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Registry No. Phenol, 108-95-2; *o*-cresol, 95-48-7; *m*-cresol, 108-39-4; *p*-cresol, 106-44-5; 2-ethylphenol, 90-00-6; 4-ethylphenol, 123-07-9; 2,6-dimethylphenol, 576-26-1; ionol, 128-37-0; guaiacol, 90-05-1; 4-methylguaiacol, 93-51-6; 4-ethylguaiacol, 2785-89-9; 4-propylguaiacol, 2785-87-7; 4-isopropylguaiacol, 53587-16-9; 4-vinylguaiacol, 7786-61-0; eugenol, 97-53-0; *cis*-isoeugenol, 5912-86-7; *trans*-isoeugenol, 5932-68-3; 6-methylguaiacol, 2896-67-5; 6-ethylguaiacol, 90534-46-6; vanillin, 121-33-5; acetovanillone, 498-02-2; 2,6-dimethoxyphenol, 91-10-1; 4-methyl-2,6-dimethoxyphenol, 6638-05-7; 4-ethyl-2,6-dimethoxyphenol, 14059-92-8; 4-propyl-2,6-dimethoxyphenol, 6766-82-1; γ-valerolactone, 108-29-2; γ-hexalactone, 695-06-7; γ-nonalactone, 104-61-0; Δ-hexalactone, 823-22-3; Δ-heptalactone, 3301-90-4; Δ-octalactone, 698-76-0; Δ-nonalactone, 3301-94-8; 2,4-methyl-2-buteneolide, 5584-69-0; cyclotene, 80-71-7; 3,5-dimethylcyclopentane-1,2-dione, 13494-07-0; 3,4-dimethylcyclopentane-1,2-dione, 13494-06-9; 3-ethylcyclopentane-1,2-dione, 13494-08-1; acetic acid, 64-19-7; propionic acid, 79-09-4; octanoic acid, 124-07-2; decanoic acid, 334-48-5; 2-ethylhexanoic acid, 149-57-5; 2,4,5-trimethyl-3-(2*H*)-furanone, 64880-73-5.

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Functional and Physical Property Characterization of Peanut Milk Proteins Partially Hydrolyzed by Immobilized Papain in a Continuous Reactor

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A laboratory-scale reactor was designed in which immobilized papain was used to continuously hydrolyze peanut flour milk (PFM) at 52 °C and pH 7.2 for 10 h of operation time. The reaction volume was 150 mL, and the flow rate was 100 mL/h. Enzyme activity was maintained for 6 h under continuous purging with nitrogen and then decreased with time. A reduction in molecular weight of proteins in PFM was observed by subjecting samples to gel electrophoresis and gel filtration chromatography. The hydrolyzed peanut flour milk (HPFM) had improved nitrogen solubility at pH 4.5–7.0 and thermal stability at pH 5.0. The emulsion capacity of HPFM was slightly less than that of PFM. Freeze-dried HPFM incorporated (1%, w/v) into grape and apple juices resulted in nitrogen solubilities of 59.6 and 65.4%, respectively.

INTRODUCTION

Batch processes using papain to partially hydrolyze defatted peanut flour protein have been reported (Sekul and Ory, 1977; Sekul et al., 1978). In these studies, complete hydrolysis of peanut protein was observed in a 10% peanut flour slurry treated with a 0.5% aqueous solution of peptidase-free papain. Consequently, the formation of bitter peptides caused by excessive hydrolysis of protein did not occur. Hydrolysis had a desired effect in that protein functionality was modified, resulting in advantages when hydrolyzates were incorporated into certain foods. A similar batch process using bromelain to hydrolyze defatted peanut flour suspension followed by incorporation of hydrolyzates into cookie formulas has been studied (Beuchat et al., 1975; Beuchat, 1977a,b). Improvement of cookie-baking properties as well as fortification of wheat flour with high-protein peanut flour were achieved. These reports gave further encouragement to investigating the use of plant proteases (papain, bromelain, ficin) to modify vegetable protein for increased usage in the food industry. However, in batch processes, enzymes can be used only once, and most systems are energy and labor intensive.

A continuous system using a protease in an ultrafiltration reactor to modify soy protein isolates has been reported (Deeslie and Cheryan, 1981). In a previous study in our laboratory, immobilization of papain on an anion-exchange resin (Dowex MWA-1, 20–50 mesh) was achieved with considerable success. In that study, both immobilized papain and soluble papain were labile to air, which indicated that oxygen inactivation of immobilized papain through formation of disulfide bonds with sulfhydral groups of papain is a hindrance for application in a continuous system. However, in a long-term storage test, the activity of immobilized papain could be satisfactorily maintained by depletion of oxygen in a closed system followed by nitrogen purging. Theoretically, in a continuous system, depletion of oxygen could be obtained by a similar procedure. A laboratory-scale continuous reactor was therefore designed so that immobilized papain activity

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on an aqueous suspension of peanut flour (peanut flour milk, PFM) under continuous nitrogen purging could be studied. The operation procedures, extent of protein hydrolysis, solubilities of hydrolyzates, and changes in protein functionalities as related to utilization in foods are reported here.

MATERIALS AND METHODS

Immobilized papain was prepared according to a method described elsewhere (Chiou and Beuchat, 1986). Untoasted partially defatted peanut flour (Flavored Nuts, Inc., Albany, GA) was used as the raw material to prepare peanut flour milk (PFM). Bio-Gel A-0.5 m (100–200 mesh) was obtained from Bio Rad Laboratories, Richmond, CA. Tris base, acrylamide, bis(acrylamide), TEMED (tetramethylethylenediamine), glycine, and standard proteins (carbonic anhydrase, bovine albumin, phosphorylase *b*, and β-galactosidase) were from Sigma Co, St. Louis, MO. Pasteurized apple and grape juices (Food Giant Inc., Atlanta, GA) were purchased from a local grocery. All other reagents and chemicals were analytical grade.

Preparation of PFM. The method used to prepare PFM was modified from those described by Rhee et al. (1972, 1973) and Lawhon et al. (1981). Peanut flour suspension (5%, w/v) in deionized water at 65 ± 2 °C was prepared, and the pH was adjusted to 7.6 ± 0.1 with 1 N NaOH. The suspension was blended with a homogenizer (Tekmar Co., Cincinnati, OH) set at high speed for 1 min. The temperature was maintained at 65 ± 2 °C and pH at 7.6 ± 0.1 through the addition of 1 N NaOH for a 30-min period following homogenization. The suspension was then filtered through 16 layers of cheesecloth. The filtrate was separated into three phases by centrifuging at 4000*g* for 30 min at 20 ± 2 °C. The bottom (insoluble components) and top (oil) phases were discarded, and the middle phase (PFM) was used as the substrate for continuous reactor experiments.

Partial Proteolytic Hydrolysis of PFM in a Continuous Reactor. Immobilized papain was prepared according to a method described by Chiou and Beuchat (1986). Papain (150 mg, twice crystallized; Sigma Chemical Co., St. Louis, MO) was immobilized on 6.0 g of Dowex MWA-1 resin (Dow Chemical Co., Midland, MI) through a covalent linking reaction with glutaraldehyde for 10 min of reaction time. A laboratory-scale reactor for continuous hydrolysis of proteins in PFM was designed (Figure 1). A 250-mL wide-mouth glass bottle set in a 400-mL glass beaker was used as a reactor. The beaker was wrapped with a plastic tube that circulated hot water from a water bath to control the reactor temperature at 52 ± 2 °C. Water in the beaker was maintained at a higher level than that of the substrate in the reactor by addition of warm tap water as required. The pH was measured and controlled manually at 7.2 ± 0.2 by addition of 1 N NaOH. Nitrogen purging (oxygen-free nitrogen, Matheson Gas Products, Inc., East Rutherford, NJ) was at a rate of 20 mL/min. Agitation was achieved with use of a stirring bar and a magnetic mixer set a high speed. The reaction volume was maintained and controlled at 150 mL by locating a sampling tube with a sintered-glass head at the surface of the substrate in the reactor. The pumping power of the sampling (suction) pump was operated at a speed faster than that of the substrate feeding pump in order to maintain a constant reaction volume. The outflow was about 100 mL/h. Foaming was controlled manually by adding 1-octanol, not exceeding a level that interfered with enzyme activity. New substrate (PFM) was added after the initial 150 mL of substrate had reacted with immobilized papain for 30 min. The amount of TCA-soluble

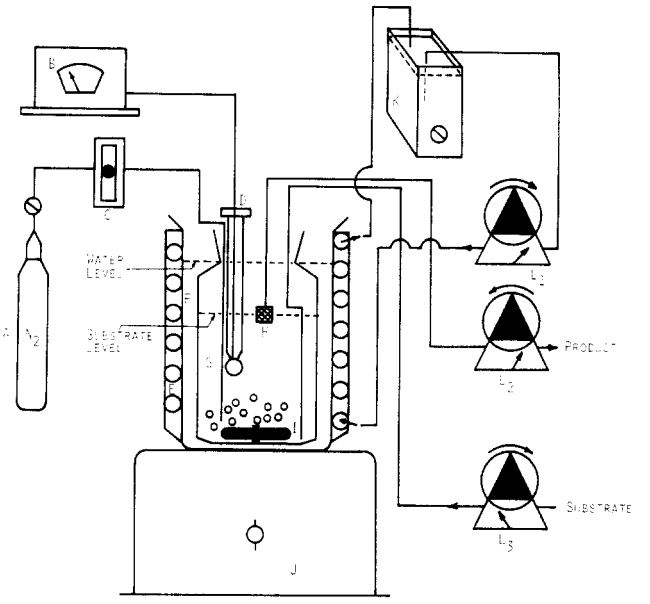

Figure 1. Schematic illustration of a continuous reactor: A, nitrogen tank; B, pH meter; C, flow meter; D, pH electrode; E, plastic tube heat exchanger; F, 400-mL glass beaker; G, wide-mouth glass bottle; H, sintered-glass head; I, stirring bar; J; magnetic stirrer; K, water bath; L, peristaltic pump.

protein in the outflow of the reactor was determined at 30-min intervals for 10 h by the Biuret method (Robinson and Hogden, 1940; Gornall et al., 1949). The outflow product (hydrolyzed peanut flour milk, HPFM) was collected over the 1.5–10-h reaction period, freeze-dried, and stored in a desiccator at room temperature until analyzed. Freeze-dried PFM was treated similarly.

Gel Electrophoresis. Disk polyacrylamide gel electrophoresis methods (Ornstein, 1964; Davis, 1964) were used to detect changes of protein patterns resulting from immobilized papain treatment. Samples of PFM from the reactor were analyzed initially and after 10, 20, 30, 60, 90, and 120 min of reaction time. Protein (~75 μg) in 50 μL of Tris–glycine buffer solution (6 g of Tris base and 28.8 g of glycine made to 1 L, pH 8.3) containing 5 μL of sucrose–dye solution (50% sucrose, 0.1% bromophenol blue) was loaded on the top of the gel. Electrophoresis was carried out by using a power supply (Bio-Rad Model 500/200, Bio Rad Laboratories, Richmond, CA) for 150 min at 450 V. Carbonic anhydrase, bovine albumin, phosphorylase *b*, and β-galactosidase were run concurrently as standard protein references (~5 μg of each protein in 50 μL of buffer solution containing 5 μL of sucrose–dye). The gels were fixed, stained, and destained with trichloroactic acid (50%, w/v) solution, coomassie blue (0.05% in methanol–acetic acid–H_2O, 5:1:4, v/v/v) and methanol–acetic acid (methanol 15%, acetic acid 7%, in water, v/v) solutions, respectively.

Gel Filtration. The Bio-Gel A-0.5 m (100–200 mesh) was packed into a 2.7 × 60 cm glass column and washed thoroughly with 0.01 M borate buffer (pH 8.5) containing 1 M NaCl and 0.02% sodium azide. Protein samples were prepared by dissolving PFM and HPFM powders (2%, w/v) in the buffer solution. Protein solution (2.5 mL mixed with 0.6 mL of glycerine) was layered on the gel column and allowed to be absorbed. The column was then eluted with the same buffer solution at a rate of 36 mL/h. Fractions (7.2 mL) were collected, and absorbance at 280 nm was measured (ISCO Collector and Monitor, Model 1850; Instrumentation Specialties Co., Lincoln, NE).

Nitrogen Solubility. The procedure for measuring soluble nitrogen was modified from methods described by

Beuchat (1977a) and McWatters and Holmes (1979). Suspensions (1% w/v) of PFM and HPFM were prepared by suspending the freeze-dried powders in distilled water. Solutions (15 mL) were then adjusted to pH values ranging from 1.9 to 8.8 over a 30-min period at room temperature by addition of 1 N NaOH or HCl followed by centrifugation at 13000*g* for 15 min at 20 ± 2 °C. The nitrogen contents in the supernatants were determined by the Kjeldahl method (AOAC, 1975). Solubility was expressed as a percentage by dividing the nitrogen content in supernatants at a given pH value by the nitrogen content in supernatant at pH 8.8 and multiplying by 100.

To investigate heat stability, 15 mL of PFM and HPFM was adjusted to pH 5.0, 7.8, and 9.0 separately and autoclaved at 121 °C for 15 min. Centrifugation and nitrogen determination were then carried out as described above. Nitrogen solubility calculations were made by using solubility values of unheated samples at pH 8.8 as a reference.

Emulsion Capacity. Oil-in-water emulsions were prepared in triplicate in a model system described by McWatters and Cherry (1975). Peanut milks (2%, w/v) were prepared by dissolving 0.5 g of freeze-dried PFM and HPFM in 25 mL of distilled water. Commercial peanut oil (Nugget Brand Peanut Oil, Atlanta, GA) was delivered from a buret during emulsion formation. Emulsion capacity was considered to be the point at which a sudden drop in viscosity occurred due to oil–water phase separation. The capacity was expressed as milliliters of oil emulsified per milligram of protein.

Incorporation of PFM and HPFM in Fruit Juice. Commercial grape and apple juices were supplemented (1%, w/v) with freeze-dried powders at room temperature. After the mixtures were blended for 2 min, the pH values of the solutions as well as the pure juices were measured. The solutions were centrifuged at 13000*g* for 15 min, and the supernatants were filtered through Whatman GF/A paper. Nitrogen content in 15 mL of the filtrates was analyzed by the Kjeldahl method (AOAC, 1975). The nitrogen solubilities (percentage) of PFM and HPFM in juices were calculated by dividing the total increase of nitrogen in the juices after protein incorporation by the total nitrogen introduced by PFM and HPFM samples and then multiplying by 100.

RESULTS AND DISCUSSION

Papain immobilization on anion-exchange resins (Dowex MWA-1, 20–50 mesh) is described in another report (Chiou and Beuchat, 1986). One of the advantageous properties of immobilized papain is that the enzyme molecules are covalently bound on fairly large macroporous resin beads. The size of the beads facilitates easier handling when used in a continuous-flow stirred-tank reactor (CSTR). The observation that immobilized papain is labile to air would detract from its successful application in a continuous reactor. However, activity of the immobilized papain can be maintained by depletion of oxygen in a closed-batch system followed by nitrogen purging (Chiou and Beuchat, 1986).

Continuous Reactor. The advantage of nitrogen purging in a continuous reactor to maintain enzyme activity for a long-term use lead to the design of a laboratory-scale unit (Figure 1). Basically, the system consists of a CSTR (Wingard et al., 1976). The advantages of this design are that it is easy to use, is inexpensive to construct and operate, and has the main essential functions of up-to-date reactors. A sintered-glass head at the end of a suction tube was installed at a strategic position in the reactor to separate the beads from breakdown products as well as to maintain a constant reaction volume. Continued nitrogen purging was used to deplete oxygen from the reaction mixture.

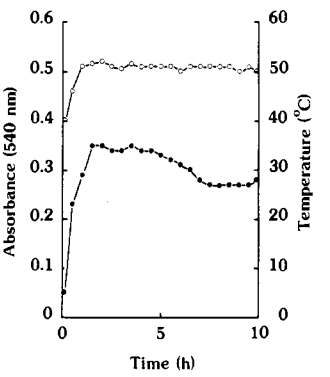

Figure 2. Temperature (○) and reaction activity of immobilized papain expressed as absorbance at 540 nm (●) based on TCA-soluble protein in the outflow of a continuous reactor with peanut flour milk (PFM) protein as a substrate.

Table I. Proximate Composition of Freeze-Dried Peanut Flour Milk (PFM) and Hydrolyzed Peanut Flour Milk (HPFM)

component	compn,[a] % PFM	HPFM
protein[b]	58.0	55.9
moisture	2.6	3.6
oil[c]	21.2	19.3
ash	3.9	8.5
carbohydrate[d]	14.3	12.7

[a] Average value obtained from duplicate samples. [b] Kjeldahl nitrogen × 5.46. [c] Petroleum ether soluble fraction. [d] By difference, excludes fiber.

The temperature of the reactor during operation was recorded (Figure 2). Except for the first hour required for reaching the operating temperature, 52 ± 2 °C was maintained by circulating water (73 °C) from a water bath through the tube (heat exchanger) wrapped around the 400-mL beaker. The reaction temperature could be adjusted by changing the temperature of the water bath or the flow rate of the circulating water.

Immobilized papain was continuously used in the reactor for 10 h to hydrolyze PFM protein at 52 ± 2 °C, pH 7.2 ± 0.2, 100 mL/h outflow rate, and 20 mL/min nitrogen purging. The reaction volume was 150 mL. Enzyme activity expressed as absorbance at 540 nm (Figure 2) indicates the amount of TCA-soluble protein in the outflow. A volume of 150 mL of PFM initially at 40 °C was hydrolyzed by immobilized papain during the first 30 min before introducing new substrate for a continuous operation. The sharp increase in absorbance during the first 90 min revealed that extensive hydrolysis was occurring. This high activity (≥0.30 AU) continued until 7 h of operation and then decreased with time to about 0.27 AU after 10 h. The decrease in papain activity during continuous reaction from 7 to 10 h implies that operational design has not been optimized.

Protein Hydrolysis. Hydrolyzed peanut flour milk (HPFM) was collected from 1.5 to 10 h of reaction period. The approximate composition of the freeze-dried PFM and HPFM powders is listed in Table I. Composition of the powders is essentially the same except for an increase in ash content in HPFM. This may be due to the continued addition of NaOH to increase pH during operation.

Changes in protein gel patterns in PFM during hydrolysis by immobilized papain in the CSTR are illustrated in Figure 3. The protein fractions were separated by a nondenaturing method of disk polyacrylamide gel elec-

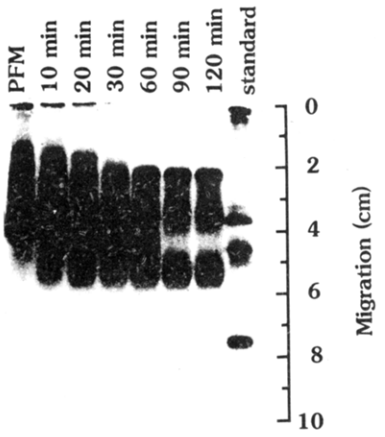

Figure 3. Disk polyacrylamide gel electrophoresis patterns of proteins in PFM and in HPFM collected after 10, 20, 30, 60, 90, and 120 min of reaction time.

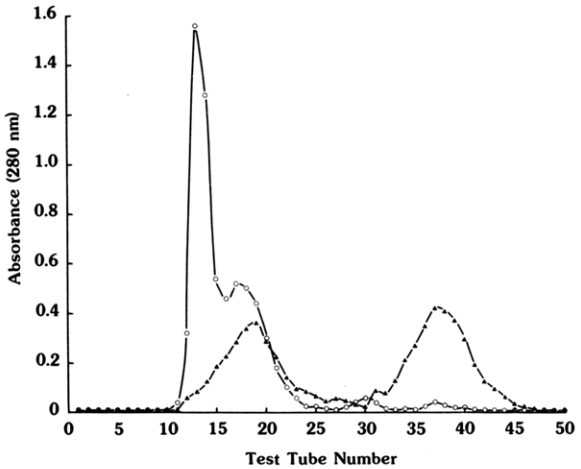

Figure 4. Gel filtration chromatography patterns of PFM (○) and HPFM (▲). Five tubes were collected during each hour of elution time.

trophoresis (Ornstein, 1964; Davis, 1964). Protein standards with molecular weights of 515 000 (β-galactosidase), 250 000 (phosphorylase *b*), 66 000 (bovine albumin), and 29 000 (carbonic anhydrase) run concurrently had migration distances of about 0.2, 3.7, 4.5, and 7.5 cm, respectively. Except for some immobile proteins in PFM and HPFM samples collected after 10–30 min of reaction time, peanut proteins are in the molecular weight range of 29 000–515 000. A very obvious change in electrophoretic pattern indicates that considerable hydrolysis of proteins occurred. A hydrolytic product with a migration distance of 5–6 cm appeared in substantial quantity during the course of reaction. There was no change in protein pattern from 90 to 120 min of reaction time. However, protein patterns were not the same as those reported by Sekul and Ory (1977) and Sekul et al. (1978) or those reported by Beuchat et al. (1975) who used pepsin, bromelain, and trypsin to hydrolyze peanut protein. This may be due to differences in milling conditions for preparing raw materials, protein extraction methods, assay conditions, enzyme residues, or the specificities of various enzymes.

A further comparison of protein patterns of PFM and HPFM was done by gel filtration chromatography. Data from these experiments are plotted in Figure 4. The trend of shift in size from large to small molecules was similar to that observed in polyacrylamide gel electrophoresis.

Nitrogen Solubility. Nitrogen solubility profiles of PFM and HPFM in distilled water at pH 1.9–8.8 are presented in Figure 5. In general, nitrogen in unheated

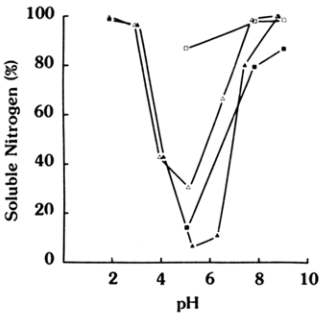

Figure 5. Percentage of soluble nitrogen of PFM and HPFM in a 1% suspension at various pH values: ▲, PFM; △, HPFM; ■, 1% PFM suspension after heat treatment (121 °C, 15 min); □, 1% HPFM suspension after heat treatment.

Table II. Emulsion Capacity and Nitrogen Solubility of PFM and HFPM in Fruit Juice

sample	emulsn capacity, mL oil/mg protein	apple juice[a]		grape juice[a]	
		N_2 sol, %	pH[b]	N_2 sol, %	pH[c]
PFM	0.141 ± 0.007	14.9 ± 1.2	4.2	18.6 ± 1.2	3.5
HPFM	0.121 ± 0.005	65.4 ± 0.5	4.3	59.6 ± 2.1	3.6

[a] With standard deviation. [b] Initial pH 4.0. [c] Initial pH 3.1.

HPFM was more soluble than nitrogen in PFM at the same pH. Increased nitrogen solubility due to hydrolysis was especially notable at pH 4.5–7.0, which encompasses the isoelectric points of most peanut proteins. In batch systems, similar results were reported by Sekul et al. (1978), who used soluble papain to hydrolyze peanut protein. Beuchat et al. (1975) and Beuchat (1977a), using bromelain, pepsin, and trypsin to modify defatted peanut flour, also observed a dramatic increase in nitrogen solubility at pH values near the isoelectric point of peanut proteins.

Heat denaturation of PFM and HPFM expressed as a change of nitrogen solubility in distilled water after autoclave treatment (121 °C, 15 min) at three pH values (5.0, 7.8, 9.0) was investigated. At pH 7.8 and 9.0, substantial heat denaturation of both PFM and HPFM occurred. Nitrogen solubility of PFM protein was decreased to a greater extent than was that of HPFM. Heated HPFM protein was more soluble than heated PFM at all three pH values tested.

Usually, the severity of heat denaturation of proteins and consequent insolubility of nitrogen are exhibited most clearly at pH values of their isoelectric points. The surprising observation in these experiments was that, at pH 5.0, heat treatment caused an increase in nitrogen solubility in both PFM and HPFM. It is theorized that perhaps heat treatment of samples at pH 5.0 resulted in further hydrolysis. This may be occurring to a greater extent in HPFM due to increased vulnerability of partially hydrolyzed proteins to further breakdown under acid conditions. From a viewpoint of incorporating peanut proteins into foods, increased nitrogen solubility of HPFM when subjected to elevated temperature may be advantageous in some instances.

Emulsion Properties. Values for emulsion capacities of PFM and HPFM are listed in Table II. PFM has a slightly higher emulsion capacity than does HPFM, with values of 0.141 and 0.121 mL of oil/mg of protein, respectively. This is in agreement with Sekul et al. (1978) who reported that the emulsifying capacity of partially hydrolyzed peanut protein at various pH values was generally lower than that of unmodified flours. Beuchat (1977a) reported that only slight differences existed be-

tween emulsifying capacities of bromelain-hydrolyzed peanut flours and controls. However, it was observed that treatment with pepsin restored emulsifying capacity. In an earlier study, Beuchat et al. (1975) reported that trypsin completely destroyed the emulsifying capacity of the modified peanut proteins.

While it appears that a simple conclusion cannot be drawn concerning the effects of proteolysis on emulsion capacity of peanut protein, these reports do indicate that effects depend upon the nature of the enzyme employed and conditions under which hydrolysis is carried out. As stated by Crenwelge et al. (1974), the action of proteins as emulsifiers is influenced by protein conditions, speed of mixing, and type of oil. All of these variables make comparison of data derived by various investigators difficult.

Supplementation of Fruit Juices. An acidic naturally cloudy beverage (pineapple juice) was selected by Conkerton and Ory (1976) as a vehicle for peanut flour supplementation. The protein content of the juice could be tripled and turbidity was slightly increased but flavor, texture, and aroma of the juice were not affected. In the present study, PFM and HPFM were incorporated into commercial grape and apple juices (Table II). These juices are clear acidic beverages. Consequently, any protein incorporated into the juices must not result in the development of cloudiness or turbidity. Incorporation of 1% (w/v) freeze-dried PFM and HPFM increased the pH of both grape and apple juices. About 59.6% of the nitrogen in HPFM was soluble in grape juice; 65.4% was soluble in apple juice. The difference in pH of the juices does not appear to significantly influence the solubility of PFM and HPFM proteins. The visible color and turbidity of both juices after incorporation of HPFM and clarification by centrifuging or filtration were only slightly changed.

In summary, the application of immobilized papain in a continuous system for modifying peanut flour milk proteins resulted in changes in functional properties that would enhance performance in some food systems. However, conditions for operating the continuous reactor system have not been optimized. Additional research is needed to define operating conditions and reactor size most satisfactory for economically producing hydrolyzed peanut proteins for use as functional ingredients in a variety of foods.

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