An Inducible Ferrichrome A–degrading Peptidase from *Pseudomonas* FC-1*

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ABSTRACT: Growth of *Pseudomonas* FC-1 on ferrichrome A or exposure of the cells to this cyclic peptide induced the formation of an alkaline peptidase. Enzyme induction, which was inhibited by chloramphenicol, could not be obtained with general proteinaceous substrates such as peptone. The peptidase opens the ring of ferrichrome A at an acyl serine bond. The product, a linear hexapeptide, was isolated and characterized. An assay for the peptidase was developed, based on periodate oxidation of the newly formed N-terminal serine residue, followed by determination of formaldehyde via chromotropic acid. The enzyme, which is intracellular, was isolated in soluble form from ferrichrome A–grown cells by treatment of an acetone powder with snake venom in slightly alkaline medium. A 40-fold purification was achieved by ammonium sulfate precipitation and gel filtration on Sephadex G-100. The purified enzyme was neither strongly activated nor inhibited by a series of divalent metal ions; sulfhydryl reagents did not destroy the activity. The *p*H optimum was about 9.0.

Enzymes which hydrolyze cyclic peptides are of special biochemical interest. Cyclic peptides are known to be quite resistant to the common plant and animal proteolytic enzymes (Bodanszky and Perlman, 1964), and the existence of enzymes capable of attacking such peptides is of obvious significance in microbial metabolism. In addition, these enzymes afford a tool for structure determination in peptides which are especially difficult to characterize by chemical methods.

In a previous report we demonstrated that a soil isolate, designated *Pseudomonas* FC-1, is capable of growth on the ferrichrome compounds (Figure 1) as sole source of carbon and nitrogen (Warren and Neilands, 1964). The organism appears to execute its initial attack by means of a peptidase which opens the ring, in the case of ferrichrome A, at the nitrogen atom of the "first" serine residue (Warren and Neilands, 1965). In this paper we described the assay, induction, isolation, and partial characterization of the enzyme. A subsequent communication will deal with the substrate specificity and other properties of *Pseudomonas* FC-1 peptidase.

Materials and Methods

Pseudomonas FC-1. The liquid inoculum was grown with vigorous aeration at 30° in the Barnett and Ingram (1955) medium supplemented with 0.2% each of glucose and ammonium sulfate. Cultures were stored at 5° following growth on the same medium with agar added at the level of 1.5%.

Substrate. Ferrichrome A was crystallized from *Ustilage sphaerogena* fermentations according to published procedures (Garibaldi and Neilands, 1955).

Other Materials. Chemicals used were of the highest quality commercially available. The *Crotalus adamanteus* venom was purchased from the Ross Allen Reptile Institute, Silver Springs, Fla.

Analytical. Colorimetric analyses were performed with the Bausch and Lomb Spectronic 20; for more accurate measurements the Gilford-Beckman spectrophotometer was employed. Protein was measured by the method of Lowry *et al.* (1951). Paper electrophoresis was conducted with the apparatus of Crestfield and Allen (1955).

Peptidase Activity. Gross qualitative evidence for cleavage of the peptide ring was obtained through paper electrophoretic analysis at extremes of *p*H (Figure 2). Further qualitative evidence for degradation of the polyhydroxamate center of ferrichrome A was obtained by taking advantage of the diminished absorbancy coefficient and red spectral shift displayed by ferric monohydroxamates as the *p*H is depressed from neutrality to more acidic values (Warren and Neilands, 1964); this assay was used in experiments dealing with induction of the peptidase. A quantitative assay for peptidase action on ferrichrome A was developed and was used as a basis for isolation of the enzyme (*vide infra*).

One unit of enzyme is defined as that amount which forms 1 mμmole of product per minute under the assay conditions employed. Specific activity is expressed as units per mg protein.

Experimental and Results

Quantitative Assay. In order to isolate the enzyme it was first necessary to develop a specific assay. Although whole cells of *Pseudomonas* FC-1 attack ferrichrome and a number of other cyclic polypeptides such

* From the Department of Biochemistry, University of California, Berkeley. *Received February 18, 1965.* Supported in part by grants from the Office of Naval Research (222-939) and the U.S. Public Health Service (E-4156).

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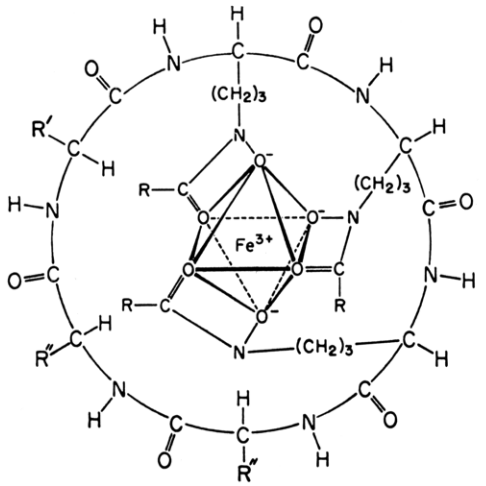

FIGURE 1: Structures of the ferrichromes. (Emery and Nielands, 1961; Rogers *et al.*, 1963; Rogers and Neilands, 1964; Zalkin *et al.*, 1964). Ferrichrome A: R = $\begin{matrix}\text{HOOC}\cdot\text{CH}_2\\ \text{H}_3\text{C}\end{matrix}\!>\!\text{C}\!=\!\text{C}\!<^{\text{H}}$; R′ = H—; R″ = HOCH_2—. Ferrichrome: R = CH_3—; R′ = H—; R″ = H.

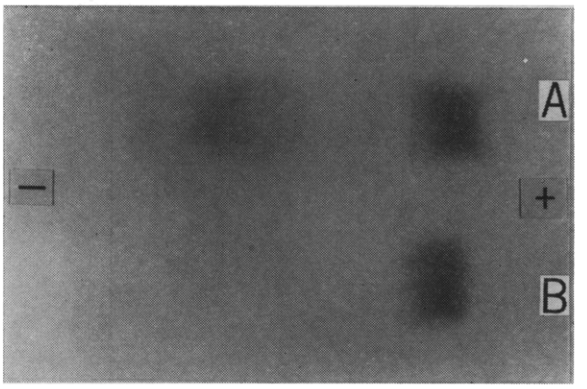

FIGURE 2: Paper electrophoretic demonstration of the action of *Pseudomonas* FC-1 peptidase on ferrichrome A. A 2-μmole sample of ferrichrome A dissolved in 0.2 ml of 0.05 M Veronal–0.01 M $MgCl_2$ buffer, *p*H 8.5, was incubated at 30° with 0.2 ml of 0.1 M $NaHCO_3$–0.2 M NaCl containing 3 units of enzyme which had been purified through Sephadex G-100. The blank consisted of 0.2 ml of the enzyme buffer added to an equivalent amount of ferrichrome A. At the end of 2 hours 10 μl of the experimental (A) and blank (B) preparations were analyzed by electrophoresis in 4% formic acid on Whatman No. 1 paper at 1200 v and 25 ma for 30 minutes. Ferrichrome A remained at the origin while the split product migrated about 5 cm to the cathode.

as circulin and polymixin, ferrichrome A was selected as the substrate of choice since (a) it is more readily available to us than ferrichrome or the other substrates, (b) it is the preferred growth substrate for *Pseudomonas* FC-1, and (c) the product of the reaction, a linear hexapeptide with serine in the N-terminal position, is amenable to a relatively specific analytical technique. The last involves periodate oxidation of the N-terminal serine residue followed by the determination of the liberated formaldehyde with the very sensitive chromotropic acid reagent. The method adopted was essentially the same as that devised by Frisell *et al.* (1954).

A test tube was charged with 0.9 ml of 0.05 M Veronal–0.01 M $MgCl_2$ buffer, *p*H 8.5, containing 2.0 μmoles recrystallized ferrichrome A (trisodium salt). The tube was then placed in a water bath at 30° and the suitably diluted enzyme dissolved in 0.1 M $NaHCO_3$–0.2 M NaCl was introduced. At zero time and at intervals of 20, 40, and 60 minutes 0.1-ml aliquots of the incubation mixture were withdrawn and placed in a tube which was held in a boiling-water bath for 5 minutes. Blanks consisting of enzyme and substrate alone were included. At low levels of enzyme the rate remained zero order up to 60 minutes.

The analysis for N-terminal serine was begun by adding 0.1 ml of *p*H 8.5 0.05 M Veronal buffer and 0.05 ml of 0.075 M sodium metaperiodate to each tube. After 10 minutes a drop of dilute methyl red indicator solution and 0.05 ml of 10% $NaHSO_3$ solution were added. If the reaction was not acidic to the indicator, a small amount of 1% trichloroacetic acid was introduced. Following the addition of 2.5 ml of chromotropic acid reagent the tubes were placed in a boiling-water bath for 45 minutes. The tubes were then cooled, 0.3 ml of saturated thiourea solution was added, the volume was diluted to 3.3 ml, and the absorbancy was measured at 570 mμ. Three aliquots of a standard solution of serine to provide 30, 90, and 150 mμmoles of the amino acid were analyzed simultaneously with the unknown samples. The reported millimolar absorbancy coefficient of 16 was confirmed (Frisell *et al.*, 1954). Figure 3 illustrates the enzyme-time-product relationship obtained with this assay.

Verification of the Quantitative Assay. A number of control experiments were performed in order to check the reliability of the method. The blank with either ferrichrome A or deferri-ferrichrome A incubated alone was consistently found to be zero. Incubation of ferrichrome A with the *Crotalus adamanteus* venom, as used in the isolation of the enzyme, did not give formaldehyde-yielding material on periodate oxidation nor did it give rise to the split product shown in Figure 2. In crude extracts blanks of up to 30% were encountered but these were reduced essentially to zero after ammonium sulfate precipitation or dialysis. Since even intact cells do not produce formaldehyde from ferrichrome A unless the periodate-oxidation step is included, the assay appears to be quite specific for the ring-opening peptidase.

The following experiment was performed in order to examine the stoichiometric relationship between the appearance of the split product (Figure 2) and the liberation of N-terminal serine. A 0.2-ml volume of enzyme solution containing 6 units of activity (purified through

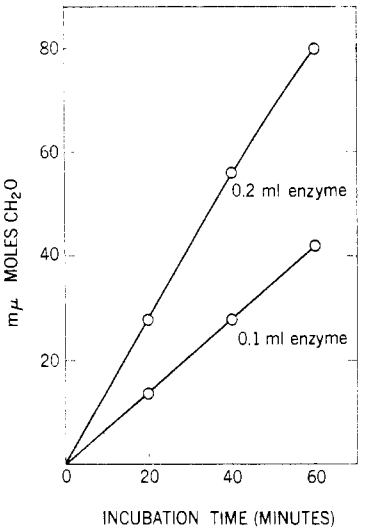

FIGURE 3: Enzyme-time-product relationship for *Pseudomonas* FC-1 peptidase. The assay is based on the determination of formaldehyde liberated by periodate oxidation of the initial product, a serine N-terminal peptide. Blanks with enzyme alone or substrate alone gave zero net reaction. Conditions given in the text.

the ammonium sulfate stage) was incubated with 0.9 ml of Veronal buffer, *p*H 8.5, containing 2 μmoles of ferrichrome A. Analysis for amino-terminal serine in the usual way indicated 20% hydrolysis after 1 hour at 30°. At that time a further 0.1-ml aliquot of the reaction mixture was spread across a strip of Whatman No. 1 paper and an electrophoretic separation carried out for 30 minutes at 1000 v and 25 ma in 4% formic acid. Under these conditions the cyclic peptide remained at the origin while the linear peptide, having now a positively charged group, moved some 5 cm to the cathode (see Figure 2). The two colored zones were eluted with 0.02 M phosphate buffer, *p*H 7.0, and the absorbancies were read in 1-ml cuvets at 440 mμ in the Gilford-Beckman spectrophotometer. The values found were 0.423 and 0.110 cm^{-1} for the cyclic and linear peptides, respectively. Assuming equivalence of absorbancy these data indicate 21% hydrolysis, a value in excellent agreement with the analysis for N-terminal serine.

Product of Hydrolysis of Ferrichrome A. A 22-mg (20 μmoles) sample of recrystallized ferrichrome A was dissolved in 0.5 ml of 0.05 M Veronal–0.01 M $MgCl_2$ buffer. The *p*H was readjusted to 8.5 by addition of 60 μmoles of NaOH. After the introduction of 0.5 ml of enzyme purified through the ammonium sulfate stage and containing about 50 units of activity, the reaction mixture was incubated at 30° for 4 hours. The solution was then lyophilized, the residue was dissolved in a few drops of water, and the entire volume was streaked across a sheet of Whatman 3MM paper impregnated with 0.05 M ammonium acetate buffer, *p*H 9.0. Electrophoretic separation was carried out at 25 ma and 1000 v for several hours. Under these conditions the split product, still carrying the iron atom, moves to the anode as a colored band traveling just ahead of the residual ferrichrome A. This can be taken as presumptive evidence for the presence of an additional carboxyl group per mole of the former compound. Serine and other neutral amino acids remain near the origin.

Elution of the split product with water afforded 2.5 ml of solution with an absorbancy of 5.4 cm^{-1} at 440 mμ corresponding to 1.4 μmoles/ml (assumed millimolar absorbancy coefficient equivalent to that of ferrichrome A, i.e., 3.74). Serine analysis by periodate oxidation of an aliquot gave 1.5 μmoles/ml. The remainder, about 1.0 μmole, was allowed to stand for 24 hours after admixture with an equal volume of methanol containing 50 mg of 8-hydroxyquinoline. The solvent was then removed, and the residue was suspended in a 1:1 mixture of chloroform-water and repeatedly extracted with chloroform until the excess 8-hydroxyquinoline and its ferric coordination compound had been completely removed. The iron-free peptide solution was evaporated to dryness, and the residue was dissolved in 1 ml of Eastman 95% hydrazine and sealed in a test tube. Hydrazinolysis was performed at 100° for 10 hours. After removal of the excess hydrazine in a desiccator, analysis by paper electrophoresis in 4% formic acid showed ornithine to be the sole free amino acid present (*N*-δ-hydroxyornithine is reduced to ornithine during the hydrazinolysis procedure: Warren and Neilands, 1964).

A 0.25-ml aliquot of a solution of the iron-free peptide, containing about 0.8 μmole/ml and prepared as described, was hydrolyzed with an equal volume of 12 N HCl at 100° for 16 hours in a sealed tube. After removal of the excess HCl an electrophoretic analysis in 4% formic acid revealed the presence of serine, glycine, and *N*-δ-hydroxyornithine in what appeared to be a ratio of 2:1:3 (ninhydrin spray). Through the courtesy of Dr. Roger D. Cole, the remainder of the preparation was analyzed using the automatic amino acid analyzer (Spackman *et al.*, 1958) and was found to contain 0.234 μmole serine and 0.158 μmole glycine. A further 0.25-ml aliquot of the original peptide solution (about 0.2 μmole) was treated with fluorodinitrobenzene for amino end-group determination (Sanger and Thompson, 1953). The hydrolysate, upon analysis by descending paper chromatography in 1.5 M phosphate buffer, *p*H 6.0, revealed the presence of a component with R_F of 0.47 (authentic dinitrophenylserine, 0.45; dinitrophenylglycine, 0.32).

Together with the information adduced earlier (Warren and Neilands, 1965), and in spite of the inherent difficulties in working with serine-containing peptides, these data strongly suggest the structure of the split product to be the ferric complex of $(seryl)_2$-glycyl-$(N$-δ-acylhydroxyornithyl$)_3$.

Evidence for Induction. Pseudomonas FC-1 was grown for 24 hours as given previously. The cells were collected in a Sharples centrifuge and suspended in 0.05 M phosphate buffer, *p*H 7.0. The suspension was divided into equal portions, designated A and B, and spun at high speed in the Sorvall RC-2 centrifuge. About 5 ml of packed cells was obtained in each tube. The cells in tube

A were suspended in 50 ml of 0.05 M phosphate buffer containing 100 μmoles of ferrichrome A and shaken at 30° for 18 hours. Cells in tube B were incubated under similar conditions in the absence of ferrichrome A. At the end of the incubation period the cells were centrifuged, washed, suspended in 50 ml of buffer containing 100 μmoles of ferrichrome A, and shaken at 30°. The rates of digestion, as recorded in Figure 4, demonstrate the superior capacity of induced cells to attack ferrichrome A.

The remaining aliquots of the incubation mixtures were centrifuged to remove the cells. The supernatant solution was then concentrated to a very small volume under reduced pressure and examined for ninhydrin-positive components by paper chromatography in 1-butanol–acetic acid–water (63:10:27). In tube B material no ninhydrin-positive spots appeared until after the 15th hour of incubation, while in the product from tube A such spots became visible after 1 hour and were very intense after 2 or 3 hours.

The above results were confirmed by use of chloramphenicol, an inhibitor of protein synthesis. A 100-ml batch of 24-hour culture of *Pseudomonas* FC-1 grown on glucose-salts medium was centrifuged and the packed cells (about 0.5 ml) suspended in 10 ml of phosphate buffer, *p*H 7.0. Exactly 5 ml of the cell suspension was placed in each of two Erlenmeyer flasks and 20 μmoles of trisodium ferrichrome A salt was added to each flask. To one flask was added 2 mg of chloramphenicol; both flasks were made up to 10 ml with phosphate buffer and shaken at 30° for 20 hours. After clarification by centrifugation a 0.2-ml aliquot from each mixture was diluted with 3.8 ml of *p*H 2.0 maleate buffer and the absorbancy was measured at 440 mμ. A control without cells and the supernatant containing chloramphenicol both gave an absorbancy of 0.370 cm^{-1} while the experimental solution gave a value of 0.122 cm^{-1}. A control in which chloramphenicol had been added to preinduced cells demonstrated that the antibiotic did not inhibit the activity of the peptidase.

Purification Procedure. ACETONE POWDER. *Pseudomonas* FC-1 was cultured in 5-liter volumes in the salts medium of Barnett and Ingram (1955) supplemented with 0.2% ferrichrome A. After incubation for 18–20 hours at 30° on the reciprocal shaker the cells were collected by centrifugation in the cold and washed with cold distilled water. Prolonged incubation for 2 or 3 days yielded cells which were less active. The pellet was homogenized in 300 ml of cold acetone and allowed to stand at 4° for 6 hours; the supernatant was then decanted and replaced by cold acetone. This procedure was repeated two more times in the course of 24 hours. The solid was collected on a Buchner funnel and dried over $CaCl_2$ in a desiccator at 4°. The powder, which weighed about 2 g, was stored over $CaCl_2$ in the deep freeze. An activity assay indicated 1.8 units/mg powder.

As an alternative to growing the organism on ferrichrome A it was found possible to obtain active preparations by inducing glucose-grown cells with ferrichrome A (see Figure 4). In this case, however, the peptidase could not be extracted by any of the methods used in this work (*vide infra*).

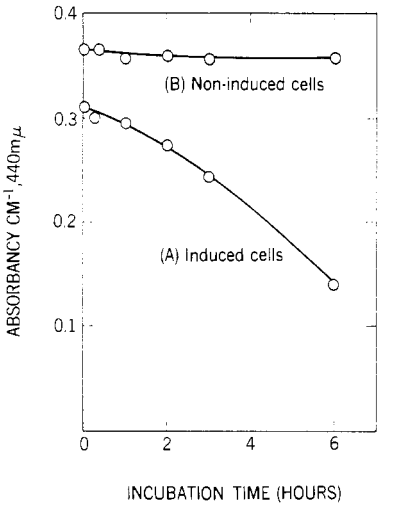

FIGURE 4: Rate of digestion of ferrichrome A by induced and noninduced cells of *Pseudomonas* FC-1. Induced cells (A) and noninduced cells (B), prepared as described in the text, were shaken at 30° in the presence of 2 μmoles of ferrichrome A per ml in 0.05 M phosphate buffer, *p*H 7.0. At various time intervals 0.2-ml aliquots were centrifuged to remove the cells, the supernatant was mixed with 3.8 ml of 0.1 M maleate buffer, *p*H 2.0, and the absorbancy was read at 440 mμ.

CRUDE EXTRACT. The enzyme is strictly intracellular and it could not be extracted from the acetone-dried cells by autolysis at room temperature, shaking with butanol, sonication, repeated freezing and thawing, digestion with bacterial proteinase, or treatment with lysozyme. Finally, it was discovered that the activity could be efficiently brought into solution by extraction in slightly alkaline media, especially after addition of the venom of *Crotalus adamanteus*.

Exactly 300 mg of acetone powder was dispersed in 10 ml of cold glass-distilled water, stirred for 3 hours at 4°, and centrifuged at 16,000 rpm for 30 minutes. The supernatant, which contains a substantial amount of protein and formaldehyde-yielding material but no peptidase, was discarded and the residue was suspended in 10 ml of 0.1 M $NaHCO_3$–0.2 M NaCl. After the addition of 15 mg of *C. adamanteus* venom the mixture was shaken at 30° for 1 hour and then stirred overnight at 4°. Centrifugation at 16,000 rpm for 30 minutes yielded a clear supernatant which contained 416 units of enzyme with a specific activity of 14.

Alkaline *p*H is to be preferred for enzyme extraction. At neutral or acidic *p*H the enzyme is either inactivated or is not brought into solution. The extracted enzyme is in true solution since the activity is not sedimented by centrifugation at 40,000 rpm for 1 hour.

AMMONIUM SULFATE PRECIPITATION. To 10 ml of the crude extract was added enzyme-grade ammonium sulfate to 60% saturation. The *p*H was maintained at

TABLE I: Summary of Isolation Procedure for Pseudomonas FC-1 Peptidase.

Stage	Volume (ml)	Activity (units/ml)	Yield (total units)	Protein[a] (mg/ml)	Specific Activity (units/mg)
Acetone powder (300 mg)			554		1.8[b]
Crude extract	10.8	38.5	416	2.8	14
Ammonium sulfate precipitate	2.3	101.3	233	4.6	22
Effluent from Sephadex G-100	5.0	37.8	189	0.5	76

[a] Determined according to Lowry *et al.* (1951). [b] Per mg acetone powder.

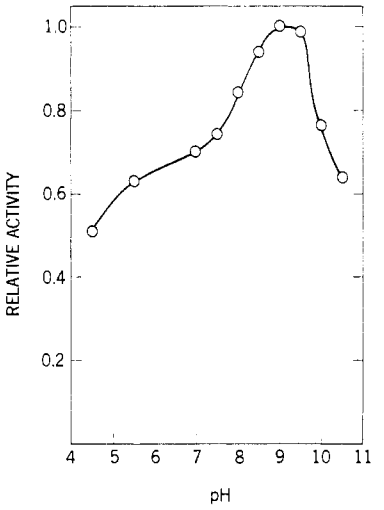

FIGURE 5: The *p*H-activity curve for *Pseudomonas* FC-1 peptidase. The enzyme from dialyzed crude extract was used in conjunction with the regular (serine) assay medium in 0.05 M acetate, Veronal, and glycine buffers at *p*H values of 4.5–5.5, 7.0–9.0, and 9.0–10.5, respectively.

7.6 with dilute ammonium hydroxide and the temperature at 4° during the salt addition. The precipitate was collected 30 minutes later at 16,000 rpm, suspended in a small volume of 0.1 M $NaHCO_3$–0.2 M NaCl, and centrifuged to yield 2.3 ml of enzyme solution containing 233 units with a specific activity of 22.

GEL FILTRATION. The entire 2.3-ml volume of enzyme solution, minus trace amounts removed for analysis, was placed on a 1.3- × 7.2-cm column of Sephadex G-100 which had a holdup volume of 3.0 ml. The column was previously equilibrated with 0.1 M $NaHCO_3$–0.2 M NaCl at 4°. The major portion of the activity appeared in the second holdup volume, yielding 5.0 ml of solution containing 189 units with a specific activity of 76. Elution with phosphate buffer led to extensive loss of enzyme.

MOVING-BOUNDARY ELECTROPHORESIS. In one experiment a pooled batch of enzyme which had been passed through Sephadex G-100 was concentrated by precipitation with 60% saturated ammonium sulfate. After dialysis for 16 hours against 0.06 M NaCl–0.04 M Veronal buffer, *p*H 8.2, in the cold, the specific activity was measured and found to be 78. The preparation was then subjected to electrophoretic analysis on the Model 38A Perkin-Elmer apparatus using the 2-ml cell. After 3 hours at a power level of 2 watts two major components appeared, both of which were moving to the anode. A portion of the slower-moving component was extracted from the cathode limb by means of a traveling syringe and found to have a specific activity of 163. The central compartment was then displaced so that the material in the anode limb could be recovered. Analysis indicated the latter to have a specific activity of 56. Table I shows a summary of the isolation procedure.

Properties. MOLECULAR. An insufficient amount of the highly purified enzyme has thus far been obtained to allow charting of the molecular constants. The fact that it passes so readily through Sephadex G-100 would indicate that the molecular weight is scarcely less than 100,000. Also, the very slow migration to the anode at *p*H 8.2 suggests that the isoelectric point is on the alkaline side of neutrality.

CATALYTIC. (1) Effect of *p*H. Figure 5 illustrates the *p*H versus activity profile for *Pseudomonas* FC-1 peptidase. Since the activity at *p*H 9.0 was essentially the same in both Veronal and glycine buffers the negative slope above this value cannot be attributed to inhibition by glycine anion.

(2) Activators and inhibitors. A dialyzed preparation of the crude extract was used to test the effect of added metal ions, chelating agents, and sulfhydryl reagents on the enzyme activity. Of the divalent metal ions tested, Zn^{2+}, Mn^{2+}, Co^{2+}, and Cd^{2+} gave weak inhibition (about 50%) at a final concentration of 10^{-3} M. At this concentration Ca^{2+} and Fe^{2+} showed little effect while Mg^{2+} gave a slight stimulation (about 10%). Ethylenediaminetetraacetate at 5×10^{-3} M concentration and iodoacetate, iodoacetamide and *p*-mercuriphenylsulfonate, each at a level of 10^{-3} M, did not produce significant changes in activity.

(3) Stability. The enzyme retains essentially full activity for at least several days at 4° in the buffers used in this work. There is a gradual loss of activity upon storage in the frozen state in 0.1 M $NaHCO_3$–0.2 M NaCl.

The acetone powder maintains what appears to be full potency over several months in the deep freeze, and this is the recommended procedure for storage.

Discussion

In the assay of *Pseudomonas* FC-1 peptidase a buffer of *p*H 8.5 was employed. This is sufficiently close to the optimum while, at the same time, it avoids the possibility of excessive alkaline denaturation. In view of the rather high *p*H optimum, the peptidase certainly could be assayed by direct and continuous monitoring in a *p*H-stat. Such an assay, however, is less sensitive than the one used here, and it would provide no information on the nature of the bond broken in ferrichrome A. Of the discontinuous methods, the quantitative ninhydrin and N-terminal serine assays are both very sensitive, but the former again suffers from a lack of specificity. The loss of absorbancy at 440 mμ at low *p*H has not been developed as a quantitative method of assay because of the lack of both sensitivity and specificity.

In contrast to the insignificant rate of reaction of periodate with intact ferrichrome A, the linear peptide produced by the petidase is quite rapidly degraded at the iron-binding center as evidenced by the loss of yellow color. While periodate is consumed in this particular reaction, no formaldehyde is produced, and in the amino-terminal serine oxidation assay described it is necessary only to ensure that a sufficient excess of the oxidizing agent has been added. Tris buffer cannot be used in this assay because of its reaction with periodate.

The main possibility for error in the present assay derives from the fact that there are two serine residues in ferrichrome A. Thus scission of the ring at the N atom of the first serine followed by removal of that amino acid would provide an additional residue capable of reacting with periodate. Incubation of crude preparations of the enzyme with ferrichrome A over protracted periods did in fact provide chromatographic evidence for the appearance of free serine.

Pseudomonas FC-1 is incapable of growth on deferrideoxyferrichrome (Rogers and Neilands, 1964), tyrocidin, bacitracin, polymixin, circulin, and gramicidin (Warren and Neilands, 1964). Whole cells of the organism grown on glucose-salts medium are capable of hydrolyzing circulin, polymixin, tyrocidin, and gramicidin as well as ferrichrome and ferrichrome A upon prolonged incubation. A complex pattern of ninhydrin-positive components emerge, indicating extensive degradation (Warren, 1964). An acetone powder of cells from the ferrichrome A medium was capable of rapid cleavage of ferrichrome; the product was not identified. Unfortunately, growth of the organism on peptone and various proteinaceous substrates did not induce the peptidase.

Recently, Saz and Lowery (1964) reported that in *Bacillus cereus* and *Staphylococcus aureus* ferrichrome and ferrichrome A act as inducers of and substrates for the penicillinase enzymes. Unfortunately, we have not yet been able to determine whether or not purified *Pseudomonas* FC-1 peptidase has penicillinase activity.

In view of the limited number of enzymes known to be capable of opening cyclic peptides, the specificity requirements of the *Pseudomonas* peptidase will prove to be quite interesting. Experiments dealing with the specificity can best be performed with preparations for which criteria of purity are available. An additional interesting feature of the specificity is the fact that the enzyme attacks at an acyl serine bond. Although, as the crystallographic structure shows (Zalkin *et al.*, 1964), this particular peptide linkage is quite exposed in ferrichrome A, there is nonetheless an unlikely possibility that the peptidase exhibits a preference for peptide bonds containing serine as the amino component. The peptidase might then be used for opening of the polypeptide chains of certain enzymes via preferential hydrolysis at the active center. Again, such questions could be most satisfactorily answered through studying the action of the highly purified enzyme on synthetic substrates and on small proteins and peptides of known structure.

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