

PURIFICATION AND ACTIVITY OF PROTEINASE OF *STREPTOCOCCUS FAECALIS* VAR. *LIQUEFACIENS*

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ABSTRACT

SHUGART, LEE R. (University of Tennessee, Knoxville) AND RAYMOND W. BECK. Purification and activity of proteinase of *Streptococcus faecalis* var. *liquefaciens*. J. Bacteriol. 88:586-590. 1964.— A proteolytic enzyme from *Streptococcus faecalis* var. *liquefaciens* was purified 480-fold by ammonium sulfate fractionation and treatment with calcium phosphate gel. Approximately 20% of the original enzyme activity was recovered in the purified fraction. Optimal enzyme activity was found to be at pH 7.6 and 35 C. The enzyme is apparently more susceptible to heat denaturation when complexed with substrate than when heated in the absence of substrate. Michaelis-Menten constants were found to be 0.655% for hemoglobin and 0.133% for casein. Apparent energies of activation on these substrates were calculated to be 9,060 and 12,020 cal, respectively.

Reports concerning proteolytic enzymes of microorganisms appear frequently in current literature (Hurley, Gardner, and Vanderzant, 1962; Morihara, 1963; Hampson, Mills, and Spencer, 1963). Various members of the genus *Streptococcus* have been observed to possess such enzymes (Frobisher and Denny, 1928; Elliott, 1950; Williamson, Tove, and Speck, 1964); typical strains of *S. faecalis* var. *liquefaciens* are known to coagulate and hydrolyze milk proteins at acid pH (Dudani, 1950; Grutter and Zimmerman, 1955; Rabin and Zimmerman, 1956). In the present investigation, an apparent extracellular proteinase of a member of this group was purified, and a number of its properties were determined.

MATERIALS AND METHODS

Organism. *Streptococcus* R64A, the organism used throughout this investigation, was originally isolated from the fecal droppings of a box turtle by J. O. Mundt, Department of Bacteriology, University of Tennessee, Knoxville, and is similar to the *S. faecalis* var. *liquefaciens* reported

in the 7th edition of *Bergey's Manual of Determinative Bacteriology* (Breed, Murray, and Smith, 1957). Stock cultures of the organism were carried at 7 C in sodium azide-dextrose broth containing 0.5% CaCO₃.

Enzyme assay. Hemoglobin (Anson, 1938) and casein (Kunitz, 1947) were the substrates used for the estimation of the proteolytic activity of the enzyme. The assay procedure was that of Northrop, Kunitz, and Herriott (1948). The activity was expressed by the arbitrary unit of the change in optical density at 280 m μ that was brought about by 1 ml of the enzyme solution in 1 min per milliliter of assay mixture (Δ OD min⁻¹ ml⁻¹). Unless otherwise stated, the assay temperature was 35 C and the assay time was 10 min for hemoglobin substrates and 30 min for casein substrates. All concentrations of enzyme and substrates are expressed as per cent (w/v).

Protein concentration was measured spectrophotometrically (Layne, 1957) with a Beckman model DB spectrophotometer.

Buffers were prepared as outlined by Gomori (1955) and the concentrations employed were as stated in the reference(s) cited unless otherwise noted; pH measurements were made with a Beckman model G pH meter. Centrifugations were performed in a Servall RC-2 refrigerated centrifuge set at 4 C.

Procedure for the purification of enzyme. Twenty liters of sterile 10% skim milk (Starlac; The Borden Co., New York, N.Y.) were inoculated aseptically with 50 ml of a 24-hr starter culture of *Streptococcus* R64A grown on the same medium. After approximately 110 hr of incubation at room temperature (24 to 26 C), 19 liters of whey were recovered with the use of a Szent-Gyorgyi and Blum continuous-flow system at 25,000 \times g.

The whey was brought to approximately 55% saturation by the addition of solid ammonium sulfate (350 g per liter), and the mixture was allowed to stand in the cold overnight.

The precipitate that resulted was recovered by

passing the $(\text{NH}_4)_2\text{SO}_4$ solution twice through a Sharples super-centrifuge operating at a speed of 25,000 rev/min with a flow rate of 100 ml/min. The precipitate recovered was dissolved in 1 liter of 0.01 M phosphate buffer (pH 7.0), and 10 ml of Thimerosal (1:1,000 solution of Merthiolate; Eli Lilly & Co., Indianapolis, Ind.) were added as a preservative.

After standing covered for 3 weeks in the cold, the enzyme solution was clarified by centrifugation at $10,000 \times g$ for 60 min. The supernatant fluid was dialyzed against 18 liters of deionized distilled water in the cold for 24 hr, and 10 ml of Thimerosal were added to the recovered dialyzed solution.

To each 150 ml of enzyme solution, 30 ml of calcium phosphate gel (64.5 mg/ml) prepared according to Levine (1962) were added, mixed well, and allowed to stand at room temperature for 10 min; the gel was removed by centrifugation and discarded. This procedure was repeated twice with the use of 20 and 10 ml of the gel per 150 ml of enzyme solution. The final clarified supernatant fluid was brought to approximately 60% saturation with $(\text{NH}_4)_2\text{SO}_4$ (400 g per liter) and was placed in the cold. After several days, a fine, brownish colloidal suspension collected at the surface of the $(\text{NH}_4)_2\text{SO}_4$ solution, and the more dense solution was removed with a Pasteur pipette. The precipitate was resuspended in about 800 ml of 0.2 M saline solution, placed in the cold overnight, and recovered by centrifugation at $35,000 \times g$ for 60 min. The supernatant fluid was retained and constituted solution no. 1 obtained from the second $(\text{NH}_4)_2\text{SO}_4$ fractionation.

The precipitate was washed several times with

0.1 M NaCl solution, and the first supernatant fluid recovered was designated solution no. 2 obtained from the second $(\text{NH}_4)_2\text{SO}_4$ fractionation.

The remaining supernatant fluids were combined and were designated solution no. 3 obtained from the second $(\text{NH}_4)_2\text{SO}_4$ fractionation.

RESULTS

Purification. Table 1 was prepared according to the recommendations of Dixon and Webb (1958), and shows in a condensed form the progress of the purification of the enzyme through the different stages.

Solution no. 2 obtained from the second $(\text{NH}_4)_2\text{SO}_4$ fractionation showed the highest purification and was used exclusively for the remaining determinations.

Effect of pH. The effect of hydrogen ion concentration on the enzyme was determined with casein substrate. The buffer systems employed were (i) citrate for pH below 5.0, (ii) glycine-NaOH for pH above 9.0, and (iii) tris(hydroxymethyl)-aminomethane-maleate-NaOH for pH 5.0 to 9.0.

To determine the proteolytic activity at various hydrogen ion concentrations, 1 ml of 0.98% casein substrate was diluted with 4 ml of appropriate buffer and was equilibrated to 35 C; the activity was measured after the addition of 1 ml of diluted enzyme solution. The final casein and enzyme concentrations of the assay mixture were 1.63 mg/ml and 15 μg /ml, respectively.

Proteolytic activity after exposure of the enzyme to various hydrogen ion concentrations was determined by adding 0.5 ml of the undiluted enzyme to 4.5 ml of buffer of the appropriate pH. This mixture was allowed to stand for 15 min at

TABLE 1. *Purification of proteolytic enzyme*

Procedure	Vol	Activity*	Total units	Protein	Purity ($\times 10^3$)	Yield	Purification
	<i>ml</i>			<i>mg/ml</i>		<i>%</i>	
Recovered whey	19,000	0.217	4,123	46.1	4.71	100	1
First $(\text{NH}_4)_2\text{SO}_4$ fractionation	1,010	3.697	3,734	75.0	49.29	90.5	10.4
3 weeks at 7 C followed by centrifugation and dialysis	1,150	3.137	3,608	30.0	104.56	87.5	22.2
Calcium phosphate gel fractionation	1,200	3.060	3,672	20.0	153.00	89.1	32.5
Second $(\text{NH}_4)_2\text{SO}_4$ fractionation:							
Solution 1	800	2.295	1,836	3.50	655.71	44.5	139.2
Solution 2	160	5.100	816	2.25	2266.66	19.8	481.2
Solution 3	520	2.461	1,280	2.30	1070.00	31.0	227.2

* Expressed as $\Delta\text{OD min}^{-1} \text{ ml}^{-1}$.

room temperature, after which 7.5 ml of 0.1 M phosphate buffer (pH 7.5) were added. The diluted enzyme mixture was equilibrated to 35 C, 2 ml were added to 2 ml of 0.98% casein substrate, and the activity was measured. Concentrations of casein and enzyme in the assay mixture were 4.89 mg/ml and 45 μ g/ml, respectively.

Figure 1 shows the effect of pH on the enzyme.

Effect of temperature. The proteolytic activity at various temperatures was determined with hemoglobin used as the substrate. A mixture of 2.5 ml of 2.47% hemoglobin and 1 ml of enzyme solution was exposed to the specified temperature for 5 min before the enzyme activity was determined. The hemoglobin and enzyme concentrations were 17.6 mg/ml and 26 μ g/ml, respectively.

For the determination of proteolytic activity after exposure to various temperatures, a diluted solution of the enzyme was exposed to the specified temperature for 15 min, and then was quickly equilibrated to 35 C. A 1-ml portion of the heat-treated enzyme solution was added to 2.5 ml of 2.47% hemoglobin substrate previously equilibrated to 35 C, and the activity was determined. The concentrations of hemoglobin and enzyme in the reaction mixture were 17.6 mg/ml and 26 μ g/ml, respectively.

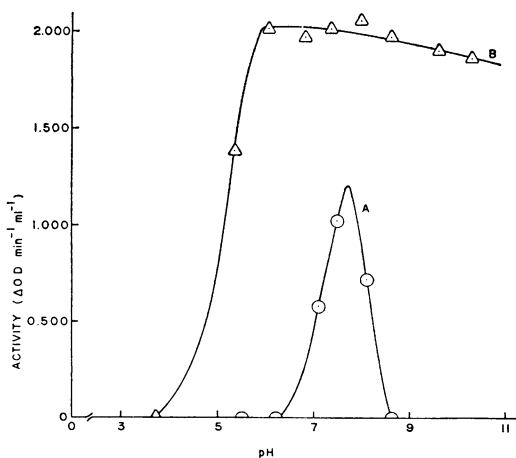


FIG. 1. Effect of pH on proteinase activity. For curve A, the activity was tested at the pH given. The assay mixture contained 1.63 mg/ml of casein and 15 μ g/ml of enzyme. For curve B, the enzyme was exposed for 15 min at the pH given, and the activity was tested at pH 7.5. The assay mixture contained 4.89 mg/ml of casein and 45 μ g/ml of enzyme.

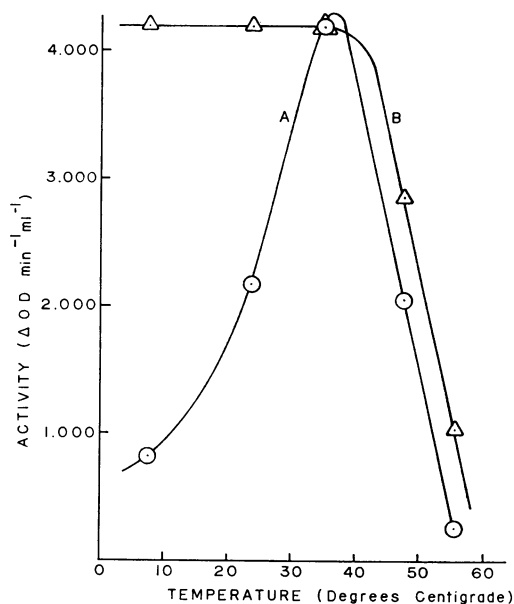


FIG. 2. Effect of temperature on proteinase activity. For curve A, the activity was tested after 5-min exposure of the assay mixture at the temperature given. For curve B, the enzyme was exposed for 15 min at the temperature given, and the activity was tested at 35 C. Assay mixtures for both curves A and B contained 17.6 mg/ml of hemoglobin and 26 μ g/ml of enzyme.

Figure 2 demonstrates the effect of temperature on the enzyme.

Michaelis-Menten constant. For the determination of the Michaelis-Menten constant, casein substrate was diluted with 0.1 M phosphate buffer (pH 7.5), and hemoglobin substrate was diluted with the same buffer which was 10% (w/v) with respect to urea. The enzyme concentration was held constant in the assay mixture, and was 45 μ g/ml for casein substrate assay mixtures and 26 μ g/ml for hemoglobin substrate assay mixtures.

Figure 3 is a graphical determination of the Michaelis-Menten constant (K_m) according to the method of Lineweaver and Burk (1934) for the two proteolytic substrates, hemoglobin and casein. Michaelis-Menten constants of 0.655 and 0.133% were found for hemoglobin and casein, respectively.

Apparent energy of activation. For the determination of the apparent energy of activation (E), the velocity constant (k) was measured at two different temperatures for casein and hemo-

globin substrates and the value for E was determined according to the following formula (Crockford and Knight, 1959):

$$\log k_2/k_1 = 0.219 E T_2 - T_1/T_1T_2 \quad (1)$$

The final concentrations of hemoglobin and enzyme in the assay mixtures were 17.6 mg/ml and 26 μ g/ml, respectively. The final concentrations of casein and enzyme in the assay mixtures were 4.89 mg/ml and 45 μ g/ml, respectively.

E was found to be 9,060 cal for hemoglobin and 12,020 cal for casein according to equation 1 (Table 2).

DISCUSSION

Although the proteolytic enzyme studied in this work was obtained from the whey of hydrolyzed milk, no attempt was made to establish whether this enzyme was truly extracellular (Pollock, 1962).

The purification procedure resulted in a recovery of 20% of the original activity and a 480-fold increase in purity.

An interesting observation is the decrease in protein concentration of the enzyme solution after the first $(\text{NH}_4)_2\text{SO}_4$ fractionation (Table 1). Part

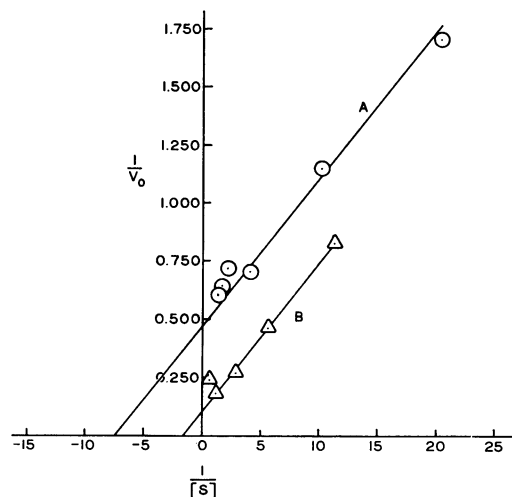


FIG. 3. Lineweaver-Burk plots of reaction velocities vs. hemoglobin concentration (curve A) and casein concentration (curve B). Substrate concentrations are expressed in per cent. Enzyme concentration was 26 μ g/ml for hemoglobin and 45 μ g/ml for casein. K_m for hemoglobin was 0.655% and for casein was 0.133%.

TABLE 2. Effect of temperature on reaction rate

Temp	Substrate velocity constant k^*	
	Casein	Hemoglobin
<i>C</i>		
13.15	0.300	—
23.80	—	2.195
35.00	1.382	3.853

* Initial velocity ($\Delta\text{OD min}^{-1} \text{ ml}^{-1}$) of enzyme-catalyzed reaction at temperature indicated.

of this decrease can be attributed to the hydrolysis of extraneous protein by the proteolytic enzyme upon standing for 3 weeks in the cold.

The shapes of curves A and B of Fig. 1 demonstrate the enzyme to be active over a limited pH range with an optimum near pH 7.6. Further, the fall-off of enzyme activity as the hydrogen ion concentration is increased from the optimum is probably due to the irreversible destruction of the enzyme, whereas the fall-off of activity as the hydrogen ion concentration is decreased from the optimum is probably due to a decreased saturation of enzyme with substrate.

The shapes of curves A and B of Fig. 2 demonstrate the typical increase in velocity of enzyme-catalyzed reaction with increase in temperature, followed by the irreversible destruction of the enzyme above 40 C. In addition, the enzyme is more susceptible to heat destruction when complexed with its substrate at temperatures above 40 C.

The apparent energies of activation found for the substrates, casein and hemoglobin, are in the range normally reported for enzyme-catalyzed reactions (Setlow and Pollard, 1962).

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LITERATURE CITED

- ANSON, M. L. 1938. The estimation of pepsin, trypsin, papain, and cathepsin with hemoglobin. *J. Gen. Physiol.* **22**:79-89.

- BREED, R. S., E. G. D. MURRAY, AND N. R. SMITH. Bergey's manual of determinative bacteriology, 7th ed. The Williams & Wilkins Co., Baltimore.
- CROCKFORD, H. D., AND S. B. KNIGHT. 1959. Fundamentals of physical chemistry, p. 371-373. John Wiley & Sons, Inc., New York.
- DIXON, M., AND E. C. WEBB. 1958. Enzymes, p. 40-41. Academic Press, Inc., New York.
- DUDANI, A. T. 1950. Proteolytic and coagulating enzymes of enterococci. Ph.D. Thesis, Iowa State College, Ames.
- ELLIOTT, S. D. 1950. The crystallization and serological differentiation of a streptococcal proteinase and its precursor. *J. Exptl. Med.* **92**: 201-218.
- FROBISHER, M., JR., AND E. R. DENNY. 1928. A study of *Micrococcus zymogenes*. *J. Bacteriol.* **16**:301-314.
- GOMORI, G. 1955. Preparation of buffers for use in enzyme studies, p. 138-146. *In* S. P. Colowick and N. O. Kaplan [ed.], *Methods in enzymology*, vol. 1. Academic Press, Inc., New York.
- GRUTTER, F. H., AND L. N. ZIMMERMAN. 1955. A proteolytic enzyme of *Streptococcus zymogenes*. *J. Bacteriol.* **69**:728-732.
- HAMPSON, S. E., C. L. MILLS, AND T. SPENCER. 1963. Observations on a protease from *Proteus mirabilis*. *Biochim. Biophys. Acta* **73**:476-487.
- HURLEY, W. C., F. A. GARDNER, AND C. VANDERZANT. 1962. Some characteristics of a proteolytic enzyme system of *Pseudomonas fluorescens*. *J. Food Technol.* **28**:47-54.
- KUNITZ, M. 1947. Crystalline soybean trypsin inhibitor. *J. Gen. Physiol.* **30**:291-310.
- LAYNE, E. 1957. Spectrophotometric and turbidimetric methods for measuring proteins. IV. Protein estimation by ultraviolet absorption, p. 451-454. *In* S. P. Colowick and N. O. Kaplan [ed.], *Methods in enzymology*, vol. 3. Academic Press, Inc., New York.
- LEVINE, O. 1962. Column chromatography of proteins. Calcium phosphate, p. 27-32. *In* S. P. Colowick and N. O. Kaplan [ed.], *Methods in enzymology*, vol. 5. Academic Press, Inc., New York.
- LINEWEAVER, H., AND D. BURK. 1934. The determination of enzyme dissociation constants. *J. Am. Chem. Soc.* **56**:658-666.
- MORIHARA, K. 1963. *Pseudomonas aeruginosa* proteinase. *Biochim. Biophys. Acta* **73**:113-124.
- NORTHROP, J. H., M. KUNITZ, AND R. M. HERRIOTT. 1948. Crystalline enzymes, 2nd ed., p. 303-310. Columbia University Press, New York.
- POLLOCK, M. R. 1962. Exoenzymes, p. 129-130. *In* I. C. Gunsalus and R. Y. Stanier [ed.], *The bacteria*, vol. 4. Academic Press, Inc., New York.
- RABIN, R., AND L. N. ZIMMERMAN. 1956. Proteinase biosynthesis by *Streptococcus liquefaciens*. I. The effect of carbon and nitrogen sources, pH, and inhibitors. *Can. J. Microbiol.* **2**:747-756.
- SETLOW, R. B., AND E. C. POLLARD. 1962. Molecular biophysics, p. 241-242. Addison-Wesley Publishing Co., Inc., Reading.
- WILLIAMSON, W. T., S. B. TOVE, AND M. L. SPECK. 1964. Extracellular proteinase of *Streptococcus lactis*. *J. Bacteriol.* **87**:49-53.