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Reasons for the High Stability of Fumarase Activity of *Brevibacterium flavum* Cells Immobilized with κ-Carrageenan Gel

ISAO TAKATA,* TETSUYA TOSA, AND ICHIRO CHIBATA

Department of Biochemistry, Research Laboratory of Applied Biochemistry, Tanabe Seiyaku Co., Ltd., 16-89, Kashima-3-chome, Yodogawa-ku, Osaka, Japan

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Abstract

Whole cells of *Brevibacterium flavum* having high fumarase activity were immobilized using κ-carrageenan. The reason for the high stability of fumarase activity of immobilized cells was investigated.

One of main reasons for stabilizing fumarase activity by immobilization using κ-carrageenan against organic solvents such as ethanol and acetone was the lower concentration of these solvents in the carrageenan gel compared with that in outer bulk solution. The stabilization of fumarase activity in the immobilized cells against protein-denaturing reagents was found to be related to rheological properties of κ-carrageenan gel. Another reason for stabilizing fumarase activity by immobilization with κ-carrageenan was to protect the cells from lysis.

When immobilized cells were freeze-thawed, their fumarase activity increased and operation stability decreased. Therefore, one reason for the high decay of fumarase activity caused by the freeze-thawing may be a change in the pore size of the κ-carrageenan gel.

Fumarase activity and the operational stability of immobilized cells was found to depend on gelling conditions. Therefore, the steric structure of the κ-carrageenan gel may be related to the decay of fumarase activity.

Index Entries: κ-Carrageenan; immobilized cells, of *Brevibacterium flavum;* fumarase, activity in immobilized cells; stabilization of enzyme, by immobilization with κ-carrageenan; *Brevibacterium flavum,* immobilized.

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Introduction

It is well known that the immobilization of microbial cells enhances both their enzyme stabilities against heat (*1–5*) and storage (*6, 7*) and their operational stabilities (*2, 5, 6, 8–12*). The mechanisms for the stabilization of target enzyme in the immobilized cells, however, have not been determined.

In 1977, we succeeded in the industrial production of L-malic acid using *Brevibacterium flavum* cells immobilized with κ-carrageenan (*13*). The operational stability of these immobilized cells was very high; that is, the half-life of fumarase (fumarate hydratase; 4.2.1.2) activity was 160 d at 37°C (*14*). In the previous paper (*15*), we compared the stabilities of fumarase activity found in the native enzyme, in free cells, and in immobilized cells. From these results, "gel-state" κ-carrageenan and cellular components were found to affect the stability of fumarase activity considerably.

In this paper, the reason for the stabilization of fumarase in *B. flavum* immobilized with κ-carrageenan gel is presented.

Materials and Methods

Chemicals

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

Fumarase Preparations

B. flavum cells, native fumarase, and cells immobilized with κ-carrageenan were prepared in the same manner as described in a previous paper (*15*). Immobilization of *B. flavum* cells using polyacrylamide was carried out according to a method given in our previous paper (*6*). In the present studies, free cells and immobilized cells were treated with bile extract to activate fumarase activity and to suppress succinic acid formation.

Standard Assay of Fumarase Activity

The enzyme assay, using fumarase preparations such as native fumarase, free cells, and immobilized cells, was carried out by a previously reported method (*15*).

Estimation of Operational Stability of a Column Packed with Immobilized Cells

Immobilized cells (6.2 g gels) were packed into a column (17 × 159 mm), and a solution of 1*M* sodium fumarate (pH 7.0) was charged into the column at a flow

rate of space velocity = 0.3 h^{-1}. The first rate of fumarase activity at 37°C was estimated from the concentration of L-malic acid in effluent after the substrate solution was flowed at a rate between 20 and 30% of the maximum conversion rate (space velocity = 1.8 h^{-1} for 4 h). The apparent half-life was estimated by assuming exponential decay of the enzyme activity with time.

Partition Coefficient

A volume of 25 mL of gel containing 3.4% carrageenan and 16% (wet wt %) *B. flavum* were added to 25 mL of 2% potassium chloride aqueous solution containing organic solvent or protein-denaturing reagents, and the resulting mixtures were incubated at 37°C for 48 h under shaking. Concentrations of organic solvents in outer bulk solution were determined using gas chromatography.

The partition coefficients of organic solvents or protein-denaturing reagents between the gel and outer bulk solution were obtained according to the following equation:

$$K_p = \frac{C_0V_0 - C_1V_1}{V_2} \cdot \frac{1}{C_1}$$

In this equation, K_p is partition coefficient, C_0 and C_1 are initial and final concentrations of organic solvent or protein denaturing reagent in outer bulk solution, respectively; V_0 and V_1 are the initial and final volumes of outer bulk solution, respectively; and V_2 is the final volume of gel.

Gel Strength

The gel strength of samples was measured by using a NRM 2002J rheometer (Fudo Kogyo Co. Ltd., Tokyo, Japan) with a disk plate plunger of 10 mm diameter, and evaluated with load for gel-crush when a sample was pressed by the plunger.

Number of Cells

The number of cells was counted with a hemocytometer (Thoma Type; Erma Optics Co. Ltd., Tokyo, Japan) mounted under the microscope. *B. flavum* cells were dead because they had been treated with bile extract. In the case of immobilized cells, gels were solubilized by soaking in physiological saline, and the number of cells was counted.

Electron Micrographs of Gel

Wet gel was replaced in series of water/ethanol (50–70%), and absolute isoamylaceteate, and dried at the critical point of carbon dioxide. The resultant dried gel was cross-sectioned with a clean razor blade. The sections were successively sputter-coated with carbon and gold. The coated sections were observed in a JSM-35C scanning electron microscope (JEOL LTD. Tokyo, Japan).

Results

Stabilities of Fumarase Activity of Free Cells and Immobilized Cells

In a previous paper (*15*), it was found that the stabilities of fumarase activity of *B. flavum* cells against various external factors, such as heat, pH, organic solvents, and denaturing reagents, were enhanced by immobilization using "gel-state" κ-carrageenan.

Thus, the reason for the stabilization of fumarase activity by "gel-state" κ-carrageenan was investigated in detail.

Stabilization Against Organic Solvents. As shown in Table 1, the stabilizing effect of κ-carrageenan gel against treatment with ethanol and acetone was recognized, but the effect was not found against tetrahydrofuran. In order to examine why the stabilizing effect against organic solvents by κ-carrageenan gel was influenced by the type of organic solvent used, concentrations of these organic solvents in κ-carrageenan gel and outer solution bulk were investigated. As shown in Table 2, concentrations of ethanol and acetone in the gel phase were lower than those in outer bulk solution, but concentration of tetrahydrofuran was nearly equal in both phases.

Stabilization Against Protein-Denaturing Reagents. The stabilities of fumarase activity in "gel-state" κ-carrageenan against treatment with protein denaturing reagents such as urea and guanidine hydrochloride were compared with those in the "sol-state." As shown in Table 3, fumarase activity in "gel-state" κ-carrageenan after treatment with 3*M* urea was higher than that in "sol-state" κ-carrageenan. The same protective effect against guanidine hydrochloride was observed in the case of "gel-state" κ-carrageenan. Then the partition coefficient of these reagents between gel-phase and bulk solution was investigated. As the result,

TABLE 1
Effect of Organic Solvents on the Stability of Fumarase Activity

Organic solvent and its concentration[a]		Relative remaining activity[b]	
		Free cells	Immobilized cells
Acetone	1*M*	100	100
	3*M*	100	100
	5*M*	7	80
Ethanol	1*M*	92	100
	3*M*	33	100
	5*M*	11	75
Tetrahydrofuran	1*M*	90	94
	3*M*	4	4
	5*M*	3	3

[a]Treatment was carried out at 37°C for 30 min.
[b]Initial activity was taken as 100%.

TABLE 2
Partition Coefficient of Organic Solvents Between κ-carrageenan Gel and Outer Bulk Solution

Organic solvent	Partition coefficient
Acetone	0.61
Ethanol	0.75
Tetrahydrofuran	0.98

TABLE 3
Effect of Protein-Denaturing Reagents on the Stability of Fumarase Activity

Protein denaturing reagent and its concentration		Relative remaining activity[a] in Gel-state κ-carrageenan	Relative remaining activity[a] in Sol-state κ-carrageenan	Partition coefficient	Relative gel-strength, %
None		100	100		100
Urea	3*M*	55	37	0.98	49
Guanidine	0.6*M*	80	69	1.08	74

[a]Activity and gel-strength of nontreated preparation were taken as 100%.

we found that the concentrations of these reagents were not different between the gel phase and the outer bulk solution. On the other hand, the gel strength of immobilized cells decreased after treatment with these reagents. Specifically, the hydrophilic interaction between κ-carrageenan gel and the outer membrane of *B. flavum* cells was found to be decreased by these reagents.

Decay of Fumarase Activity During Repeated Batchwise Reaction and Continuous Reaction. Fumarase activity and the number of immobilized cells were compared with the same properties of free cells obtained by the repeated batchwise enzyme reaction, and the results are shown in Table 4. In the case of free cells, after five repeated batchwise reactions, both fumarase activity and the number of cells decreased to approximately 75% of their initial values. On the other hand, immobilized cells were protected from decreases in both fumarase activity and number of cells. In other words, cell lysis did not occur in the case of immobilized cells because a decrease in the number of cells was not observed. In the case of continuous enzyme reactions for periods as long as 88 d, values for the decrease of fumarase activity and the cell lysis of immobilized cells were less than those in the cases of five batchwise reactions using free cells. From these results, κ-carrageenan gel was suggested to have protective effect on cell lysis. Then the protective effect of κ-carrageenan gel on cell lysis was examined by scanning electron microscope. As shown in Fig. 1, it is clear that the shape of the cells and the number found in the gel matrix scarcely changed during continuous enzyme reaction.

TABLE 4
Variations of Fumarase Activity and Number of Cells After Enzyme Reaction

Treatment		Relative fumarase activity, %	Number of cells ($\times 10^{-11}$/g of cells)
Preparation	Enzyme reaction		
Free cells	First batchwise	100	1.10 (100%)[a]
	Fifth batchwise	74	0.80 (73%)
Immobilized cells	First batchwise	100	1.11 (100%)
	Fifth batchwise	100	1.12 (100%)
Immobilized cells	Continuous for 88 d	83[b]	1.02 (92%)[b]

[a]Value at first batchwise reaction was taken as 100%.
[b]Values of initial preparation were taken as 100%.

Effect of κ-Carrageenan Gel on the Operational Stability of Fumarase Activity

As described above, κ-carrageenan gel had a protective effect on cell lysis. This phenomenon may be related to the main reason for the stabilization of fumarase activity in immobilized cells during a continuous enzyme reaction over a long period. Then, we investigated the relationship between the steric structures of the κ-carrageenan gel and their stabilizing effect on fumarase activity.

Effect of Freeze-Thawing on Operational Stability. The effects of freeze-thawing immobilized cells on their fumarase activity and their operational stability were investigated at different freeze-treatment temperatures. As shown in Table 5, the fumarase activity of immobilized cells increased as a result of the freeze-thawing treatment, but the operational stability decreased compared with that of nontreated preparation, and this phenomenon was independent of the freeze-treatment temperature. On the other hand, as shown in Fig. 2, the freeze-thawing treatment for free cells did not affect the fumarase activities of free and immobilized cells, and the operational stability of immobilized cells. But the same treatment for immobilized cells increased fumarase activity and decreased its operational stability. As shown in the photograph of scanning electron microscopy in Fig. 3, the pore size of the carrageenan gel was widened by the freeze-thawing treatment.

Effect of Gelling Conditions on Operational Stability. As described in a previous paper (*16*), κ-carrageenan solutions became gels under various conditions. Thus, the effect of gelling conditions on fumarase activity and the operational stability of immobilized *B. flavum* cells was investigated. As shown in Table 6, the fumarase activities and the operational stabilities of immobilized preparations were different from each other because of the gelling conditions. The immobilized cells prepared by a cooling procedure were highest in activity and their stability. When immobilized cells prepared under other gelling conditions were ground with a mortar, those fumarase activities were equal to the activities found in free cells, except for the preparation gelled with acetone. Recovery of the fumarase activity in

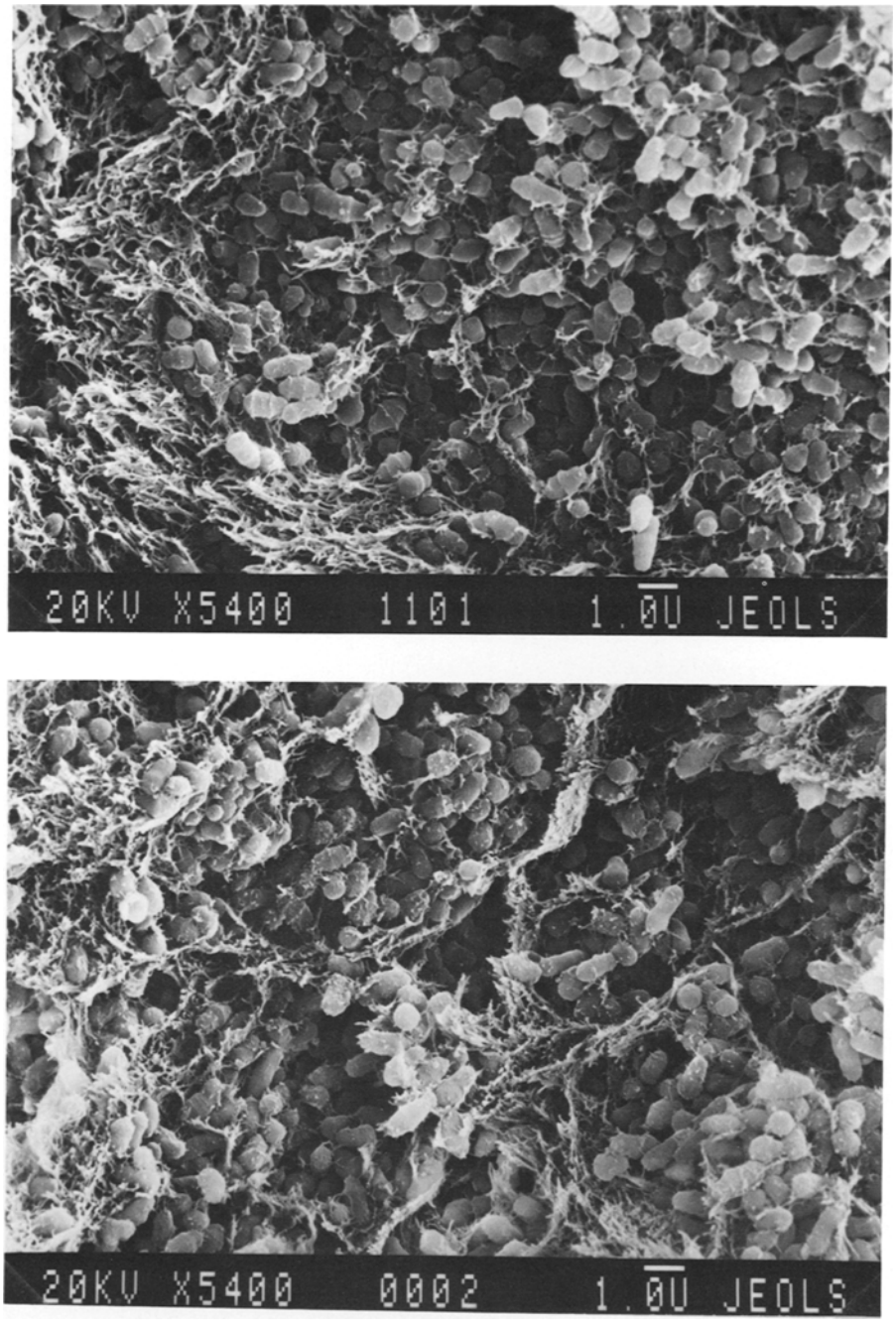

Fig. 1. Scanning electron micrograph of immobilized *B. flavum:* the initial preparation was a gel immediately after immobilization: (A) initial preparation; (B) preparation used for a continuous enzyme reaction for 88 d.

TABLE 5
Effect of Freeze-Thawing on Fumarase Activity and Operational Stability of Immobilized Cells

Freezing temperature, °C	Fumarase activity, μmol/h/g of cells	Operational stability, half-life, d
No treatment	9,920	160
−10	12,500	123
−20	11,400	132
−40	11,620	132

the preparation gelled with acetone was 68% of all other preparations. Therefore, it is obvious that the fumarase protein was denatured by acetone, but not by other gelling agents. Further activity yield was different in each gelling condition, as shown in Table 6.

Discussion

In the previous papers of this series (*16, 17*), we disclosed that κ-carrageenan is a very suitable matrix for the immobilization of microbial cells, and that the enzyme activities of cells immobilized with this matrix had generally high stabilities during continuous enzyme reaction.

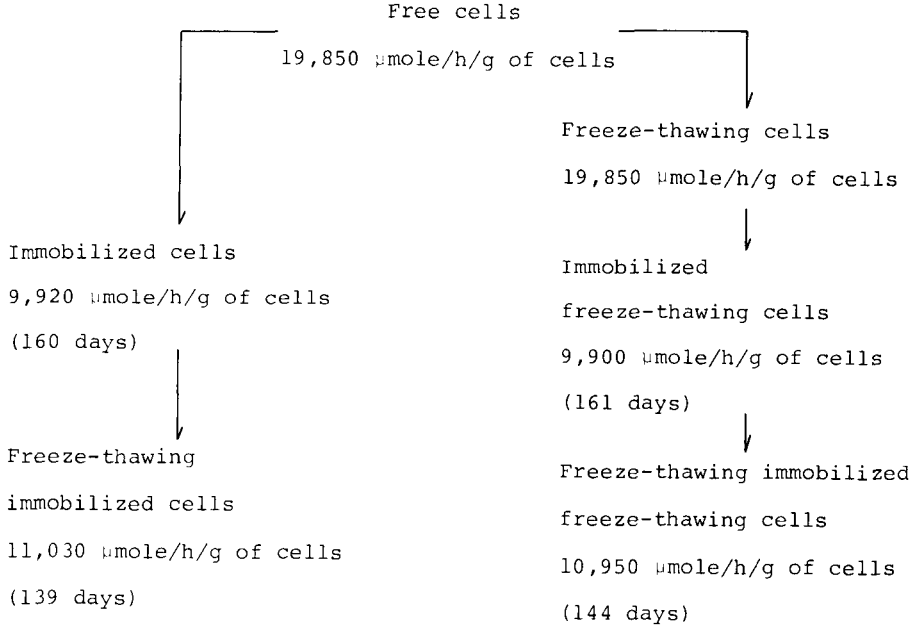

Fig. 2. Fumarase activity and operational stability of freeze-thawed preparations: Free cells and immobilized cells were frozen at −20°C for 48 h, and thawed at room temperature. Freeze-thawed cells were immobilized by standard method described in the previous paper (*14*). Values in parenthesis show the half-life of fumarase activity at 37°C.

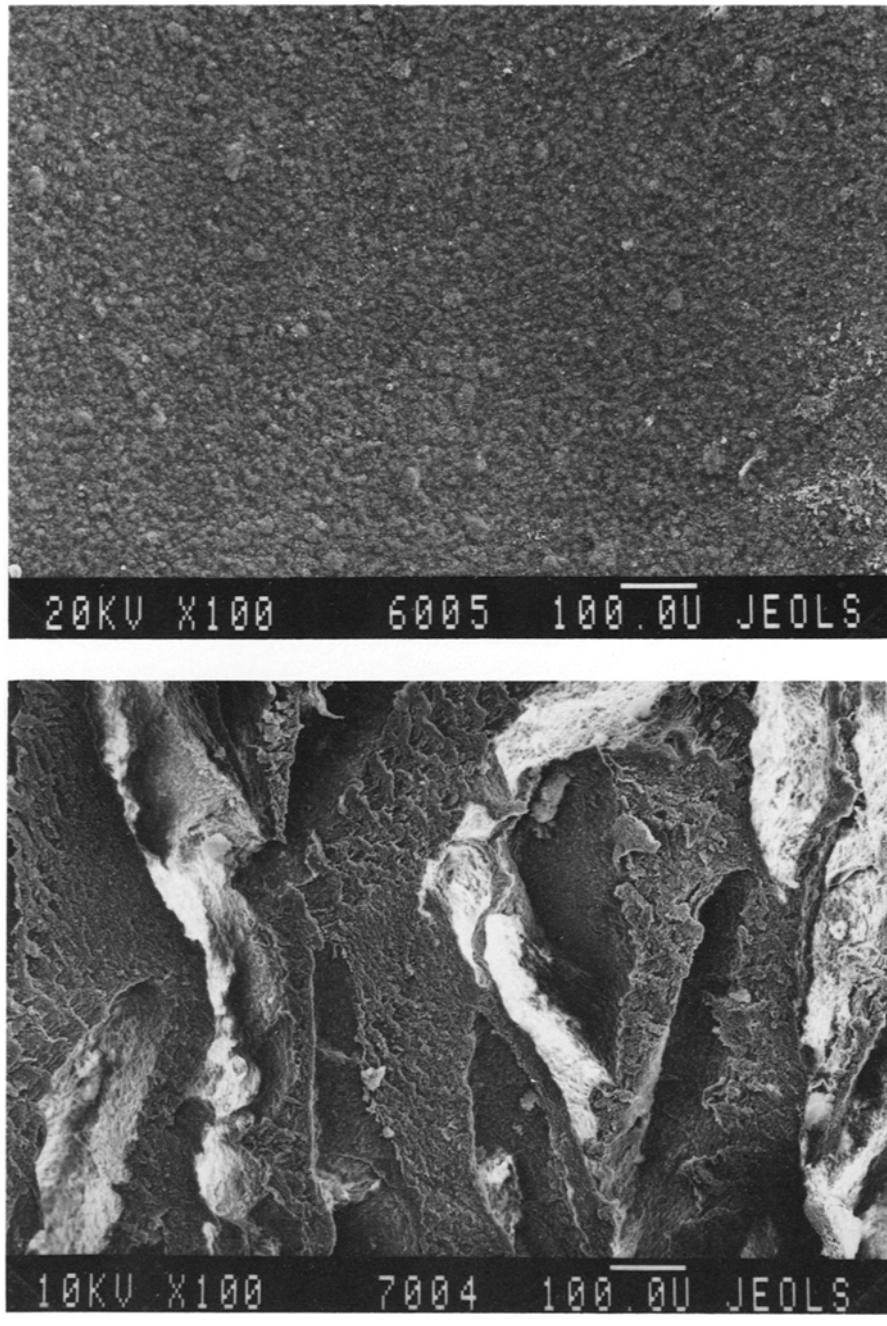

Fig. 3. Effect of freeze-thawing on the steric structure of κ-carrageenan gel: (A) cross-section of normal immobilized cells; (B) cross-section of immobilized cells after freeze-thawing.

TABLE 6
Effect of Gelling Conditions of κ-Carrageenan on Fumarase Activity and the Operational Stability

Gelling condition[a]	Fumarase activity,[b] μmol/h/g of cells: Immobilized cells	Fumarase activity,[b] μmol/h/g of cells: Ground immobilized cells	Operational stability at 37°C, half-life, d
Cooling	9,920	19,840	160
Contact with			
KCl	8,830	19,400	105
$CaCl_2$	8,200	19,480	116
NH_4Cl	8,750	19,420	117
Dimethylenediamine	8,130	19,390	120
p-Phenylenediamine	6,290	19,280	36
Acetone	6,770	13,310	73

[a]Concentration of salts and amines was 0.3*M*, and absolute acetone was used.
[b]Fumarase activity of free cells was 19,850 μmol/h/g of cells.

Further, we investigated the stabilities of fumarase activity of *Brevibacterium flavum* having high fumarase activity in detail (*15*). As the result, we found that the stabilities of fumarase activities against external factors influencing enzymic protein denaturation were enhanced by immobilization using κ-carrageenan gel.

In order to clarify the extent of stabilization by immobilization, the results of a previous paper (*15*) were summarized in Table 7. The fumarase activity of cells immobilized with κ-carrageenan is seen to be far more stable than that of free cells. Furthermore, in the previous paper (*15*), the stabilizing effect on fumarase activity of "gel-state" κ-carrageenan was presented as markedly higher than that of "sol-state" reagent. Therefore, in this paper, the reasons for the higher stability of fumarase activity in "gel-state" κ-carrageenan were investigated.

TABLE 7
Effect of External Factors on the Stability of Fumarase Activity

Treatment: External factor	Treatment: Conditions	Relative remaining activity,[a] %: Free cells	Relative remaining activity,[a] %: Immobilized cells
Heat	55°C, 1 h	18	100
Hydrogen ion	pH 4.5, 37°C, 1 h	5	100
Ethanol	3*M*, 37°C, 2 h	33	100
Urea	3*M*, 37°C, 2 h	37	55
Storage	37°C, 30 days	51	100

[a]Initial activity was taken as 100%.

At first, we considered the main factors in the stabilization of fumarase activity by "gel-state" κ-carrageenan to be:

1. Steric structure of κ-carrageenan gel.
2. Rheological properties of the gel.
3. Buffer action of sulfate groups in the carrageenan molecule.
4. Partition coefficient of denaturing reagent between the gel and outer bulk solution.
5. Mild gelling conditions during immobilization.

The evaluation and rejection of some of these assumptions are discussed as follows.

Steric Structrue of κ-Carrageenan Gel

κ-Carrageenan has sulfate and hydroxy groups in its molecule, and the steric structure of the gel consists of both double helix and kink zones (*18*). Further, gellation of κ-carrageenan is induced by cooling or contact with metal ions, amines, or water-miscible organic solvents, and the strength of the gels obtained under those various gelling conditions is not equal (*16*). From these facts, the steric structures of gels prepared under different gelling conditions are presumed not to be equivalent. In order to confirm this assumption, the several experiments described in "Results" section of this article were carried out. At first, as shown in Table 6, the fumarase activity yields of *B. flavum* immobilized under various gelling conditions were not equal, but when their gels were ground with mortar to remove the effects of diffusion and permeability of substrate and/or products, recoverys of fumarase activity were 100%, except for the case of gellation contacting with acetone. Therefore, the diffusion rate of substrates and/or products is suggested to be different under each gelling condition because of the different steric structures of the gels. The different steric structure is considered to prepare unequivalent mutual steric distance of sulfate and hydroxy groups in κ-carrageenan gel, and interactions between these functional groups of κ-carrageenan and some functional groups of the cell wall of *B. flavum* may become different under each gelling condition.

In the immobilized cells, the difference of interaction between κ-carrageenan and the cell wall is considered to affect the stabilities of fumarase activity. Then continuous enzyme reaction was carried out using *B. flavum* cells immobilized under various gelling conditions over a long time. As shown in Table 6, the operational stabilities of immobilized cells prepared under different gelling conditions were unequivalent.

The immobilized cells providing the highest operational stability were prepared with gelation by cooling and then contact with aqueous potassium chloride solution. When using these immobilized cells, only a small amount of cell-lysis was observed even if the immobilized cells were then used in a continuous enzyme reaction for a long time, such as 88 d (Table 4). Further, as shown in Fig. 1, scarcely any transformation and withering of cells was observed. On the other hand, when batchwise enzyme reaction was repeated using free cells, cell lysis occurred within five reaction runs (Table 4). The operational stability of fumarase activity was affected by freeze-thawing the immobilized cells. As shown in Fig. 2, both the

fumarase activity and the operational stability of immobilized freeze-thawed cells were nearly equal to those of immobilized cells. But when those gels of two kinds of immobilized cells were frozen and thawed, both fumarase activities increased and both operational stabilities decreased to same extent. Then pore size of κ-carrageenan gel was observed by electron microscope. As shown in Fig. 3, the pore size was widened by freeze-thawing the gel. Therefore, widening of pore size is considered to provide an increase in the diffusion rates of substrate and product, and to provide an increase in fumarase activity both by the leakage of cells from the gel matrix and by enzyme denaturation. However, such alteration of steric structure of κ-carrageenan gel induced a decrease in the operational stability of fumarase activity. From these data, the steric structure of κ-carrageenan gel may be assumed to play an important role in the stabilities of fumarase activity of *B. flavum*.

Rheorogical Properties of κ-Carrageenan Gel

In general, the stabilities of activity found in immobilized enzymes are higher than those of native enzymes. As the main reason for this stabilization, Martinek (*19*) and Klibanov (*20*) reported that the conformation of the enzyme molecule becomes too rigid by immobilization, and that the rigidity of enzyme molecule is attained by some interactions between enzyme and matrices. Also in the case of immobilized cells, the same kinds of interaction may occur between some outer components of the cell wall and functional groups of matrices. If the conformation of cell wall components is supposed to be rigidly held as a result of the interaction, and the conformations of some components of the inner cellular membrane adjacent to the cell-wall are influenced by the rigidity in the conformations of cell-wall components, fumarase protein-binding cellular membrane is considered to be stabilized by the rigid conformation of inner cellular membrane. From these considerations, the rheological properties of the κ-carrageenan gel are considered to be one of the important factors in the stability of enzyme activity in immobilized cells. In the rheological properties of κ-carrageenan, a parameter affecting rigidity and elasticity of steric structure of the gel is gel-strength (*21*). Therefore, a conformation of fumarase protein binding to cellular membrane is supposed to be influenced indirectly by the gel-strength. Furthermore if the favorable gel-strength of κ-carrageenan plays an important role in holding the native conformation of many cellular components in immobilized cells, the gel-strength may also relate to maintaining the native binding situation between fumarase and cellular membrane, and may prevent the microbial cells from autolysis.

From the following results, the rheological properties of κ-carrageenan gel were confirmed to relate to the stability of immobilized cells. That is, as shown in Table 3, when the immobilized cells were treated with protein-denaturing reagents, both the deactivation of fumarase and a decrease in gel-strength were observed. However, even if the gel-strength decreases, as long as κ-carrageenan keeps its "gel-state," the fumarase activity of immobilized cells remained higher than that of free cells.

Buffer Action of Sulfate Groups in the κ-Carrageenan Molecule

It is expected that the fumarase activity of immobilized cells becomes apparently stable at lower pH because of the buffer action of the sulfate groups in κ-carrageenan. If the buffer action affects microbial cells, the apparent stabilization of fumarase activity should be observed with "sol-state" as well as "gel-state" κ-carrageenan. However, as described in a previous paper (*15*), the protective effect of fumarase activity at pH 4.5 in the immobilized cells, namely "gel-state" κ-carrageenan, was recognized, but the protective effect was scarcely recognized by "sol-state" κ-carrageenan. Therefore, stabilization by the buffer action of sulfate groups is impossible, and the stabilization against pH treatment was considered to be caused by the rigidity of the fumarase protein structure induced by the interaction between κ-carrageenan gel and cells.

Partition Coefficient of Denaturing Reagent Between κ-Carrageenan Gel and Outer Bulk Solution

If the concentration of a denaturant in gel is lower than that in outer bulk solution, the stabilities of fumarase activity of the immobilized cells in the indicated concentration of the denaturant should be higher than those of free cells. Tables 1 and 2 show the justice of above assumption. That is, when the denaturant was an organic solvent such as ethanol and acetone, their concentrations in gel were lower than those of outer bulk solution, and the fumarase activity of immobilized cells was more stable than that of free cells. But the stabilization was not observed in tetrahydrofuran, and the concentration of the organic solvent was nearly equivalent in gel and in outer bulk solution. Therefore, the main reason for the stabilization of fumarase activity against such organic solvent is the apparent phenomenon induced by partition coefficient of organic solvents between κ-carrageenan gel and the outer bulk solution.

Mild Gelling Conditions in Immobilization

The standard carrageenan method is mild, because yeast cells for production of ethanol were immobilized with κ-carrageenan gel to maintain them in the living state (*22*). And as shown in Table 6, the fumarase activity is considered not to be denatured during the immobilization procedure. Further as shown in Fig. 4, in the case of the standard immobilization method using κ-carrageenan, many cellular components of *B. flavum* may be kept in their intact conformation, because the fumarase activity of the ground, immobilized cells was nearly equal to that of intact cells.

To assay total fumarase activity in *B. flavum* cells, they were sonicated, or treated with bile extract or cetylpyridinium chloride, and the enzyme activity was found to be 19,850 μmol of L-malic acid formed/h/g of cells in each procedure. Therefore, the whole fumarase activity of *B. flavum* cells was considered to have resulted from these treatments. Then the immobilized cells were treated with bile extract, and the gels were ground for measurement of total fumarase activity. From the result, fumarase activity was found to be kept in immobilized cells without any

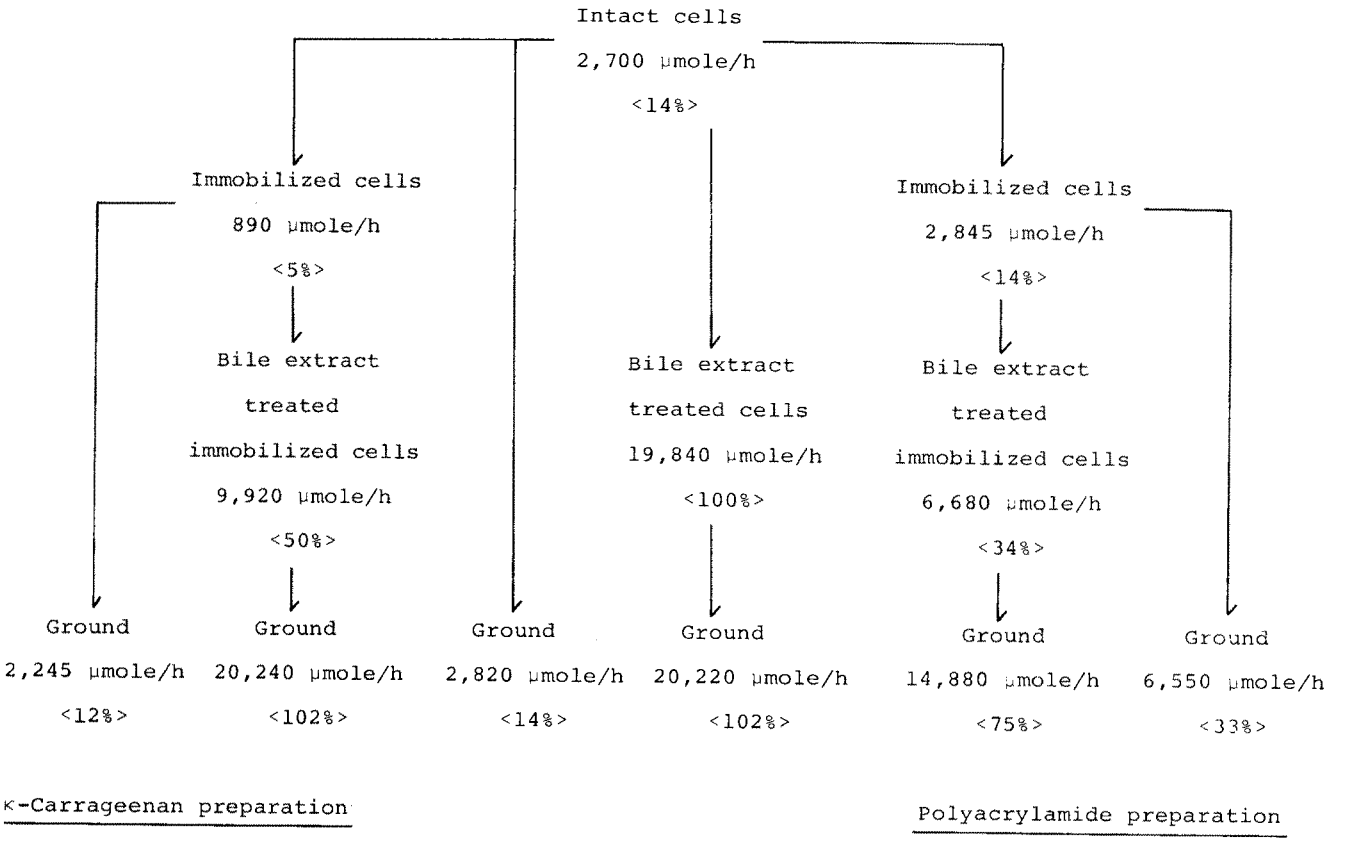

Fig. 4. Comparison of fumarase activity of *B. flavum* cells immobilized with κ-carrageenan or polyacrylamide: The activity indicated is μmol of L-malic acid formed/h/g of cells. Bile extract-treated cells and bile extract-treated immobilized cells correspond to free cells and immobilized cells in the text. Values in parenthsis show the relative fumarase activity, taking the activity of bile extract-treated cells as 100%.

loss of enzyme activity. Therefore fumarase and the cellular components around fumarase are considered to be held intact in conformation during the immobilization procedure.

On the other hand, in the case of the polyacrylamide method, it is considered from following results that some cellular components, as well as fumarase, were partially denatured during the immobilization procedure. That is, the apparent fumarase activity of immobilized cells became higher than that of intact cells. The higher activity indicates an increase in the diffusion rates of substrate and product owing to the removal, denaturation, and modification of some cellular components during the immobilization procedure. Further, when the immobilized cells were treated with bile extract and then ground by mortar, the fumarase activity became lower than that of free cells treated with bile extract. This fact showed that the fumarase is more exposed to denaturing conditions when *B. flavum* cells were immobilized using polyacrylamide. Thus, it is considered that the operational stability of fumarase activity of *B. flavum* immobilized with polyacrylamide was lower than that of fumarase activity of *B. flavum* immobilized with κ-carrageenan (*14*).

In conclusion, the main reasons for the stabilization of the fumarase activity of *B. flavum* by the κ-carrageenan method were considered to be mild immobilization conditions, interaction between κ-carrageenan gel and *B. flavum* cells, suitable pore size of κ-carrageenan gel, and the partition coefficient of the denaturant.

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References

1. Chibata, I., Tosa, T., and Sato, T. (1974), *Appl. Microbiol.* **27,** 878.
2. Yamamoto, K., Sato, T., Tosa, T., and Chibata, I. (1974), *Biotechnol. Bioeng.* **16,** 1589.
3. Saif, S. R., Tani, Y., and Ogata, K. (1975), *J. Ferment. Technol.* **53,** 380.
4. Ohmiya, K., Ohashi, H., Kobayashi, T., and Shimizu, S., (1977), *Appl. Environ. Microbiol.* **33,** 137.
5. Uchida, T., Watanabe, T., Kato, T., and Chibata, I. (1978), *Biotechnol. Bioeng.* **20,** 225.
6. Yamamoto, K., Tosa, T., and Chibata, I. (1976), *European J. Appl. Microbiol.* **3,** 169.
7. Hayashi, T., Tanaka, Y., and Kawashima, K. (1979), *Biotechnol. Bioeng.* **21,** 1019.
8. Tosa, T., Sato, T., Mori, T., and Chibata, I. (1974), *Appl. Microbiol.* **27,** 886.
9. Yamamoto, K., Sato, T., Tosa, T., and Chibata, I. (1974), *Biotechnol. Bioeng.* **16,** 1601.
10. Tosa, T., Sato, T., Nishida, Y., and Chibata, I. (1977), *Biochim. Biophys. Acta* **483,** 193.

11. Omata, T., Tanaka, A., Yamane, T., and Fukui, S. (1979), *European J. Appl. Microbiol. Biotechnol.* **6,** 207.
12. Sonomoto, K., Tanaka, A., Omata, T., Yamame, T., and Fukui, S. (1979), *European J. Appl. Microbiol. Biotechnol.* **6,** 325.
13. Takata, I., Yamamoto, K., Tosa, T., and Chibata, I. (1979), *European J. Appl. Microbiol. Biotechnol.* **7,** 161.
14. Takata, I., Yamamoto, K., Tosa, T., and Chibata, I. (1980), *Enzyme Microb. Technol.* **2,** 30.
15. Takata, I., Tosa, T., and Chibata, I. (1983), *Appl. Biochem. Biotechnol.* **8,** 31.
16. Tosa, T., Sato, T., Mori, T., Yamamoto, K., Takata, I., Nishida, Y., and Chibata, I. (1979), *Biotechnol. Bioeng.* **21,** 1679.
17. Takata, I., Tosa, T., and Chibata, I. (1977), *J. Solid-Phase Biochem.* **2,** 161.
18. Dea, I., McKinnon, A., and Rees, D. (1972), *J. Mol. Biol.* **68,** 153.
19. Martinek, K., and Berezin, I. V. (1977), *J. Solid-Phase Biochem.* **2,** 343.
20. Klibanov, A. M. (1979), *Anal. Biochem.* **93,** 1.
21. Morris, E. R., Rees, D. A., Robinson, G. (1980), *J. Mol. Biol.* **138,** 349.
22. Wada, M., Kato, J., and Chibata, I., (1980), Abstracts of Annual Meeting of the Agricultural Chemical Society of Japan, p. 18.