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Determination of Kinetic Parameters for Immobilized Xanthine Oxidase and Its Application to a Post-Column Enzyme Reactor in High-Performance Liquid Chromatographic Analysis of Hypoxanthine, Xanthine and Uric Acid[1)]

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Immobilized xanthine oxidase (XO) was prepared by intermolecular cross-linking to controlled-pore-glass (CPG) using glutaraldehyde, and the enzymic properties (pH optimum, stability change on immobilization and apparent Michaelis constant) were studied. The performances of enzyme reactors packed with the immobilized XO were considered theoretically and investigated experimentally. Using a post-column reactor packed with the immobilized enzyme, simultaneous determinations of hypoxanthine, xanthine and uric acid by high-performance liquid chromatography (HPLC) were achieved with detection at 290 nm. The coefficients of variation after 4 runs were 1.7—13.4, 0.3—9.0 and 3.7—31.4% in the determination of 0.405—1.620 μg/ml of hypoxanthine, 0.450—1.799 μg/ml of xanthine and 0.495—1.980 μg/ml of uric acid, respectively.

Keywords——immobilized xanthine oxidase; controlled-pore-glass; apparent Michaelis constant; reaction capacity; simplified complementary tristimulus colorimetry; hypoxanthine; xanthine; uric acid; high-performance liquid chromatography; post-column enzyme reactor

Hypoxanthine, xanthine and uric acid are the end products of the breakdown of purine nucleotides. There is a great need for simple, sensitive and specific assays of hypoxanthine, xanthine and uric acid in plasma or serum, for correlation with clinical signs of xanthinuria, toxemia of pregnancy and renal failure.[2)] It would also be of interest to be able to detect the oxypurines simultaneously in plasma in relation to the diagnosis of haemorrhagic shock and intrauterine hypoxia of newborn infants. In recent years, attempts have been made to introduce high-performance liquid chromatographic (HPLC) techniques for the determination of these metabolites, but HPLC analysis is often complicated in the presence of other nucleosides and their bases.[3)] The use of an immobilized xanthine oxidase (XO) would be a simple means for the highly sensitive and simultaneous detection of hypoxanthine and xanthine which are the substrates of XO, due to its high specificity. Although immobilizations of XO on cellulose and Sepharose have been reported,[4)] the immobilized enzyme packed in a column connected with the usual HPLC column has never been applied to the simultaneous determination of the oxypurines. Therefore, in this study, the preparation of XO coupled to controlled-pore-glass (CPG) was developed, because CPG is more appropriate than Sepharose or cellulose as a support material for enzyme immobilization for use in an enzyme reactor after HPLC. The characteristics of reactors packed with the immobilized enzyme were studied to provide a basis for the development of a simultaneous analysis of the oxypurines by HPLC. The method developed was simple and specific.

Experimental

Materials——XO (EC 1.2.3.2, cow milk, about 0.4 U/mg) was purchased from Boehringer-Mannheim-Yamanouchi Co., Ltd. Aminopropyl-CPG (80—120 mesh, 530-Å mean pore diameter) was obtained from Electro-Nucleonics, Inc. (Fairfield, U.S.A.). Hypoxanthine, xanthine and uric acid were obtained from E. Merck (Darmstadt, G.F.R.) and Nakarai Chemicals, Ltd. (Kyoto, Japan). All other reagents used were of guaranteed grade, and distilled water was used through out the work.

Apparatus——The stopped-flow injection system used included a pneumatic amplifier pump (DuPont LC-830 liquid chromatograph), a Shimadzu UV-202 spectrophotometer with two micro flow-cells of 8 μl volume and a Shimadzu SIL-1A sampling valve having 200 μl sample loops (see Fig. 3). The liquid chromatograph was a Shimadzu LC-3A, equipped with a SPD-2A variable-wavelength UV detector and a C-R1A integrator.

Preparation of an Immobilized XO——A mixture of 1 ml of 5% glutaraldehyde in 0.1 M phosphate buffer containing 10 mM ethylenediamine tetraacetic acid (EDTA) (pH 7.0) and 0.5 g of aminopropyl-CPG was allowed to react at 25°C under reduced pressure for 1 h. Excess glutaraldehyde was removed by filtration and exhaustive washing with cold water. Aldehyde-CPG produced and 4 mg of XO dialyzed overnight against 500 ml of 0.1 M phosphate buffer containing 10 mM EDTA (pH 7.2) were added in 2 ml of 0.1 M phosphate buffer containing 10 mM EDTA (pH 7.0), and allowed to react at 4°C overnight. The glass beads carrying the enzyme were filtered off and washed with 100 ml of cold water to remove any extraneous enzyme, followed by washing with 100 ml of 1 M sodium chloride and 1 l of cold water.

HPLC Procedure——The reversed-phase column employed (20×0.4 cm i.d.) was packed with 5 μm Nucleosil C_{18} (Macherey, Nagel and Co., Düren, G.F.R.). The eluent, 0.01 M potassium phosphate (pH 4.8), was pumped through the column connected to a mixing tee at a flow rate of 0.7 ml/min and the effluent from the column was mixed with 0.01 M sodium borate (pH 9.2) at a flow rate of 0.23 ml/min to adjust the pH to 7.4. The chromatographic set-up used is shown in Fig. 6.

Simplified Complementary Tristimulus Colorimetry (SCTS Method)[5]——The absorbances at wavelengths u (290 nm), v (270 nm) and w (245 nm) were recorded against a reagent blank for each injection of solutions. The color point corresponding to wavelength r ($r=u$, v and w) was calculated from the relation

$$Q_r = A_r/J \tag{1}$$

where A_r is the absorbance at wavelength r, and J is the sum of absorbances at the three wavelengths. The mol fraction of product at any time, q, can be calculated from the color points of a reaction mixture of substrate and product at the same time, Q_{tr},:

$$q = E_S(Q_{tr}-Q_{Sr})/[E_S(Q_{tr}-Q_{Sr})+E_P(Q_{Pr}-Q_{tr})] \tag{2}$$

where E_S and E_P are the overall absorptivities of substrate and product, and Q_{sr} and Q_{pr} are their color points at wavelength r, respectively.

Results and Discussion

The characteristics of the free and the immobilized enzyme system were determined in batch operations. The oxidation of xanthine by both enzymes was studied using xanthine of 5.4×10^{-5} M at 25°C. All assys were performed in 50 mM phosphate buffer containing 10 mM EDTA by the same method as reported previously.[6]

The pH dependence of the immobilized enzyme activity was identical with that of the soluble enzyme, and the pH optima were 7.4.

Thermal stability of the free and the immobilized enzyme was studied by incubation for 30 min at various temperatures in 3.2 M ammonium sulfate containing 10 mM EDTA. The residual activity was determined under the conditions used above. It was generally observed that the immobilized enzyme had a higher thermal stability than the free enzyme in solution.

The operational stability (long term) of the immobilized enzyme was investigated and the results are shown in Fig. 1. The initial activity of the enzyme was taken as 100 arbitrary units. After each assay, the immobilized enzyme was kept at 4°C in 3.2 M ammonium sulfate containing 10 mM EDTA (pH 8). While it was used in continuous assays, the activity decreased very slowly over a long period of time.

The apparent Michaelis constant, K_m', for xanthine was determined at pH 7.4 (25°C) with the immobilized enzyme. Data from each initial velocity experiment were fitted to the Michaelis–Menten equation and K_m' was estimated to be 2.5×10^{-5} M (Fig. 2), which is in good agreement with the value measured by Coughlan and Johnson.[4a]

Though the observed overall initial rate of substrate depletion or product formation in an immobilized enzyme system includes rate constants due to the enzyme reaction and the bulk diffusion of the substrate, the reaction kinetics in certain concentration ranges of substrate may be described by Michaelis–Menten kinetics.[7] The integrated Michaelis–Menten equation for an immobilized enzyme in a batch reactor is given by

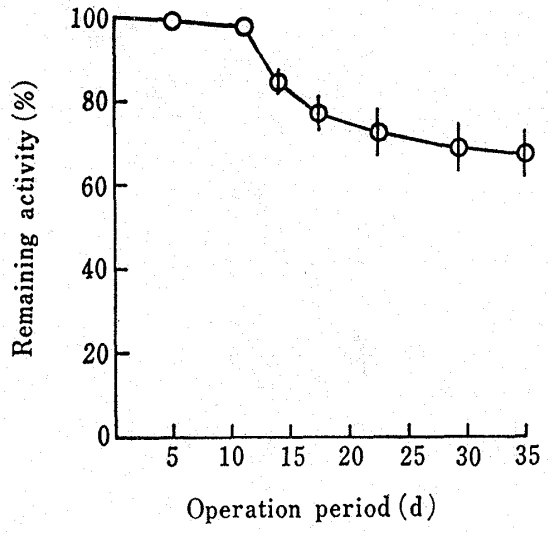

Fig. 1. Stability of the Activity of the Immobilized XO

Experimental details are given in the text.

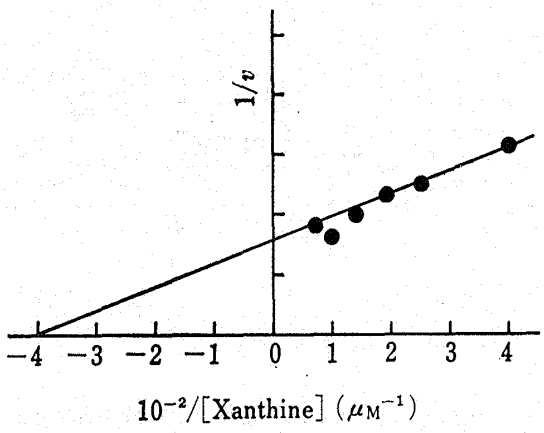

Fig. 2. Determination of the Apparent K_m Value for the Immobilized XO (Lineweaver-Burk Plots)

Activity was measured with xanthine as a substrate at pH 7.4 (25°C).[5]

$$S_0 - S_t = k_2 E_0 t - K_m' \ln(S_t/S_0) \quad (3)$$

where S_0 and S_t are the concentrations of substrate initially and at time t, and k_2 and E_0 are the rate constant for the decomposition of the enzyme–substrate complex and the concentration of total enzyme, respectively. The application of Eq. (3) to a reaction catalyzed by an immobilized enzyme in a packed-bed reactor requires that all elements of fluid move through the reactor at equal velocities. Under these conditions, Eq. (3) applies to each infinitesimal cross-sectional volume of the reactor and therefore expresses the total reaction taking place in each such volume during its passage through the reactor. Then t is the residence time, which is given by

$$t = V_l/F \quad (4)$$

where V_l and F are the void volume and the flow rate through the reactor packed with the immobilized enzyme, respectively. By considering that an immobilized enzyme exerts its catalytic action through the void volume of the reactor, the concentration of enzyme is given by

$$E_0 = [E]/V_l \quad (5)$$

where $[E]$ is the total amount of enzyme packed in the reactor. Substituting Eqs. (4) and (5) into Eq. (3), the following equation is obtained.

$$S_0 - S_t = k_2[E]/F + K_m' \ln(S_t/S_0) \quad (6)$$

where S_0 and S_t indicate the concentrations of substrate entering and leaving the reactor, and K_m' is the apparent Michaelis constant at a given flow rate through the reactor, respectively. The performance of an enzyme reactor can be judged from the degree of reaction (mol fraction of the product), q, defined as

$$q = (S_0 - S_t)/S_0 \quad (7)$$

Then Eq. (6) is written as

$$qS_0 = C/F + K_m' \ln(1-q) \quad (8)$$

where $C = k_2[E]$ is called the reaction capacity of the enzyme reactor indicating the degree of substrate conversion at unit time.

Performance characteristics of immobilized XO in reactor operation were determined on columns packed in stainless-steel tubing (4.2×0.4 cm i.d., reactor I) and Teflon tubing (4.2×0.1 cm i.d., reactor II), and each reactor was used in a continuous-flow system (Fig. 3).

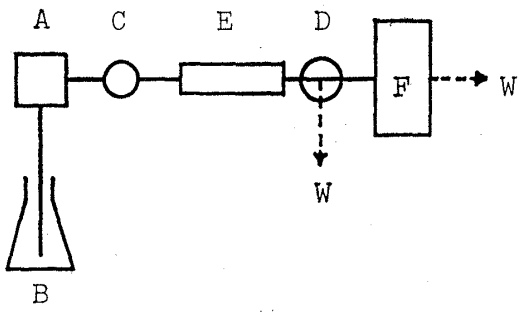

Fig. 3. A Block Diagram of the Stopped-Flow Scanning System

A, pump; B, carrier (50 mM phosphate buffer containing 10 mM EDTA, pH 7.4); C, sampling valve; D, 3-way valve; E, enzyme reactor; F, UV spectrophotometer; W, waste.

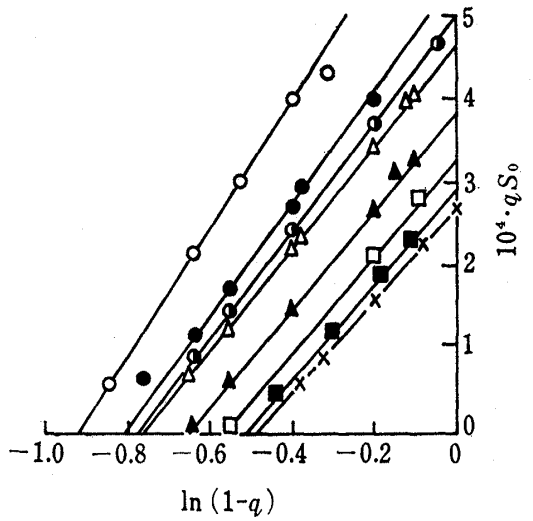

Fig. 4. Relationship between qS_0 and $1n(1-q)$ for the Oxidation of Xanthine by the Immobilized XO

Reactor II (4.2×0.1 cm i.d.). Flow rate (ml/min): 0.37 (○); 0.50 (●); 0.52 (◑); 0.69 (△); 0.90 (▲); 1.08 (□); 1.13 (■); 1.30 (×).

The relationships between qS_0 and $1n(1-q)$ are shown in Fig. 4, in which the value of q was calculated by means of Eq. (2) using the color point which was recorded from each measurement of the stopped-flow scanning spectrum for the reaction solutions. It is clear that good straight lines could be drawn at given flow rates. Plots of K_m' *vs.* flow rate through reactors I and II are shown in Fig. 5. They indicate that K_m' values in both reactors were always higher than that in a stirred suspension (2.5×10^{-5} M), that the influence of bulk diffusion on K_m' decreased at higher flow rates, and that K_m' approaches the apparent K_m value of the free enzyme. This dependence on flow rate may be explained by regarding each immobilized enzyme as being surrounded by a diffusion layer.[8] This would mean that the rate of diffusion of xanthine to the immobilized enzyme would be strongly dependent on flow rate, especially in reactor I.

C values for both enzyme reactors used were also estimated from Fig. 4. A single C value for each reactor was observed for all the flow rates studied, as shown in Table I. It is interesting to compare the observed value of C with V_l of the reactors used. A presumption of the

Table I. Comparison of C Values for Reactors I and II

Reactor I[a]			Reactor II[b]		
F (ml/min)	$10^6C/F$ (mol/min)	10^5C (mol/min)	F (ml/min)	$10^7C/F$ (mol/min)	10^7C (mol/min)
0.61	17.74	1.08	0.37	7.12	2.63
	18.55	1.13	0.50	5.32	2.66
0.72	14.58	1.05	0.52	4.76	2.48
0.86	12.34	1.06	0.69	4.75	3.28
0.90	12.42	1.12	0.90	3.81	3.43
	12.94	1.17			2.90±0.43[c]
1.00	11.46	1.15			
1.33	8.53	1.13			
		1.11±0.04[c]			

a) $V_l=0.25$ ml[9]; P_r value for the oxidation of xanthine to uric acid was estimated to be 4.44×10^{-5} mol/ml·min using the C and V_l values.
b) $V_l=6.5\times10^{-3}$ ml[9]; P_r value for the oxidation of xanthine to uric acid was estimated to be 4.46×10^{-5} mol/ml·min using the C and V_l values.
c) The mean values±S.D.

value of C for reactor II is possible by use of the known value of V_l: considering that reactor I (having V_l of about 0.25 ml) gave C of $1.11\pm0.04\times10^{-5}$ mol/min at pH 7.4 (25°C), C for reactor II with V_l of 6.5×10^{-3} ml should be about 2.8×10^{-7} mol/min under the same conditions. This is in good agreement with the observed value (see Table I). Therefore it is suggested that the value of C can be predicted from the given V_l of a reactor, if C for another reactor packed with the immobilized enzyme is estimated from correlations such as the one above. The catalytic activity per void volume of the reactor and time, P_r, which is an important parameter in the practical use of an enzyme reactor can also determined.[8b] Table I also gives the calculated values of P_r for reactors I and II.

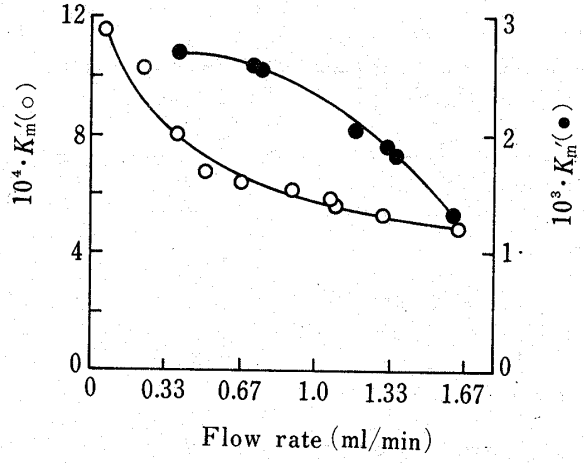

Fig. 5. Dependence of K'_m on Flow Rate for Reactor I (4.2×0.4 cm i.d.) and Reactor II (4.2×0.1 cm i.d.) packed with the Immobilized XO

Reactor I (●); Reactor II (○).

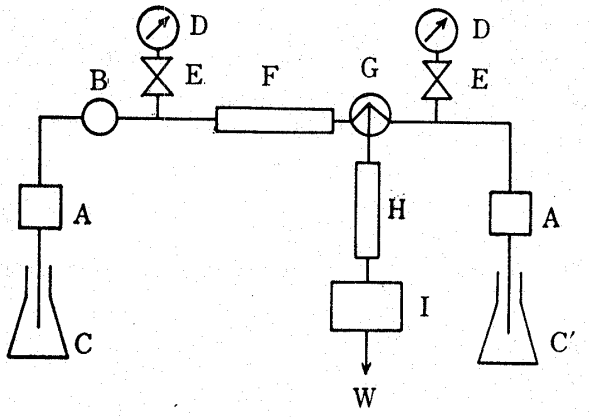

Fig. 6. Schematic Diagram of the Chromatographic System

A, pump; B, sampling value; C, carrier (0.01 M potassium phosphate); C', carrier (0.01 M sodium borate); D, pressure monitor; E, pulse damp; F, analytical column; G, mixing tee; H, enzyme reactor; I, UV detector; W, waste.

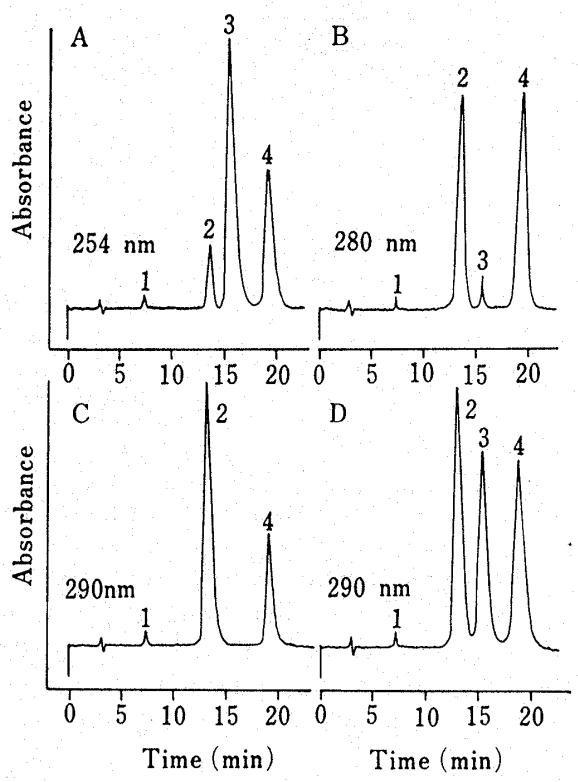

Fig. 7. HPLC Profiles of a Mixture of Hypoxanthine, Xanthine and Uric Acid (Chromatograms A, B and C), and with the Use of Reactor II (Chromatogram D)

Chromatographic conditions: Nucleosil $5C_{18}$; mobile phase (0.01 M KH_2PO_4 and 0.01 M $Na_2B_4O_7$); flow rate 0.93 ml/min. 1, unknown; 2, uric acid; 3, hypoxanthine; 4, xanthine.

Simultaneous determination of hypoxanthine, xanthine and uric acid was achieved by HPLC using reactor II packed with the immobilized XO (Fig. 6). The separation of hypoxxanthine, xanthine and uric acid is shown in Fig. 7. The chromatograms A, B and C were obtained at 254, 280 and 290 nm under the present experimental conditions, when the enzyme reactor II had been removed. The chromatogram D shows the separation of each component

in which the flow had passed through the enzyme reactor under the same conditions, and shows a simultaneous detection of hypoxanthine, xanthine and uric acid with satisfactory sensitivity. The retention times and peak shapes were reproducible, and the added peak broadening brought about by connecting the reactor II was 5.2%. The peak area for each component was proportional to its concentration, and good straight lines were obtained. Based on these initial separations, a series of samples containing hypoxanthine, xanthine and uric acid were chromatographed. The results are summarized in Table II. The coefficients of variation after 4 runs were 1.7—13.4, 0.3—9.0 and 3.7—31.4% in the determination of 0.405—1.620 μg/ml of hypoxanthine, 0.450—1.799 μg/ml of xanthine and 0.495—1.980 μg/ml of uric acid, respectively.

Table II. HPLC Analysis of Oxypurines

Compound	Amount present (μg/ml)	Amount found[a] (μg/ml)	Recovery (%)
Hypoxanthine	0.405	0.397±0.053	98.0
Xanthine	0.450	0.498±0.045	110.7
Uric acid	0.495	0.493±0.155	99.6
	0.648	0.543±0.063	83.8
	0.720	0.745±0.050	103.5
	0.792	0.785±0.029	99.1
	0.810	0.717±0.048	88.5
	0.900	0.921±0.059	102.3
	0.990	0.991±0.136	100.1
	1.214	1.048±0.018	89.3
	1.350	1.334±0.019	98.8
	1.485	1.498±0.118	100.9
	1.620	1.420±0.041	87.7
	1.799	1.760±0.006	97.8
	1.980	1.983±0.144	100.2

a) The mean values±S.D. of four determinations.

The studies in this paper indicate that the method presented may be successfully used to determine hypoxanthine, xanthine and uric acid in sera or urine from patients simultaneously without employing arduous and time-consuming techniques to overcome interference from compounds such as other purine bases, nucleosides and nucleotides. Further studies on clinical samples by this method are in progress.

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References and Notes

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