B. SUBTILIS NEUTRAL PROTEASE, A ZINC ENZYME OF HIGH ACTIVITY*

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There have been a few reports suggesting that certain bacterial proteases are metalloenzymes (1,2). However, these studies are subject to criticism since the evidence was based on the fact that these enzymes are inhibited by EDTA. Since EDTA has been shown to inhibit nonmetalloenzymes (3), the reports of these investigators do not conclusively show that these bacterial proteases are metalloenzymes. Furthermore, the results of the earlier workers did not demonstrate the actual prosthetic group of the enzyme. The object of the present communication is to show that a B. subtilis protease which was purified from a commercial starting material (Pacific Enzyme Laboratories, Honolulu, Hawaii) is a zinc enzyme. In addition, the B. subtilis protease is one of the most active protease ever reported.

In order to demonstrate that the B. subtilis protease was a zinc enzyme, the specific activity and zinc content were followed during the purification procedure of the enzyme. The results are summarized in Table I and clearly demonstrate a direct proportionality between the zinc content and specific activity of the enzyme during the purification procedure. Spectrographic and microchemical analyses of the purified enzyme of specific activity 13,400 disclosed that Fe, Cu, Mn, Ni, Cr, Mo, Co, Mg, V, W and Sr were not the prosthetic group. Calcium salts were

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TABLE I

Proportionality of Zinc Content and Specific Activity of the Enzyme During Purification

Step	Specific Activity** units/mg protein	Zn Content μg/mg protein
1. Crude	930	.109
2. DEAE-cellulose treated enzyme	1426	.303
3. 0 to 60% acetone precipitate	3077	.494
4. CM-cellulose chromatography	11310	1.93
5. Rechromatography on CM-cellulose*	12520	2.10

*The enzyme obtained in Step 5 is at least 90% homogeneous as judged by electrophoresis and ultracentrifuge experiments at pH 5.5. The details of the purification procedure will be published elsewhere (4).
**The protease activity was determined essentially by the casein digestion method described by Hagihara (5). Ammonium phosphate buffer was replaced by Tris buffer since phosphate inhibits the enzyme. A unit of enzyme is defined as the μg of tyrosine liberated per ml per min at 30°.

added to the enzyme preparation at each stage of the purification procedure and therefore, it was detected in the purified enzyme. However, evidence will be shown later that Ca cannot reactivate the apoenzyme to any significant extent. The average zinc content for the enzyme (specific activity, 13,500) which was monodisperse by a number of physicochemical criteria contained 2.1 μg Zn per mg of enzyme. On the basis that there is one gram atom of zinc per mole of enzyme, a minimum molecular weight of about 31,100 is obtained. Since the sedimentation coefficient of the enzyme was 3.4S (uncorrected), it is very likely that the molecular weight of the enzyme will be in the neighborhood of 30-40,000.

As further evidence of the zinc-protein nature of the enzyme, Table II summarizes the inhibition of the enzyme by a number of known zinc chelating agents.

TABLE II

Inhibition of Enzyme by Zinc Chelating Agents

Chelating Agent	Conc. Required for 50% Inhibition (M/L)
1. Ethylenediaminetetraacetic acid	2.2×10^{-6}
2. O-phenanthroline	5.0×10^{-5}
3. Dithizone	4.5×10^{-4}
4. Sodium cyanide	1.8×10^{-3}
5. Na-diethyldithiocarbamate	2.5×10^{-3}

The enzyme was preincubated at 4° and pH 7.0 for 10 min prior to the addition of substrate.

In order to show that zinc is essential for activity, the apoenzyme was prepared as follows. The enzyme (20-100 mg) was treated with 2×10^{-4} M EDTA for 2 min. at 4° and then passed through a mixed bed resin (1 x 25 cm) consisting of an equal mixture of Dowex 1 and Dowex 50 X-8 under suction. The enzyme thus obtained was assayed for activity and zinc content and the results are summarized in Table III. It can be seen that both the zinc content and activity dropped in parallel. The apoenzyme thus produced was then treated with 1×10^{-4}M solutions of Ca, Zn, Co and Mn salt solutions. Reactivation varied from 38-82% in the various experiments when zinc sulfate was added and zinc ions always the most effective in reactivating the apoenzyme followed by Co and Mn in that order. Little or no reactivation occurred when Ca salts were added.

The purified enzyme (specific activity 13,600) was 5 to 16 times more active than crystalline preparations of trypsin, chymotrypsin, Nagarse, papain and chymopapain in hydrolyzing casein.

The B. subtilis protease was unable to hydrolyze L-leucine amide, a typical leucineaminopeptidase substrate (6) nor carbobenzoxy-glycyl-L-

TABLE III

Preparation of the Apoenzyme and Reactivation by Some Metal Ions

Treatment	Specific Activity units/mg	% of Initial Activity	Zn Content μg/mg Protein
1. None	12,400	100	1.92
2. After EDTA and ion exchange resin treatment			
a. Diluted with water	334	2.7	.06
b. Diluted with $MnSO_4$	1,435	11.6	--
c. Diluted with $CaCl_2$	1,230	9.9	--
d. Diluted with $CoCl_2$	4,240	34.2	--
e. Diluted with $ZnSO_4$	6,900	55.7	--
3. Expt. 2e after dialysis against $ZnSO_4$ and then Zn free buffer	7,100	57.2	1.09

Details of the experimental conditions are described in the text.

phenylalanine, a typical carboxypeptidase substrate (7). However, it hydrolyzed casein and hemoglobin very rapidly (4).

In summary, evidence is presented to show clearly that a B. subtilis protease is a zinc enzyme which probably contains one gram atom of zinc per mole of enzyme. The enzyme is extremely active and should be useful in protein structural studies. The demonstration that this endopeptidase is a zinc enzyme makes it possible to compare the structure function relationships of two zinc enzymes, namely carboxypeptidase which is an exopeptidase (8) and the B. subtilis neutral protease which is an endopeptidase.

REFERENCES

1. Hagihara, B., in P. D. Boyer, H. Lardy and K. Myrback (Editors), The Enzymes, Vol. 4, Academic Press, Inc., New York (1958), p. 193.
2. Fukumoto, J., Yamamoto, T., and Ichikawa, K., J. Agr. Chem. Soc. Japan, 31, 331 (1957); 32, 233 (1958).
3. Lamy, F., Craig, C. P., and Tauber, S., J. Biol. Chem., 236, 86 (1961).
4. McConn, J. D., Tsuru, D., and Yasunobu, K. T., J. Biol. Chem., submitted.
5. Hagihara, B., Matsubara, H., Nakai, M., and Okunuki, K., J. Biochem., 45, 185 (1958).
6. Smith, E. L., and Spackman, D. H., J. Biol. Chem., 212, 271 (1955).
7. Neurath, H., and Schwert, G. W., Chem. Revs., 46, 69 (1950).
8. Vallee, B. L., and Neurath, H., J. Biol. Chem., 217, 253 (1955).