10. Hawk, P. B., Osler, B. L., Summerson, W. H., in *Practical Physiological Chemistry,* 11th ed., Philadelphia, 1937.

11. Berry, J. P., Woolf, L. I., *Nature,* 1952, v169, 202.

12. Armstrong, M. D., Shaw, K. N. F., Robinson, K. S., *J. Biol. Chem.*, 1955, v213, 797.

13. Weissbach, H., King, W., Sjoerdsma, A., Udenfriend, A., *ibid.,* 1959, v234, 81.

14. Udenfriend, S., Titus, E., Weissbach, H., *ibid.,* 1955, v216, 499.

15. Tashian, R. E., *Science,* 1959, v129, 1553.

16. Woolley, D. W., Edelman, P. M., *ibid.,* 1958, v127, 281.

Received October 30, 1959. P.S.E.B.M., 1960, v103.

Some Characteristics of Proteolytic System of a Marine Bacterial Species.* (25539)

J. M. PRESCOTT AND CHARLES R. WILLMS

Dept. of Biochemistry and Nutrition, Texas Agric. Exp. Station, College Station

The desirability of increasing present limited knowledge of bacterial proteolytic enzymes may be inferred from work of previous authors(1-4), some of whom suggested potential usefulness of these enzymes in investigations of protein and enzyme structure and in industrial processes(3,4). Investigations of bacterial proteinases have dealt principally with enzymes from terrestrial species; marine organisms have been largely neglected, even though they have been reported more proteolytic than either soil or fresh water forms(5). In view of established proteolytic ability, marine bacteria appear to offer considerable promise in investigations designed to increase our overall information on bacterial proteolytic enzymes. This report describes characteristics of a proteolytic system of an organism isolated from a marine environment.

Materials and methods. The organism was a marine bacterial species isolated by Merkel and Traganza(6) from the alimentary canal of the marine isopod, *Limnoria,* and reported by them to be highly proteolytic.† Stock cultures were grown on slants consisting of 1% peptone and 2% agar in artificial sea water, and were transferred at monthly intervals. A transfer was made from stock culture to a slant identical to stock medium, except that artificial sea water diluted 1:10 with distilled water was used. After 12 to 24 hours incubation, a loop transfer was made to 50 ml Erlenmeyer flask containing 6 ml of growth medium (Table I); this subculture was incubated 12 to 24 hr before use to inoculate 1 liter of growth medium. Incubation was at 25°C with shaking. After 54 to 56 hours incubation, cells were removed by centrifugation and passage through a Seitz filter; the resulting culture filtrate was used as source of enzyme. One ml of enzyme solution was incubated at 37°C with 5 ml of denatured hemoglobin substrate.‡ At end of desired incubation time, reaction was stopped and residual protein precipitated by addition of 10 ml of 5% trichloroacetic acid. The mixture was filtered through Whatman No. 3 paper, and determination of acid-soluble split products was made by the method of Northrup *et al.* (7) by reading at 280 mμ in Beckman Model DU Spectrophotometer. Enzyme concentration and time of incubation were always chosen so that activity was proportional to

* Supported, in part, by grants from Nat. Inst. Allergy and Infect. Diseases and from Robert A. Welch Fn.

† We are indebted to Dr. Joseph R. Merkel and Eugene D. Traganza for this culture, and for information on its characteristics prior to publication. Morphological, physiological, and biochemical characteristics of the organism are to be published by these workers.

‡ Prepared by suspending 5 g salt-free lyophilized hemoglobin (Mann Research Labs.) in 80 ml of water, adding 8 g urea, and incubating at 37° C for 45 to 60 minutes. Ten g urea and 125 ml of 0.2 M phosphate buffer (pH 8.0) were then added. Other pH values were employed where indicated.

TABLE I. Composition of Growth Medium.

Component	Amount/l
Peptone	10.0 g
NaCl	8.12
$MgCl_2 \cdot 6H_2O$	4.22
K_2HPO_4	1.0
KH_2PO_4	1.0
TRIS*	1.0
$MgSO_4 \cdot 7H_2O$	.4
Trace mineral solution†	5.0 ml

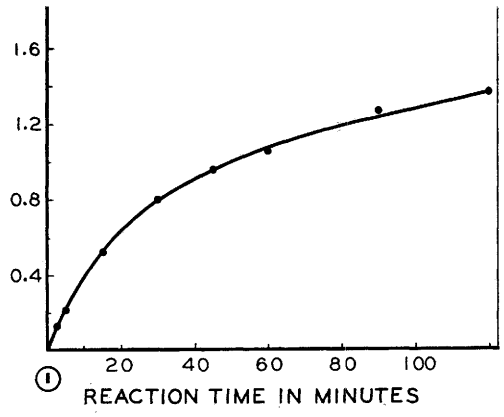
* Trishydroxymethylamino methane.

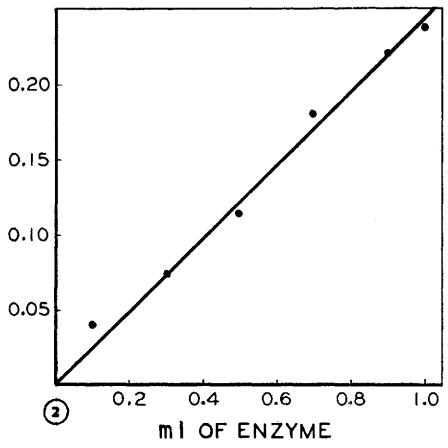
† Components, and concentrations in g/l: Ethylenediaminetetraacetic acid (tetrasodium salt), 1; H_3BO_3, 1.14; $FeCl_3 \cdot 6H_2O$, .049; $FeSO_4 \cdot 7H_2O$, 4; $MnSO_4 \cdot H_2O$, 3.08; $ZnSO_4 \cdot 7H_2O$, .022; $CuSO_4 \cdot 5H_2O$, .00016; $CoSO_4 \cdot 7H_2O$, .00048.

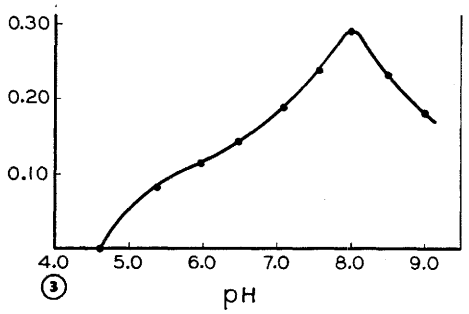
amount of enzyme and to time of incubation. Five minute incubation was commonly used; however, assays were frequently performed at several different time values so that activity could be plotted as a function of time. Appropriate blanks were used in all experiments.

Results. Culture filtrates prepared as described were so active proteolytically that it was necessary to dilute them 1:10 to obtain accurate assays at 5 minutes incubation. Filtrates could be frozen and stored several weeks without appreciable loss of activity. Our experiments did not permit us to determine whether proteinase is truly extracellular, or whether it appears in growth medium through autolysis of cells(2). At short periods of incubation, rate of proteolysis was proportional to reaction time (Fig. 1) and to enzyme concentration (Fig. 2). Other experiments showed that rate of reaction was affected by substrate concentration, rising rapidly to maximum value at 8 mg of hemoglobin/ml, and decreasing slightly at higher levels. Similar behavior has been reported for other bacterial proteinases(8,9).

Optimum pH for enzyme activity was determined by buffering samples of substrate at desired pH values with phosphate buffer. These experiments revealed a single, sharply defined optimum at pH 8 (Fig. 3). This is slightly more alkaline than the optima of some extracellular bacterial proteinases previously reported(3,9), but is distinctly more acidic than that of subtilisin(10). It is perhaps significant that the optimum pH for the proteinase system of the marine organism coincides almost exactly with the pH of sea water(11). Effects of pH on enzyme stability were tested by determining activity at different incubation times (up to 30 min) in samples of substrate adjusted to pH values of 7.0,







All ordinates: Optical density at 280 mμ.

FIG. 1. Effect of reaction time on hydrolysis of hemoglobin by culture filtrate of the marine bacterium.

FIG. 2. Effect of enzyme concentration on proteolysis. Incubation time, 5 min.

FIG. 3. Influence of pH on activity of proteolytic system. Samples of substrate were buffered with phosphate buffer at values shown.

7.5, 8.0, 8.5, and 9.0. The enzyme system was stable at all of these pH values at incubation times tested, and comparative slopes of the activity plots were identical to those which one would predict from data in Fig. 3.

Effects of temperature on enzyme activity. Six temperatures of incubation from 17°C to 55°C were tested at incubation times ranging from 5 to 60 minutes. Activity increased with temperature through 48°C, but declined somewhat at 55°C, indicating partial inactivation of the proteinase system at the highest temperature. From these data, minimum energy of activation for the proteinase system was calculated to be about 8300 cal. To determine effects of temperature on stability of the enzyme system, samples of the culture filtrate were heated at 70°C for varying times, chilled instantly in ice water, then assayed for remaining enzymatic activity. Five minute exposure at 70°C reduced activity to 87% of original, 7.5 minutes to 50%, and 13 minutes of heating virtually destroyed enzymatic activity.

Effects of activators and inhibitors. Prolonged dialysis of culture filtrate against 0.01 M phosphate buffer (pH 8) resulted in sharp reduction of enzymatic activity, indicating a requirement for one or more activators normally present in the growth medium. Approximately 80% of original activity was restored by rendering the dialyzed enzyme 0.005 M with respect to Co^{++} ions. Similar activating effects of Co^{++} ions on bacterial proteinases have been observed by Weil and Kocholaty (12), and by Hunt and Moore(13). Activity was restored to about 60% of the original by adding Zn^{++} ions to a concentration of 0.0005 M, but higher levels reduced activity. Undialyzed enzyme was inhibited by 0.005 M concentrations of $HgCl_2$, $FeCl_3$, $NiCl_2$, and $CuSO_4$. No decrease in activity occurred when *p*-chloromercuribenzoate was added, indicating that sulfhydryl bonds are not required for activity. Furthermore, reducing agents (cysteine, glutathione, KCN) produced marked reduction of activity at a concentration of 0.005 M, thus indicating a necessity for disulfide linkages. Previous investigators have also observed some inhibition in activity when reducing agents were added to proteolytic enzymes of aerobic bacteria(14,15). Damodaran *et al.*(3) reported that reducing agents were without effect on the proteinase of *Bacillus licheniformis,* while Hunt and Moore(13) found inhibition by both *p*-chloromercuribenzoate and cysteine on a proteinase from a facultatively aerobic rod.

Summary. Filtrates from pure cultures of a marine bacterial species contain a proteolytic enzyme which rapidly hydrolyzes denatured hemoglobin. Enzymatic activity after 54 to 56 hours of growth is so high that culture filtrates must be diluted for accurate assay even with short reaction periods. The enzyme system has optimum activity at pH 8, is activated by Co^{++} and Zn^{++} ions, and is inhibited by Fe^{++}, Hg^{++}, and Cu^{++} ions. Disulfide linkages appear to be required for activity, since reducing agents inhibit activity and *p*-chloromercuribenzoate is without effect. The enzyme is highly active at 48°C during incubation periods to 40 minutes, but temperatures above 50°C reduce activity even at shorter incubation periods. Heating at 70°C for 7.5 minutes before assay reduces enzyme activity to one-half of original.

1. Smith, E. L., *The Enzymes,* Sumner and Myrbäck, Eds., Academic Press, N. Y., 1951, v1, part 2, p862.

2. Fry, B. A., *The Nitrogen Metabolism of Microorganisms,* Methuen, London, 1957, pp112-125.

3. Damodaran, M., Govindarajan, V. S., Subramanian, S. S., *Biochim. Biophys. Acta,* 1955, v17, 99.

4. Mills, G. L., Wilkins, J. M., *ibid.,* 1958, v30, 63.

5. ZoBell, C. E., *Marine Microbiology,* Chronica Botanica Co., Waltham, Mass., 1946, pp117-118.

6. Merkel, J. R., Traganza, E. D., *Bact. Proc.,* 1958, p53.

7. Northrup, J. H., Kunitz, M., Herriott, R. M., *Crystalline Enzymes,* 2nd ed., Columbia Univ. Press, N. Y., 1948.

8. Friedman, M. E., Nelson, W. O., Wood, W. A., *J. Dairy Sci.,* 1953, v36, 1.

9. Husain, I., McDonald, I. J., *Canad. J. Microbiol.,* 1958, v4, 237.

10. Güntelberg, A. V., Otteson, M., *Comp. Rend. Trav. Lab. Carlsberg* (Ser. Chim), 1954, v29, 36.

11. Harvey, H. W., *The Chemistry and Fertility of Sea Waters,* Cambridge Univ. Press, 1955, p155.

12. Weil, L., Kocholaty, W., *Biochem. J.,* 1937, v31, 1255.

13. Hunt, W. G., Moore, R. O., *Applied Microbiol.*, 1958, v6, 36.

14. Maschmann, E., *Biochem. Z.*, 1937, v294, 1.

15. Weil, L., Kocholaty, W., Smith, L. D., *Biochem. J.*, 1939, v33, 893.

Received November 4, 1959. P.S.E.B.M., 1960, v103.

Spontaneous Activity in Gamma Efferents of a Deafferented Spinal Cord Segment. (25540)

DAVID F. LINDSLEY AND EARL ELDRED

Dept. of Anatomy, School of Medicine, University of California, Los Angeles, and Vet. Admin. Hospital, Long Beach

Isolation of spinal cord segment from descending and afferent impulses leads in puppies(1), cats(2) and monkeys(3) to contractile inactivity of parts innervated by the segment. Even the spinal dogfish, which has incessant spontaneous swimming movements when dorsal roots are intact, becomes quiet when sufficient roots are cut(4). Absence of overt contractions, of course, indicates that there is no firing of the large alpha motoneurons which innervate extrafusal muscle fibers. Not eliminated, however, is the possibility of unrevealed activity in the small fusimotor neurons or gamma efferents leading to intrafusal fibers of muscle spindles. Under many conditions of physiological and experimental stimulation, spindle loops may be in vigorous fluctuating activity without alpha motoneuron participation(5). The firing of these fusimotor neurons can be influenced from both segmental and supraspinal levels(6). Bilateral exclusion of dorsal root inflow does not extinguish unevoked activity in these neurons in animals with intact supraspinal connections(5); nor does interruption of descending pathways prevent continuing activity if segmental inflow is intact(7). In our experiments, both segmental afferent and descending influences have been excluded from the cat's lumbosacral cord to see if activity in fusimotor fibers may be fully independent. Evidence of spontaneous activity of fusimotor neurons under these conditions has been obtained in acute and chronic preparations.

Methods. The lumbosacral segment of spinal cord in 16 cats was isolated from impinging nervous impulses by transecting at L_1 and cutting all dorsal roots to the segment. Transection in acute experiments was preceded by freezing of the cord at immediate site of sectioning; in chronic preparations, either the same method was used, or dura was left intact and a cut made between ligatures tied around the dural sac. Dorsal roots were clamped with silver clips and cut extradurally if deafferentation was performed at time of initial operation, or if left to terminal experiment, the roots were cut along line of their entrance into the cord, at which level it was generally possible to spare major radicular vessels. When cutting extradurally, ventral roots from about S_3 caudalward also had to be sacrificed. Close nursing care involving treatment with antibiotics, forced feeding, and bladder expression was given to chronic animals over 2-30 days. At time of terminal observation, cats were electrolytically decerebrated, the lumbosacral cord bathed in mineral oil maintained within 1° of normal body temperature by thermistor-controlled radiant heat, and observations begun 2-6 hours after surgery was completed. Potentials were detected by suspending fine filaments of either ventral or dorsal roots on submerged silver electrodes. When dorsal root fibers were monitored, the hind leg was denervated except for the nerve to triceps surae, and units were identified as spindle afferents by presence of a pause in discharge during induced twitch. Checks were routinely made for completeness of deafferentation by inspection for reflex responses upon stimulation of the skin, through search for afferent activity in dorsal roots in preparations with extradurally cut roots, and by postmortem inspection of root and cord lesions.