Physicochemical Properties of the Native, Zinc- and Manganese-Prepared Metalloprotease of *Bacillus polymyxa*

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The neutral protease of *Bacillus polymyxa* had a broad pH optimum (6.0 to 7.2) for activity at 37°C. The enzyme was most stable at pH 5.6 to 5.8. The protease had an optimum temperature of 37°C and was quite thermostable up to 35°C, but at higher temperatures the stability decreased rapidly. The substrate specificity of the protease was similar to that of the neutral proteases of other members of the genus *Bacillus*. The enzyme was shown to be a zinc metalloprotease. However, manganese ions had a greater activating and stabilizing influence on the activity of this enzyme than zinc. Replacement of zinc in the native enzyme by manganese resulted in a 50% increase in activity. In addition, the prepared manganese metalloprotease had higher temperature and more alkaline pH optima than the native enzyme.

Microbial metalloproteases all have pH optima around pH 7.0 and are sensitive to metal chelating agents such as ethylenediaminetetraacetic acid (EDTA) and o-phenanthroline, but are not inhibited by diisopropyl-fluorophosphate or thiol reagents. The molecular weights of these neutral proteases have been found to be in the range 35,000 to 45,000. The most extensively studied enzymes of this group are produced by *Bacillus subtilis* (7, 8), *Bacillus thermoproteolyticus* (7), *Bacillus megaterium* (6, 10), *Bacillus cereus* (3), *Streptomyces griseus* (14), *Aspergillus oryzae* (11), and *Serratia* (12).

Conditions relating to the production of a neutral protease by *Bacillus polymyxa* have been described (4). The enzyme was purified by ammonium sulfate fractionation, acetone precipitation, and gel filtration on Sephadex G-100 and behaved as a homogeneous entity on rechromatography on a Sephadex G-100 (2.5 by 80 cm) column and also when eluted from a CM-cellulose ion exchange column by a sodium chloride gradient (4). In this paper we report on some of the physicochemical properties of the enzyme. A number of metalloenzymes were prepared from the native enzyme, and some of their properties were studied. A preliminary account of this work has been recorded (5).

**MATERIALS AND METHODS**

**Enzyme and assay methods.** The protease and protein assays together with the method of production and purification of the enzyme have already been described (4).

**Reagents.** A range of peptides, peptide derivatives (8), and natural protein substrates were obtained from Sigma Chemical Co. (London). Hide powder was obtained from Calbiochem (Los Angeles, Calif.). Britton and Robinson's universal buffer is the acetate-phosphate-borate buffer described by Campbell (2). All reagents used were of "Analar" grade.

**Specificity of the protease towards natural substrates.** The natural substrates were added to 2.0 ml of 0.1 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer, pH 7.0, to give a final concentration of 1.0% (wt/vol) and then allowed to equilibrate for 5 min in a water bath at 37°C. 1.0 ml of the purified protease solution containing 4,000 U of activity was added, and the digestion was allowed to proceed for 2 h. The reaction was stopped (except in the case of gelatin) by adding an equal volume of 10% (wt/vol) trichloroacetic acid. In the case of gelatin, the reaction was stopped by adding an equal volume of 30% (wt/vol) trichloroacetic acid. After standing for 10 min, the precipitate was removed by filtering through a Whatman no. 4 filter paper. The degraded protein left in the filtrate was determined by the ninhydrin method as described by Moore and Stein (13) and expressed as a percentage of the total protein in the starting material. Blanks were prepared by adding heat-inactivated enzyme to the substrate after the addition of trichloroacetic acid. The percent of actual
hydrolysis was obtained by subtracting readings of blanks from those obtained with the enzymatically digested proteins.

**Specificity of the protease towards peptides and peptide derivatives.** A 15-mg amount of the substrate was added to 1.0 ml of 0.01 M Tris-hydrochloride buffer, pH 7.0, containing 10⁻⁴ M EDTA, for 3 h at 0 to 4 C, followed by dialysis against three changes of the buffer alone for a further 12-h period. Spectroscopic analysis of the apoenzyme showed that it contained less than 0.01 μg of Zn²⁺ per mg of protein. Mn²⁺ (as MnCl₂) was then added to the apoenzyme solution to a final concentration of 10⁻⁴ M. After 1 h at 0 to 4 C, this solution was then dialyzed against 0.01 M Tris-hydrochloride buffer, pH 7.0, for 6 h to remove any free Mn²⁺ from the protein solution. The external buffer system was changed twice during this period. The zinc enzyme was prepared in a similar manner, substituting 10⁻⁴ M zinc for the manganese. The manganese-prepared enzyme contained 1.14 μg of Mn²⁺ and 0.02 μg of Zn²⁺ per mg of protein. The zinc-prepared enzyme contained 1.01 μg of Zn²⁺ per mg of protein. Manganese was not detected in this preparation.

**RESULTS**

**Specificity towards natural substrates.** The degree of hydrolysis of natural substrates was measured by determining the amount of protein soluble in 5% (wt/vol) trichloroacetic acid (Table 1). Highest activity was detected towards casein and hide powder. No activity was detected towards collagen or elastin under the conditions used. Seventy percent of bovine albumin, but only 42% of egg albumin was converted to protein products soluble in 5% (wt/vol) trichloroacetic acid. When the specificity of the B. polymyxa protease was examined toward the synthetic substrates listed by McConn, Tsuru, and Yasunobu (8), only the dipeptide L-histidine-L-leucine was hydrolyzed.

**Effect of inhibitors, chelating agents, and metal ions on protease activity.** Metal chelating agents such as EDTA, o-phenanthroline, αα'-dipyridyl, citrate, and oxalate inhibited the protease to varying degrees (Table 2). The sulfhydryl reagents, iodoacetate and p-chloromercuribenzoate, showed no inhibitory effects. Similarly, reducing or oxidizing agents had no effect with the exception of the reducing agent, sodium thiosulfite, which caused a 70% reduction in activity.

At 10⁻⁴ M final concentration, Cu²⁺ had an inhibitory effect of 31% and zinc had a slight activating effect on protease activity (Table 3). Mn²⁺ and the other metals had no effect on

| Substance      | % solution in 5% (wt/vol) trichloroacetic acid |
|----------------|-----------------------------------------------|
| Fibrinogen     | 66                                            |
| Egg albumin    | 42                                            |
| Hemoglobin     | 69                                            |
| Fibrin         | 70                                            |
| Collagen       | 0                                             |
| Elastin        | 0                                             |
| Hide powder    | 82                                            |
| Gluten         | 59                                            |
| Casein         | 83                                            |
| Bovine albumin | 70                                            |
| Keratin        | 67                                            |
| Gelatin        | 73a                                           |

*Percent soluble in 30% (wt/vol) trichloroacetic acid.

**Table 2. Effect of various inhibitors on protease activity**

| Inhibitor                  | Final conc | Relative activity |
|----------------------------|------------|-------------------|
| None                       |            | 100               |
| EDTA                       | 1 x 10⁻⁴ M | 0                 |
| αα'-Dipyridyl              | 1 x 10⁻⁴ M | 80                |
| 34                         | 2 x 10⁻⁴ M | 34                |
| o-Phenanthroline           | 1 x 10⁻⁴ M | 0                 |
| Sodium oxalate             | 5 x 10⁻⁴ M | 93                |
| Sodium citrate             | 66         |                   |
| Sodium iodoacetate         | 100        |                   |
| Sodium p-chloromercuribenzoate | 100       |                   |
| L-Cysteine                 | 99         |                   |
| Potassium ferricyanide     | 100        |                   |
| Sodium thiosulfite         | 100        |                   |
| Iodine                     | 30         |                   |
| N-bromosuccinimide         | 100        |                   |

*The various inhibitors were added to the enzyme at the final concentrations shown. After standing the mixture at 37 C for 10 min the activity was determined in the usual way.
enzyme activity. Zn\(^{2+}\) at 10\(^{-3}\) M gave a 50% reduction in activity and at 10\(^{-2}\) M gave complete inhibition. Calcium had no effect at 10\(^{-3}\) M, but this ion gave a 60% reduction in activity when added at a final concentration of 10\(^{-2}\) M. In contrast to the other metals, Mn\(^{2+}\) caused a small increase (10%) in activity at a final concentration of 10\(^{-3}\) M but gave a 33% increase at 10\(^{-2}\) M.

When the activity of the enzyme was determined at 45 C in the presence and absence of various metal ions, calcium and cobalt were shown to stabilize the enzyme to a slight extent. Manganese had the greatest influence of the metals tested. Thus, at 10\(^{-3}\) M the Mn\(^{2+}\) prepared enzyme had an activity double that of a control to which no metal was added. At 10\(^{-3}\) M no significant effect on stabilizing the enzyme could be detected with Ba\(^{2+}\), Mg\(^{2+}\), Na\(^{+}\), and K\(^{+}\), while Zn\(^{2+}\) caused a small reduction in activity. The metallic composition of the B. polymyxa protease and the neutral protease of B. subtilis were similar. The level of zinc present in both enzymes was the same (Table 4). However, it is important to note that there is no manganese present in either enzyme.

Temperature and pH characteristics of the native protease and the manganese- and zinc-prepared enzymes. The native and zinc enzymes were most stable at pH 5.6 to 5.8, but the manganese enzyme was most stable at pH 6.0 (Fig. 1). Figure 2 shows that the optimum pH for activity of the native and zinc-prepared enzymes is identical in that both have broad plateaus ranging from 6.0 to 7.0. However, the manganese-prepared enzyme exhibited a much broader plateau for optimum pH and this ranged from pH 6.0 to 8.2. The optimum temperature of the manganese enzyme was 45 C as compared to 37 C for the native and zinc-prepared proteases (Fig. 3). It is interesting to record that the action patterns (i.e., the substrate specificity) of the manganese- and zinc-prepared enzymes were similar. It was observed, however, that when zinc was replaced by manganese in the native enzyme a 50% increase in activity resulted.

**DISCUSSION**

The neutral proteases of Bacillus species examined to date are metalloenzymes. B. megaterium (6), B. subtilis (8), B. cereus (3) and others are known to contain 1 g-atom of zinc per g-mol of purified enzyme. If the zinc component is removed by dialysis against chelating agents, such as EDTA, an inactive apoenzyme is obtained. This apoenzyme can be reactivated in full by the addition of zinc, and it may also be

| Treatment | Relative protease activity |
|-----------|---------------------------|
|           | 10\(^{-4}\) M | 10\(^{-3}\) M | 10\(^{-2}\) M |
| None      | 100       | 100       | 100       |
| Ca\(^{2+}\) | 40        | 100       | 100       |
| Mg\(^{2+}\) | 73        | 100       | 102       |
| Ba\(^{2+}\) | 40        | 94        | 97        |
| Co\(^{2+}\) | 83        | 100       | 100       |
| Mn\(^{2+}\) | 133       | 110       | 100       |
| Cu\(^{2+}\) | 0         | 20        | 79        |
| Zn\(^{2+}\) | 0         | 50        | 107       |
| Hg\(^{2+}\) | 0         | 0         |            |
| Fe\(^{3+}\) | 0         | 0         |            |
| Na\(^{+}\) | 80        | 100       | 100       |
| K\(^{+}\)  | 73        | 100       | 100       |
| EDTA      | 0         | 0         | 0         |

*The various metals and EDTA were added to the enzyme solution at the final concentrations shown; after incubation for 10 min at 37 C the enzyme activity was determined.

*Final concentrations of metal.

**TABLE 3. Effect of metals and EDTA on protease activity**

| Enzyme source | Metal (μg/mg protein) |
|---------------|-----------------------|
|               | Calcium | Zinc | Manganese |
| **B. polymyxa** | 12.5   | 1.0  | 0         |
| **B. subtilis (dialyzed)** | 14.5  | 1.0  | 0         |
| **B. subtilis** | 15.5   | 1.5  | 0         |

*For comparative purposes the neutral protease of B. subtilis (Sigma Chemical Co.) was also analyzed.

![Fig. 1. Influence of pH on the stability of the B. polymyxa native, zinc-, and manganese-prepared enzymes. The influence of pH on the stability of the protease was determined by holding the enzyme solutions in 0.01 M universal buffer at the different pH values for 1 h at 37 C. The residual activity was subsequently measured at pH 7.0.](image)
reactivated to varying degrees by other divalent metals, such as cobalt and manganese (3, 8). Calcium stabilized the activity of the B. subtilis neutral protease considerably (8). Strontium, magnesium, manganese, and barium also increased the stability of the enzyme. McConn, Tsuru, and Yasunobu (8) found that manganese was absent from the neutral protease of B. subtilis. These workers showed that the apoenzyme could be reactivated by manganese. However, the reactivation was only 50% of that obtained with zinc. Manganese has a much greater activating effect in the case of B. polymyxa protease than on any of the other neutral proteases of the Bacilli.

The identical pH and temperature characteristics of the native and zinc-prepared proteases are consistent with the results obtained on the spectroscopic analyses of the enzyme, i.e., the native protease is a zinc metalloenzyme.

As well as having pronounced effects on the temperature, activity, stability, and pH characteristics of the neutral protease, manganese could prove to be a useful tool in the elucidation of the nature, structure, and properties of the active site of the neutral protease of B. polymyxa due to the important magnetic properties of this cation.

The broad plateau of pH activity exhibited by the B. polymyxa protease is similar to that of thermolysin (7). However, this is in contrast to the sharp peaks of 7.3 observed for B. megaterium (6) and 7.0 to 7.2 for B. cereus (3). Calcium was detected in the B. polymyxa protease on spectroscopic analysis. This cation is believed to play a role in maintaining the structural configuration of some enzymes, particularly at elevated temperatures. It is also believed to prevent enzyme autolysis. One could envisage such a function for this cation in relation to the protease described here.

B. polymyxa protease caused considerable degradation of hide powder (82%), but failed to degrade collagen. The hide powder (fine grind) used in this investigation was obtained from Calbiochem and the collagen (from bovine achilles tendon) was obtained from Sigma Chemical Co. The apparent discrepancy observed here could be due to the effect of the extraction procedure and to variation in the composition and structure of these materials. It is more likely, however, that these differences may be due to the fact that collagen from different sources can vary considerably in composition and physical structure (1, 9, 15).

The hydrolysis of synthetic substrates by the protease is in agreement with the results of Keay and Wildi (7). However, in contrast to the results of McConn, Tsuru, and Yasunobu (8), N-CBZ-glycinephenylalanine was not hydrolyzed. Keay and Wildi (7) explained the result obtained by these workers as being probably due to the presence of a small amount of alkaline protease as a contaminant in the digestion mixture.

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