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Formation and use in enzyme immobilization of cellulose acetate–metal alkoxide gels

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A homogeneous transparent gel was formed when an organic solution of cellulose acetate was in contact with a metal alkoxide solution. The gel is insoluble in common solvents and electrolyte solutions. It seems that the reaction is due to coordination bonding between hydroxy groups on the pyranose ring and the metal. Enzyme could be immobilized in the gel fibres. The immobilized enzyme is stable for a long period and can be used in a packed column.

INTRODUCTION

We have been investigating the formation of a polymer–inorganic composite and its properties (Kurokawa & Sasaki, 1982). It was noted that a homogeneous gel is formed when an organic solution of cellulose acetate is in contact with an alkoxide solution. It has been reported that an oil-like substance is formed from contact of the polysaccharide solution and titanium triethanolamine (Kramer & Prud'homme, 1986), but the reaction mechanism is not well established. Hydrated TiO_2 and ZrO_2 have been shown to be suitable as matrices on which enzymes are immobilized with retention of enzyme activity (White & Kennedy, 1980). The immobilized enzyme on TiO_2 is prepared by hydrolysis of a $TiCl_4$ solution coexisting with the enzyme. The hydrated TiO_2 is very fine, and a finite amount of enzyme then leaks from the matrix by washing or during reactions. In addition, it is difficult to form it into different shapes. Alginate and carrageenan gels are widely employed to immobilize enzymes (Chibata, 1990). The lack of stability of alginate gels to phosphate buffer is a serious problem. Here, we report the gel formation of cellulose acetate (CA) with an organometallic compound and its application in enzyme immobilization.

EXPERIMENTAL

All chemicals were of commercially available reagent grade and used without further purification. Urease (1·5 units mg^{-1} from Jackbean) and glucose oxidase (15 units mg^{-1} from *Aspergillus niger*) were purchased from the Sigma Co. Cellulose acetate (acetyl content, 38·8%), metal alkoxide (Ti–iso-propoxide, Zr–*n*-butoxide), D-glucose, and urea were commercially obtained from Wako Pure Chemicals Co. 2, 2′-Azinobis (3-ethyl benzothiazoline-6-sulphonic acid) diammonium salt (ABTS) and Tashiro reagent (0·03 g methyl red/100 ml of 60% ethanol solution + 15 ml of 0·1% aqueous methylene blue solution), bovine haemoglobin and cytochrome C (from horse heart) were obtained from Tokyo Kasei Co. Prior to the experiments, acetone was dehydrated using molecular sieves (3Å 1/16). Buffer solutions were prepared from sodium phosphate. The enzyme reaction and leakage of protein from gel fibres were followed spectrophotometrically using a SHIMADZU UV-2200. Gel formation was examined by mixing a 10% cellulose derivative organic solution and a 10% metal alkoxide or metal acetylacetonate organic solution. CP/MAS-^{13}C-NMR spectra were recorded using a JEOL GX-270 spectrometer (resonance frequency 67·8 MHz). Samples were cut into small pieces in order to fill a rotor. Spectra were accumulated for 1000–2000 runs. IR spectra were recorded using a SHIMADZU FTIR-8100 by the KBr disk method. Ten wt % cellulose acetate acetone solution dispersed with enzyme or protein (3 wt % for the former solution) was slowly fed through the nozzle into a 10 wt % $Zr(O\text{-}n\text{-}C_4H_9)_4$ acetone solution. After standing for 30 min, the entrapped acetone was washed from the gel fibres with pure water and cold 0·1 M phosphate solu-

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tion (pH 7·0). The fibres were stored in a freeze-dried state. One gram of fibre containing trapped coloured protein was added to 300 ml of phosphate buffer solution (pH 7·0) and stirred at 25 ± 1°C. Increases in absorbance at 405 or 408 nm were followed for haemoglobin or cytochrome C, respectively. Glucose was determined using a Wako glucose kit. Ten ml of D-glucose solution was mixed with 50 ml of 0·1 M NaH_2PO_4 buffer solution (pH 6·0) containing ABTS (0·5 mg ml^{-1}). Gel fibre (0·5 g) was added to this solution thermostated at 25 ± 0·1°C. The enzyme reaction was determined from the change in absorbance at 414 nm. The molar absorptivity was adopted as ε (414 nm) = $3{\cdot}6 \times 10^4\ M^{-1}\ cm^{-1}$ (Ichimura, 1984). The hydrolysis of urea was assayed using the neutralization titration method (Kobatake & Shimizu, 1983). One gram of fibre was added to a mixture of 5 ml urea solution and 5 ml 0·1 M NaH_2PO_4 solution (pH 7·0) thermostated at 25 ± 0·1°C. After 10 min, the reaction was stopped by adding a few drops of 1% $HgCl_2$ to the solution. NH_3 produced by the reaction was titrated with a standard HCl solution using Tashiro reagent as an indicator.

RESULTS AND DISCUSSION

Gel formation of the cellulose derivative with a metal alkoxide or metal acetyl acetonate were examined in various solutions. The results for acetone solutions are given in Table 1. The alkoxides listed in the upper column gave good gel formation. The alkoxides listed given in the middle column and acetyl acetonate in the lower column did not produce gelation. Since the interaction between a metal and acetyl acetone is strong, the coordination bonding between an OH group on the pyranose ring and metal, i.e. ligand exchange, may not easily proceed. In addition, alkoxides in the upper column are easily hydrolyzed by water, but alkoxides in the middle column and acetyl acetonate in the lower column are not. Similar results were obtained for THF and dioxane solutions. The IR spectra are shown in Fig. 1. Ti–CA does not give any new bands due to gel formation. However, Zr–CA gives a new band at 1560 cm^{-1} which may be ascribed to the Zr–O–C bond (Izumi *et al.*, 1989). It is denoted in the figure by an arrow. Figure 2 shows the NMR spectra of a CA gel film prepared by casting the CA acetone solution and the Zr–CA gel fibre. The distinction between the C_1 and C_6 signals is clearly observed, but the C_2, C_3, C_4 and C_5 signals are superposed. No substantial displacement of the signals was seen among these spectra. The relaxation time (s) data are as follows: C=O 30, C_1 18, C_{2-5} 24, C_6 5·0, CH_3 14 for CA film, 37, 24, 31, 9.3, 16 for the CA–Zr(ZrO_2;5 wt %) fibre and 33, 22, 30, 8.4, 17 for the CA–Zr(ZrO_2;15 wt %) fibre. This indicates that the relaxation time, generally, tends to increase when gel formation occurs. This fact may explain why hydrogen

Table 1. Gel formation of cellulose acetate with organometallic compounds

Compound	Formation
Zr (O-*n*-C_4H_9)$_4$, Zr (O-*n*-C_3H_7)$_4$ Ti (O-*n*-C_4H_9)$_4$, Ti (O-iso-C_3H_7)$_4$, Ti (OC_2H_5)$_4$ Y (O-iso-C_3H_7)$_3$, Fe (O-*n*-C_4H_9)$_3$	○
Si (O-*n*-C_4H_9)$_4$, Si (OC_2H_5)$_4$, Si (OCH_3)$_4$, Al (O-iso-C_3H_7)$_3$	×
Ar (acetyl acetone)$_4$ Ti (acetyl acetone)$_4$	×

○ — Good gel formation.
× — No gelation.

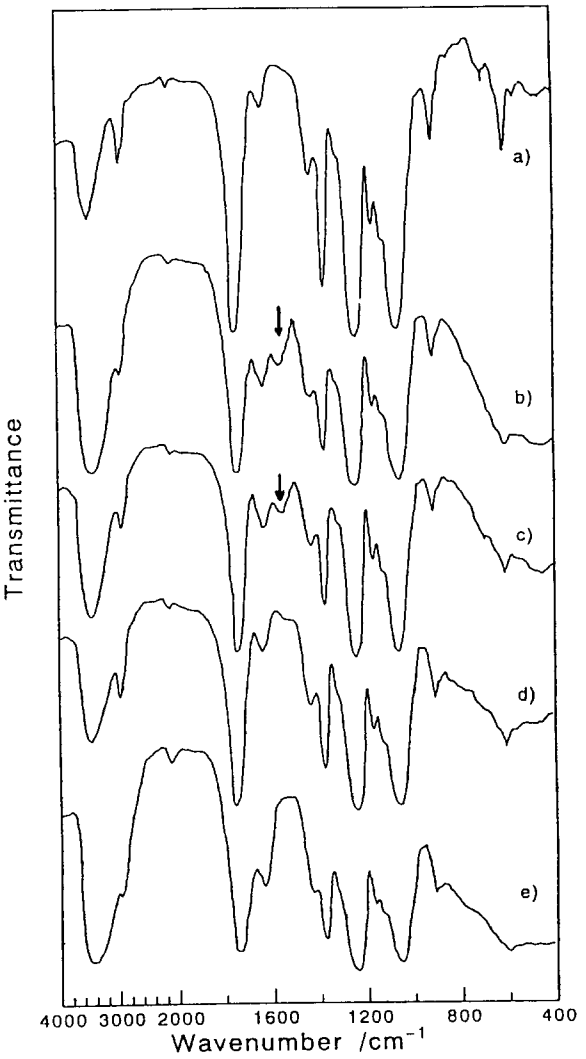

Fig. 1. IR spectra of cellulose acetate (CA) and CA–Metal oxide. (a) CA; (b) Zr–CA (ZrO_2 is derived from Zr(O-*n*-C_4H_9)$_4$); (c) Zr–CA (ZrO_2 is derived from Zr(O-*n*-C_3H_7)$_4$); (d) Ti–CA (TiO_2 is derived from Ti(O-*n*-C_4H_9)$_4$); (e) Ti–CA (TiO_2 is derived from Ti(O-iso-C_3H_7)$_4$). Arrow indicates band ascribed to Zr–O–C bond.

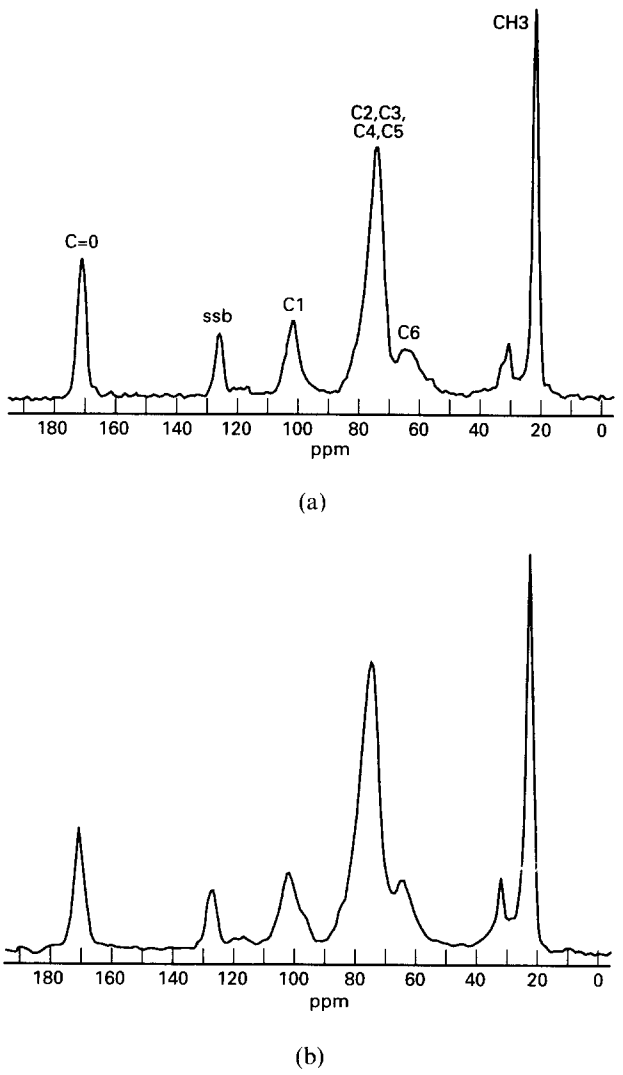

Fig. 2. CP MAS ^{13}C NMR spectra of (a) CA and (b) Zr–CA. Zr–CA contains 15 wt % ZrO_2.

bonds between the OH groups on CA break after gel formation occurs.

This gel formation was applied to the immobilization of an enzyme. In this case, the enzyme is immobilized solely physically; hence there exists the possibility that the enzyme can escape from the fibre. To estimate the magnitude of this effect, the leakage of coloured proteins (haemoglobin, MW = $6{\cdot}5 \times 10^4$, cytchrome C, MW = $1{\cdot}04 \times 10^4$) was followed at given time intervals. The results are shown in Fig. 3. The leakage increases during the initial period and then reaches a steady state. Since the molecular weight of the enzyme (urease: $4{\cdot}8 \times 10^5$, glucose oxidase: $1{\cdot}8 \times 10^5$) is larger than that of the coloured proteins, it seems that the leakage of these enzymes will be small. In fact, the solution decanted from the fibre immersed and in a flask did not give the enzyme reaction after several washings. Many factors such as pH, temperature, product, etc., affect enzyme stability. The repeated run effect for frequently

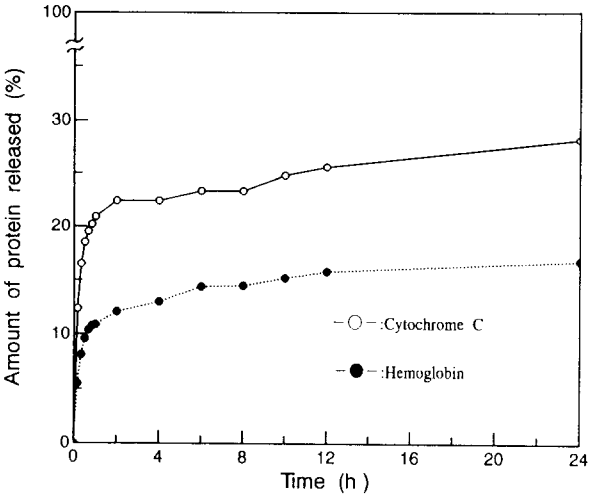

Fig. 3. Leakage of coloured protein from fibre.

immobilized enzymes and the product inhibition effect for urease were examined to see if this method could be used practically. The other effects on stability are now under investigation in columns packed with the immobilized fibre. Effects of repeated runs on activities of the immobilized enzyme are shown in Fig. 4 for urease and glucose oxidase. The change in activity was calculated under the assumption that the enzyme is totally entrapped and sustains the initial activity. Activity tends to increase with run number. This may be due to the formation of a new reaction path, or the manifestation of a new reaction site as a result of the rearrangement of the enzyme through use. Activity of an immobilized enzyme is decreased by about one order of magnitude over the native activity. The lower activity of the enzyme immobilized in the gel fibre does not seems to be related to any diffusion effect. It may be due to the fact that an enzyme entrapped inside the gel fibre does not participate in the reaction, whereas an enzyme entrapped on the surface of the fibre is involved in the reaction. An enhancement of activity may be improved

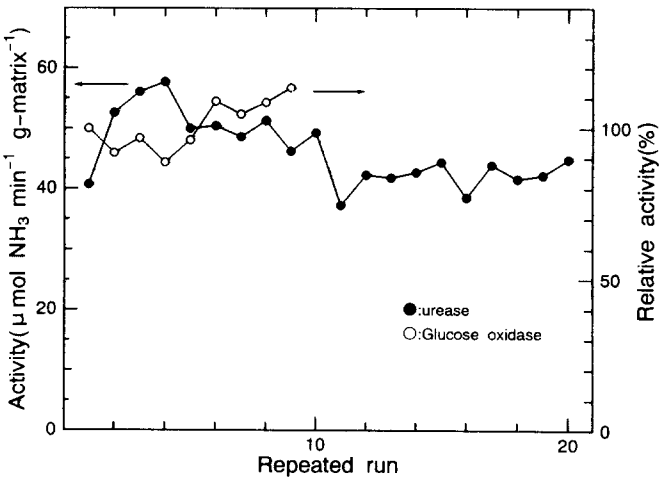

Fig. 4. Change in activity with repeated runs. ●: Zr–CA fibre containing urease; ○: Zr–CA fibre containing glucose oxidase.

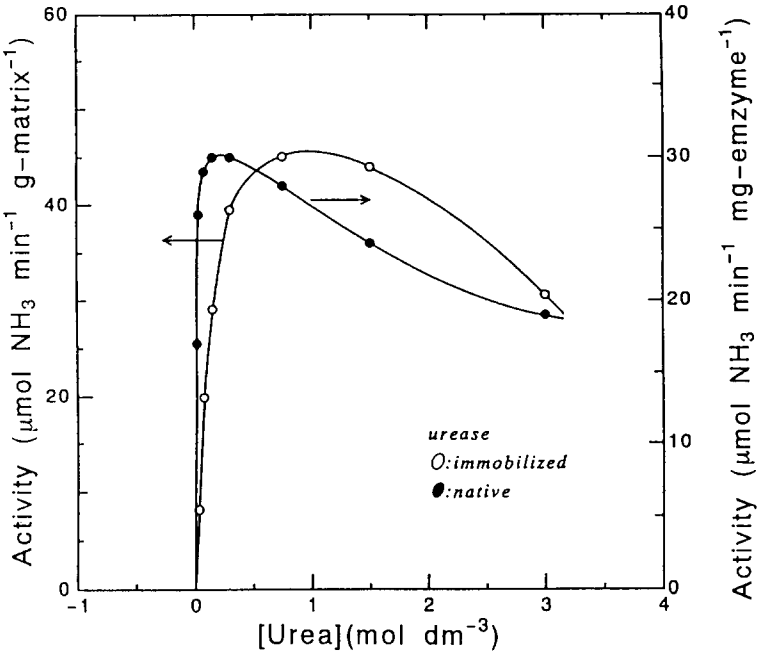

Fig. 5. Effect of substrate concentration on urease activity. Zr–CA fibre contains urease.

by making the fibre thinner or coentrapping a hydrophilic active reagent into the fibre. The effect of substrate concentration on urease activity is shown in Fig. 5. Activity is expressed as units per gram of dried fibre. There is a rapid increase in activity up to 0·1 mol litre^{-1} for the immobilized enzyme. Beyond this concentration, the activity decreases with concentration. It appears that the enzyme has undergone inhibition due to products of its own reaction. A similar trend is observed with the native enzyme. Highly purified urease is known to be very unstable in solution (Gorin & Chin, 1966). The optimum concentration of the immobilized enzyme shifts to the higher concentration side compared to the native enzyme. This indicates that the enzyme becomes more stable after being immobilized.

It seems that gel formation of CA with a metal alkoxide is attributed to the possible coordination of the metal with OH groups present in CA. The tetra-functional titanate or zirconate ($M(OR)_4$) can undergo multiple hydrolysis reactions. Then, OH at CA may easily take part in this reaction. An enzyme possesses a side-chain amine, carboxy and thiol groups. These must be considered to act as ligands to the metal as suggested by J.F. Kennedy (White & Kennedy, 1980). This immobilization can be simply carried out *in situ*, which could then be conveniently extended to the immobilization of other enzymes.

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