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Characterization of a Heat-Stable Protease of *Pseudomonas fluorescens* P26[1]

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A heat-stable, extracellular proteolytic enzyme was isolated from *Pseudomonas fluorescens* P26. Brain heart infusion broth (Fisher Scientific Co.), pH 7.5, and incubation at 21 C provided the optimal conditions for bacterial growth for enzyme production. The organism had a D value of 2.6 min at 62.8 C (145 F). The enzyme, however, was quite heat-stable, requiring 15 hr at 62.8 C, 8 hr at 71.4 C, and 9 min at 121 C for complete inactivation. Milk, whey, and casein each had a protective effect on the enzyme against heat inactivation. Purification was accomplished by growth of organisms in broth, centrifugation, sterilization by filtration, ammonium sulfate precipitation (55% saturation), dialysis (against six changes of water), and protein separation by passage through a Sephadex G-100 column. Ultracentrifugation revealed a single band with a sedimentation coefficient of 1.51 which suggested a molecular weight of approximately 23,000. As little as 0.2 unit of the purified enzyme caused detectable flavor defects during 30 days of storage at 4 C, and the Hull test for liberation of tyrosine compared favorably in sensitivity with the sensory method in milk.

Many refrigerated foods are heated during processing to inactivate enzymes and microorganisms. This is of particular importance in foods such as pasteurized milk in which psychrophilic bacteria are the major cause of spoilage. These bacteria are normally killed by pasteurization. The major genera are *Pseudomonas*, *Achromobacter*, *Escherichia*, and *Aerobacter* (5, 6, 7), and the most common species are *P. putrefaciens*, *P. fragi*, and *P. fluorescens* (9).

Extracellular enzymes, produced by microorganisms in refrigerated foods prior to heat treatment, may not be completely inactivated by the heating and may be active in the stored product. This paper reports on the characteristics of a proteolytic enzyme produced by *P. fluorescens* P26, an isolate from refrigerated foods. We studied conditions for optimal production of the enzyme, heat stability, the protective effect of milk during heating, methods of purification, and effects on the organoleptic characteristics of milk.

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MATERIALS AND METHODS

Selection of protease producers. Psychrophilic bacteria from the stock culture collection of the Department of Food Science and Nutrition were screened for production of protease by streaking on casein digest agar (15% skim milk in plate count agar). Cultures which were proteolytic were grown in nutrient broth at 21 C for 48 hr. Cells were removed by centrifugation, and the supernatant fluid was sterilized by filtration. Filtrates (0.1 ml) were absorbed in 12-mm filter paper discs which were placed on surfaces of casein digest agar plates. Zones of proteolysis were measured after incubation at 21 C for 48 hr.

Quantitation of protease activity. Enzyme activity was determined by the method of Hull (3), in which the quantitites of free tyrosine and tryptophan are measured spectrophotometrically after incubation of the enzyme with the substrate. The standard curve was based on analyses of solutions of tyrosine. One unit of enzyme was defined as the milligram of purified protein that would produce one milligram of tyrosine (equivalent) per milliliter from a solution of 2.5% casein in 48 hr at 21 C. The quantity of protein per unit was determined by adding 2.5 ml of two fold serial dilutions (1:1 through 1:256) of purified enzyme in sterile phosphate buffer (pH 7.45) to 7.5 ml of 2.5% casein solution. After 48 hr at 21 C, the enzyme was inactivated with trichloroacetic acid and the quantity of tyrosine and tryptophan was determined.

Optimal medium, pH, and temperature. The following media were tested for ability to support protease production: brain heart infusion (Fisher Scientific Co.), nutrient (Difco), Trypticase soy (Difco), and tryptone glucose extract broth (Difco). Each was adjusted to *p*H levels of 5.5, 6.5, 7.5, and 8.5, with 1 N HCl or NaOH. Incubation temperatures of 10, 21, 32, and 37 C were used for each combination of medium and *p*H. Also, the organism was grown in brain heart infusion broth from three different manufacturers, and the enzyme concentration in each was determined.

Enzyme preparation and examination. Brain heart infusion broth (Fisher Scientific Co.) was inoculated with 1% of an actively growing culture of *P. flourescens* P26 and incubated for 48 hr at 21 C. Cells were removed by centrifugation (4,500 $\times$ *g* for 30 min) and filtration (0.45-μm membrane filter). The filtrate (crude enzyme) was stored at -10 C in 5-ml portions.

Purified enzyme was prepared by ammonium sulfate precipitation from the crude enzyme. Initial stepwise addition of ammonium sulfate indicated best yields of enzyme at 55% of saturation during storage at 4 C for 12 hr. The precipitate was removed by centrifugation and resuspended in phosphate buffer at *p*H 7.45. Salts were removed by dialysis against six changes of water over a 24-hr period and against phosphate buffer for 12 hr. Dialysate (320 mg of protein) was filtered through a 4- by 78-cm column of Sephadex G-100 which had previously been equilibrated against the eluent buffer (phosphate buffer, *p*H 7.45). Eluate was collected in 3-ml fractions, and protein content was determined on a Hitachi Perkin-Elmer model 124 double-beam spectrophotometer at 280 nm (bovine serum albumin standard curve). Fractions were screened for proteolytic activity by using saturated filter paper discs on casein digest agar plates. Proteolytic fractions were further evaluated by the Hull test, and strongly proteolytic fractions were pooled and stored at -20 C.

Homogeneity of purified enzyme was confirmed by agar plates. Proteolytic fractions were further evaluated by the Hull test, and strongly proteolytic as follows: cyanogum 41 gel (7%), 0.4 M trisglycine buffer at *p*H 9.3, 200 v for 3.5 hr, amido black stain, methanol, and glacial acetic acid dye solvent. Ultracentrifugation was accomplished in a Beckman model L analytical ultracentrifuge by using a 4- to 12-mm sector valve type synthetic boundary cell operated at 59,780 rev/min at 20 C. Purified enzyme was acid hydrolyzed under nitrogen at 110 C for 21 hr (4). The hydrolysate was taken to dryness, redissolved in 2 ml of 0.125 M norleucine in *p*H 2.2 citrate buffer, and then analyzed by automated cation exchange chromatography using a Bio Cal BC200 analyzer (1).

D value for P. fluorescens P26. Sterile skim milk (10 ml) was inoculated with 5.0×10^6 organisms per ml and heated at 62.8 C. Tubes were removed to an ice bath at "come up time" and each minute thereafter for 30 min. Plate counts were made in duplicate.

Inactivation of enzyme by heat. Samples (3 ml) of crude enzyme at 4 C were placed in screw-cap tubes (12 by 125 mm) which were immersed in a water bath at 62.8 $\pm$ 0.2 C. Time for temperature to reach 62.8 C ("come up time") was 3.8 min, as determined by repeated measurement with a thermister (model 42SF Telethermometer, Yellow Springs Instruments) immersed in the liquid. When the temperature reached 62.8 C, and each hour thereafter for 18 hr, one tube was removed. Upon removal, tubes were immediately immersed in an ice bath and held until heating was completed for the lot. The same technique was used for determining the rate of inactivation at 71.4 C ($\pm$ 0.2 C) except that samples were removed at "come up time," at 15, 30, 60, 90, and 120 min and at 4, 6, 8, and 10 hr of heating. Tests for inactivation at 121 C were done in thermal death time tubes to which 6.0 ml of crude enzyme were added prior to sealing the tubes with an oxygen flame. The thermister was placed in one tube, and the opening was sealed with epoxy. All tubes were submerged in a thermostatically controlled mineral oil bath at 121 $\pm$ 0.2 C. Tubes were removed to an ice bath at "come up time" and at 1-min intervals up to 25 min. Activity of each sample was determined by the Hull test.

Three milliliters each of skim milk, whey (from acidified pasteurized skim milk), casein solution (2.5% acid casein), and water were placed in separate test tubes. Three milliliters of crude enzyme was added to each tube, and the contents were pasteurized at 71.4 $\pm$ 0.2 C. One tube from each group was removed and placed immediately in an ice bath at "come up time," after 15, 30, 45, 60, 90, and 120 min of heating, and at hourly intervals to 10 hr.

Off-flavor production. Skim milk was sterilized by heating in flowing steam for 30 min on 3 consecutive days. Purified enzyme was added to 50-ml samples to give final concentrations of 4, 2, 1, 0.5, 0.4, 0.2, 0.1, 0.05, and 0.04 units per ml. Controls were the sterilized skim milk and sterilized skim milk plus 4 ml of buffer, the amount added with the enzyme. Samples were randomly coded and stored at 4 C for 30 days, after which the amount of proteolysis was determined by the Hull test, and flavor and odor were determined by two experienced judges.

RESULTS AND DISCUSSION

Selection of protease producers. Tests were conducted on 47 different strains of psychrophilic bacteria: 3 *Achromobacter*, 3 *Aerobacter*, 3 *Enterobacter*, 1 *Escherichia*, 1 *Flavobacterium*, 1 *Proteus*, and 36 *Pseudomonas*. The *Escherichia* strain and 14 of the pseudomonads were proteolytic according to the disc assay method. Six of the pseudomonads produced proteases that remained active after heating at 62.8 C for 30 min. Each belonged to the fluorescent group of aerobic pseudomonads (8). The most heat-stable of these enzymes was produced by *P. fluorescens* P26, which was studied in detail.

Optimal medium, pH, and temperature. Brain heart infusion broth was slightly better than Trypticase soy broth for enzyme production. The organism produced little enzyme in nutrient broth or tryptone glucose meat extract broth. In the latter, growth was excellent. Slightly more activity was found at *p*H 7.5 than at 6.5, but broths adjusted to *p*H 5.5 or 8.5 had less than half of this activity. Incubation at 21 C proved far superior to 37, 32, or 10 C. Enzyme activity in broths produced at 37 C was only 3% of that associated with incubation at 21 C. The generation times for *P. fluorescens* P26 at 4, 10, and 21 C were 10, 5.5, and 4.5 hr, respectively.

The three brain heart infusion media, BBL, Difco, and Fisher, supported growth equally well. Average plate counts were within 10% of each other. But enzyme activity was nearly four times higher in the sterile filtrate from the Fisher broth than in filtrate from Difco broth, and activity of the latter was 10 times that of filtrate from the BBL broth.

TABLE 1. *Changes in total protein concentration and enzyme activity during enzyme purification*

Enzyme	Total protein (mg/ml)	Tyrosine produced by enzyme (μg/ml)	Specific activity
Crude	43.6	8.8	0.202
Dialysate	32.0	404.0	12.625
Pure	0.5	39.6	79.200

Characteristics of the enzyme. We recovered 5.7% of the protein applied to the Sephadex column (Table 1). Specific activity of the crude and purified preparations indicated concentration by some 392 times. The single band observed after polyacrylamide electrophoresis indicated the preparation was electrophoretically pure (Fig. 1). All protein migrated from the spot, as evidenced by the lack of stain at that location. A zone of proteolysis developed when an unstained slice of the polyacrylamide gel was placed on casein agar. The zone developed 12.5 cm from the slot, the same distance at which we observed the stained band.

Ultracentrifugation also revealed a single band (Fig. 2) which had a sedimentation coefficient of 1.51, suggesting a molecular weight of 23,000.

Amino acid analysis (Table 2) revealed the absence of cystine. Tryptophan is destroyed by acid hydrolysis; hence, it does not appear in the table. Proline (17.9%), glycine (17.9%) and glutamic acid (12.9%) were present in greatest quantities.

D value for P. fluorescens P26. The test organism was rapidly killed in skim milk heated at 62.8 C. The D value was 2.6 min, which agrees closely with the D value of 2.5 min

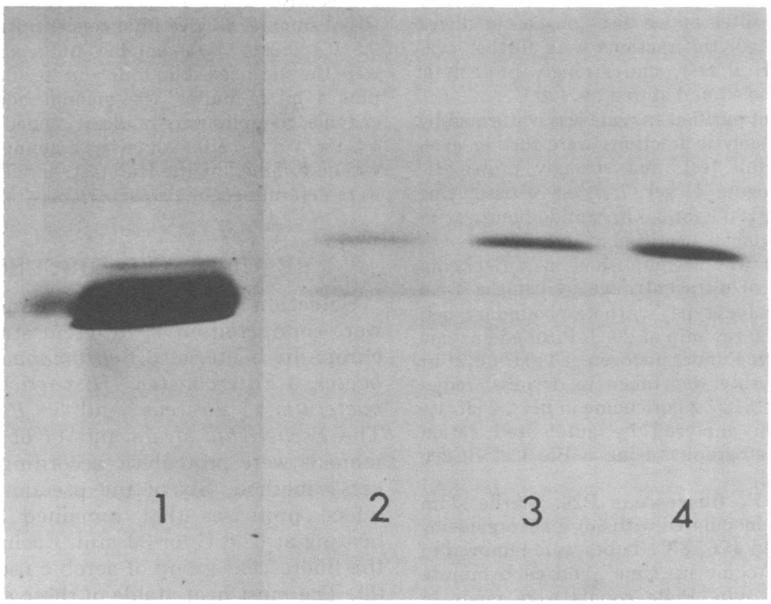

FIG. 1. *Polyacrylamide gel electrophoresis of purified enzyme. 1, Blood serum albumin; 2 and 3, heat-stable enzyme from two other strains of Pseudomonas; 4, enzyme from P. fluorescens P26.*

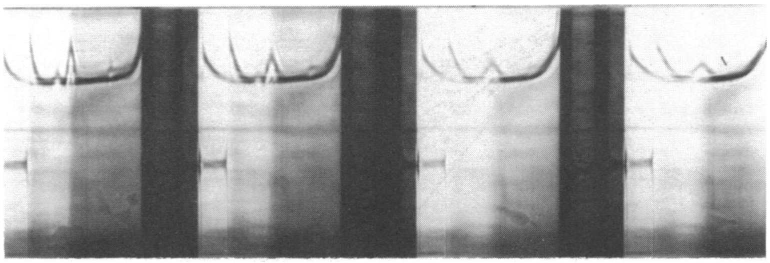

FIG. 2. *Ultracentrifugal sedimentation pattern of purified protease from P. fluorescens P26.*

TABLE 2. *Amino acid composition of purified protease isolated from Pseudomonas fluorescens strain P26*

Amino acid	total amino acids (relative %)
Aspartic acid	8.3
Threonine	3.2
Serine	6.2
Glutamic acid	12.9
Proline	17.9
Glycine	17.9
Alanine	7.1
Cystine	
Valine	3.9
Methionine	1.2
Isoleucine	2.7
Leucine	5.0
Tyrosine	1.9
Phenylalanine	2.9
Histidine	2.1
Lysine	3.9
Arginine	3.1

reported by Davis and Babel (2) for organisms producing slime on cottage cheese.

Inactivation of enzyme by heat. "Come up times" for heating at 62.8, 71.4, and 120 C were 3.8, 4.6, and 8.5 min, respectively. The enzyme lost about 30% of its initial activity in this time during heating to 62.8 and 71.4 C. However, about 14 hr were required to inactivate 90% of the enzyme at 62.8 C (Fig. 3). More than 8 hr of exposure were required for complete inactivation at 71.4 C (Fig. 3). About 9 min were required when the temperature was 120 C (Fig. 4). The lengthy "come up time" at the highest temperature caused a loss of about 70% of the initial activity.

Skim milk, whey, and 2.5% casein protected the enzyme from heat denaturation at 71.4 C (Fig. 5). Skim milk was significantly more protective than whey or casein, and they produced significant protection ($P < 0.05$) compared to water. The data suggested that the effect of skim milk was due to the additive effects of whey and casein.

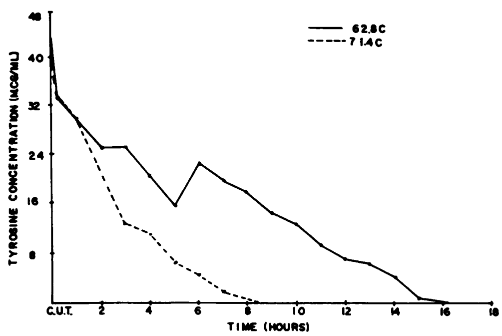

FIG. 3. *Changes in enzyme activity, expressed as quantities of tyrosine liberated, with increasing time of heat treatment at 62.8 C and 71.4 C. (Activity tested at 21 C for 72 hr. C.U.T., "come up time" to respective temperature.)*

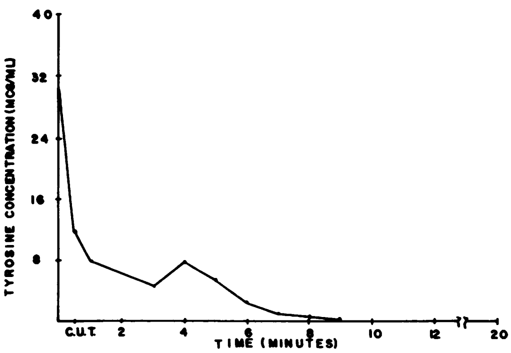

FIG. 4. *Changes in enzyme activity, expressed as quantities of tyrosine liberated, with increasing time of heat treatment at 121 C. (Activity tested at 21 C for 72 hr. C.U.T., "come up time" to 121 C.)*

Off-flavors produced. The purified enzyme caused an unclean flavor to develop, followed by a bitter flavor (Table 3). Evidence of proteolysis, the presence of free tyrosine and tryptophan in the milk, was directly related to the presence of bitterness. As little as 0.2 unit of enzyme was able to produce an off-flavor during storage of the milk for 30 days at 4 C.

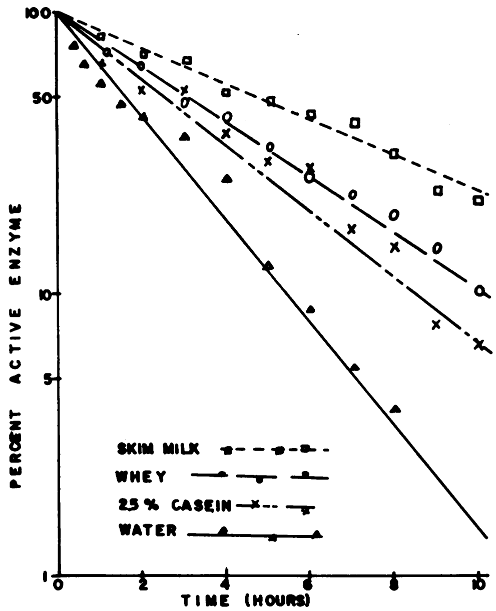

FIG. 5. *Percentage of enzyme remaining active after heating at 71.4 C in the presence of water, skim milk, whey, and 2.5% casein.*

TABLE 3. *Amount of purified enzyme required for off-flavor development in sterile skim milk during 30 days of storage at 4 C, with corresponding enzyme activity*[a]

Enzyme concn (units/ml)	Sample coding	Remarks		Tyrosine produced by enzyme (μg/ml)
		Judge 1	Judge 2	
4	4	Extremely bitter	Very bitter	40.4
2	11	Very bitter	Bitter	26.8
1	9	Bitter	Bitter	16.4
0.5	6	Bitter	Bitter	7.8
0.4	8	Slightly bitter	Bitter	5.8
0.2	1	Slightly astringent	Slightly bitter	1.6
0.1	7	No criticism	Astringent	<1.0
0.05	3	No criticism	No criticism	<1.0
0.04	10	No criticism	No criticism	<1.0
Buffer control	2	No criticism	Slightly medicinal	<1.0
Control	5	No criticism	No criticism	<1.0

[a] Activity tested by the Hull test after incubation at 21 C for 72 hr.

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