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Determination of Urea in Serum Based on the Combination of an Enzymatic Reaction with Immobilized Urease and Ion Chromatographic Analysis

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A quantitative method for the determination of urea in serum was studied. An ion chromatograph (IC) with a conductivity detector was used in this method, where the chromatograph was modified by placing an immobilized urease column between the injection loop and a guard column of the cation analysis column. Immobilized urease was prepared by the adsorption of urease on cedar sawdust with triethylenetetramine. The adsorption capacity of urease was 190 mg g^{-1}, and its activity was 3500 U g^{-1}. The conversion efficiency of urea to ammonium ion was 100%, and the half life of immobilized urease was 60 days. It was possible to use the immobilized urease in a pH range of 3.0 to 9.0, and at temperatures up to 60°C. The determination of urea was attempted by IC attaching an immobilized urease column. The limit of detection of urea was 0.2 mg L^{-1}, and the calibration curves of urea were very linear over 0.8 – 25 mg L^{-1}. The urea concentration in the human serum could be determined with a standard deviation of 0.06 – 0.13 within 5 min after injecting the serum sample.

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Introduction

Many studies have been performed concerning use, as chemical materials, of lignin and cellulose extracted from woody biomass.[1-5] However, few studies have addressed the conversion of cedar sawdust possessing a higher-order structure into chemical materials.[6-8] Cedar sawdust is a biopolymer that possesses a carbon framework microstructure. Through the introduction of various functional groups with chemical processing, cedar sawdust can be converted into chemical materials possessing novel characteristics. Such materials include adsorbents for dyes and enzymes, and chelating resins with metal-ion selectivity.

In our previous paper, we reported on the synthesis and characterization of cedar sawdust-derived functional materials with sulfo-groups and with polyamines.[9-12] The former was found to have good adsorption for basic dyes, whereas the latter had a high selectivity for mercury ions. This paper focuses on cedar sawdust-derived functional materials as supports for the adsorption of enzymes.

Various methods of urea determination using an enzyme reaction have been reported.[13,14] Enzymatic methods are based on measurements of the ammonium ion or carbon dioxide produced by the hydrolysis of urea. As a rule, the concentration of ammonium ion is measured by spectrometry,[15] fluorometry,[16,17] liquid chromatography[18,19] or standard ion chromatography (IC).[20,21] In the case of liquid chromatography or standard IC, an immobilized urease column was placed between the sample syringe and the injection loop.[18-21] This configuration, where an immobilized urease column was off-line of the IC system, helped to maintain the activity of the immobilized enzyme for a long period of time.

In this study, urea was determined by IC with a conductivity detector (CD); however, the immobilized urease column was placed between the injection loop and the guard column of the cation analysis column, *i.e.* on-line to the IC system. The advantages of placing the enzyme column within the IC system include the following: a) the temperature of the enzyme column can be controlled by the system controller with IC equipment, b) a small amount of injection sample (25 μL) is sufficient, c) urea can be measured rapidly, because the immobilized urease column is connected to the cation analysis column and d) it is not necessary to clean the immobilized enzyme column. Before placing the immobilized urease column in the IC system, the operational conditions of the enzyme column, such as its shelf-life, conversion efficiency, pH range, flow rate of the eluent as well as the operating temperature range were examined. Then the immobilized urease column was placed on-line to the IC system, and the determination of urea in human serum was attempted.

Experimental

Chemicals and apparatus

Cedar sawdust (60 – 80 mesh) was received as a gift sample from Toyo Jushi Co., Ltd. Triethyleneteramine (TETA) and urea were purchased from Kanto Chemical Co., Inc.

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Urease (EC.3.5.1.5; activity, 130 U mg^{-1}; from jack bean) was purchased from Wako Pure Chemical Industries, Ltd. A buffer solution was prepared by creating a mixed solution of monopotassium phosphate–dipotassium phosphate, and citric acid–dipotassium phosphate. The eluent used on the IC system was a mixed solution of tartaric acid–picolinic acid–boric acid. All other chemicals used were commercial extra-pure reagents. A UV/Vis spectrophotometer (JASCO Co., V-570) was used to measure the absorbance, and a disposable cell with a 10-mm optical path length was used. IC was a Shimadzu CTO-20A with Shodex IC YK-421 column.

Pre-processing of cedar sawdust

Cedar sawdust was first strengthened with acid or alkali in the following manner. Concentrated hydrochloric acid (150 mL) was gradually added to 30 g of cedar sawdust. The mixture was heated to about 100°C for 4 h with stirring, and then the mixture was washed with water and acetone. After drying, acid-treated cedar sawdust (CSH) was obtained (crude yield: 23 g). For alkali processing of cedar sawdust, 100 mL of a 4 mol L^{-1} sodium hydroxide solution was gradually added to 30 g of cedar sawdust over a 30-min interval, with stirring. The mixtures were heated at about 100°C for 2 h, and then neutralized with a 10 v/v% of acetic acid solution. After washing with water and acetone, alkali-treated cedar sawdust (CSOH) was obtained (crude yield: 27 g).

Introduction of sulfo group to cedar sawdust

Ten grams of CSH and CSOH, respectively, were immersed in 100 mL of concentrated sulfuric acid (98%) for 30 min. Then, 15 mL of fuming sulfuric acid was slowly added with stirring and allowed to stand for 30 min. The products were washed with water and acetone. After drying at 100°C, acid-treated sulfo-type cedar sawdust (CSH-SO_3H) and alkali-treated sulfo-type cedar sawdust (CSOH-SO_3H) were obtained (crude yield: 6 g, 6 g, respectively).

Introduction of TETA into cedar sawdust

Twenty milliliters of TETA were gradually added to 10 g of CSH and CSOH, respectively, with stirring. The mixtures were heated for 20 min in about 100°C, with stirring. The products were then washed with dilute hydrochloric acid, followed by water and acetone. After drying at 100°C, acid-treated TETA-type cedar sawdust (CSH-TETA) and alkali-treated TETA-type cedar sawdust (CSOH-TETA) were obtained (crude yield: 8 g, 8 g, respectively).

Adsorption of urease on chemically modified cedar sawdust

The adsorption of urease was carried out by the column method. One gram of modified cedar sawdust (particle size: 250 – 325 mesh) was weighed and packed into a glass column of 8-mm internal diameter and 200-mm length. After flushing with the buffer solution (pH 7.5), a 0.01 w/v% urease solution adjusted to pH 7.5 with the buffer solution was passed through a column, and the eluent was collected at each of 10-mL by a fraction collector. The concentration of urease contained in each fraction was measured by the Bradford method to observe whether urease was adsorbed or not. The saturated absorption capacity of urease was obtained by a calculation based on the total volume of the urease solution developed until the leakage of urease was detected.

The activity of immobilized urease

The activity of immobilized urease was measured using the Hogan & Done method.[22] Fifty milliliters of *n*-propanol, 20 mL of the buffer solution (pH 7.5) prepared by monopotassium phosphate and dipotassium phosphate, and 12 g of urea were placed in a 1-L volumetric flask, and then the total volume was made up to 1 L with pure water. Next, 200 mL of the prepared substrate solution was placed into a 300-mL Erlenmeyer flask, and kept at 30°C in a drying oven. To this solution was added 5 mg of dried immobilized urease. At 5-min intervals, ammonium ion produced was determined by the indophenol method.[23] The concentration of ammonium ion was plotted against the reaction time. The activity of immobilized urease was calculated from the initial rate. In this paper, the amount of immobilized urease hydrolyzing 1 μmol of urea in 1 min is specified as 1 unit. The activity of free urease was also measured by the above mentioned procedure.

Serum samples

Blood samples were collected from 10 healthy human males, and the serums were obtained by the centrifugation of blood samples without the addition of a coagulating agent. The resulting serums were kept in a freezer and diluted by 50 fold prior to the measurement.

Table 1 Properties of cedar sawdust with a sulfo group and with polyamine

Material	Adsorption of urease/mg g^{-1}	Activity of immobilized urease/U g^{-1}
CSH-SO_3H	50	160
CSH-TETA	190	3500
CSOH-SO_3H	30	100
CSOH-TETA	35	110

Results and Discussion

Adsorption of urease on chemically modified cedar sawdust

The adsorption behavior of urease on cedar sawdust with sulfo-groups and with TETA was investigated. The results are given in Table 1. For CSH-SO_3H, CSOH-SO_3H, and CSOH-TETA, the adsorption capacity of urease was low, namely, between 30 and 50 mg g^{-1}. In contrast, for CSH-TETA, a favorable adsorption capacity was seen, namely, 190 mg g^{-1}. The activities of immobilized urease are given in Table 1. The activity of immobilized urease on CSH-TETA was 3500 U g^{-1}. This activity is approximately 23-times lower than the activity of free urease (80 U mg^{-1}). Nevertheless, we found it to be sufficient for the hydrolysis of urea to ammonium ion in the proposed IC system.

The most suitable pH range for the adsorption of urease was found to be 6.5 to 8.0. In this pH range, the amino group in TETA may form a quarternary ammonium ion, and terminal carboxyl groups of urease may also dissociate into ion, assuming the isoelectric point of urease to be 5.5. This indicates that the adsorption of urease on CSH-TETA is due to ionic bonding between terminal amino group of TETA and the terminal carboxyl group of urease. Next, the operational conditions of immobilized urease, such as the conversion efficiency, pH, the shelf-life, the flow rate, and the temperature, were studied.

Conversion efficiency

The apparent conversion efficiency of urea to ammonia in a column packed with immobilized urease was obtained by the following equation:

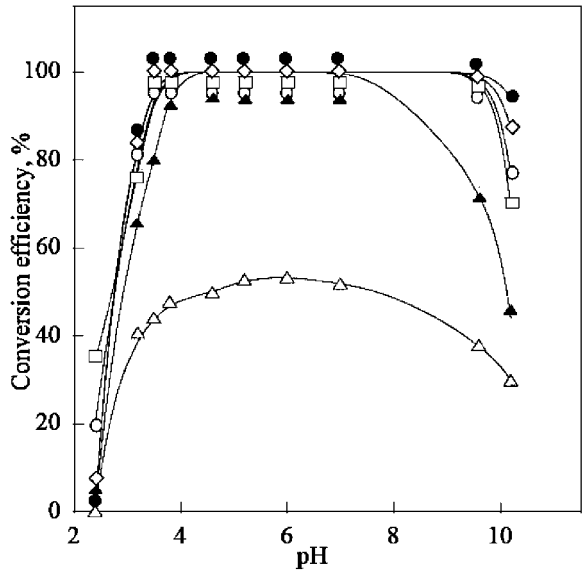

Fig. 1 Effect of the concentration of urea and pH on the conversion efficiency when 5 mmol L^{-1} to 2 mol L^{-1} of urea solution adjusted to various pH was developed in a column packed with 1 g of immobilized urease at 2.8 mL min^{-1} of the flow rate. Concentration of urea: ○, 5 mmol L^{-1}; □, 0.01 mol L^{-1}; ◇, 0.1 mol L^{-1}; ●, 0.2 mol L^{-1}; ▲, 1 mol L^{-1}; △, 2 mol L^{-1}.

$$\text{Conversion efficiency (\%)} = ([NH_4^+]_{obs}/[NH_4^+]_{cal}) \times 100$$

where $[NH_4^+]_{obs}$ is the observed concentration of ammonium ion produced, and $[NH_4^+]_{cal}$ is the calculated concentration of ammonium ion produced upon the complete hydrolysis of urea. The measured errors due the dispersal of urea and ammonium ion in the column were hypothesized to be almost identical.

The conversion efficiency of urea to ammonium ion was affected by the urea concentration and the pH of the urea solution (Fig. 1). The influence of the urea concentration was studied in the range between 5 mmol L^{-1} and 2 mol L^{-1}. When 2 mol L^{-1} of the urea solution was developed in a column packed with immobilized urease, the conversion efficiency was 58%. However, for 1 mol L^{-1} of the urea concentration, the conversion efficiency was approximately 100%, and a suitable pH for the enzyme reaction was in the range between 4.5 and 7.5. When the concentration of urea was reduced to 0.2 mol L^{-1} or lower, a suitable pH range was 3.0 to 9.0.

Shelf-life of immobilized urease

A urea solution (0.02 mol L^{-1}) adjusted to pH 7.5 was passed continually through the column with 1 g of immobilized urease during about 3 months, and the conversion efficiency of urea to ammonium ion was measured daily. The results are shown in Fig. 2. Up to the 45-day point, the conversion efficiency of urea was 100%, and the half life was 60 days; hence, immobilized urease was excellent in long-term stability. The authors hypothesize that this excellent shelf-life is due to the formation of multiple hydrogen bonds between urease and hydroxyl groups in cellulose and lignin contained within cedar sawdust.

Influence of the eluent flow rate

With passing the eluent (pH 7.5) through an immobilized urease column at a flow rate in the range of 2.8 to 10 mL min^{-1}, 100 μL of urea solution in the range between 5 mmol L^{-1} and 1 mol L^{-1} was injected into the column. Figure 3 shows the

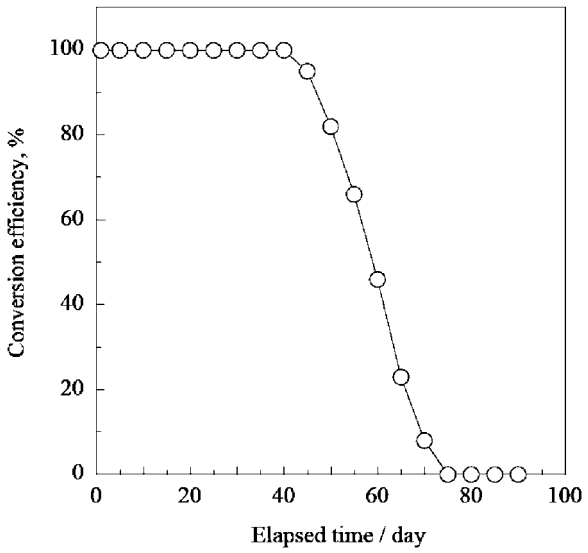

Fig. 2 Shelf life of immobilized urease when 0.02 mol L^{-1} of urea solution (pH 7.5) was passed continually through the column with 1 g of immobilized urease at 2.8 mL min^{-1} of the flow rate during about 3 months.

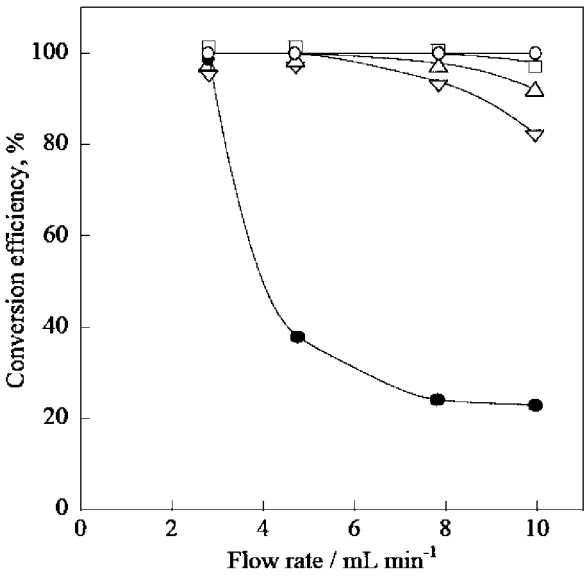

Fig. 3 Effect of the flow rate of the eluent on the conversion efficiency. With passing the eluent (pH 7.5) through an immobilized urease column at various flow rates, a urea sample was injected into the column. Concentration of the urea sample: ○, 5 mmol L^{-1}; □, 0.01 mol L^{-1}; △, 0.1 mol L^{-1}; ▽, 0.2 mol L^{-1}; ●, 1 mol L^{-1}.

influence of the flow rate on the conversion efficiency. When 1 mol L^{-1} of the urea solution was injected, the eluent could be developed at 2.8 mL min^{-1} of the flow rate, and its conversion efficiency was about 100%, but at over 2.8 mL min^{-1} the conversion efficiency decreased remarkably. For 0.01 to 0.2 mol L^{-1} of the urea solution, each of conversion efficiency was 100% at 8 mL min^{-1} of the flow rate. In addition when the concentration of urea solution was reduced to 5 mmol L^{-1}, the eluent could be developed even at 10 mL min^{-1} of the flow rate.

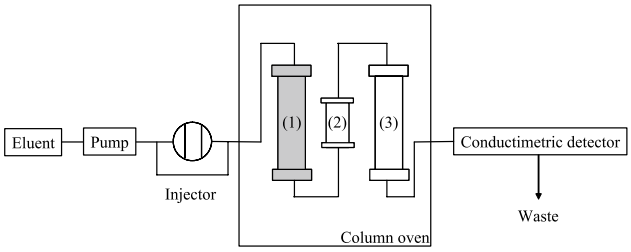

Fig. 4 Schematic diagram of urea determination by an immobilized urease column on-line to the IC system. (1) Immobilized urease column, (2) guard column, (3) YK-421 column.

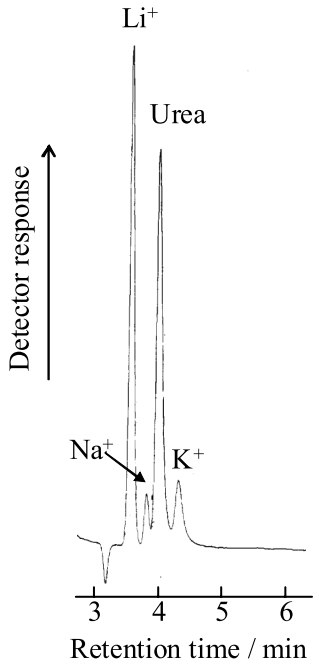

Fig. 5 Chromatograms for the determination of urea by an IC attaching immobilized urease column (150 mm × 4.6 mm). Operational condition: injection volume, 25 µL; flow rate, 1.0 mL min^{-1}; oven temperature, 40°C; eluent, tartaric acid–picolinic acid–boric acid. The concentration of urea was 6.0 mg L^{-1} and that of Li^+, Na^+ and K^+ was 1.0 mg L^{-1}, respectively.

Permissible temperature

The effect of temperature on the conversion efficiency of urea to ammonium ion was examined by using the eluent fixed at approximately 25, 40, and 60°C. A conversion efficiency of 100% was observed at 25 and 40°C, and of approximately 90% at 60°C. Hence, immobilized urease could be used over a wide range of temperatures.

Determination of urea using the IC system with immobilized urease column

Immobilized urease had excellent operational characteristics, such as the conversion efficiency (100%) for the hydrolysis of urea, a long shelf-life (half life; 60 days), pH stability (from 3.0 – 9.0), and thermal stability (25 – 60°C). From these operational characteristics, we considered that the immobilized urease column would be possible to be on-line in the IC system.

Figure 4 shows an illustration of the IC system attaching an immobilized ureae column (150 mm × 4.6 mm) between the injection loop and guard column of a cation analysis column (Shodex IC YK-421). Using this IC system the determination of urea was attempted following the operational conditions. The eluent was a tartaric acid–picolinic acid–boric acid buffer solution (pH 3.0). The temperature in the oven was set at 40°C, and the flow rate of the eluent was 1.0 mL min^{-1}. A urea sample (25 µL) was injected through the injection valve. Figure 5 shows a chromatogram for the determination of 6.0 mg L^{-1} urea in the presence of 1.0 mg L^{-1} of Li^+, Na^+ and K^+, respectively. The relative retention time of ammonium ion produced, (NH_4^+/Li^+), was 1.3, which was almost the same as that of ammonium ion measured by standard IC without an immobilized urease column. When the area of the ammonium ion was a plotted against the concentration of urea, a good linear relationship was observed over 0.8 – 25 mg L^{-1} of urea. The detection of urea permitted a sensitivity of 0.2 mg L^{-1}.

The configuration of Fig. 4, where the immobilized urease column was on-line in the IC system, maintained the activity of immobilized urease for a long period of time (Table 2). Under the condition of urea determination described above, when 25 µL of the urea solution (10 mg L^{-1}) was injected 20 times per day, the conversion efficiency of urea to ammonium ion was 100% up to the 30-day point.

After then, by using this IC system, the concentration of urea in serums taken from ten 22-year old males was measured. The standard deviation of the individual observed values was 0.06 – 0.13. The values of the urea concentration in the serums are given in Table 3. The measured values were 216 to 444 mg L^{-1}. These results were within the range of the standard value (192 – 450 mg L^{-1})[24] of the urea concentration contained in human serum. This method for the determination of urea is based on measurements of ammonium ion produced by urea hydrolysis. Therefore, if urea is to be determined accurately, the influence of ammonium ion contained in the serum must be taken into account. The concentration of ammonium ion in human serum is from 0.07 to 0.9 mg L^{-1}.[24] However, as the serum sample was diluted 50 fold to the measurement, ammonium ion in serum is present in just trace amounts.

Table 2 Shelf-life of immobilized urease on-line to IC system[a]

	Elapsed time/day				
	0	7	30	60	90
Conversion efficiency, %	100	100	98	95	92

a. Urea concentration used for the test was 10 mg L^{-1}.

Table 3 Determination of urea in serum by an immobilized urease on-line to IC system

Sample No.	Determined urea concentration/mg L^{-1}
1	330
2	438
3	366
4	444
5	336
6	216
7	294
8	384
9	282
10	282

Hence the concentration of ammonium ion in serum could be disregarded. Accordingly, the IC system described above (Fig. 4) was deemed appropriate for the analysis of urea in serum.

Conclusion

Cedar sawdust chemically modified with TETA after pre-processing by hydrochloric acid had an excellent absorption ability for urease. The activity of immobilized urease on CSH-TETA was 3500 U g^{-1}, which was sufficient for the hydrolysis of urea to ammonium ion. The prepared immobilized urease had excellent operational characteristics, such as long shelf-life, pH stability, high flow rate, and good thermal stability. In conclusion, the above results indicate that the immobilized urease column could be placed on-line to the IC system, and using this IC system urea in human serum could be determined.

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