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Fermentation without Multiplication of Cells Using Microcapsules That Contain Zymase Complex and Muscle Enzyme Extract

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In an earlier article, the present authors described that such enzymes as catalase, urease, lipase, and hemoglobin could be microencapsulated into sphere particles from 10 to 1000 μ in diameter by the use of some synthetic polymers, without much loss of enzymatic activity.[1] An enzyme solution was enveloped with a semi-permeable inactive polymer membrane which protected the enzymes, macromolecular protein, from leaking out, but which did not affect the permeation of substrates and products of a low molecular weight.

These enzyme-containing microcapsules are regarded as a kind of immobilized enzymes,[2] but they are essentially different from the others in that the enzymes themselves are intact and retain their normal properties. One of the merits of the encapsulation in comparison to other methods of chemical or physical immobilization is expected to lie in its applicability to multi-enzyme systems.

More than one enzyme of any kind, with reagents if necessary, can be microencapsulated at the same time in a capsule. In this paper we will discuss the microencapsulation of enzyme extracts obtained from yeast (zymase complex) and the muscle of a rabbit, and will investigate alcoholic and lactic fermentation using these microcapsules.

Experimental

Enzyme Extracts. Zymase Complex: A freshly-fermented yeast solution (supplied by the Toyo Jozo Co., Ltd.) was treated with a refrigerated cell fractionator (Sorvall-Ribi, RF-1) to destroy the cells. The homogenated solution was centrifuged at 2000 rpm for 30 min, and then the supernatant was filtered with a filter paper to remove the cell wall and the undestroyed cells. The filtrate was turbid and contained plasmic granules.

The Muscle Enzyme Extract. About 100 g of leg muscle of a rabbit was cut into one-centimeter blocks, and then it was homogenated by a homoblender for 15 min while 200 m*l* of a 0.05M pH 7.0 phosphate buffer solution was added. After the fibrous materials had been taken off, the solution was centrifuged at 9000 rpm for 20 min. The supernatant, the enzyme extract, was clear and colorless. All these treatments were carried out at temperatures below 10°C.

Microencapsulation. 3.0 g of an enzyme extract were emulsified in 15 m*l* of a 5% benzene solution of a ladder polymer of sesqui phenylsiloxane (supplied by the Shinetsu Kagaku Co., Ltd.) by the use of a homoblender for 3 min. This W/O-type emulsion was added quickly to 150 m*l* of a 3% gelatine solution phosphate-buffered at pH 7.0 under brisk agitation at 20°C. At this stage, a (W/O)/W-type complex emulsion was formed. The temperature was gradually raised to 37°C over a 1-hr period, after which the agitation was continued for an additional 1.5 hr at the same temperature. The benzene was evaporated gradually during the process so that a hard, solid capsule wall of the silicone was formed around a droplet of the enzyme extract. The microcapsules thus formed were recovered by centrifugation and were washed with the buffer solution three times.

In all the experiments, the amount of the capsules was weighed after incubation and drying in order to minimize the denaturation of the enzymes.

Incubation and Determination of the Reaction Product. Alcoholic Fermentation: In a 50 m*l* flask we placed microcapsules containing the zymase complex and 30 m*l* of a 400 mM glucose solution. Then it was put in an air incubator kept at 30°C.

The concentration of the reaction product, ethyl alcohol, was determined by gas chromatography using a Hitachi-Perkin Elmer Model F6-D gas chromatograph. The calibration curve was obtained by the use of an alchohol-water mixture as the standard solution.

Lactic Fermentation: In a test tube we placed about 150 mg of microcapsules containing the muscle enzyme extract, 1 m*l* of 100 mM glucose, 1 m*l* of 1 mM NAD, 1 m*l* of 10 mM ATP, and 2 m*l* of the 50 mM pH 7.0 tris-HCl buffer solution. Then it was incubated in a water bath kept at 39°C.

At the end of the incubation, the microcapsules were removed by filtration, then, to the filtrate obtained above, we added 5 m*l* of a 10% trichloroacetic acid solution. (No preciptitation was observed upon this addition, as there was no free enzyme in the filtrate). After filtration, the amount of the reaction product, lactic acid, was determined according to the Barker and Summerson method.[3] The optical density was measured at 560 nm using a Hitachi Type 139 spectrophotometer. The control solution was prepared by removing the microcapsules from the incubation solution. The calibration curve was prepared by the use of lithium lactate as the standard material.

Results and Discussion

Figure 1 shows a photomicrograph of the microcapsules that contain the zymase complex. Each looks like a ping-pong ball containing the enzyme solution.

Table 1 gives the results for alcoholic fermentation using the microcapsules containing the zymase complex. About 1% of alcohol was produced in 10 days. The reaction rate is calculated as 10—50 μmol of alcohol per gram of capsule per hour. We could not observe any multiplication of yeast cells outside the capsules in any of the experiments.

Table 2 gives the results for the lactic fermentation using microcapsules containing the muscle-enzyme ex-

1) M. Kitajima, S. Miyano, and A. Kondo, *Kogyo Kagaku Zasshi*, **72**, 493 (1969).

2) A. S. Lindsey, *J. Macromol. Sci., Revs. Macromol. Chem.*, **C(1)**, 1 (1969).

3) S. B. Barker and W. H. Summerson, *J. Biol. Chem.*, **138**, 535 (1941).

Table 1. Alcoholic fermentation using the microcapsules that contain the zymase complex

Sample	Diameter (μ)	Weight (mg)	Time (day)	Alc. Conc. (%)	Alcohol (mmol)	Reaction rate (Alc. μmol/ caps-g·hr)
Caps-1	100—500	734	6	0.55	3.6	34
Caps-2	20—200	648	7	0.95	6.2	57
Caps-3	200—800	374	7	0.05	0.3	5.0
Caps-4	100—500	602	11	0.76	5.0	32
Untreated[a)]	—	1.0 (ml)	1	0.16	0.11	46[b)]

a) Untreated zymase complex solution.
b) Alc. μmol/ml·hr

Table 2. Lactic fermentation using the microcapsules that contain the muscle enzyme extract

Sample	Diameter (μ)	Weight (mg)	Time (hr)	Lactic acid (μmol)	Reaction rate (L.a. μmol/ caps-g·hr)
Caps-1	100—500	168	1	0.14	0.82
Caps-1	100—500	162	40	5.15	0.79
Caps-2	100—500	147	15	0.59	0.27
Caps-3	20—200	180	15	0.17	0.63
Untreated[a)]	—	1.0 (ml)	1	2.82	2.82[b)]

a) Untreated muscle enzyme extract.
b) L.a. μmol/ml·hr

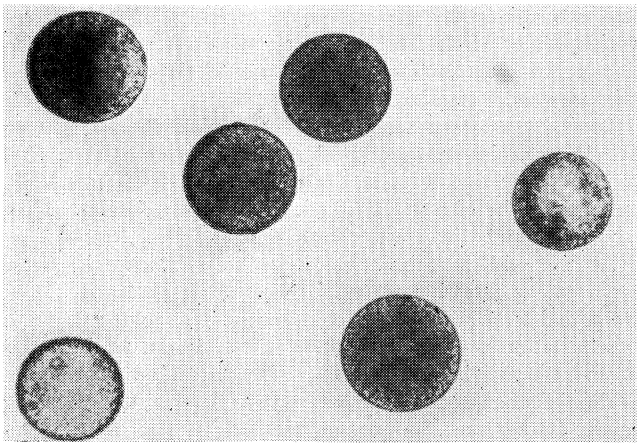

Fig. 1. A photomicrograph of the microcapsules containing the zymase comples.

tract. In these cases, ATP and NAD were added to facilitate the reaction. It is calculated from the results that about 1 μmol of lactic acid is procuced in an hour. The reaction time was chosen according to the sensitivity of the analysis for the products.

As was indicated by Emden, Mielhof, and Parnas, 13 enzymes are necessary to convert glucose into alcohol or into lactic acid. If any one of these enzymes is lost or denatures during the microencapsulation process, the reaction cycle cannot be completed and the final products can not be obtained. Although many encapsulation methods have been reported,[4)] most of them are unsuitable for enzymes. The method described above is believed to be the most suitable one for the encapsulation of enzymes, since no reactive reagent is necessary and the reaction conditions are quite mild throughout the process.

The present authors previously reported that modified red cells that have a reinforced membrane with normal hemoglobin and enzymes in it were obtained by treating red cells with some isocyanates.[5)] These modified cells can also be used as an immobilized multi-enzyme system, since they exibit many complicated enzymatic activities.

The microcapsules obtained here will be useful for the industrial and medical use of enzymes, since they are easy to recover and handle, and can be used in continuous-column reactions that are more complicated than that made possible by the immobilized monofunctional enzymes. It is expected that the enzymes are aligned close to each other in these capsules, so multi-stage reactions can proceed effectively, as in natural cells. It is also expected that they can be used as model samples in studying cellular reactions or membranes.

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4) A. Kondo, "Microcapsules," Nikkan Kogyo Shinbun, Tokyo (1970).
5) M. Kitajima, W. Sekiguchi, and A. Kondo, This Bulletin, **44**, 139 (1971).