Enzymatic Solubilization of Fish Protein Concentrate: Batch Studies Applicable to Continuous Enzyme Recycling Processes

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Batch experiments were performed to obtain information for a continuous solubilization process of fish protein concentrate (FPC) by proteolytic enzymes. The batch kinetic data were gathered with the intention of converting this system into a continuous reactor capable of enzyme reusage through the use of an ultrafiltration membrane. Methods were adapted in order to assay proteolytic activity, particularly in FPC hydrolyzates. Among the relative activities of various proteases for FPC solubilization, pepsin and Pronase were particularly effective. The effects of pH, temperature, substrate, and enzyme concentrations on the rate and extent of FPC proteolysis and solubilization by Pronase were tested. The stability of Pronase was determined during FPC hydrolysis. After 8 hr of proteolysis at 34° C, Pronase degradation was approximately 30%, and Pronase inhibition by the products of proteolysis was 60%. Gel chromatography showed the molecular weights of the soluble peptides to be less than 2000; those of the proteolytic enzymes of Pronase were close to 20,000.

Fish protein concentrate (FPC) prepared by solvent extraction of whole fish is not readily soluble or dispersible in foods. Since FPC is a protein source of high biological value, it is desirable to have it in useful forms. Soluble FPC's could be used in a variety of foods, including soups, weaning foods, and protein beverages.

FPC solubilization can be accomplished by several different processes. The use of direct biological conversion by microorganisms has been reviewed by Roels (1969). An alkaline process has been investigated by Tannenbaum *et al.* (1970a,b), and direct use of enzymes most recently by Hale (1969). This paper presents an approach to the solubilization of FPC by proteolytic enzymes, including those of plants, animals, and microorganisms. The use of FPC as the substrate instead of fish eliminates stability problems normally associated with residual oxidizable lipid.

For an economical solubilization process, desirable features include capability of continuous operation and use of high enzyme concentrations to achieve rapid rate and high conversion efficiency. However, these goals are not compatible with conventional reactors if the cost of enzyme is high. A continuous enzymatic reaction with high concentration has been demonstrated in a membrane reactor (Wang *et al.*, 1970). The system employed an ultrafiltration membrane which retained the enzyme but passed the reaction products, and under ideal conditions the enzyme could be used indefinitely. Factors particularly important for this enzyme recycle, extent of substrate proteolysis, and stability of the enzyme were studied in detail. In this initial phase, results applicable to both batch and continuous recycling processes are reported.

MATERIALS AND METHODS

Substrates. Fish protein concentrate (FPC) from the Bureau of Commercial Fisheries, College Park, Md., was prepared by grinding whole fresh red hake (*Urophycis chuss*), extracting water and lipids with isopropanol at 95° C, and drying (Table I). The lipid content was less than 0.5%.

Enzymes. The following commercial enzyme preparations were employed in our studies: Pronase, B grade, activity 45,000 proteolytic units per g (*Streptomyces griseus*); pepsin, 3x crystallized, B grade; papain (Calbiochem); bromelin and ficin (Nutritional Biochemical Corp.); Rhozyme P11, diluent salt; Rhozyme 41, diluent corn starch; enzyme No. 56 (Rohm and Haas); Monzyme PA-1™ (*Bacillus subtilis*, Monsanto).

Standard FPC Proteolysis and Solubilization Procedure. All the experiments reported here were batch experiments in which 5 g of FPC were suspended in water and subjected to the action of a proteolytic enzyme. The solubilization experiments were conducted in a jacketed vessel, maintained at a constant temperature, and under constant conditions of magnetic stirring. The pH was maintained at a desired value with 1.0*N* NaOH or 1.0*N* HCl using a Radiometer pH stat. The amount of alkali or acid addition *vs.* time was recorded automatically.

The standard conditions for Pronase study were the following: temperature = 34° C, pH = 8.0, FPC concentration = 10% w/v, Pronase:FPC = 1% w/w. Five percent v/v ethanol was generally added to the system as a preservative (Nomoto *et al.*, 1960). Pronase was dissolved in 2 ml of water shortly before addition.

After a given time of proteolysis, the suspension was centrifuged for 5 min at 6000 × *g* or greater; the pellet was resuspended in 50 ml of water and recentrifuged. The clear supernatants were pooled and the absorbance at 210 and 280 nm was recorded. The total amount of solubilized protein was determined by micro Kjeldahl (A.O.A.C., 1965). The supernatants were also tasted, after neutralization. Neutralization does not cause appreciable precipitation of nitrogeneous material.

Gel Chromatography. After supernatant neutralization, the molecular weight distribution of the soluble peptides was estimated by chromatography of samples in a range of 0.5 to 1.5 ml on a Biogel P10 column, 200- to 400-mesh (BioRad). The samples were prepared in 0.05 *M*, pH 6.9 ammonium acetate. Fractions were collected and absorbance at 280 nm was determined. The column size was 150 × 1.5 cm with a void volume of 37 ml. The flow rate was maintained at 15 ml per hr. The Pronase preparation was chromatographed in a similar manner and the proteolytic activity was assayed in the fractions from the column by the azoalbumin test.

Estimation of the Extent and Rate of Proteolysis. In addition to gel chromatography studies, the extent of proteol-

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Table I. Composition of FPC Batches

Sample Number	Bureau of Commercial Fisheries Reference	Nitrogen, %	Volatiles, %	Ash, %
1	BH-14/0	13.4	5.54	11.9
2	62.1 H26.3	13.8	1.6	...
3	41 B	13.0	4.7	...
4[a]	71.500	14.9	4.2	3–4

[a] Made from deboned fish.

ysis was also estimated in a number of ways. The amino nitrogen to total nitrogen ratio was determined in some cases by the ninhydrin method; this showed a good correlation with the reciprocal of the 210 nm absorbance (peptide bond absorption). In most cases, the equivalence of NaOH added during proteolysis was assumed to be proportional to the number of peptide bonds cleaved. However, this was not absolutely correct due to the increasing buffering capacity of the hydrolyzate, but nevertheless yielded an approximate indication of the degree of hydrolysis. Using the NaOH addition in this manner as an indication of degree of hydrolysis, a plot of the logarithm of the relative concentration of remaining peptide bonds

$$\frac{(\text{ml NaOH}_{max} - \text{ml NaOH at time } t)}{\text{ml NaOH}_{max}}$$

vs. time should allow an estimate of the overall reaction rate constant (ml NaOH_{max} taken as 8 ml of 1*N* NaOH per 5 g of FPC at a pH of 8.0). If proteolysis were first-order with respect to the concentration of peptide bonds, the slope from this plot should remain constant with time. Furthermore, the slope should also be proportional to the enzyme concentration. However, this type of analysis was not valid over the entire range of hydrolysis. The reasons could be multiple enzymes and substrates, changes in the nature of the substrates during reaction, product inhibition, or enzyme inactivation. It was found, however, that the estimated slope during an initial time interval of 15 min could be used for an approximate assay of the enzyme concentration at the conditions and in the ranges tested.

Estimation of Inhibition and Degradation of Pronase During FPC Proteolysis. This determination of the overall reaction rate constant was used to estimate the inhibition and degradation of Pronase during FPC proteolysis. The following FPC addition technique was adopted. Five grams of FPC was hydrolyzed for a given length of time; at this time, an additional 5 g of FPC was added to the reaction mixture. The initial rate constants for the first as well as the second FPC additions were compared again using the 15-min interval evaluation period. The enzyme activity measured from the slope during hydrolysis after the second addition was compared with the initial activity. This ratio allowed an estimate of the remaining enzyme activity during hydrolysis and was designated as percentage A.

The following experiment permitted a rough estimation of the respective roles of enzyme inhibition (by the products of proteolysis) and of enzyme degradation (thermal denaturation or autolysis). Enzyme-hydrolyzed FPC was first boiled 5 min and cooled; 5 g of fresh FPC was then added. The pH was adjusted to 8.0 and an identical amount of fresh enzyme was added. Again the slope was estimated over a 15-min interval and compared with the initial activity. Percentage of enzyme activity was recorded as B, and degree of inhibition was calculated by obtaining the difference of B from 100%. Percentage of enzyme degradation during FPC proteolysis was taken as $[1 - A/B] \times 100$. A standard curve was constructed by adding various amounts of fresh enzyme (10, 30, 50 μg) in the inhibition control experiment, which proved initial reaction rate constant approximately proportional to enzyme concentration.

Assays of Protease Activity. UNITS OF PROTEOLYTIC ACTIVITY. One unit of proteolytic activity is defined as the amount of enzyme which yields, per min, the same absorbance as 1 μg of tyrosine under the same conditions. Units of proteolytic activity were determined in the following manner: 20 to 50 μg of enzyme was added to 5 ml of a 0.6% casein solution in an appropriate buffer. After 10 min at 40° C, the reaction was stopped by adding 5 ml of 10% trichloroacetic acid solution. The mixture was centrifuged; 1 ml of Folin-Ciocalteau reagent (Fisher Scientific), diluted 1 to 3, and 1 ml of 1.3 *M* sodium carbonate were added to 2 ml of the supernatant. The mixture was allowed to stand for 30 min at 30° C and the absorbance was then measured at 660 nm. The absorbance was proportional to the enzyme concentration.

AZOALBUMIN TEST. The proteolytic activity of Pronase was also assayed using a 0.75% w/v solution of azoalbumin as substrate (Sigma Chemicals) in a 0.05 *M* phosphate buffer, pH 8.0. Pronase, ranging from 10 to 30 μg, was added to 2 ml of the substrate solution. After 10 min at 40° C, 2 ml of a 10% trichloroacetic acid solution was added and the mixture was allowed to stand for 20 min at 40° C. The protein precipitate was filtered on Schleicher and Schull paper No. 576. The filtrate was made alkaline with an equal volume of 1*N* NaOH. The amino acids and small peptides released from azoalbumin by proteolysis were soluble in trichloroacetic acid. Their absorbance at 400 nm (due to the attached azoic dye) was measured in the alkaline filtrate. The absorbance measurements were made at 0° C to minimize changes in the intensity of the developed color. The absorbance is a direct indication of the proteolytic activity and was proportional to the enzyme concentration. However, when Pronase was present in FPC hydrolyzates, the assay procedure outlined above was not reliable due to the interference of other colors developed.

^{14}C ACTOMYOSIN TEST. ^{14}C actomyosin prepared from rat skeletal muscle after injection of ^{14}C amino acids was used. Eleven mg of actomyosin (6000 dpm) in suspension in 1 ml of water were mixed with 0.5 ml of 0.1*M*, pH 8.0 sodium phosphate buffer. A sample containing 10 to 30 μg of Pronase was added. After 10 min at 40° C, 0.4 ml of a 10% trichloroacetic acid solution was added. The mixture was centrifuged and the radioactivity of the supernatant was counted. When the radioactivity from a blank was subtracted, the net radioactivity was proportional to the enzyme concentration. This test was used for the determination of Pronase degradation during FPC proteolysis.

RESULTS AND DISCUSSION

Relative Activities of Various Proteolytic Enzymes on FPC. The relative activities of various enzyme preparations are presented in Tables II and III. On a weight basis, Pronase and pepsin preparations appeared to be the most effective with respect to the extent as well as the rate of solubilization of FPC. These findings are similar to those published by Hale (1969). Our results further showed that the extent and the rate of proteolysis are both higher for Pronase than for pepsin or the plant enzymes. This could be due to the broad

Table II. Solubilization of FPC Proteins by Various Proteolytic Enzymes

FPC Batch No. 1. FPC:H_2O = 5% w/v. 5% of ethanol unless otherwise indicated. 37° C

Enzyme:FPC % w/w	Hydrolysis Time (hr)	pH	Other Conditions	Nitrogen Solubilized, %	Peptide Bonds Broken, % (From NH_2 Analysis)
Pepsin					
0.5	24	2.0	...	95	25
0.5	3	2.0	...	67	...
0.25	6	2.0	...	77	...
0.25	5	3.0	...	18	...
0.25	12	2.0	...	90	...
0.05	24	2.0	...	87	...
0.0	12	1.5	no ethanol	32	...
Papain					
5	24	7.5–6.5	with or with-out ethanol	84	38
0.5	24	7.5–6.5	...	28	...
0.5	24	7.5–6.5	no ethanol	56	...
0.5	24	5.1	...	32	...
Ficin					
5	24	7.5–6.5	with or with-out ethanol	86	40
0.5	24	7.5–6.5	...	49	...
0.5	24	7.5–6.5	no ethanol	76	...
0.5	24	5.1	...	44	...
Bromelin					
5	24	7.5–6.5	with or with-out ethanol	81	43
0.5	24	7.5–6.5	...	55	...
0.5	24	7.5–6.5	no ethanol	64	...
0.5	24	6.3	...	67	...
Rhozyme P11					
5	24	7.5–6.5	no ethanol	47	...
0.5	48	7.5–6.5	...	36	...
Rhozyme 41					
5	24	7.5–6.5	no ethanol	49	...
0.5	48	7.5–6.5	...	41	...
Pronase					
0.5	24	8.5	...	95	53
0.5	5	8.5	...	82	...
0.5	5	7.5–6.4	...	71	...
0.5	3	8.5	...	67	26
0.25	12	8.5	...	90	...
0.05	24	8.5	...	87	...

specificity of Pronase or to a relatively small degree of enzyme inhibition by the products of hydrolysis. The hydrolyzates from pepsin and Pronase digestion demonstrated the most and the least bitterness, respectively, of all those samples tasted. Precooking or pretreatment of FPC with acid did not facilitate enzymatic solubilization. The use of ethanol as a preservative inhibited Pronase activity to the extent of approximately 10% (Table III).

Adjustment of an aqueous slurry containing 1 g of FPC to pH 8.0 or to pH 2.0 required 0.21 ml of 1*N* NaOH or 2.6 ml of 1*N* HCl, respectively. For pH maintenance during proteolysis (reaching 95 or 80% nitrogen solubilization), 1.6 ml of 1*N* NaOH or 0.4 ml of 1*N* HCl were required, respectively. Neutralization further increased the ion content of the final product.

Comparing the activity of the various enzymes for FPC hydrolysis, our findings indicated that Pronase would be particularly promising for a continuous proteolytic process with enzyme reutilization. This is true, especially considering the nearly complete solubilization of FPC nitrogen. We therefore selected Pronase for further evaluation; however, other enzymes may be found that satisfy a greater number of criteria desirable for either batch or for recycling processes.

Effect of pH on the Rate of FPC Proteolysis and Solubilization by Pronase. The influence of pH on the rate of FPC proteolysis was studied at 40° C (Table IV). Although NaOH addition at different pH values yielded only a rough estimate of the extent of proteolysis, this was sufficient for comparative purposes. The results showed that in the pH range tested (7.8 to 8.5) there was little difference in the degree of hydrolysis. Solubilization of FPC protein was also quite similar at pH

Table III. Solubilization of FPC Proteins by Various Proteolytic Enzymes

FPC Batch Nos. 2, 3, and 4. FPC:H_2O = 10% w/v. 5% of ethanol, unless otherwise indicated. 34° C

Enzyme: FPC % w/w	Hydrolysis Time (hr)	pH	Other Conditions	Nitrogen Solubilized, %
		Pronase[a]		
1	23	8.0	FPC No. 2	94
1	2	8.0	FPC No. 2	73
1	2	8.0	FPC No. 3	67
1	2	8.0	FPC No. 3, no ethanol	74
1	2	8.0	FPC No. 4	65
1	2	9.0	FPC No. 3	72
1	2	9.0–8.0	FPC No. 3, pH control with $Ca(OH)_2$, no ethanol	69
0.5	2	8.0	FPC No. 2	50
0.5	5	8.0	FPC No. 2	82
0.2	5	8.0	FPC No. 3, 50° C, no ethanol	70
		Monzyme[a]		
5	23	7.3–6.4	FPC No. 3	80
1	2	7.3–6.4	FPC No. 3, no ethanol	46
		Rohm and Haas No. 56		
1	2	7.4	FPC No. 2, no ethanol	≃50
1	2	10	FPC No. 2, no ethanol	≃50

[a] Pronase (pH 8.0) was found to have 89,000 units of proteolytic activity per g and Monzyme (pH 7.0–6.5), 59,000 units per g.

Table IV. Influence of pH on FPC Proteolysis by Pronase

Values presented are milliliters of 1*N* NaOH added per 5 g of FPC for various pH's and durations of proteolysis. Pronase:FPC = 1% w/w. No ethanol was added. 40° C. FPC batch No. 2

Time (min)	pH 7.8	pH 8.0	pH 8.2	pH 8.5
15	1.6	1.9	2.1	2.0
60	3.1	3.7	3.9	3.7
120	4.0	4.7	4.9	4.8

Table V. Influence of Temperature on FPC Proteolysis by Pronase

Values are milliliters of 1*N* NaOH added per 5 g of FPC for various temperatures and durations of proteolysis. Pronase:FPC = 1% w/w. No ethanol added. FPC batch No. 2

Time	26° C	34° C	40° C	48° C	55° C
15 min	0.85	1.3	1.9	2.5	2.9
30 min	1.3	2.0	2.7	3.3	3.7
1 hr	1.9	2.8	3.7	4.2	4.5
2 hr	2.7	3.8	4.7	5.0	5.3
3 hr	3.4	4.5	5.4	5.5	5.7
8 hr	4.8	5.7	6.3	6.1	6.2

7.5 to 6.4, pH 8, or pH 9 (Tables II and III). In view of these findings, we arbitrarily selected a pH of 8.0 for the remainder of our studies.

Effect of Temperature on the Rate of FPC Proteolysis by Pronase. The study of the influence of temperature on the rate of FPC proteolysis is shown in Table V. The degree of proteolysis increased for reaction times of less than 2 hr as the temperature was increased from 26° to 55° C. However, beyond this reaction time, and for temperatures above 40° C, there was no appreciable rate enhancement. This may be due to the rapid degradation of Pronase at the higher temperatures.

Effect of Substrate Concentration on the Rate of FPC Protein Solubilization by Pronase. Protein solubilization has been studied for FPC batch No. 2 for 2 hr at 34° C and 1% enzyme to substrate ratio. The degrees of solubilization obtained were 76, 73, and 63% for FPC concentrations of 5, 10, and 15%, respectively. The reduced rate of solubilization observed at higher FPC concentrations may stem from a less efficient substrate to enzyme transfer, or to inhibition of Pronase by FPC. It will be shown later that the products of the reaction partly inhibit further FPC proteolysis. An FPC concentration of 10% has been selected for further experiments.

Influence of Enzyme Concentration on the Rates of FPC Proteolysis and Solubilization by Pronase. The effect of enzyme concentration on FPC proteolysis and protein solubilization is reported in Table VI. For a reaction time of 2 hr, the amount of protein solubilized due solely to enzyme action was roughly proportional to the enzyme concentration. The importance of the effect of enzyme concentration for proteolysis is especially relevant when considering enzyme recycle. For example, assuming a relatively stable enzyme which can be recycled, a high enzyme concentration would maximize enzyme reactor productivity. However, one must also consider the effect of enzyme concentration on enzyme stability.

Considering the amount of NaOH added with respect to the extent of solubilization, an efficiency ratio of solubilization: proteolysis appears to be highest within the range of 40 to 70% solubilization. One can visualize that soluble peptides can be further proteolyzed without any increase in the efficiency of solubilization.

Rate of FPC Proteolysis and Solubilization by Pronase. The extent of proteolysis and solubilization of FPC proteins were studied as a function of time. These results, shown in Table VII, were all derived from duplicate samples. In addition, the nonsolubilized residue was dried, homogenized, and analyzed to obtain a detailed material balance. The results from this material balance were found to agree within 5% with the protein added initially. It was also observed that the 280 nm absorbance of proteolysis supernatants was not an accurate measure of their nitrogen content.

From the data in Table VII the overall reaction rate constant was calculated on the basis of NaOH added. However, according to these data, the rate constant decreased with time. Therefore, proteolysis did not follow first-order kinetics with respect to the concentration of peptide bonds. This is not surprising if one considers the multitude of possible substrates, the number of proteolytic enzymes, as well as the different specificities which are known to be present in Pronase. In addition, the formation of products of hydrolysis which may be inhibitory, as well as enzyme deactivation during the course of FPC proteolysis, could also contribute to the decrease in the rate constant. However, in the initial time interval of 15 min, the reaction rate constant is approximately proportional to the enzyme concentration, provided the enzyme concentration is less than 0.5 mg per ml.

For a reaction time of 5 hr at a Pronase:FPC ratio of 0.5%, the degree of solubilization as measured by soluble nitrogen was found to be identical to that obtained with a ratio of 1%. This indicates that a complex relationship exists between the enzyme:substrate ratio and the residence time in the reactor,

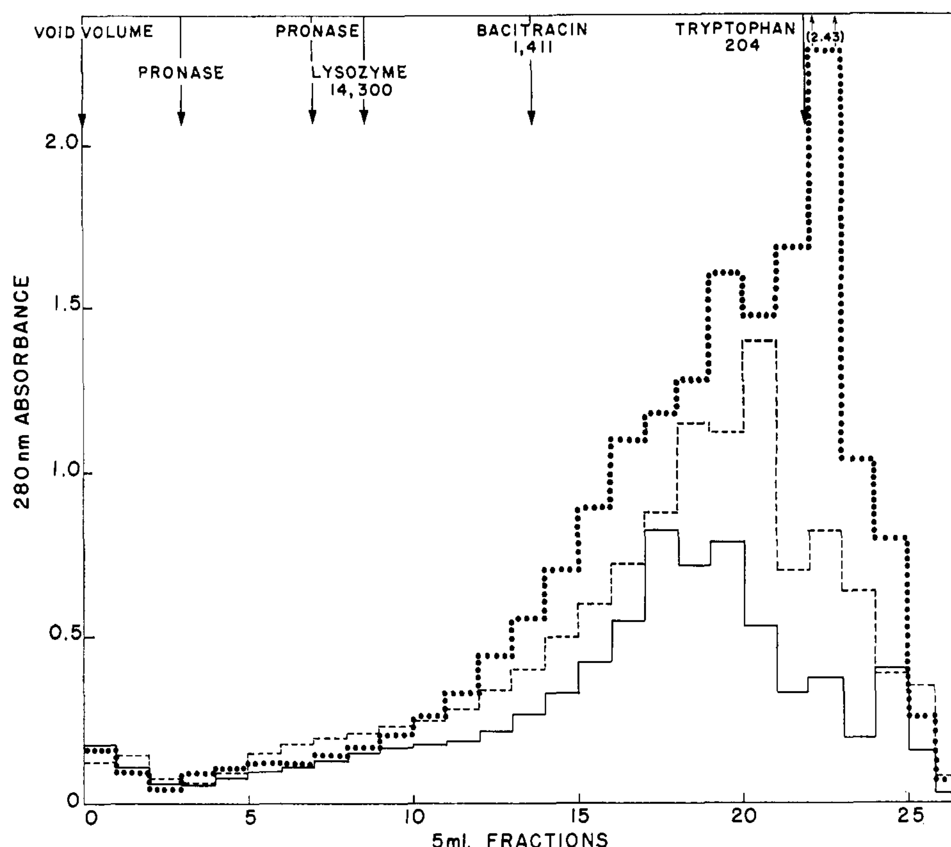

Figure 1. Chromatography of supernatants from FPC hydrolyzates on Biogel P10. The supernatants are those from the experiments described in Table VII; 20 min (—), 2 hr (– –), and 23 hr (. . .) proteolyses. Conditions of chromatography are given in "Methods." Chromatography of Pronase, lysozyme, bacitracin, or tryptophan was run independently

Table VI. Influence of Pronase Concentration on FPC Proteolysis and Protein Solubilization

2 hr of proteolysis. 34° C. FPC batch No. 2

Pronase:FPC % w/w	No. ml 1*N* NaOH Added During 2 hr of Proteolysis (values per 5 g of FPC)	Protein Solubilization, %
0.0	...	≃20
0.2	1.9	31
0.5	3.1	50
1.0	3.8	73

and that the optimal enzyme:substrate ratio can only be determined for a given residence time.

Composition and Molecular Weight Distribution of the Fractions Obtained from FPC Hydrolysis by Pronase. The insoluble material after enzyme hydrolysis consisted of two layers which were separated by decantation: a dense white bottom layer, probably of bone origin, which showed sharp-angled particles upon microscopic examination; and a less dense layer, dark, and probably originating from the skin. The amount of this second layer was dependent on the extent of proteolysis.

From an experiment in which 84% of FPC nitrogen was solubilized, the neutralized supernatant and the insoluble fractions were spray-dried. Table VIII summarizes the material balance from this experiment, as well as the composition of the different fractions.

The soluble supernatants of FPC hydrolyzates were also analyzed by chromatography on Biogel P10, as shown in Figure 1. The 280 nm absorbance of the fractions, although not exactly proportional to the nitrogen content, permitted an estimation of the molecular weight distribution of FPC peptides. A very small percentage of the 280 nm absorbing material was found to be excluded from the column. The molecular weight of most soluble peptides was approximately equal to or less than 2000. This behavior was also true even with short reaction times of FPC in the presence of Pronase.

Table VII. Rate of FPC Proteolysis and Solubilization

Pronase:FPC = 1% w/w. 34° C. FPC batch No. 2

Time	No. ml 1*N* NaOH Added (per 5 g of FPC)	Protein Solubilization, %
20 min	1.6	39
2 hr	3.8	73
5 hr	5.2	83
23 hr	8.1	94

Table VIII. Soluble and Insoluble Constituents of an FPC Hydrolyzate

Pronase:FPC = 0.125%. 1 kg of FPC batch No. 1. 37° C, ≃20 hr, pH 8.5

	Dried Soluble Fraction	Dried White Insoluble Fraction	Dried Black Insoluble Solids
Weight (g)	795	120	81
FPC nitrogen, %	84	≃4[a]	12
Nitrogen content, %	14.2	4.1	14.0
Ash content, %	9.6[b]	72	8
Volatile content, %	4.2	...	...

[a] This value is probably constant; thus when 95% of FPC nitrogen is solubilized, the black fraction accounts for very little nitrogen. [b] Including 5% NaCl formed by the process.

Table IX. Degradation of Pronase by Heat

0.1% Solution of Pronase in H_2O. Azoalbumin test

Temperature	−15° C	4° C	25° C	55° C	85° C	100° C
Duration	55 hr	55 hr	25 hr	4 hr	40 min	3 min
Degradation, %	0	3	15	65	100	100

Table X. Degradation of Pronase at 34° C and pH 8.0

Azoalbumin test

Pronase concentration (% in H_2O)	0.1	0.1	0.1[a]	0.2
Duration (hr)	5	3	3	3
Degradation, %	54	35	15	41

[a] In the presence of $10^{-2}M$ Ca^{2+}.

Table XI. Inhibition and Degradation of Pronase in FPC Hydrolyzates

Pronase:FPC = 1% w/w; pH = 8.0; 8 hr of proteolysis. FPC batch No. 2. No ethanol added

Temperature	26° C	34° C	40° C	48° C	55° C
Remaining enzymatic activity, %	31	27	26	24	16
Enzyme inhibition, %	56	61	58	...	...
Enzyme degradation, %	30	31	38	...	...

The peptides, however, shifted toward even lower molecular weight products of hydrolysis, including free amino acids, as a result of longer lasting proteolysis. Similar results were found with soluble peptides derived from FPC proteolysis by Monzyme.

Lastly, the Protein Efficiency Ratio (PER) (NAS-NRC, 1963) of the dried soluble fraction was measured in young male rats fed a 10% protein diet; their PER was equivalent to the starting PER of the FPC material and slightly greater than that of casein.

The results on molecular weight distribution of the soluble products indicated that separation of these peptides from the proteolytic enzymes by ultrafiltration may be possible.

Pronase Stability During FPC Proteolysis; Inhibition of Proteolytic Activity by the Products of Hydrolysis. The stability of a 0.1% solution of Pronase in water was examined at various temperatures for various periods of time (Table IX). The results indicated that temperatures somewhere between 25° and 55° C were extremely detrimental to enzyme stability, and that loss of enzymatic activities may be encountered in a prolonged continuous proteolytic process. The possible protective effects of some factors on enzymes must also be considered and are presented below.

To examine further the nature of enzyme inactivation, enzyme stability experiments were performed at pH 8.0, 34° C, with constant stirring. The results for a 0.1% Pronase solution are shown in Table X.

In these experiments it was necessary to add NaOH to maintain the pH at 8.0. These results, especially at high enzyme concentration, suggested that the enzymes of Pronase were self-digesting, which in turn accounted for portions of the decrease in enzymatic activities. Thermal denaturation may also occur. Since calcium ions have been reported to increase Pronase stability, experiments to verify this were also performed (Table X). A $0.01M$ Ca^{2+} solution did offer some protection against enzyme deactivation.

To elucidate the possible role of FPC in protecting the enzymes from deactivation, a series of experiments was performed in the presence of substrates and proteolysis products at various temperatures. In order to assess the true degree of protection, possible inhibition by the products of proteolysis was also estimated. In the conditions of our assays, enzyme inhibition reached 60% (Table XI).

In another inhibition test, performed with a 17-hr (34° C) hydrolyzate, the percentage of inhibition was 41%. From these results and the previously reported effect of FPC concentrations on the rate of protein solubilization, it seems likely that the rate of proteolysis of FPC by Pronase is inhibited both by the products of hydrolysis and, to a lesser extent, by a high concentration of FPC itself.

From a comparison of the percentages of degradation of Pronase at pH 8.0 without Ca^{2+} (Table X), and the percentages of degradation in the presence of FPC (Table XI), it seems that the stability of the enzymatic activity is increased by FPC. The percentage of degradation still was found to be close to 30% after 8 hr at 26° C (Table XI). It is probably much higher at 55° C. It is likely that below 55° C Pronase degradation is mainly due to its own proteolytic activity. It would be useful to know if some of the proteolytic enzymes of Pronase remain stable for longer periods of time, and also if enzyme degradation in the presence of FPC is a function of enzyme concentration.

Tests with ^{14}C actomyosin confirmed the degradation values reported in Table XI. Thirty-five percent degradation was found after 8 hr of proteolysis at 34° C.

From the data presented, we have selected a temperature of 34° C for most of the present studies, because of enzyme stability considerations. The percentage of enzyme degradation observed does not preclude the feasibility of Pronase recycling by ultrafiltration of the small FPC peptides. Preliminary results with ultrafiltration membranes showed that it was indeed possible to separate the proteolytic enzymes from the soluble FPC peptides, and that most inhibitors of Pronase were not retained by the membranes (Cheftel *et al.*, 1970). It is also possible that in a continuous solubilization process, Pronase may be better protected because of the continuous addition of fresh FPC.

Estimation of the Molecular Weights of Pronase Proteases. Recent studies on the separation and characterization of the various constituents of Pronase have shown that this enzyme preparation is a mixture of peptidases, neutral proteinases,

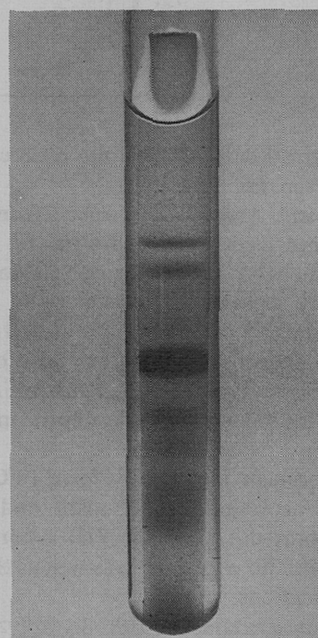

Figure 2. Polyacrylamide gel electrophoresis of Pronase in the presence of sodium dodecyl sulfate. Staining with Coumassie blue; 100 μg of Pronase

and alkaline proteinases with various specificities and inhibitors (Narahashi *et al.*, 1968; Wählby, 1968; Narahashi and Fukunaga, 1969; Trop and Birk, 1969). Molecular weights were determined by ultracentrifugation or estimated by gel filtration and amino acid composition, and were found to be in a range of 15,000 to 20,000 (Narahashi and Yanagita, 1967; Narahashi *et al.*, 1968; Morihara *et al.*, 1968; Trop, and Birk, 1970).

Chromatographic examination of the Pronase preparation used on Biogel P10 showed three main 280 nm absorbing peaks. Only the first two displayed proteolytic activity against azoalbumin at pH 8.0; their position is indicated in Figure 1. The third peak probably consisted of small peptides and free amino acids. It was not possible to state accurately the molecular weights of the two enzymatic fractions, since self-proteolysis of Pronase may have occurred.

Pronase was also analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate according to Weber and Osborn (1969). This method permitted a determination of molecular weights which is both reproducible and reliable. For this determination, dry Pronase was first dissolved in boiling 6*M* guanidine, reduced, and carboxymethylated according to standard procedures. The protein was then precipitated by extensive dialysis, centrifuged, and redissolved in sodium dodecyl sulfate. After electrophoresis and staining, we observed a major band representing 70 to 90% of the protein material. This band was calculated to correspond to a molecular weight of 18,500 to 19,000 (Figure 2). Other minor bands corresponded to molecular weights close to 36,000, 31,000, and 15,000. The method, however, did not permit estimation of molecular weights less than 10,000. Molecular weight calibrations were performed concurrently using standard proteins.

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