Screening System for Urease Inhibitors Using ^{13}C-NMR

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Urease inhibitors are candidate drugs to treat infection with the human pathogen, *Helicobacter pylori*, which produces a potent urease [urea amidohydrolase; EC 3.5.1.5]. We developed a screening system based on ^{13}C-NMR measurement of the time course of decrease in the signal of ^{13}C-urea in the presence of urease. The effect on urease activity of known inhibitors, hydroxamic acids, L-ascorbic acid, 2,2′-dipyridyl disulfide and ninhydrin, was speedily and conveniently measured by this method.

Key words ^{13}C-NMR; ^{13}C-urea; screening; urease; urease inhibitor

Helicobacter pylori plays an important role in the pathogenesis of gastritis and peptic ulcer. It produces a potent urease which catalyzes the hydrolysis of urea in gastric juice to produce ammonia and carbamate. The carbamate spontaneously hydrolyzes to form carbonic acid and a second molecule of ammonia. This production of ammonia makes it possible for *H. pylori* to inhabit the stomach (higher animals have no endogenous urease activity in the stomach). We have developed a ^{13}C-urea breath test (^{13}C-UBT) as a non-invasive method for the detection of *H. pylori* infection utilizing this urease activity.[1)]

Eradication therapy of *H. pylori* at present usually consists of combination therapy with an antibiotic and a proton pump inhibitor. However, the therapy may have side effects, including the induction of drug-resistance, and therefore, alternatives are needed. One possibility is eradication therapy by inhibition of the potent urease activity of *H. pylori*. A screening method is required to find suitable inhibitors. At present, the urease enzyme reaction is usually followed by indirect detection of ammonia produced by hydrolysis of the substrate. However, the available methods are complex and time-consuming; usually ammonia is transformed to another compound by chemical reaction for detection. For example, the indophenol method requires about 30—60 min to transform ammonia to indophenol by chemical reaction.

We describe here a rapid and easy method of screening for urease inhibitors using ^{13}C-NMR to monitor the time course of ^{13}C-urea reduction due to the urease enzyme reaction.

Results and Discussion

One of the best-studied ureases is that from jack bean. This plant urease was the first enzyme to be crystallized[2)] and also the first shown to contain nickel at the active site.[3)] The X-ray crystal structure of the urease from *Klebsiella aerogenes* was reported by Jabri *et al.* in 1995,[4)] and the active site was shown to consist of two nickel atoms bridged by the carbamate side chain of the modified ε-amino residue of Lys^{217}.

We used jack bean urease for our assay system. The time course of the decreasing ^{13}C-NMR signal of ^{13}C-urea (10 mg) is shown in Fig. 1. The signal of ^{13}C-urea was observed at 165.0 ppm and decreased with enzyme reaction time, while that of ^{13}C-carbonate produced in the reaction at 162.8—164.2 ppm appeared and increased. The signal of ammonium ^{13}C-carbamate was observed at 167.8 ppm. The disappearance time of the ^{13}C-urea signal was increased by addition of urease inhibitors. Thus, screening for urease inhibitors can be done by comparing the disappearance time of the ^{13}C-urea signal in the presence of a test compound with that in its absence (control). Since it is convenient to use ethanol to dissolve aliphatic test compounds, we examined the effect of ethanol on the disappearance of the ^{13}C-urea signal (Fig. 2A). The signal of ^{13}C-urea was observed at 165.0 ppm, that of ^{13}C-carbonate at 162.8 ppm and that of monoethyl ^{13}C-carbonate at 161.8 ppm. The effect of ethanol on the inhibition of urease by acetohydroxamic acid is shown in Fig. 2B. The relation between the disappearance time of the ^{13}C-urea signal and the acetohydroxamic acid concentration was linear in both the presence and absence of ethanol.

The enzyme reaction velocity was calculated from the relation between ^{13}C-urea concentration and the disappearance time of ^{13}C-urea signals in the presence of various inhibitors and the results were analyzed using Lineweaver-Burk plots. Figure 3 shows the results for acetohydroxamic acid, a competitive inhibitor, and ninhydrin, a noncompetitive inhibitor, in the presence and absence of ethanol. Table 1 summarizes the results for all the inhibitors tested.

Hydroxamic acids have been reported to be potent and specific competitive inhibitors of urease.[5)] The results obtained by our method were fully consistent with the reported data. Urease contains a thiol group that is essential for activity and is inactivated by disulfide and alkylating agents.[6)] We confirmed that 2,2′-dipyridyl disulfide was a noncompetitive inhibitor; L-ascorbic acid and ninhydrin were also noncompetitive inhibitors. Ninhydrin may inactivate urease *via* a mechanism similar to that of its color generation with amino acid, and other compounds with a 1,2,3-tricarbonyl moiety (such as alloxan) may also inhibit urease. In the case of L-ascorbic acid, there are two possible mechanisms. Firstly, it may reduce Ni ions at the urease active site. Secondly, it is readily oxidized to dehydroascorbic acid, which has a 1,2,3-tricarbonyl group and may inactivate urease in the same manner as ninhydrin.

Ethanol was a competitive inhibitor of urease, and the

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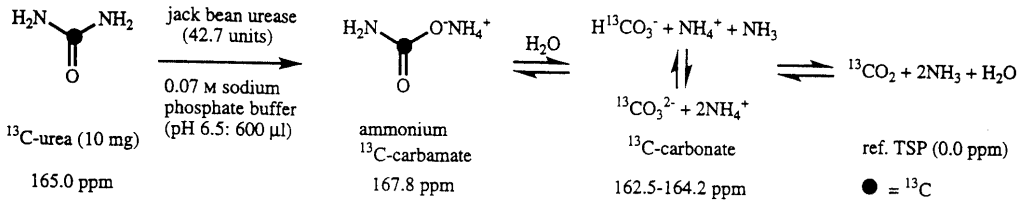

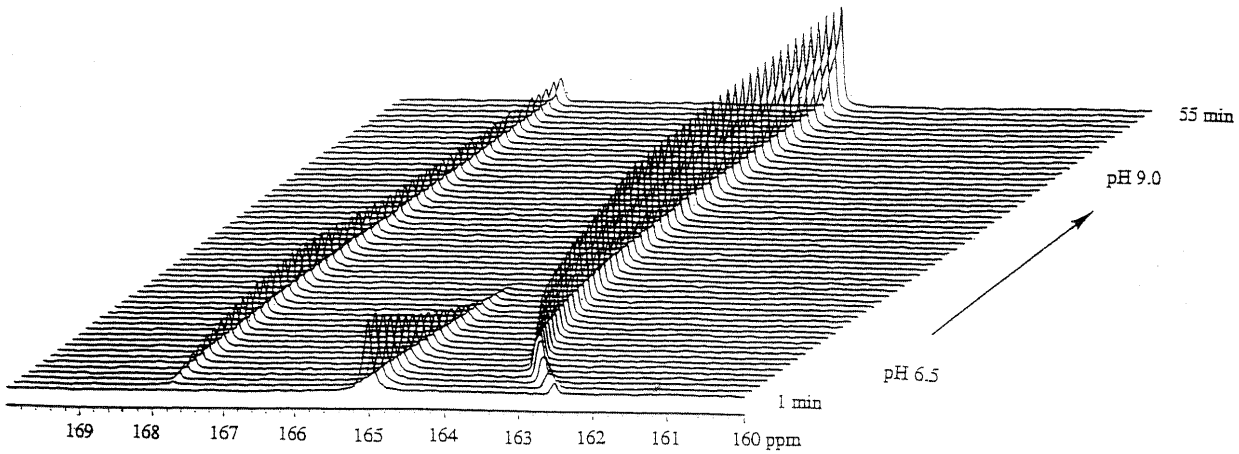

Fig. 1. Time Course of Urease Enzyme Reaction with ^{13}C-Urea (10 mg) Followed by ^{13}C-NMR

A solution of 42.7 units of urease in 500 μl of 0.07 M sodium phosphate buffer (pH 6.5) was preincubated at 22 °C for 30 min, then at 0 °C for 10 min. A solution of ^{13}C-urea (10 mg) in 100 μl of the same buffer (total volume 600 μl) was added, and the disappearance of ^{13}C-urea was followed by ^{13}C-NMR. 162.8—164.2 ppm; ^{13}C-carbonate, 165.0 ppm; ^{13}C-urea, 167.8 ppm; ammonium ^{13}C-carbamate.

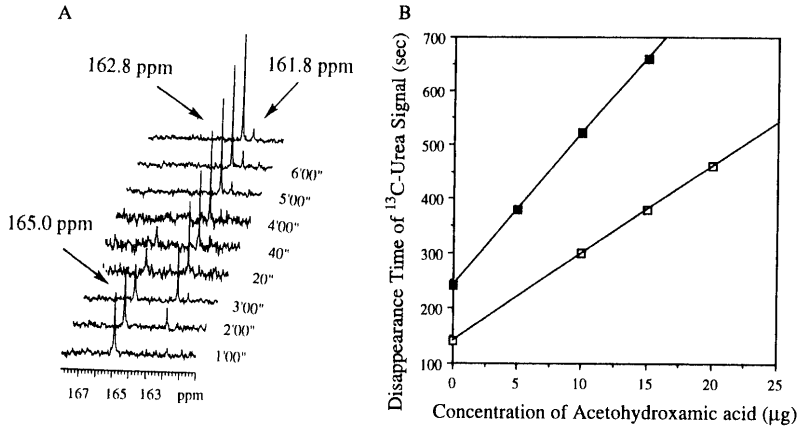

Fig. 2. Relation between Concentration of Acetohydroxamic Acid and Disappearance Time of ^{13}C-Urea Signal

A solution of 28.2 units of urease (A; in 0.07 M sodium phosphate buffer (pH 6.5) 400 μl and ethanol 100 μl. B; in the presence of acetohydroxamic acid. □, in 500 μl of the above buffer; ■, in 400 μl of the buffer and 100 μl of ethanol) was preincubated at 22 °C for 30 min, then at 0 °C for 10 min. ^{13}C-Urea (1 mg) in 100 μl of the same buffer (total volume 600 μl) was added, and the disappearance of ^{13}C-urea was followed by ^{13}C-NMR.

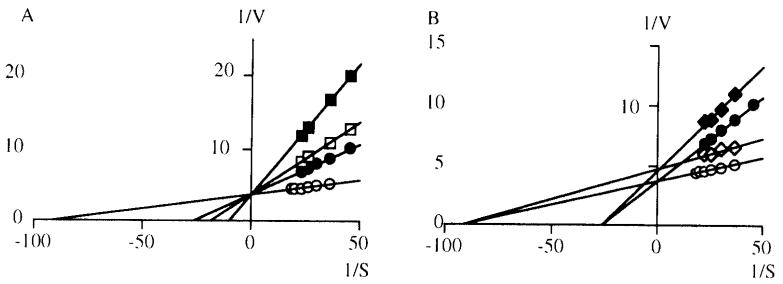

Fig. 3. Effect of Acetohydroxamic Acid and Ninhydrin on Urease Activity in the Presence and Absence of Ethanol

A solution of 28.2 units of urease (A: in the presence of acetohydroxamic acid 10 μg. □, in 500 μl of 0.07 M sodium phosphate buffer; ■, in the same buffer with 400 μl and 100 μl of ethanol. B; in the presence of ninhydrin 100 μg. ◇, in the same buffer without ethanol; ◆, with 100 μl of ethanol) was preincubated at 22 °C for 30 min, then at 0 °C for 10 min. ^{13}C-Urea (0.8—1.6 mg) in 100 μl of the same buffer (total volume 600 μl) was added and its disappearance was followed by ^{13}C-NMR. Controls: ●, with ethanol; ○, without ethanol.

Table 1. Effect of Various Inhibitors on Urease Activity in the Presence and Absence of Ethanol

Inhibitor	With ethanol		Without ethanol		Mechanism[a]
	I_{50} (M)	K_i	I_{50} (M)	K_i	
Acetohydroxamic acid	1.90×10^{-4}	1.11×10^{-4}	1.94×10^{-4}	5.55×10^{-5}	c
Benzohydroxamic acid	3.65×10^{-5}	2.13×10^{-5}	ND	ND	c
Nicotinohydroxamid acid	1.81×10^{-5}	1.06×10^{-5}	ND	ND	c
2,2′-Dipyridyl disulfide	1.14×10^{-3}	1.14×10^{-3}	ND	ND	n
L-Ascorbic acid	1.62×10^{-2}	1.62×10^{-2}	1.67×10^{-2}	1.67×10^{-2}	n
Ninhydrin	3.74×10^{-3}	3.89×10^{-3}	3.28×10^{-3}	3.18×10^{-3}	n

a) c, competitive inhibition; n, non-competitive inhibition.

enzyme activity in the assay mixture containing 100 μl of ethanol was 58.3% of that in its absence. Further, the value of K_i of acetohydroxamic acid was changed by ethanol. This may reflect different binding abilities of ethanol and water at the urease active site. Nevertheless, ethanol is a convenient solvent to use for screening of many compounds, and its effect on urease activity can be easily compensated for by the use of an appropriate ethanol-containing control run. We consider that the present assay can be applied as a rapid and convenient screening system for urease inhibitors as candidate drugs for the treatment of *H. pylori* infection.

Experimental

Materials Acetohydroxamic acid and 2,2′-dipyridyl disulfide were supplied by Tokyo Chemical Industry Co., Ltd. Benzohydroxamic acid and nicotinohydroxamic acid were supplied by Sigma Chemical Co. L-Ascorbic acid and ninhydrin were supplied by Wako Pure Chemical Industries, Ltd. ^{13}C-Urea (99 atom% ^{13}C) was supplied by Cambridge Isotope Laboratories. Jack bean urease (141 units/mg) was supplied by Wako.

Instrument ^{13}C-NMR spectra were taken on a Varian GEMINI 300 spectrometer (75 MHz). Chemical shifts are given downfield from sodium 3-trimethylsilylpropionate-d_4 (TSP) as an internal standard at 0 ppm.

^{13}C-NMR Conditions Acquisition time was 1.0 sec and the pulse delay time was 0.5 sec. The number of scans was 8—30. Probe temperature was 20 °C. Spectral width was 18102.9 Hz, with 36192 data point. Pulse angle was 27°.

Time Course Measurement of ^{13}C-Urea Disappearance by ^{13}C-NMR Jack bean urease (42.7 units) was added to 0.07 M sodium phosphate buffer (500 μl) in a 5 mm NMR tube. The NMR tube was allowed to stand at 22 °C for 30 min, then was kept under ice-cooling for 10 min. ^{13}C-Urea in the same buffer solution (100 μl: ^{13}C-urea 10 mg) was added to the NMR tube. The ^{13}C-NMR spectrum was measured at 20 °C at intervals of 1 min.

Screening of Urease Inhibitors by ^{13}C-NMR Test compounds were dissolved in ethanol or 0.07 M sodium phosphate buffer (pH 6.5) and diluted to the desired concentration. Jack bean urease (28.2 units; 0.2 mg) and a test compound were added to a mixture of ethanol (100 μl) and the above buffer (400 μl) in a 5 mm NMR tube; the tube was allowed to stand at 22 °C for 30 min, then was kept under ice-cooling for 10 min. ^{13}C-Urea in the same buffer solution (100 μl: ^{13}C-urea 1 mg) was added to the NMR tube. The ^{13}C-NMR spectrum was measured at 20 °C at intervals of 20—60 sec.

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