

Study of the Mechanism of Lipase-Catalyzed Methanolysis of Sunflower Oil in *tert*-Butanol and Heptane

[Lipaz-Katalizli Ayçiçek Yağının Metanoliz Reaksiyon Mekanizmasınının *tert*-Bütanol ve Heptanda İncelenmesi]

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ABSTRACT

Objectives: *tert*-Butanol has been reported to be a novel reaction medium for lipase-catalyzed biodiesel production due to its ability to eliminate negative effects of excess methanol, yet the molecular basis has remained to be solved.

Methods: In this study, we investigated the effect of *tert*-butanol on the mechanisms of biodiesel production catalyzed by two different immobilized lipases from *Candida antarctica* lipase B (Novozym 435) and from *Thermomyces lanuginosus* lipase (Lipozyme TL IM). For comparison, similar experiments were also carried out in heptane.

Results: In *tert*-butanol, the ping-pong bi-bi mechanism represents the kinetic data of both Novozym 435- and Lipozyme TL IM-catalyzed reactions and no methanol inhibition of lipase activities occurs. On the other hand, in heptane, the kinetic model of Novozym 435-catalyzed reaction agrees with the ping-pong bi-bi with alcohol inhibition mechanism. Whereas the kinetic model of Lipozyme TL IM-catalyzed reaction obeys ping-pong mechanism at low methanol (≤ 200 mM); it shows ping-pong mechanism with alcohol inhibition at high concentrations of methanol (≥ 300 mM).

Conclusion: These results demonstrate that *tert*-butanol alters the mechanisms of lipase-catalyzed reactions, presumably by causing conformational changes on the enzyme structure, which also eliminates inhibition by methanol.

Key Words: Biodiesel, immobilized-lipases, Novozym 435, Lipozyme TL IM, mechanism, *tert*-butanol, heptane, sunflower oil

ÖZET

Amaç: *tert*-Bütanol lipaz-katalizli biyodizel üretiminde, ortama eklenen fazla metanolün olumsuz etkilerini giderdiği için, yeni bir solvent sistemi olarak sunulmuştu; ancak enzim üzerinde moleküler düzeyde nasıl bir etki oluşturduğu henüz belirlenmemiştir.

Yöntem: Biz bu çalışmada, *tert*-bütanolün iki farklı immobilize lipaz sisteminin, *Candida antarctica* lipase (Novozym 435) ve *Thermomyces lanuginosus* lipase (Lipozyme TL IM), katalizlediği ayçiçek yağının metanoliz reaksiyon mekanizmalarına etkisini inceledik. Ayrıca, karşılaştırma amacıyla aynı deneyleri heptanda da gerçekleştirdik.

Bulgular: *tert*-Bütanolde, her iki lipazın kinetik dataları pin-pon çift-çift mekanizmasına uygunluk göstermekte ve metanolün inhibisyon etkisi görülmemektedir. Ancak, heptanda metanol Novozym 435'in aktivitesini inhibe etmekte ve reaksiyonun kinetik dataları alkol inhibisyonlu pin-pon çift-çift mekanizmasına uygunluk göstermektedir. Lipozyme TL IM aktivitesi ise yüksek metanol konsantrasyonlarında (≥ 300 mM) inhibe olmakta ve kinetik data düşük metanol konsantrasyonunda (≤ 200 mM) pin-pon çift-çift mekanizması, fakat yüksek metanol konsantrasyonlarında alkol inhibisyonlu pin-pon çift-çift mekanizmasına uygunluk göstermektedir.

Sonuç: Bu sonuçlar, *tert*-bütanolün enzimlerin yapılarına etki ederek mekanizmalarını değiştirdiği ve bundan dolayı da alkol inhibisyonunu önlediğini göstermektedir.

Anahtar Kelimeler: Biyodizel, immobilize-lipaz, Novozym 435, Lipozyme TL IM, mekanizma, *tert*-butanol, heptan, ayçiçek yağı

Introduction

Lipases (E.C.3.1.1.3) are triacylglycerol hydrolases that catalyze hydrolysis of lipids at a water/lipid interface. Because of some of its advantages over chemical catalysis such as producing no chemical waste and thus being environmentally friendly, use of lipases in variety of processes has been described (1, 2). Lipase-catalyzed biodiesel (fatty acid methyl esters, FAMES) production from vegetable oils by transesterification reaction with methanol or ethanol in organic solvents as well as in solvent-free media also has drawn a great attention. A number of immobilized-lipase systems such as Novozym 435 from *Candida antarctica*, Lipozyme TL IM from *Thermomyces lanuginosus*, Lipozyme RM IM from *Rhizomucor miehei* and Amano AK from *Pseudomonas fluorescens* have been used as model enzymes in biodiesel production from various vegetable oils (3-7).

Lipases have a similar molecular architecture consisting of a predominantly parallel central β -sheet and a catalytic triad of serine, histidine and a carboxylic acid, yet their active site structures can be quite different, which determine the substrate specificity and even the mechanism of the reaction (8-11). The ping-pong bi-bi mechanism with competitive inhibition by the alcohol is the most widely accepted model for lipase-catalyzed (trans)esterification reactions in non-aqueous media (12-14). Only a limited number of kinetic studies using triacylglycerols and methanol have been found in the literature (15-16). The kinetic model of Al-Zuhair is based on the classical ping-pong bi-bi mechanism with alcohol inhibition but also includes in an extra parameter taking into account the release of fatty acids first (15). The kinetic model of Pilarek *et al.* (16) considers ping-pong bi-bi mechanism with competitive inhibition by alcohol with the assumptions of an irreversible acyl bond cleavage in glycerides, a reversible monoglyceride isomerization and an irreversible enzyme deactivation.

Methanol is the choice of alcohol in biodiesel production as an acyl acceptor, yet at the same time it inactivates the lipases when used more than $\frac{1}{2}$ molar equivalents with respect to oil (4-5). Although the molecular basis of methanol inhibition has not been elucidated yet, it is assumed that methanol droplets forming in the oil at high concentrations deactivate the lipases by denaturing them (4-5). Nevertheless, it has been shown that Novozym 435 displays considerable activity after a long reaction period (24 h or more) even when four molar equivalent of methanol is used (17). The inhibitory effect of methanol on the activities of Novozym 435 and Lipozyme TL IM was eliminated by using *tert*-butanol as a reaction medium (18-19). Although it has been assumed that *tert*-butanol increases the solubility of methanol, which eliminates its inhibitory effect on lipases, the molecular basis has remained to be solved. Therefore, in this study, using oil and methanol as substrates, we investigated the mechanisms of biodiesel production catalyzed by Novozym 435 and Lipozyme TL IM, which have different active site structures, in *tert*-butanol as well as in heptane. Our results show that *tert*-butanol significantly affects the

mechanism of lipase-catalyzed reaction, presumably by causing conformational changes on the enzyme structure, which also eliminates inhibition by alcohol.

Materials and Methods

Materials

Novozym 435 and Lipozyme TL IM were generous gifts from Novo Nordisk (Bagsvaerd, Denmark). All solvent used were HPLC grade and from Merck (Darmstadt, Germany). Refined sunflower oil was purchased from a local store and the fatty acid composition was reported elsewhere (20).

Initial rate determinations in organic solvents

Batch reactions were carried out in 50 mL Erlenmeyer flasks containing sunflower oil (varied between 25 mM and 500 mM) and methanol (at specified concentrations) in a 5 mL total volume of heptane or *tert*-butanol. The reactions were initiated by adding the enzyme (10 mg TL IM or 50 mg Novo 435) last after equilibrating the reaction mixture at 40 °C for 30 min. The reactions were agitated in an orbital shaker (250 rpm) at 40 °C. At regular intervals of time (usually 5-10 min), 30 μ L samples were taken from the flask (three samples per reaction) and diluted with acetone to the final volume of 500-1000 μ L, and subjected to HPLC analysis as described below. The initial rates were determined under 10 % substrate consumption to assure steady-state conditions.

HPLC analyses

HPLC analyses of the reaction samples were performed as essentially described by Türkan and Kalay (20) using an Agilent HP1100 series (Agilent Tech., USA) liquid chromatograph with a UV-Vis diode array detector at 210 nm. Acquisition and processing of data was made using the HP Chemstation software provided with the instrument. The quantitative analysis of double-bond containing methyl ester products was also performed as described before (20). Mainly double bond-containing lipids can be measured quantitatively with UV detection at 210 nm. The sunflower oil used in this study was composed of 90 % double bond-containing fatty acids (linoleic and oleic) and 10 % saturated fatty acids (palmitic and stearic). Since saturated fatty acids do not have significant absorbance at 210 nm and the lipases are not specific for a particular fatty acid molecule as discussed by Türkan and Kalay (20), the total amount of methyl esters (biodiesel) can be calculated by adding 10 % to the sum of methyl esters of linoleic and oleic acids.

Results and Discussion

Steady-state kinetics studies in *tert*-butanol

It has been reported that the organic solvents can induce dramatic changes in enzyme activity, rate determining

step and specificity by simply changing the physicochemical properties of the reaction medium such as the polarity and hydrophobicity (21-22). As mentioned above, *tert*-butanol (2-methyl-2-propanol) has been reported to be a good solvent for biodiesel production due to its ability to eliminate negative effects of excess methanol on lipases (18-19). Hence, we wanted to study the mechanisms of lipase-catalyzed biodiesel production in *tert*-butanol. Figure 1A demonstrates the initial velocities of Novozym 435-catalyzed transesterification reaction as a function of oil concentration at various fixed concentrations of methanol (MeOH) in *tert*-butanol. There was no alcohol inhibition of Novozym 435 activity in *tert*-butanol even at MeOH concentrations as high as 500 mM. The double reciprocal plot (Figure 1B) depicts parallel lines agreeing with ping-pong bi-bi mechanism (23). Since methanol concentrations above 200 mM did not cause further changes in the initial activities (Figure 1A), only the data obtained at 50, 100 and 200 mM methanol are depicted in the double reciprocal plot (Figure 1B). Similarly, in *tert*-butanol methanol had no inhibitory effect

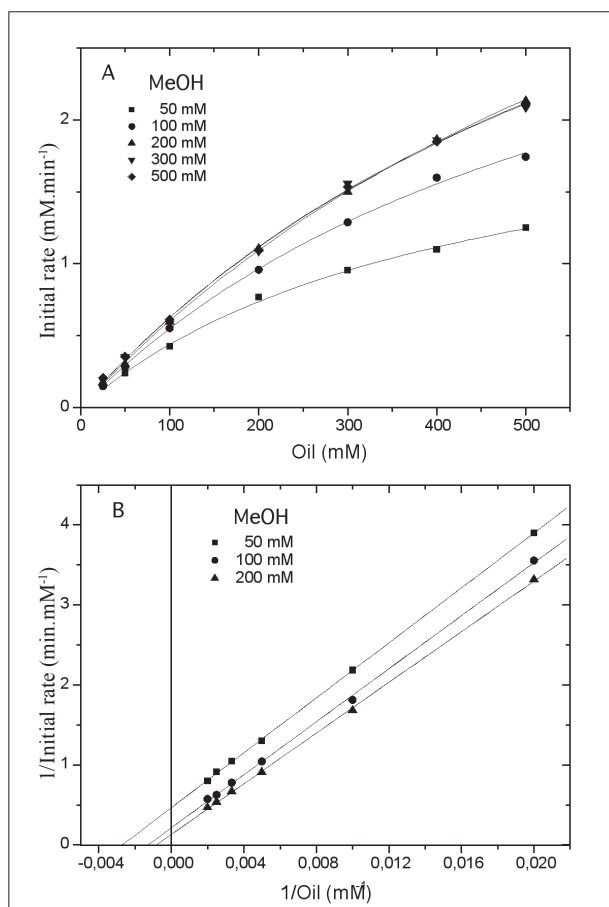


Figure 1. Effect of oil concentration on initial reaction rates of Novozym 435-catalyzed transesterification of sunflower oil at indicated fixed concentrations of methanol in *tert*-butanol (A) and the reciprocal ($1/v_i$ vs. $1/\text{oil}$) plot (B). The reactions were carried out in 5 mL *tert*-butanol containing sunflower oil (varied between 25 and 500 mM) and indicated fixed concentrations of MeOH with 50 mg of immobilized enzyme. The other conditions are as described in the Experimental Section.

on Lipozyme TL IM activity up to 500 mM (Figure 2A). The double reciprocal plot (Figure 2B) indicates two sets of parallel data: one at low MeOH (50 and 100 mM) and one at high MeOH (200 and 300 mM). Parallel slopes agree with ping-pong bi-bi mechanism (23). However, why parallel slopes differ at low and high methanol concentrations is not clear. It could be due to the fact that high concentrations of methanol change the polarity of the solution which may induce conformational changes on the enzyme structure.

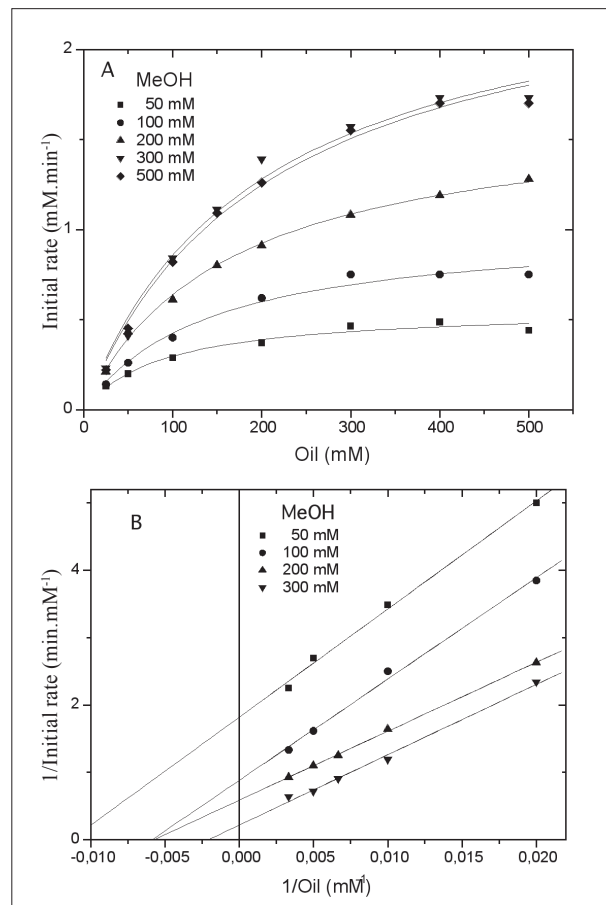


Figure 2. Effect of oil concentration on initial reaction rates of Lipozyme TL IM-catalyzed transesterification of sunflower oil at indicated fixed concentrations of methanol in *tert*-butanol (A) and the reciprocal ($1/v_i$ vs. $1/\text{oil}$) plot (B). The reactions were carried out in 5 mL *tert*-butanol containing sunflower oil (varied between 25 and 500 mM) and indicated fixed concentrations of MeOH with 10 mg of immobilized enzyme. The other conditions are as described in the Experimental Section.

Steady-state kinetics studies in heptane

The effect of the concentrations of both substrates on the rate of reaction was also investigated in heptane. Figure 3A illustrates the initial velocity of Novozym 435-catalyzed reaction as a function of oil concentration at different fixed concentrations of methanol. As can be seen from Figure 3A, the initial velocity of Novozym 435-catalyzed reaction was lower as the concentration of MeOH was increased up to 200 mM, but there was no further significant decrease in the initial activity as the MeOH concentration was further increased up to 500 mM. This

also can be seen in the double reciprocal plot (Figure 3B), which shows that as the fixed concentration of methanol increases, the slope increases indicating inhibition by methanol. The increase in the slope with increasing alcohol concentration is in agreement with an assumed ping-pong bi-bi mechanism with dead-end inhibition by one substrate as described by Segel (23). In the transesterification reactions between fatty acids and long chain alcohols, the competitive alcohol inhibition of Novozym 435 in organic solvents have been reported previously (21). In the structure of *Candida antarctica* lipase B (CALB), the active site is composed of one large pocket containing both the acyl- and the alcohol-binding regions (8). Using molecular modeling calculations, Foresti *et al.* (24) demonstrated that when alcohol binds at the active site of CALB first, it prevents binding of the acyl-donor (i.e., oleic acid). However, these molecular modeling calculations ignore solvent effect on the enzyme. It seems that this may be true in heptane but not in *tert*-butanol, in which no alcohol inhibition of lipase activities occurred.

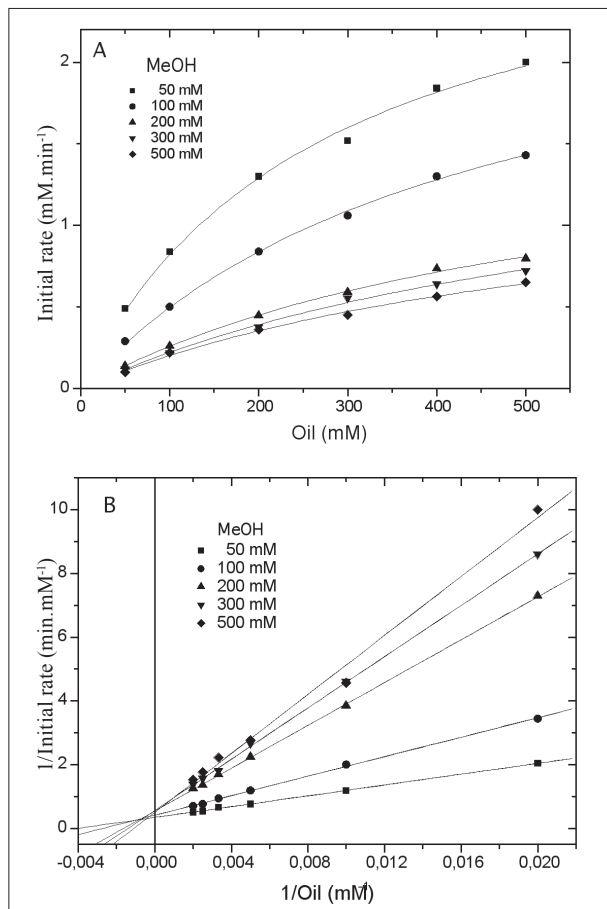


Figure 3. Effect of oil concentration on initial reaction rates of Novozym 435-catalyzed transesterification of sunflower oil at indicated fixed concentrations of methanol in heptane (A) and the reciprocal ($1/v_i$ vs. $1/\text{oil}$) plot (B). The reactions were carried out in 5 mL heptane containing sunflower oil (varied between 25 and 500 mM) and indicated fixed concentrations of methanol (MeOH) with 50 mg of immobilized enzyme. The other conditions are as described in the Experimental Section.

On the other hand, the initial velocity of TL IM-catalyzed reaction increased with both oil and MeOH concentrations when MeOH concentration increased up to 200 mM (Figure 4A); at higher MeOH concentrations (≥ 300 mM), alcohol inhibition occurred at low but not at high oil concentrations resulting a sigmoidal kinetic profile at 500 mM MeOH. From the double reciprocal plot (Figure 4B), a set of parallel lines was obtained when the methanol concentration was in the range of 50 and 100 mM. This first result agrees with ping-pong bi-bi mechanism. When the methanol concentration increased more than 200 mM, the slope increased. This second result agrees with ping-pong bi-bi mechanism with alcohol inhibition. The sigmoidal profile at 500 mM methanol suggests an allosteric regulation of the enzyme. Sigmoidal kinetic profiles are obtained for random bi-bi systems and some hybrid ping-pong mechanisms (23). The active site of *Thermomyces lanuginosus* lipase (TLL) is composed of two distinct pockets and buried under a helical lid and therefore not accessible to external solvent. Opening of the lid happens when the lipase binds a lipid surface (interfacial activa-

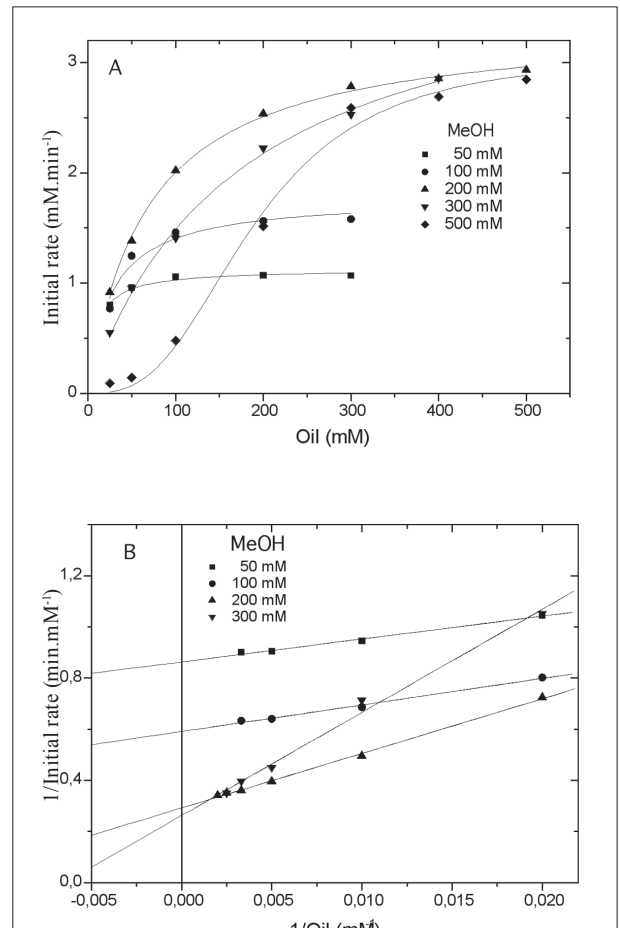


Figure 4. Effect of oil concentration on initial reaction rates of Lipozyme TL IM-catalyzed transesterification of sunflower oil at indicated fixed concentrations of methanol in heptane (A) and the reciprocal ($1/v_i$ vs. $1/\text{oil}$) plot (B). The reactions were carried out in 5 mL heptane containing sunflower oil (varied between 25 and 500 mM) and indicated fixed concentrations of MeOH with 10 mg of immobilized enzyme. The other conditions are as described in the Experimental Section.

tion) as well as in an organic solvent (9-10). It has been reported that 2-propanol induces the opening of the lid as well as the oligomerization of TLL (25). Lid opening, then, induces small changes in the active site structure influencing the enantioselectivity as shown by the work of Peters *et al.* (26) who demonstrated that substitution of the active site serine by alanine altered the dynamic properties of the lid covering the active site.

Conclusions

In conclusion, Novozym 435 and Lipozyme TL IM catalyze methanolysis of sunflower oil via different mechanisms in *tert*-butanol and heptane. *tert*-Butanol alters the mechanism of the lipase-catalyzed biodiesel production, presumably by causing a conformational change on the structures of lipases, which in turn eliminates inhibition of enzymatic activity by alcohol.

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