Immunological Specificity of *Helicobacter pylori* Urease and Identification by Immunological Detection of Its Specific Urease

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Abstract

Helicobacter pylori urease was recovered as a single peak by DEAE-Sepharose column chromatography and Sephacryl S-200 gel filtration. The purified urease was obtained by fast protein liquid chromatography using a Mono Q column. The purified urease preparation gave a single band in polyacrylamide gel disc electrophoresis.

Latex particles were sensitized with anti-urease immunoglobulin. The sensitized latex particles were agglutinated with the purified urease and by cell sonicates obtained from 55 strains of *H. pylori* which were isolated from the gastric mucosa of patients with gastric and duodenal disorders, while they did not react with those obtained from related bacteria known to be urease producers, such as *Helicobacter mustelae* and urease-positive "*Campylobacter lari* variants", or by urease of some strains of *Enterobacteriaceae*. We have developed a specific and sensitive method for detecting the urease by using the reversed passive latex agglutination technique, in order to identify of the organism.

Introduction

Recently a possible role of *Helicobacter pylori* in the etiology of chronic gastritis and gastric or duodenal ulcers has been proposed by several workers^{1)~3)}. The organism has been detected at a high frequency in biopsy specimens of gastric mucosa from patients with gastric diseases. This organism produces urease, and in this way differs from species of *Campylobacter*. The fact that *H. pylori* produces urease was first observed in a strain isolated by Langenberg et al⁴⁾ in the Netherlands in 1984. Owen et al⁵⁾ confirmed this characteristic property of *H. pylori* in strains isolated in Australia, West Germany and the United Kingdom. The urease of *H. pylori* has been described as a highly active enzyme, more active than that of urease produced by *Proteus* species. Indeed, *H. pylori* urease has been directly detected in biopsy specimens from patients with gastritis. We developed a specific and sensitive method for detecting the enzyme by using the reversed passive latex agglutination (RPLA) technique and used it to identify the organisms.

Materials and Methods

Bacterial strains: *H. pylori* strains NCTC 11637, NCTC 11638 and NCTC 11639, obtained from B.J. Marshall were clinically isolated, and 55 isolates of *H. pylori* were cultured from gastric biopsies of patients with gastritis, gastric ulcer and duodenal ulcer as previously described³⁾. As related bacteria 4 strains of *H.*

mustelae (NCTC 12032, F6, F7, F10) were received from D.S. Tompkins and R.J. Owen and 8 were isolated from ferret stomach. Two strains of urease-positive "*Campylobacter lari* variants" were provided by F.J. Bolton. As urease-positive *Enterobacteriaceae*, 4 strains of *Proteus mirabilis*, one strain of *Proteus vulgaris*, 4 strains of *Providencia rettgeri* and two strains of *Yersinia enterocolitica* isolated from human feces were used.

Bacterial growth for purification of urease and cell-free extract: *H. pylori* NCTC 11638 was cultured at 37°C for 2 days in blood agar medium under microaerophilic conditions, and the cells (3.45 g) were harvested and suspended in PEM buffer (20 mM phosphate buffer, 1 mM EDTA, 1 mM 2-mercaptoethanol, pH 7.0). The cell suspension was centrifuged, washed with PEM buffer and disrupted with a French press Model No. 550 1 M type, Ohtake Seisaku Inc., Tokyo, Japan) under 1,000 kg/cm² pressure. The suspension of disrupted cells was centrifuged (14,000 rpm, 20 min) to remove the cellular debris, and the supernatant was filtered through a 0.45- μ m Millipore filter disk. this cell-free extract was used as crude starting material.

Cultivation of the strains used for immunological investigation of urease and cell-free extracts: *H. pylori*, *H. mustelae* and the "*C. lari* variants" were grown on blood agar medium at 37°C for 2 days under microaerophilic conditions. The cells were suspended in 10 ml of PEM buffer and disrupted with an ultrasonic disrupted (model UR 200P, Tomy Seiko, Ltd., Tokyo) with 40 watts for 5 min and the filtrate was used for urease assay and for RPLA.

P. mirabilis, *P. vulgaris*, *P. rettgeri* and *Y. enterocolitica* were inoculated in to 10 ml of brain heart infusion broth (Difco) containing 2% filter-sterilized urea and cultured at 37°C for 4–5 hours with shaking. The cells were subjected to ultrasonic treatment as described above.

Column chromatography: Urease was purified by chromatography on a DEAE-Sepharose CL-6B column (2.5 $\times$ 17 cm), a Phenyl-Sepharose CL-4B column (1.5 $\times$ 32 cm), and a Sephacryl S-200 SF column (1.5 $\times$ 88 cm) at 4°C described by Haushinger⁶. Fast protein liquid chromatography on a Mono-Q column (0.5 $\times$ 5 cm) and a Superose 6 column (1.0 $\times$ 30 cm) was performed at room temperature (Fig. 1).

Gel diffusion test: The double gel diffusion test was performed by the method of Ouchterlony⁷.

Polyacrylamide gel disc electrophoresis: Purity of the purified urease was determined by disc electrophoresis on a 7.5% polyacrylamide gel with 0.1 M tris-glycine buffer (pH 8.3) as described by Davis⁸.

Assays for urease: According to Chaney and Marbach⁹, urease activity indicates the amount of ammonia released from urea. Twenty μ l of urease was added to 1 ml of PEM buffer (pH 7.0) containing 50 mmol of urea, and the mixture was incubated at 37°C for 10 min. The released ammonia was measured by the Berthelot reaction. One unit of urease activity was defined as the amount that hydrolyzes 1 ml of urea per min.

Rabbit antiserum to urease: Antiserum was produced in rabbits with purified urease plus Freund's complete adjuvant.

Purification of immunoglobulin from anti-urease serum: For this purpose a Protein A Sepharose CL-4B column (1 $\times$ 8 cm) equilibrated with 0.1 M tris-HCl buffer (pH 7.2) was used. Anti-urease serum was added to the column. After being washed with 0.1 M tris-HCl buffer (pH 7.2) to remove the non-adsorbed material, antiserum was eluted with a 3 M NH₄ CN solution to separate IgG. The IgG fraction was concentrated with a PM-10 ultrafilter (Amicon) and applied to a Sephadex G-25 column (2.6 $\times$ 40 cm) equilibrated with 1/15 M PBS (pH 7.2) to remove NH₄CNS.

Anti-urease immunoglobulin-sensitized latex: Polystyren latex particles (SDL-59, 0.9 μ m in diameter, Takeda Chemical Industries, Ltd., Osaka) were suspended in 1/15 M PBS (pH 7.2) to 0.25% concentration. This suspension was mixed with an equal volume of a 15–20 μ g/ml solution of anti-urease immunoglobulin prepared as above and sensitized at room temperature for 1 hour (Fig. 2). The sensitized latex particles

Fig. 1 Procedure for purifying urease of *H. pylori*

Fig. 2 Procedure for coupling anti-urease IgG to polystyrene latex particles

were adjusted to 0.025% concentration with the diluent.

Reversed passive latex agglutination: Various cell extracts were examined for urease by microplate agglutination. Forty-fold serial dilution of purified urease and the cell extracts were prepared and 0.025 ml of each dilution was inoculated on a flat type microtiter plate, and 0.025 ml of a 0.025% suspension of sensitized latex particles was added to each plate. The plates were allowed to react in a humidified box at room temperature for 12–16 hours. Latex particles sensitized with normal rabbit immunoglobulin were used as a control.

Results

1. Purification of urease

Cultivated of *H. pylori* NCTC 11638 cells were disrupted with the French press, and the supernatant after centrifugation was chromatographed on DEAE-sepharose equilibrated with PEM buffer. After the urease was eluted with PEM buffer. After the urease was eluted with PEM buffer containing 0.2 M NaCl, further elution was performed with PEM buffer containing 0.5 M NaCl. Urease activity was detected as a single peak in the PEM buffer fraction containing 0.2 M NaCl. The active fractions were chromatographed on a Phenyl-Sepharose CL-4B column equilibrated with PEM buffer containing 2 M NaCl, and urease was eluted with PEM buffer. The concentrated fractions were subjected to gel filtration on a Sephacryl S-200 Superfine column equilibrated with PEM buffer. To remove remaining impurities. The filtrate was subjected to fast protein liquid chromatography on a Mono-Q column. The urease showing a single peak was eluted with a linear gradient from 0 to 2 M NaCl in PEM buffer (Fig. 3). Final chromatography was on a Superose 6 column for desalting. Urease was purified 80.7-fold by the above process, and the overall recovery was 9.5%. The purified urease showed a single band on polyacrylamide gel disc electrophoresis.

2. Immunological specificity of purified urease

Antisera were prepared by immunizing rabbits with crude urease obtained by DEAE-Sepharose chromatography and with purified urease, and tested for immunological specificity by the Ouchterlony gel diffusion method. Crude urease gave three precipitin lines with crude antiserum, but purified urease gave a single precipitin line with anti-purified urease serum and anti-crude urease serum (Fig. 4).

3. Detection of *H. pylori* urease by RPLA

The latex particles sensitized with anti-urease immunoglobulin showed agglutination by purified urease on the microtiter plate. Using the purified urease, RPLA was used to determine the minimum

Fig. 3 Fast protein liquid chromatography of *H. pylori* urease on a Mono-Q column. The absorbance was monitored at 280 nm (—), and urease activity (---) was determined for aliquots of 0.5 ml fractions. (·····) linear gradient of NaCl.

Table 1 Reactivity of urease from cell extracts of *H. pylori* and other bacteria with latex particles coupled with anti-urease immunoglobulin

Organisms	RPLA titer by Anti- <i>H. pylori</i> urease	Urease activity ^a (units/ml)
<i>H. pylori</i>		
NCTC 11637	10,240 ^b	112.3
NCTC 11638	40,960	115.4
NCTC 11638	10,240	103.2
Isolates (n=55)	10,240-40,960	37.1-132.1
<i>H. mustelae</i>		
NCTC 12032	—	125.5
F 6	—	182.6
F 7	—	48.0
F 10	—	29.1
Isolates (n=7)	—	31.1-152.4
" <i>C. lari</i> vari."		
10231/7/83	—	23.0
3980/1/83	—	23.5
<i>Proteus</i> spp (n=5)	—	31.6-35.5
<i>Providencia</i> sp (n=4)	—	19.3-28.4
<i>Yersinia</i> sp (n=2)	—	7.1-30.3
Jack bean	—	355.4

a) Units of urea hydrolysed/min/ml

b) The reciprocal of the highest dilution exhibiting agglutination by RPLA

Fig. 4 Ouchterlony double gel diffusion test of purified urease of *H. pylori* (A) with anti-purified urease serum (B) and anti-crude urease serum (C).

Fig. 5 Detection of *H. pylori* urease by RPLA

Rows 1a to 4a: Anti-urease IgG-sensitized latex particles.

Rows 1b to 4b: Normal IgG-sensitized latex particle.

Rows 1a, 1b: Purified urease. Well 1, 13.0 $\mu\text{g}/\text{ml}$. Wells 2-12, 4-fold serial dilutions.

Rows 2a, 2b: Cell extract from *H. pylori* NCTC 11638.

Well 1, undiluted. Wells 2-12, 4-fold serial dilutions. Dilutions as in Rows 2a, 2b.

Rows 4a, 4b: Cell extract from *P. mirabilis*. Dilutions as in Rows 2a, 2b.

detectable amount of urease in the microtiter plate. Serial 4-fold dilutions of a concentration of 13.0 μg of purified urease per ml were agglutinated up to by the sensitized latex particles (Fig. 5). It was deduced from these results that the RPLA method can detect urease in a concentration of 1 ng per ml.

Immunological specificity of urease produced by *H. pylori* and that of ureases of other bacteria was investigated by RPLA. The sensitized latex particles showed high agglutination titer from 1:10,240 to 1:40,960, with *H. pylori* NCTC 11637, NCTC 11638 and NCTC 11639 (Table 1). Further, agglutination titer from 1:10,240 to 1:40,960 were observed in 55 strains of *H. pylori* isolated from gastric mucous. Eleven strains of *H. mustelae* showed no agglutination with the sensitized latex particles. Furthermore, two strain of "*C. lari* variants" showed 23.0 and 23.5 units of urease activity per ml, respectively, but they also did not react with these ureases.

The sensitized latex particles were neither agglutinated by urease-positive *Enterobacteriaceae* nor reacted with Jack bean urease.

Discussion

In order to develop a method for detecting *H. pylori* urease by immunological means, the authors first purified the urease of this strain. The urease was absorbed on a DEAE-Sepharose CL-6B column and eluted with PEM buffer containing 0.2 M NaCl. It was absorbed on a Phenyl-Sepharose CL-4B column with a buffer of 2.0 M NaCl and could be eluted with salt-free buffer. Urease could not be completely separated from any contaminating protein by Sephacryl S-200 SF chromatography, but it was purified by fast protein liquid chromatography on a Mono-Q column.

To study the immunological specificity of *H. pylori* urease, antiserum to the urease was prepared, since the present antiserum specifically precipitated only urease of *H. pylori*. Guo and Liv¹⁰⁾ and Jones and

Mobley¹¹⁾ reported that urease of *P. vulgaris* is immunologically related to that of *P. mirabilis* but differs from that of *Morganella morganii*. These observation suggested that ureases produced by bacteria have immunological specificity for the genus of species. Therefore, the detection of urease based on RPLA was investigated by binding anti-*H. pylori* urease immunoglobulin to polystyrene latex particles. Anti-*H. pylori* urease serum reacted with urease of *H. pylori* but showed no reaction with urease-positive *H. mustelae* isolated from ferret gastric mucous or "C. lari variants" which can produce urease or with ureases produced by *P. mirabilis*, *P. vulgaris*, *P. rettgeri* and *Y. enterocolitica*. These results show that the urease of *H. pylori* has significant immunological specificity.

Since *H. pylori* urease may play a role in gastritis and has been shown to be an important colonizer in gastric mucosa^{12,13,14)}, the enzyme urease is the most studied of all the *H. pylori* products. It was found to be an extremely active protein with a molecular mass of about 600 kDa, and isoelectric point of 5.9 and composed of two subunits^{15,16)}. The native urease enzymes of *H. mustelae*, *H. felis* and *H. nemestrinae* were recently found to be almost identical in molecular mass and isoelectric point¹⁷⁾. It is necessary to define the relationship of immunological properties to the physical characteristics of the ureases in *Helicobacter* species.

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Helicobacter pylori が産生するウレアーゼの免疫学的特異性と ラテックス凝集反応による本菌の同定

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要 旨

Helicobacter pylori のウレアーゼを免疫学的手段により検出する方法を開発するため、本菌のウレアーゼの精製を行った。*H. pylori* NCTC11638株の菌体をフレンチプレスで破壊し、菌体成分を除去した上清液から、各種のクロマトグラフィーにより精製したウレアーゼは polyacrylamide gel disc electrophoresis により単一なバンドが得られた。さらに精製したウレアーゼを家兎に免疫して作成した抗ウレアーゼ血清は *H. pylori* のウレアーゼと反応し、*Yersinia enterocolitica* のウレアーゼとは反応しなかった。本抗血清からアフィニティークロマトグラフィー

により IgG 画分を精製し、ラテックス粒子に感作させた。本感作ラテックスは *H. pylori* の精製ウレアーゼおよび *H. pylori* 55菌株のウレアーゼと明瞭に凝集反応したが、*H. mustelae* のウレアーゼとは反応しなかったし、ウレアーゼを産生する “*Campylobacter lari* variants” とも反応しなかった。さらに本感作ラテックスは *Proteus mirabilis*, *P. vulgaris*, *P. rettgeri*, *Y. enterocolitica* のそれぞれのウレアーゼとも反応しなかった。著者らは *H. pylori* の産生するウレアーゼの免疫学的な特異性を指摘するとともに、ラテックス凝集反応により *H. pylori* を簡易に同定できることを明らかにした。