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Heat Treated Hydrotalcite as Support for Lipase Immobilization

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ABSTRAK

Hidrotalsit (HT) dan hidrotalsit terawat haba (HTHT) dengan Mg^{2+}: Al^{3+} pada nisbah 4 telah disediakan melalui kaedah pemendakan bersama melalui penggoncangan secara berterusan dan pemanasan pada 200°C. Dari corak belauan sinar-X bahan-bahan ini, didapati bahawa hidrotalsit telah berjaya disintesis dan struktur berlapis gandanya tidak terurai oleh pemanasan kepada 200°C. Lipase daripada *Candida rugosa* kemudiannya disekat gerak kepada bahan ini melalui kaedah penjerapan fizikal. Didapati bahawa peratus kandungan protein meningkat daripada HT (20%) kepada HTHT (40%). Keaktifan lipase yang tersekat gerak diuji melalui tindak balas esterifikasi di antara asid oleik dan butanol di dalam heksana. Enzim tersekat gerak ini kemudiannya dicirikan dari segi kestabilan terhadap suhu, pelarut (heksana) dan penyimpanan. Kestabilan lipase tersekat gerak didapati lebih tinggi jika dibandingkan dengan lipase asli (NL).

ABSTRACT

Hydrotalcite (HT) and heat treated hydrotalcite (HTHT) with Mg^{2+}:Al^{3+} of ratio 4 were prepared by co-precipitating through continuous agitation and heating to 200 °C, respectively. From the X-ray diffraction patterns of these materials, it was found that hydrotalcite had been successfully synthesized and the layered structure was not collapsed by the heat treatment up to 200°C. Lipase from *Candida rugosa* was then immobilized onto these materials, through the physical adsorption method. It was found that percentage of protein loading increases from HT (20%) to HTHT (40%). Their activities were tested by the esterification reaction of oleic acid and butanol in hexane. The immobilized enzymes were then characterized in terms of their thermal, solvent (hexane) and storage stability. Their various stabilities were comparatively higher than native lipase (NL).

Keywords: Lipase, immobilization, hydrotalcite, heat treatment, esterification, stability

INTRODUCTION

Knowledge on lipase has increased especially in the areas of molecular structure and mechanism of action. Their wide applications are often centered on its ability to catalyze hydrolytic reactions in an aqueous medium and the reverse esterification reaction in organic solvents where ester bonds are formed (Kirchner *et al.* 1985). Lipases have been isolated from a wide variety of mammalian and microbial sources and are commercially available in quantity. In mammals, pancreatic lipase are involved in the digestion of dietary fat (Lin *et al.* 1982). *Candida rugosa* is an example of a microbial lipase, which hydrolyses all ester bonds of natural or synthetic triacylglycerols and have been widely used in the complete hydrolysis of fats as well as in the esterification reactions. Besides that, it is also known as a highly stereoselective enzyme which catalyses stereoselective esterification as well as transesterification reaction in organic medium (Triantafyllou *et al.* 1993).

However, soluble forms of lipase were reported to have low operational range and stability (Basri *et al.* 1995). In order to ease these problems, methods of immobilization of enzymes onto insoluble materials were introduced and this has formed the basis of many biotechnological processes and analytical devices. Immobilization not only eases enzyme-product separation, but also allows enzyme recyclability and persistency of its functional activities that help to bring about the increased stability of an enzyme (Fagain 1997). This has been proven, as most immobilized enzymes of various sources have been found, applicable in the pharmaceutical, cosmetic, fragrance and flavour industries (Basri *et al.* 1996).

Hydrotalcite or layered double hydroxides (LDH) is the most common member of the group of minerals known as the LDH or the so called anionic clays. It consists of layers of M^{II} and M^{III} cations with a similar layered structure to that of brucite, $Mg(OH)_2$ with Mg octahedrally surrounded by six oxygens in the form of hydroxides with the octahedral units forming infinite sheets. These sheets are then stacked together through hydrogen bonding forming layers of hydroxides (Carlino and Hudson 1995).

Hydrotalcite has long attracted interest in the variety of areas of physics, chemistry, biology and material sciences. They possess many properties comparable to clay but also add new and interesting applications (Mitchell 1990). Its heat treated and intercalated derivatives are new materials known to have extensive use and applications in areas of gas separations, pharmaceutical science, catalysis (Miyata and Hirose 1978; Miyata and Kumura 1973), anion exchanger and adsorbents (Meyn *et al.* 1990; Miyata 1983). In this study, hydrotalcite and heat treated hydrotalcite were synthesized. It was then used as support for lipase immobilization. They were then characterized and their activities were investigated.

MATERIALS AND METHODS

Lipase from *Candida rugosa* (Type VII) was purchased from Sigma Chemicals, Germany. $Al(SO_4)_3.17H_2O$, $MgSO_4.8H_2O$ were purchased from Hamburg, Germany, while $NaCO_3$ and NaOH were supplied by Fluka, Switzerland and Mallickrodt, Mexico. Substrates, oleic acid and butanol were from BDH Laboratory, England and J. T. Baker, U.S.A. All reagents and solvents used were of highest analytical grade.

Synthesis of Hydrotalcite

Mg-Al-CO_3 hydrotalcite were prepared following an aqueous precipitation and thermal crystallization method described by Reichle (1985). In this synthesis, 100 mL of a solution containing 0.25 M of $MgSO_4.8H_2O$ and 0.06 M of $Al_2(SO_4)_3.17H_2O$ was added dropwise over a vigorously stirred 100 mL solution containing NaOH (3.5 M) and Na_2CO_3 (1.0 M). The slurry obtained was aged at 65 °C for 18 h in a water bath (Memmert GmbH, Germany). After it was cooled, it was then filtered and washed with a copious amount of distilled water until the pH of the washing was neutral. The resulting white solid was dried at 120 °C for 24 h before being ground in a pestle and mortar. Powder of the refined solid (HT) was characterized using X-ray diffractometer (Siemens D-5000, Germany) with Ni filtered, CuKa radiation at 40 KV and 20 mA. The samples were mounted on glass slides and scanned at 2°-65° with 2θ/min at 0.003°. General features that are typical of all hydrotalcite are the sharp and intense lines at the low values of the 2θ angles at about 7-8 Å.

Surface characterization of the materials was carried out by nitrogen gas adsorption-desorption at 77 K using a Micromeretics ASAP 2000. Prior to the adsorption of nitrogen gas, the samples were out-gassed to at least 5 x 10^{-3} mm Hg in an evacuation-heated chamber at 120 °C, overnight.

The surface area and pore size distribution were calculated using Brunauer, Emmett and Teller (BET), and Brunauer, Johner and Halenda (BJH) equation, respectively. The specific total pore volumes were estimated from the nitrogen uptake at P/P_o = 0.99 (Gill and Montes 1994).

Preparation of Heat Treated Hydrotalcite

The powder (HT) was heated in a quartz furnace (Carbolite, Sheffield, England) for 5 h at 200 °C. Powder of HTHT was characterized as stated above.

Partial Purification of Lipase

Partial purification by water extraction was carried out by dissolving 1.5 g of crude commercial lipase from *Candida rugosa* (Type VII) in 15.0 mL of distilled water. The mixture was stirred for 30 min and centrifuged at 10000 rpm for 15 min. The undissolved solid suspension was discarded after centrifugation while the supernatant was used as partially purified lipase and stored at -20°C prior to use.

Immobilization of Lipase

The supernatant (15.0 mL) was added to 2.0 g of hydrotalcite. The mixture was incubated at room temperature for 1 h in a horizontal waterbath shaker with continuous shaking at 100 rpm. The lipase-immobilized hydrotalcite was separated from the supernatant by filtering through Whatman no. 1 filter paper. The immobilized enzymes were then lyophilized in a freeze drier (Labconco 195, Kansas City, England). Percentage of protein immobilized is calculated based on the total protein used (100%) in the immobilization reaction.

Protein Assay

The amount of protein was determined by Bradford method (Bradford 1976), with Coomassie brilliant blue assay, using bovine serum albumin as standard. In a typical protein assay, lipase solution (100 μL; 0.1 g/mL) was mixed with Bradford reagent (5.0 mL). The protein concentration was determined spectrophotometrically at a wavelength of 595 nm using the calibration curve of bovine serum albumin.

Activity Assay

The reaction system consisted of 2.0 mL hexane, 4.0 mmole 1-butanol, 2.0 mmole oleic acid and 0.3 g immobilized lipase or 50 mg of native lipase. The mixture was incubated at 30°C for 5 h with continuous shaking at 150 rpm. Reaction was terminated by dilution with 3.5 mL ethanol:acetone (1:1 v/v) and the remaining free fatty acid in the reaction mixture was determined by titration with 0.15 M NaOH using an automatic titrator (ABU90, Radiometer, Copenhagen) to an end point of pH 10.0. The activities were expressed as mmol/min/mg protein using the Equation (1) below:

$$\text{Esterification Activity} = \left[\frac{(V_c - V_s)M] \div t}{W}\right] \quad (1)$$

Whereby, V_c – titration volume of the control (mL)
V_s – titration volume of the sample (mL)
M – molarity of NaOH
t – time (min)
W – amount of protein

Effect of Temperature on the Esterification Activity

To investigate the most suitable temperature for the esterification reaction, the reaction mixtures were incubated at different temperatures (30, 40, 50, 60, 70°C) for 5 h with continuous shaking at 150 rpm. The residual esterification activities were determined. The relative activities were expressed as percentages of the activities at different temperatures as compared to the activity at optimum temperature (40°C).

Thermal Stability

Enzymes were incubated at various temperatures (30, 40, 50, 60, 70°C) in sealed vials for 1 h. The enzymes were left to cool at room temperature before determination of esterification activity was carried out. The activities were expressed as percentages of the relative activities at different temperatures compared to the activity of the untreated enzyme.

Leaching Study

To investigate the effect of washing on the immobilized lipase, 0.3 g of immobilized lipase were carefully washed with increasing amount of hexane (4.0, 8.0, 12.0, 16.0, 20.0 mL) with 4.0 mL at each wash. The activities of the immobilized lipase at each volume of hexanes were determined by esterification reaction. The activities of the lipases were expressed as percentages of the relative activities at different wash cycle compared to the activity of the unwashed enzyme.

Stability in Organic Solvent

To investigate the stability of the immobilized enzyme in organic solvent, they were incubated in hexane at room temperature from day one to day ten. After the incubation, their residual activities were determined at 30 °C. The residual activities were expressed as percentages of their activity at the different time intervals compared to the activity at day one.

Storage Stability

This study was conducted to investigate the stability of native lipase compared to immobilized lipases at different storage temperature. The enzymes were kept for 60 days. The residual activities were determined and expressed as percentages of the residual activities at 60 days compared to the initial activity at day one.

RESULTS AND DISCUSSION

Analysis of Hydrotalcite

Powder XRD patterns of HT and HTHT, are shown in *Figs. 1(a)* and *1(b)*, respectively. The patterns show that pure hydrotalcite phase was obtained. The basal spacing of HT and HTHT are similar, indicating that the layered structure of the material is still maintained and non-collapsed even after heat treatment at 200°C for 5 h. However, further heating at higher temperature leads to intensity decay of hydrotalcite and collapse of the layered structure.

Figs. 2 and *3* show the pore size distribution of HT and HTHT. A sharp pore size distribution peaks were observed for both. This sharp maximum is associated with a dip in the desorption isotherm which is not reflected in the adsorption isotherm. Such a feature is a characteristic of the laminar clay-like structure as expected from the known structure of hydrotalcite. As heat treatment proceeds this peak decreases in intensity together with gradual changing of shape to a wider one (Hussein *et al.* 1995).

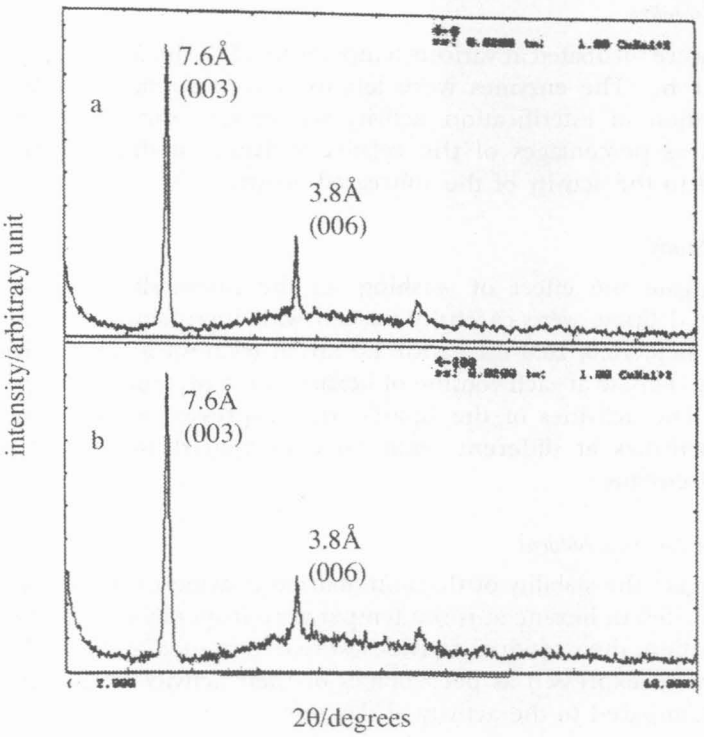

Fig. 1: (a) XRD pattern of Hydrotalcite (HT) prepared at ratio (Mg^{2+}:Al^{3+}) 4, (b) XRD pattern of Heat Treated Hydrotalcite (HTHT) prepared at ratio (Mg^{2+}:Al^{3+})4

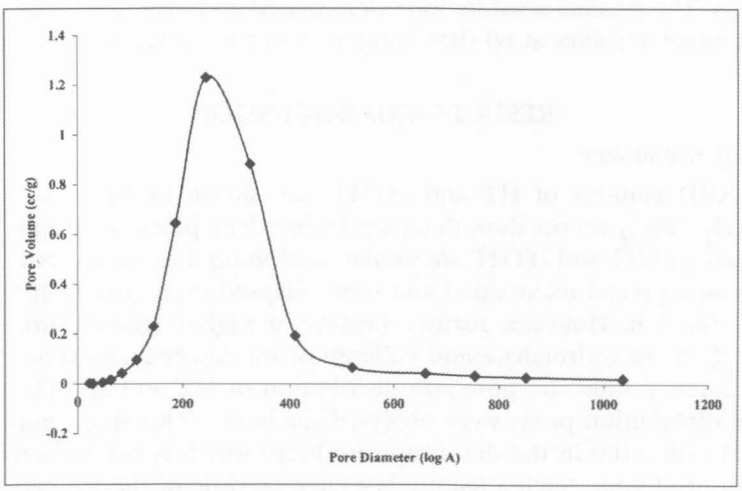

Fig. 2: Pore size distribution of Hydrotalcite (HT) of Mg^{2+}:Al^{3+} prepared at ratio 4

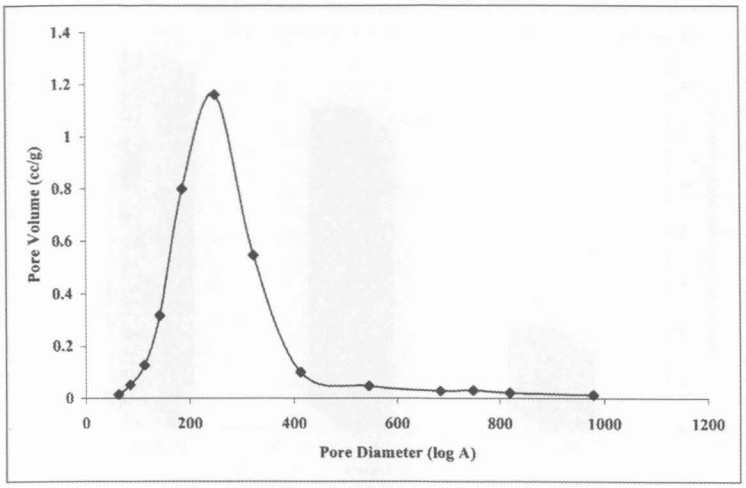

Fig. 3: Pore size distribution of Heat Treated Hydrotalcite (HTHT) of Mg^{2+}:Al^{3+} prepared at ratio 4

TABLE 1
BET surface area, micropore volume and BJH desorption pore size distribution of HT and HTHT of Mg^{2+}:Al^{3+} prepared ratio 4

LDH	BET Surface Area (m^2/g)	Micropore Volume (cc/g)	BJH Desorption (Å)
HT	53.8	0.0007	228
HTHT	62.0	0.0025	139

Summary of the BET surface area, micropore volume and BJH desorption pore size distribution of these materials are shown in Table 1.

Immobilization of Lipase

In a typical immobilization of lipase on HT and HTHT, the percentages of protein immobilized were 20% and 40%, respectively. The increase in protein immobilized on HTHT as compared to on HT is in accordance with the surface area and porosity of the materials. When the surface area of the materials increases, more protein can be adsorbed. Similarly, as heat treatment eliminates water molecules from the interlayers of the materials, larger space is obtained between the brucite sheets of the materials. This then allows more enzyme molecules to enter the interlayers.

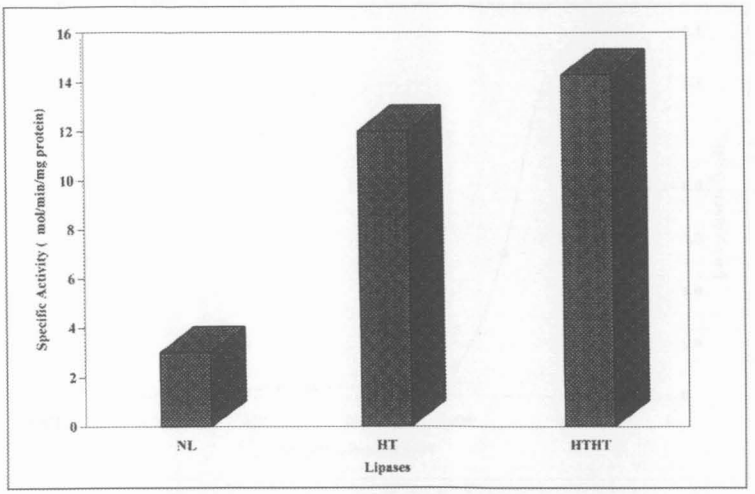

Fig. 4: Specific of immobilized (HT and HTHT) and Native (NL) lipases in the effect of heat treatment

Specific Activities of Immobilized Lipase and Native Lipase

From the plot in *Fig. 4*, HTHT seemed to have the higher specific activity compared to HT. Their specific activities are significantly higher than native lipase (NL). This shows that immobilization increases the specific activity of lipases and immobilization on the heat-treated hydrotalcite further increase lipase specific activity. In this study, the amount of the protein used was kept constant for all the enzymes. Thus, the higher activity in the immobilized lipases as compared to the native lipase may be due to the immobilized lipases being more active and stable in organic solvents. The higher activity in HTHT as compared in HT could be due to the large amount of enzymes being trapped in HTHT, with better porosity.

Effect of Temperature on the Esterification Activity

In *Fig. 5*, activities of the immobilized and native lipase were increased at temperatures between 30°C and 40°C. However, drastic decrease in activity occurred after 50°C especially for native lipase. Activities of lipase were low at 30°C which may be due to the diffusion limitation of reactant caused by increase in reactant viscosity at low temperature. The optimum temperature was at 40°C for both native and immobilized lipases. This shows that immobilization did not alter the general character of the lipase. Among these enzymes, lipase immobilized onto HTHT seemed to protect the enzyme best against denaturation by heat.

According to Fagain (1997), lipases suffer denaturation at higher temperatures. This is due to the reactivation of the peptide bonds and amino acid side chains, which causes them to participate in deleterious reactions at

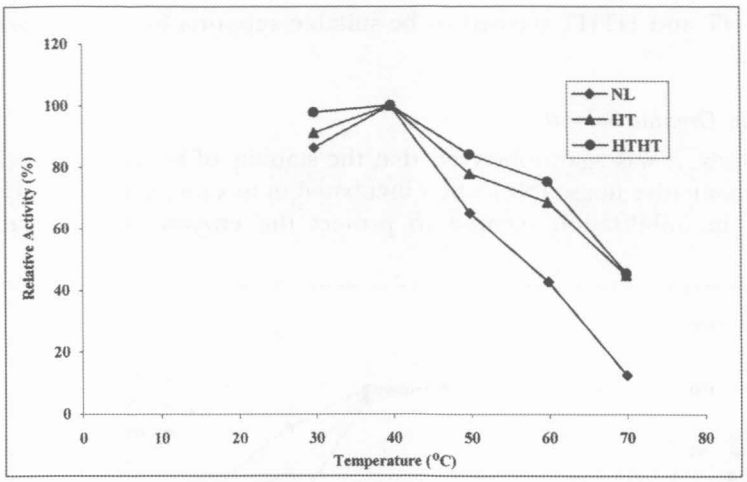

Fig. 5: Effect of temperature on esterification activity of native (NL) and immobilized (HT, HTHT) lipases

higher temperatures. This then causes the weak stabilizing interactions and a built-up conformational stability in an enzyme to be destroyed. In order to overcome this problem, lipase requires ample conformational mobility that it can achieve through immobilization or attachment on flexible solid support.

Thermal Stability

Stability of lipases immobilized onto HT and HTHT, is shown in *Fig. 6.* The derivatised lipase preparations were significantly more thermostable than native lipase even after 1 h of incubation at temperatures between 30°C to 70°C. Among these, lipase immobilized onto HTHT show highest thermal stability.

At temperatures above 30°C, enzyme resistance to adverse heat influences and persistency of its molecular integrity to face denaturants were weakened. This denaturation happens when a fully functional monomeric enzyme looses its biological activity in vitro by the unfolding of its tertiary structure to a disordered polypeptide. At this point, the key residues are no longer aligned closely enough for continued participation in functional or structure stabilizing interactions.

Immobilization had however increased thermal stability of lipase. Although heat considerably reduce conformational flexibility of native and immobilized lipase, immobilized lipase is still capable of performing its vibrational and more complex movement required for efficient catalytic activity.

Leaching Study

The immobilized lipase preparations retained their catalytic activities at 100% even after 5 washing cycles. This indicated that lipase from *C. rugosa* remained immobilized to HT and HTHT even after careful washing with 20 mL of

hexane. HT and HTHT seemed to be suitable supports for immobilization of enzymes.

Stability in Organic Solvent

In this study, it was again observed that the stability of immobilized lipase was higher than native lipase (NL) when incubated in hexane, at room temperature (*Fig. 7*). Immobilization seemed to protect the enzyme from denaturation

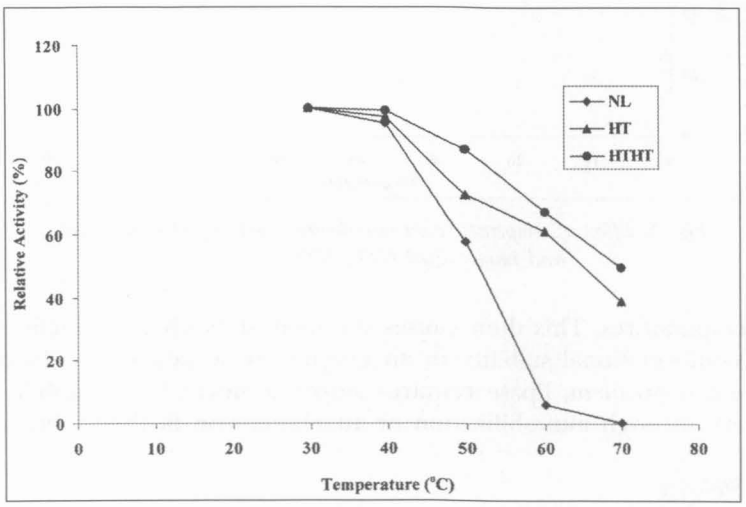

Fig. 6: Thermal stability of native (NL) and immobilized (HT, HTHT) lipases

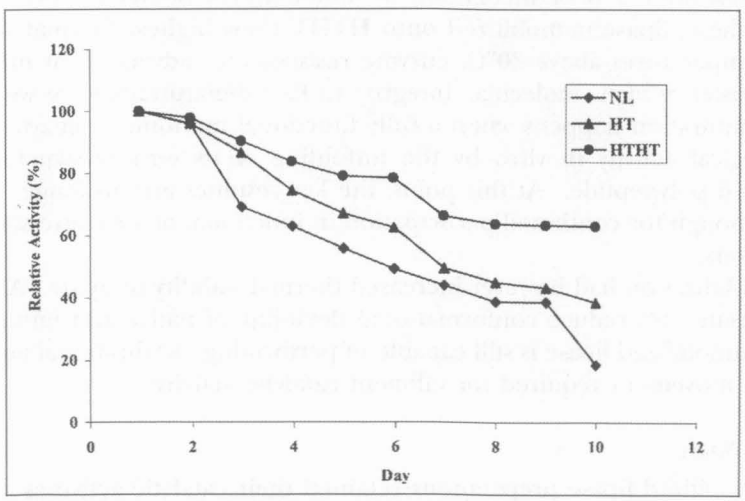

Fig. 7: Solvent stability of native (NL) and immobilized (HT, HTHT) lipases incubated in hexane at room temperature (26.5°C)

caused by the presence of organic solvent. Supports used may induce water molecules surrounding lipase, which was necessary for its catalytic activity from being stripped off by organic solvent (Basri *et al.* 1997).

Although water is needed to maintain the active conformation of enzymes, only a thin layer of it is needed. A large amount of water may result in the undesirable side reactions such as hydrolysis of acid anhydrides and halogenates. Furthermore, thermodynamic equilibrium of most processes concerning enzymes are unfavourable in water. Therefore, enzymes may be more stable in organic solvents than they are in water and this is a reason why they are used as reaction medium in catalytic activities (Klibanov 1986).

Storage Stability

The residual activities of various lipase preparations were determined after storing for 60 days under various conditions (Table 2). Native and immobilized lipases exhibited full catalytic activity after storing them at –20°C. Immobilized lipases retained their full catalytic activity when stored at 0°C. At very low temperatures, the lipase is probably locked in its native, catalytically active conformation. When stored at higher temperatures, immobilized lipases showed increase storage stability compared to native lipase. At these temperatures, the stabilization may be owing to multipoint attachment of the enzymes to the supports, creating a more rigid enzyme molecule. Hence, disruption of the active center becomes less likely to occur (Basri *et al.* 1994).

TABLE 2
Storage stability of native (NL) and immoblized (HT, HTHT) lipases after being incubated for 60 days at different storage conditions

	Residual Activity (%)			
Lipases	RT	4°C	0°C	-20°C
NL	26	36	70	100
HT	72	78	100	100
HTHT	75	82	100	100

- 100% is based on the initial synthetic activity of lipases using butanol and oleic acid.

CONCLUSION

The immobilization of lipase was successfully carried out, referring to the increase in activity and stabilities of the native lipase (NL) after immobilization. HTHT used seemed to meet the requirements of a suitable support and had satisfied a number of criteria as it allows easy lipase immobilization without having to loose its catalytic activity due to the large pore size and most importantly, it can be obtained through simple and inexpensive methods.

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