♣Continuous Hydrolysis of Olive Oil by Lipase in Microporous Hydrophobic Membrane Bioreactor[1]

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ABSTRACT

Continuous hydrolysis of olive oil by *Candida cylindracea*'s lipase was studied in a microporous hydrophobic membrane bioreactor. Olive oil and buffer solution, fed continuously through two compartments partitioned by membrane, caused reaction at the interface of lipase-adsorbed membrane and buffer solution. Fatty acid was obtained in a single phase without being mixed with components of other phases. At all mean residence times, countercurrent flow mode was superior to cocurrent one. The lipase was adsorbed onto the membrane, and its adsorption was suggested to be partially specific from the experiments with enzymes having various levels of purity. The percent hydrolysis depended hyperbolically on the interfacial enzyme concentration. The hydrolysis seemed to be limited by diffusion of fat or fatty acid through the micropores of the membrane at higher interfacial enzyme concentrations. The lipase was stabilized significantly by glycerol added to the buffer solution. Satisfactory performance of the membrane bioreactor was obtained in a longterm continuous operation which lasted for 24 days by feeding buffer-glycerol (18.0%) solution over the adsorbed lipase. The operational half-life of the adsorbed enzyme was 15 days at 40 C.

INTRODUCTION

Enzyme technological approaches for the modification of fats and oils are a subject of intense interest. Modifications of starch and protein by enzymatic means are well established as industrial processes. Unfortunately, the use of enzymes to modify lipids is still in a developing state.

According to Stirton (1), a process using castor bean lipases for fatty acid production was operated on a commercial scale in the past. Recently it was reported that a Japanese company (2) used *Candida cylindracea*'s lipase to hydrolyze oils for the production of soaps. The company claimed that the enzymatic method yielded products having better odor and color and gave a cheaper overall process than the conventional uncatalyzed splitting method (Colgate-Emery Process). It has been pointed out that enzymatic hydrolysis could provide a useful method of generating fatty acids from unstable oils containing conjugated or highly unsaturated fatty acid residues (3,4).

A variety of literature has come out on the hydrolysis of fat by lipase either in free (5-8), immobilized (9-12) or cell-bound (13,14) states. All these studies (5-14) except reference (13) were carried out in conventional emulsion systems.

The conventional emulsion systems have certain drawbacks with respect to maintaining fine emulsion, product separation, continuous operation, control of water/glycerol concentration, restricted access to the immobilized lipase, etc. Their details have been described in our previous paper (15). In view of these drawbacks, we have developed a microporous hydrophobic membrane bioreactor with which continuous production of glycerides by lipase was successfully carried out (15,16).

Here, we also have attempted continuous hydrolysis of olive oil by lipase in a non-emulsion system using the membrane bioreactor with a little modification. In the present report, the configuration of the modified bioreactor, the optimal condition of operation, the phenomenon of spontaneous adsorption of lipase on the membrane and performance of the bioreactor system are described.

[1] Bioreactor for Enzymatic Reaction of Fat and Fatty Acid Derivatives (III).

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CONFIGURATION OF MEMBRANE BIOREACTOR

As the microporous membrane bioreactor, one unit of a flat plate type dialyzer (Hospal Hemodialyzer RP-6) was used with modification described previously (15). The unit was composed of two fat phases (fat compartments) and an enzyme solution phase, and these compartments were partitioned by two membranes. The upper and lower supports were made of 5 mm thick stainless steel plates, unlike the supports of the previous one (15). The upper and lower plates and frame were made of plastic. The membrane was thin polypropylene film with numerous micropores (Juragurd 2500, thickness of 25 μm, elliptically shaped pores with maximum pore diameter of 0.4 μm, a void fraction of 55%, Polyplastic Co. Ltd., Tokyo). The effective area of total membrane was $11.6 \times 31.3 \times 2$ cm^2.

First, the frame was covered on both side with two membranes, making a narrow compartment. The plate surfaces adjacent to the membrane were grooved (312 mm long bifurcated grooves having 0.5 mm depth and 0.8 mm width, the total number of grooves was 115). The plates and membrane frame were placed between upper and lower supports and fastened firmly by 24 bolts and nuts using a torque wrench (12 kgf · cm). Thus, the reactor set-up resulted in three closed compartments leaving no room to hold air inside when it was filled with reactants. The enzyme-glycerol solution was passed through the narrow compartment formed by two membranes, while liquid fat was passed on the other sides of the membrane compartment through the grooves of the plates. The hold-ups of fat and glycerol-enzyme solution were ca. 20 ml and 8 ml, respectively.

Figure 1 shows the neighborhood of the membrane where schematic micropores are drawn. Because of the hydrophobic nature of the membrane, fat can readily penetrate the micropores while enzyme solution cannot. Thus, fat came in contact with lipase adsorbed on the membrane surface as shown in Figure 1. The reaction took place at the interface between the membrane-lipase and fat. The main product, fatty acid, diffused back into the bulk flow of fat while the other product, glycerol, diffused into the bulk flow of enzyme solution or buffer solution, to which glycerol often was added as the enzyme stabilizer.

A process flow diagram of the reaction system is shown in Figure 2. In most short-term experiments, the enzyme-glycerol solution preserved in an ice-chamber was fed continuously at a desired flow rate into the membrane compartment through c-a inlet connection with a peristaltic pump (Minipuls-2, Gilson, France SA, Viller Le Bel, France) for a required experimental period. In a longterm experiment, the enzyme-glycerol solution was fed for 20-24 hr (t_s), which was the saturation period for lipase adsorption. Then, the feeding of enzyme-glycerol solution was switched to the feeding of buffer-glycerol solution through b-a inlet connection with the same peristaltic pump and continued for a desired period. The liquid fat (olive oil) was fed into

the bioreactor from the opposite side with a reciprocal pump (Micro pump, Model SP-100, Sibata Chemical Apparatus Manufacturing Co. Ltd., Tokyo). Fatty acid formed was obtained at the outlet in a single phase with no component of other phase. Glycerol, the other product of fat hydrolysis, thus flew out with enzyme solution or buffer solution through the other outlet as shown in Figure 3. The membrane bioreactor was placed in a constant temperature water bath (40 C).

EXPERIMENTAL PROCEDURE

Materials

Lipases of different purity grades from *Candida cylindracea* (Lipase-MY, Lipase-OF and Lipase-CC), produced by Meito Sangyo Co. Ltd., Japan, were used. The specific activities of these enzymes, Lipase-MY, Lipase-OF and Lipase-CC were 24, 240 and 1350 unit/mg powder, respectively. Unless otherwise stated, Lipase-OF was used in most of the experiments. Olive oil having a saponification value of 190, purchased from Wako Pure Chemical Industries Ltd., Japan, was used without any solvent throughout the experiments. Glycerol of 99.7% purity from Nippon Oil and Fats Co. Ltd., Amagasaki Works, Japan, was used.

Enzyme and Buffer Solution

Required amounts of lipase powder were dissolved in deionized distilled water. In case of crude lipases (Lipase-MY and Lipase-OF), the enzyme solution was sonicated (in Branson-Sonifier, Cell disruptor 200) for 30 sec to 1 min. The sonicated solution was then centrifuged for 10 min at 12,000 rpm; the clear solution thus obtained was used as enzyme solution. Unless otherwise stated, glycerol as a stabilizer of lipase was dissolved at a concentration of 18% in the clear enzyme solution. The resulting solution is referred to as "enzyme-glycerol solution" in this article. The pH of this solution was 6.8-7.0. Buffer solution used in the long-term experiment was 0.1 M KH_2PO_4 plus 0.1 M K_2HPO_4, pH 7.0 in which glycerol was dissolved to a level of 18.0%. This solution is referred to as "buffer-glycerol solution."

Start-up of Continuous Experiment

The enzyme-glycerol solution placed in an ice-chamber was fed into the bioreactor by a peristaltic pump. During this feeding, the outlet and inlet of fat phases were kept closed by pinchcocks to let air pressure up to ensure no leakage of enzyme solution through the membrane. After the bioreactor was filled with enzyme-glycerol solution, olive oil feeding was started by a reciprocal pump. When fat came out of the outlets, the reactor was dipped into a constant temperature water bath (40 C). Initially, both fat and enzyme-glycerol solution were fed at a higher flow rate for filling the bioreactor rapidly to save time. After the bioreactor was dipped in the constant water bath, the flow rate was adjusted at the required level and kept constant throughout each experiment. The flow rates of olive oil, enzyme solution, enzyme-glycerol solution or buffer-glycerol solution are specified in the caption of each figure.

Adsorption of Lipase onto the Hydrophobic Membrane

The enzyme-glycerol solution was fed continuously into the membrane compartment of the bioreactor by a peristaltic pump at a constant flow rate (5.5-6.0 ml/hr). The initial activity of this solution was assayed as 1216 units/ml. After the bioreactor was filled with enzyme-glycerol solution (holdup 8.0 ml), olive oil feeding was started and continued throughout the experiment. To get a breakthrough curve, 1.5 or 3.0 ml (at 15 min or 30 min interval) enzyme glycerol solution was collected through outlet in sample vials of

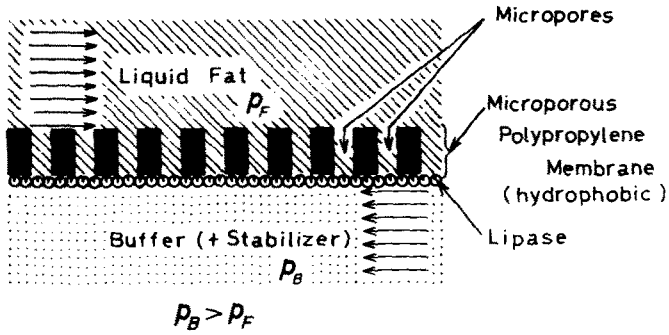

FIG. 1. The neighborhood of the microporous hydrophobic membrane. The micropores are drawn schematically.

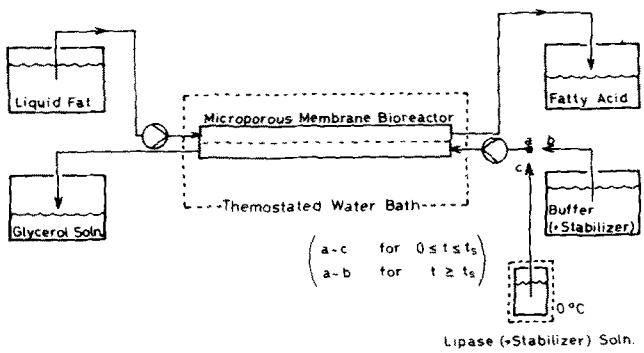

FIG. 2. Process flow diagram of the continuous hydrolysis of fat. t, Elapsed time in the continuous experiment. t_s, Time of switching over. From the start of the experiment to the time t_s, the tubes a and c were connected. From t_s on, the tubes a and b were connected.

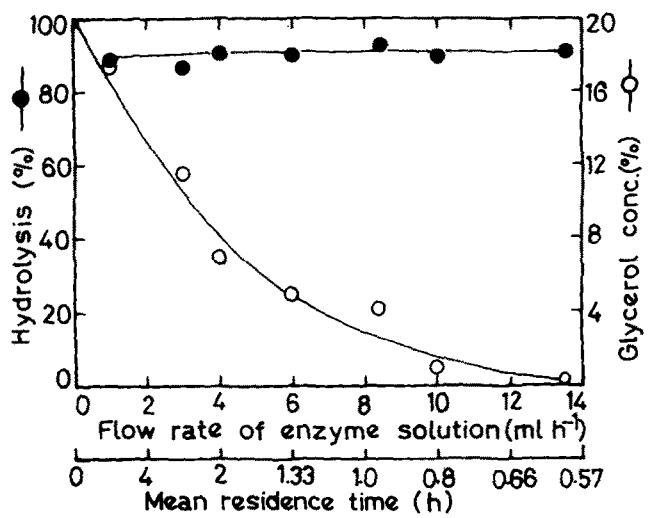

FIG. 3. Accumulation of glycerol in the enzyme solution during the continuous olive oil hydrolysis in the membrane bioreactor. The values of glycerol concentration and percent hydrolysis were the maximum ones at each experiment. Each experiment was carried out for 30 hr at a fat flow rate of 2.3 ml/hr. Enzyme solution without glycerol was fed in all the experiments.

a fraction collector. Immediately after the outlet enzyme-glycerol solution was collected in a sample vial, it was transferred to a refrigerator (4 C) followed by the assay of its residual enzyme activities. Enzyme-glycerol solution feeding was continued until the outlet enzyme activity equaled that of inlet one, monitored during the course of feeding. The

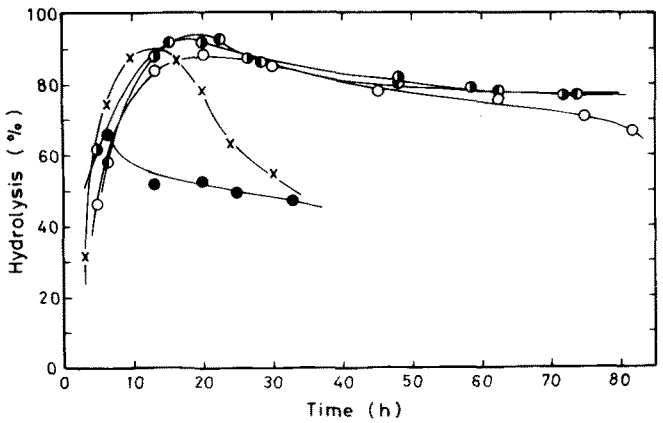

FIG. 4. Stabilizing effect of glycerol in lipase solution on hydrolysis in the membrane bioreactor system. Fat flow rate = 2.4 ml/hr. Enzyme-solution flow rate = 14 ml/hr. Glycerol concentrations in the enzyme solution feed were: X, 0%; ○, 9.0%; ◑, 18.0%; ◐, 23.0%, and ●, 29.0%.

amount of adsorbed enzyme was calculated from the total amount of supplied enzyme minus the sum of the outlet enzyme minus total free enzyme that remained in the bioreactor.

To know the dependency of the purity level of lipase on adsorption, experiments have been conducted using enzymes of specific activities 17 (a mixture of Lipase-MY + albumin, 2:1, w/w), 24 (Lipase-MY), 240 (Lipase-OF) and 1350 (Lipase-CC) per mg powder enzyme. For each experiment, sufficient amounts of enzyme were dissolved in 18.0% glycerol solution so as to get almost the same initial enzyme activity ranging from 667-690 units per ml. In all these experiments enzyme-glycerol solution was recycled between a reservoir placed in an ice chamber and the bioreactor, unlike in the breakthrough experiment. It was recycled for at least 16 hr. The amount of adsorbed enzyme was calculated from the difference of total initial and final enzyme activities of enzyme-glycerol solution. Interfacial enzyme concentration was calculated as the total amounts of enzyme adsorbed divided by the total area of the membrane. Thermal inactivation of the enzyme was found insignificant for the experimental period.

Analytical Procedure

The activity of lipases was determined by the olive oil emulsion method (17). One unit of activity is defined as the amounts of the enzyme which liberate 1 μmole equivalent of fatty acid from olive oil in one min at 37 C.

The degree of hydrolysis of olive oil was estimated by acid value determination of output sample through alkalimetric titration. For this analysis, about 190-240 mg of the sample (hydrolyzed product) was weighed in a 125-ml conical flask. The sample was then dissolved in about 75 ml neutralized mixture of ethanol and diethylether (1:1, v/v) and titrated with standardized 0.1 N potassium hydroxide ethanolic solution using phenophthalein as an indicator. Hydrolysis (%) was calculated from the following equation:

$$\text{Hydrolysis (\%)} = \frac{(AV)_{out} - (AV)_{in}}{SV - (AV)_{in}} \times 100$$

where $(AV)_{in}$ is the acid value of the olive oil supplied into the bioreactor, $(AV)_{out}$ is the acid value of the outlet (product) and (SV) is the saponification value of supplied olive oil.

Glycerol concentration of the enzyme-glycerol solution and buffer-solution was measured by the same method as described previously (15). The calibration curves were made for the enzyme-glycerol solution and buffer-glycerol solution, respectively.

RESULTS AND DISCUSSION

Accumulation of Glycerol in Enzyme Solution Phase

In the present membrane bioreactor system of continuous olive oil hydrolysis, glycerol accumulated in the enzyme solution phase. The extent of its accumulation depends mainly on the flow rates of enzyme solution. Accordingly, the experiments were carried out in a wide range of flow rates of glycerol-free enzyme solution. The results are shown in Figure 3. At the lowest flow rate (the highest mean residence time) of enzyme solution, glycerol accumulated by 18.0% in the enzyme solution phase. However, the percent hydrolyses were almost constant in the ranges of 0-18%. This means that glycerol exerts no adverse effect upon the hydrolysis rate in this range. This information is also useful for conventional emulsion systems. As far as we know, no report has been made concerning the effect of glycerol concentration on the hydrolysis rate of fat.

Stabilizing Effect of Glycerol

Enzymes generally are stabilized by polyhydric alcohols. In order to improve the operational stability of lipase in the present reaction system, pure glycerol was added to an aqueous enzyme solution. Investigation was carried out at several glycerol concentrations ranging from 0-29%. The flow rate of enzyme solution was set at 14 ml/hr, where accumulation of glycerol was negligible (Fig. 3). The experimental results are shown in Figure 4. When glycerol was not added to the enzyme solution, no appreciable steady state was achieved. The sharp decline of conversion is assumed to be due to instability of the lipase. On the other hand, glycerol addition up to 23.0% of the aqueous enzyme solution significantly improved the prolongation of steady state conversion. However, 29.0% of glycerol solution lowered the conversion level, which may be because the esterification reaction became prominent. Half life of the enzyme in the absence of glycerol was about 2 days. The exact half life value of the enzyme during the reaction will be stated in a later section. When one considers that glycerol is one of the products but it exerts no adverse effect on the hydrolysis rate in the range of 0-18% (Fig. 3), and that the configuration of the bioreactor makes it easy to control its concentration, it is apparent that glycerol is an effective stabilizer of the lipase at almost the same concentration range (9-23%).

A similar stabilizing effect of glycerol on the same lipase in aqueous solution with no hydrolysis reaction was reported previously by Murakami et al. (18).

Effect of Co- and Countercurrent Flow Modes of Reactants

Figure 5 depicts the time courses of hydrolysis of olive oil at two flow modes of reactants, i.e. cocurrent and countercurrent flows. A significant difference in the hydrolysis percentage was observed in the two flow modes. This was investigated further at several mean residence times of olive oil flow. Figure 6 shows the results of hydrolysis in the steady state at various olive oil flow rates. The significant difference in hydrolysis rate which was observed with increasing flow rate of olive oil revealed the superiority of the countercurrent over the cocurrent flow. The exact reason for this phenomenon is not clear yet and is under investigation.

Adsorption Phenomenon of Lipase onto the Hydrophobic Microporous Membrane

It was observed that the lipase supplied to the bioreactor

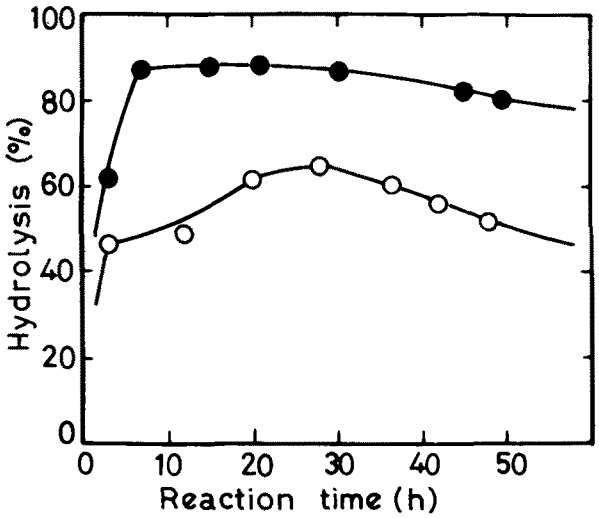

FIG. 5. Time course of hydrolysis in two opposite flow modes of reactants: —●—, countercurrent mode; —○—, cocurrent mode. Fat flow rate = 4.3 ml/hr, flow rate of enzyme-glycerol solution = 5.5 ml/hr.

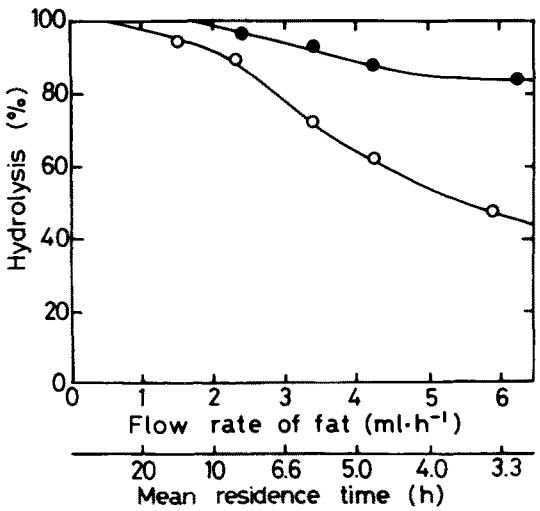

FIG. 6. Comparative efficiency of two opposite flow modes on hydrolysis at different flow rates of olive oil in the membrane bioreactor system. The values of highest conversion were taken. Flow rate of enzyme-glycerol solution = 5.5 ml/hr. —○—, cocurrent flow; —●—, countercurrent flow.

was adsorbed spontaneously onto the hydrophobic microporous membrane impregnated with olive oil. Figure 7 illustrates the typical breakthrough curve of adsorption. About 16 hr was required to complete adsorption of lipase in the continuous operation under experimental conditions. This adsorption phenomenon is consistent with the explanation cited in the literature that microbial lipases have exceptionally high surface activity, resulting in a strong adsorption onto hydrophobic surfaces (19,20). Characteristics of such adsorption were further investigated by adsorption experiment using lipases of different purities. The results are shown in Table I, indicating little variation in enzyme amounts adsorbed onto the membrane interface (6-19 units/cm^2) with widely increasing purities of enzyme (17-1350 units/mg). This small variation of adsorption may be explained by partial specificity of adsorption of the lipase due to its higher hydrophobicity in comparison with other inert proteins, or limiting capacity of the surface of the

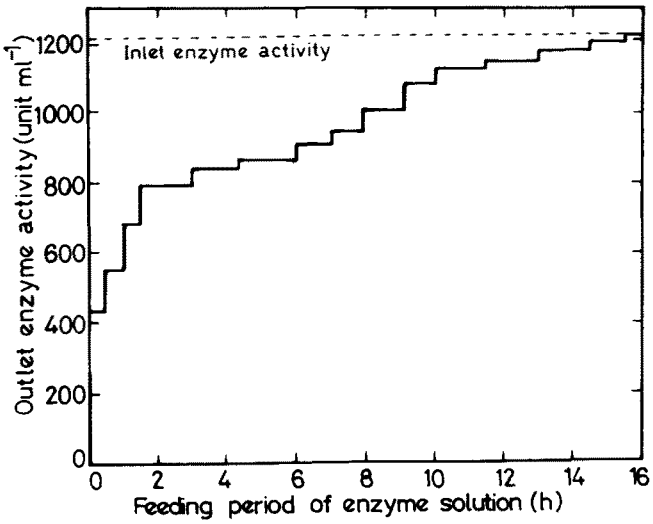

FIG. 7. Time course of lipase adsorption onto the olive oil impregnated microporous hydrophobic membrane (breakthrough curve). Olive oil was being fed at the flow rate of 2.8 ml/hr throughout the experiment. Flow rate of enzyme-glycerol solution = 5.5 ml/hr.

TABLE I

Dependency of Purity of Lipase upon the Adsorption onto the Olive Oil-Impregnated Hydrophobic Microporous Membrane

Purity unit/mg	Adsorbed amount / Initial amount %	Interfacial enzyme conc. unit/cm^2
17	12.1	5.8
24	17.6	7.5
240	28.3	12.4
1350	41.8	19.1

membrane interface for the adsorption of enzyme and other inert proteins. It was found that the value of the interfacial enzyme concentration (15 units/cm^2) calculated from Figure 7 was nearly the same as that (12.4 units/cm^2) shown in Table I.

Figure 8 shows a relationship between percent hydrolysis and the interfacial enzyme concentration (units/cm^2) (Table I). At lower interfacial enzyme concentrations, percent hydrolysis was almost proportional to the interfacial enzyme concentrations, but at higher interfacial enzyme concentrations the dependence reached a plateau though there was far less than 100% hydrolysis. This phenomenon can be explained in terms of both reaction limitation in the lower interfacial enzyme concentrations and diffusion limitation in the higher interfacial enzyme concentrations. It is quite plausible that the molecular diffusions of substrate (fat) and product (fatty acid) in the narrow (ca. 0.1 μm) and long (25 μm) micropore path limit the local overall hydrolysis rate when the interfacial enzyme concentration is high. Figure 8 exhibits the idea that excess adsorption of the enzyme on the membrane interface is ineffective. Careful enzyme loading will economize the amount of enzyme used in this type of bioreactor.

Performance of Long Term Continuous Operation of the Bioreactor

In order to know both the strength of the lipase's adsorption and half life of the adsorbed lipase in the bioreactor, a specially designed long-term continuous experiment was carried

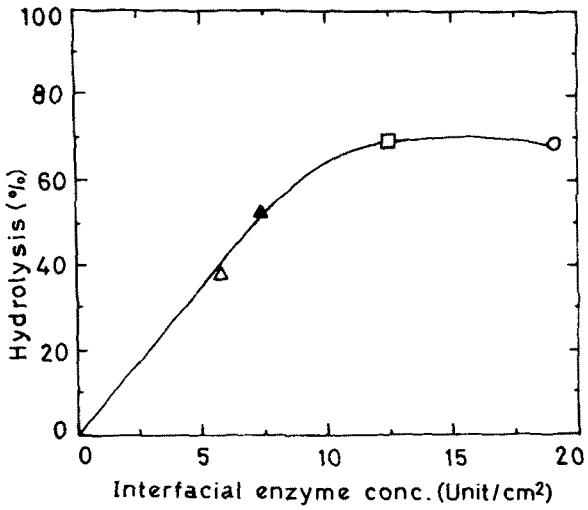

FIG. 8. Relationship between interfacial enzyme concentration and hydrolysis in the membrane bioreactor system. Fat flow rate = 5.5 ml/hr. △, A mixture of Lipase-MY + albumin (2:1, w/w); ▲, Lipase-MY; □, Lipase-OF; ○, Lipase-CC.

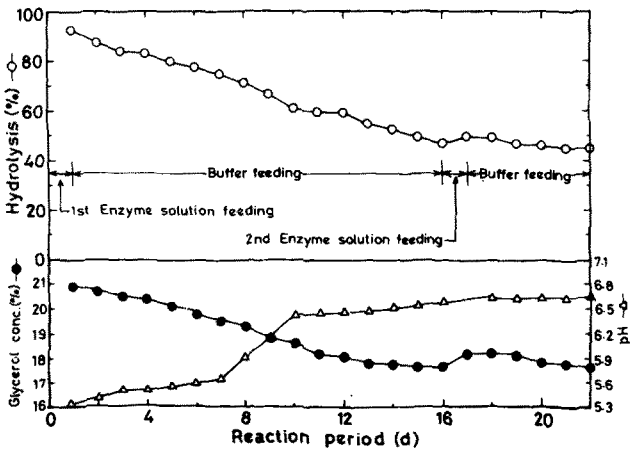

FIG. 9. Performance of the membrane bioreactor system on olive oil hydrolysis (long-term continuous operation). Fat flow rate = 2.8-3.2 ml/hr. Flow rate of enzyme-glycerol solution = 6.0 ml/hr.

out for a period of 24 days (Fig. 9). After the enzyme glycerol solution was fed for one day to immobilize the lipase on the olive oil-impregnated membrane through adsorption (first enzyme solution feeding in Fig. 9), it was switched over to the buffer-glycerol feeding at the same flow rate as with the enzyme-glycerol solution. The hydrolysis reaction proceeded with a gradual decrease in percent hydrolysis. No desorption of lipase was observed through outlets tested by ninhydrin reaction of protein. To determine whether hydrolysis rate can be recovered by reloading new enzyme, the fresh enzyme-glycerol solution was again fed for one day after an operation period of 16 days (second enzyme solution feeding in Fig. 9). Negligible increase in hydrolysis was observed (Fig. 9). This indicates that adsorption was strong and the gradual decrease in hydrolysis during the period of buffer feeding was not due to desorption of the enzyme from the membrane interface but due to thermal inactivation of the adsorbed enzyme. From the profile of hydrolysis in Figure 9, the half life of the adsorbed enzyme was calculated as about 15 days, which was much longer than that obtained without glycerol addition (2 days, Fig. 4).

Changes of outlet glycerol concentration and pH were quite consistent with the observed time course of hydrolysis. As glycerol liberation is in parallel with the hydrolysis, outlet glycerol concentration leveled down approaching the inlet glycerol concentration. At the highest hydrolysis, the pH of the outlet buffer-glycerol solution decreased most, and with a gradual decrease in hydrolysis it approached the inlet value. The decrease in pH of the outlet glycerol-buffer solution may be due to dissolution of some fatty acid(s) into the solution from the oil phase. The details are under investigation and will be reported in a forthcoming publication. It should be noted here that the decrease in pH of the buffer-glycerol solution inside the bioreactor had no adverse effect on hydrolysis rate. First, the lipase from *Candida cylindracea* is very stable at pH 3-7 and shows higher activity in the acidic range (21). Second, being a continuous buffer-glycerol feeding system, the pH of all microenvironments was not the same lower pH as that of outlet solution.

To obtain more prolonged high conversion, experimentation at lower temperature and reduced fat flow rate or with more thermostable lipase may be preferable.

General Discussion

The new bioreactor with hydrophobic microporous membrane was used for continuous hydrolysis of olive oil by lipase. The advantages of the bioreactor for the fat hydrolysis are summarized as follows:

- The system does not require making of an emulsion, obviating the need for a surfactant and stirring.
- No dilution of fat by organic solvent that reduces the enzyme inactivation is required.
- The immobilization of enzyme is simple and does not require conventional carrier material.
- Control of glycerol concentration is simple.
- Product can be obtained in a single phase with no component of another phase.
- Autooxidation of fatty acid is avoidable simply by freeing the fed substrate from oxygen, because the bioreactor has no free space to hold air inside when it is filled with reactants.
- The flow of fatty acid in the present bioreactor system is close to plug flow (more strictly speaking, laminar flow), whereas the emulsion system is always operated in perfect mixing.

In the present study, glycerol was added to stabilize the lipase. It is conceivable that in addition to the stabilizing effect, high glycerol content (ca. 20%) has two other merits. First, higher glycerol concentration reduces separation costs when glycerol is later purified by distillation. Dilute solution must be concentrated by such a method as reverse osmosis or evaporation prior to distillation (6). Second, high glycerol content excludes any chance of microbial contamination. In fact, no microbial contamination was encountered in the continuous experiment which lasted for 24 days. This would be due to reduced water activity or increased osmotic pressure or both. On the other hand, conceivable disadvantages of the membrane bioreactor are:

- A large area of membrane is required.
- The enzyme-coated membrane must be cleaned for reuse.
- Pressures of the two phases must be carefully controlled.

In this study, only one unit of a flat membrane bioreactor was used. This configuration is very convenient to obtain basic data under various conditions. However, hollow fiber module may be better as an industrial bioreactor configuration in terms of area per volume performance. Continuous hydrolysis of oil by lipase in a bioreactor having hollow fibers made of microporous polypropylene will be reported elsewhere.

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♣Cottonseed Oil Estimation by Pulsed Nuclear Magnetic Resonance Technique

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ABSTRACT

Seed asymmetry and moisture associated with the seeds are known to affect seed oil estimation by pulsed nuclear magnetic resonance (NMR) technique employing free induction decay or single spin echo (SE) pulse sequence. Using *Gossypium* (cottonseeds) as experimental material, it is shown that transverse relaxation times (T_2) of seed oil, in different varieties of seeds, measured in vivo, are not the same. The mean T_2 value of tetraploid seeds is found to be significantly higher than that of diploids. The effect of T_2 variation and other problems on oil estimation by the free induction decay and SE methods can be avoided by using the Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence to monitor the signal intensities of a certain number of selected echoes and processing them to yield the extrapolated signal intensity at zero time. The oil content values thus estimated are found to agree well with those obtained by Soxhlet method. The agreement between the two methods might depend upon the presence of gossypol and other pigments present in the samples. Neither delinting nor dehydrating the seeds is necessary in the present method. Even with the CPMG sequence, use of individual echoes is not recommended, as the T_2 variations give rise to erroneous values.

INTRODUCTION

Pulsed nuclear magnetic resonance (NMR) provides a quick and convenient method for the determination of solid fat content in partially crystallized fats (1), seed oil in oil bearing seeds (2,3) and oil and water determinations in emulsions (4). Recently, this technique has been used to estimate the moisture content in paddy seeds (5,6). A detailed study on *Gossypium* has substantiated our earlier contention (7) that among the various pulse sequences available in NMR spectroscopy, the Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence (8-10) is by far the best so far as the effects of seed orientation, seed asymmetry and seed moisture are concerned. The various aspects investigated were: (i) measurement of transverse relaxation times (T_2) of oil protons; (ii) the effect of T_2 variation on oil estimation; (iii) the effect of lint and moisture on oil estimation, and (iv) the estimation of oil content by CPMG pulse sequence and comparison with chemically estimated values.

BASIC PRINCIPLE

The basic principles involved in the different pulse sequences have been described elsewhere (7,8). In the simple, two-pulse spin echo sequence, the amplitude of the echo at 2τ is given by:

$$A = M_0 \text{ EXP } [-(2\tau/T_2) - 2\gamma^2 G^2 D\tau^3/3] \qquad [1]$$

where γ = magnetogyric ratio of protons; D = diffusion coefficient; G = magnetic field gradient; τ = pulse separation between 90 and 180 pulses; T_2 = transverse relaxation time of the protons, and M_0 = proportionality constant.

The departure from simple exponential decay due to diffusion (second term) becomes pronounced for large values of τ. Moreover, the shape and amplitude of the echo are highly influenced by magnetic field instabilities. The effect of diffusion can be arbitrarily minimized by applying the pulse sequence $(90)_x$-τ-$(180)_y$-2τ-$(180)_y$-2τ-$(180)_y$..... etc., and reducing the pulse spacing to less than a msec. Such a pulse train is known as CPMG sequence. Under these conditions, a series of spin echoes with decreasing amplitudes are formed and, neglecting the diffusion term, the echo amplitude corresponding to any echo can be represented by

$$A = M_0 \text{ EXP } (-2n\tau/T_2) \qquad [2]$$

n being the echo number. The nondestructive estimation of oil content in seeds consists essentially of evaluating the