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Stabilization of Fumarase Activity of *Brevibacterium flavum* Cells by Immobilization with κ-Carrageenan

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Abstract

Whole cells of *Brevibacterium flavum* having fumarase activity were immobilized using κ-carrageenan. The stabilities of fumarase activity in the immobilized cells against external factors, including heat, pH, organic solvents, and protein denaturing reagents, were compared with those of free cells and native enzyme.

The stabilities of fumarase activity in immobillized cells against external factors were highest, and those of native enzyme were lowest. In the "gel-state," κ-carrageenan-immobilized cells showed a much higher stabilization effect for external factors than "sol-state" immobilized cells.

Index Entries: κ-Carrageenan, immobilization of fumarase on; immobilized cells, fumarase activity of; fumarase, activity on immobilized cells; enzyme stability, and immobilized cells; *Brevibacterium flavum*, fumarase activity in immobilized cells of; stability, of fumarase in immobilized cells

Introduction

Within the last several years, many papers on immobilized whole microbial cells have been published (*1*, *2*).

One of the advantages occurring from the use of immobilizaed enzymes and microbial cells is enhancement of the stability of the target enzyme. Recently, several reviews of the stabilization mechanisms of enzyme immobilization have been published (*3*, *4*). These reviews are useful for analyzing the mechanisms, and some of

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the reasons cited for stabilization can be adapted to the case of immobilized microbial cells. However, it is very difficult to clarify the stabilization mechanism for such immobilized microbial cells, whereas the stability of immobilized enzymes can easily be discussed from the viewpoint of the interaction between the enzyme and the immobilization carrier. On the other hand, in the case of immobilized microbial cells, it is necessary to consider the complicated system of enzyme–cell membrane–cell wall–gel matrix.

In previous papers, we reported that κ-carrageenan, a polysaccharide, is a suitable polymer for the immobilization of microbial cells. Since the enzymic activities of cells immobilized with κ-carrageenan were very stable, we changed two processes for the industrial productions of L-malic acid (*7, 8*), and L-aspartic acid (*9*) from the conventional immobilization method using polyacrylamide gel to this new method in 1977 and 1978, respectively.

In this paper, we present studies on the stabilities of fumarase (L-malate hydrolyase, fumarase hydratase, EC.4.2.1.2) of immobilized *Brevibacterium flavum* against various external factors and compare the effect on the stabilization of fumarase activity of these cells using "gel-state" and "sol-state" κ-carrageenans as the immobilizing agents.

Materials and Methods

Chemicals

κ-Carragenan was obtained from Sansyo Co. Ltd. (Osaka, Japan). Bile extract was obtained from Inolex Pharmaceutical Division, Wilson Pharmaceutical and Chemical Corporation (Illinois, USA). Fumaric acid was obtained from Kawasaki Kasei Kogyo Co. Ltd. (Kanagawa, Japan). Other reagents were obtained from Katayama Chemical Industries Co. Ltd. (Osaka, Japan).

B. flavum Cells

B. flavum ATCC 14067 cells were obtained by the cultural method previously reported (*7*).

Native Fumarase

Native fumarase was prepared by the method previously reported (*7*). This preparation formed 9.3 μmol of L-malic acid/min/mg of protein under the standard assay conditions; 1 mL of the enzyme solution contained 2.8 mg of protein.

Free Cells

In order to initiate fumarase activity and to suppress L-succinic acid formation, 2.5 g (wet weight) of *B. flavum* cells were treated with 150 mL of 1*M* sodium fumarate (pH 7.0) containing 900 mg of bile extract at 37°C for 24 h. The cells were col-

lected by centrifugation (3000*g*), washed with 1*M* sodium fumarate, and suspended in 40 mL of 1*M* sodium fumarate.

Immobilized Cells

A 16 g quantity of *B. flavum* cells was suspended in 16 mL of physiological saline at 45°C and 3.4 g of carrageenan were dissolved in 34 mL of physiological saline. Both were mixed together at 50°C, and the mixture was cooled at 10°C for 30 min. In order to increase the gel strength, the gel was soaked in cold 0.3*M* potassium chloride solution for 4 h. After this treatment, the rigid gel obtained was granulated to particles of 3 mm diameter. In order to initiate fumarase activity and to suppress L-succinic acid formation, 6.2 g of the immobilized cells were added to 15 mL of 1*M* sodium fumarate containing 0.6% bile extract. The mixture was incubated at 37°C for 24 h, and the gel was thoroughly washed with 0.3*M* potassium chloride.

Standard Assay of Fumarase Activity

(1) Native Fumarase A mixture of 0.1 mL of native fumarase and 1.9 mL of 1*M* sodium fumarate (pH 7.0) was incubated at 37°C for 10 min. After the reaction was stopped by the addition of 2 mL of 2*N* hydrochloric acid, the precipitates were removed by centrifugation (3000*g*). The L-malic acid formed in the supernatant was determined colorimetrically by the method of Goodman and Stark (*10*). A 1 mL volume of the native fumarase solution formed 26.0 μmol of L-malic acid/min.

(2) Free Cells A mixture of 0.1 mL of cell suspension and 1.9 mL of 1*M* sodium fumarate was incubated at 37°C for 10 min. The reaction was stopped by the addition of 2 mL of 2*N* hydrochloric acid, and the L-malic acid formed was determined by the same manner as in the case of native fumarase. A 1 mL volume of the cell suspension formed 26.8 μmol of L-malic acid/min.

(3) Immobilized Cells. To 20 mL of 1*M* sodium fumarate (pH 7.0) were added 2 g of immobilized cells. The mixture was incubated with shaking at 37°C, and L-malic acid in the reaction mixture after 15 and 30 min was determined by the same method used in the case of native fumarase. The fumarase activity was calculated from increase in L-malic acid concentration during 15 min. A 1 g quantity of the immobilized cells formed 26.2 μmol of L-malic acid/min.

Results and Discussion

In the previous papers of this series (*5*, *6*), we reported that κ-carrageenan is a suitable matrix for immobilization of microbial cells, and that enzyme activities of cells immobilized with this matrix generally had sufficiently high stabilities to permit continuous enzyme reaction.

Therefore, in order to determine the stabilization mechanism of immobilized cells, we investigated the stability of fumarase activity of *B. flavum* cells immobilized with κ-carrageenan.

Stabilities of Fumarase Activity of Native Enzyme, Free Cells, and Immobilized Cells

At first, we tested the stabilities of fumarase activity towards external factors such as heat, pH, organic solvents, and protein denaturing reagents using native fumarase, free cells of *B. flavum,* and the immobilized cells.

(1) Thermal Stability The thermal stabilities of fumarase activity of the three preparations were compared with each other. As shown in Fig. 1, the thermal stability of fumarase of immobilized cells was the highest among these three preparations, and the stability of free cells was higher than that of the native enzyme.

(2) pH Stability The stabilities of fumarase activity against hydrogen ion were compared in these preparations, and the results are shown in Fig. 2. The stabilities of fumarase activity of free cells and immobilized cells over an extensive pH range were higher than that of the native enzyme.

(3) Stability Against Organic Solvents The stability of fumarase activity was investigated in various concentrations of ethanol and acetone. As shown in Fig. 3, the stabilities of fumarase activity of immobilized cells against ethanol and acetone were highest among these three preparations. The stability of free cells was obviously higher than that of the native enzyme.

(4) Stability Against Protein Denaturing Reagents The stabilities of the fumarase activity of immobilized cells against protein denaturing reagents such as urea and guanidine hydrochloride were compared with those of the free cells and the native enzyme. The fumarase activity of immobilized cells was the most stable

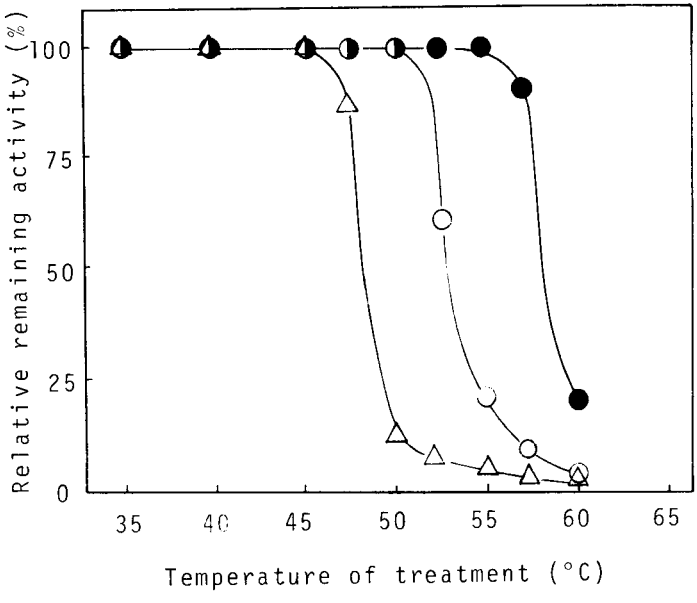

Fig. 1. Thermal stabilities of fumarase activity of native enzyme, free cells, and immobilized cells: Fumarase preparations were incubated in 0.1*M* phosphate buffer (pH 7.0) at the indicated temperature for 1 h. The remaining fumarase activity was determined by the method described in the text. Native enzyme (△), free cells (○), immobilized cells (●).

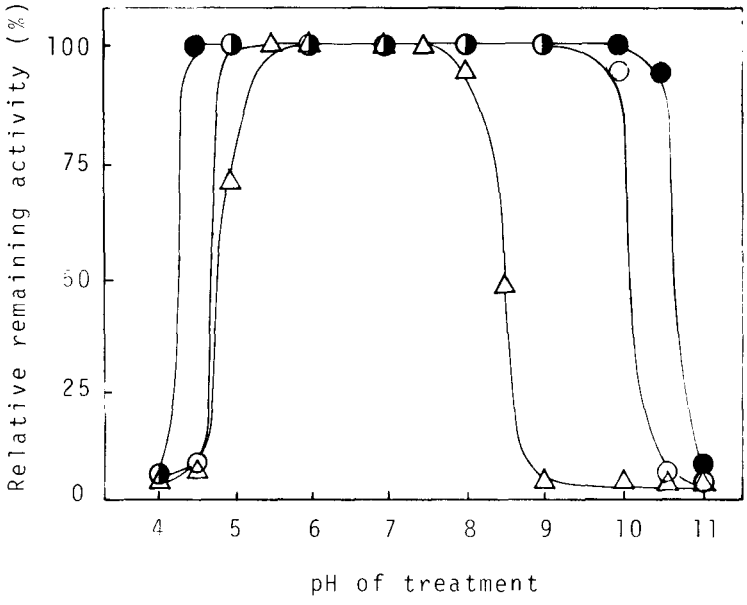

Fig. 2. The pH stability of fumarase activity of native enzyme, free cells, and immobilized cells: Fumarase preparations were incubated in 0.5*M* buffer of various pH at 37°C for 1 h. The buffers were acetate, pH 4.0–6.0, phosphate, pH 6.0–7.5, and borate, pH 8.0–11.0. The remaining fumarase activity was determined by the method described in the text. Native enzyme (△), free cells (○), immobilized cells (●).

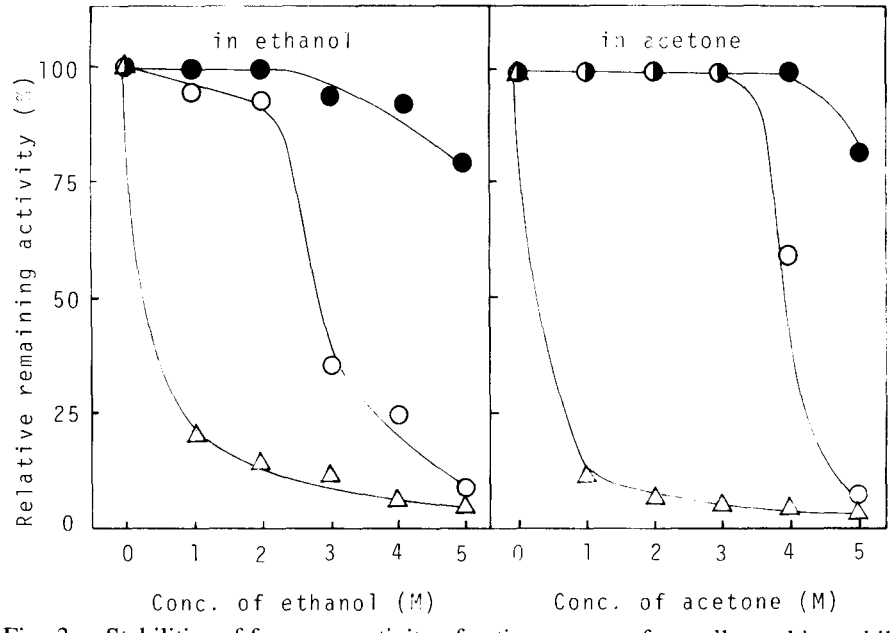

Fig. 3. Stabilities of fumarase activity of native enzyme, free cells, and immobilized cells against organic solvents: Fumarase preparations were treated in 0.1*M* phosphate buffer (pH 7.0) containing ethanol or acetone of indicated concentration at 37°C for 2 h. Immediately after the treatment a substrate was added into the mixture, and the remaining fumarase activity was determined by the method described in the text. Native enzyme (△), free cells (○), immobilized cells (●).

among these three preparations, and fumarase activity of free cells was more stable than that of native enzyme.

Under the protein denaturing conditions just mentioned, the fumarase activity of the immobilized cells was the most stable among the three preparations, and native enzyme was the least stable. These facts showed that the fumarase activity of immobilized cells is stabilized by κ-carrageenan and some cellular components of *B. flavum*.

Furthermore, in order to understand the stabilizing effect of κ-carrageenan matrix, the stabilities of fumarase activity were investigated under enzyme protein denaturing conditions using "gel-state" and "sol-state" κ-carrageenan.

Effect of "Gel-State" κ-Carrageenan on the Stability of Fumarase Activity

In general, polysaccharides have a protective effect with respect to the denaturation of enzymes. As described above, κ-carrageenan gel markedly affected the stabilities of fumarase activity of immobilized *B. flavum* cells against various external factors. Further, in order to reveal stabilization of fumarase activity by κ-carrageenan, fumarase activities of free cells and native enzyme were tested in the presence of κ-carrageenan in "sol-state" and "gel-state." "Sol-state" means "liquid-state carrageenan," and "gel-state" is the same material used in immobilized preparations.

(1) Thermal Stability Because thermal stability of the native enzyme in the absence of κ-carrageenan was ~5°C lower than that of free cells (Fig. 1), the thermal stability of fumarase activity of the native enzyme and that of the free cells in "sol-state" and "gel-state" κ-carrageenans were investigated at 50 and 55°C, respectively. As shown in Fig. 4, the fumarase activity of free cells was markedly stabilized by "gel-state" κ-carrageenan. "Sol-state" κ-carrageenan, however, had little stabilizing effect.

Stabilizing effect of fumarase activity by "gel-state" κ-carrageenan was also confirmed with native fumarase.

Therefore, it is reasonable to assume that some cellular components should be stabilized by "gel-state" κ-carrageenan in the same manner in the case of native fumarase, and that those cellular components should influence stability of fumarase in *B. flavum* cell.

(2) pH Stability As shown in Fig. 2, the remaining fumarase activity of immobilized cells after 1 h incubation was significantly higher than that of free cells at pH 4.5. Therefore, the stability of fumarase activity of free cells for treatment at pH 4.5 was investigated with "gel-state" and "sol-state" κ-carrageenans. The fumarase activity was markedly stabilized by "gel-state" κ-carrageenan. On the other hand, "sol-state" κ-carrageenan had little stabilizing effect.

(3) Stability Against Ethanol The protective effect of "gel-state" κ-carrageenan against ethanol treatment on free cells was investigated. The fumarase activity maintained its initial value in the case of "gel-state" κ-carrageenan. However, "sol-state" κ-carrageenan had little stabilizing effect compared with the case of no addition of κ-carrageenan.

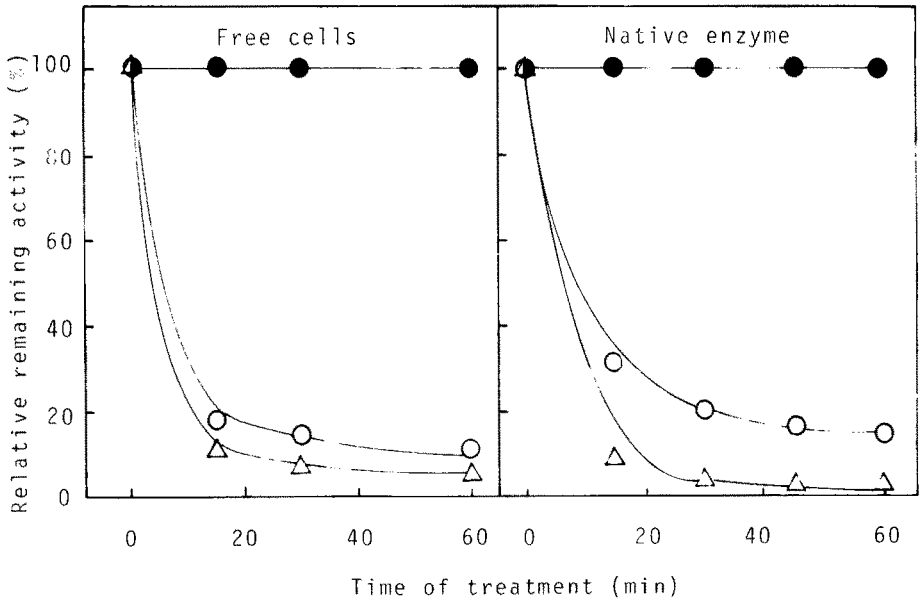

Fig. 4. Effect of κ-carrageenan in the "sol-state" or "gel-state" on the thermal stability of fumarase activity of cells and native enzyme: Stability in "sol-state" κ-carrageenan: 4 mL of free cells or native enzyme were added to 8.5 mL of a 5% κ-carrageenan solution in physiological saline, and the mixture was incubated for the indicated time at 55 or 50°C, respectively.
Determination of the fumarase activity was carried out as follows. Substrate was added into each treated mixture, and the fumarase activity was determined by the method described in the text. Stability in "gel-state" κ-carrageenan: The mixture in sol-state κ-carrageenan described above was cooled in an ice–water bath for 30 min to become gel, and 2% potassium chloride solution was added to the above gel. The potassium chloride solution was discarded. The gel was ground with a mortar and the thermal stability of the fumarase activity of the crushed gel was investigated by the same manner as that used in the case of "sol-state." Sol-state (○), gel-state (●), absence of κ-carrageenan (△).

(4) Stability Against Urea The stability of the fumarase activity of free cells toward a protein denaturing reagent such as urea was investigated. Fumarase activity in "gel-state" κ-carrageenan was 30% higher than that in "sol-state" material after treatment with 3*M* urea for 2 h.

(5) Stability for Repeated Use. The operational stability of the fumarase activity of free cells on repeated use in enzyme reactions was investigated in the presence of "gel-state" and "sol-state" κ-carrageenans and in the absence of κ-carrageenan. Fumarase activity in "gel-state" κ-carrageenan was the most stable.

In conclusion, the results presented in this paper suggest that high stabilities of *B. flavum* immobilized with κ-carrageenan were induced by "gel-state" κ-carrageenan and some cellular components of *B. flavum*.

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