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Implantable Enzyme Capsules for Cancer Chemotherapy from Bakers' Yeast Cytosine Deaminase Immobilized on Epoxy-Acrylic Resin and Urethane Prepolymer

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ABSTRACT

For trial use in the local chemotherapy of cancer by a combination of cytosine deaminase (EC 3.5.4.1) and 5-fluorocytosine (*J. Biotechnol.*, (1985), **2**, 13–21), 40 U of partially purified cytosine deaminase was obtained from 500 g of commercial compressed bakers' yeast. The enzyme, which is unstable, was immobilized to stabilize it by the use of commercial epoxy-acrylic beads (Eupergit C). The immobilized enzyme was made into enzyme capsules with cellulose tubing for dialysis to encapsulate it or urethane polymer to entrap it, which materials are biocompatible. The activity of the intact cellulose capsules thus made was 0.4% that of the immobilized enzyme inside. The enzyme capsules also were stable. Ten days after the cellulose capsules were implanted in rats, 25% of the starting activity remained. When the polyurethane capsules were tested in vitro for 9 mo for thermostability at 37°C, the activity decreased rapidly (with a half-life of 28 d) during the first 4 mo, and then slowly (half-life, about 100 d) during the next 5 mo. A calculation to transform the biphasic decline into a sum of the exponential decline of two components of

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enzymic activities with different strengths and half-lives showed that the larger half-life was 5 mo.

Index Entries: Bakers' yeast, cancer chemotherapy; cytosine deaminase (EC 3.5.4.1); enzyme capsule; 5-fluorocytosine; 5-fluorouracil; immobilized enzyme; implantable bioreactor; *Saccharomyces cerevisiae*; targeting chemotherapy.

INTRODUCTION

Local chemotherapy for cancer may be possible with the combined use of 5-fluorocytosine (5FC) given orally and cytosine deaminase (EC 3.5.4.1) implanted locally (*1,2*). The idea came from the therapeutic use of 5FC against fungal and yeast infections, such as candidiasis and cryptococcal meningitis (*3*). The compound is not antineoplastic (*4*), unlike its analog, 5-fluorouracil (5FU). The antifungal activity of 5FC arises from the formation of 5FU by deamination of the drug at the site of infection by the cytosine deaminase of the fungus itself (*5*). 5FC is the only effective agent against many systemic fungal diseases that has no serious side effects (*6*) and can be given by mouth.

The cytosine deaminase of *Saccharomyces cerevisiae* can deaminate 5FC (*7*). This enzyme can be obtained readily from compressed bakers' yeast (*8*), but it is unstable at 37.5°C (*9*), so it would be rapidly inactivated, and we could not use it for long-term therapy without immobilization, which has a stabilizing effect. The cytosine deaminase from *Escherichia coli* (*1*) deaminates 5FC to 5FU, and is thermostable (*1*). When 5FC is given to rats with experimental brain tumors grown under the skin, and cytosine deaminase from *E. coli* is encapsulated in a semipermeable membrane (dialysis bag) and implanted near the tumors, tumor growth is inhibited (*2*). 5FU also appears in moderately high concentration near the enzyme bags (*2*), and the activity of the enzyme is stable (*1*). In this system, the cutaneous passive anaphylaxis test gives negative results (*2*).

One problem in the use of the *E. coli* enzyme is in its large-scale cultivation (*10*), needed to obtain much activity (*1*). Also because of possible pyrogenic reactions with microbial extracts, it is preferable to use highly-purified preparation in therapy even for an enzyme used in encapsulated form. So we decided to try to immobilize the yeast enzyme both to stabilize it and to prevent allergic reactions. Of the various immobilization techniques we tried, that with a commercial acrylic resin with terminal epoxy groups, Eupergit C, was the easiest; immobilization involved pouring an enzyme solution over the resin and leaving the mixture overnight.

The immobilized enzyme might be encapsulated for therapy in cellulose membrane (*1*) or polyurethane (*11*), which are easily handled and biocompatible with animal tissues (*12*).

MATERIALS AND METHODS

Materials

The buffer used was 50 m*M* potassium phosphate buffer, pH 7.0, unless otherwise noted. The phosphate-buffered saline was a 0.42% NaCl solution containing 10 m*M* potassium phosphate buffer, adjusted to pH 7.4. Compressed bakers' yeast was obtained from the Oriental Yeast Co. (Tokyo). Eupergit C is beads of an acrylic resin with epoxy (oxirane) groups, manufactured by Röhm Pharma GmbH (Darmstadt, FRG). DEAE-Sephacel was from Pharmacia K. K. (Tokyo), and Toyopearl HW-65C from Tosoh Corp. (Tokyo). The cellulose tubing had a mol wt cutoff of 1000 (Spectra/Por 6, flat width, 8 mm; Spectrum Medical Industries, Los Angeles). The urethane prepolymer used was PU-3, which was developed by Fukui et al. (*11,12*) and manufactured by the Toyo Tire & Rubber Co. (Osaka). A similar kind of commercial prepolymer is available from W. R. Grace & Co. (Columbia, Md.; Hypol EHP-3000), and from Bayer's subsidiaries over the world (Desmodur T-80) (*13–15*).

Enzyme Solution

A typical procedure for preparation of the enzyme was as follows. Compressed yeast (500 g) was broken into flakes with the fingers and stirred with a spatula with 25 mL of ethyl acetate and then with a magnetic stirrer for 30 min to cause plasmolysis of the cells. To the autolysate was added 1.7 L of the buffer containing ammonium sulfate to 15% saturation. The mixture was adjusted to pH 7.0 and left in a cold room with stirring once daily for a few minutes. Cell debris was removed by centrifugation after 3 d. From 1.92 L of the clarified lysate (containing 710 U of activity and 14.2 g of protein), the enzyme was precipitated with ammonium sulfate between 60 and 75% saturation. The precipitate obtained was dissolved in 500 mL of buffer (400 U, 1.93 g of protein), brought to 50% saturation of ammonium sulfate and centrifuged to remove the precipitate formed. The enzyme solution was put on a column of Toyopearl HW-65C (4.4 × 28 cm) equilibrated with the same buffer with ammonium sulfate, and chromatographed with 1000 mL of buffer containing a reverse linear gradient of ammonium sulfate of 50–30% saturation. The active fraction (170 U, 58 mg of protein) was dialyzed thoroughly against buffer, and the dialysate was chromatographed on a column of DEAE-Sephacel (4.4 × 16 cm) with 800 mL of buffer containing a linear gradient of potassium chloride of 0–0.50 *M*. The active fraction (33 mL) contained 40 U of activity and 1.74 mg of protein (specific activity, 23 U/mg).

Enzyme and Protein Assay

Activity and amount of protein in enzyme solutions and enzymic activity in enzyme immobilized on Eupergit C were assayed as described

previously (*1*). Enzymic activity in the polyurethane gel was assayed as apparent activity as follows. The gel was cut into cubes several millimeters to a side with scissors, put into a screw-capped test tube, and incubated with several changes at intervals of a few hours of 1.0 mL of 1.0 m*M* cytosine (or 5FC) in phosphate-buffered saline. The solutions removed were analyzed spectrophotometrically for changes in cytosine (5FC) and uracil (5FU) concentrations during incubation as described previously for enzyme capsules (*1*) to find the enzymic activity in the gel.

Immobilization of Enzyme on Eupergit C

The enzyme solution was mixed with 1.0 *M* potassium phosphate buffer, pH 7.0, poured evenly over dry Eupergit C, and left overnight in the cold. The completed preparation was washed with saline on a sintered-glass filter under reduced pressure, weighed, and suspended.

Sterilization of Immobilized Enzyme

The immobilized enzyme was brought into contact with saline containing 0.01% NaN_3 for 1 h and then washed thoroughly over a sintered-glass filter with sterile saline on a clean bench. In a preliminary experiment, this agent did not inactivate the enzyme, unlike benzalkonium chloride (alkyldimethylbenzylammonium chloride; Osban) and $HgCl_2$.

Urethane Polymerization

Prepolymer was polymerized as follows. First, 1 g of prepolymer was weighed into a 100 mL beaker, and heated in an oven at 110°C with aluminum foil over it if sterilization is necessary, or warmed to melt the prepolymer if it was solidified, and then cooled to a moderate temperature. The prepolymer was mixed with a spatula with 2 mL of suspension of the immobilized enzyme in buffer, stirred further until gelation started (for several minutes after foaming started), and kept in a refrigerator for a few hours to complete gelation.

RESULTS

Immobilization of Enzyme

Eupergit C

The enzyme solution was mixed with dry Eupergit C, commercial acrylic beads with epoxy groups for covalent-bond immobilization (Table 1). Two lots of the resultant wet gel contained nearly half of the initial activity used.

In Vitro Stability of Immobilized Enzyme

The above two lots of immobilized enzyme were sterilized with azide and suspended in phosphate-buffered saline in vials, which were

Table 1
Immobilization of Cytosine Deaminase on Eupergit C

Enzyme solution used, mL[a]	Completed gel, wet, Weight, g	Enzymic activity, U/g gel	Yield, %
1.0	3.3	24	44
2.5	3.3	65	46

[a]The portion indicated of enzyme solution containing 180 U of activity and 9.7 mg of protein/mL was brought to 4.0 mL with 1 *M* potassium phosphate, pH 7.4, and mixed with 1.0 g of dry Eupergit C.

sealed tightly and incubated at 37°C for 1 mo. At times, portions were withdrawn to assay the enzymic activity remaining. Activity decreased exponentially to about a quarter of the initial activity, with a half-life of 18 and 44 d (correlation coefficient, $r = -0.87$ and -0.69), respectively (Fig. 1, left). With longer incubation of up to 3 mo, activity did not decrease much further (Fig. 1, right). The half-life then was 220 and 140 d ($r = -0.77$ and -0.84), respectively, for the above two lots.

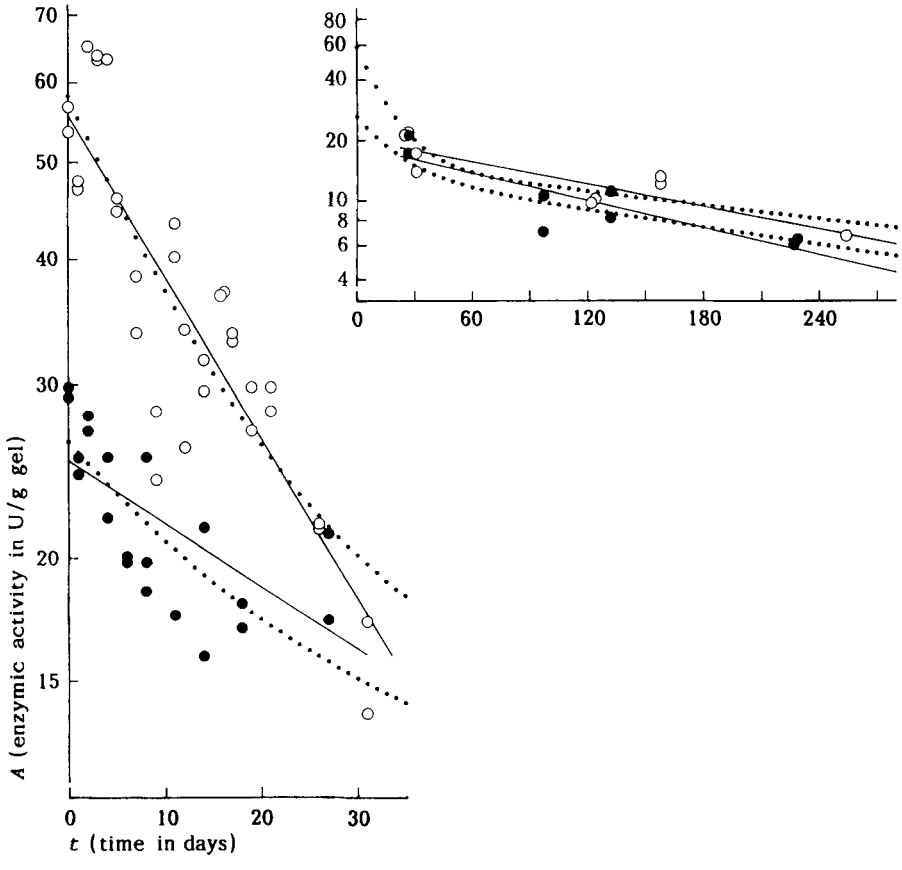

Fig. 1. Stability at 37°C of cytosine deaminase immobilized on Eupergit C. Two lots of the immobilized enzyme were used. Solid lines were obtained by linear regression; dotted lines by least-squares calculation using Eq. 1.

Biphasic Decline

As described above, the decrease in the enzymic activity of Eupergit C-immobilized cytosine deaminase was biphasic. So, we assumed that the activity of the immobilized enzyme, A, at time t was the sum of the activity of two kinds of immobilized enzyme residues with the activities of A_1 and A_2 at time zero, and with the half-lives of T_1 and T_2, respectively, where the dimensions were U/g gel for A, A_1, and A_2; and days for t, T_1, and T_2. This relationship is represented by the following equation:

$$A = \frac{A_1}{2^{t/T_1}} + \frac{A_2}{2^{t/T_2}} \qquad \text{(Eq. 1)}$$

Through the least-squares calculation for the curve of log$A$ vs t, suitable choices of the sets of parameters A_1, A_2, T_1, and T_2 were found: 43, 15, 11, and 270, respectively, for one lot and 13, 14, 14, and 210 for the other.

Encapsulation of Immobilized Enzyme

Enclosure in Dialysis Membrane.

The enzyme immobilized on Eupergit C with activity of 65 U/g of gel was enclosed in a tubular cellulose membrane for dialysis with 0.20 g of the gel (13 U) in each capsule and with round-ended silicone caps on both ends of the tubing as described before (*1*).

In Vivo Stability of Cellulose-Enzyme Capsule

An enzyme capsule was implanted in four rats bearing experimental brain tumors (*1,2*), in three under the skin, and in one near the tumor. After 10 d, the rats were killed. The capsules were removed, cleaned with saline, and assayed for enzymic activity with 1.0 m*M* cytosine (or 1.0 m*M* 5FC) as the substrate. The activity of the intact capsule was 0.014 ± 0.002 U (mean ± standard deviation, SD) for cytosine (0.013 ± 0.001 U for 5FC; both, $n = 4$). The capsules then were cut with scissors, and the gel removed and assayed for enzymic activity, which was 3.3 ± 0.1 U in each capsule, corresponding to 25% of the initial activity (half-life, 5d).

Entrapment in Polyurethane

The enzyme immobilized on Eupergit C then was trapped into polyurethane upon polymerization of the prepolymer. That is, the Eupergit C-immobilized enzyme was mixed with urethane prepolymer to make a urethane foam-like gel and cut by scissors into cubes several millimeters to a side. We assayed the six gels we made for enzymic activity (Table 2), and the yield of apparent activity was 36 ± 8% (mean ± SD).

In Vitro Stability of Polyurethane-Enzyme

Cubes weighing several dozens of milligrams made of the above six gels were put into vials containing 1.0 mL of saline and incubated at 37°C

Table 2
Entrapment of Eupergit C-Immobilized Cytosine Deaminase upon Polymerization of Urethane Prepolymer

	Immobilized enzyme used [a]		Yield of enzymic activity	
Gel no.	g	U	U/g gel	%
1	0.050	0.42	0.061	22
2	0.050	0.42	0.12	42
3	0.10	0.84	0.22	40
4	0.10	0.84	0.26	47
5	0.20	1.7	0.34	30
6	0.30	2.5	0.57	34

[a]Eupergit C-immobilized enzyme (8.4 U/g wet) weighing as indicated was suspended in 1.0 mL of saline and mixed with 0.5 g of PU-3, resulting in 1.5 g of polymer.

for 9 mo. At times, the remaining enzymic activity was assayed. Figure 2 shows the profile of decreases in the activity of gel no. 4 from Table 2. This was a typical result (others not shown); the activity decreased rapidly in the first 4 mo, and then slowly in the next 5 mo. The half-life of activity was read from the linear graph and found to be 28 d ($r = -0.98$) during the first 4 mo, but 99 d ($r = -0.94$) during the next 5 mo. Equation 1 was also applied to the curve in Fig. 2. The least-squares calculation led to similar results, which are listed in Table 3 together with those for the other five gels.

DISCUSSION

The enzyme capsules we designed can be regarded as therapeutic bioreactors when embedded in a patient's body. We attempted stabiliza-

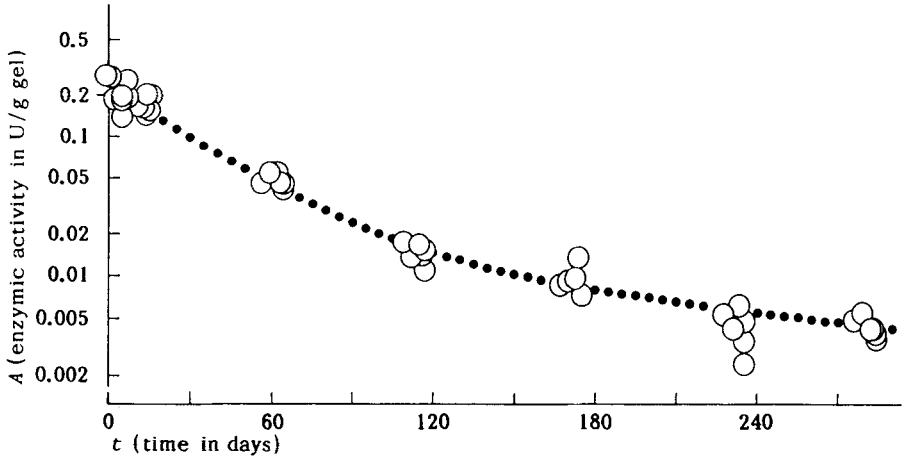

Fig. 2. Stability at 37°C of cytosine deaminase immobilized on Eupergit C and entrapped in polyurethane. Gel no. 4 from Table 2 is shown as a typical example.

Table 3
Stability of Immobilized Cytosine Deaminase in Polyurethane[a]

Gel no.	A_1	T_1	A_2	T_2	r
1	0.063	8.5	0.019	110	0.973
2	0.12	24	0.0046	3400	0.988
	(0.12	19	0.012	160	0.984)
3	0.21	22	0.018	140	0.993
4	0.23	22	0.012	190	0.994
5	0.35	21	0.013	220	0.992
6	0.59	22	0.030	120	0.993

[a]Gels from Table 2 were tested as described in the text. Stability is expressed by the parameters described in Eq. 1. r is the correlation coefficient. Parameters given in parentheses are those obtained assuming T_2 to be 160, which is the mean of T_2 for the remaining five gels (gels other than no. 2), because T_2 found was too large. r Did not become much smaller.

tion of the yeast enzyme, which is very unstable, by the use of immobilization techniques. The enzyme protein must be isolated from macromolecules in the body fluid to prevent allergic reactions; this is probably achieved by the immobilization of the enzyme protein. Here we used cellulose tubing (*1*) or urethane prepolymer to entrap, within gel blocks, the enzyme that had been already immobilized on a resin. The advantage of urethane is that the shape of the capsule could be custom-tailored (*12*) as follows. Polymer can be cast into any shape desired, and the completed gel can be reshaped with a knife or scissors. The polymerization reaction is mild, and the process is simple.

Biocompatibility of the materials and of the completed enzyme capsules with the patient's body is the second necessity for therapy. Polyurethanes were biocompatible in experiments with rats and rabbits (*16*).

Urethane prepolymer is often used to immobilize biocatalysts such as microbial, plant, and animal cells, cellular organelles, immobilized enzyme gels, and microcapsules by entrapment (*12*). The possibility of immobilization of soluble enzymes with such prepolymers has been mentioned by Wood et al. (*13*), who used Hypol EHP-3000. In a related experiment with this enzyme and PU-3, we found that the method was usable, but that the enzymic activity of the resultant gel was not very stable.

SUMMARY

We made enzyme capsules from cytosine deaminase (EC 3.5.4.1) from bakers' yeast and biocompatible materials for trial use for the local chemotherapy of cancer in combination with 5-fluorocytosine (*J. Biotechnol.*, (1985), **2**, 13–21). The enzyme, which is unstable, was immobilized on epoxy-acrylic beads (Eupergit C), and then encapsulated in cellulose

tubing for dialysis or entrapped in urethane polymer, which are biocompatible. The enzyme capsules thus made were stable. When the cellulose capsules were tested for stability in rats, the activity remaining after 10 d was about 25% of the starting activity. When the polyurethane capsules had been incubated for 4 mo at 37°C, the half-life of their activity then was calculated to be about 100 d.

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