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PHOTOCONTROL OF UREASE-COLLAGEN MEMBRANE ACTIVITY

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Summary

(1) Urease (EC 3.5.1.5) was modified with β-1-[3,3-dimethyl-6′-nitrospiro-(indoline-2,2′-2*H*-benzopyrene)] propionic anhydride. Three amino acid residues of urease were modified by the anhydride at a molar ratio of 2000.

(2) The activity of modified urease was decreased with ultraviolet irradiation and then restored to the initial activity with visible light irradiation.

(3) Modified urease was used to prepare a urease-collagen membrane. The apparent Michaelis constant (K_m') of the modified urease-collagen membrane under ultraviolet light was identical to that of the membrane under visible light.

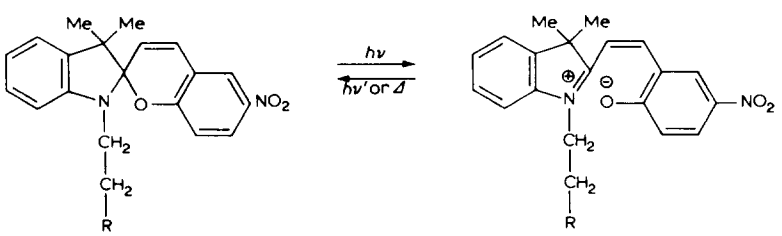
(4) The optimum pH of the modified urease-collagen membrane was displaced toward lower pH values with ultraviolet irradiation. At higher ionic strength, the pH activity curve of the membrane was displaced toward higher pH values.

(5) The thermostability of urease was increased with its modification.

Introduction

The control of enzyme systems has practical importance in metabolic processes of cells. Activity control of immobilized enzymes also has a potential application in a switch system or as a controller of a bioreactor.

Substances that undergo reversible color formation under irradiation with light are called photochromic compounds. A number of spiropyran compounds show photochromism represented by the following reaction scheme [1]:



Here $h\nu$ and $h\nu'$ are the ultraviolet and visible light energies required for the coloration and decoloration reactions, respectively. Colored spiropyrans are also converted into colorless forms when they are placed in the dark (Δ). The authors previously found that photosensitive enzymes could be prepared by modifying α-amylase with such photochromic spiropyran compounds [2]. Substrate affinity of enzymes was found to be changed by the modification, possibly because the polarity of spiropyran compound drastically changes, as shown.

A method for the preparation of enzyme-collagen membranes has been developed by the authors [3,4]. Collagen membrane has been shown to be a useful carrier for enzyme immobilization [5]. In the present study photosensitive urease (EC 3.5.1.5) prepared by modification of urease with a photochromic spiropyran compound was entrapped in a collagen membrane.

Physicochemical property changes of the modified urease-collagen membrane with light irradiation are described in this paper.

Materials and Methods

Collagen. Collagen was obtained from bull calf skin. The calf skin collagen was purified by the method of Karube et al. [6].

Enzyme. Urease (from Jack bean, 2900 units/g) was obtained from Sigma Chemical Co.

Photochromic spiran. 1-(β-carboxyethyl)-3,3-dimethyl-6'-nitrospiro[indoline-2,2'-2*H*-benzopyran] (m.p. 109—110.5°C) was obtained by the reaction of 1-(β-carboxyethyl)-3,3-dimethyl-indolenium iodide and 5-nitrosalicylaldehyde in the presence of piperidine, and then condensed to the corresponding anhydride in dioxane with *N,N'*-dicyclohexylcarbodiimide [7].

Modification of Urease. 60 mg of urease in 2 ml of distilled water were added to 20 ml of dioxane solution containing 180 mg of the anhydride. The reaction mixture was allowed to stand at 10°C for 2 h with stirring. 200 ml of cooled acetone were added to the reaction mixture. Then precipitates were separated by centrifugation at 2000 × *g* for 10 min and thoroughly washed with acetone and dioxane. Modified urease was isolated by DEAE-Sephadex column chromatography.

Preparation of modified Urease-collagen membrane. The suspension for preparation of the modified urease-collagen membrane contained 5.3 g of 1% collagen fibrils at pH 5.3 and 5 mg modified urease. The modified urease-collagen membrane was prepared by casting the suspension on a Teflon plate at 4°C. Modified urease-collagen membranes were treated with 0.1% glutaraldehyde solution for 1 min and dried at 4°C.

Enzyme activity. Unless otherwise noted, standard assays of the native urease and the urease-collagen membrane were carried out as follows. A reaction mixture of 5 ml of 3% urea/0.2 M phosphate buffer (pH 7.0) containing 25 μg of native urease or 3—5 mg urease-collagen membrane (300—500 μg urease) was incubated for 15 min at 25°C with shaking. The reaction mixture was irradiated with visible light (Olympus slide projector kp-8) or ultraviolet light (ultrahigh pressure arc light USH-250 D, Ushio Electric Inc. Tokyo) during the reaction. The reaction was stopped by the addition of saturated potassium

carbonate. The urease-collagen membrane was removed from the reaction mixture. Produced ammonia was determined by the method of Van Slyke and Archibald [8].

Preparation of modified collagen membrane. 1 g of collagen was added to 100 ml of dioxane solution containing 0.38 g of the anhydride. The reaction mixture was allowed to stand for 20 h with stirring at room temperature. Then precipitates were separated and thoroughly washed with acetone and dioxane. Modified collagen membrane (30 μm) was prepared by casting the modified collagen fibril on a Teflon plate at room temperature.

Diffusion measurement. Diffusion of NH_4^+ was measured as described previously [9].

Results

Activity of native urease was not affected by irradiation with ultraviolet or visible light.

Fig. 1 shows the relationship between the amount of anhydride and the activity of modified urease. As shown by the dotted line, modified urease retained 80% of native urease activity under visible light irradiation. 15% of the amino groups, that is three amino acid residues, of urease were modified by the anhydride at molar ratio of 2000. Further increase of the molar ratio decreased the activity of modified urease. Modified urease obtained at molar ratio 2000 was used in the following experiments.

Spiropyran compound bound to urease showed an absorption band around 500 nm after ultraviolet irradiation. Absorbance around 500 nm was reduced in the dark. The bound spiropyran showed the same photoreversible change in absorbance as free spiropyran. The activity of modified urease was decreased with ultraviolet irradiation and then restored with visible light irradiation. The activity change is reversible.

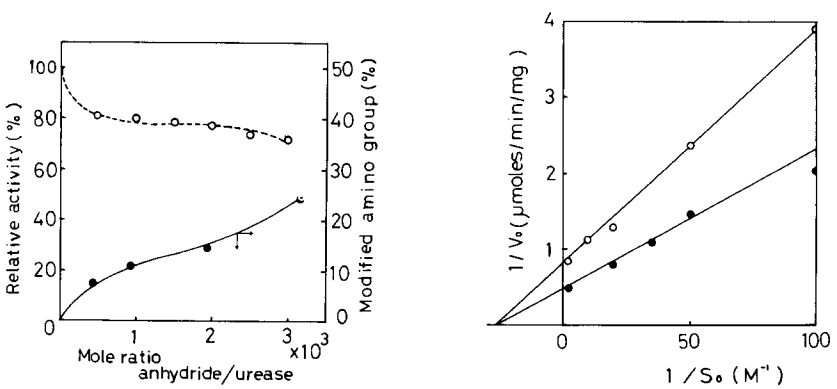


Fig. 1. Modification of urease with the anhydride. The enzyme activity was measured under visible light irradiation. (○) Relationship between the amount of anhydride and the activity of modified urease. (●) Modified amino groups (determined by the method of Matushima et al. [10]).

Fig. 2. Lineweaver-Burk plots for the modified urease-collagen membrane. The enzyme assay was carried out under standard conditions at various substrate concentrations. (○) Under ultraviolet irradiation. (●) Under visible light irradiation.

TABLE I

RELATIVE ACTIVITY OF MODIFIED UREASE-COLLAGEN MEMBRANE

	Relative activity (%)
Dark, or visible light irradiation	100
Ultraviolet irradiation	62

TABLE II

KINETIC PARAMETERS OF MODIFIED UREASE-COLLAGEN MEMBRANE

	K'_m (M)	V (μmol NH_3/min per mg membrane)
Modified urease-collagen membrane, ultraviolet light	0.04	1.37
Modified urease-collagen membrane, visible light	0.04	2.10

The activity of modified urease-collagen membrane was also decreased with ultraviolet irradiation, and then restored with visible light irradiation (Table I).

Fig. 2 shows the Lineweaver-Burk plots of the modified urease-collagen membrane. Table II shows kinetic parameters of the modified urease-collagen membrane. As shown, the apparent Michaelis constant (K_m') of the modified urease-collagen membrane under ultraviolet light was identical to that of the membrane under visible light.

The maximum velocity (V) of the modified urease-collagen membrane was decreased with ultraviolet irradiation.

Fig. 3 shows the pH-activity curves of soluble modified urease. The modified urease was labilized in the basic condition with ultraviolet irradiation. The pH-activity curve of modified urease was bell-shaped. No significant shift of the optimum pH of modified urease was observed with ultraviolet irradiation.

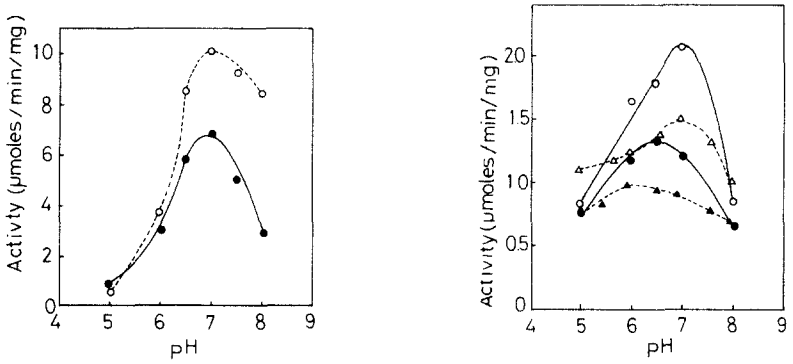


Fig. 3. pH-activity curves of soluble modified urease. The enzyme assay was carried out under standard conditions except for buffer employed. At pH 5.0—6.0 and pH 6.0—8.0, 0.1 M acetate buffer and 0.1 M phosphate buffer were employed, respectively. (○) under visible light irradiation, (●) under ultraviolet irradiation.

Fig. 4. pH-activity curves of the modified urease-collagen membrane. The enzyme assay was carried out under standard conditions except for buffer employed. At pH 5.0—6.0 and pH 6.0—8.0, 0.1 M acetate buffer and 0.1 M phosphate buffer were employed, respectively. (○) Under visible light irradiation, (●) under ultraviolet irradiation, I = 0.5. (△) Under visible light irradiation, (▲) ultraviolet irradiation, I = 0.05.

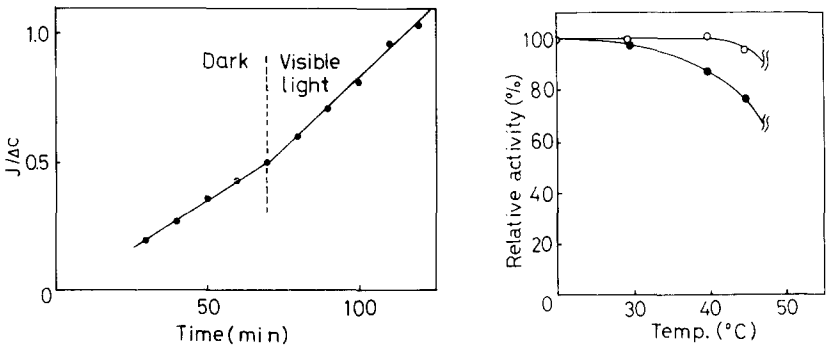


Fig. 5. Permeation of NH_4^+ in modified collagen membrane. The initial concentration of $(NH_4)_2SO_4$ was 0.1 M in compartment I and zero in compartment II. 25°C.

Fig. 6. Thermostability of the modified urease-collagen membrane. The enzyme assay was carried out under standard conditions modified by varying the incubation temperature. Incubation time of the membrane was 15 min. (○) Modified urease-collagen membrane, (●) native urease-collagen membrane under visible light irradiation.

Fig. 4 shows the pH-activity curves of the modified urease-collagen membrane.

The optimum pH of the modified urease-collagen membrane was displaced toward lower pH values with ultraviolet irradiation. However, at higher ionic strength, the pH activity curve of the membrane was displaced toward higher pH values.

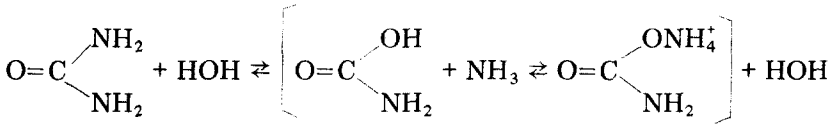
Fig. 5 shows the effect of visible light irradiation on the permeation of NH_4^+. Spiropyran-collagen membrane was used for the experiment. As spiropyran-collagen membrane shows reverse photochromism, the membrane is colored in the dark and bleached with visible light irradiation. As shown, the permeation coefficient of NH_4^+, which is $1.9 \cdot 10^{-6}$ cm^2/s in the dark, increases to $2.5 \cdot 10^{-6}$ cm^2/s with visible light irradiation. Therefore, it was concluded that NH_4^+ may interact with negative charges of spiropyran bound to the collagen membrane.

Fig. 6 shows the thermostability of the modified urease-collagen membrane. Modified urease was more stable than unmodified urease in the collagen membrane below 45°C. However, the collagen membrane shrank above 45°C and the thermostability of the urease-collagen membrane could not determined.

Discussion

Photocontrol of modified urease-collagen membranes was examined. Spiropyran compounds change in polarity with isomerization. The spiropyran compound bound to urease was hydrophobic under visible light. However, spiropyran derivative has charges after ultraviolet irradiation. Therefore, it was assumed that the affinity of modified urease for substrate urea increased with ultraviolet irradiation. However, the results obtained did not bear out this speculation (Tables I and II).

The work of Gorin [11] and of Blakely et al. [12] has provided convincing evidence that carbamate is the intermediate in a two-step reaction.



$\rightarrow \rightarrow H_2CO_3 + 2NH_3$

Presumably urease forms a carbomoyl complex as one of the ES complexes. The intermediate carbamoyl complex has a positive charge. On the other hand, as mentioned above, spiropyran becomes charged with ultraviolet light. The negative charge of the spiropyran derivatives may interact with the positive charge of the intermediate. Furthermore, NH_4^+, a product of the reaction, is an inhibitor. NH_4^+ may also interact with the negative charge of the spiropyran bound to urease (Fig. 5). These interactions may decrease the velocity of urea hydrolysis.

The net charge of a matrix can have a profound influence on the characteristics of immobilized enzymes, owing to the creation of a microenvironment around the immobilized enzyme [13]. It is known that the isoelectric point of collagen is around 7. In the neutral condition, the net charge of collagen fibril is almost "zero". The charges on the collagen fibrils probably do not influence the nature of the enzyme. This phenomenon can also be seen commonly with other enzyme-collagen membranes [5]. It seems that the altered pH-activity profile is due to the creation of charges in the microenvironment of the enzyme when exposed to ultraviolet irradiation (Fig. 4). As mentioned above, NH_4^+ may increase local pH near the immobilized enzyme in the membrane. The increase of local pH causes the displacement of the pH-activity curve of the membrane toward lower pH values. Partial or complete elimination of the displacement of pH-activity curves with increasing ionic strength was reported for some systems [13,14]. As shown in Fig. 4, a clear shift towards the native pH-activity profile is observed, although a difference of about 0.5 pH units still remains. Goldstein et al. [14] reported that the pH-activity curve approached that of the soluble enzyme at an ionic strength of 1. Therefore, higher ionic strength may be needed for the complete elimination of the displacement of the pH-activity curve of the membrane.

The thermostabilization of modified urease may be caused by the hydrophobic intermolecular interaction between spiropyran derivatives.

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