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Stabilization of a Psychrotrophic *Pseudomonas* Protease by Calcium Against Thermal Inactivation in Milk at Ultrahigh Temperature[1]

JEFF T. BARACH,* DANIEL M. ADAMS, AND MARVIN L. SPECK

Department of Food Science, North Carolina State University, Raleigh, North Carolina 27607

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The heat-stable extracellular protease of *Pseudomonas* sp. (isolate MC60) was investigated. Heat resistance of the enzyme in milk at sterilization temperature was dependent on the presence of Ca^{2+}. The half-life of the enzyme at ultrahigh temperature (149 C) in skim milk or milk-salts buffer with Ca^{2+} was approximately 7.0 s. Treatment of milk with chelators completely removed the heat-stabilizing effect of milk. The enzyme was partially purified by ammonium sulfate precipitation and column chromatography on Sephadex G-100. At 21 C the enzyme retained >85% activity after exposure to pH values between 5 and 10. Enzyme activity was reduced by metal chelating agents. Both Ca^{2+} and Zn^{2+} were required for optimal enzyme activity. Molecular weight was estimated at 48,000 by gel filtration.

Proteases produced by psychrotrophic species of *Pseudomohas* have been reported to be extremely heat resistant (1, 4; A. C. Malik and A. M. Swanson, J. Dairy Sci. 57:591, 1974). These enzymes are not inactivated in milk by conventional pasteurization or ultrahigh temperature treatments at 120 to 149 C. The extracellular protease of *Pseudomonas fluorescens* P26 was found to be more stable, at pasteurization temperatures, when heated in milk than when heated in water. The enzyme's heat stability was attributed to both the whey and casein components of milk (12). Association with other proteins might have produced changes in the enzyme's thermal stability; however, small molecules or ions also could have contributed heat resistance. Calcium, a major milk ion, has been shown to increase thermal stability of several proteolytic as well as other types of enzymes (7, 8, 15, 16). However, most of these enzymes were rapidly inactivated at temperatures approaching 100 C compared to MC60 protease, which remained active after at least 40 min (1) at 100 C.

The purpose of this investigation was to determine the basis for heat resistance of *Pseudomonas* sp. (isolate MC60) protease in milk at ultrahigh temperature and to compare properties of the partially purified enzyme with those of other *Pseudomonas* proteases.

MATERIALS AND METHODS

Organism and culture conditions. A psychrotrophic *Pseudomonas* species (isolate no. MC60), isolated from raw milk and selected for high proteolytic activity (1), was maintained by monthly transfers into reconstituted 10% nonfat dried milk and held at 4 C. For protease production, a 0.1% inoculum of a 24-h, room temperature (21 C), nonfat dried milk culture was made into nutrient broth (BBL, Cockeysville, Md.), which contained 4% lactose. Flasks were incubated with shaking at 21 C for 72 h.

Reagents. Milk-salts buffer I was the milk ultrafiltrate of Jenness and Koops (11) and was composed of: KH_2PO_4, 11.6 mM; potassium citrate, 1.6 mM; magnesium citrate, 1.6 mM; K_2CO_3, 2.2 mM; KCl, 26.4 mM; sodium citrate, 6.1 mM; adjusted to pH 6.7 with 1 N KOH. Milk-salts buffer II was identical to buffer I, except that potassium citrate was substituted on an equimolar basis for magnesium citrate to eliminate all divalent cations. When required, 10 or 15 mM $CaCl_2$ was added to buffer I or II, respectively.

Sephadex G-100 and blue dextran 2000 were products of Pharmacia Fine Chemicals (Piscataway, N.J.). Ribonuclease A and ovalbumin were obtained from Sigma Chemical Co. (St. Louis, Mo.). Bovine serum albumin was purchased from Mann Research Lab. (New York, N.Y.). All chemicals were of analytical or reagent grade.

Enzyme isolation. The culture supernatant was obtained by continuous-flow refrigerated centrifugation at 27,000 × *g* with a flow rate of 150 ml/min. Particles larger than 0.45 μm were removed by filtration through a membrane filter disk (Millipore Corp., Bedford, Mass.).

(i) Ammonium sulfate was added slowly to 11.5 liters of supernatant while stirring to obtain 65% saturation. The precipitate that had formed during 24 h at 4 C was collected by continuous-flow refrigerated centrifugation at 27,000 × *g* with a flow rate of 30 ml/min.

(ii) The precipitate was dissolved in 100 ml of milk-salts buffer I and dialyzed at 4 C, first against

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30 volumes of distilled-deionized water for 48 h and then against 30 volumes of milk-salts buffer I for 48 h.

(iii) The enzyme solution was concentrated to 20 ml by ultrafiltration and placed on a Sephadex G-100 column (2.5 by 87 cm) equilibrated with buffer I. The column, maintained at 4 C, was eluted with the same buffer at a flow rate of 25 ml/h. Fractions containing proteolytic activity were pooled, frozen, and stored at −10 C.

Enzyme assay. Proteolytic activity was measured spectrophotometrically by the method of Hull using the Folin-Ciocalteau reagent (9). Enzyme solution was added to 3.5% vitamin-free casein solution (Nutritional Biochemicals Corp., Cleveland, Ohio), pH 7.5, and incubated at 45 C. When Zn^{2+} was added to the substrate solution, 0.5 mM $ZnSO_4$ was used. The reaction was terminated by addition of 0.72 N trichloroacetic acid to the reaction mixture. One unit of activity is defined as the amount of enzyme that produces 1 μg of trichloroacetic acid-soluble material (as tyrosine) per ml of reaction mixture per h at 45 C.

Determination of protein. Protein was estimated spectrophotometrically by comparing the ratio of absorbance at 280 and 260 nm of the enzyme solution with that of standard ovalbumin solutions.

Heat treatment. Enzyme in buffer or milk was treated in sealed capillary tubes in an oil bath at 149 C. The come-up time for the fluid (50 μl) in the tube was approximately 10 s, as measured by a thermocouple implanted in a separate capillary tube. The correction for lag in heat penetration was made by subtracting 4 s from the total exposure time. Heated tubes were cooled quickly in an ice-water mixture, and the contents were added to casein substrate.

Molecular weight estimation. The molecular weight of the purified enzyme was estimated by gel filtration on a Sephadex G-100 column (1 by 60 cm) (2). The column was calibrated with blue dextran and globular proteins of known molecular weights, namely: ribonuclease A, 13,700; ovalbumin, 45,000; and bovine serum albumin, 68,000. The column was equilibrated with buffer I, maintained at 10 C, and run at a flow rate of 4 ml/h.

Electrophoresis. Vertical slab electrophoresis was performed in a 3 M urea-10% polyacrylamide gel made with 0.12 M tris(hydroxymethyl)aminomethane-citrate buffer, pH 2.9. The enzyme was treated with 6 M urea before application. The electrode buffer was 2.7 M glycine adjusted to pH 4.0 with citric acid. After electrophoresis towards the cathode for 4.5 h at 300 V (100 mA), the protein band was stained with amido black and destained with 7% acetic acid.

Milk free of divalent cations. Divalent cations were removed from milk by two methods. Approximately 5.0 g (dry weight) of Amberlite-IR-120 cation-exchange resin (in H+ form) was slowly added in 100 ml of skim milk. The pH was maintained between 5.5 and 7.5 by addition of 10 N NaOH. After 30 min, the resin was removed by centrifugation. The supernatant solution was adjusted to 6 M urea and held for 30 min at 21 C. Additional resin (5.0 g) was added and, after 30 min, was removed as described. The solution was dialyzed for 24 h against 400 volumes of deionized water and then against the same volume of buffer II for an additional 24 h at 4 C. Alternatively, solid disodium ethylenediaminetetraacetate (EDTA) was added to skim milk to a concentration for 20 mM. The pH was adjusted to 6.9, and the solution was dialyzed for 24 h against 400 volumes of buffer II at 4 C.

RESULTS

Isolation of *Pseudomonas* sp. (MC60) protease. The isolation procedure is summarized in Table 1. The increased specific activity of the partially purified preparation as compared to that of the crude culture indicated a 141-fold purification with 32% recovery of the initial activity.

Two protein peaks were obtained by gel filtration with Sephadex G-100, with only the fractions of the second peak containing proteolytic activity (Fig. 1).

The final enzyme preparation appeared homogeneous as judged by polyacrylamide gel electrophoresis. A low pH gel was chosen to prevent autolysis. The purified protein migrated toward the cathode 9 mm from the slot as a single band.

Effect of heating protease in milk treated with chelating agents. Milk that was treated with EDTA or Amberlite-IR120 cation exchanger did not appear to protect the protease against thermal inactivation. Protease given an effective heat treatment of 6 s at 149 C was protected 100% by raw skim milk, whereas it was protected <1% in cation-free milk. The addition of calcium to cation-free milk prepared with EDTA or Amberlite-IR120 restored 91 and 75% of the protective effect seen with untreated milk, respectively.

Effect of heating on protease activity. As was observed for P26 protease by Mayerhofer et al. (12), skim milk appeared to give protection to MC60 protease against thermal inactivation. However, it was Ca^{2+} and not the milk proteins that gave the protease stability. The heat resistance of MC60 protease at 149 C in milk or milk-salts buffer I with Ca^{2+} was greater than in buffer alone or water (Fig. 2). Zinc offered no protection during heating (data not shown). The half-life ($t_{1/2}$) of the protease in milk at 149 C was 7.0 s. In water or buffer I the enzyme was rapidly inactivated with a $t_{1/2}$ of <1 s. From the regression lines it appeared the rate of inactivation in buffer I with Ca^{2+} was the same as in milk, except for an initial rapid loss of approximately 55% activity.

Reactivation of ultrahigh temperature-treated protease with Zn^{2+}. Activity of the pro-

TABLE 1. *Isolation of protease from Pseudomonas sp. (MC60)*

Step	Total protein (mg)	Total activity (units × 10^{-3})	Recovery of activity (%)	Sp act (units/mg of protein)	Purification (fold increase)
Culture supernatant	66,372	17,589	100	265	1
Ammonium sulfate precipitation and dialysis	350	8,930	50.8	25,514	96
Sephadex G-100	153	5,700	32.4	37,225	141

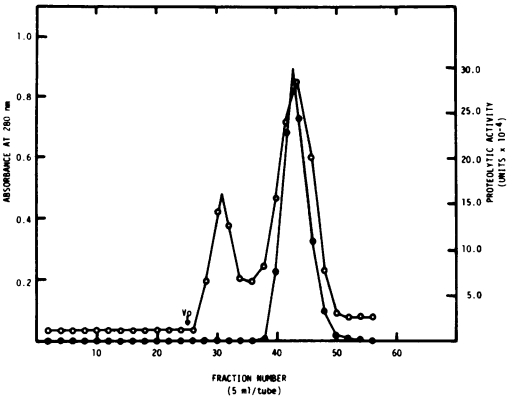

FIG. 1. *Gel filtration of partially purified Pseudomonas sp. (MC60) protease on Sephadex G-100. Void volume (V_0) was 125 ml. Symbols: proteolytic activity (●); absorbance at 280 nm (○).*

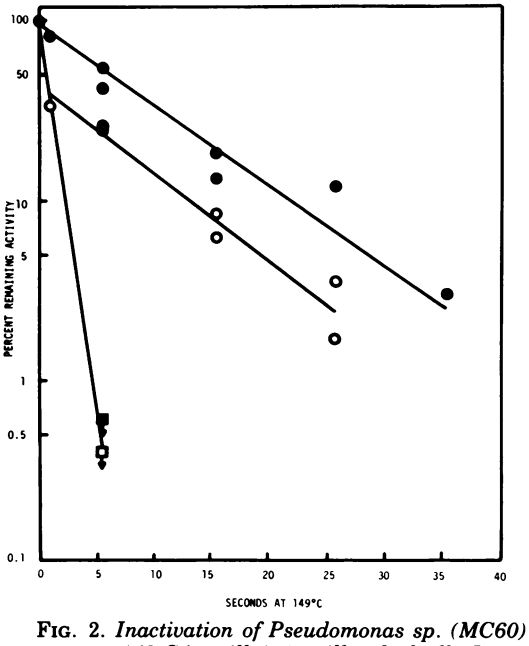

FIG. 2. *Inactivation of Pseudomonas sp. (MC60) protease at 149 C in milk (●), milk-salts buffer I (□), water (■), and milk-salts buffer I with 10 mM $CaCl_2$ (○).*

tease heated in milk or water at 149 C was unaffected by the addition of Zn^{2+} to the substrate (Fig. 2 and 3). Activity of unheated protease also was unaffected by Zn^{2+} addition. However, activity of protease heated in milk-salts buffer II with Ca^{2+} increased when Zn^{2+} was added to the substrate (Fig. 3). The change was manifested as the elimination of the initial loss of activity. The $t_{1/2}$ of protease heated in milk or buffer II with Ca^{2+} and assayed in casein supplemented with Zn^{2+} was 7.4 s.

Molecular weight determination. The molecular weight, estimated by gel filtration, was 48,000.

Effect of pH on protease stability. The enzyme was stable to irreversible loss of activity in a pH range of 5 to 10 (Fig. 4). Greater than 85% activity remained after a 2-h exposure in this range at 21 C. The enzyme appeared most stable around pH 7.5. This was the same pH that Adams et al. (1) reported for optimum proteolytic activity with a casein substrate.

Effect of metal chelating agents on protease activity. The enzyme was completely inactivated by EDTA and partially inactivated by *o*-phenanthroline (Fig. 5). *o*-Phenanthroline was more effective at concentrations below 1 mM.

Reactivation of EDTA-treated enzyme with metal ions or milk. EDTA-treated enzyme could be partially reactivated with selected metal cations or half-strength milk (Table 2). It appeared the apoenzyme required both Zn^{2+} and Ca^{2+} for maximum activity, since either one alone gave less reactivation than a mixture of the two. Reactivation occurred in milk as well as in the Zn^{2+} plus Ca^{2+} mixture. To determine if Ca^{2+} stabilized the protease during the normal assay at 45 C, the enzyme assay was run at a lower temperature as well as at 45 C. Little difference in activity with assay temperature indicated Ca^{2+} was not required for stability during the assay; however, it was required, as was Zn^{2+}, for optimal activity.

DISCUSSION

The divalent cations of milk play an important role in the heat stability and activity of MC60 protease heated at ultrahigh temperatures in milk. The heat stability of MC60 pro-

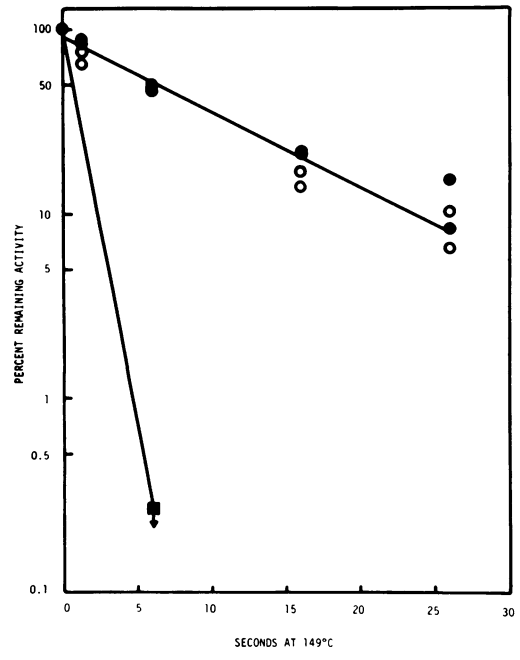

FIG. 3. *Inactivation of Pseudomonas sp. (MC60) protease at 149 C in milk (●), milk-salts buffer II with 15 mM $CaCl_2$ (○), and water (■). Substrate was supplemented with $ZnSO_4$ to 0.5 mM.*

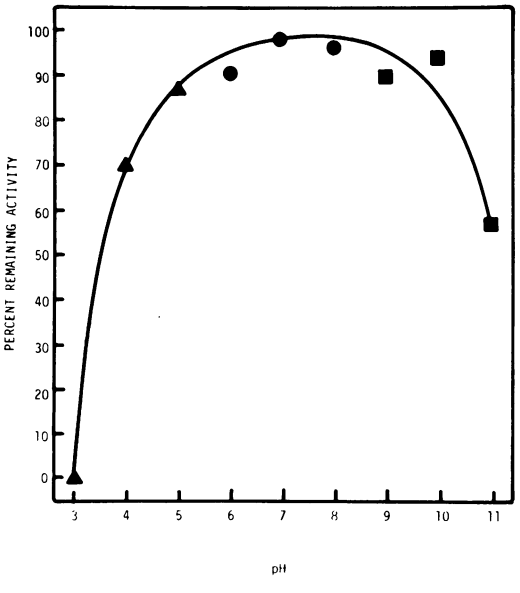

FIG. 4. *Effect of pH on the stability of Pseudomonas sp. (MC60) protease. Remaining activity was determined as a percentage of the initial activity before a 2-h exposure at 21 C. The buffers used were: pH 3 to 5, 50 mM acetate buffer (▲); pH 6 to 8, milk-salts buffer I (●); and pH 9 to 11, 50 mM glycine-NaOH buffer (■).*

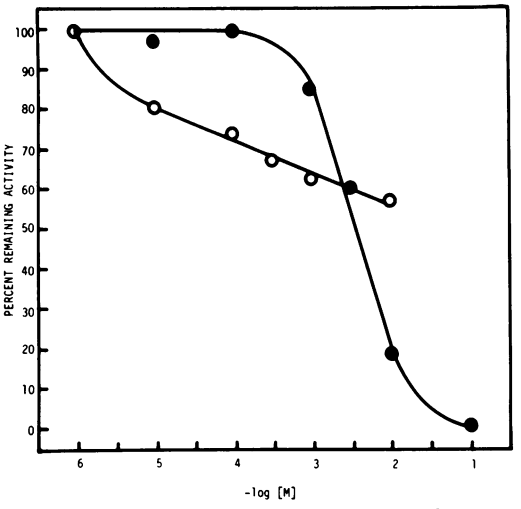

FIG. 5. *Effect of metal chelators on Pseudomonas sp. (MC60) protease. Stock protease solution was diluted 1:10 into EDTA, pH 7.0 (●), or o-phenanthroline, pH 7.0 (○), and held for 20 min at 21 C. Remaining activity was a percentage of initial activity before exposure. o-Phenanthroline in the substrate mixture did not interfere with absorbance readings at 650 nm.*

TABLE 2. *Metal ion reactivation of apoenzyme*[a]

Metal cation[b]	% Original activity[c]	
	Assay at 30 C	Assay at 45 C
None	5.8	11.5
Ca^{2+}	52.9	50.1
Zn^{2+}	52.9	50.1
Ca^{2+} (5 mM)/Zn^{2+} (5 mM)	60.8	72.5
Fe^{2+}	21.2	10.6
Co^{2+}	8.8	6.0
Mg^{2+}	7.9	19.5
Milk	63.5	71.4

[a] Enzyme was treated with 10 mM EDTA for 20 min at 21 C and then dialyzed against 300 volumes of deionized water at 4 C.

[b] Apoenzyme was treated with 10 mM metal ion or sterilized milk diluted 1:1 with water for 20 min at 21 C.

[c] Based on activity of untreated enzyme assayed at the respective temperatures.

tease in milk at 149 C was associated with its interaction with Ca^{2+}. It is likely that the basis for the heat resistance of other psychrotrophic bacterial proteases in milk (4, 12) is similar, since Ca^{2+} has been shown to stabilize many proteolytic enzymes. Removal of cations from milk by either of two methods resulted in a medium in which MC60 protease was rapidly inactivated at ultrahigh temperatures. The protease exhibited high heat stability in cation-free milk to which Ca^{2+} had been added.

Enzyme-associated Zn^{2+} was displaced when protease was heated in milk-salts buffer with Ca^{2+}. The resulting decrease in activity was regained by addition of Zn^{2+} to the substrate. The dependence of full activity on Zn^{2+} as well as on Ca^{2+} was confirmed by several observations. The ordinate intercept (45% remaining activity) of the second phase of the biphasic inactivation curve (Fig. 2) indicated a 55% activity loss by heating in Ca^{2+} buffer. The percentage of activity observed for the Ca^{2+}-holoenzyme or the Zn^{2+}-holoenzyme was only 50%. At high concentrations of *o*-phenanthroline, a loss of only 50% activity was observed.

The reactivation of the apoenzyme in half-strength milk to the extent observed in the Ca^{2+} plus Zn^{2+} solution was consistent with the effects of Ca^{2+} and Zn^{2+} in milk during heating at ultrahigh temperatures. Milk contains approximately 30 mM Ca^{2+} and 50 μM Zn^{2+} (17). Apparently there was adequate Ca^{2+} in milk for thermal stability and subsequent activity, and either the loss of Zn^{2+} was prevented or provision of supplementary Zn^{2+} for reactivation occurred.

The sensitivity of MC60 protease to EDTA and *o*-phenanthroline was typical of metalloproteases (5, 6, 14, 15). The incomplete inactivation of the enzyme by *o*-phenanthroline suggested the removal of Zn^{2+} rather than Ca^{2+} from the enzyme, since *o*-phenanthroline is rather specific in chelating Zn^{2+}.

Several psychrotrophic *Pseudomonas* species produce extracellular caseinolytic proteases, which reportedly differ in heat stability, pH optima and stability, molecular weight, and other properties (1, 10, 12, 13, 15). Of the different enzymes characterized, the metalloprotease of *Pseudomonas* sp. (MC60) very closely resembles that of *P. fluorescens* P26 (12) in heat stability. Both proteases were active after several minutes of heating in milk at 121 C (1, 12). However, since the molecular weight of the P26 protease was approximately one-half of the MC60 protease, it is unlikely that the enzymes are the same. The *P. fragi* protease studied by Porzio and Pearson (15) had the same apparent molecular weight and pH optimum as the MC60 protease. The optimal temperatures for activity and the pH stability range were also similar for the two, and both were stabilized by Ca^{2+}. The *P. fragi* protease was reported to be heat sensitive because it was inactivated rapidly at temperatures around 55 C. Recently, Barach et al. (3) reported that MC60 and eight other proteases that were heat resistant at 149 C were inactivated at 55 to 60 C by some mechanism other than thermal inactivation. It seems likely that the *P. fragi* protease and other Ca^{2+}-stabilized proteases that appear heat sensitive also may prove to be heat resistant when tested at high temperatures. The loss of activity at temperatures just above the optimum for activity might not reflect the true heat resistance of these enzymes.

ACKNOWLEDGMENT

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LITERATURE CITED

1. Adams, D. M., J. T. Barach, and M. L. Speck. 1975. Heat resistant proteases produced in milk by psychrotrophic bacteria of dairy origin. J. Dairy Sci. 58:828-834.
2. Andrews, P. 1964. Estimation of the molecular weights of proteins by Sephadex gel-filtration. Biochem. J. 91:222-233.
3. Barach, J. T., D. M. Adams, and M. L. Speck. 1975. Low temperature inactivation in milk of heat-resistant proteases from psychrotrophic bacteria. J. Dairy Sci. 59:391-397.
4. Bergtsson, K., L. Gardhage, and B. Isaksson. 1973. Gelation in UHT treated milk, whey and casein solution. The effect of heat resistant proteases. Milchwissenschaft 28:495-499.
5. Boethling, R. S. 1975. Purification and properties of a serine protease from *Pseudomonas maltophilia*. J. Bacteriol. 121:933-941.
6. Coleman, J. E., and B. L. Vallee. 1960. Metallocarboxy-peptidases. J. Biol. Chem. 235:390-395.
7. Feder, J., L. R. Garrett, and B. S. Wildi. 1971. Studies on the role of calcium in thermolysin. Biochemistry 10:4552-4555.
8. Friedberg, F. 1974. Effects of metal binding on protein structure. Q. Rev. Biophys. 7:1-33.
9. Hull, M. E. 1947. Studies on milk proteins. II. Colorimetric determination of the partial hydrolysis of the proteins in milk. J. Dairy Sci. 30:881-884.
10. Hurley, W. C., F. A. Gardner, and C. Vanderzant. 1963. Some characteristics of a proteolytic enzyme system of *Pseudomonas fluorescens*. J. Food Sci. 28:47-54.
11. Jenness, R., and J. Koops. 1962. Preparation and properties of a salt solution which simulates milk ultrafiltrate. Neth. Milk Dairy J. 16:153.
12. Mayerhofer, H. J., R. T. Marshall, C. H. White, and M. Lu. 1973. Characterization of a heat-stable protease of *Pseudomonas fluorescens* P26. Appl. Microbiol. 25:44-48.
13. Morihari, K., and H. Tsuzyki. 1964. *Pseudomonas aeruginosa* peptide peptidohydrolase. III. Some characters as a Ca^{++} metalloenzyme. Biochim. Biophys. Acta 92:351-360.
14. Nakajima, M., K. Mizusawa, and F. Yoshida. 1974. Purification and properties of an extracellular proteinase of psychrophilic *Escherichia freundii*. Eur. J. Biochem. 44:87-96.
15. Porzio, M. A., and A. M. Pearson. 1975. Isolation of an extracellular neutral proteinase from *Pseudomonas fragi*. Biochim. Biophys. Acta 384:235-241.
16. Seigel, S., and W. M. Award, Jr. 1973. The proteolytic enzymes of the K-1 strain of *Streptomyces griseus* obtained from a commercial preparation (Pronase). J. Biol. Chem. 248:3233-3240.
17. Webb, B. H., and A. H. Johnson. 1965. Fundamentals of dairy chemistry, p. 16-22. Avi Publishing Co., Inc., Westport, Conn.