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IMMOBILIZATION OF LIPASE IN COLLAGEN MEMBRANES

The hydrolysis of sunflower oil by lipase immobilized on collagen membranes is described. The enzyme was adsorbed on the membranes and then cross-linked with glutaraldehyde or immobilized by peptide binding via amine or carboxylic groups. The immobilized lipase showed a very high initial activity, but the activity decreased markedly during successive reuse. Calcium, ethanol and detergents were used to improve the transport of hydrolysis products (free fatty acids) from the membrane surface. None of these substances improved the lipase activity. The rate of oil hydrolysis catalyzed by immobilized lipase was of the same order as that of soluble enzyme when calculated as the surface hydrolysis rate, but the operational stability of the lipolytic membrane must be improved.

1. INTRODUCTION

Lipases catalyze the hydrolysis of triacylglycerides of animal fats and vegetable oils. Recently, the potential for using lipase for industrial purposes has significantly broadened [1]–[3]. Immobilization of lipases allows reuse of the enzymes and is expected to make possible the continuous enzymatic hydrolysis of triacylglycerides. Immobilization of lipase has been performed by several authors on various carriers [4]–[8]. This work describes the hydrolysis of sunflower oils by lipase immobilized on collagen membranes. Three methods of immobilization were applied and what was done was a comparison of their influence on the enzyme properties.

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2. MATERIALS AND METHODS

2.1. REAGENTS

Lipase from *Candida rugosa* (690 U/g) was obtained from Sigma. Edible sunflower oil was supplied by Zakłady Przemysłu Tłuszczowego (Plant of Fat Industry) Brzeg, Poland. Collagen membranes were made from nondissolved bovine collagen. All other chemicals were of reagent grade and were purchased from POCh, Poland.

2.2. ENZYME IMMOBILIZATION

Sorption of lipase was carried out in the following way: 25 mg of lipase powder was dissolved in 20 cm^3 of 0.05 M tris-HCl buffer of pH = 8, and the membrane (5 cm in diameter) was immersed in this solution for 24 hours. Then the membrane was washed with water and immersed in a solution of 8% glutaraldehyde for the next 24 hours. After washing, the membrane was used for sunflower oil hydrolysis.

Covalent binding via amine groups was performed according to the following method: the membrane was activated by immersion in a solution of glutaraldehyde (8% in tris-HCl buffer, pH = 8, 0.05 M) for 24 hours and, after washing, the procedure described above for adsorption was used.

Covalent binding via carboxylic groups was done according to the procedure described previously [9].

2.3. MEMBRANE PREPARATION

A collagen pulp was extruded through a ring-shaped spinneret and then the membrane was cross-linked with formaldehyde and dried in air stream. The obtained membrane kept the fibrillar structure of collagen. It's hydraulic flux was 0.003 m^3/m^2d MPa, the cut-off measured with azide dyes was 460 Da.

2.4. ANALYSIS

Enzyme activity was measured by the determination of free fatty acids (FFA) in both oil and water phases according to KWON and RHEE [11]. The protein concentration was determined by the method of LOWRY et al. [12].

One unit of lipase activity (U) was defined as the amount of enzyme that liberates 1 μM of free fatty acids per minute. Membrane activity is given as enzyme activity in units U of 1 m^2 of membrane area. The activity of lipolytic collagen membranes was measured in a diffusion cell in which the membranes separated oil and water phases. A detailed description of the apparatus as well as the procedure applied can be found in our previous work [10].

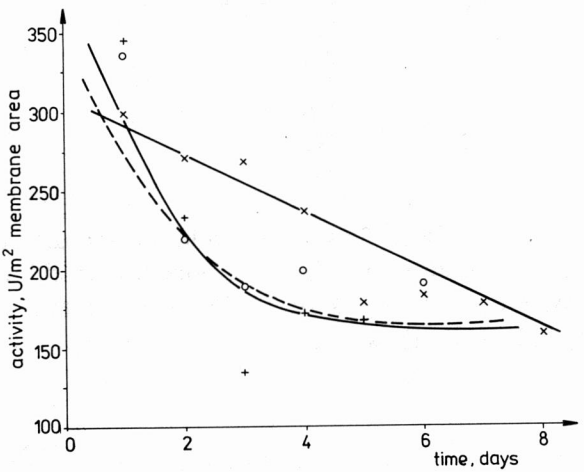
3. RESULTS AND DISCUSSION

Lipolytic membranes were used for hydrolysis of sunflower oil. The comparison of the three methods of immobilization is given in table 1. The values of activity yield (η), e.g. activity of immobilized enzyme divided by the activity of an equivalent quantity of free enzyme, indicates that the activity of lipase has not been reduced substantially by immobilization or diffusional restriction.

Table 1

Hydrolysis of sunflower oil

Immobilization method	Initial rate μM FFA/min m^2	Activity yield η	Operational stability $T/2$, days	Model
Sorption	361	1.0	3.5	Exponential
Covalent, –COOH	330	0.91	7.4	Exponential
Covalent, $-NH_2$	301	0.90	6.4	Linear



Lipase activity versus time
–×– enzyme immobilized by amine groups, –+– enzyme immobilized by carboxylic groups, –o– enzyme immobilized by sorption

Although the immobilized lipase showed a very high initial activity, the activity decreased markedly during successive reuse. The half-life time o enzyme activity, which is the time required for half of the original activity to be lost, is rather short, especially for lipase sorbed on collagen membrane. The relationship between the enzymatic activity and time is shown in the figure. For lipase immobilized by sorption as well as for lipase immobilized by peptide binding via –COOH groups,

the activity decays exponentially in time, whereas for covalent binding via $-NH_2$ groups, the activity decays linearly in time. The loss of the activity is linear when diffusion limits activity, whereas concave decay can be caused by a leakage of the enzyme from reactor [13]. Lipase immobilization via amine groups results in the alteration of membrane properties caused by an additional cross-linking of collagen with glutaraldehyde. This supposition could be verified by testing membrane properties.

From the data in table 1 it can be seen that the lipolytic membranes exhibited similar properties irrespective of the immobilization method employed.

Table 2

Dependence of lipase activity on pH
(enzyme immobilized on collagen membranes)

pH	Activity U/m^2 membrane area	
	Immobilized covalently	Immobilized by sorption
5	299	335
6	295	320
7	316	342
8	330	360
9	292	407
10	307	351

The comparison of the pH profiles of sorbed lipase and the one covalent binding (table 2) confirms the suggestion that the immobilization methods employed did not influence the enzyme properties. The activity of both of the immobilized preparations is practically independent of pH, whereas the highest lipolytic activity of the soluble enzyme was observed at pH = 6.

Table 3

Influence of calcium, ethanol and detergents on lipase activity
(enzyme immobilized on collagen membranes)

Additive	Activity U/m^2 membrane area		
	24 h	48 h	72 h
EtOH, 10%	200	221	–
$CaCl_2$, 0.05 M	273	204	180
Tween 60, 0.01 M	176	178	190
Triton X-100, 0.01%	173	164	151

Some additives were chosen to the buffer phase for improving lipase activity. The data obtained are shown in table 3. The effect of calcium on the rate of lipase-catalyzed oil hydrolysis is controversial. Calcium has been reported to be a stimulator [15] or an inhibitor [16] of lipolysis. Detergents and ethanol can affect the transport of hydrolysis products (FFA) from the membrane surface, which can influence enzyme activity. None of the compounds applied improve lipase activity, on the contrary, all of them bring about a decrease in the activity tested. The latter was particularly noticeable when detergents were employed. The accumulation of their molecules at the interfaces may cause a decrease of lipolysis.

It ought to be emphasized that the rate of oil hydrolysis catalyzed by immobilized lipase was of the same order as that of the soluble enzyme when calculated as the surface hydrolysis rate. For soluble lipase it was found to be 180 $\mu M/m^2$ min [15].

4. CONCLUSIONS

All the immobilized methods applied prove that the immobilization of lipase on collagen membranes did not change enzyme properties such as activity and pH. None of additives of water phase increased the enzyme activity. Ethanol, calcium chloride as well as both surface active agents (Tween and Triton) decreased the lipase activity.

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REFERENCES

[1] ANTONIAN E., Lipids, 23 (1988), 1101–1106.
[2] O'CONNOR K.C., BAILAY J.E., Enzyme Microb. Technol., 10 (1980), 352–356.
[3] HUGE-JENSEN B., GALLUZZO D.R., JENSEN R.G., Lipids, 22 (1987), 559–565.
[4] HOQ M.M., YAMANE T., SHIMITZU S., FUNADA T., ISHIDA S., J. Amer. Oil Chem. Soc., 62 (1985), 1016–1021.
[5] LAVAYRE J., BARATTI J., J. Biotechnol. Bioeng., 24 (1982), 1007–1013.
[6] KIMURA Y., TANAKA A., SONOMOTO K., NIHIRA T., FUKUI S., Eur. J. Appl. Microbiol. Biotechnol., 17 (1983), 107–112
[7] PRONK W., KERKHOF P.J.A.M., VAN HELDEN C., VAN'T RIET K., Biotechnol. Bioeng., 32 (1988), 512–518.
[8] RUCKA M., TURKIEWICZ B., TOMASZEWSKA M., CHLUBEK N., Biotechnol. Lett., 11 (1989), 167–174.
[9] WOODWARD J. (ed.), *Immobilized cell and enzymes. A practical approach*, IRL Press Limited, Oxford, Washington 1985.
[10] RUCKA M., TURKIEWICZ B., Enzyme Microb.Technol., 12 (1990), 52–55.

[11] KWON Y.D., RHEE J.S., J. Amer. Oil Chem. Soc., 63 (1986), 89–92.
[12] LOWRY O.H., ROSENBROUGH N. J., FAN A.L., RANDALL R.J., J. Biol. Chem., 193 (1951), 265–275.
[13] WISEMAN A. (ed.), *Handbook of Enzyme Biotechnology*, p. 143, Ellis Horwood Limited, Chichester 1985.
[14] LEE Y.K., CHOO Ch.L., Biotechnol. Bioeng., 33 (1989), 183–190.
[15] KHOR H.T., TAN N.H., CHUA C.L., J. Amer. Oil Chem. Soc., 63 (1986), 538–540.
[16] WANG Y.J., SHEN J.Y., WANG F.F., SHAW I.F., Biotechnol. Bioeng., 31 (1988), 628–633.

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UNIERUCHOMIENIE LIPAZY W MEMBRANACH KOLAGENOWYCH

Opisano hydrolizę oleju słonecznikowego przez lipazę unieruchomioną na membranach kolagenowych. Enzym był adsorbowany na membranach, a następnie usieciowany aldehydem glutarowym albo unieruchomiony przez wiązanie peptydowe amin lub grup karboksylowych. Unieruchomiona lipaza wykazuje bardzo dużą aktywność początkową, która jednak znacznie zmniejsza się podczas kolejnych reakcji. Użyto wapnia, etanolu i detergentów, aby poprawić transport produktów hydrolizy (wolne kwasy tłuszczowe) z powierzchni membrany. Żadna z tych substancji nie poprawiła aktywności lipazy. Szybkość hydrolizy oleju, katalizowanej przez unieruchomioną lipazę, była tego samego rzędu, co rozpuszczonego enzymu, gdy obliczono ją jako szybkość hydrolizy powierzchniowej. Stabilność membran lipolitycznych musi być poprawiona.

ОСТАНОВКА ЛИПАЗЫ В КОЛЛАГЕНОВЫХ МЕМБРАНАХ

Описан гидролиз подсолнечного масла липазой, остановленной на коллагеновых мембранах. Энзим был адсорбирован на мембранах, а затем структурирован глутарным альдегидом или посредством пептидной связи аминов или карбоксильных групп. Остановленная липаза обнаруживает очень большую начальную активность, которая однако значительно уменьшается во время дальнейших реакций. Были употреблены кальций, этанол и детергенты для улучшения транспорта продуктов гидролиза (свободныв жирные кислоты) из поверхности. Все вти вещества не улучшили активности липазы. Быстрота гидролиза масла, катализированного остановленной липазой, была такого же порядка как растворенного энзима, когда ее рассчитывали в качестве повиерхностного гидролиза. Стабильность липолитических мембран необходимо улучшить.