Caucasian Journal of Science
ISSN: 2148-6840
https://doi.org/10.48138/cjo.968723
Year: 2021
Volume: 8 Issue: 2

Investigation of The Activity of Lipase Variants on Different 4-Nitrophenyl Esters by Spectrophotometric Assay

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Makalenin Alanı: Biyoloji

Makale Bilgileri

Geliş Tarihi
09.07.2021

Kabul Tarihi
02.12.2021

Anahtar Kelimeler
Mikrobiyal enzim
Lipaz
Protein mühendisliği
Spektrofotometri
4-nitrofenol

Öz

Mikrobiyal enzimler biyoteknoloji alanında sıklıkla kullanılan önemli araçlardır. Hücre içi biyolojik reaksiyonlarda rol oynayan bu mikrobiyal enzimler çok farklı endüstrilerde kullanılmaktadır. Lipaz, proteazlar ve amilazlar hidrolitik enzimlerin önemli birer üyelerindendir. Hidrolitik enzimler arasında en yaygın kullanım alanına sahip olan lipaz enzimi, lipit molekülü arasındaki ester bağlarının hidrolizini katalizleyen bir enzimdir. Lipaz enziminin aktivitesi yaygın olarak spektrofotometrik yöntemle ölçülmektedir. Kinetik analizler için yaygın olarak p-nitrofenol esterleri tercih edilmektedir. Spektrofotometrik analiz yönteminde p-nitrofenol ester substratların lipaz enzimi tarafından hidroliz edilmesi sonucunda renkli ürün ölçüm yapılmaktadır. Bu çalışmada farklı lipaz varyantların aktivite ve kinetik parametrelerin ölçümü için p-nitrofenil asetat (Asetik asit 4-nitrofenil ester) , p-nitrofenil bütirat (Bütirik asit 4-nitrofenil ester), p-nitrofenil oktanoat (Oktanoik asit 4-nitrofenil ester, 4-Nitrofenil kaprilat), p-nitrofenil dodekanoat (Dodekanoik asit 4-nitrofenil ester), p-nitrofenil palmitat (p-Nitrofenil palmitat, Hekzanoik asit 4-nitrofenil ester) substratları kullanılmıştır. Farklı karbon uzunluklarına sahip substratların lipaz enzimi ile belli sürelerde inkübasyon sonucu oluşan ürünler spektrofotometrik olarak ölçülmüştür. Denemeler 25C'de 5dk-120dk aralığında gerçekleştirilmiştir. Dört tekrar yapılan deneyler sonucunda lipaz enzimin aktivitesinin substratların karbon zincirinin uzunluğuna göre değişim gösterdiği tespit edilmiştir. Lipaz enzimin p-nitrofenil palmitat üzerindeki aktivitesinin çok düşük olduğu belirlenmiştir. Yabanıl lipaz enzimin Vmax değerleri pNP-asetat, pNP-bütirat, pNP-oktanoat, pNP- dodekanoat, pNP-palmitat için sırasıyla 0.42 U/mg protein, 0.95 U/mg protein, 1.1 U/mg protein, 0.78 U/mg protein, 0.18 U/mg protein olarak hesaplanmıştır. Yabanıl lipaz enzimin 8 karbon zincir uzunluğunda olan pNP- oktanoat substratı üzerindeki aktivitesinin diğer substratlara göre daha yüksek olduğu belirlenmiştir.

Article Info

Received
09.07.2021

Accepted
02.12.2021

Keywords
Microbial enzyme
Lipase
Protein engineering
Spectrophotometry
4-nitrophenol

Abstract

Microbial enzymes are important tools that are frequently used in the field of biotechnology. These microbial enzymes, which play a role in intracellular biological reactions, are used in many different industries. Lipase, proteases and amylases are important members of hydrolytic enzymes. Lipase enzyme, which has the most common usage area among hydrolytic enzymes, is an enzyme that catalyzes the hydrolysis of ester bonds between lipid molecules. The activity of lipase enzyme is commonly measured by spectrophotometric method. P-nitrophenol esters are commonly preferred for kinetic analysis. In the spectrophotometric analysis method, the colored product is measured as a result of the hydrolysis of p-nitrophenol ester substrates by the lipase enzyme. In this study, p-nitrophenyl acetate (Acetic acid 4-nitrophenyl ester), p-nitrophenyl butyrate (Butyric acid 4-nitrophenyl ester), p-

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nitrophenyl octanoate (Octanoic acid 4-nitrophenyl ester, 4 -Nitrophenyl caprylate), p-nitrophenyl dodecanoate (Dodecanoic acid 4-nitrophenyl ester), p-nitrophenyl palmitate (p-Nitrophenyl palmitate, Hexadecanoic acid 4-nitrophenyl ester) substrates were used. The products formed as a result of incubation of substrates with different carbon lengths with lipase enzyme periods were measured spectrophotometrically. Trials were carried out at 25°C between 5min-120min. As a result of the experiments carried out in four repetitions, it was determined that the activity of the lipase enzyme varies according to the length of the carbon chain of the substrates. Vmax values of wild lipase enzyme were calculated as 0.42 U/mg protein, 0.95 U/mg protein, 1.1 U/mg protein, 0.78 U/mg protein, 0.18 U/mg protein for pNP-acetate, pNP-buritate, pNP-octanoate, pNP-dodecanoate, pNP-palmitate, respectively. It was determined that the activity of lipase enzyme on p-nitrophenyl palmitate was very low. It was determined that the activity of wild lipase enzyme on the eight-carbon chain pNP-octanoate substrate was higher than the other substrates.

INTRODUCTION

Enzymes are biocatalysts that regulate most of the metabolic reactions in living cells and ensure the maintenance of biological activities. Today, some enzymes are used in many industrial areas, especially in the food, textile, detergent, pharmaceutical and cosmetic industries. Proteases and lipases are the most commonly used enzymes commercially. One of the most widespread group of enzymes for industrial use are lipases (Dror et al., 2014). Generally, lipases hydrolyze oils and fatty acid esters (Sharma et al., 2001). Their biotechnological potential is enormous, due to their resistance to organic solutions, broad substrate specificity, regioselectivity, stereoselectivity, and lack of cofactors. (Jaeger & Reetz, 1998; Jaeger & Eggert, 2002). The ability of lipase enzyme to be resistant to high temperatures and to remain stable in alkaline environments has provided many advantages in bioprocesses (Hasan et al., 2006; Rua et al., 1997). Especially the small size of bacterial lipases provides ease of use in enzyme studies and their substrate specificity makes these enzymes valuable in biotechnological fields. It has many application areas in industry. This reveals the importance of the lipase enzyme.

For example, biodiesel production, biopolymer synthesis, paper manufacturing, yogurt and cheese making, washing powder production, biosensor manufacturing, cosmetics and perfume industry, leather industry, tea making, applications in medicine, detergent and textile industry, waste water treatment, food and pharmaceutical industry (Jaeger & Reetz, 1998; Schmid & Verger, 1998; Califano et al., 2015; Hasan et al., 2006; Kimtun et al., 2015; Maharana & Ray, 2015; Speranza et al., 2016). The most commonly used bacterial lipases are Novo Nordisk A/S (Bagsværd, Denmark), Novozyme (Bagsværd, Denmark), Amano (Nagoya, Japan), Sigma-Aldrich (St. Louis, United States), Boehringer Mannheim (Mannheim, USA). It is

produced and marketed by biotechnology companies such as Germany), Genencor (New York, United States), Asahi (Tokyo, Japan), and Biocatalysts (Wales, United Kingdom). Examples of the most widely used commercial lipases in biodiesel production are Novozym 435 (Deng et al., 2005), Lipopan 50BG (Verdugo et al., 2011) and Lipase AK (Soumanou & Bornscheuer, 2003) (Hwang et al., 2014). Among European countries, especially Denmark is leading in the field of lipase production and marketing. Since the use of lipase enzymes in various chemical and bioprocess industries is a billion dollar trade, there is great pressure to isolate new lipase enzymes with improved properties. There is a great need for a new generation of these enzymes with more advantageous features such as increased activity against various natural substrates, resistance to extreme temperature and pH conditions, and easier and cheaper production. For example, the availability of more effective lipases could enable the development of process technologies that produce biodiesel from vegetable oils.

Rapid developments in protein engineering techniques enable the production of lipase enzymes with desired properties (Hwang et al., 2014). Examples of commercial lipase variants whose properties have been improved by protein engineering methods are Lipolase Ultra and LipoPrime manufactured by Novo Nordisk A/S (Walsh, 2002). Protein engineering is a process that can improve substrate specificity and stability, as well as increase the catalytic function of enzymes. There are three main areas in protein engineering: rational design, quasi-rational design, and directed evolution. The rational design method, which requires detailed information about the three-dimensional structure and function of the enzyme, and using this information, the position to be mutated and which amino acid will be predicted by computer methods before proceeding to the experimental stage (Steiner & Schwab, 2012). In the directed evolution method, which requires no prior knowledge of the enzyme's three-dimensional structure and function, a large number of mutations are randomly added to the enzyme structure, and then a functional scan is performed for the desired feature (Bloom & Arnold, 2009; Goldsmith & Tawfik, 2012; Turner, 2009; Wang et al. ., 2012; Packer & Liu, 2015; Denard et al., 2015). The quasi-rational design method was created by combining rational design and directed evolution techniques (Bornscheuer & Kazlauskas, 2011; Bornscheuer & Pohl, 2001; Bornscheuer et al., 2012; Böttcher & Bornscheuer, 2010). With this method, the amino acid position to be mutated is predetermined and 19 other possible amino acids are placed in this position. In this way, the best amino acid required to be in a specific position for the desired trait can be discovered after functional screening.

In this study, kinetic analysis and characterization of new lipase enzymes, which will support the development of bioprocess industries with protein engineering methods, were carried out by partial purification. In this study, different substrares such as p-nitrophenyl acetate (Acetic acid 4-nitrophenyl ester), p-nitrophenyl butyrate (Butyric acid 4-nitrophenyl ester), p-nitrophenyl octanoate (Octanoic acid 4-nitrophenyl ester, 4 -Nitrophenyl caprylate), p-nitrophenyl dodecanoate (Dodecanoic acid 4-nitrophenyl ester), p-nitrophenyl palmitate (p-Nitrophenyl palmitate, Hexadecanoic acid 4-nitrophenyl ester) substrates were used to determine the enzyme activity.

Tables and Figures

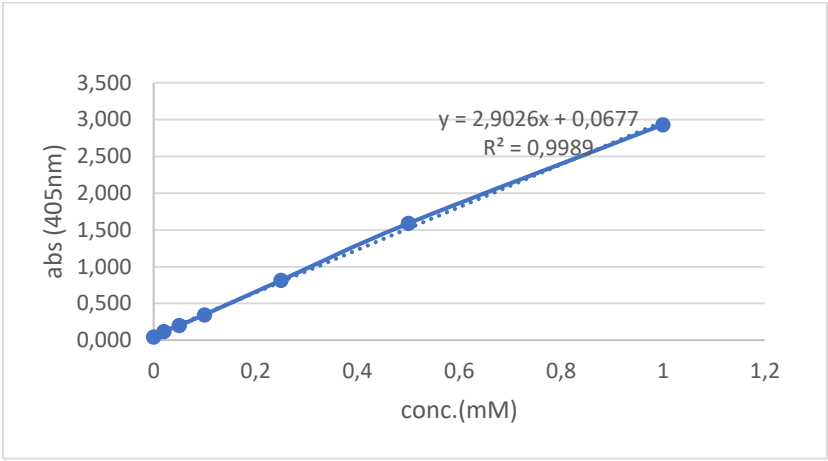

Figure1. P-nitrophenol (pNP) calibration curve

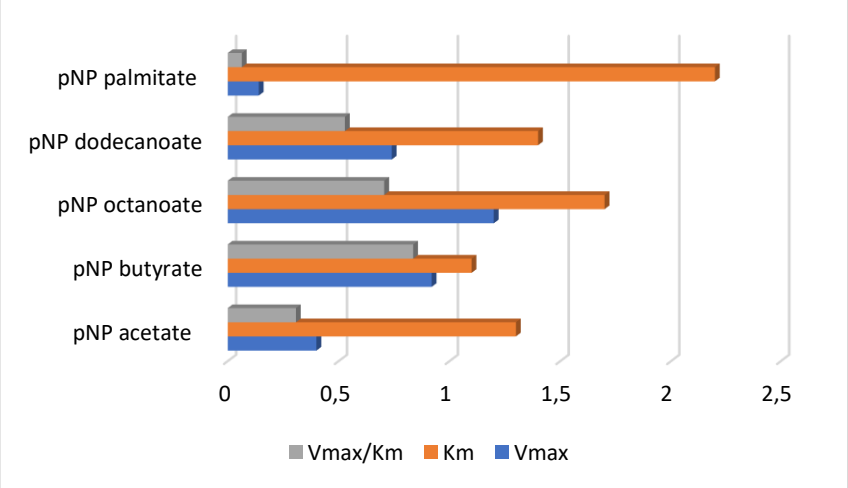

Figure2. Vmax, Km, Vmax/Km values of lipase enzyme on different 4-Nitrophenol esters

Table1. 280 nm absorbance values of three different variant and wild type samples and corresponding mprotein/ml value

	280nm	**mprotein/ml**
V1	3,81	4,953
V2	5,57	7,241
V3	2,96	3,848
Wt	3,01	3,913

Table2. Vmax, Km, Vmax/Km values of wild type and variants

	Vmax (U/mg total protein)	Km (mM)	Vmax/Km
Wt	0,92	1,1	0,83
V1	0,48	1,6	0,3
V2	1,09	1,2	0,9
V3	0,22	1,5	0,14

MATERIALS AND METHODS

Materials

Environmentally isolated lipase enzyme was developed by protein engineering method in our previous study and cloned into *E.coli* BL21 to use in this study. p-nitrophenol (pNP), p-nitrophenyl acetate (Acetic acid 4-nitrophenyl ester), p-nitrophenyl butyrate (Butyric acid 4-nitrophenyl ester), p-nitrophenyl octanoate (Octanoic acid 4-nitrophenyl ester, 4 -Nitrophenyl caprylate), p-nitrophenyl dodecanoate (Dodecanoic acid 4-nitrophenyl ester), p-nitrophenyl palmitate (p-Nitrophenyl palmitate, Hexadecanoic acid 4-nitrophenyl ester), acetonitrile, protein assay kit were obtained from Sigma Aldrich and Merck companies. Multiskan go microplate spectrophotometer and SkanIt Software 4.1 for Microplate Readers RE, ver. 4.1.0.43 from thermo scientific was used for spectrophotometric analysis

Enzyme purification

A single colony was selected from the culture incubated overnight at 37°C in LB medium containing ampicillin. BL21(DE3) cells containing *E.coli* wild-type plasmid and variants were grown in 50 mL of LB-ampicillin broth at 37 °C. Protein expression was induced by adding

isopropyl-β-D-thiogalactopyranoside (IPTG) to the medium when the OD600 value was 0.2-0.3. The induced cultures were incubated for an additional 18 hours at 25 °C. Grown culture centrifuged at 6500 rpm for 5 minutes in a cooled centrifuge and the supernatant was transferred to Amicon Ultra-15 tubes. It was concentrated by centrifugation at 4500 rpm for 20 minutes at 4 °C. The concentrated enzyme was washed with 25 mM NaPO4 buffer, pH 7.0. Protein concentration was measured using the Pierce Coomassie Protein Assay Kit (Thermo Scientific).

p-Nitrophenol calibration

Eight different concentrations (0mM, 0.02mM, 0.05mM, 0.1mM, 0.25mM, 0.5mM, 1mM, 2mM) p-nitrophenol (pNP) solutions were prepared and their absorbance at 405 nm was obtained. The standard curve graphic was drawn according to the concentration and absorbance value (Figure1).

Protein Assay

To determine the amount of protein, the absorbance at 280 nm of the eluates collected from chromatographic studies was investigated. The Bradford protein method was used to determine the amount of protein for both the high activity tubes collected from the column and the homogenate. Ready-to-use Bradford Reagent containing Coomassie Brilliant Blue G-250 was used as paint. To prepare Standard Protein solution, bovine serum albumin (BSA) solution at a concentration of 1 mg mL^{-1} was prepared. Then, six eppendorf tubes were taken and 0.0, 2.5, 5.0, 7.5, 10.0, 12.5µL of 1 mg mL^{-1} concentration of BSA solution were put into the tubes, respectively. Different volumes were taken for the homogenate and column samples and added to the new eppendorf tube. The volume of each tube content was made up to 100 µL with distilled water. Then, 1 mL of ready-to-use Bradford Reagent was added to all tubes and mixed with vortex. After waiting in the dark for 20 minutes, the absorbance values against the blank were read at 595 nm. Protein concentrations of 1/20 dilution wild type and variants samples are shown in Table 1.

Lipase activity measurement

Lipase activity using pNP substrates determined by spectrophotometric method by using Microplate Spectrophotometer. The amount of p-nitrophenol (pNP) released from pNP-

substrates were measured periodically at 405 nm at 25 °C. 20mM pNP substrate solutions were made by mixing with acetonitrile. 25mM NaPO4, pH 7.0 solution was used as buffer solution. Seven different concentration of substrate (0mM, 0.05mM, 0.25mM, 0.5mM, 1mM, 2mM, 4mM) were examined with 10 µL of the enzyme concentrate. The plate was designed to have a total volume of 200 µl. All experiments were repeated three times. 25mM NaPO4 buffer and acetonitrile mixture were used as blank. For kinetic analysis, measurements were taken at ten different time points. For kinetic analysis, five different substrate concentrations were studied as 0.05, 0.25, 0.5, 1, 2, 4mM. Ten different time points were used to calculate the most appropriate values. While performing the spectrophotometric analysis, measurements were made at the 1st, 5th, 10th, 15th, 30th, 45th, 60th, 90th, 120th and 150th minutes after the enzyme was added to the mixture at last.

Initial velocity (V_0) was calculated from time-pNP concentration graph. Km and Vmax values were determined with the help of Lineweaver-Burk plot drawn between velocity values and substrate concentration. Wild type and different variants were compared to obtain relative results. Standard deviation was also calculated.

RESULTS AND DISCUSSION

In this study, the absorbance of the pNP product formed as a result of the degradation of the pNPP ester by the effect of lipase was measured spectrophotometrically. Many studies have used the spectrophotometric method for the activity and kinetic analyzes of the lipase enzyme (Özarslaner&Albayrak, 2013; Pencreac'h& Baratti, 1996; Smeltzer et al., 1992; Mosmuller et al., 2009; Šibalić et al., 2020; Ciuffreda et al., 2009).

The activity of the same lipase enzyme on substrates of different carbon lengths was investigated. In addition, the effects of lipase variants with different activating properties on similar substrates were examined and a compared. Five different 4-Nitrophenol esters were used in this study. P-nitrophenyl acetate (Acetic acid 4-nitrophenyl ester), p-nitrophenyl butyrate (Butyric acid 4-nitrophenyl ester), p-nitrophenyl octanoate (Octanoic acid 4-nitrophenyl ester, 4 -Nitrophenyl caprylate), p-nitrophenyl dodecanoate (Dodecanoic acid 4-nitrophenyl ester), p-nitrophenyl palmitate (p-Nitrophenyl palmitate, Hexadecanoic acid 4-nitrophenyl ester) substrates were used. The products formed as a result of incubation of substrates with different carbon lengths with lipase enzyme periods were measured spectrophotometrically. Trials were carried out at 25°C. Figure 2 shows the Vmax, Km and

Vmax/Km values of lipase enzyme on different substrates. The Vmax value of the wild type lipase enzyme increased up to the eight-carbon (C8) substrate, p-NP octanoate, and decreased with the twelve-carbon (C12) pNP dodecanoate. The lowest Vmax value was obtained with the sixteen-carbon (C16) pNP palmitate substrate, while the highest Vmax value was obtained with the pNP octanoate substrate (Figure2). The catalytic efficiency values were also calculated on the Lineweaver-Burk plot for different substrates. Considering the Vmax/Km value, p-NP butyrate (C4) gives the highest result among the other substrates with a value of 0.83. The catalytic activity for the pNP palmitate substrate was measured at the lowest value of 0.063.

It has also been seen in similar studies that different substrates have effects on the catalytic activity of lipase enzyme. The activity of the lipase enzyme obtained from *Thermomyces lanuginosus* has been shown to be greatly affected by substrate hydrophobicity. In this study, the activity of substrates with different aliphatic chain lengths (p-nitrophenyl benzoate, p-nitrophenyl acetate, p-nitrophenyl butyrate, p-nitrophenyl hexanoate, p-nitrophenyl octanoate, p-nitrophenyl laurate and p-nitrophenyl palmitate) examined on lipase enzyme. It has been shown that the effect of the modified enzyme on short chain substrates is higher than on long chain substrates (Noro et al., 2020). The activity and catalytic efficiency of wild type and three different lipase variants (V1, V2, V3) were analyzed spectroscopically. Among the variants, the Vmax value of the V2 type lipase enzyme was found to be 1.3 times higher than that of the wild type. Since Km values are similar to wt, the catalytic efficiency Vmax/Km value increased 1.18 times for the V2 type. Both Vmax values of V1 and V3 variants were calculated as 0.48 and 0.22 respectively (Table2). Although the Vmax value of these variants is lower than the wild type, the Km values are also higher, so the Vmax/Km values are much lower than both wild type and V2 types. (Table2). Since the activities of wild type and different variants will be compared in this study, pNP butyrate was used as the substrate, which gave the best results compared to the results obtained from the previous study.

Enzymes have a great use in scientific studies. Metabolic enzyme can be used as marker to measure cellular stress (Tunçsoy et al., 2021). In addition, enzymes are widely used in many biotechnological fields. Lipases have a wide range of applications, especially in the food, pharmaceutical, textile, detergent, paper and cosmetic industries. Lipase enzymes are widely used in the processing of fats and oils in the food industry. In addition to the food industry, lipase enzymes are widely used in detergent formulations. Lipases are of great

importance to the detergent industry, which are very helpful in removing oil stains, oily food stains and greasy soiling from fabrics. Lipases obtained from different organisms can show activity at different temperatures. Alkaline yeast lipases can operate at lower temperatures than bacterial and mold lipases. Lipases, which are active in cold environments, are preferred because they are advantageous in both energy consumption and textile durability. Protein engineering methods are frequently used to enable lipase enzymes to show activity at low temperatures (Hasan et al., 2006). In order to increase the usage areas of enzymes in the industry, many studies are carried out such as increasing the catalytic activities of enzymes, changing the enantioselectivity, changing the substrate selectivity, increasing the thermal stability, changing the pH stability. For these purposes, mostly protein engineering methods are applied. Although the changes as a result of mutation on the enzyme are mostly unlikely to increase the activity, the protein engineering method is a good method for all of these purposes mentioned above (Rigoldi et al., 2018; Rotticci et al., 2001; Ruslan et al., 2012; Rubingh, 1997; Widersten, 2014; Mala et al., 2001; Patkar et al., 1998).

As a conclusion, with this study, the importance of the lipase enzyme in the industry has been emphasized once again. The activity of wild type and variant enzymes was analyzed spectrophotometrically and the results were compared with the variants. The catalytic activity of the V2 type, which is one of the lipase variants developed by protein engineering, was increased compared to the wild type. It is planned to carry out genetic studies on the basis of the results obtained from these studies. It is planned to determine the region of the enzyme where the mutation is located, and to examine the effects of amino acid changes on the three-dimensional structure of the protein.

Acknowledgement

This research project was supported by Altınbaş University Scientific Research Fund. Project Number: PB2020-SHMYO-4

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