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Flavor of Enzyme-Solubilized Fish Protein Concentrate Fractions

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The soluble portion of fish protein concentrate (FPC) hydrolysates, obtained by treatment with the proteolytic enzymes bromelain, ficin, or Pronase, were fractionated by size and charge. All fractions were tasted. Bitterness and glutamic acid taste (acid-like) were the main contributors to off-flavors in the hydrolysates. Pronase hydrolysates were less bitter than those from bromelain or ficin. Pronase hydrolysates had a higher proportion of low molecular weight peptides (mol wt ~300) than those obtained with bromelain or ficin. The last two exhibited a higher proportion of peptides in the 800 molecular weight region where bitterness was also predominantly found. Paper electrophoresis showed that the bitter fractions had more basic peptides while the acid-like fractions had more acidic peptides. A basic bitter mixture isolated from the 800 molecular weight region of a ficin hydrolysate contained glycine, isoleucine, leucine, phenylalanine, and valine as N-terminal residues. Further separations of this bitter fraction suggested the presence of a basic bitter tripeptide or peptides with the following structures: N-terminus, leucine or glycine; middle, asparagine; C-terminus, lysine.

Proteolytic enzyme hydrolysis of fish protein concentrate (FPC) improves the solubility of the product (Cheftel et al., 1971; Hevia et al., 1976). However, the formation of an undesirable bitter taste is a consistent side effect of the treatment (Cheftel et al., 1971; Fujimaki et al; 1973, 1974; Noguchi et al., 1975). The degree of bitterness in the soluble product differs with the enzyme used. The bacterial protease Pronase has been reported to produce less bitterness than ficin or bromelain (Cheftel et al., 1971; Hevia et al., 1976). In the present study, soluble fish protein concentrates obtained by treatment with Pronase, ficin, and bromelain were compared with regard to molecular weight, charge, and taste of the resultant mixture of peptides. The purpose was to determine the nature of the bitter components.

EXPERIMENTAL MATERIAL AND METHODS

Fish protein concentrate hydrolysates containing equal amounts of soluble products, formed in equal time periods, were obtained by incubation of solvent-extracted FPC with bromelain (25 mg/g of FPC), ficin (40 mg/g of FPC), or Pronase (10 mg/g of FPC) for 1 and 7 h as previously described (Hevia et al., 1976).

The molecular weight distribution of the different hydrolysates was determined by gel filtration through a column of Bio-Gel P-2 polyacrylamide gel (100–200 mesh, Bio-Rad). A total gel bed of 450 mL was packed into a K 26/100 Sephadex column (100 × 2.6 (i.d.) cm). Two hundred milligrams of each of the solubilized fractions was dissolved in 2.5 mL of 10% acetic acid, added to the column, and eluted with 10% acetic acid (0.5 mL/min). Ten-milliliter fractions were collected and freeze-dried. The void volume of the column was determined by the elution of bovine serum albumin (OD at 280 nm). The elution patterns of the hydrolysates or of calibration standards (oxytocin, oxidized glutathione, reduced glutathione, tetraglycine, and glycine) were monitored manually by reaction with ninhydrin as described by Hirs (1967).

The charge distribution of the peptides in the whole hydrolysates and in their Bio-Gel P-2 fractions was determined by paper electrophoresis, conducted in a horizontal Savant high-voltage electrophoresis apparatus provided with a cooling plate on the bottom. The sample was applied to the center of the paper (Whatman No. 3) and covered with a siliconized glass plate. The buffer (10% pyridine adjusted to pH 6.5 with glacial acetic acid) was allowed to reach the application point by capillarity.

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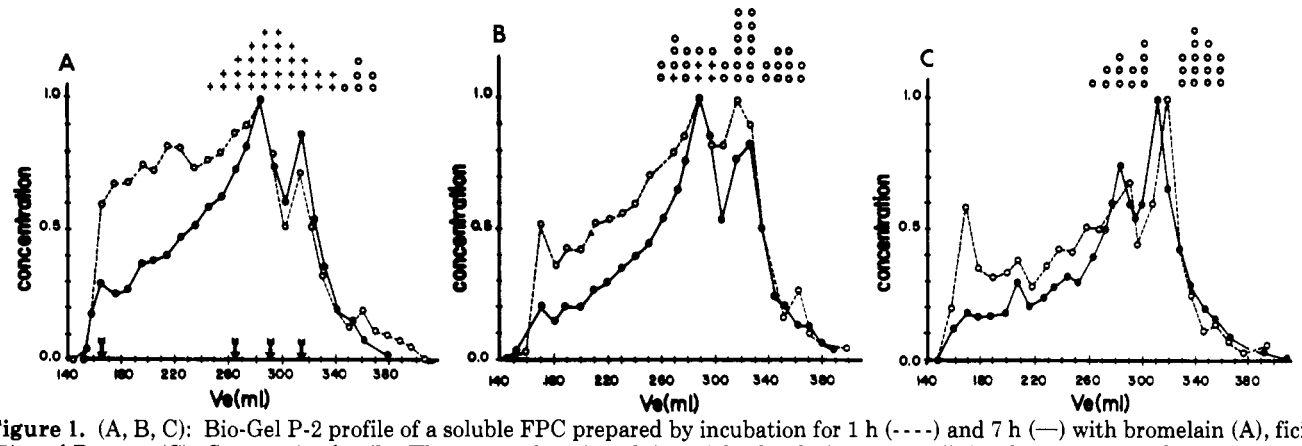

Figure 1. (A, B, C): Bio-Gel P-2 profile of a soluble FPC prepared by incubation for 1 h (----) and 7 h (—) with bromelain (A), ficin (B), and Pronase (C). See text for details. The arrows show from left to right the elution volume (V_e) of bovine serum albumin, oxytocin (mol wt 1225), oxidized glutathione (mol wt 600) and reduced glutathione (mol wt 300). At top are the flavor profiles (after 7-h incubation); bitter (+), acid-like (O), or bland in taste (no symbol).

Electropherograms were developed with 0.1% ninhydrin in acetone.

Whole hydrolysates, water-resuspended Bio-Gel P-2 fractions, and samples eluted from the electropherograms were tasted as follows: the solution was placed directly on the center of the tongue with a Pasteur pipet. Two or more persons were involved in each taste determination.

N-Terminal amino acids were determined by dansylation as described by Gray (1972). The time of hydrolysis of the danysl peptides was 4 h as recommended by Gross and Labouesse (1966). The dansyl amino acids were identified by two-dimensional thin-layer chromatography on Polyamide sheets (Gray, 1972). Peptide sequence was determined by the rapid degradation procedure described by Gray (1972). The reagents were phenylisothiocyanate (PITC solution) and trifluoroacetic acid, both Sequenal grade (Pierce). Ion-exchange chromatography was conducted with a jacketed 22 × 0.8 (i.d.) cm column of Bio-Rad AG 50W-X8 cation-exchange resin (Schroeder, 1972). The pyridine acetate gradient consisted of 250 mL of pyridine acetate, pH 2.8 (8.1 mL of pyridine and 278 mL of glacial acetic acid per liter), in the mixing chamber and 250 mL of pyridine acetate, pH 5 (161 mL of pyridine and 143 mL of glacial acetic acid per liter), connected by siphon. The temperature of the column was 38 °C. Four-milliliter fractions were collected and monitored with ninhydrin (Hirs, 1967).

RESULTS AND DISCUSSION

Figure 1 (A, B, C) illustrates the Bio-Gel P-2 elution profiles of FPC hydrolysates that had been obtained by incubation for 1 and 7 h, with bromelain, ficin, and Pronase. All showed two well-resolved peaks in the low molecular weight region of the chromatograms. The first had elution volume of 280 mL, between that of oxytocin and oxidized glutathione. The second had an elution volume of 310 mL similar to that of reduced glutathione. Thus large fractions of the peptides in the hydrolysate had molecular weights in the range of 800 and of 300 (Table I). A shift toward smaller peptides with time of hydrolysis was also described for Pronase-solubilized FPCs by Cheftel et al. (1971).

The hydrolysates prepared with bromelain and ficin contained more peptides in the region of 800 molecular weight than in the 300 molecular weight region, whereas the opposite was observed with Pronase-treated FPC (Table I).

The top part of Figure 1 (A, B, and C) shows the flavor profiles of the Bio-Gel P-2 fractions of the hydrolysates obtained by incubation of FPC for 7 h with the enzymes.

Table I. Relationships between the Peak Areas (in cm^2) and Total Areas in the Bio-Gel P-2 Fractionations

Digest[a]	1st peak area	% of total	2nd peak area	% of total
B1	2.3	33	0.6	9
B7	1.8	33	1.0	19
F1	2.0	32	0.9	14
F7	2.2	42	1.2	23
P1	1.1	22	1.2	24
P7	1.1	26	1.4	33

[a] B1, B7, bromelain digests, 1 and 7 h; F1 and F7, ficin digests; P1 and P7, Pronase digests.

Fractions in the high molecular weight region (V_e 140–240 mL) were bland in taste. Off-flavors, mainly bitterness (+) and acid-like taste (O) (similar to the taste of glutamic acid), were associated with fractions in the low molecular weight region (V_e 240–380 mL). The bromelain hydrolysate exhibited bitter fractions mainly in the region of the first peak (V_e 260–290 mL) while the acid-like taste was found at the lower end of the chromatogram. In the ficin hydrolysate, the acid-like taste was the predominant flavor. However, when bitter flavor was detected, it was also associated with the first of the two peaks (V_e 260–290 mL). The Pronase hydrolysate was not bitter (Figure 1C). When the concentration of the high molecular weight fractions (V_e 140–240 mL) was doubled to give a level similar to that of concentrates obtained in the peak region of the chromatogram, increased off-flavors, mainly bitterness, were observed. However, these off-flavors were much less pronounced than those observed with the first (V_e 280 mL) and second (V_e 310 mL) peaks of the chromatogram. Fujimaki et al. (1974) reported similar results for a Pronase-solubilized fish protein concentrate.

These observations indicate that off-flavors in enzyme-treated FPC are related mainly to the peptides with molecular weight in the region of 800 and smaller. Bitter peptides are predominantly found in the 800 molecular weight region while acid taste peptides are lighter (mol wt ~300). The degree of bitterness or acid-like taste found in a particular hydrolysate depends upon the proportion of peptides found in either of these two regions. This could explain the lower level of bitterness observed in Pronase-treated FPC. This conclusion is somewhat obscured by the fact that bitterness was not found in any of the molecular weight fractions in our Pronase-treated FPC. Fujimaki et al. (1974) reported bitterness in a 1000 molecular weight fraction of a Pronase-treated FPC.

The charge distribution at different regions of the Bio-Gel P-2 profile for the hydrolysates obtained by in-

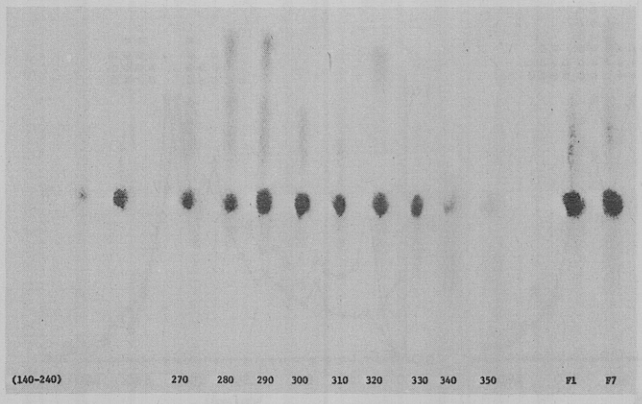

Figure 2. Charge distribution of selected Bio-Gel P-2 fractions and soluble FPC obtained by incubation with ficin. From left to right: 25 μL (first spot) and 50 μL (second spot) of the pooled bland tasting fractions (V_e 140–240 mL); 25 μL fraction V_e 270 through 350 mL (spots 3 to 11); 20 μL of a 0.6% (w/v) whole hydrolysate obtained by incubation with ficin for 1 and 7 h (last two spots). Neutral peptides are in the center of the figure. Basic and acidic peptides are in the upper and bottom parts of the figure, respectively.

Table II. Known Basic Bitter Peptides, Source and Molecular Weight

Peptide	Source	Mol wt	Reference
Lys-Gly		221	Kirimura et al. (1969)
Lys-Ala		235	Kirimura et al. (1969)
Arg-Pro		289	Kirimura et al. (1969)
Leu-Lys	Soy protein	277	Fujimaki et al. (1971)
Arg-Leu	Soy protein	305	Fujimaki et al. (1971)
Arg-Leu-Leu	Soy protein	437	Fujimaki et al. (1971)
Ser-Lys-Gly-Leu	Soy protein	458	Fujimaki et al. (1971)
Arg-Gly-Pro-Pro-Phe-Ile-Val	Casein	893	Miniamura et al. (1972)

cubation with ficin for 7 h is shown in Figure 2. The pooled bland fractions (V_e 140–240 mL) contained mainly neutral peptides while fractions in the off-flavor region (V_e 270–350 mL) exhibited more charged peptides. The figure also shows that the bitter region (V_e 270–280 mL) exhibited a higher proportion of basic peptides than the bland (V_e 140–240 mL) or the acid-like tasting region (V_e 310–350 mL). The latter showed an increase in acidic peptides. Similar electrophoretic behaviors were observed for the Pronase and bromelain hydrolysates (not shown).

Figure 3 indicates the flavor of isolated zones of pooled bitter fractions (V_e 270–280 mL) of the same ficin hydrolysate. The neutral (E3) and one of the basic (E2) zones were bitter, while the acid-like taste was found in the neutral (E3) and acidic (E4) zones. The fact that zone E2 was bitter even although the concentration of ninhydrin-positive components in E3 were higher than E2 suggests that there is a lower bitter threshold level in the basic than in the neutral zone. It also indicates that basic bitter peptides are important contributors to the total bitter taste of the V_e 270–280 mL fraction and the whole hydrolysate. Dansylation and separation of the dansyl amino acids showed glycine, leucine, phenylanine, valine, and isoleucine as N-terminal residues in the basic bitter zone E2. The first four have been found as terminal amino acids in bitter peptides isolated from other food stuffs (Kirimura et al., 1969; Matoba et al., 1969; Fujimaki et al., 1971; Minamiura et al., 1972). Examples of bitter peptides isolated from hydrolysates of other food stuffs are shown in Table II.

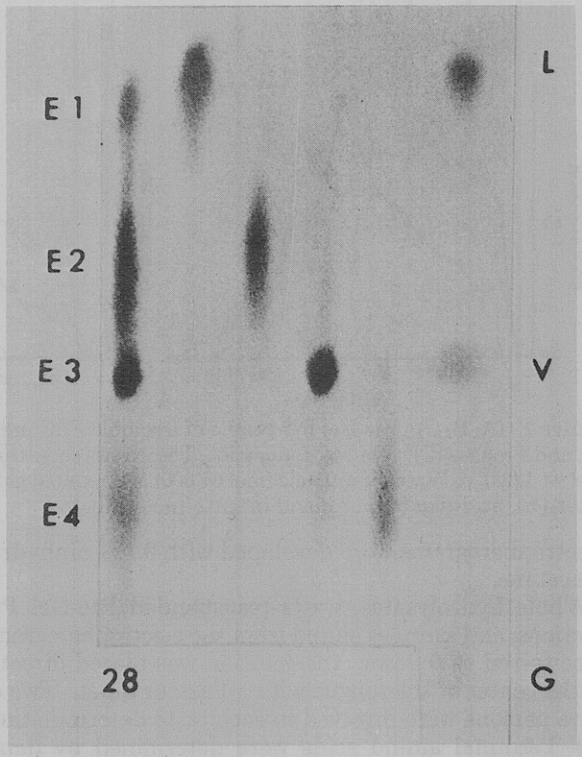

Figure 3. Isolation of the basic (E1 and E2), neutral (E3), and acidic (E4) zones of the Bio-Gel P-2 fraction 28 (V_e 280 mL) of a soluble FPC obtained by incubation with ficin for 7 h. The four zones were tasted after isolation by preparative electrophoresis. Two hundred microliters had been applied on a 10 cm streak. After electrophoresis, the four isolated zones were eluted from the paper with 10% acetic acid, dried, redissolved in deionized water, and tasted. The flavor was: E1, sweet; E2, bitter; E3, acidic and bitter; and E4, acidic (like glutamic acid). L = lysine, V = valine, G = glutamic acid.

Approximately 3 mg of the material in the basic zone E2 (Figure 3) was isolated by paper electrophoresis and rechromatographed on a AG 50W-X8 cation-exchange column. A bitter peak was detected in fractions 25–27. Dansylation prior to and after several cycles of Edman degradation indicated that the peak contained a mixture of free amino acids and a tripeptide containing asparagine and lysine as the second and C-terminal residues, respectively. The free amino acids were mainly leucine and glycine. The N-terminal residue of the tripeptide is probably leucine or glycine, not yet identified. Possibly two tripeptides are involved. The total concentration of ninhydrin-positive components in this bitter peak was lower than the threshold level of any of the bitter amino acids as reported by Kirimura et al. (1969).

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Effect of Maillard Reaction Products on Disaccharidase Activities in the Rat

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Effect of nonenzymatically browned products on the activities of mucosal disaccharidase in the small intestine of young rats both in vivo and in vitro was studied using browned products prepared from a natural food system (apricot) and model systems (glucose, egg albumin, and glucose–tryptophan). Disaccharidase activities of rats fed a diet containing browned products were found to be significantly reduced. Using a model system (glucose–tryptophan), the in vitro study on the mode of inhibitory effect of browning products on maltase activity revealed that the fructose–L-tryptophan (Amadori compound) fraction showed a competitive inhibitory effect (K_i = 3.5 mol or 1.28 mg), whereas the fraction free from fructose–L-tryptophan exhibited a noncompetitive inhibitory effect (K_i = 0.42 mg). The fructose–L-tryptophan free fraction contained mostly colored and fluorescent compounds. This fraction appeared to increase in quantity with an increase in reaction time and temperature.

Mucosal carbohydrases in the small intestine play a fundamental role in mediating digestion and absorption of carbohydrates, being responsible for the hydrolysis of poorly absorbable carbohydrate into easily absorbable monosaccharides. Most studies of dietary effects on carbohydrases have been focused on their activity in response to dietary carbohydrate. The feeding of maltose to rats caused a specific increase in maltase activity and similarly the feeding of sucrose increased sucrase activity (Deren et al., 1967; Reddy et al., 1968). However, contradictory results were observed when similar attempts were made with a lactose diet. Some reports showed increased lactase levels in rats after feeding a high lactose diet (Bolin et al., 1969; Bolin et al., 1971; Goldstein et al., 1971; Jones et al., 1972), while others showed no adaptive increase in the lactase activity upon increasing dietary lactose (Cuatrecasas et al., 1965; Bayless and Huang, 1969; Keusch et al., 1969; Leichter, 1973). Furthermore, an adverse effect of antibiotics on carbohydrase activities was observed and the decreased intestinal carbohydrase activities resulting from antibiotic treatment caused intestinal malabsorption (Paes et al., 1967; Cain et al., 1968).

In our previous study, it was observed that diets containing Maillard reaction products formed from a reaction mixture of reducing sugar and protein (or amino acid) caused diarrhea as well as depressed growth in rats (Lee et al., 1977). The extent of diarrhea varied with the nature of Maillard products and became pronounced with the increased intensity of browning. It was speculated that such diarrhea could be developed as a result of reduced activity of mucosal enzymes due to the ingestion of browned products in such a way that the poorly hydrolyzed products cause increased retention in the lumen contents. The present work was undertaken to verify the effect of the Maillard reaction products on the activities of mucosal disaccharidase and to reveal the underlying mechanisms by which such an effect would occur.

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Table I. Composition of Control and Browned Apricot Diet

Ingredients	% control	% test
Casein	10	13[a]
Apricot powder	74	71
Corn oil[b]	10	10
Salt mixture[c]	5	5
Vitamin mixture[d]	1	1
Choline chloride[e]	0.01	0.01
Total	100.01	100.01

[a] 3% casein was added to the test diet at the expense of carbohydrate in order to supplement protein in browned apricot which is assumed to be nutritionally unavailable (Sgarbieri et al., 1973). [b] Mazola Pure Corn Oil (Best Foods, Division of CPC, International Inc.). [c] Jones and Foster (1942), Salt Mixture (Nutritional Biochemicals Corp.). [d] Vitamin mixture (Nutritional Biochemicals Corp.). [e] Additional requirement, in addition to the quantity in the vitamin mixture, which has been recommended for rats by the National Academy of Science (1972).

MATERIALS AND METHODS

In Vivo Study. *Preparation of Diets.* Sundried apricots (Royals variety, received no chemical treatment and were purchased from the Prune and Apricot Growers Association, Calif.) were rehydrated in a growth chamber (Sherer Gillett, Marshall, Mich.) in order to bring the moisture content to 12% which facilitated maximum browning according to our previous study (Lee, 1974). The rehydrated apricots were then stored at 45 °C and 70% relative humidity (RH) for 3 months. The apricots browned as such were dehydrated by hot-air blast until