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A simple method to determine urea concentration using intact *Helicobacter pylori* and BromoCresol Purple as a pH indicator

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Abstract

A modified method for urea quantification, by measuring the ammonia formed by urease, used the urease-positive *Helicobacter pylori* in place of purified urease with a pH indicator dye, BromoCresol Purple, to provide a color change. The color formed was stable for 20 min and could be read at 588 nm for urea quantification. Using this method, urea standard curves were linear up to 8.3 mM. As there was no need for centrifugation or precipitation, the assay was developed for use with 96-well microplates.

Introduction

A common method to quantify urea in solution is analysis of the ammonia formed by the urease (E.C. 3.5.1.5) reaction. Methods for quantifying ammonia released from urea use either acid titration, or the Nesslerization, or Berthelot reaction to form a colored product (Natelson 1971, Henry *et al.* 1974, Imler *et al.* 1979). However, the Nesslerization and Berthelot reactions are rarely used due to their long reaction times. The precision of Nesslerization and Berthelot of ammonia determination are dependent on the range of ammonia concentration expected (Barnes & Sugden 1990). The titration method is commonly used for the determination of urea, and is performed by titrating the released ammonia from the urease reaction with diluted HCl in the presence of a colored pH indicator solution (Gorin *et al.* 1962). Recently, the coupled enzyme system of purified urease and glutamate dehydrogenase has often been employed for urea assay (Sampson & Baird 1980). In this study, we developed a simple method for urea determination by using intact bacterial cells instead of isolated urease. The gastric bacterium, *Helicobacter pylori* was chosen for this purpose due to its high urease activity (Mobley *et al.* 1988). The urease of *H. pylori* has the lowest K_m (0.3 to 0.5 mM) of all bacterial ureases, and is present in the cell's outer membrane. This allows it to detect low concentrations of urea (Dunn *et al.* 1990).

In order to simplify the analytical system for released ammonia determination, the modified approach has also employed the color changing between ammonia and a pH indicator dye BromoCresol Purple (BCP) for directly quantifying ammonia (Chandler *et al.* 1982).

Material and methods

Chemicals and reagents

The stock solution for use in urea determination was made up as follows: 8 mg BCP was dissolved in 1.48 ml 10 mM NaOH and made to 100 ml with deionized water. EDTA was added to give 0.2 mM. The pH of the stock solution was adjusted to 5.1 using 0.1 M NaOH. The solution was stored at 4 °C until use.

Bacterial strains and cultivation

Helicobacter pylori ATCC49503 was cultured on chocolate agar plates (Difco) at 37 °C CO_2/air (10:90, v/v) and 98% (v/v) humidity for three days. Cells were washed from the agar plates with phosphate buffer saline (PBS, 0.05 M, pH 7.2). Then washed three times with PBS, centrifuged at 12 000 × *g* for 10 min and stored at −70 °C until use.

Urease assay

The procedure to determine the urease activity of *H. pylori* cells was previously described (Gorin *et al.* 1962), and the ammonia formed was determined by acid titration rather than Nesslerization. The substrate solution contained 30 mg urea ml^{-1}, 2 drops of pH indicator and 0.5 mg BSA ml^{-1} in 0.75 M phosphate buffer, pH 7.0. The enzyme was diluted in 0.05 M PBS buffer, then added to substrate solution. The pH indicator in titration solution was BromoCresol Green/Methyl Red. The titration solution was titrated to first purple color by 0.1 M HCl. One unit of urease activity was defined as that form of 1 μmol ammonia min^{-1} at 25 °C under the assay conditions.

Urea and serum urea nitrogen determination

To determine urea in solution, 10 μl sample solution was mixed with 990 μl urea determination solution in a small test tube, and the pH of the mixture was adjusted to 5.1 with 0.1 M HCl. The reaction was started by adding 2 μl *H. pylori* suspension (about 2 × 10^5 cells). After incubating 20 min at room temperature, the color of reaction solution was read at 588 nm. To establish a standard curve, solutions containing urea from 0 to 50 μM were prepared.

To quantifying urea nitrogen in serum, the sample was pretreated by mixing with an equal volume of 5% (w/v) trichloroacetic acid and incubated at 4 °C for 20 min to precipitate most proteins. After being centrifuged at 1700 × *g* for 5 min, the supernatant was used for urea determination described as above procedures, and also by commercial blood urea nitrogen (BUN) diagnosis kit (end-point) which was purchased from Sigma and performed according the instruction manual. It was to compare the concentration of urea nitrogen in serum determined by these two methods.

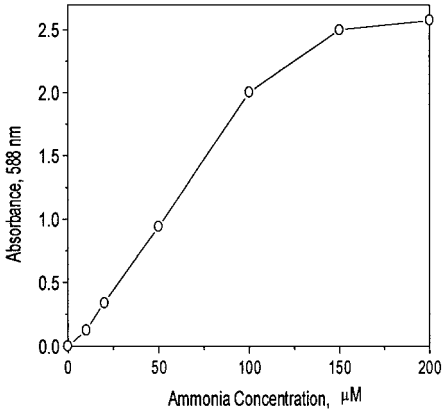

Fig. 1. Relationship between ammonia concentration and the color change of BromoCresol Purple profile. Reactions were carried out in the presence of 80 μg BCP ml^{-1} and various concentration of ammonia (0–200 μM) and read at 588 nm which was the wavelength of maximum absorbancy.

Results and discussion

Performance of ammonia with BCP at pH 5.0–6.5 produced a colored product with the absorption maximum at 588 nm. However, there is no interference to influence the color changing determination in our study except initial pH effect. Figure 1 shows the linearity of the standard curve was maintained at ammonia concentrations up to 100 μM. In this study, we used intact *H. pylori* cells as the crude urease, and the performance of BCP/ammonia providing urea quantification. Ammonia formed from urease reaction and BCP indicates shift of pH due to evolving ammonia to produce color change. In Figure 2, the absorbance reached its maximal intensity in 8 to 20 min depending on the urea concentration, and remained constant for at least 3 h. According the report of Chandler *et al.* (1982), the BCP provided a clear, vivid and linear color change between pH 5.2 and 6.8. It should be possible to adjust the initial pH of reaction solution as near pH 5.2 as possible for quantifying urea with greater sensitivity.

Figure 3 shows the dependence of absorbance on *H. pylori* cells at a constant pH and concentrations of urea and BCP. In this study, the total activity of urease used in the reaction was 1.1 units, which was prepared by adding 2 μl of *H. pylori* cell suspension (about 2 × 10^5 cells) into 1 ml reaction solution. To reproduce the same assay in other laboratories, we suggest that cells suspension be diluted to obtain 1 unit urease activity as the total activity in the reaction solution. The pellets of *H. pylori* cells maintain urease activity at −20 °C for at

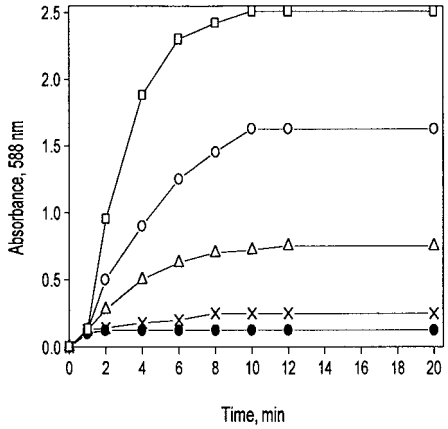

Fig. 2. Relationship between absorbance versus time profiles for the mixtures with 80 μg BCP ml^{-1}, 2×10^5 cells ml^{-1} of *H. pylori* and various concentrations of urea; 0 mM (●), 0.83 mM (×), 1.66 mM (△), 4.98 mM (○), and 8.3 mM (□) urea.

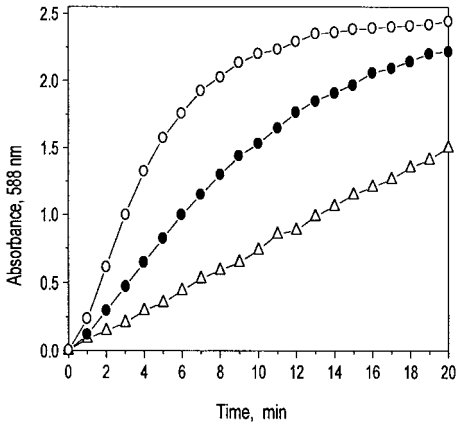

Fig. 3. Effect of various amounts of *H. pylori* cells on the rate of color change measured at 588 nm of ammonia formation from urea and BCP presence. 5×10^4 cells (△), 1×10^5 cells (●), and 2×10^5 cells (○). Various amounts of 1 μl cell-suspension were added into reaction mixtures of 1 ml final volume.

least one year. This urea assay was also developed for use with 96-well microassay plates. Since there was no need for a centrifugation and precipitation step, the same wells were used for both reaction and absorbance measurements using a microassay plates reader. Due to microscale determination, the time for the color formation was less than 2 min, and the linearity of the standard curve obtained up to 16.6 mM of urea.

This modified method was also employed for quantifying urea in serum. Serum proteins must first be removed by precipitation to eliminate the buffering effect. To evaluate the performance of this modified method in the serum urea nitrogen determination, a commercial BUN end point diagnosis kit was used for the comparison. Table 1 shows results, comparing the serum urea nitrogen determination using the method described in this study and a commercial BUN diagnosis kit. Both methods yielded essentially the same serum urea nitrogen values in each serum sample. There is no significant difference between two methods of serum urea nitrogen determination. In this report, we demonstrated a simple, rapid, and specific method for quantifying urea.

Table 1. Comparing of BCP/Urea method and BUN commercial diagnostic kit in serum urea nitrogen quantification.

Serum sample	Serum urea nitrogen (mg dl^{-1})	
	Commercial Kit	BCP/urea method[a]
1	8.6	7.9
2	10.4	10.0
3	16.7	15.0
4	13.5	12.0
5	13.5	12.3
6	12.2	11.1
7	9.1	9.0

[a]The values of serum urea nitrogen were obtained by multiplying the urea amount with a converting factor of 0.44.
All samples were assayed in triplicate.
The statistic method was by *t*-test and confidence level $p < 0.01$, with no significant difference between two methods.

References

Barnes AR, Sugden JK (1990) Comparsion of colourmetric methods for ammonia determination. *Pharmaceut. Acta Helvetica* **65**: 258–261.

Chandler HM, Cox JC, Healey K, MacGregor A, Premier RR, Hurrell JGR (1982) An investigation of the use of urease-antibody conjugates in enzyme immunoassay. *J. Immunol. Method* **53**: 187–194.

Dunn BE, Campbell GP, Perez-Perez G, Blasé MJ (1990) Purification and characterization of urease from *Helicobacter pylori. J. Biol. Chem.* **265**: 9464–9469.

Gorin G, Fuchs E, Butler LG, Chopra S, Hersh RT (1962) Some properties of urease. *Biochemistry* **1**: 911–915.

Henry RJ, Cannon DC, Wildelmann JW (1974) *Clinical Chemistry: Principles and Techniques*, 2nd edn. New York: Harper & Row, pp. 504–606.

Imler M, Frick A, Schlienger JL, Stahl A (1979) An automated microassay for blood ammonia. *J. Clin. Chem. Clin. Biochem.* **17**: 247–250.

Mobley HL, Cortesia MJ, Rosenthal LE, Jones BD (1988) Characterization of urease from *Camylobacter pylori. J. Clin. Microbiol.* **26**: 831–835.

Natelson S (1971) In: Thomas CC, ed. *Techniques of Clinical Chemistry*, 3rd edn. Springfield, IL, pp. 728–745.

Sampson EJ, Baird MA (1980) A coupled-enzyme equilibrium method of measuring urea in serum: optimization and evaluation of AACC study group on urea candidate reference method. *Clin. Chem.* **26**: 816–826.