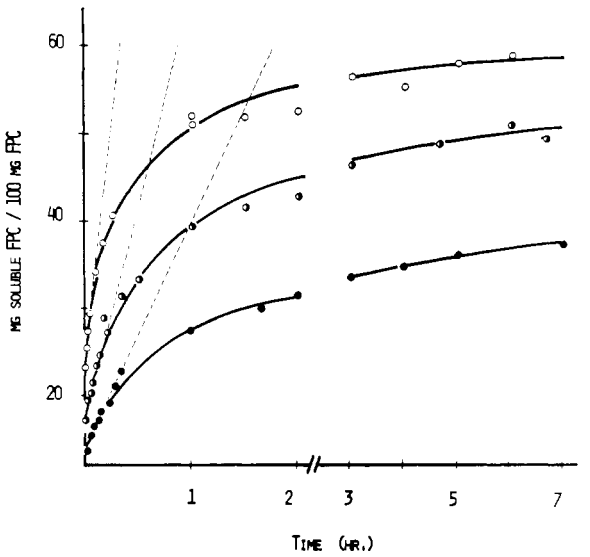

Figure 1. Effect of concentration of ficin on the rate of hydrolysis of FPC. Reaction conditions: concentration of FPC 10% (w/v); ratio of ficin to FPC (w/w) (○) 1:25, (◑) 1:100, (●) 1:400; temperature, 40 °C. Broken lines represent initial velocities (V_0) of 19, 9, and 4, respectively, expressed as milligrams of soluble FPC per 100 mg of FPC formed in the first 10 min.

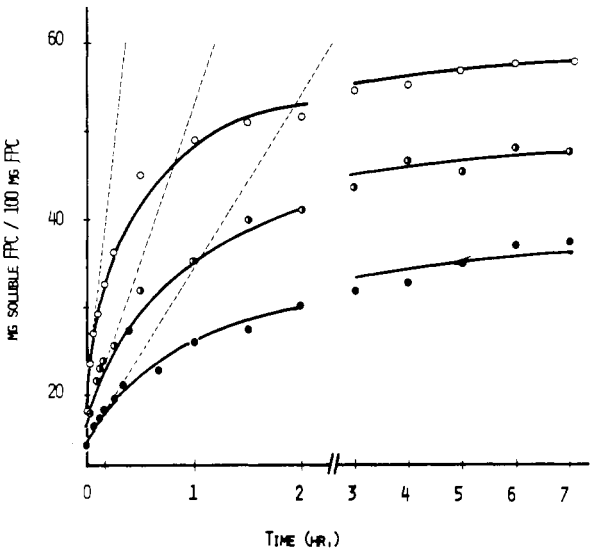

Figure 2. Effect of concentration of bromelain on the rate of hydrolysis of FPC. Reaction conditions: concentration of FPC 10% (w/v); ratio of bromelain to FPC (w/w) (○) 1:25, (◑) 1:100, (●) 1:400; temperature, 50 °C. Broken lines represent initial velocities of 22, 7, and 3, respectively (see Figure 1).

indicates that the concentration of available substrate is limiting at high enzyme concentrations (Whitaker, 1972, p 214), to be expected since FPC is quite insoluble in water.

Activation of Ficin by Cysteine. Cysteine activates ficin toward FPC (Figure 4) as it does for casein (Whitaker, 1957) and elastin (Yatco-Manzo and Whitaker, 1962). Activated ficin at 40 °C is slightly less effective than Pronase at 50 °C toward FPC. The initial rate constants for activated and nonactivated ficin show that activated ficin is almost twice as active as nonactivated ficin. This is also apparent in Figure 5, where the enzyme concentration–velocity relationships for Pronase and nonactivated ficin are summarized.

Although the increase in activity observed was marked, the four- or fivefold activation of ficin toward casein observed by Whitaker (1957) was not achieved. One reason might be that the FPC is rich in connective tissue. Tannenbaum et al. (1970) indicated that FPC contains about 20% collagen. Yatco-Manzo and Whitaker (1962) found less activation of ficin by cysteine when elastin was the substrate; however, the effect of added cysteine on the solubilization of collagen by ficin was not reported (Hinrichs and Whitaker, 1962).

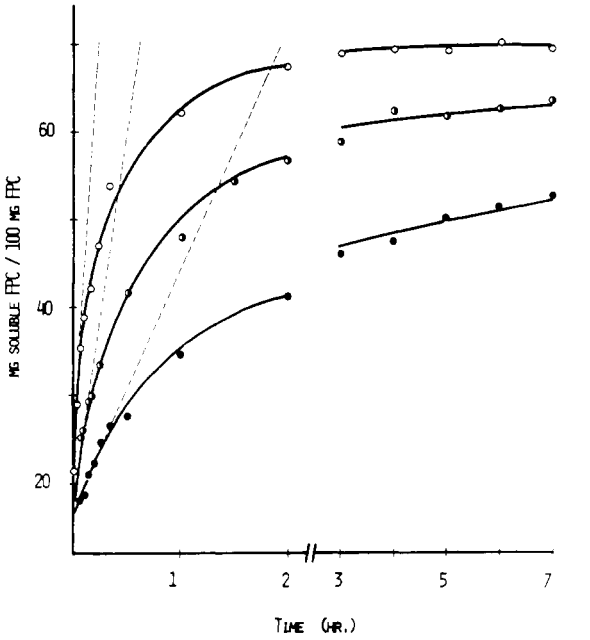

Figure 3. Effect of concentration of Pronase on the rate of hydrolysis of FPC. Reaction conditions: concentration of FPC 10% (w/v); ratio Pronase to FPC (w/w) (○) 1:25, (◑) 1:100, (●) 1:400; temperature, 50 °C. Broken lines represent initial velocities of 38, 16, and 5, respectively (see Figure 1).

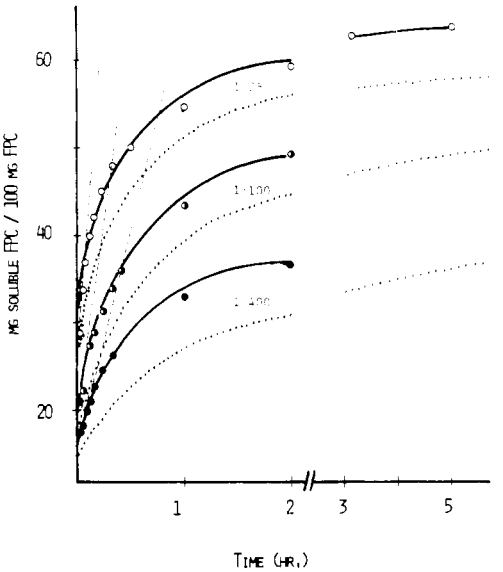

Figure 4. Effect of concentration of activated (—) and nonactivated (···) ficin on the rate of hydrolysis of FPC. Reaction conditions: concentration of FPC 10% (w/v); ratio of ficin to FPC (w/w) (top) 1:25, (middle) 1:100, (bottom) 1:400; temperature, 40 °C; activator, 0.025 M cysteine. Broken lines represent initial velocities of 32, 17, and 7, respectively (see Figure 1).

Validity of the Enzyme Concentration–Velocity Relationship. Three digests were prepared with the following amounts of enzyme per milligram of FPC: 4×10^{-2} mg of ficin, 2.5×10^{-2} mg of bromelain, and 1×10^{-2} mg of Pronase. These enzyme concentrations were cal-

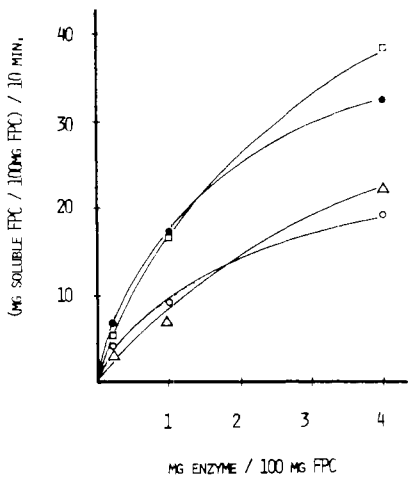

Figure 5. Enzyme concentration-velocity relationship for bromelain (△), ficin (○), activated ficin (●), and Pronase (□) toward FPC.

Table I. Product Formed after 1 and 7 h by Concentrations of Bromelain, Ficin, and Pronase Estimated from Figure 5 to Give Equivalent Amounts of Product[a]

Enzyme	Concn, mg/mg of FPC	Enzyme: FPC, w/w	Product, mg of sol FPC/100 mg of FPC	
			1 h	7 h
Bromelain	2.5×10^{-2}	1:40	47	58
Ficin	4.0×10^{-2}	1:25	53	64
Pronase	1.0×10^{-2}	1:100	51	65

[a] The reaction temperatures were 40 °C for ficin and 50 °C for bromelain and Pronase.

culated from the data shown in Figure 5 to give similar amounts of product formation by the three enzymes.

The results obtained after 1 and 7 h (Table I) show that similar amounts of product were obtained with enzyme concentrations derived from the enzyme concentration–velocity relationships, thus validating the interpretation of the curves. Hence, even though FPC is not an ideal substrate for enzyme kinetic studies, its behavior during proteolysis can be defined by the conventional methods used here. This is in contrast to the suggestions of Archer et al. (1973a) who proposed more sophisticated analytical considerations.

The limited value of long periods of hydrolysis is also illustrated. The gain in solubility was only about 20% when the hydrolysis time was increased from 1 to 7 h.

The increase in activity of activated ficin to the level of Pronase is a promising result. Although Pronase has been reported as a useful enzyme for solubilizing fish protein because of its high activity (Hale, 1969; Cheftel et al., 1971), its potential is limited by a high market price (Hale, 1969).

Several reducing agents and chelating agents have been reported as possible activators of commercial ficin by Whitaker (1957). Selection of the best activators of ficin and other sulfhydryl enzymes such as papain and bromelain with respect to their proteolytic activity toward FPC needs further study.

The soluble fractions of the 1- and 7-h digests already described were tasted at 6, 3, and 1.5% (w/v) by four people and all were reported to exhibit an unpleasant flavor. The flavor was characterized as bitter and acid-like, bitterness being the more important contributor. The Pronase hydrolysates were found less bitter than the ficin or bromelain hydrolysates in agreement with Cheftel et al. (1971). The bromelain and ficin hydrolysates were found to be similar in taste. No marked taste difference was found among the 1 and 7 h digests for any of the enzymes.

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