Biochemical Pharmacology, Vol. 26, pp. 639–641. Pergamon Press, 1977. Printed in Great Britain.

INHIBITION OF PSEUDORENIN BY PEPSTATIN

RODNEY L. JOHNSON and ALAN M. POISNER

Department of Pharmacology, University of Kansas Medical Center, College of Health Sciences and Hospital, Kansas City, KS 66103, U.S.A.

(*Received* 1 *June* 1976; *accepted* 10 *September* 1976)

Abstract—The pentapeptide pepstatin was found to inhibit the ability of rat spleen pseudorenin to form angiotensin I from tetradecapeptide renin substrate. Dixon and Webb plots showed that this inhibition was noncompetitive in nature. Lineweaver–Burk analysis also showed noncompetitive inhibition. K_i values determined by the three graphical methods ranged from 1.8 to 3.8 $\times 10^{-10}$ M. The K_m for pseudorenin was determined to be between 0.82 and 1.23 μM. The concentration of enzyme used was estimated to be 3.1 $\times 10^{-10}$ M. Pepstatin should prove useful in the future for the analysis and purification of pseudorenin.

Pseudorenin is an enzyme which like renin is capable of producing angiotensin I from tetradecapeptide renin substrate and purified hog renin substrate. The enzyme is, however, chromatographically distinct from renin, has its optimum enzymatic activity at a lower pH value, and is unable to produce angiotensin I from substrate occurring naturally in plasma [1]. We now have found that pepstatin (isovaleryl-L-valyl-L-valyl-4-amino-3-hydroxy-6-methylheptanoyl-L-alanyl-4-amino-3-hydroxy-6-methylheptanoic acid), a pentapeptide produced by certain species of *Streptomyces* [2] and previously shown to be a potent inhibitor of pepsin [2–4], cathepsin D [4, 5] and renin [4, 6, 7], is also an inhibitor of pseudorenin isolated from rat spleen. In the present study, we wish to report on the kinetic characteristics of this inhibition.

MATERIALS AND METHODS

Materials. Pepstatin was obtained from the Protein Research Foundation, Tokyo, Japan. Tetradecapeptide renin substrate (TDP) and [^{125}I]angiotensin I were obtained from Schwarz/Mann, Orangeburg, N.Y., and New England Nuclear, Boston, MA, respectively. Angiotensin I for the radioimmunoassay was obtained from the National Institute for Biological Standards, Holly Hill, London.

Preparation of rat spleen pseudorenin. Spleens from Holtzman rats were frozen, thawed, and homogenized in 10 vol. water. After centrifugation for 20 min at 12,000 *g*, the supernatant was treated with ammonium sulfate to give a fraction precipitating between 1.3 and 2.5 M. The precipitate was resuspended in water and dialyzed overnight against water. The solution was clarified by centrifugation and treated with DEAE cellulose (20 mg moist resin/mg of protein). The suspension was stirred for 30 min, vacuum filtered, and the residue resuspended in 5 vol. water and adjusted to pH 4.8 with 1 N acetic acid. The suspension was stirred for 10 min and filtered. The residue was resuspended in 5 vol. NaCl (100 mM) and the pH adjusted to 4.0 with acetic acid. The suspension was stirred for 10 min and filtered. The elution at pH 4.0 was repeated once more, and the pH 4.0 eluates were combined and precipitated with ammonium sulfate (2.5 M). The precipitate was resuspended in water and dialyzed overnight against water. The protein concentration of the final solution was 2.0 mg/ml.

Pepstatin inhibition of pseudorenin. Twenty-five μl of a 200-fold dilution of the above pseudorenin preparation was incubated at 37° in a final volume of 100 μl containing 50 mM sodium citrate buffer (pH 5.0), 500 pmoles/ml of TDP, and 0–5 ng/ml pepstatin. After 30 min, the enzymatic reaction was stopped by placing the incubation mixtures on ice and diluting them with 0.8 ml of 0.1 M Tris–acetate buffer, pH 7.4. The angiotensin I produced was then assayed by radioimmunoassay as described below.

Radioimmunoassay of angiotensin I. A modification of the method described by Haber *et al.* [8] was used in this study. Samples (50 μl) of the incubation mixtures to be assayed were mixed with 50 μl of [^{125}I]angiotensin I (4500 cpm) in Tris–acetate buffer, pH 7.4, containing bovine serum albumin (5.0 mg/ml). Rabbit antiserum, 100 μl of a 1:10,000 dilution in 0.1 M Tris–acetate buffer, pH 7.4, was added and the mixture allowed to equilibrate at 4° for 24 hr. The antiserum was prepared as described by Haber *et al.* [8]. A charcoal suspension (0.8 ml) containing 3.9 g/l of charcoal and 0.39 g/l of dextran in 0.1 M Tris–acetate buffer, pH 7.4, was then added. The mixtures were thoroughly mixed and then centrifuged at 7000 *g* for 10 min. The supernatant was decanted, mixed with 4.5 ml of scintillation fluid (Aquasol) and counted in a Packard Tri-Carb liquid scintillation counter. Known amounts of angiotensin I varying from 25 to 1600 pg were treated in a similar manner to produce a standard curve.

Kinetic studies. Data for Dixon and Webb plots were obtained by measuring the reaction velocity of pseudorenin at two substrate concentrations (0.5 and 1.0 μM TDP) and in the presence of varying amounts of pepstatin (2.9 $\times 10^{-10}$ to 20.4 $\times 10^{-10}$ M). The enzymatic reaction and radioimmunoassay were carried out as described above. For each substrate level a plot of $1/V$ vs pepstatin concentration (Dixon plot see Fig. 2) was made and the lines calculated by linear regression analysis. A plot of $1/i$ vs $1/[pepstatin]$, where $i = 1 - V_i/V_c$, provided a Webb plot (see Fig. 3).

The results obtained in the Dixon and Webb plots were also used to estimate the concentration of pseu-

dorenin present in the incubation mixture. In cases where an inhibitor has a high affinity for an enzyme, the molarities of the inhibitor and enzyme are essentially the same. In such cases a plot of I/i vs $1/1 - i$ will yield a straight line in which the slope of the line is equal to the K_i and the intercept on the y-axis is equal to the concentration of the enzyme [9].

Lineweaver–Burk analysis of pseudorenin activity was carried out by determining the reaction velocities under conditions in which the concentration of TDP was varied from 0.2 to 0.5 μM in either the absence or presence of 3.65×10^{-10} M pepstatin. The results were used in a plot of $1/V$ vs $1/S$.

RESULTS

The pseudorenin preparation isolated from rat spleen was found to generate 103 μg angiotensin I/ml/hr when incubated at 37 in the presence of 0.5 μM TDP. As shown in Fig. 1, pepstatin concentrations ranging from 0.125 ng/ml (1.8×10^{-10} M) to 5 ng/ml (7.3×10^{-9} M) inhibited the formation of angiotensin I from TDP. Fifty per cent inhibition was observed at a pepstatin concentration of 3.3×10^{-10} M, while complete inhibition was observed at 7.3×10^{-9} M. The ID_{50} obtained is similar to that obtained by McKown *et al.* [9] for human renin and porcine pepsin.

The determination of the reaction velocities at two TDP concentrations (0.5 and 1.0 μM) with pepstatin concentrations ranging between 2.9×10^{-10} M and 20.4×10^{-10} M provided data for the Dixon and Webb plots, Figs. 2 and 3, respectively. A Dixon plot of $1/V$ vs [pepstatin] gave two lines which intersected the x-axis at -2.06 and -1.78 respectively. The difference between these two values is not statistically significant. This type of plot is indicative of noncompetitive inhibition. Since the point on the x-axis where the two lines intersect is equal to $-K_i$, the K_i value for pepstatin is between 1.78 and 2.06×10^{-10} M. In a Webb plot of $1/i$ vs 1/[pepstatin], the two lines were superimposable and intersected the y-axis at a value close to 1.0. Such a plot, which is indicative of noncompetitive inhibition [10], gave a K_i value of 3.8×10^{-10} M.

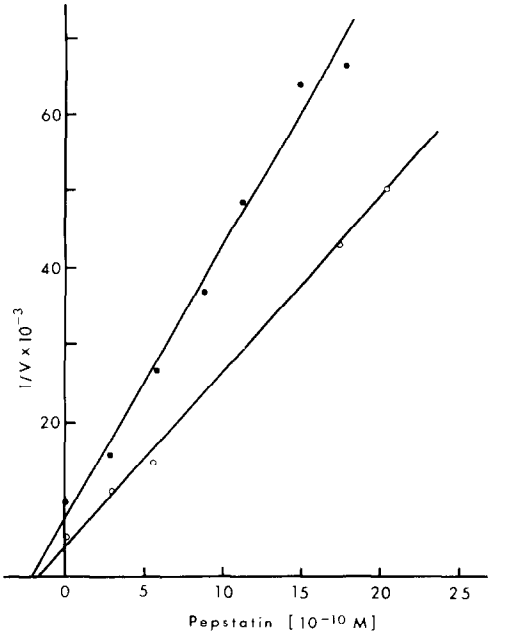

Fig. 2. Dixon plot ($1/V$ vs [pepstatin]) of the inhibition of rat spleen pseudorenin by pepstatin. V is the reaction velocity (μg angiotensin I/ml/hr) determined at two substrate concentrations: S_1, 0.5 μM (●) and S_2, 1.0 μM (○) TDP, in the presence of pepstatin. K_i is equal to 2.06 and 1.78×10^{-10} M respectively.

The results obtained from the Dixon and Webb plots when plotted as I/i vs $1/1 - i$ gave a straight line as shown in Fig. 4. This line intercepted the y-axis at a value of 3.13×10^{-10} M and has a slope equal to 2.09×10^{-10} M. These values are equal to the concentration of pseudorenin in the system and to the K_i respectively.

The reaction velocities of pseudorenin were determined at substrate concentrations ranging between

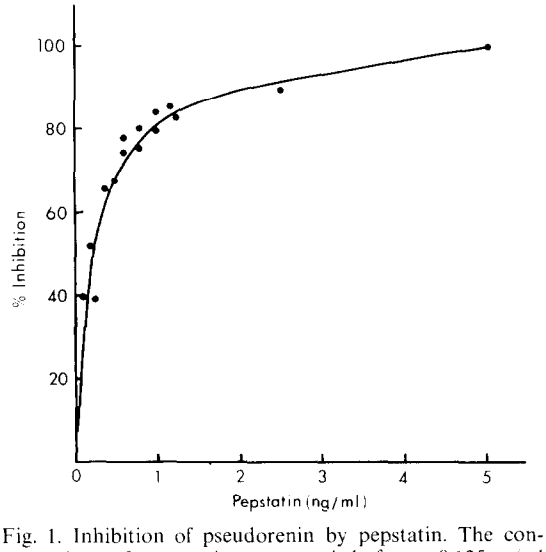

Fig. 1. Inhibition of pseudorenin by pepstatin. The concentration of pepstatin was varied from 0.125 ng/ml (1.82×10^{-10} M) to 5 ng/ml (7.3×10^{-9} M).

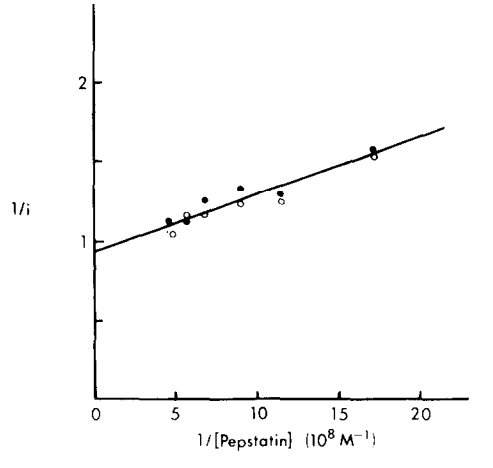

Fig. 3. Webb plot ($1/i$ vs 1/[pepstatin]); i is the fractional inhibition and is equal to $1 - V_i/V_c$ where V_i and V_c are the reaction velocities in the presence and absence of pepstatin respectively. Concentrations of TDP are: S_1, 0.5 μM (●) and S_2, 1.0 μM (○). K_i (x-intercept) equals 3.8×10^{-10} M.

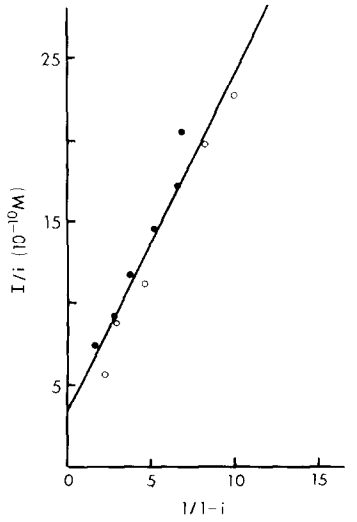

Fig. 4. Determination of the concentration of pseudorenin. Substrate concentrations are: S_1, 0.5 μM, and S_2, 1.0 μM. From the y-intercept of a plot of I/i vs $1/1 - i$, the concentration of pseudorenin is determined to be 3.13×10^{-10} M.

0.2 and 0.5 μM in the absence or presence of pepstatin (3.65×10^{-10} M). The values obtained were plotted by the Lineweaver–Burk method (Fig. 5) and showed that the inhibition of pseudorenin by pepstatin was noncompetitive. The maximum rate of angiotensin I formation was 374 μg/ml/hr. In the presence of 3.65×10^{-10} M pepstatin, the V_{max} fell to 144 μg/ml/hr. The K_i for pepstatin in this case was found to be 2.31×10^{-10} M, a value similar to that obtained in the Dixon and Webb plots described earlier. The K_m for pseudorenin was determined to be between 0.82 and 1.23 μM. These values are similar to the K_m value of 1.85 μM reported by Skeggs *et al.* [1] for human kidney pseudorenin.

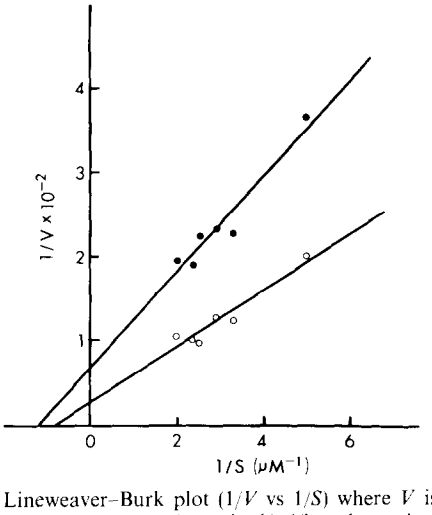

Fig. 5. Lineweaver–Burk plot ($1/V$ vs $1/S$) where V is the reaction velocity (μg angiotensin I/ml/hr) determined at varying concentrations of TDP (S) in the absence (○) or presence of pepstatin (3.64×10^{-10} M) (●). $K_m = 0.82$ to 1.22 μM.

DISCUSSION

Since the first report by Skeggs *et al.* [1] on the isolation of pseudorenin, very little additional work has been reported on this enzyme. The results obtained in this study demonstrate that the ability of pseudorenin to form angiotensin I from tetradecapeptide renin substrate is inhibited by pepstatin in a noncompetitive manner. Although early reports [7, 11] in the literature suggested that pepstatin inhibition of pepsin and renin was competitive in nature, later work [9, 12] has shown it to be noncompetitive. Thus, the inhibition of pseudorenin by pepstatin is similar to that observed with pepsin and renin. Whether or not an aspartic acid residue is involved in the catalytic function of pseudorenin, as has been shown for renin [13] and pepsin [14], remains to be demonstrated.

Since the spleen has an extremely high concentration of pseudorenin (Ref. 1, and A. Poisner and S. Strom, unpublished observations), it was chosen for the source of enzyme used in this study. However, ongoing work in our laboratory has shown that pseudorenin from other tissues and body fluids (including human and rat plasma) is also inhibited by pepstatin at very low concentrations.

The potent inhibitory nature of pepstatin on pseudorenin should provide a useful tool in determining the physiological function of this enzyme and also in its purification by affinity chromatography.

REFERENCES

1. L. T. Skeggs, K. E. Lentz, J. R. Kahn, F. E. Doser and M. Levine, *Circulation Res.* **25,** 451 (1969).
2. H. Umezawa, T. Aoyagi, H. Morishima, M. Matsuzaki, M. Hamada and T. Takeuchi, *J. Antibiot., Tokyo* **23,** 259 (1970).
3. T. Aoyagi, S. Kahimoto, H. Morishima, T. Takeuchi and H. Umezawa, *J. Antibiot., Tokyo* **24,** 687 (1971).
4. T. Aoyagi, H. Morishima, R. Nishizawa, S. Kunimoto, T. Takeuchi and H. Umezawa, *J. Antiobiot., Tokyo* **25,** 689 (1972).
5. A. J. Barrett and J. T. Dingle, *Biochem. J.* **127,** 439 (1972).
6. F. Gross, J. Lazar and H. Orth, *Science, N.Y.* **175,** 656 (1972).
7. R. P. Miller, C. J. Poper, C. W. Wilson and E. De Vito, *Biochem. Pharmac.* **21,** 2941 (1972).
8. E. Haber, T. Koerner, L. B. Page, B. Kilman and A. Purnode, *J. clin. Endocr. Metab.* **29,** 1349 (1969).
9. M. M. McKnown, R. J. Workman and R. I. Gregerman, *J. biol. Chem.* **249,** 7770 (1974).
10. J. L. Webb, *Enzymes and Metabolic Inhibitors*, Vol. 1, p. 153. Academic Press, New York (1963).
11. P. Corvol, C. Devaux and J. Menard, *Fedn Euro. Biochem. Soc.* **34,** 189 (1973).
12. H. Orth, E. Hackenthal, J. Lazar, U. Mikshe and F. Grass, *Circulation Res.* **35,** 52 (1974).
13. M. M. McKown and R. I. Gregerman, *Life Sci.* **16,** 71 (1975).
14. S. Kunimoto, T. Aoyagi, H. Morishima, T. Takeuchi and H. Umezawa, *J. Antibiot., Tokyo* **25,** 251 (1972).