Immobilization of Lipase from *Rhizopus delemar* on Polyethylene Membrane

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ABSTRACT

Rhizopus delemar lipase was immobilized by physical adsorption onto polyethylene membranes. The influence of membrane pore size and thickness on enzyme activity was studied. The immobilization efficiency was higher for the thicker membrane than thin one, this related to the large excess of area that the enzyme can occupy. The immobilization efficiency was also affected by enzyme loading, in which suppression was occurred at high enzyme loading. At the initial rate of hydrolysis reaction, the amount of enzyme bound, concentration of substrate, and membrane's thickness as related to the limitation of the substrate transfer affected the production of fatty acid. The thin polyethylene membrane was the best support since the enzyme immobilized on this support was stable during storage and possessed higher degree of hydrolysis and ability for subsequent reuses. Both membranes were regenerable by washing for fresh enzyme immobilization.

INTRODUCTION

Immobilized lipases were widely applied on industrial processes that have a major commercial importance, such as fat hydrolysis, triacylglycerol modification and ester synthesis. One example was the immobilized lipase catalyzes production of structured triacylglyserols under essentially nonaqueous conditions, such as medium chain triacylglycerol for nutritional purposes (Moore and McNeill 1996, Foglia and Villeneuve 1997, Soumanou et al., 1997).

Immobilized technology gave the benefits of the ability to reuse enzyme, easy product separation from enzyme, and the potential to run continuous processes by using packed bed reactor. Furthermore, the mild reaction conditions used lead to high quality products that require the minimum of refining (Bosley and Peilow 1997). Studies also have shown that immobilization enhances lipase activity, as well as thermal and chemical stability (Al-Duri et al., 1995).

Method for immobilization was varied, but the simplest one is an adsorption technique. By introducing the enzyme into material support, it could be adsorbed on such materials and immobilized. Selection of the right support for enzyme immobilization was the most important, as enzyme efficiency depends largely on the support and its linkage to it. The support should possess a well-developed internal structure, a large surface area, provided by high porosity, and reasonable pore size distribution (Gemeiner 1992). Hydrophobic supports (in form of microporous powders, membranes, or fibbers), such as polyethylene, were found to give the best performance, as little of the lipase's activity was lost upon immobilization (Brady et al., 1988, Malcata et al., 1992). A microporous membrane was an enzyme's support that could separate the bulk phases where a large surface area of contact between the two phases was provided. This support had some advantages, such as stable interface, ease of scale-up, and elimination of emulsification problems (Rucka et al., 1990). Al-Duri and his co-workers (1995) have reported that porosity and composition of the hydrophobic support was more important than the surface area. However, according to Rucka et al (1990) the porosity and hydrophobicity of the membranes did not influence the lipase activity. In this paper we reported the results of studies on an immobilized lipase on polyethylene membranes of different properties, and discussed on the influence on the enzyme activity.

MATERIALS AND METHODS

Enzyme and membrane

Lipase D from *Rhizopus delemar* was kindly giving from Amano Pharmaceuticals (Nagoya, Japan). Two polyethylene membranes (Hipore H-1100 and H-6022) were manufactured and kindly given by Asahi Kasei (Japan). These materials differed in pore size, thickness, and permeability (see Table 1).

Table 1. **Physical characteristics of polyethylene membranes**

Properties	Unit	Type H-1100	Type H-6022
- Thickness	μ	100	27
- Porosity	%	55	50
- Pore size	μ	0.05	0.10
- Permeability	sec/100 cm^2	600	90
- Tensile strength	Kg/cm^2 (M)	50	1400
	Kg/cm^2 (D)	40	190
- Stretch	% (M)	200	45
	% (D)	250	450

Immobilization of lipase

Lipase was immobilized by adsorption on the membrane surface (2 x 2 cm) at 4°C for 24 h. The amount of enzymatic protein adsorbed was varied and calculated from the difference between the concentrations before and after the enzyme immobilization. The efficiency of lipase immobilization was calculated by the following equation:

$$\text{Immobilization efficiency (\%)} = \frac{E_i}{E_o V_o} \times 100$$

where E_o was the lipase activity of the original lipase solution (U/ml), V_o was its volume (ml); E_i was the lipase activity of the immobilized enzyme.

Enzyme reaction

The reaction was carried out at 30°C for 2 h (unless otherwise stated) with shaking in a 100 ml Erlenmeyer flask containing immobilized lipase (2 x 2 cm) corresponding to 0.052 to 0.655 mg/cm^2 of enzyme powder immobilized initially, 5 ml of 60% (v/v) olive oil in isooctane, and 5 ml of 0.1 M phosphate buffer pH 7.0. In some cases, other reaction conditions were varied. Free fatty acid produced was measured according to the method of Marseno et al (1998). The degree of hydrolysis of the oil was calculated by the following equation:

$$\text{Degree of hydrolysis (\%)} = \frac{\mu\text{mol fatty acid liberated}}{(\text{SV})\ (1000/56.1)\ (\text{g oil})} \times 100$$

SV was the saponification value of olive oil, which was 192.

Repeated batch hydrolysis

The experimental conditions were similar to those described above, with 0.21 mg enzyme powder immobilized initially per cm^2 membrane. After 15 h for each run, the hydrolysis degree was determined, and the immobilized lipase preparations were reused with fresh substrate.

Variation of the substrate concentration

The membrane was loaded with 0.21 mg lipase/cm^2 membrane. Initial rates were determined at different initial olive oil concentration (40 - 100%) in isooctane.

Storage stability

Storage stability was estimated from measurement of activities of immobilized enzyme (0.21 mg protein loading per cm^2 membrane) that were stored at 4 °C. The activity measurement was continuous over a period of 20 days.

Membrane regeneration

Membrane status was monitored by cleaning the used membranes and reused them for immobilization. Membrane cleaning was done with ethanol, 0.1 M sodium hydroxide, 1 M NaCl, and phosphate buffer 0.1 M pH 7.0. After cleaning, a fresh lipase solution was immobilized on the membrane.

Analysis

Protein amount was measured by the method of Lowry et al (1951) which was modified by Peterson (1977) using bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Effect of protein loading

This study was carried out using a 2 x 2 cm membrane and the reaction time was 20 min. The

immobilized lipases were assessed for hydrolysis activity and plot of activity and efficiency (activity/ loading) vs. loading were shown in Fig. 1 and 2, respectively. This plot shows that the activity (as µmole fatty acid produced) of immobilized enzyme increased as more lipase was loaded onto the membrane, but when converted to an efficiency plot, it decreased as enzyme loading was increased (Fig.2). According to Bosley and Peilow (1997) this was a typical system of immobilized enzyme that mass transfer limitation hinders the diffusion of substrate through the pores, caused in a decrease of concentration of substrate toward the centre of the particle. This especially important at high enzyme loading where the enzyme will be distributed throughout the particles. At low loading, most of the enzyme will be located at or near the surface of the particles, and mass transfer limitation will less occur (Bosley and Peilow 1997).

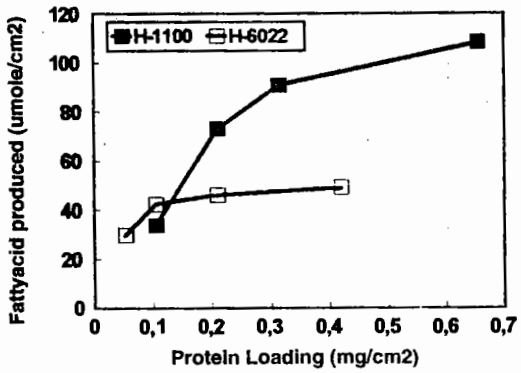

Figure 1. Effect of various proteins loading for lipase immobilization on the fatty acid formation. Reaction time was 20 min.

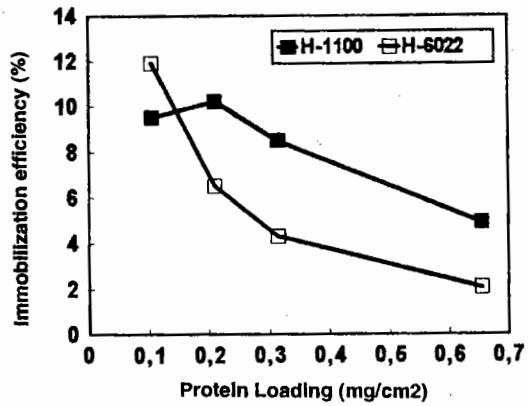

Figure 2. Immobilization efficiency of lipase on polyethylene membranes at 4°C for 24 h. Reaction time was 20 min.

In comparation between membrane type H-1100 (thick) and type H-6022 (thin), the efficiency was better for the thick one, except at low loading (Fig.2). At thick membrane, there was a large excess of area that the enzyme can occupy. However, as loading was increased, less area was available for the lipase to spread (Fig.1), so the activity was constant and the efficiency fell. From these data it seem that the saturation loading for thin and thick membranes was about 0.1 and 0.3 mg/cm^2, respectively.

Initial rate of olive oil hydrolysis

Fatty acid production with lipase immobilized on polyethylene membranes type H-1100 and H-6022 as a function of reaction time at various enzyme loading was shown in Fig. 3 and 4, respectively. At the initial rate of

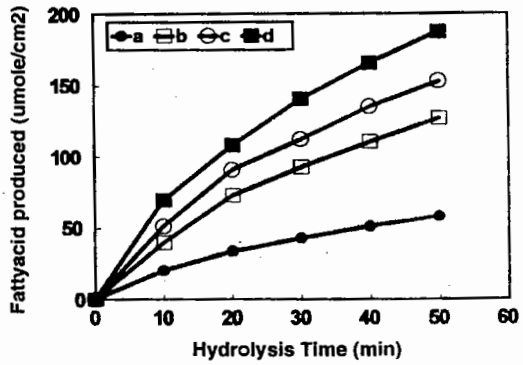

Figure 3. Amount of fatty acid produced as a function of reaction time at various bound lipase concentration on polyethylene membrane H-1100 : a, 0.105; b, 0.210; c, 0.314; d, 0.655 mg/cm^2 membrane.

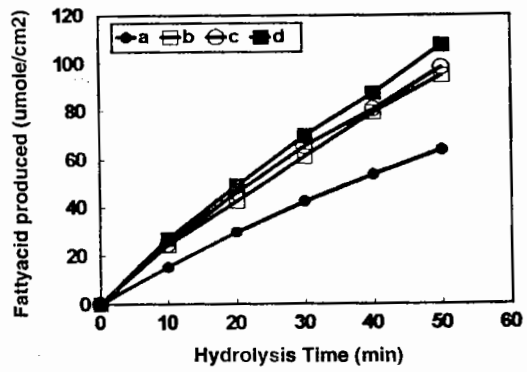

Figure 4. Amount of fatty acid produced as a function of reaction time at various bound lipase concentration on polyethylene membrane H-6022 : a, 0.052; b, 0.105; c, 0.210; d, 0.419 mg/cm^2 membrane.

lrolysis reaction, the amount of fatty acid produced ...s linearly proportional to the enzyme bound, both at lower to higher concentration, particularly for membrane H-6022 (thin). The production rate for membrane type H-1100 (thick) deviated from the straight line, which probably because of saturated adsorption of the enzyme on the support or the limitation of the substrate transfer. In this study, the reason mention later might have occurred since this result was also happened at low enzyme loading (Fig.3). This character of membrane type H-1100 was a disadvantage particularly for prolonged time of hydrolysis.

Degree of hydrolysis

Fig. 5 shows the degree of hydrolysis of olive oil using a 2 x 2 cm immobilized membrane of different types at 0.21 mg enzyme loading/cm^2. Olive oil was approximately 65 % hydrolyzed in 24 h when immobilized lipase on polyethylene type H-6022 (thin) was used. The degree of hydrolysis fell when the thick membrane was used. These results were in accordance with the data mentioned above.

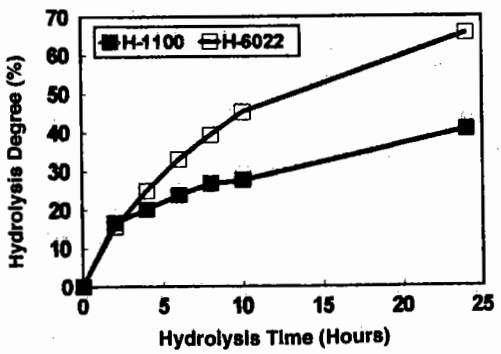

Figure 5. **Degree of olive oil hydrolysis by lipase immobilized on polyethylene membranes at various times. Protein loading was 0.21 mg/cm^2 membrane.**

Effect of storage on lipase stability

The immobilized lipases were dry incubation at 4 °C in a closed bottle. The enzymes immobilized on a polyethylene membrane type H-6022 were more stable than those immobilized on that type H-1100 as shown in Fig.6. The half life of the enzymes adsorbed on membrane type H-1100 were about 9 days, while on membrane type H-6022 was failed to be measured since up to 24 days of storage their activity was no change.

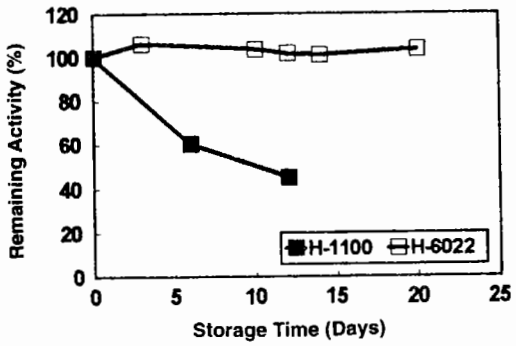

Figure 6. **Storage stability of lipase immobilized on polyethylene membranes. Initial activity was calculated as 100% when incubation was started. Reaction time was 2 h.**

Reusability

The immobilized lipases were used for hydrolysis of olive oil for 15 h. Lipase immobilized on a polyethylene membrane type H-6022 displayed good stability in this process (Fig.7). After 5 subsequent reuses, the fatty acid produced was 70 % of the first use, showing the effectiveness of immobilization and reusability of the immobilized enzyme. For membrane type H-1100, enzyme inactivation occurred fast, with only 13 % of fatty acid produced after the second used as compared to the first use. Even for the first use of the immobilized enzyme, only 57 % of fatty acid was achieved as compared to that membrane type H-6022.

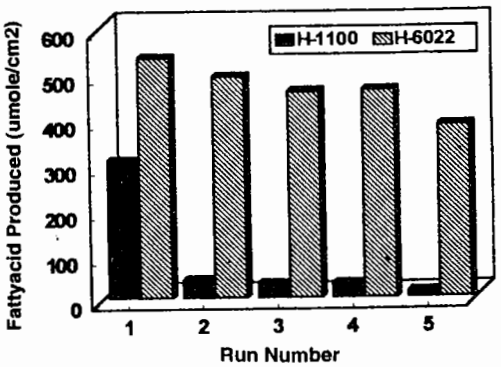

Figure 7. **Reusability of polyethylene membrane-immobilized lipases on hydrolysis of olive oil at 30 °C. Hydrolysis time was 15 h.**

Regeneration

For economic application, it is necessary to use the membrane repeatedly for enzyme immobilization. Regeneration of the membranes was attempted by

cleaning as mentioned on the Materials and Methods. As shown in Fig. 8, either membrane type H-1100 or H-6022 showed the same profiles of hydrolysis (15 h) using the new or washed membranes. In addition, hydrolysis with lipase immobilized on membrane type H-1100 increased after first washing, probably washing could change the membrane so the limitation of the substrate transfer decreased. From these results it seems that the used of membranes were regenerable. Since hydrolysis degree, storage stability, and reusability in polyethylene membrane type H-6022 were superior to those in type H-1100, membrane type H-6022 was used as the selected support.

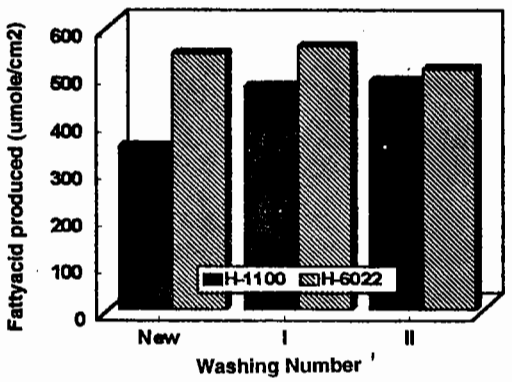

Figure 8. **Washing and repeated use of polyethylene membranes for lipase immobilization. Twice washing and repeated uses were carried out, and the results were compared to the new membrane.**

Effect of substrate concentration

Fig. 9 shows the results of fatty acid production from the hydrolysis of olive oil of various substrate concentrations using immobilized lipase on polyethylene membrane type H-6022. On the initial velocity

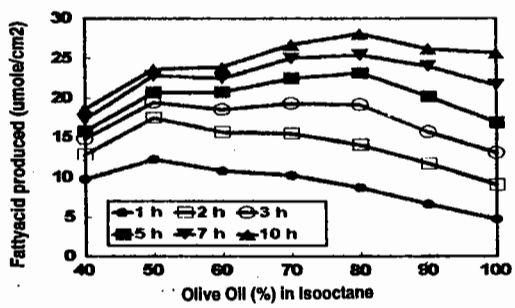

Figure 9. **Effect of olive oil concentration on fatty acid formation at various reaction times. Protein loading was 0.21 mg/cm² membrane polyethylene type H-6022.**

of hydrolysis reaction (1 h), the production of fatty acid increased with concentration of olive oil to 50 %, and gradually decreased thereafter the increase of substrate concentration. This result was the same with that reported by Kwon and Rhee (1984), but was differed with that of Kang and Rhee (1989) where concentration of substrate more than 60 % did not cause any change in activities. Fig. 9 also shows that the production plots were similar for various times of incubation, which the maximum fatty acid production shifted to 80 % of olive oil concentration as hydrolysis time was prolong to 10 h. Therefore, the determination of initial rate of hydrolysis by immobilized lipase on polyethylene membrane was better carried out by using 50 % olive oil.

CONCLUSION

Lipase adsorbed on a polyethylene membrane type H-6022 (the thin one) was found effectively catalyzed olive oil hydrolysis, which after 24 h it hydrolyzed approximately 65 % of the oil. The catalytic degree depended on the substrate concentration, in which at initial rate of hydrolysis reaction olive oil at a concentration of 50 % was the optimum. The immobilization efficiency was greatly affected by enzyme loading, with apparent suppression of efficiency at high loading for both types of membrane. This immobilized enzyme was very stable during storage as well as on repeated used. The membrane was also regenerable so it could be used again for fresh lipase immobilization.

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