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Notes

Preparation of reusable enzyme strips using alkylamine and arylamine glass beads affixed on plastic strips for urea determination

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Reusable strips of urease were prepared by covalently immobilizing it onto alkyl and arylamine glass beads, affixed on a plastic strip with a non-reactive adhesive. The immobilized urease retained its initial activity, prior to immobilization about 56% on alkylamine and 33.3% on arylamine. Maximum activity of alkylamine conjugated urease was attained at *p*H 6.5, 25°C for 1 min, while arylamine conjugated urease showed maximum activity at *p*H 7.0, 40°C, 15 min. The K_m for urea was 4.65 and 16.66 mM, for alkyl and arylamine conjugated urease respectively. A method for determination of serum urea developed using these strips is based on measurement of NH_3 generated with Nessler's reagent. The minimum detection limit permitted by the method was 10 and 5 mg/dL using alkyl and arylamine conjugated urease, respectively. Urea value in serum of healthy persons determined by this method, was in the range of 5-55 mg/dL with a mean of 26 mg/dL. The recovery of added urea was 82% for alkylamine and 85% for arylamine glass beads. A fair correlation (r=0.821) was found between urea values obtained by the standard urease kit and the present method. The alkylamine and arylamine conjugated urease stored at 4°C in 0.02 M potassium phosphate buffer *p*H 7.0 were reused 100 and 90 times respectively, during the span of one month. The method offers advantage of enormous ease in handling of immobilized urease.

Keywords: Urea, Urease, Serum, Alkyl and arylamine glass, Immobilization

Measurement of urea in serum is required for clinical diagnosis of renal diseases[1]. The analysis of urea is important in agricultural field, it being the choice nitrogen fertilizer and pollutant in agricultural waste water. Among various methods available for measurement of urea, enzymatic colorimetric method, employing urease is simple, specific, sensitive and hence suitable for routine analysis[2]. However, the method is expensive for a large number of samples due to huge requirement of urease. Its immobilization onto insoluble support permits its reuse, thus reducing the cost of the analysis. Among various insoluble supports available for immobilization of enzymes[3-11], alkyl and arylamine glass are attractive being resistant to microbial attack, stable over a wide *p*H range and in various solvents (such as ethanol and acetone) and possess a reasonable working life[12]. The present study describes the preparation of reusable strips of urease by immobilizing it onto alkyl and arylamine glass beads affixed on plastic strips and its advantages for reuse.

Experimental Procedure

Materials and Methods

Zirconia coated alkyl and arylamine glass beads (pore diameter 55Å) were a gift from Prof. H.H. Weetall (Corning Glass Work, New York). Urease from jack bean, urea from SRL, Mumbai, Nessler`s reagent from Hi-Media Laboratory Mumbai and enzymic kit for urea from Bayer's Diagnostics India Ltd., Baroda were used. All other chemicals were AR grade.

Assay of free/ soluble urease

The assay of free urease was carried out as described by Sumner[13] based on colorimetric determination of NH_3 generated by hydrolysis of urea (3% in 0.6 M potassium phosphate buffer, *p*H 7.0) by urease (1 mg/4 mL of 0.02 *M* potassium phosphate, *p*H 7.0) with Nessler's reagent, upon incubation at 40°C for 15 min and terminating it by 1.0 mL of 1 M HCl. A_{405} of yellow orange colour was read. The blank was prepared in the same manner, except that enzyme solution was replaced by 0.02 M potassium phosphate buffer *p*H 7.0. One unit of urease is defined as the amount of enzyme bound to glass beads, which produces 1 µmole of NH_3/min/mL under standard assay conditions.

Preparation of reusable strips of urease

Affixation of glass beads on plastic strips

The plastic strips of 15×1 cm size (length × width) was rounded at one end. A 0.2 mm thickness layer of commercial 'Araldite' was applied uniformly on both sides of rounded strips up to a height of 2 cm. Fifty mg each of alkylamine and arylamine glass beads were sprinkled on the fixative and spread uniformly using aluminum foil. The strips were kept for 24 h at room temperature for affixation of the glass beads.

Immobilization of urease onto affixed alkyl and arylamine glass beads

This was performed as per Lynn with modification[12]. The affixed alkylamine and arylamine glass beads were activated by glutaraldehyde coupling (3.0 mL 2.5% glutaraldehyde in 0.6 M potassium phosphate buffer *p*H 7.0, 2 h room temperature, constant shaking). The strips were subsequently washed repeatedly in distilled water until *p*H 7.0, to ensure complete removal of free glutaraldhyde. To diazotize the activated beads, plastic strips were dipped into 2 mL of chilled 2 N HCl (where 50 mg $NaNO_2$ was added and kept for 30 min in ice bath). The excess HCl was decanted and diazotized beads washed with 0.1 M sodium phosphate buffer (*p*H 7.0) until *p*H 7.0. The plastic strips containing glutaraldhyde activated and diazotized alkylamine/ arylamine glass beads were separately dipped into urease solution (13.50 and 14.0 units of urease added to 50 mg alkyl/ arylamine glass beads, 4°C, 48 h) with occasional shaking. After immobilization, the strips were taken off and the remaining urease was assayed for activity[13] and protein[14]. The strips were dipped into 0.02 M potassium phosphate buffer (*p*H 7.0) several times until no urease activity was detected in the washing. These strips were assayed for urease activity by determining the loss of protein from the solution during immobilization[15].

Assay of strip bound urease

The assay of immobilized urease (strips termed as Alkylamine and Arylamine enzyme strips) was carried out in the similar manner as described for free enzyme, except that enzyme solution was replaced by strip bound enzyme and volume of reaction mixture was increased by 1 mL by adding reaction buffer. The mixture was stirred continuously during incubation. The control was prepared in the same manner, except that strip had only affixed glass beads. Both the experimental and control strips were washed in distilled water after the assay and stored in 0.02 M potassium phosphate buffer *p*H 7.0 at 4°C until reuse.

Kinetic properties of strip bound urease

The kinetic properties of immobilized urease (optimum *p*H and temperature, incubation period, effect of urea concentration and apparent K_m and V_{max} from L-B plot) were also studied.

Determination of serum urea with 'Enzyme strip'

Collection of blood and preparation of serum

Blood samples (2 mL) from persons of different age groups and sex were collected using sterilized needle and syringe and kept at room temperature for 1 h. After centrifuging (2000 × g, 5 min, room temperature), the supernatant (serum) was collected and stored at 4ºC until use.

Determination of serum urea

It was carried out in the same manner as described for assay of immobilized urease under optimum assay conditions, except that urea solution was replaced by serum. The concentration of urea was extrapolated from the standard curve between urea concentrations ranging from 10 to 250 mg/dL, A_{405} prepared under similar assay conditions (Fig. 1).

Reuse and storage of enzyme strips

After each assay, enzyme strips were washed 4-5 times in 0.02 M potassium phosphate buffer (*p*H 7.0), stored in the same buffer at 4°C, until their reuse in the next assay.

Results and Discussion

Reusable strips of urease were prepared by immobilizing commercial jackbean urease onto alkyl and arylamine glass beads affixed on a plastic strip, through glutaraldehyde activation and subsequent diazotization, with 88% and 90% retention of initial activity of free enzyme, respectively. The conjugation

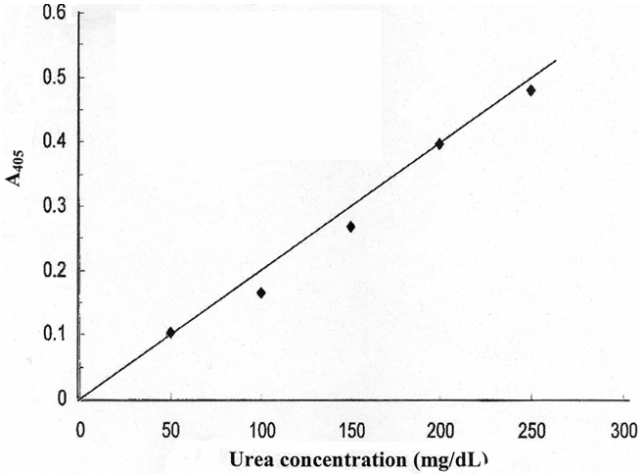

Fig. 1—Standard curve of urea at A_{405} by using immobilized urease on a plastic strip. The reaction contained 1 mL, 0.02 M potassium phosphate buffer (*p*H 7.0) and the strip containing immobilized urease. After incubation at 30°C for 30 s in case of alkylamine and 40°C for 15 min in case of arylamine glass beads, 1.0 mL of 1 M HCl was added. To 0.2 mL aliquot of this reaction mixture, 8.8 mL distilled water and 1.0 mL Nessler's reagent added to develop the colour.

Table 1—Immobilization of jack bean urease on alkyl and arylamine glass beads affixed on a plastic strip.

Type of glass beads	Urease added to 50 mg glass beads (mg)	Urease coupled to 50 mg glass beads (mg)	Units of urease added to 50 mg glass beads	Conjugation yield (mg/g)	Total sp. activity of free urease	Total sp. activity of immobilized urease	% Retention of sp. activity
Alkylamine	1.68	0.90	13.50	18.0	8.03	7.06	88
Arylamine	1.60	1.20	14.00	24.0	8.00	7.20	90

One unit of urease = 1 μNH_3 liberated/min/mg

yield of enzyme on alkylamine and arylamine glass beads was 18 and 24 mg/g support, respectively (Table 1).

Kinetic properties of immobilized urease

Compared to free urease, the alkylamine conjugated urease exhibited decrease in optimum *p*H from 7.0 to 6.5, while arylamine conjugated urease showed no change in optimum *p*H. Optimum *p*H of an enzyme is displaced upon immobilization, particularly, when the support matrix is charged.

The alkylamine and arylamine conjugated urease showed maximum activity at 30 and 40°C, respectively, which is higher than that of free urease (25°C). This indicated stabilization of urease upon immobilization. The period of incubation was also increased from 30 s to 1 min and 15 min after immobilization on alkylamine and arylamine glass beads, respectively. K_m for urea was 4.65 and 16.66 mM for alkylamine and arylamine conjugated urease, respectively, which is higher than that of free urease (3 mM) (Table 2). These changes in kinetic properties of urease after immobilization are controlled by (i) change in enzyme conformation (ii) its micro-environment, (iii) steric effects and (iv) diffusional effect[15].

Determination of serum urea

A discrete method for analysis of urea in serum was developed using alkylamine and arylamine conjugated urease strips. It is based on the measurement of NH_3, generated in reaction mixture from serum urea by immobilized urease, using Nessler's reagent. This method has the advantage of reuse of enzyme and enormous ease. The minimum detection limit of the method is 10 and 5 mg/dL, employing alkylamine and arylamine conjugated urease strip, respectively. The urea value in serum of apparently healthy persons was determined by both the strips and found in the range 5 to 55 mg/dL with a mean of 26 mg/dL (n=20). Following analytical parameters were studied to evaluate the method.

Table 2—A comparison of various kinetic parameters of free, alkyl and arylamine glass bead bound jack bean urease.

Parameters	Free urease	Alkylamine glass beads bound urease	Arylamine glass beads bound urease
Optimun *p*H	7.0	6.5	7.0
Optimum temperature (°C)	25.0	30.0	40.0
Incubation period (min)	0.5	1.0	15.0
K_m for urea (mM)	3.00	4.65	16.66
V_{max} (μ mol/min)	6.2	5.8	0.7

Linearity

A linear relation was found between A_{405} versus urea concentration ranging from 10-250 mg/dL (Fig. 1).

Detection limit

The lower detection limit of the method is 10 mg/dL employing alkylamine conjugated urease strip, which is similar to that by DEAE-Cellulose paper strip bound pigeon pea urease[6].

Analytical recovery

The analytical recovery of added urea in sample was 82 and 85%, employing alkylamine and arylamine conjugated urease strips, respectively.

Accuracy

There was a fair correlation (r=0.821) between the serum urea values obtained by the standard enzymatic kit (x) and the present method (y): employing alkyl and arylamine conjugated urease strip method, indicating the high accuracy of the method (Fig. 2).

Reusability and storage stability

The alkylamine and arylamine conjugated urease strips showed 50% loss of its activity after 100 and 90 uses, respectively, over 1 month period, when stored in 0.02 M potassium phosphate buffer *p*H 7.0, at 4°C.

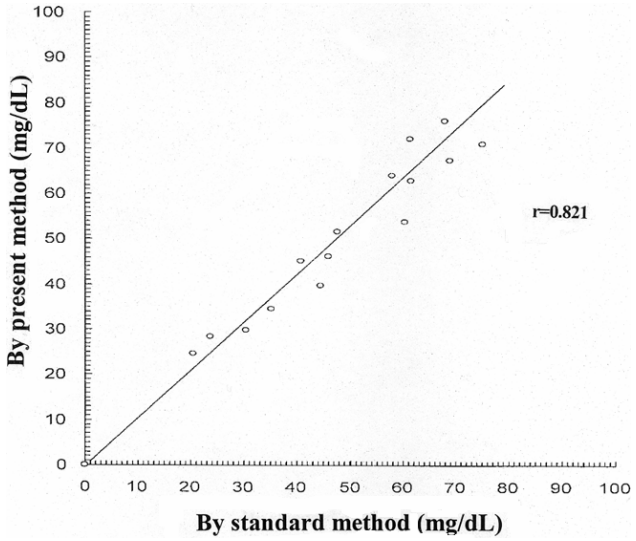

Fig. 2—Correlation between serum urea as determined by the commercial enzymatic colorimetric method (x) and the present method employing alkyl/aryl amine conjugated urease strip (y).

Conclusion

Reusable strips of urease prepared by immobilizing commercial jack bean urease onto alkyl and arylamine glass beads affixed on plastic strips can be successfully used for the determination of serum urea with enormous ease. These strips can be employed 90-100 times for the determination of serum urea.

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