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Characteristics of Urease from *Vibrio parahaemolyticus* Possessing *tdh* and *trh* Genes Isolated in Korea

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***Vibrio parahaemolyticus* is a halophilic bacterium associated with seafood gastroenteritis. An unusual strain of Kanagawa-positive urease producing *Vibrio parahaemolyticus* O1 : K1 was isolated from the environment and identified. A polymerase chain reaction assay revealed that this strain harbored both the *tdh* and *trh* genes. The urease from this strain was studied. Maximum urease production was induced in LB medium containing 0.2% urea, 0.5% glucose, 2% NaCl and pH 5.5 with 6 h of cultivation at 37°C under aeration. Purification of urease was achieved by the process of whole cell lysate, 65% ammonium sulphate precipitation, DEAE-cellulose ion exchange column chromatography, Sepharose CL-6B gel filtration and oxirane activated Sepharose 6B-urea affinity chromatography with 203 fold purification and 2.2% yield. Analysis of the purified enzyme by SDS-PAGE demonstrated the presence of the subunits with a molecular weight of 85 kDa, 59 kDa, 41 kDa and the molecular weight for the native enzyme by nondenaturing PAGE and gel filtration chromatography was 255 kDa. The purified urease was stable at pH 7.5 and the optimal pH in HEPES buffer was 8.0. The enzyme was stable at 60°C for 2 h with a residual activity of 32%. The addition of 10 μM of $NiCl_2$ maintained stability for 30 min. The *Km* value of the purified enzyme was 35.6 mM in urea substrate. The TD_{50} (median toxic dose) of the purified urease was 2.5 μg/ml on human leukemia cells.**

***Key words*:** urease, *Vibrio parahaemolyticus*

Urease is an enzyme which hydrolyzes urea to yield ammonia and carbon dioxide (30). It is produced by over one hundred species of bacteria and has been used in taxonomic identification; the pathogenic role of this enzyme has become appreciated (6, 19, 21, 23). Urease is an important enzyme in relation to pathogenicity and in consequence, its pathogenic role in bacteria is of interest. Since urease is a virulence factor in human infection of the urinary and gastrointestinal tracts, it presumably contributes to the pathogenesis and inhibition of mucus biosynthesis. It also causes intestinal mucus to dissemble at the mucosal surface. These changes that occur facilitate colonization and possibly promote the formation of ulcers (7, 9, 19) by releasing a high concentration of ammonium ions from urease.

Early studies of the pathogenicity of halophilic bacterium *Vibrio parahaemolyticus* discussed the Kanagawa-phenomenon, a zone of beta-type hemolysis around colonies on special blood agar and production of thermostable direct hemolysin (TDH) or TDH related hemolysin (TRH) (1, 11, 12). TDH and TRH encoded by the *tdh* and *trh* genes, respectively, are considered important virulence factors in the pathogenicity of this strain (35). Since the outbreak of gastroenteritis of a urease-positive *V. parahaemolyticus* was reported by Huq *et al.* (13), urease has been recognized as an important virulence factor in the pathogenesis of this strain. It has been suggested that the ability to hydrolyze urea can be used as a marker to predict potential pathogenicity of this bacterium (14, 16, 25-27). The relationship between urease activity and the presence of either *tdh* or *trh* genes has been recognized. There is evidence that urea hydrolysis is not a reliable marker for identifying *tdh*-carrying *V. parahaemolyticus*. However, it may be a marker for *tdh*-carrying strains (27) or urea hydrolysis and supressed production of TDH by this strain associated with the presence of a TDH-related hemolysin gene with unknown mechanisms (26).

There have been limited reports on *V. parahaemolyticus* urease (3). In the present study we examined some physicochemical properties of urease produced by an environmental isolate of *V. parahaemolyticus* which contains *tdh* and *trh* genes, Kanagawa-positive, which was isolated in Korea.

Materials and Methods

Organism and culture conditions

The organism used throughout this study was isolated from

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sea water and sediment from the southern part of Korea. The bacteriological screening procedures adopted were described by Kaysner *et al.* (14). It was identified by plate medium, sucrose-negative isolates on thiosulfate citrate bile salts sucrose agar (Eiken Chemical Co., Ltd., Tokyo, Japan) and beta-hemolysin test (thermostable direct hemolysin production) on Wagatsuma agar. Further biochemical testing was performed by Biolog Microstation (E-10136, Biolog Microstation, Biolog, USA). Urea hydrolysis was confirmed on Christensen's urea agar and urea broth (Difco Laboratories, Detroit, MI., USA) supplemented with 2% NaCl after 18 h at 37°C cultivation by color change (pinkish) seen by the naked eye. Serotyping (O and K antigens) was also performed with a commercially available rabbit antiserum (Denka Seiken Co, Ltd., Tokyo, Japan). The broth medium employed for urease production was LB containing 0.2% urea, 0.5% glucose and 2% NaCl with initial pH 5.5, for 6 h cultivation with aeration at 37°C. Cells were harvested by centrifugation (3,000 × *g*, 10 min, 4°C) and stored as cell pellets at -20°C. The buffer used for the purification process was 20 mM phosphate buffer (pH 7.0) unless indicated.

Preparation of template DNA

The cells were cultured in LB broth containing 3% NaCl incubated at 37°C overnight. Chromosomal DNA was extracted by the method described by Sambrook (31).

Polymerase chain reaction (PCR)

A polymerase chain reaction (PCR) assay was performed to detect both *tdh* and *trh* genes. *V. parahaemolyticus* thermostable direct hemolysin gene detection primer was VPD-1/2 (S001) and VPS-1/2 (S002) purchased from Takara (Takara Code R001, Japan) was used. The predicted sizes of the amplified DNA from the *tdh* and *trh* genes, respectively, were 251- and 211-bp. The total volume of the PCR mixture was 30 μl, including 3 μl of template DNA. The PCR mixture was covered with 50 μl of mineral oil and subjected to PCR on a programmable incubator (Perkin Elmer, GeneAmp PCR system 2400, Norwalk, CT., USA). Thirty-five cycles of amplification were done at 94°C, 55°C, and 60°C (1-min each), with a final 5-min extension at 72°C. The amplified PCR products were analyzed using 2.0% agarose gel.

Preparation of crude extracts

The pellets in 5-ml aliquots were washed two times with saline and suspended in phosphate buffer. The final pellet was subjected to sonication in a cell sonicator (Sonifer 250, Branson, USA) and resuspended in phosphate buffer. Cell-free extracts were then obtained by centrifugation (10,000 × *g*, 20 min, 4°C).

Assay of urease activity

Urease activity was measured by the modified methods proposed by Weatherburn (36). Enzyme preparations of the appropriate dilution (50 μl) were added to 200 μl UHEP buffer (20 mM HEPES buffer[N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid], pH 7.5 containing 30 mM urea, 1 mM EDTA, 1 mM 2-mercaptoethanol) and incubated for 30 min at 37°C. Then 400 μl of phenol nitroprusside and alkaline hypochlorite each were added and incubated for 10 min at 50°C. Absorbance at 625 nm was read. One unit of urease activity (U) was defined as the amount of enzyme required to hydrolyze 1 μM of urea per min at 37°C and pH 6.8. Specific activities were expressed as μM of urea hydrolyzed per min per mg of protein. Protein was measured by Lowry's (20) method, with bovine serum albumin as the standard.

Purification of urease

All the steps in the purification of the enzyme were carried out between 25°C and 28°C, except size exclusion chromatography, which was performed at 4°C (5, 28, 31). The supernatant was saturated to 65% ammonium sulfate. The precipitates from the previous stage were applied to a DEAE-sephadex column chromatography (2 × 15 cm) equilibrated with phosphate buffer and eluted with a linear gradient of 0.2 M to 0.5 M NaCl in phosphate buffer at a flow rate of 20 ml/h. Throughout the procedure, column eluates were monitored for 280 nm absorbance. The active fractions were pooled and concentrated with 80% ammonium sulfate saturation. The precipitates were collected by centrifugation (10,000 × *g*, 20 min) and dialyzed 2-3 times at 4°C with phosphate buffer. The supernatant was pooled and loaded onto a Sepharose CL-6B gel column (2.5 × 73 cm) and eluted with a flow rate of 25 ml/h. Fractions of 5 each were collected and assayed for urease activity. The most active fractions were pooled and applied to an Oxirane activated Sepharose 6B-urea affinity column chromatography (1.5 × 10 cm) and eluted with a stepwise gradient of 10-50 mM urea at a flow rate of 20 ml/h (34, 37). Each fraction of 2 ml was pooled for activity.

Determination of subunits and native molecular mass

The molecular mass of subunits of the urease was determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) in 10% polyacrylamide gels with the Bio-Rad gel system according to Laemmli (18). The samples and molecular mass standards were denatured prior to electrophoresis for 3 min at 100°C in SDS buffer. Samples containing 5 μg/ml of protein and molecular mass standard was loaded onto 3.5-10% gradient acrylamide gels (Bio-Rad), electrophoresed at 25 mA for 1 h and Coomassie blue-stained. The native molecular mass was determined by Sephadex G-200 gel filteration. Molecular standards (Sigma) included carbonic anhydrase (29,000), albumin (66,000), alcohol dehydrogenase (150,000), β-amylase (200,000), apoferritin (443,000), and thyroglo-

bulin (669,000).

Kinetic properties

Fractions from the last purification step were assayed for heat stability after 10 min of heat treatment at different temperatures between 20°C and 100°C and the remaining enzyme activity was then determined. Optimum temperature for the purified urease was examined between 20°C and 70°C. To ensure buffering capacity at a wide pH range (5.5 to 9.0) the purified urease was put into citrate buffer, phosphate buffer and glycine-NaOH buffer each at a 20 mM concentration. Then each activity was determined. $NiCl_2$ and metal ions were added to the reaction mixture and was kept at 37°C for 5 min prior to the addition of the substrate.

Cytotoxic test

Cytotoxity was examined by the modified methods described by Mosmann (22). Human erythro leukemia cell line (K562) was cultured in complete RPMI 1640 medium (Sigma, USA) containing 10% fetal calf serum, 2 mM *L*-glutamine, 100 IU/mg penicillin, 100 mg/ml streptomysin, and 0.25 μg/ml amphotericin B (Gibco, USA). Serial cultivation was continued with final concentration of 0.5×10^6 cells/ml at 37°C in a 5% CO_2 incubator. Cultured cells were incubated for 4 days in minimum essential medium containing 10% fetal calf serum containing 10 mM urea and control without urea but with urease. After two washings with PBS (Mg^{2+}, Ca^{2+} free), two ml trypsin-versene was added, followed by harvesting. One hundred microliters of 1×10^3 cells/0.2 ml were transferred to a 96 well microplate and incubated for 18-24 h. Serially diluted purified urease was applied onto a microplate. Cellular viability was assayed by MTT (3-[4,5-dimethylthiazol-2-yl]2,5-diphenyl tetrazolium bromide, Sigma) dye reduction assay. Median toxic dose was determined by employing 50 μl of MTT (2 mg/ml in PBS) into each well and incubating for 4 h. The supernatant was discarded and 100 μl of dimethyl sulfoxide (DMSO) was added and mixed for 20-30 min at room temperature. The cell number was quantified by using a multiscanner (Titertek multiscan mcc/340, Flow Laboratories, USA) absorbance at 540 nm. TD_{50} was determined by student's test; p was <0.05.

Results

Selection of strain

Among the 30 samples examined, only 17 strains with six different somatic groups of O1, O2, O3, O4, O5, O8 with 17 different K antigen types were confirmed. All these serovars showed Kanagawa-positive but urease-negative, and only one serotype of O1 : K1 showed urease-positive. This strain was used for subsequent purification.

Polymerase chain reaction

The possession of both *tdh* and *trh* genes in our urease-positive strain is shown in Fig. 1 with the amplified products of 251 bp for *tdh* and 211 bp for *trh*.

Purification of urease

The urease was purified from the cell extract by sequential steps with 65% ammonium sulfate fractionation, DEAE-cellulose ion exchange column chromatography, Sepharose-CL-6B gel filtration, and final Oxirane activated Sepharose 6B-urea affinity chromatography. The purification results of urease are summarized in Table 1.

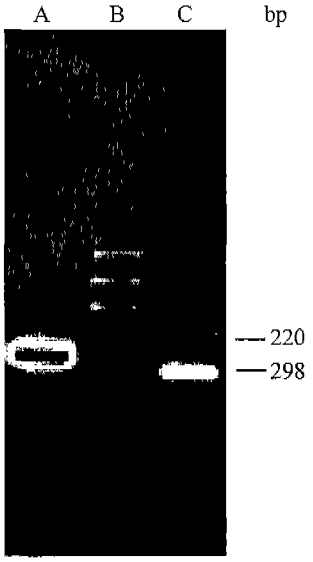

Fig. 1. PCR product of *tdh* gene and *trh* gene in *V. parahaemolyticus* O1 : K1. A; amplification product of *tdh* gene, B; 1 kb ladder marker, C; amplication product of *trh* gene.

Table 1. Purification of urease from *V. parahaemolyticus*

Purification step	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Whole cell lysate	1,491,000	10,080	148	1	100
Ammonium sulfate precipitation	1,349,000	1,137	1.186	8	91
DEAE-cellulose Chromatography	638,000	144	4,431	30	43
Sepharose CL-6B gel filtration	461,000	35	13,171	89	31
Oxirane-activated Sepharose 6B-urea affinity	33,000	1.1	30,000	203	2.2

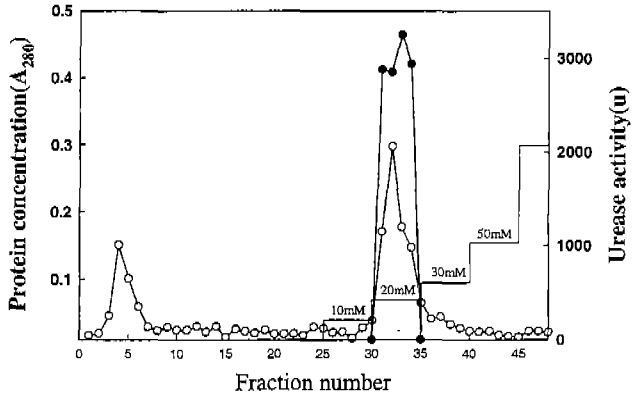

Fig. 2. Oxirane-activated Sepharose 6B urea affinity chromatography of *V. parahaemolyticus* urease. Active enzyme solution was loaded onto a column equilibrated with 20 mM phosphate buffer, pH 7.0. Enzyme was eluted with a stepwise gradient of 10-50 mM urea. Column size: 1.5 cm × 10 cm, Flow rate: 20 ml/hr, Fraction volume: 2 ml. Symbols: –○–, Protein concentration (OD at 280 nm); –●–, Urease activity (U).

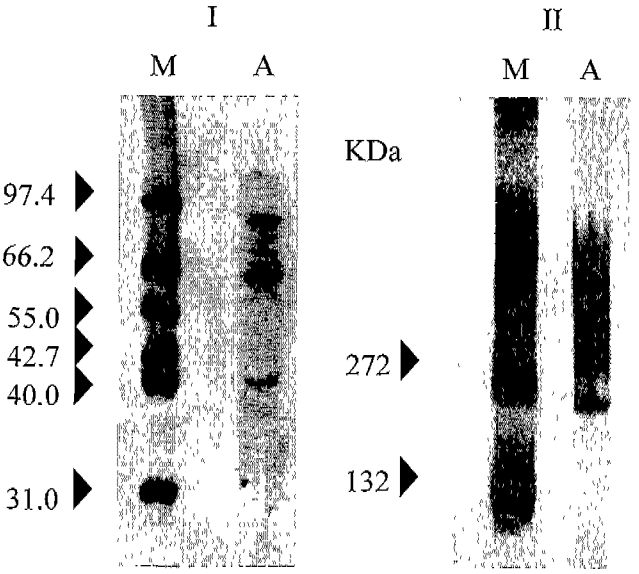

Fig. 3. SDS-PAGE (I) and nondenaturing PAGE (II) of the purified urease from *V. parahaemolyticus*. 1 μg of protein was loaded on a 10% SDS-PAGE system and Coomassie blue-stained. Lanes: M, molecular mass marker; A, purified urease.

The active fractions were pooled and chromatographed on a step of oxirane-activated sepharose 6B-urea affinity chromatography by a stepwise gradient of 10 mM-50 mM urea as shown in Fig. 2. The urease activity was detected at 20 mM of urea with specific activity of 30,000 units/ml. Four steps of purification employed by column chromatography led to 203-fold purification with an overall recovery of 2.2%.

Kinetic properties and heat stability

Analysis of the purified enzyme from the final purification step on SDS-PAGE demonstrated the presence of the three subunits with molecular masses of 85, 59 and 41 kDa. The native molecular mass of the purified urease was 255 kDa by Sephadex G200 gel filtration (Fig. 3, 4). The urease exhibited a *Km* value of 35.6 mM urea measured at pH 6.8 in HEPES buffer at room temperature. This value is consistent with the result of Nagano (23) since his microbial urease *Km* value was 18-72 mM depending on used buffer pH. Urease production by this strain was influenced by the initial pH of the basal medium. pH 5.5 rapidly increased the production of the enzyme and it was maintained up to pH 8.0. Below pH 5 or above pH 9.0 production fell rapidly. The purified enzyme was heat-stable. No remarkable loss of activity was observed upon incubation between 40°C and 50°C. There was a residual activity of 32% after 2 hr at 60°C; at temperatures above 70°C, rapid inactivation occurred (Fig. 5). The heat stability of our strain was a little lower than that of other bacterial urease (3); ours was stable at 90°C for 10 min. The purified urease lost approximately 50% of activity within 5 days upon storage at 4°C. Subsequent storage over a period of 2 weeks under the same conditions resulted in a loss of activity of 10%. We observed our urease was stable in the range of pH between 6.5 and 8.0 in 20 mM phosphate buffer system. Below or above these values, an irreversible inactivation occurred, and this buffer somewhat increased the activity. Complete loss of

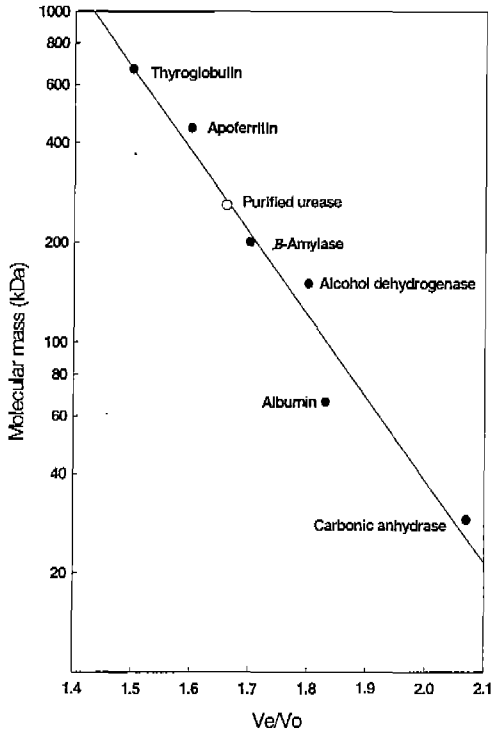

Fig. 4. Estimation of molecular mass of the purified *V. parahaemolyticus* urease on the gel filtration chromatography. Symbols : ○, Purified urease; ●, Molecular mass marker. Ve/Vo, elution volume/void volume.

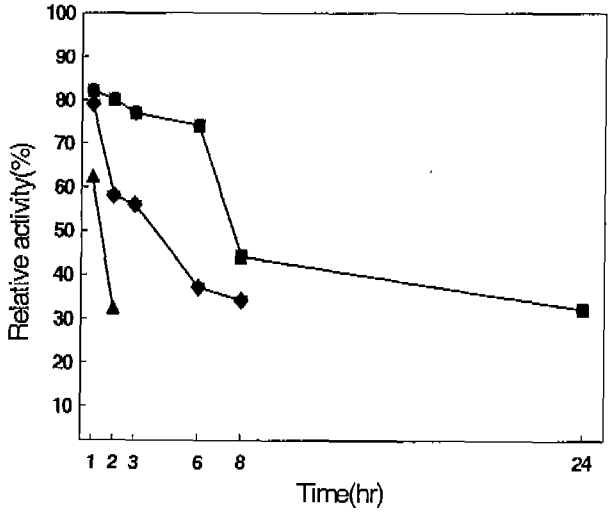

Fig. 5. Temperature stability of the purified urease from *V. parahaemolyticus*. To determine the temperature stability of urease, the enzyme was incubated for 15 min at the temperature indicated for 3 min prior to the urease activity determination. Symbols: ●, 40°C; ■, 50°C; ▲, 60°C.

activity occurred during storage at -20°C in phosphate buffer. The optimum pH was 7.5 in 20 mM phosphate buffer (Fig. 6). Urease is the first enzyme containing Ni^{2+} (6). $NiCl_2$ on urease remained active for 30 min at the concentration of 100 μM (data not shown). The addition of the metal ions did not affect urease activity when tested at an equimolar concentration (data not shown).

Cytotoxicity

Determination of cytotoxicity by growth inhibition was assayed by cellular viability. The cellular morphological

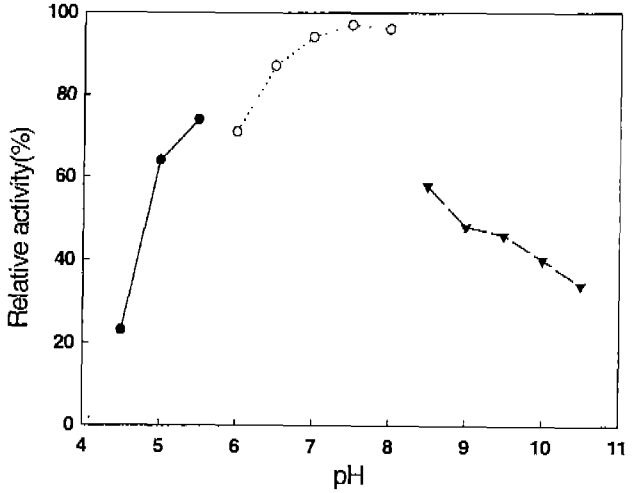

Fig. 6. pH stability of the purified urease from *V. parahaemolyticus* in buffer systems. For the determination of pH stability, the enzyme was incubated for 15 min at the indicated buffer. Symbols: –●–, 20 mM citrate buffer; –○–, 20 mM phosphate buffer; –▼–, 20 mM glycine-NaOH buffer.

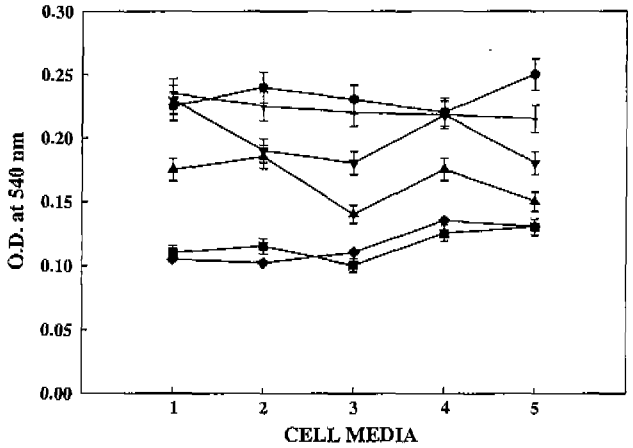

Fig. 7. MTT assay of urease. Five cell media with human leukemia cells (K562) containing 10 mM urea. Each concentration of urease was added and incubated at 37°C for 18-24 h in a 5% CO_2 incubator. Absorbance at 540 nm was read. Urease concentration (μg/ml): ◆, 10; ■, 5; ▲, 2.5; ▼, 1.25; +, 0.625; ●, control.

modifications and induced toxicity in the culture were compared with the control. A median toxic dose (TD_{50}) on human leukemia cells of the purified urease was 2.5 μg/ml as shown in Fig. 7. Significant p was 0.0001.

Discussion

Early epidemiological studies have shown that the highest isolation frequency of urease-positive *V. parahaemolyticus* was from either patients or frozen seafoods and rarely from sea water (1, 13, 16, 24). From our isolation of sea water and sediment, we obtained 6 different somatic groups with 17 different K antigen types. They were all Kanagawa-positive, urease-negative except for O1 : K1 serovar which was the only one urease-positive. It has been suggested that the serovars (O : K) associated with urease-positive strains may vary by geographic area (35). Other studies (14) have shown that serogroups O4 and O5 are the most common and are also urease-positive. Nevertheless, our isolate showed 5 out of 17 strains belonged to the somatic group of O4 but are urease-negative. Our isolate of O1 : K1 can be considered as an unusual isolate because it is the first in Korea, even though this serovar may be endemic in other areas or it was predominant in diarrheal isolation as well as being one of the most common serovars found in Asia (35). However, the frequency of this serovar in Korea has yet to be determined.

Suthienkul (35) reported that urease production correlates with possession of the *trh* gene regardless of the presence of the *tdh* gene in *V. parahaemolyticus*. He also suggested that the *trh* gene might have only transferred into a unique ancestor of *V. parahaemolyticus* with a urease-positive phenotype or the genes for urease activity and *trh* might be in proximity on the chromosomal DNA

of *V. parahaemolyticus*, enabling genetic linkage of the genotype. It was difficult to interpret with our discovery due to its carrying both the *tdh* and *trh* genes and being urease-positive with TDH production. It may be due to the unique source of isolation, diversity of the geographic distribution or genetic linkage of the phenotypes. This fact may explain the difference in the relationship between urease production and genes among somatic antigen groups of *V. parahaemolyticus* or the high frequency of urea-hydrolyzing strains in certain areas (35). It seems that full understanding of the regulation of gene expression in *V. parahaemolyticus* requires more work (26).

Purification of urease from *V. parahaemolyticus* has been limited (3) even though isolation of urease-positive strains have been conducted by several workers (10, 14, 16). We made an attempt to purify urease with our isolate to examine some physicochemical properties. We found that previous studies (8, 15) mentioned that the growth of some bacteria depended on the defined amount of urea, but it was not a growth limiting factor for our strain. With added urea, the production of urease was directly related to its concentration and 0.2% of urea induced maximum production of the enzyme indicating this is an inducible enzyme. From our study (17) above 0.5% urea as substrate decreased the production of urease by a facter of 50. It was remarkable to see that 6 hr of cultivation under aeration at 37°C was critical in urease production by this strain. To purify urease the single method of water extraction step was applied (3, 7), but we could not increase specific activity by this procedure.

Since urease is an important indicator of pathogenesis in other bacteria, *V. parahaemolyticus* urease can act as an important virulence factor like other known toxic factors (29, 32, 33, 38) and presumably contributes to the pathogenesis. Many other methods (3, 4) can be used to evaluate the pathogenicity; we employed a tetrazolium salt (MTT) to determine cytotoxicity. This assay carried out in microplates saved time, labor and expenses. We assume that ammonia generated by urea hydrolysis might have produced severe cytotoxic effects on human leukemia cells (2, 4). We expect to evaluate if urease itself or a urease coregulated factor(s) could be involved since not only urease has been identified as a critical virulence factor but also the specific mechanisms of pathogenesis are still quite unclear (21).

In this study we used a strain O1 : K1 of urease-positive *V. parahaemolyticus* to examine the presence of *tdh, trh* genes and explained some properties of urease. We hope that further studies of urease-positive *V. parahaemolyticus* with different serotypes containing the *tdh* or the *trh* genes will be done to validate our results on physicochemical properties as well as pathogenicity in order to support our conclusion.

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