Note

Fe²⁺ Inhibition of Extracellular Protease Synthesis in *Aeromonas hydrophila*

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Received July 30, 1984

Iron is known to play a dual role in microbial metabolism, serving as an essential micronutrient as well as an inhibitory agent for some vital processes, it represses phenolic acid synthesizing enzymes in *Bacillus subtilis*,¹⁾ pigment formation in *Pseudomonas aeruginosa*,²⁾ outer membrane proteins synthesis in *Escherichia coli*³⁾ and synthesis of diphtheria toxin in *Corynebacterium diphtheriae*.⁴⁾

Secretion of extracellular proteases by contaminant microorganisms is detrimental to the keeping qualities of flesh foods, since these enzymes produced by psychrotrophic organisms could act on muscle even during refrigerated storage.⁵⁾ It was, therefore, of interest to study the influence of endogenous nutritional factors present in muscle on extracellular protease synthesis by spoilage organisms. *Aeromonas hydrophila* has been recognized as an active spoiler⁶⁾ as well as an enteric pathogen.⁷⁾ We recently observed that extracellular protease synthesis by the bacterium was supported by several individual amino acids while the enzyme was repressed by ammonium and glutamine.⁸⁾ The present report pertains to inhibition of protease synthesis in *A. hydrophila* by iron.

Aeromonas hydrophila, isolated from Indian mackerel (*Rastrelliger kanagurta*), was used for the experiment. The culture was maintained on Nutrient Agar (Difco) slants. An active culture of the bacterium was grown aerobically at 28°C in 0.8% Nutrient Broth (Difco) on a rotary shaker. The culture (18 h) was inoculated at the 0.5% level in 500 ml Erlenmeyer flasks containing 250 ml of the 0.8% Nutrient Broth. The flasks were incubated at 28°C on a rotary shaker for 24 hr. The cultures were centrifuged at 5,920 × g for 15 min. The cells were washed once with 100 mM phosphate buffer (pH 7.5).

The cell pellet was suspended in 10 mM phosphate buffer (pH 7.5), to obtain turbidity equivalent to a Klett Summerson spectrophotometer reading of 550 nm.

This cell suspension was used for protease synthesis studies as described by Boethling.⁹⁾ The cell suspension was diluted with an equal volume of 0.4% yeast extract

(Difco) and incubated at 28°C on a rotary shaker. The diluted cell suspension had a Klett reading of 450. Aliquots of the cell suspension were withdrawn at regular intervals, centrifuged at 5,920 × g for 15 min and then the protease activity of the cell free supernatant was determined using hemoglobin (Difco) as substrate. The assay system contained 1.5 ml of 100 mM Tris-HCl buffer (pH 8.5), 12.5 mg of hemoglobin and 0.5 ml of cell free supernatant in a total volume of 2.0 ml. The assay system was incubated at 50°C for 30 min and the reaction was stopped by the addition of 1 ml of 20% TCA. The mixture was filtered through Whatman No. 1 filter paper and tyrosine was determined.¹⁰⁾ One unit of protease activity was expressed as the μmol tyrosine formed during 30 min per mg cell nitrogen. Cell nitrogen was determined by Nesslerisation.¹¹⁾

Aeromonas hydrophila was found to produce two types of extracellular proteases differing in molecular weight and certain other properties but having pH optima of 8.5 to 9.0 and temperature optima of 45 to 50°C. Protease activity was therefore monitored at 50°C and pH 8.5.

Figure 1 shows the secretion of protease during in-

FIG. 1. Effect of Iron on Secretion of Protease by *A. hydrophila*.

FeSO₄ concentrations: ●, 0 mM; ○, 1 mM; △, 2 mM; ▲, 5 mM.

FIG. 2. Effect of Varying Concentrations of Iron on Secretion (●—●) and *in Vitro* Inhibition (●---●) of *A. hydrophila* Protease.

cubation of resting cells in dilute yeast extract. Secretion of the enzyme by the cells increased up to an incubation time of 3 hr. The Klett reading of the cell suspension was 450 throughout the incubation period indicating there was no increase in cell growth. Incorporation of ferrous sulfate in the secretion system resulted in a decreased amount of protease in the supernatant. An about 40% decrease in enzyme yield was observed at a concentration of iron as low as 1 mM. The effect of the metal on the enzyme yield could be due to the effect of the cation on enzyme synthesis and secretion, or due to its *in vitro* inhibition of the enzyme. This possibility was examined by determining the activity of the cell free supernatant in the presence of Fe^{2+} . The results are shown in Fig. 2. It can be seen that at 1 to 5 mM iron, the extent of inhibition of secretion was much more pronounced than the *in vitro* inhibition caused by equivalent amounts of iron. Incorporation of FeCl_3 in the cell system at a concentration of 2 mM did not affect the enzyme yield suggesting that the above inhibitory effect was specific to Fe^{2+} . Other divalent metals like Ca^{2+} , Mg^{2+} and Mn^{2+} at concentrations of 2 mM each had no effect on the enzyme secretion.

The requirement of certain cations like Mg^{2+} and Mn^{2+} on the synthesis of extracellular proteins has been recently reported.^{12,13} The inhibitory effect of Fe^{2+} on secretion of toxin in *Corynebacterium diphtheriae* has been reported.⁴ The role of this cation in influencing certain cellular morphological properties has also been suggested.⁴ Neilands¹⁵ has proposed a scheme for iron assimilation and its influence on protein synthesis. Klebba *et al.*³ showed that iron deprivation induced five proteins in the outer membrane of *E. coli* whereas these proteins were repressed when iron was available to the bacterium. It has been well established that extracellular proteins including the diphtheria toxin are synthesized on membrane bound ribosomes and secreted cotranslationally.¹⁶ As proposed for *C. diphtheria* toxin production,¹⁷ it is likely in the present situation also that Fe^{2+} influences the synthesis of the protease. The inhibitory effect of iron on protease synthesis could indirectly play a significant role in food

preservation since iron is one of the key microelements present in flesh foods. Recently, protease secretion by the bacterium has also been shown to be inhibited by certain food preservatives.¹⁸

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