Inhibition of *Helicobacter pylori* Urease Activity by Hydroxamic Acid Derivatives

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Helicobacter pylori (HP) produces strong urease [EC 3.5.1.5], which is considered to play a role in the pathogenesis of gastritis and peptic ulcers. Inhibitions against this enzyme have been studied with hydroxamic acid (HXA) derivatives of aliphatic or aromatic carboxylic acids, amino acids and dipeptides. A number of HXAs potently inhibited the urease (I_{50} values were near the order of 10^{-6} M), and H-Ile-Gly-NHOH ($I_{50}=0.20 \times 10^{-6}$ M) was the most potent inhibitor among the derivatives. HP urease was inhibited more potently, in general, than Jack bean (JB) urease by HXAs, and a correlation between the chemical structures of HXA derivatives and their inhibitory effects on HP urease was observed, in comparison with JB urease.

Keywords *Helicobacter pylori* urease; inhibition; hydroxamic acid; Jack bean urease

Helicobacter pylori (HP) is a microaerophilic spiral bacterium and infection by it in the human stomach causes gastritis, furthermore, it is considered to be involved in the pathogenesis of peptic ulcers and the development of gastric carcinoma.¹⁾ This bacterium produces strong urease which hydrolyzes urea in gastric juice to produce ammonia.²⁾ Recently, several studies about this enzyme have been reported. A urease-deficient HP strain which does not produce urease did not colonize on the gastric epithelial cell surface of gnotobiotic piglets.³⁾ Ammonia produced by HP urease elevated the level of pH in the stomach and broke gastric mucus,⁴⁾ and the ammonia itself inhibited the consumption of oxygen and reduced the production of ATP in gastric mucous cells or in the mitochondria.⁵⁾ In the culture of gastric epithelial cells, the addition of HP urease and urea significantly reduced the survival rate of the cells.⁶⁾ These reports suggested that HP urease plays a critical role in the infection of HP on the gastric epithelial cell surface of the human stomach, but also in the pathogenesis of the diseases caused by HP. We think that it is useful, in order to establish these theories, to develop specific and potent inhibitors of HP urease, and to study the therapeutic application of the inhibitors *in vivo*.

Hydroxamic acids (HXAs, R-NHOH: R = acyl groups) were found to be potent and specific inhibitors of urease activities of plant and bacterial origins, and the correlation between the chemical structures or the physicochemical properties of the acyl residues (R) and their inhibitory effects on urease activity, mainly of Jack bean (JB) or *Proteus mirabilis* ureases, has been established.⁷⁾ On the basis of this research, intensive efforts have been made to develop HXAs as a therapeutic agent against diseases caused by urease, for example, urolithiasis.⁸⁾ However, only acetohydroxamic acid was used to inhibit HP urease activity.⁶⁾

In this study, we investigated the inhibitory effects of HXA derivatives of aliphatic or aromatic carboxylic acids, amino acids and dipeptides on the urease activity of HP, and compared their inhibitory activities with those against

JB urease.

MATERIALS AND METHODS

Preparation of Urease HP (ATCC 43504) was kindly donated by the Research Institute for Microbial Disease, Osaka University. HP was inoculated into Brucella HK agar plates (Kyokuto Seiyaku Kogyo, Japan) and was cultured micro-aerobically at 37°C for 3 d. Cells were flooded with 2 ml of saline, and the suspension was vortexed for 30 s. Then, the cell suspension was inoculated into 50 ml of Brucella broth (BBL, U.S.A.) supplemented with 10% fetal bovine serum and was cultured micro-aerobically at 37°C for 3 d. The cultured medium was centrifuged at 20000 *g* for 20 min at 4°C and the supernatant was discarded. The harvested cells were suspended in 5 ml of 20 mM phosphate buffer pH 7.0 containing 1 mM EDTA and 1 mM 2-mercaptoethanol, and then disrupted by a sonicator (Heat Systems, U.S.A.). The sonicated suspension was centrifuged at 20000 *g* for 20 min at 4°C. The supernatant fluid was used for the measurement of urease activity. The specific activity of the enzyme was 14 IU/mg protein.

Measurement of I_{50} Values of HXAs Urease activity of the crude extract (5.7 units/mg protein) of JB powder was assayed according to the method of van Slyke and Archibald.⁹⁾ A mixture of JB urease (140 units) in 0.3 ml of 0.1 M phosphate buffer, pH 7.7, and of HXA to be tested of various concentrations in 0.3 ml of the same buffer, was preincubated for 15 min at 37°C. After preincubation, 5.0 ml of 3% urea solution in 0.1 M phosphate buffer pH 6.7 and 0.05 ml of alcoholic solution of 0.1% phenol red were added to the mixture. The time (min) it took the mixture to change from pH 6.7 to 7.7 was measured colorimetrically.

The assay mixture, containing 50 μ l of HP urease and 50 μ l of HXA solution to be tested, was preincubated for 15 min at 37°C. After preincubation, 300 μ l of 100 mM phosphate buffer pH 7.0 containing 400 mM urea was added and incubated for 30 min at 37°C. The reaction

was terminated by the addition of 1 N H₂SO₄ (100 μ l) and the amount of ammonia production was determined by the indophenol method.¹⁰⁾

The I₅₀ value of the compounds, *i.e.*, the final molar concentration which caused 50% inhibition, was determined by computer analysis.

Inhibitors HXA derivatives have been synthesized in our laboratory according to the previous study.¹¹⁾

RESULTS

Inhibition of Aliphatic or Aromatic Acyl HXAs The *n*-aliphatic and aromatic acyl HXAs were used to investigate the structure-activity relationships between acyl residues and their inhibitory effects on HP urease, and their I₅₀ values were listed in Table I.

Among aliphatic acyl HXAs, the *n*-hexanoyl (C₆) derivative was the most potent inhibitor for HP urease, whereas the analogs with shorter (C₂) or longer (C₈ and C₁₀) chains than C₆ showed lower activity than the C₆ analog. Benzoyl HXA inhibited HP urease with an I₅₀ value of 3.3 μ M, stronger than JB urease, with an I₅₀ value of 29 μ M, under the same assay conditions. Nicotinoyl HXA inhibited HP urease (I₅₀ value: 4.1 μ M), but the isonicotinoyl and picolinoyl analogs did not show any inhibition at 1 mM.

Inhibition of HXA Derivatives of Amino Acids The I₅₀ values of eighteen α -aminoacyl HXAs (H-X-NHOH):

TABLE I. Inhibition of HP Urease by Acyl HXAs (R-NHOH)

R	I ₅₀ (μ M)	R	I ₅₀ (μ M)
Aliphatic		Aromatic	
Acetyl	15	Benzoyl	3.3
<i>n</i> -Hexanoyl	6.8	Nicotinoyl	4.1
<i>n</i> -Octanoyl	14	Isonicotinoyl	>1000
<i>n</i> -Decanoyl	31	Picolinoyl	>1000

TABLE II. Inhibition of HP and JB Ureases by HXA Derivatives of Amino Acids (H-X-NHOH)

Compd. No.	X	I ₅₀ (μ M)	
		HP urease	JB urease
AA- 1	Phe ^{a)}	0.38	5.4
AA- 2	Ile	0.68	20
AA- 3	Ala ^{a)}	1.4	7.8
AA- 4	Gly	2.4	9.0
AA- 5	Pro	3.1	N.D.
AA- 6	Met ^{a)}	3.5	3.9
AA- 7	Leu	4.2	13
AA- 8	Trp	5.9	24
AA- 9	Ser ^{a)}	7.1	6.2
AA-10	His	7.9	10
AA-11	Tyr	11	24
AA-12	Arg	13	16
AA-13	Thr ^{a)}	15	12
AA-14	Val ^{a)}	25	43
AA-15	Lys	40	90
AA-16	Asp	63	53
AA-17	Glu	100	620
AA-18	Gln	790	N.D.

a) D, L compound. N.D.: not determined.

X=amino acid residues) against HP urease are shown in Table II, comparing them with those of the HXA derivatives against JB urease.

A number of α -aminoacyl HXAs showed potent inhibition against HP urease, and the I₅₀ values of the Phe and Ile analogs (AA-1 and 2) were less than 1.0 μ M. Lys (AA-15), Asp (AA-16), Glu (AA-17), and Gln (AA-18) analogs, however, were much less inhibitory, compared with the Phe and Ile analogs. The inhibition of α -aminoacyl HXAs against HP urease was almost more potent than those against JB urease, and remarkable differences were observed in the inhibitions by two potent analogs, Phe and Ile (the ratios of I₅₀ values of JB urease to those of HP urease were 14:1 and 29:1, respectively).

Inhibition of HXA Derivatives of Dipeptides The inhibitory activity of a series of dipeptidyl HXAs (H-X-Gly-NHOH) against HP and JB ureases was examined and the results are listed in Table III.

Seven out of thirteen dipeptidyl HXAs tested here inhibited HP urease with an I₅₀ of the order of 10⁻⁷ M. Similarly to the inhibitions of α -aminoacyl HXAs, Glu (DP-12) and Gln (DP-13) analogs, in descending order, were much less inhibitory, and the inhibitions of a number of dipeptidyl HXAs against HP urease were more potent than those against JB urease.

TABLE III. Inhibition of HP and JB Ureases by HXA Derivatives of Dipeptides (H-X-Gly-NHOH)

Compd. No.	X	I ₅₀ (μ M)	
		HP urease	JB urease
DP- 1	Ile	0.20	15
DP- 2	Leu	0.22	1.8
DP- 3	Phe	0.71	2.4
DP- 4	Met	0.31	2.6
DP- 5	Gly	0.78	21
DP- 6	Ala	0.79	21
DP- 7	Arg	0.97	8.4
DP- 8	Pro	1.1	6.0
DP- 9	Ser	1.3	9.3
DP-10	Tyr	1.6	3.3
DP-11	Lys	7.0	20
DP-12	Glu	37	6.0
DP-13	Gln	370	16

TABLE IV. Inhibition of HP Urease by HXA Derivatives of Dipeptides (R-X-Gly-NHOH)

Compd. No.	R	X	I ₅₀ (μ M)	Compd. No.	R	X	I ₅₀ (μ M)
DP-14	Boc	Ile	95	DP-25	Boc	Phe	28
DP-15	Boc	Leu	21	DP-26	Bz	Phe	14
DP-16	Boc	Met	20	DP-27	Ac	Phe	16
DP-17	Boc	Gly	41	DP-28	Z	Phe	29
DP-18	Boc	Ala	32				
DP-19	Boc	Pro	87	DP-29	H	D-Phe	1.2
DP-20	Boc	Ser	36	DP-30	H	Ser(Bzl)	0.21
DP-21	Boc	Tyr	110	DP-31	H	Tyr(Bzl)	1.2
DP-22	Boc	Lys	>1000				
DP-23	Boc	Glu	710			H-Phe-Gly-NH ₂	>1000
DP-24	Boc	Gln	520			H-Phe-Gly-NHEt	>1000

Boc, *tert*-butoxycarbonyl; Bz, benzoyl; Ac, acetyl; Z, benzyloxycarbonyl; Bzl, benzyl.

As shown in Table IV, the inhibitory activities of the dipeptidyl HXA derivatives of protected or D- amino acids against HP urease have also been examined.

Blocking of an α -amino group by *tert*-butoxycarbonyl (Boc), benzoyl (Bz), acetyl (Ac) and benzyloxycarbonyl (Z) groups reduced the inhibitory activity (refer to Table III). By contrast, blocking of the hydroxy group of Ser and Tyr by benzyl (Bzl) group increased their inhibitory activities (DP-30 and 31). The inhibitory activities of D-Phe (DP-29) and L-Phe (DP-3) were almost equal. Amide derivatives of DP-3 had no inhibitory activity, even at concentrations of 1 mM.

DISCUSSION

A number of HXA derivatives tested here inhibited HP urease potently; therefore, it has been certified that HP urease is inhibited by HXA as well as other ureases from plant and bacterial origin.

The I_{50} values of two α -aminoacyl (AA-1 and 2) and seven dipeptidyl HXAs (DP-1—7) against HP urease reached the order of 10^{-7} M. Such potent inhibition was not observed in the inhibition against JB urease, and the inhibitory activities of almost all HXAs against HP urease were more potent than those against JB urease (Tables II and III). In our previous study, dipeptidyl HXAs inhibited urease activity of *Proteus mirabilis* with an I_{50} of the order of 10^{-5} M.¹¹⁾ Thus, HP urease was inhibited by dipeptidyl HXAs about one hundred-fold more potently than *Proteus mirabilis* urease was, though both ureases are from bacteria. These results indicate that HP urease has a higher affinity for the HXA derivatives than JB and *Proteus mirabilis* ureases, as well as for urea (K_m values for urea: HP; 0.2—0.3 mM, JB; 10.5 mM, *Proteus mirabilis*; 13 mM). A difference between HP and JB ureases was also observed in the order of inhibitory potency of HXAs, for example, among dipeptidyl HXAs, the Ile analog (DP-1) was a relatively weak inhibitor against JB urease, but was the most potent inhibitor against HP urease (Table III), and, among aliphatic acyl HXAs, *n*-hexanoyl analog was the most powerful inhibitor for HP urease, though, *n*-octanoyl HXA was the most potent inhibitor against JB urease (I_{50} value: 5.4 μ M) in the previous study.⁷⁾

A number of dipeptidyl HXAs (H-X-Gly-NHOH) were more inhibitory than the corresponding α -aminoacyl HXAs (H-X-NHOH) against HP urease, as well as against JB urease. This result suggested that the glycino group is favorable for the inhibition on HP urease, which is in agreement with the previous investigation of the inhibitory effects of acylglycino-HXAs on JB urease activity.⁸⁾

The results shown in Tables II and III suggest that hydrophobic amino acid derivatives, in general, are stronger than hydrophilic amino acid derivatives for the inhibition of HP urease. This tendency was also observed in the inhibitions against JB urease, and is agreement with previous results in which the hydrophobicity of acyl residues (R) of acyl HXAs (R-NHOH) had considerable effect on the inhibitory activity against urease.^{7g)}

Glycyl HXA (AA-4, $\text{NH}_2\text{-CH}_2\text{-CONHOH}$) was more inhibitory than acetyl HXA (Table I, $\text{CH}_3\text{-CONHOH}$) against HP urease, indicating the participation of the

amino group in the binding between the urease and HXAs. This suggestion is supported by the results in which the blocking of the α -amino group of dipeptidyl HXAs reduced their inhibitory activity, while the blocking of the hydroxyl group of Ser and Tyr by a benzyl (Bzl) group increased their inhibitory activities (Table IV).

The requirement of a hydroxamic acid moiety and no enantiomeric effect on inhibitory activity against JB urease by dipeptidyl HXA have been reported previously,¹¹⁾ and both of these were also observed in the inhibition against HP urease (Table IV).

The stability of HXA derivatives in the inhibition assay for HP urease was examined, since the decomposition of HXA derivatives affects their inhibition powers. No decomposition was observed after 45 min incubation of HXA derivatives with HP cell lysate (data not shown).

It has been reported that HP urease was not necessary for the growth of HP itself *in vitro*,¹²⁾ and ammonia produced by HP urease was not toxic to gastric epithelial cells *in vivo* or *in vitro*,¹³⁾ though several studies have suggested the toxicity of ammonia.⁴⁻⁶⁾ On the other hand, it has also been proposed that the association of HP urease with heat shock protein¹⁴⁾ or vacuolating toxin¹⁵⁾ produced by HP revealed pathogenicity for gastritis or peptic ulcers. Thus, it is unclear whether this urease is essentially involved in the pathogenesis of the diseases caused by HP. In this study, we found that several HXA derivatives of amino acids and dipeptides inhibited HP urease with an I_{50} of the order of 10^{-7} M, and their inhibitions were much more potent than the inhibition by aceto-HXA (Table I, R = acetyl), which has traditionally been used as an inhibitor for HP urease. We also obtained information about structure-activity relationships between HXAs and their inhibitory effects on HP urease; moreover, it is known that the hydroxamic acid moiety is stable under acidic conditions such as gastric juices. Therefore, the hydroxamic acids reported here or developed in reference to this study are expected to be examined *in vivo* to clarify the role of HP urease in the infection of HP and in the pathogenesis of diseases caused by HP.

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