Food constituents enhance urease activity in *Helicobacter pylori.*

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Abstract

Urease activity of *Helicobacter pylori* recovered from the stomach of H. pylori-infected Mongolian gerbils was affected by the diet used after infection. The effect of dietary components on urease activity was investigated by growth of *H.pylori* in Brucella broth with the addition of calcium, collagenpeptide and proline. Urease activity was about 10 times higher after growth of *H. pylori* in the presence of collagenpeptide. The activity of urease gene cluster components was measured in cell-free extracts of transformed *E.coli* DH5a cultured in the presence of calcium, collagenpeptide and proline. *UreA* activity was not affected whereas *ureI* promoter activity increased on the addition of collagenpeptide or proline to the medium. Due to the important role of urease in infection by *H. pylori* these results suggest the possibility that diet may influence the appearance of symptoms in *H.pylori* infected *Mongolian gerbils.*

Keywords: *H. pylori,* urease activity, proline

1. Introduction

Helicobacter pylori is a well-known bacterium which induces a chronic inflammatory response in the stomach and causes gastritis, peptic ulcer disease, gastric adenocarcinoma[4, 21] and gastric lymphoma[12, 25]. This microorganism has a spiral body with unipolar sheathed flagella, which is well adapted for motility in a viscous environment[7]. The essential bacterial enzyme responsible for survival in the strongly acidic conditions in the stomach is urease[15, 23], which is expressed at high levels in *H. pylori*[3]. This enzyme converts urea into ammonia and bicarbonate. The ammonia produced protects *H. pylori* against the acid conditions[14, 24], however ammonia causes damage to the gastric epithelium[17] of the host, so that urease is an important factor in the pathogenicity of this microorganism.

The relationship between diet and *H. pylori*-induced gastritis is still largely unknown. *Mongolian gerbils* are easily colonized with *H. pylori* with associated development of chronic gastritis, gastric ulcers and intestinal metaplasia after long-term infection[8]. Using this animal model a diet containing fishmeal was found to enhance *H. pylori* induced gastritis in comparison with a control diet in which the fishmeal component was replaced by casein[20].

We focussed at this point on an investigation of whether induction of urease activity in *H. pylori* could be influenced by dietary components. In this study we demonstrate a relationship between food ingredients and enhancement of urease activity *in vivo.* We also show that these food components can influence the activity of *H.pylori* gene cluster promoters.

2. Materials and Methods

2.1 Bacterial strains, plasmids and growth conditions.

Bacterial strains used in this study included *H. pylori* ATCC43504 and *E. coli* DH5α and XL1Blue. *H. pylori* ATCC43504 was cultured in Brucella Broth containing 3% heat inactivated horse serum (GIBCO BRL) supplemented with 12.5 μg / ml of vancomicin and 2.5 μg / ml of amphotericin B. Cells were incubated at 37 ℃ under microaerophilic conditions of 10% CO_2, 5% O_2 and 85% N_2. *E. coli* DH5α and XL1Blue were cultured in Luria Broth (LB) medium. When required

Table 1. Bacterial strains and plasmids used in this study.

Strains or plasmids	Relevant characteristics	Source or references
H.pylori		
ATCC43504	Typed laboratory strain	NCTC11637
E.coli		
DH5 α	F'/*endA1 hsdR1 supE44 thi-1 recA1 gyrA relA1* Δ(*lacZYA argFJU169 deoR*)	Woodcock,D.M *et al.* 27) Raleigh,E.A *et al.* 16)
Plasmids		
pBlueScript II SK(−)	Amp	
pXEK10	pX1918 plus Km^r *Eco*R I fragment of pUC4K at the partial site of *Eco*R I	This study
pHPT17	12.8kb fragment with the urease gene cluster	Takeuchi *et al.* 20)
pJ43	pBlueScript II SK(−) plus 1.3kb *Bam*H I fragment of pHPT177 with *ure*I	Akada *et al.* 1)
pJ58	pBlueScript II SK(−) plus 671bp *Mlu* I −*Hind*III fragment of pHPT177 with *ure*A	Akada *et al.* 1)
pS33	pBlueScript II SK(−) plus 250bp PCR gene fragment of pJ43	This study
pSUA20	pJ58 plus *xyl*E-kan *Xba* I fragment from pXEK10	This study
pSUA25	pJ33 plus *xyl*E-kan *Xba* I fragment from pXEK10	This study

kanamycin and chloramphenicol were added to a final concentration of 25 μg / ml and 10 μg / ml, respectively. Plasmids used in this study are listed in Table1.

2.2 Urease activity

H. pylori cells in 5 ml of culture were harvested and washed twice with 10 mM sodium phosphate buffer (pH 7.5). The pellet was resuspended in 2 ml of the same buffer and sonicated. 0.1 ml of crude cell extract was used for the reaction to measure the release of ammonia according to the procedure of Ferrero & Lee[5]. One unit of activity is defined as the amount of enzyme required to hydrolyze 1 mmol of urea per min at 37 ℃. Specific activity is defined as unit per milligram of protein. The urease activity of *H. pylori* recovered from the mucus layer of the stomach in *H. pylori*-infected Mongolian gerbils which were fed a controlled diet, the ingredients of which are summarized in Table 2, was measured as follows; *H. pylori* in the mucus layer was recovered, after a brief wash in PBS, by homogenisation in 3ml PBS (pH7.0), half of the homogenized mixture was sonicated and centrifuged and the urease activity in the supernatant was measured as above. Urease activity per cell was determined by dividing the measured enzyme activity by the CFU, determined using the remaining half of the homogenized mixture.

2.3 Construction of the tools for measuring promoter activity.

The reporter gene *xylE* was used to measure promoter activities in the urease gene cluster. The *ureA* promoter region was amplified by PCR using primers, 5'- TACCAGCTCTCGCTTCTT-3', 5'-TAATGCGCAAAAGGGAGCT-3'. The PCR

Table 2. Composition of diets used for H. pylori-infected Mongolian gerbils.

Ingredient	CE-2 (%)	10% casein diet (%)
Protein	25.0	28.0
Fat	4.7	3.6
Fiber	4.0	3.0
Ash	6.0	3.8
Nitrogen free extracts	53.0	53.4
Sodium chloride	0.9	0.7
Water	7.3	8.2

＊10 % fish meal was contained in CE-2.

reaction was performed using KOD-Plus DNA polymerase (TOYOBO) for 30 cycles with each cycle consisting of 30 sec at 94 ℃, 1 min at 54 ℃ and 1 min at 68 ℃. The PCR product was applied to a 0.8% agarose gel to purify a 0.6 kb DNA fragment containing the *ureA* promoter region. The DNA fragment was digested by *MluI*, at a site newly created in the primer, and blunted with T4 DNA polymerase. The DNA fragment was further digested by *Hind*III. The fragment was inserted between *Eco*RV and *Hind*III sites of pBlueScript SKII (-). This recombinant plasmid was recut with *Hind*III and blunted by T4 DNA polymerase to create an *Nhe*I site, this was designated pS33. The KM cassette from pUC4K, the orientation of which was in the same direction as the reporter gene, *xylE*, was inserted at the *Eco*RI site of px1918 which was partially digested. pXEK10 that carried *xylE*::KM cassette in this order was cut with *Xba*I and inserted into the compatible *Nhe*I site of pS33, which was designated pSUA20. The *ureI* promoter region was amplified by PCR using primers, 5'-GACGAA TTCGCGCAACTCTTTAGC-3' and 5'-GACTCTA GACGCAAATCCCATTGC - 3', which were designed to add *Eco*RI and *Xba*I sites. KOD-Plus DNA polymerase was used for the PCR for 30 cycles with each cycle consisting of 30 sec at 94 ℃, 1 min at 58 ℃ and 1min at 68 ℃. The PCR product was applied to a 0.8% agarose gel and a 300 bp DNA fragment that included the *ureI* promoter region was purified. This was digested with *Eco*RI and *Xba*I and the fragment was inserted into pUC19 at the same restriction site. The reporter gene, *xylE*, with KM cassette was inserted downstream of *ure*I promoter region at the XbaI, site, this was designated pSUI25.

2.4 Catechol 2,3-dioxygenase assay.

To prepare samples for enzymatic assay overnight cultures were diluted 1:50 in 50 ml of 56 minimal medium modified by Low[9] containing 10^{-8}M thiamine and appropriate antibiotics and cultured at 37 ℃. When testing the effect of sugars on the promoter activity, glucose was replaced by other sugars; fructose, galactose, mannose, sorbitol, saccharose and xylose, at a concentration of 15 mM. In other cases appropriate compounds; 1 mM amino acids, 0.1 - 10 mM calcium chloride or 1 - 10 % (W/V) collagenpeptide, enzyme hydrolyzed (Wako Pure Chemical Industries, LTD.) were added to medium containing glucose. Harvested cells were washed twice with 25 μl ice-cold 0.1 M potassium phosphate buffer (pH 7.5). The pellet was resuspended in 1.5 μl of 0.1 M potassium phosphate buffer containing 10 % acetone, sonicated and centrifuged at 8000 rpm for 5 min. Enzyme activity was measured by the addition of 25 ml of supernatant and 100 μl of 10 mM catechol to 2.9 ml 50 mM potassium phosphate buffer, pH7.0, after incubation for 5 min at 27 ℃ the OD_{375} was measured[10].

One milliunit is equivalent to the formation of 1.0 nmol of 2-hydroxymuconic semialdehyde per min at 27 ℃. Specific activity is defined as milliunit per milligram of protein.

3. Results

3.1 Urease activity of single cell of *H. pylori* isolated from *Mongolian gerbils.*

The number of *H. pylori* cells recovered from the stomach of infected *Mongolian gerbils* fed with different diets (Table 2) was measured. Using the CE-2 diet (A group) 657.5 ± 211.8 *H. pylori* cells / ml were recovered, using a casein based diet (B group), 611.7 ± 133.4 cells / ml were recovered. Urease activities of *H. pylori* recovered from the A group and B group were 118 ± 128 munit / cell and 2.62 ± 2.89 munit / cell, respectively. The DNA type of recovered *H. pylori* was confirmed by RAPD methods[2], all recovered samples showed similar finger printing patterns to that of H.pylori ATCC43504, which was used for infection (Fig.1).

3.2 Urease activity of *H. pylori* cultured with collagenpeptide.

The urease activities of *H. pylori* grown in Brucella broth with the addition of various concentrations of collagenpeptide were measured. The results are presented in Fig. 2. Growth in the presence of collagenpeptide was found to increase the *H.pylori* urease activity. Urease activity reached its highest level, 47.9 ± 2.0 unit / mg of protein, at 7 % collagenpeptide concentration, at this concentration enzyme activity was approximately 5 times higher than that of *H. pylori* cultured in Brucella broth not containing collagenpeptide.

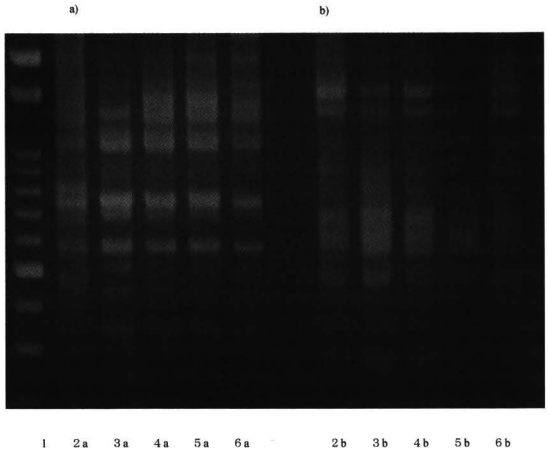

Fig. 1. Identification of *H. pylori* isolated from *Mongolian gerbils* using RAPD Method. 1:100bp marker, 2: *H. pylori* ATCC43504, 3,4: H. pylori isolated from A group (fish meal diet), 5,6: *H. pylori* isolated from B group (casein diet). Primers used for fingerprinting were a) AACGCGCAAC and b) CCGCAGCCAA.

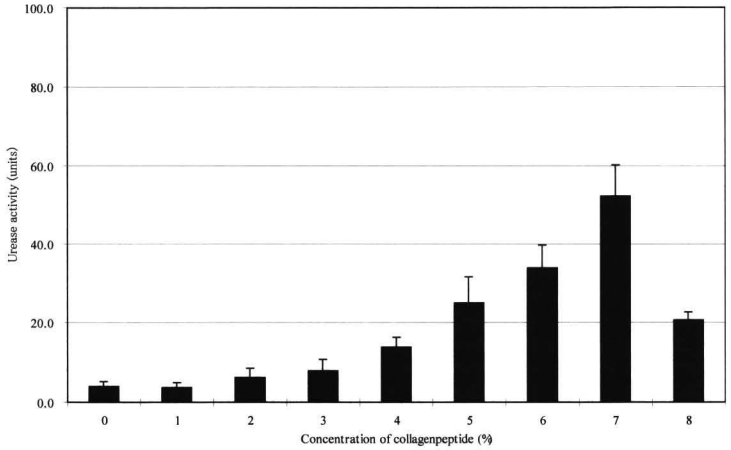

Fig. 2. Urease activity of *H. pylori* cultured in the presence of collagenpeptide. Urease activity was measured as described in Materials and Methods (micromoles of NH_4 released per minute per milligram of protein, n=3, ± SD).

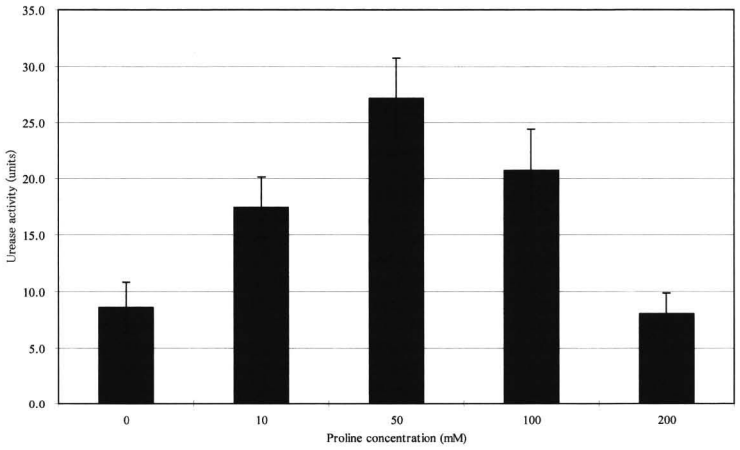

Fig. 3. Effect of proline on urease activity of *H. pylori.* Addition of 50 mM proline showed the highest urease activity. (micromoles of NH_4 released per minute per milligram of protein, n=3, ± SD). per minute per milligram of protein, n=3, ± SD).

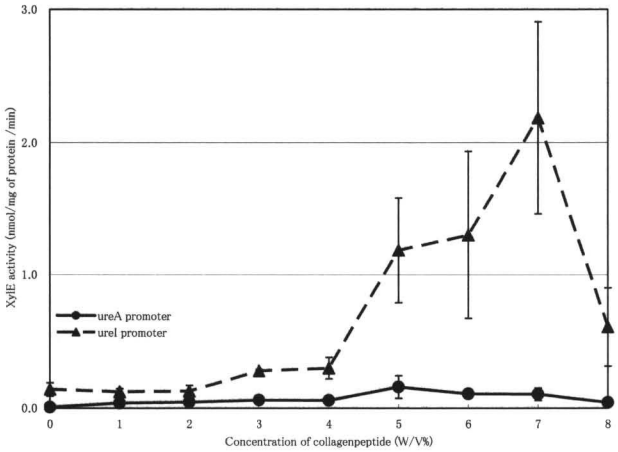

Fig. 4. The effect of collagenpeptide concentration on *ureI* (▲) and *ureA* (▲) promoter activities of *H. pylori* expressed as XylE fusions in *E. coli*. Both *ureA* and *ureI* promoter activities were measured as XylE activity as described in Materials and Methods (nmole of 2-hydroxymuconic semialdehyde formed per minute per milligram of protein, n=3, ±SD).

3.3 Urease activity of *H. pylori* cultured in excess proline.

H. pylori was cultured in Brucella broth with the addition of proline to a final concentration of 0 - 200 mM. The urease activity of cell-free extracts of *H. pylori* cultured in each of these conditions is summarized in Fig. 3. The peak urease activity was found at 50 mM proline.

3.4 Effect of calcium, collagenpeptide and proline on *ure* promoter activities.

Promoter activities were measured at calcium concentrations up to 10mM. Calcium was not found to affect either *ureI* or *ureA* promoter activity. The *ureI* promoter activity was strongly influenced by the addition of collagenpeptide (Fig. 4) or proline to the medium, while these additions did not affect *ureA* promoter activity. As shown in Fig. 4 *ureI* promoter activity was maximally affected by collagenpeptide at a concentration of 7% (w/v), the same concentration as had been found to maximally enhance urease activity.

4. Discussion

The survival of *H.pylori* in the acidic gastric environment is dependent on the presence of active urease. The enzyme is composed of two types of polypeptide, *ureA* and *ureB*, which form a large macromolecular complex containing numerous active sites, the proximity of which may provide mutual protection against acid inactivation[6]. Other products of the urease gene cluster, *ureEFGH* probably have roles in the assembly of the enzyme complex and in the incorporation of nickel at the active sites[11, 22]. The *ureI* gene may encode an acid activated urea transporter[14]. Transcription of the urease gene cluster occurs from two promoter regions, one upstream of *ureA* and the second upstream of *ureI*[1, 16, 18]

Cell-free extracts of *H. pylori* recovered from infected Mongolian gerbils were found to contain different levels of urease activity depending on the diet fed after *H. pylori* infection. Higher levels of urease activity were found when feeding with the CE-2 diet, which contains fishmeal, relative to the control diet. Based on the hypothesis that some dietary components might enhance urease activity, we proceeded to examine the effect of ingredients present in the fish diet, but absent from the casein diet, on urease activity. In addition the possible effect of dietary components on gene expression levels was studied using *xylE* fusions containing *ureA* and *ureI* promoters of *H. pylori*.

The addition of collagenpeptide to the Brucella medium used to culture *H. pylori* was found to result in an enhancement of urease activity. Since collagen contains approximately 10% proline the

effect of proline on urease activity was also measured. The addition of proline to the growth medium caused an increase in the measured urease activity which peaked at around 50mM proline.

The effects of collagenpeptide and of proline on promoter activities were examined. No effect was observed on *ureA* promoter activity. On the other hand both collagenpeptide and proline were found to increase *ureI* promoter activity. This result suggests that collagenpeptide and proline might exert their effect on urease activity through induction of expression of *ure* accessory proteins.

In order to further understand dietary influences on *H. pylori* infection, studies should be performed to measure *ure* promoter activities in *H. pylori* using an *H. pylori-E.coli* shuttle vector. *In vivo* studies of *H. pylori* infected *Mongolian gerbil* fed on appropriate diets containing collagenpeptide and proline could determine whether diet causes diagnostic variation in the stomach.

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