Application of Polyelectrolyte Complex Gel Composed of Xanthan and Chitosan to the Immobilization of *Corynebacterium glutamicum*

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SYNOPSIS

Polyelectrolyte complex (PEC) gel prepared from xanthan and chitosan was applied to the immobilization of *Corynebacterium glutamicum* (IAM 12433) having fumarase activity. A *C. glutamicum* cells suspension was mixed with xanthan and chitosan solution in the presence of NaCl below room temperature. Thereafter, the mixture was rinsed in distilled water and the PEC immobilized cells were obtained. The fumarase activity of the immobilized cells or intact ones was estimated by the amount of malate produced. The fumarase activity of immobilized cells was about 5 times that of intact cells. It was suggested that the interaction between the polycation (chitosan) and the *C. glutamicum* cells contributed to the enhancement of fumarase activity. In the column experiment, the immobilized cells were stable in an electrolyte solution of high concentration; about 90% of the initial fumarase activity was retained for 240 h. In addition, the pore size distribution for the xanthan/chitosan gel was measured by a solute exclusion method. As the result, the pore size of the xanthan/chitosan gel was similar to that of polysaccharide gels. © 1996 John Wiley & Sons, Inc.

INTRODUCTION

Immobilization is effective for using biocatalysts (such as enzymes or microbial cells) as recoverable, stable, and specific industrial catalysts. For microbial cells, several immobilization techniques were known, such as covalent bonding or electrostatic bonding to a support, a copolymerization method or an entrapment method.[1] For successful immobilization, the following factors should be considered: (1) The biocatalyst must not be inactivated; therefore immobilization must be carried out under mild conditions, such as at low temperature. (2) The mechanical properties, in particular the mechanical stability[2] and physical form,[3] and the chemical or physical stability[4] of the support must be considered. The discovery of a support or a method for immobilization of biocatalysts that satisfies these factors is important.

By mixing two oppositely charged polyelectrolytes in an aqueous solution, a polyelectrolyte complex (hereafter, PEC) is formed by the electrostatic attraction between the polyelectrolytes.[5] A support coated with PEC is suitable for immobilization of enzymes or microorganisms due to their high permeability, excellent chemical stability, and biological compatibility.[6] However, because PECs are generally obtained as precipitates, it is difficult to prepared bead- or fiber-shaped immobilized cells.[7] Sakiyama and colleagues[8] and Chu and coworkers[9] prepared moldable chitosan/κ-carrageenan and xanthan/chitosan complex gels in the presence of NaCl during the mixing of polyelectrolytes, respectively. In addition, they investigated the pH-dependent swelling behavior of the PEC gels. Ikeda and coworkers[10] analyzed the swelling behavior of the xanthan/chitosan complex gel using a model based on the Donnan equilibrium theory, with special attention to the dissociation behavior of the polyelectrolytes. These PEC gels are usually insoluble in aqueous media. In particular, the xanthan/chitosan complex gel can be prepared below room temperature; thus it is

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promising in the field of immobilization of microorganisms.

In this study, a microorganism having fumarase activity was immobilized with xanthan/chitosan gel, and the fumarase activity was measured.

EXPERIMENTAL

Reagents

Corn steep liquor, malate dehydrogenase (MD, EC 1.1.1.37, 5000 units), and glutamate–oxaloacetate transaminase (GOT, EC 2.6.1.1, 1000 units) were purchased from Sigma Chemical Co., St. Louis, Missouri. Gall powder was obtained from Wako Pure Chemical Ind., Ltd., Tokyo, Japan. Nicotinamide adenine dinucleotide free acid (NAD) was purchased from Boehringer, Germany. Polyethylene glycol polymers (PEG) were purchased from Nacalai Tesque Inc., Tokyo, Japan.

Xanthan and chitosan were purchased from Fuji Syoji Co., Ltd., and Kimitsu Chemical Industry Co., Tokyo, Japan, respectively, and were used without further purification. The carboxyl group content of xanthan was determined to be 0.00153 mol/g of xanthan (by Ikeda and colleagues[10]), and the amino group of chitosan to be 0.00432 mol/g of chitosan.

All other reagents were of analytical grade and obtained from commercial sources.

Microorganisms

Corynebacterium glutamicum (IAM 12433) having fumarase activity was obtained from the Institute of Molecular and Cellular Biosciences, University of Tokyo.

Cultivation and Harvest of *C. glutamicum*

C. glutamicum was cultured by the following method, which is similar to that employed by Takata and coworkers.[11]

The medium contained 2.0% malonic acid, 2.0% corn steep liquor, 0.5% diammonium citrate, 0.2% KH_2PO_4, and 0.05% $MgSO_4 \cdot 7H_2O$. The pH of the medium was adjusted to 7 by adding 5*N* KOH solution. A loopful of *C. glutamicum* was inoculated in a 500-mL Erlenmeyer flask (the amount of medium was 100 mL). The microorganism was cultured at 30°C for 48 h with shaking (140 cpm., 8 cm). The cells were harvested by centrifugation (8,500 rpm, 20 min) under 10°C and washed with 0.1*M* phosphate buffer (pH 7.0).

To enhance the fumarase activity and suppress succinic acid formation, 1 g (wet wt) of the cells of *C. glutamicum* was suspended in 15 mL of 1*M* potassium fumarate (pH 7.0) solution containing 0.6% gall powder, and the mixture was incubated at 37°C for 24 h. The cells were washed with 0.1*M* phosphate buffer twice at 5°C.

Immobilization of *C. glutamicum*

C. glutamicum was immobilized with the PEC gel prepared by Chu and colleagues.[9] These immobilized cells were used mainly for investigating the properties of immobilized *C. glutamicum* (this PEC gel will be referred to as 2x/2c PEC gel, because it contains 2% xanthan and 2% chitosan). The procedure is as follows: Eight grams (wet wt.) of *C. glutamicum* were suspended in 10 mL of 6% aqueous NaCl solution. Twenty milliliters of 5.0% xanthan solution containing 5.7% NaCl, and 20 mL of 5.0% chitosan solution containing 5.7% NaCl, were mixed at 70°C, then kept at 5°C for 1 day. The cells suspension and the polyelectrolyte complex solution were mixed at 5–10°C. The mixture was centrifuged at 5,000 rpm for 20 min to remove air bubbles and kept at 5°C for 1 day. Then the mixture was added dropwise into distilled water through a syringe with a 16-gauge (1.6 mm × 1.2 mm) needle. The gel beads of 3 mm in diameter obtained by this procedure were rinsed in distilled water for 2 days; the water was replaced each day. The immobilization was carried out under sterile conditions.

To investigate the effect of PEC gel composition on fumarase activity of the immobilized cells, "3x/c PEC gel" was prepared by mixing the cell suspension (which contained 8 g wet cells and 10 mL 6% NaCl solution) and PEC solution, which contained 30 mL of 5% xanthan solution and 10 mL of 5% chitosan.

Assay of Fumarase Activity

The fumarase activity of immobilized cells was measured by the following method, which was similar to that used by Takata and coworkers.[11]

Substrate solutions were prepared by dissolving sodium fumarate in 0.2*M* of specific buffer solution (acetic buffer, pHs 4 and 5; phosphate buffer, pHs 6, 7, and 7.5; borax buffer, pHs 8, 8.5, and 9) to form 0.5*M* or 1.0*M* fumarate solution.

In the assay for the intact cells, 0.2 mL of cell suspension (1.0 of wet cells suspended in 5 mL of biophysic saline solution) was added to 2.8 mL of substrate solution, and the mixture was incubated

at a specific temperature with shaking. The reaction was stopped by adding 3 mL of 2N–HCl to remove the remaining fumaric acid.

In the assay for immobilized cells, a reaction system consisting of 0.5 g of immobilized cells and 5 mL of substrate was incubated at a specific temperature with shaking. The reaction was stopped by removing the gel beads by filtration with a 0.45-μm filter unit (MILLEX-SV, Millipore, Tokyo, Japan).

The ratio of the amount of malate produced in 30 min to that produced in 30 min at 37°C and pH 7 by intact cells is defined as "relative activity" in this study. The malate concentration was measured by the method explained later in this study. Fumarase activity was evaluated mainly by the relative activity. The relative activity was measured three times for each experimental condition, and the averaged value was used.

Malate Concentration Measurement

The malate concentration was measured similarly to the method of McCloskey.[12] This assay method uses malate dehydrogenase and glutamate–oxaloacetate transaminase to accelerate the dehydrogenation reaction of malate. In the assay reaction, NADH is formed in a concentration equal to that of malate. The NADH can be quantified by a spectrophotometer at 340 nm. The assay procedures were carried out as follows.

Glycine–glutamate buffer (pH 9.8), 1.2 mL, and 20 μL of NAD solution were mixed in a 1-cm cuvette; then 10 μl of a sample, diluted appropriately, was added and mixed. The absorbance of the mixture was read in a spectrophotometer (Beckman DU-65) at 340 nm at room temperature and noted as E_1. Next, 3 μl of an enzyme solution (mixture of GOT and MD in equal volumes) was added, and the final absorbance at 340 nm was read as E_2 after 5 min at room temperature. The concentration of malate was calculated by the difference in the values E_2 and E_1.

Glycine–glutamate buffer was prepared by dissolving 11.4 g of glycine and 3.0 g of glutamic acid in about 250 mL of distilled water, then adjusting the pH to 9.8 with 5*N* KOH solution and bringing the volume to 300 mL.

Column Experiment

To test the operational stability of the immobilized *C. glutamicum* cells, a column experiment was carried out. The immobilized cells (7.25 g gel) were packed into a column (17 mm in diameter, 150 mm in height), and a solution of 1*M* fumarate, pH 8.0, was fed into the column at a flow rate of 9 mL/h. The malic acid concentration at the column exit was measured by the method described above. The column experiment was done at 37°C.

Measurement of Pore Size Distribution for PEC Gel

Because information about the network structure (pore size distribution) of the support for immobilization is important, the pore size (pore volume) distribution of the 2x/2c PEC gel was measured by a solute exclusion method[13,14]: The gel was considered as a porous body, and its pore size distribution was obtained using molecular probes of given diameter.

Glucose and a series of PEG (6,000 to 4,000,000 in molecular weight) were used as molecular size probes. The diameters of the PEG series were measured by a dynamic light scattering (DLS) method with a DLS apparatus (Malvern 4700 at 633 nm). The data are shown in Table I. As the diameter of glucose, the literature value of 0.72 nm[15] was used.

A probe solution (2.5 mL) and PEC gel (1 g) were added to a vial filled with nitrogen gas, and the vial was shaken at 25°C. After equilibrium was reached, the concentration of the probe solution was measured with a differential refractometer (Shodex RI SE-61). The pore volume per unit weight of gel for a given solute molecule, v, is calculated by the following equation based on the technique of Stone and Scallan.[13]

$$v = (C_0 - C_\infty)V_0/m/C_\infty \quad (1)$$

where C_0 is the initial solute concentration of a probe solution. When the diffusion of solute into the gel reaches an equilibrium state, the final solute concentration is C_∞. The weight of gel added is m, and V_0 is the volume of solution added. The data for the pore volume with a series of different size PEG give a cumulative curve.

Table I Molecular Probe Diameter of PEGs Measured by Dynamic Light Scattering Method

Probe	Diameter [nm]
PEG6000	7
PEG20000	10.8
PEG50000	17.2
PEG500000	64
PEG2000000	155
PEG4000000	309

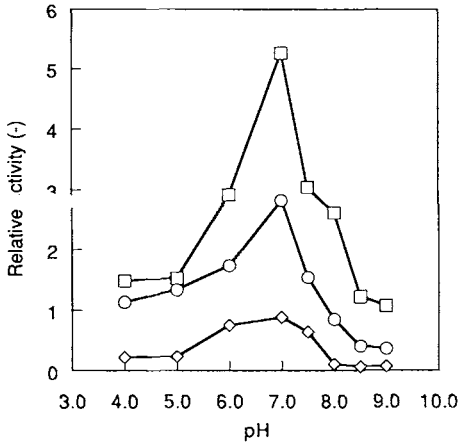

Figure 1 pH dependence of relative fumarase activity in 1*M* sodium fumarate solution at 37°C for (◇) intact cells, (□) immobilized cells with 2x/2c PEC gel, and (○) immobilized cells with 3x/c PEC gel.

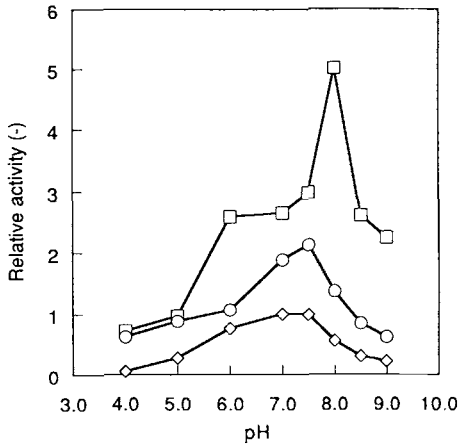

Figure 2 pH dependence of relative fumarase activity in 0.5*M* sodium fumarate solution at 37°C for (◇) intact cells, (□) immobilized cells with 2x/2c PEC gel, and (○) immobilized cells with 3x/c PEC gel.

RESULTS

Fumarase Activity of Immobilized Cells

Figure 1 shows the dependence of pH on the relative fumarase activity in 1.0*M* substrate solutions at 37°C. At identical pH, the values of relative activity of both immobilized cells were higher than that of intact cells, the activity value of 2x/2c immobilized cells being higher than that of 3x/c immobilized cells. The relative activity of the intact cells and the immobilized cells was the maximum at pH 7; the maximum for 2x/2c immobilized cells, 3x/c immobilized cells, and intact cells being 5.3, 2.8, and 1, respectively.

Figure 2 shows the dependence of pH on the relative fumarase activity in 0.5*M* substrate solutions at 37°C. At identical pH, the values of relative activity of both immobilized cells were higher than that of intact cells, the activity value of 2x/2c immobilized cells being higher than that of 3x/c immobilized cells as well as that of the 1.0*M* substrate solution shown in Figure 1. However, the optimal pH for 2x/2c shifted to pH 8.

Figure 3 shows the effect of reaction temperature on the fumarase activity for intact cells and 2x/2c PEC gel immobilized cells at pH 7 in a 1.0*M* substrate solution. The relative activity of intact cells was the maximum, 1.8, at 60°C. At identical temperatures, the values of the relative activity of the immobilized cells were higher than that of intact cells, and the maximal relative activity for the immobilized cells also occurred at 60°C.

Stability of Fumarase Activity

Figure 4 shows data for the continuous flow experiment. About 90% of the initial fumarase activity of immobilized cells was retained for at least 240 h. During the continuous-flow experiment, the PEC gel in the column apparently did not dissolve.

Pore Size Distribution of 2x/2c PEC Gel

Figure 5 shows the cumulative pore volume curves for the 2x/2c PEC gel measured by the solute exclusion method. The cumulative curves for pH 7 and pH 12 almost coincided, within experimental error.

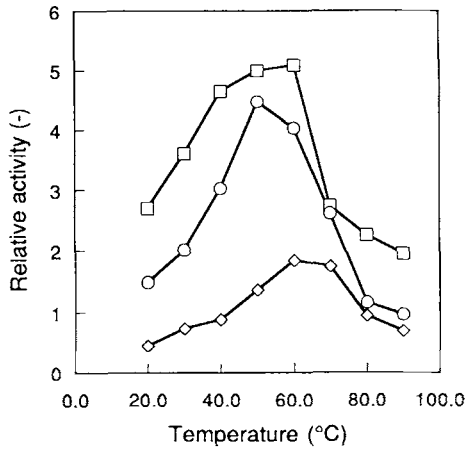

Figure 3 Temperature dependence of relative fumarase activity in sodium fumarate solution of pH 7 (0.2*M* phosphate buffer) for (◇) intact cells, (□) immobilized cells with 2x/2c PEC gel, and (○) immobilized cells with 3x/c PEC gel.

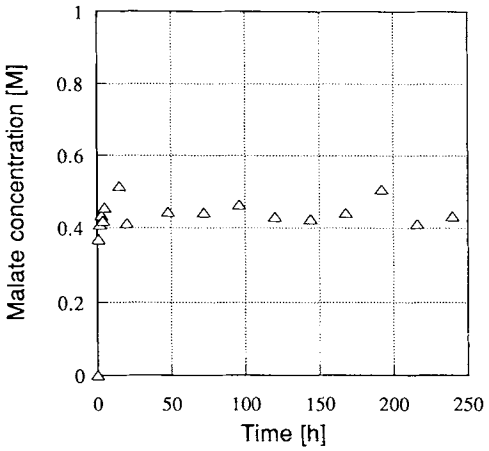

Figure 4 Continuous production of malate in the column of immobilized cells at a constant flow rate (9 mL/h) of 1*M* sodium fumarate solution of pH 8 at 37°C.

The amount of pore diameter above 100 nm at pH 10 was larger than that at pHs 7 and 12. Chu and colleagues[9] reported that the equilibrium swelling ratio of xanthan/chitosan gel was maximum at pH 10 and was unity at pH 2–9 and above pH 12.[9] The result in Figure 5 indicate that the average distance between neighboring crosslinks in the xanthan/chitosan gel increased as the swelling ratio increased. Fifty-percent diameters were 50 to 150 nm, similar to the values of polysaccharide gels such as agarose[16] and Isogel.[17]

DISCUSSION

PEC has been applied to immobilization of cells or enzymes by the usual PEC-entrapping[18–22] or encapsulating[3,4,23–25] methods. For the PEC-entrapping, immobilization is carried out by mixing a polycation solution and a polyanion solution in the presence of microorganisms. However, aggregation of PEC occurs rapidly during the mixing; thus it is difficult to prepare bead- or fiber-shaped immobilized cells for facilitating handling in a bioreactor process. For the encapsulating method, immobilization is carried out by adding dropwise a polyanion solution containing microbial cells to a polycation solution to form a layer of PEC complex on the surface of a support. Kokufuta and coworkers[3] prepared a PEC-stabilized calcium alginate gel using a method which was similar to the encapsulating method. However, the PEC-stabilized calcium alginate gel beads were broken in a 5 m*M* K_2HPO_4 solution.[3] In the support matrix prepared by the encapsulating method, a multilayer assembly with a liquid core was observed by electron microscope.[26] This heterogenous structure of support formed by the encapsulating method would have caused instability of the support in a buffer solution. On the other hand, the immobilized cells prepared in this study were moldable and easily packed into a column for the continuous-flow experiment in Figure 4. Furthermore, as shown in Figure 4, 1*M* fumarate solution (0.2*M* phosphate buffer) was charged continuously into a column packed with PEC immobilized cells. As the result, the fumarase of immobilized *C. glutamicum* with PEC gel retained about 90% of the initial activity, and the gel beads apparently did not dissolve for 240 h. The xanthan/chitosan gel is macroscopically homogenous; it is probably stable in the electrolyte solution due to electrostatic attraction between the neighboring polyanion (xanthan) and polycation (chitosan).

As shown in Figures 1 and 2, the fumarase activity of immobilized *C. glutamicum* was higher than that of the intact cells. Takata and coworkers[27] used 3% κ-carrageenan with the addition of 0.15% polyethyleneimine (polycation) to immobilize the *Brevibacterium flavum* (now included under *C. glutamicum*) cells. As a result, the activity and stability of fumarase increased by a factor of about 1.1 and 1.3, respectively, compared to that of cells immobilized with κ-carrageenan only. In their other study,[28] these same investigators used κ-carrageenans modified chemically with amines to immobilize *B. flavum*. As a result, the activity and stability of fumarase also

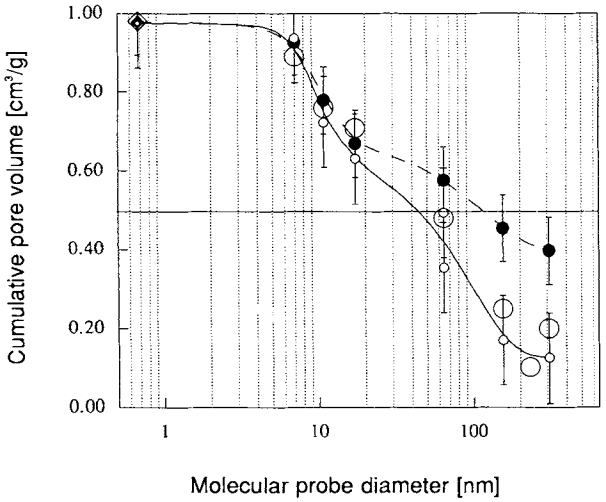

Figure 5 Pore size distribution of xanthan/chitosan gel in (——) H_2O of pH 7, (– – –) $NaOH_{aq}$ of pH 10, and (——) $NaOH_{aq}$ of pH 12. (◇, ○) Glucose and PEG at pH 7, respectively; (◆, ●) glucose and PEG at pH 10, respectively; (◆, ○) glucose and PEG at pH 12, respectively.

increased by about 1.1 and 1.3 compared to that of immobilized cells with natural κ-carrageenan. According to Takata and colleagues,[29] the enhancement of the activity and stability of fumarase by the addition of polycation was caused by the increase in interaction between the support and the outer membrane of *B. flavum* in the presence of amino groups.[29] In Figures 1 and 2 of this study, the fumarase activities were higher in the order of the 2x/2c PEC immobilized cells, the 3x/c PEC immobilized cells, and the intact cells. These results also suggest that the increase in the amount of amino groups (polycation) was effective for enhancing the activity of fumarase. However, as shown in Figures 1 and 2, the activity enhancement by polycation addition in our study was more prominent than those in Takata's studies. This activity enhancement might be ascribed to the macroscopic homogeneity of the PEC gel used in this study.

As can be seen in Figure 2, the optimal pH for the 2x/2c immobilized cells was higher than that for the intact cells in 0.5*M* substrate solution. In general, if the Donnan equilibrium is valid in the gel–solution system, all mobile ion species in the system are distributed between the gel and the ambient solution according to the identical Donnan ratio.[29] Ikeda and coworkers[10] have confirmed that the Donnan equilibrium was valid for the 2x/2c PEC gel by measuring the concentration of sodium ion in the gel, though they have not investigated the 3x/c complex gel. As discussed by Sakiyama and colleagues[8] and Chu and coworkers,[9] the sodium ion concentration in the 2x/2c PEC gel was expected to be higher than that in the ambient NaOH solution in the pH range of 7 to 12. From this aspect, the proton concentration in the 2x/2c gel would also be higher than that in the ambient solution, causing the shift in optimal pH for 2x/2c gel shown in Figure 2. On the other hand, the value of the Donnan ratio for the 3x/c complex gel would be different from that for the 2x/2c gel even in the same ambient solution, perhaps causing the same optimal pH as that for the intact cells, as shown in Figure 2. As for the 1*M* substrate solution shown in Figure 1, the Donnan ratio might have been about unity, because the charges of the gel polymers were electrically shielded by the ambient solution of high concentration.

As shown in Figure 4, the fumarase activity was maintained during the continuous-flow experiment, indicating that the cells of *C. glutamicum* did not leak out of the PEC gel. As explained in Figure 5, the average diameter of the PEC gel was 80 to 150 nm, rather smaller than that of the *C. glutamicum* cells (600–1,200 × 1,500–6,000 nm[30]). The cells would be retained in the network of the PEC gel during the continuous-flow experiment. In addition, the electrostatic interaction between the polyelectrolytes and the cells may also have contributed to the retention of the cells.

The cells immobilized in xanthan/chitosan gel had some advantages: facility in preparation, high enzyme activity, stability in buffer solution, and enzyme stability. Further investigation is needed to determine whether this PEC gel can be applied to immobilization of other microbial cells.

CONCLUSIONS

In this study, *C. glutamicum* having fumarase activity was immobilized in xanthan/chitosan gel.

1. The moldable immobilized cells were obtained under moderate condition.
2. The fumarase activity of the immobilized cells was about 5 times that of intact cells.
3. The immobilized cells were stable in an electrolyte solution of high concentration.
4. In the column experiment, about 90% of the initial fumarase activity was retained for 240 h.
5. It was suggested that the interaction between the polycation (chitosan) and the *C. glutamicum* cells contributed to the enhancement of fumarase activity.
6. The pore size of the xanthan/chitosan gel was similar to that of polysaccharide gels.

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