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SCREENING OF MATRIX SUITABLE FOR IMMOBILIZATION OF MICROBIAL CELLS

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To find a suitable matrix for immobilization of microbial cells, synthetic and natural polymers were screened. As a result, *kappa*-carrageenan, *iota*-carrageenan, furcellaran, sodium alginate, ethyl succinylated cellulose, succinylated zein, and 2-methyl-5-vinylpyridine-methylacrylate–methacrylic acid copolymer were studied. These polymers were induced to gel under mild conditions. *Streptomyces phaeochromogenes* cells having glucose isomerase activity were successfully immobilized in these polymer matrices. If a gel-inducing reagent were added to a substrate solution, these gel matrices could be stabilized. The microbial cells did not leak out from the gel lattice. When these immobilized cells were treated with hardening reagents such as glutaraldehyde or tannins, the gel matrices were strengthened, and the glucose isomerase activity became stable for a long period even in the absence of gel-inducing reagents. Among these polymer matrices tested, *kappa*-carrageenan was most suitable for immobilization of microbial cells.

INTRODUCTION

Recently, the immobilization of enzymes has been the subject of increased interest, and many papers on immobilized enzymes have been published (1). More recently papers on the immobilization of microbial cells have also been published (2).

We succeeded in the industrial applications of immobilized aminoacylase in 1969 (3), immobilized *Escherischia coli* cells having high aspartase activity in 1973 (4), and immobilized *Brevibacterium ammoniagenes* cells having high fumarase activity in 1974 (5) for production of L-amino acids, L-aspartic acid, and L-malic acid, respectively.

Although a number of immobilization methods of microbial cells have appeared (2), they have advantages and disadvantages, and the ideal general methods applicable to immobilization of cells have not yet been developed. In practice, it is necessary to choose suitable methods and conditions for immobilization of target enzymes or microbial cells, respectively.

Thus, in order to arrive at versatile immobilization methods and techniques, we investigated various immobilization methods. At first we considered immobilization by the induction of polymer gelation as the best approach, because in this case an enzyme is not denatured during the polymerization reaction due to reactive monomers and/or heat of polymerization.

As a result, we learned that several polymers formed gel lattices suitable for entrapping microbial cells. Among these polymers, "*kappa*-carrageenan" was the most suitable for immobilization of microbial cells. These results are presented in this article.

MATERIALS AND METHODS

Microbial Cells

Streptomyces phaeochromogenes cells were a gift from Gōdō Shusei Company Ltd. (Tokyo, Japan).

Polymers Matrix

Kappa-carrageenan and *iota*-carrageenan were obtained from Sansho Company Ltd. (Osaka, Japan), furcellaran from Litex Company (Denmark), and sodium alginate from Kamogawa Kasei Company Ltd. (Tokyo, Japan). 2-Methyl-5-vinylpyridine-methylacrylate–methacrylic acid copolymer (MPM-47), ethyl succinylated cellulose (ESC), and succinylated zein (SZ) are coating agents for tablets, the products of Tanabe Seiyaku Company Ltd. The number of glucose residues per molecule of ESC was approximately 50, substituent numbers of succinyl ester groups were 2.7 residues per one glucose residue, and the content ratio of ethyl ester in the succinylate was 57%.

Other Reagents

Locust bean gum was obtained from Sansho Company Ltd. (Osaka, Japan), gelatin from the Merck Company (Germany), and hexamethylenediamine from Tokyo Kasei Kogyo Company Ltd. (Tokyo, Japan). Persimmon tannin was a gift from Konishi Shuzō Company Ltd. (Kōbe, Japan). All other reagents were obtained from Katayama Chemical Industries Company Ltd. (Osaka, Japan).

Gelation of Various Polymers

Gelation by Cooling. *Kappa*-carrageenan was dissolved in physiological saline at a concentration of 3.1% (wt/vol), and the solution was cooled to 10°C.

Gelation by Contact with Ions. Solutions of *kappa*-carrageenan (3.1%), *iota*-carrageenan (1.5%), furcellaran (1.1%), sodium alginate (1.0%), and MPM-47 (10%) were contacted with a 0.3 M solution containig a cation as shown in Table 1.

Gelation by Control of pH Range. Solutions of ESC (10%) and SZ (7%) in 1% sodium bicarbonate solution were contacted with a 1 M acetate buffer containing 0.3 M magnesium chloride.

Immobilization of St. phaeochromogenes Cells Using Various Polymers

Immobilization Using Kappa-carrageenan. Twenty grams of microbial cells was suspended in 20 ml of physiological saline at 37°C, and 1.24 g of *kappa*-carrageenan was dissolved in 40 ml of physiological saline. Both were mixed at 37°C, and the mixture was cooled to approximately 10°C for 30 min. In order to increase the gel strength, the gel was soaked in cold 0.3 M potassium chloride solution. After this treatment, the resulting stiff gel was granulated in particle size 3-mm diameter.

Immobilization Using Iota-carrageenan. A cell suspension of 20 ml containing 10 g of microbial cells and a solution of 0.240 g of *iota*-carrageenan in 16 ml of physiological saline were mixed at 37°C. The mixture was added dropwise to 0.1 M magnesium chloride solution with stirring. The gel beads were soaked in 0.1 M magnesium chloride solution for 30 min.

Immobilization Using Furcellaran. Four milliliters of the cell suspension just mentioned and a solution of 0.176 g of furcellaran in 16 ml of physiological saline at 37°C were mixed. The mixture was added dropwise to 0.3 M ammonium chloride solution with stirring. The gel beads so obtained were soaked in 0.3 M ammonium chloride solution for 30 min.

Immobilization Using Sodium Alginate. Ten grams of the microbial cells was suspended in 40 ml of physiological saline at 37°C, and 500 mg of sodium alginate dissolved in 50 ml of physiological saline were mixed at 37°C. The mixture was added dropwise to 0.02 M calcium chloride solution with stirring.

Immobilization Using ESC. To a solution of 1.6 g of ESC in 16 ml of 1% sodium bicarbonate was added with stirring at 37°C, 4 ml of cell suspension containing 2 g of microbial cells. The mixture was added dropwise to 1 M acetate buffer (pH 5.5) containing 0.1 M magnesium

chloride. The gel beads obtained were washed with 0.1 M magnesium chloride solution.

Immobilization Using SZ. Four milliliters of the cell suspension just mentioned and a solution of 1.12 g of SZ in 16 ml of 1% sodium bicarbonate were mixed at 37°C. The mixture was treated in the same way as in the case of ESC.

Immobilization Using MPM-47. Two grams of the microbial cells were suspended in 2 ml of physiological saline. A total of 1.6 g of MPM-47 was dissolved in 16 ml of 1% sodium bicarbonate solution. Both were mixed at 37°C. The mixture was added dropwise to 0.1 M calcium chloride solution with stirring.

Immobilization Using Polyacrylamide. The microbial cells were immobilized using polyacrylamide gel according to a method previously reported (4).

Hardening of Immobilized St. phaeochromogenes Cells

Hardening with Glutaraldehyde. Two grams of immobilized cells was added to 20 ml of 0.2 M glutaraldehyde aqueous solution. The reaction mixture was shaken for 2 h at 30°C. After the reaction, unreacted glutaraldehyde was removed by washing with cold water. During the hardening reaction, a gel-inducing reagent was added to the reaction mixture to prevent degradation of the gel.

Hardening with Gelatin and Glutaraldehyde. To the preparation of immobilized cells just mentioned, 100 mg of gelatin was added to 2 g of the gel, along with a further 20 ml of 0.2 M glutaraldehyde aqueous solution. The mixture was shaken at 30°C for 1 h, and the unreacted glutaraldehyde was washed out with cold water.

Hardening with Hexamethylenediamine and Glutaraldehyde. Two grams of the gel was added to 20 ml of an aqueous solution containing 1 mmol hexamethylenediamine and 2 mmol of glutaraldehyde. The mixture was shaken at 30°C for 2 h. After the reaction, unreacted hexamethylenediamine and glutaraldehyde were removed by washing with cold water.

Hardening with Persimmon Tannin. Two milliliters of persimmon juice containing 5% tannin was diluted to 20 ml with water. The solution was adjusted to pH 7.0 with 20% sodium hydroxide. Two grams of immobilized cells was added to this solution, and the mixture was shaken at 37°C for 2 h. After the reaction, unreacted reagent was washed out with cold water. During the reaction, a gel-inducing reagent was added to the reaction mixture.

Hardening with Chinese Gallotannin. In 20 ml of water, 800 mg of Chinese gallotannin was dissolved. The solution was adjusted to pH 7.0

with 20% sodium hydroxide. Two grams of immobilized cells was added to this solution, and the mixture was shaken at 30°C for 2 h. During the reaction, a gel-inducing reagent was added to the reaction mixture. After the reaction, unreacted reagent was removed by washing the immobilized cells with cold water.

Measurement of Gel strength

The gel strength of the samples was measured by using Rheometer NRM-2002 J type, Fudo Kogyo Company Ltd. (Tokyo, Japan) with a disk plate plunger 10 mm in diameter.

Assay of Glucose Isomerase Activity

The immobilized cells corresponding to 0.5 g of intact cells were incubated with 20 ml of 0.1 M phosphate buffer (pH 7.0) containing 0.1 M glucose and 0.02 M $MgSO_4$ at 70°C for 30 min with shaking. The enzyme reaction was terminated by adding 0.5 M perchloric acid solution. The amount of fructose in the reaction mixture was determined by the cysteine-carbazole sulfate method (6). The enzyme activity was expressed in micromoles of fructose formed per hour.

Operational Stability of Immobilized Cells

The operational stability of the immobilized cells was investigated as follows: ten grams of immobilized *St. phaeochromogenes* cells was packed into a column and a solution of 50% (wt/vol) glucose solution containing 0.1 M sodium sulfite, 0.01 M magnesium chloride, and 0.001 M cobaltous chloride, at pH 7.0, was passed through the column at a flow rate of 10 ml/h at 60°C. Activity decay curves were typically exponential, and apparent half-lives were calculated from their slopes.

RESULTS

Conditions for Gelation of Various Polymers

As a result of screening the matrices suitable for immobilization of microbial cells, seven polymers were found to have excellent characteristics for the purpose of immobilization. They were induced to gel by the conditions shown in Table 1.

These polymers are classified into three groups according to the conditions for gelation. The first group is a matrix, such as *kappa-*

TABLE 1. **Conditions for Gelation of Various Polymers**

Polymer	Concentration	Condition for gelation
Kappa-carrageenan	3.1%	Cooling at 10°C Contact with K^+, NH_4^+, or Ca^{2+}
Iota-carrageenan	1.5	Contact with Ca^{2+} or Mg^{2+}
Furcellaran	1.1	Contact with K^+, NH_4^+ or Ca^{2+}
Sodium alginate	1.0	Contact with Ba^{2+}, Ca^{2+}, Co^{2+}, Cu^{2+}, Ni^{2+}, Zn^{2+}, Fe^{3+}, or Al^{3+}
ESC	10	Adjustment to pH 5.5
SZ	7	Adjustment to pH 5.5
MPM-47	10	Contact with Ca^{2+} or Mg^{2+}

[a] All cations used were the chloride form and their concentrations were 0.3 M

carrageenan, induced to gel by cooling. The second group consists of *kappa*-carrageenan, *iota*-carrageenan, furcellaran, sodium alginate, and MPM-47 which are induced to gel by contact with cations. The third group consists of ESC and SZ which were induced to gel by control of the pH below 5.5.

These conditions for gelation are milder than known immobilizing conditions for microbial cells. The gel-strengths of these gels were nearly equal to that of polyacrylamide gel.

Immobilization of St. phaeochromogenes *Cells Using Various Polymers*

St. phaeochromogenes cells were immobilized by using the seven selected polymers. Their gel strengths and enzyme activities are shown in Table 2. *Iota*-carrageenan gel was decomposed by high temperature during the enzyme reaction, but other gels were stable when gel-inducing reagents were added to the substrate solution during the enzyme reaction.

The highest glucose isomerase activity was obtained by using *kappa*-carrageenan. The activity was nearly as good as that obtained with polyacrylamide gel.

Hardening Treatment

In order to improve characteristics of the gel, immobilized cells were treated with hardening reagents. These results are shown in Table 3. The data show that gel characteristics of immobilized cells were generally improved by hardening treatment. That is, gel strength was increased by

TABLE 2. **Glucose Isomerase Activity and Gel Strength of Immobilized *St. phaeochromogenes* Cells Using Various Polymer Matrices**

Polymer matrix	Gel strength[a]	Enzyme activity (μmol/h/g of cells)	Activity yield[b] (%)
Kappa-carrageenan	+ + +	4440	57.3
Furcellaran	+ +	3090	40.0
Sodium alginate	+ + +	300	3.9
ESC	+ +	2490	32.2
SZ	+ +	1800	23.3
MPM-47	+ +	2360	30.5
Polyacrylamide	+ + + +	4220	57.2

[a]Symbol for gel strength corresponds to respective load for gel crush as follows: + +; 200–500 g/cm^2; + + +; 500–1000 g/cm^2; + + + +; 1000–1500 g/cm^2.
[b]Activity yield = (activity of immobilized cells)/(activity of intact cells) × 100.

the treatment. The gel matrices became stable even in the absence of added gel-inducing reagents in a substrate solution.

After the hardening treatment, gel strength of the immobilized *St. phaeochromogenes* cells using *kappa*-carrageenan, *iota*-carrageenan, furcellaran, and sodium alginate was equal to that of immobilized *St. phaeochromogenes* cells with polyacrylamide gel. When immobilized cells using *kappa*-carrageenan were treated with hexamethylenediamine and glutaraldehyde, the glucose isomerase activity was highest among immobilized cells treated with hardening reagents.

Operational Stability of Glucose Isomerase of Immobilized St. phaeochromogenes *Cells Using Various Polymer Matrices*

Continuous enzyme reaction was carried out by a column packed with immobilized cells. The stability of glucose isomerase activity was investigated. As shown in Table 3, apparent half-life of the immobilized cells using *kappa*-carrageenan was 150 days. This was equal to the half-life of immobilized cells using polyacrylamide gel. When immobilized *St. phaeochromogenes* cells using *kappa*-carrageenan were treated with hexamethylenediamine or gelatin and glutaraldehyde, the operational stabilities were markedly improved. When a preparation using sodium alginate was treated with gelatin and glutaraldehyde, the apparent half-life was also improved.

TABLE 3. **Glucose Isomerase Activity, Operational Stability, and Gel Strength of Immobilized *St. phaeochromogenes* Cells after Hardening Treatment**

Polymer matrix	Hardening reagent[a]	Gel strength[b]	Enzyme activity (μmol/h/g of cells) and yield (%)[c]	Operational stability[d] at 60°C (half-life, days)
Kappa-carrageenan	None	+ + +	4440[e] (57.3)	150
	Gelatin + GA[f]	+ + +	3280 (42.4)	532
	HMDA[g] + GA	+ + + +	4530 (58.6)	260
Iota-carrageenan	GA	+ + + +	1110 (14.4)	—
Sodium alginate	None	+ + +	300[e] (3.9)	—
	GA	+ + + +	2140 (27.7)	241
	Gelatin + GA	+ + + +	1800 (23.3)	—
	Persimmon tannin	+ + +	1530 (19.2)	137
Furcellaran	None	+ +	3090[e] (40.0)	—
	GA	+ + + +	2640 (34.1)	—
ESC	None	+ +	2490[e] (32.2)	—
	Gelatin + GA	+ + +	1840 (23.8)	—

SZ	None	++	1800[e] (23.3)	—
	Persimmon tannin	+++	2840 (36.7)	—
MPM-47	None	++	2360 (30.5)	—
	Gallotannin	++++	3110 (40.2)	121
	Persimmon tannin	++++	3670 (47.4)	89
	Gelatin+GA	++++	2180 1 (28.1)	89
	Gelatin+Persimmon tannin	++++	3690 (47.1)	96
Polyacrylamide	None	++++	4220 (57.2)	150

[a] Conditions of hardening are described in text.
[b] Symbols are the same as in Table 2.
[c] Parentheses contain activity yield (%).
[d] Stability is shown with apparent half-life.
[e] During enzyme reaction, a stabilizer for gel was added to the substrate solution at a concentration of 0.3 M as follows: KCl for *kappa*-carrageenan and furcellaran: CaCl for sodium alginate; $MgCl_2$ for ESC and SZ.
[f] Glutaraldehyde.
[g] Hexamethylenediamine.

Effect of Locust Bean Gum on Characteristics of Immobilized Cells

As *kappa*-carrageenan gel is strengthened by the addition of locust bean gum, concentration of the carrageenan can be diminished without the reduction of gel strength. As a result, an aqueous solution of the carrageenan is kept in the soluble state at lower temperatures such as 30°C. As shown in Table 4, addition of locust bean gum did not influence the enzyme activity and the operational stability.

DISCUSSION

At first, we considered that a polymer matrix suitable for immobilization of cells should have the following characteristics; (1) Mixing of microbial cells and polymer solution should be easy, and the mixture should be stable at liquid state. (2) The mixture should be easily induced to the gel state from the liquid state under mild conditions which do not modify the structure of enzyme proteins or microbial cells. (3) Formed gels should have gel strength that is not destroyed during the enzyme reaction. The gel matrix should be stable at high temperatures such as 60–70°C, over a wide pH range. (4) The pore size of this gel matrix should be small enough so that high molecular weight compounds such as enzyme proteins do not leak out of the gel lattice while the substrate and products of lower molecular weight easily pass through the gel lattice. When microbial cells are immobilized using a matrix satisfying these characteristics, the immobilized preparation would be expected to show high enzyme activity and to be stable for a long period of operation.

TABLE 4. **Effect of Locust Bean Gum on Characteristics of Immobilized *St. phaeochromogenes* Cells Using *Kappa*-carrageenan**

Kappa-carrageenan (mg)	Locust bean gum (mg)	Gelling temp.[a] (°C)	Gel strength[b]	Enzyme activity (μmol/h/g of cells)	Operational stability at 60°C (half-life, days)
600	0	40	+ + +	4300	150
500	100	37	+ + +	4440	—
400	200	34	+ + + +	4750	158
300	300	30	+ + +	4440	152

[a]Gelling temperature is estimated by incubation of the solution for 30 min at the temperature.
[b]Symbols are the same as in Table 2.

St. phaeochromogenes cells were chosen as a sample to be immobilized for screening of the matrix, and they have high glucose isomerase activity which is stable at the high temperature. Because a matrix must maintain a stable gel lattice during the enzyme reaction, these cells are suitable for screening of matrix.

Based on these stated properties, we screened the most suitable matrices among gel-forming polymers. Among the matrices screened, sodium alginate was already in use for immobilization of microbial cells (7,8). But in this matrix, one of the gel-inducing reagents, such as calcium or aluminum ion, is required during the enzyme reaction. Therefore, this immobilization method cannot be used for an enzyme reaction inhibited by these metal ions. In practice, glucose isomerase used for the present screening of matrices was inhibited by these two metal ions. Fortunately, when the preparation was treated with hardening reagents such as glutaraldehyde, the aldehyde and gelatin or persimmon tannin, the gel matrix became stable in the absence of these metal ions, and higher glucose isomerase activities were observed. It is suggested that sodium alginate is applicable to many kinds of enzyme reactions after the hardening treatment. Characteristics of the immobilized microbial cells using the other six polymers were improved by the hardening treatment, and those gels became stable even in the absence of gel-inducing reagents. These polymers can also generally be applied to the immobilization of microbial cells.

Through these results it was concluded that *kappa*-carrageenan satisfies almost all the characteristics for immobilization of microbial cells among the polymers tested. It is well known that if an aqueous solution of *kappa*-carrageenan is kept in the soluble state at above 40°C, the viscosity of the liquid is lowered. Therefore, mixing of microbial cells and a carrageenan solution is easily carried out quickly, and the mixture begins to gel rapidly by cooling below 10°C or by contacting with potassium, ammonium, and calcium ions.

The immobilization of microbial cells can be successfully carried out by a simple procedure under mild conditions as described in the text. Accordingly, as shown in Table 2, enzyme activity yields of immobilized microbial cells using *kappa*-carrageenan are high.

The operational stabilities of these immobilized microbial cells were generally high. Further, if the operational stability of the immobilized microbial cells was not satisfied, treatment with hardening reagents was carried out. As a result, more stable immobilized microbial cells could be obtained, as shown in Table 3.

The gel strength of the immobilized microbial cells using *kappa*-carrageenan was as high as that of polyacrylamide gel. The *kappa*-carrageenan gel is strengthened by addition of locust bean gum, as shown in

Table 4. The concentration of the carrageenan can be lowered without reduction of gel strength by addition of the gum. As a result, an aqueous solution of the carrageenan is kept in the soluble state at lower temperatures e.g. 30°C. Therefore, microbial cells containing thermounstable enzymes may be successfully immobilized. Addition of the gum did not influence the enzyme activity and operational stability.

In conclusion, the application of *kappa*-carrageenan for the immobilization of microbial cells is considered to be an excellent method. This facile carrageenan method is applicable to the immobilization of various microbial cells containing many kinds of enzymes, and the obtained immobilized preparations can be utilized for the production of useful chemicals. These studies will be reported elsewhere.

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