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A THESIS FOR THE DEGREE OF MASTER OF SCIENCE

**Effect of intense pulsed light on the deactivation of
lipase: enzyme-deactivation kinetics and tertiary
structural changes by fragmentation**

광펄스가 라이페이스 활성 감소에 미치는 영향:

효소-불활성화 동역학적 해석 및 단편화에 의한 3 차구조 변화

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Department of Agricultural Biotechnology

Seoul National University

Jeon, Min Sik

석사학위논문

**Effect of intense pulsed light on the deactivation of
lipase: enzyme-deactivation kinetics and tertiary
structural changes by fragmentation**

지도교수 장 판 식

이 논문을 석사학위 논문으로 제출함

2015 년 8 월

서울대학교 대학원 농생명공학부

전 민 식

전민식의 석사 학위논문을 인준함

2015 년 8 월

위원장 최 상 호 (인)

부위원장 장 판 식 (인)

위원 강 동 현 (인)

Abstract

Intense pulsed light (IPL), a novel non-thermal technology, was applied to investigate the effect on activity and structure of *Chromobacterium viscosum* lipase (triacylglycerol hydrolase, EC 3.1.1.3) by employing hydrolysis of triacylglycerol in reverse micelle system. After IPL treatment, the lipase deactivation was observed, and the lipase activity was decreased with increase of pulse fluence and exposure time. The half-life of *Chromobacterium viscosum* lipase at 8.79 mJ/cm² (37.63 min) was 4.76-fold longer than that at 14.86 mJ/cm² (7.90 min). In order to determine deactivation constants (k_1 and k_2), two-step series type deactivation kinetic model was employed. As a result, k_1 (9.05 h⁻¹) and k_2 (1.64 h⁻¹) at 14.86 mJ/cm² were 3.04, 3.88-fold higher than those at 8.79 mJ/cm² (2.98 h⁻¹, 0.42 h⁻¹), respectively. Meanwhile, pulse fluence showed a dominant impact on deactivation, in that higher pulse fluence treatment with short exposure time showed more decreased activity at the same degree of total fluence. Also, no activity reduction phenomenon appeared below 2.66 mJ/cm², demonstrating that the IPL treatment to the lipase was composed of susceptible phase and resistant phase. Based on the experiments in lipase hydrolysis, structural analysis was conducted in order to elucidate the cause of deactivation. From secondary structure analysis using

circular dichroism spectroscopy, the result spectra were not fluctuated significantly. However, changes in its tertiary structure were detected through fluorescence spectroscopy. SDS-PAGE results showed that the amount of intact lipase (32 kDa) was reduced in a time dependent manner and uncertain fragmented peptides were increased below the main protein band. For validation of fragmentation sites in the sequence of lipase, the peptides were analyzed by MALDI-TOF analysis. It was revealed that the lipase was site-selectively fragmented (<5 kDa) by IPL treatment. Furthermore, evidences for peptide bond cleavage was identified using Findpept[®] tool. From these findings, it is evident that the lipase deactivation was occurred quantitatively in molecular level.

***Keywords:* *Chromobacterium viscosum* lipase; intense pulsed light; deactivation kinetics; site-selective fragmentation; peptide bond cleavage**

***Student number:* 2013-23277**

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1. Introduction

Recently, increasing food-borne illness is being major concern for the food industry because food-borne outbreaks are frequent nowadays. Several technologies have significant impact to food products, including heating, freezing, dehydration, and the use of preservatives. However, these methods can increase side effects, such as degradation of certain components which can impair food quality (Elmnasser, Guillou, Leroi, Orange, Bakhrouf, & Federighi, 2007). To prevent undesirable thermal effects on foods, major efforts have been made to develop non-thermal technologies, which can produce safe but minimally processed foods with satisfactory nutritional and organoleptic qualities (Barbosa-Canovas, Gongora-Nieto, & Swanson, 1998). Thus non-thermal technologies enhance the food safety while maintaining their nutrient content and appearance as well as lengthening their shelf life (Cheigh, Park, Chung, Shin, & Park, 2012).

Intense pulsed light (IPL) is a technique performed to decontaminate food surfaces by killing micro-organisms, and employs a flash lamp filled with inert gas, such as xenon, which emits high-frequency pulses of broad spectrum radiation containing wavelengths from 180 nm (UV) to 1100 nm (near infrared) region (Cheigh, et al., 2012; Elmnasser, et al., 2008). IPL technique has been

suggested to decontaminate the surface of shell eggs, fresh-cut vegetables, and ready-to-eat meat products as well as to kill microorganisms in milk, fruit juices, and infant food (Marquenie, Michiels, Van Impe, Schrevens, & Nicolai, 2003; Hierro, Manzano, Ordonez, de la Hoz, & Fernandez, 2009; Choi, Cheigh, Jeong, Shin, & Chung, 2010; Caminiti, et al., 2011; Hierro, Barroso, de la Hoz, Ordonez, Manzano, & Fernandez, 2011; Pataro, Munoz, Palgan, Noci, Ferrari, & Lyng, 2011; Ramos-Villarroel, Aron-Maftei, Martin-Belloso, & Soliva-Fortuny, 2012).

Enzymes are the major targets for photo-induced modifications due to the abundance of internal chromophores within their structure. Broad radiation spectrum of IPL may affect enzymes, because proteins have strong UV absorption at 280 nm due to aromatic amino acids (Moriyama, Ohta, Hachiya, Mitsui, & Takeda, 1996; Hollosy, 2002). And peptide bonds in proteins exhibit a strong and weak absorption in the Far-UV region at of 190 nm (Aitken & Learmonth, 1996) and 210 to 220 nm (Davies & Truscott, 2001), respectively (Janve, Yang, Marshall, Reyes-De-Corcuera, & Rababah, 2014). Typically, proteins absorb the light in the range of 250 to 300 nm, originated from tryptophan and tyrosine residues while phenylalanine absorbs weakly below 275 nm and disulfide bond has a broad absorbance in the near-UV region

(Wetlaufer, 1958; Mach, Middaugh, & Lewis, 1992). Both amino acid side-chains and bound prosthetic groups (for example, flavins and heme) may act as efficient chromophores (Manzocco, Panozzo, & Nicoli, 2013). Enzymes have additional ability to bind exogenous chromophores, and quickly react with other excited state species (Davies, et al., 2001). The result would thus be a modification in the protein properties because of side-chain oxidation, backbone fragmentation, and/or formation of cross-links and aggregates (Davies, 2003). It has been reported that IPL treatment reduced the amount of intact proteins, especially in case of an enzyme, decrease in activity was observed compared to untreated one (Manzocco, et al., 2013; Janve, et al., 2014). On the other hand, some inconsistent reports are found in literature. Oms-Oliu et al. (Oms-Oliu, Aguiló-Aguayo, Martín-Belloso, & Soliva-Fortuny, 2010) showed that pulsed light stimulated an increase in polyphenol oxidase (PPO) activity in fresh-cut mushrooms. And Charles et al. (Charles, Vidal, Olive, Filgueiras, & Sallanon, 2013) demonstrated the increase in PPO activity in fresh-cut mango submitted to IPL treatment.

Lipase (triacylglycerol hydrolase, EC 3.1.1.3) is one of the most important enzymes in food industry. It has been widely applied to the industrial bioconversions such as synthesizing emulsifiers and functional esters through

hydrolysis, glycerolysis, and esterification (Houde, Kademi, & Leblanc, 2004; Park, Lee, Sung, Lee, & Chang, 2011). However, the lipase is known to cause hydrolytic rancidification by lipolysis in foods such as milk, rice bran, oil products, and etc. (Ramezanzadeh, Rao, Windhauser, Prinyawiwatkul, Tulley, & Marshall, 1999; Deeth, 2006; Ngando-Ebongue, Dhouib, Carrière, Amvam Zollo, & Arondel, 2006). General treatment for deactivating lipase in foods is heat treatment, which can induce unwanted results. Therefore, IPL system could be applied to reduce the activity for food preservation without serious damages.

Although there are reports about IPL deactivation of enzymes, it is desired to confirm the possibility of quantitative analysis and visualized expression of IPL induced lipase fragmentation. Thus, the objective of this research was to investigate the effect of IPL on lipase activity and express the deactivation phenomenon quantitatively using kinetics model. By this numerical interpretations, a possibility of practical application of IPL was evaluated. After that, the reason for lipase deactivation was assessed by evaluating secondary and tertiary structure changes with various techniques.

2. Materials & Methods

2.1. Intense pulsed light (IPL) treatment system

2.1.1. IPL apparatus

The IPL treatment system used to generate pulsed light was a slight modification of Choi's method (Choi, et al., 2010). The instruments were composed of a power supply, control, chamber, and lamp (Fig.1). The treatment chamber was made of wood and acrylic. The lamp was a xenon flash lamp (Heraeus Noblelight XAP series, type NL 4006, Cambridge, UK) comprised of a quartz tube. The size of the cylindrical lamp was 145×7.14 mm [(length) × (outer diameter)], and its emission wavelength spectrum ranged from UV to infrared. The light source was placed at the top of the chamber lamp, and the chamber had a cooling fan to remove the heat generated from the lamp during IPL treatment. Moreover, a light-blocking black quartz sheet with a small hole in the center was placed under the xenon flash lamp in order to prevent any heat associated with the light generation and to control the amount of light extend to the sample.

Fig. 1. Schematic representation of IPL instrument.

2.1.2. Measurement of IPL pulse fluence

Pulse fluence of IPL was controlled in voltage at the controller, and the input energy was applied by 200 V interval. The instrument was set at the distance of 9 cm from the light source, and the measurements were conducted at least 15 times for each pulse fluence. As the pulse fluence was controlled in voltage and converted to pulse fluence by integration, it was not expressed in an integral number.

The measurement of IPL pulse fluence was conducted using the spectroradiometer (ILT-900, InternationalLight Technologies, Peabody, MA, USA). The IPL pulse fluence was displayed on a spectrum through the software (SpectrilightTM, InternationalLight Technologies). The pulse fluence of the flash lamp in chamber was measured at different input voltages of IPL treatment system (Fig. 2). Table 1 shows total energy density of IPL from 300 nm to 1,100 nm. The values in Table 1 were pulse fluence which increased along with the input voltage.

Fig. 2. Energy distribution spectrum of IPL system at 3 Hz and 0.2 ms.

Table 1. Pulse fluence of IPL system from 300 nm to 1,100 nm

Voltage (V)	Pulse fluence (mJ/cm²)
500	4.64
600	5.03
800	6.00
1,000	6.98
1,200	8.97
1,400	11.89
1,600	13.87
1,800	14.86

2.1.3. IPL treatment on lipase

Purified *Chromobacterium viscosum* lipase was purchased from Millipore (Billerica, MA, USA) and reported to have catalytic activity of 3,400 units/mg solid. The lipase solution of pre-determined concentration in 50 mM Tris-HCl buffer (pH 8.0) was loaded in a transparent vial and set in a center of water jacket connected to water circulator in the constant temperature condition (4 °C) at the distance of 9 cm. The water jacket was placed under the xenon flash lamp in the IPL chamber. The energy density was controlled depending on the experiment and 0.2 ms pulses of IPL treatment were emitted at a frequency of 3 Hz.

2.2. Lipase assay in reverse micelle system

2.2.1. Chemicals and reagents

Bis (2-ethylhexyl) sulfosuccinate sodium salt (AOT) was purchased from Sigma-Aldrich (St. Louis, MO, USA) to be used as a surfactant for the formation of reverse micelles, and purified according to the method of Tamamushi and Watanabe (Tamamushi & Watanabe, 1980). Isooctane was purchased from Daejung Chemicals (Siheung-si, Gyeonggi-do, Korea) in

spectrophotometric grade and dehydrated over a 4 Å molecular sieve (Sigma-Aldrich) with filtration through a 0.5 µm membrane filter (J050A047A, Advantec, Tokyo, Japan), prior to use as a reaction medium in the reversed micellar system. The highly refined and low acidity olive oil (Sigma-Aldrich) was used as substrates in lipase-catalyzed hydrolysis. Cupric acetate reagent was prepared according to the method of Lowry and Tinsley (Lowry & Tinsley, 1976). The cupric acetate solution was prepared by dissolving 5 g of cupric acetate (98%, Sigma-Aldrich) in 100.0 mL of distilled water. The prepared cupric acetate solution was then filtered through Whatman No. 1 filter paper, and the pH was adjusted to 6.1 using pyridine (Showa Chemical Industry Co., Ltd, Tokyo, Japan). All of other chemicals were of extra pure grade and used without further purification.

2.2.2. Preparation of reverse micelles

Reverse micelles containing the lipase were formed by adding lipase solution to a solution with 100 mM of AOT in isooctane followed by vigorous mixing for 1 min to produce a transparent micellar solution. The desired water content was defined as the *R*-value, which means the molar ratio of water to surfactant ($[H_2O]/[AOT]$) (Banerjee, Ghosh, & Datta, 2011; Kwon, Park, Choi,

& Chang, 2015). In this study, experiments were conducted with the most optimized condition using the *R*-value at 10.0 (Prazeres, Garcia, & Cabral, 1992).

2.2.3. Determination of lipase activity

Hydrolysis activity was determined according to copper-soap colorimetric method (Hong, et al., 2015). A screw-cap vial (10.0 mL) was filled with AOT/isooctane reversed micellar solution to a 4.5 mL that was pre-incubated in a water bath at 37°C for 10 min with magnetic stirring at 800 rpm. Lipase-catalyzed hydrolysis was initiated by adding 0.5 mL of olive oil as a substrate to the reversed micellar solution. Each aliquot (200 µL) of reactant was collected at predetermined intervals and diluted with 2.3 mL of isooctane. Subsequently, 0.5 mL of cupric acetate reagent and 1.0 mL of acetonitrile (used to eliminate turbidity caused by AOT) were added and mixed vigorously using a vortex mixer for 1 min. The absorbance of the upper layer (colorized isooctane layer) was measured at 715 nm to quantitatively determine the free fatty acids produced (Prazeres, Garcia, & Cabral, 1993) using UV-vis spectrophotometer (UV-2450, Shimadzu Co. Ltd., Japan).

One unit of enzyme was defined as the amount of lipase that hydrolyzed with 1 μmol of fatty acids/min under the assay condition. And the calibration curve was obtained by calculating the regression equation of the molar concentration of oleic acid and the absorbance at 715 nm.

2.3. Analysis of IPL-treated lipase activity

2.3.1. Deactivation of lipase

The lipase deactivation was conducted at different pulse fluence of IPL and different exposure time. Samples were illuminated from 1,800 V to 1,200 V (14.86, 13.87, 11.89, and 8.79 mJ/cm^2 , respectively) at predetermined time intervals. The lipase residual activity was assessed by hydrolytic activity of IPL-treated lipase to that of untreated lipase.

2.3.2. Two-step series type deactivation kinetics

The data for deactivation by the IPL treatment (14.86, 13.87, 11.89, and 8.79 mJ/cm^2 , respectively) on *Chromobacterium viscosum* lipase were fitted to a two-step series-type deactivation kinetic model (Henley & Sadana, 1985; Knezevic, Siler-Marinkovic, & Mojovic, 1998; Longo & Combes, 1999;

Moquin & Temelli, 2008), and the kinetic parameters were determined using a non-linear regression procedure based on the Marquardt-Levenberg method of iterative convergence included in a solver tool of Microsoft Office Excel 2013 software (Valerio, et al., 2009).

2.3.3. Critical point for deactivation

IPL flash lamp was set to low pulse fluence state (minimally 2.58 mJ/cm²) for the assessment of a critical point. IPL treatment on *Chromobacterium viscosum* lipase was performed with lowering the IPL pulse fluence. Lipase assay was conducted as described above. The activity change of $\pm 10\%$ was considered to be no effect of IPL on the lipase.

2.4. Structural analysis of IPL-treated lipase

2.4.1. Circular dichroism (CD)

Measurements were performed on homogeneous *Chromobacterium viscosum* lipase at a concentration of 1.33 mg/mL (10,000 units/mL). CD spectroscopy (ChirascanTM-plus, Applied Photophysics, Ltd., Leatherhead, Surrey, UK) was performed using 10, 20, and 30 min IPL-treated (14.86

mJ/cm²) *Chromobacterium viscosum* lipase in the Far-UV regions (190-260 nm), path length (0.5 mm), and bandwidth (1.0 mm), and the temperature was set to 37°C. The structural changes of lipase were analyzed from the CD spectra using the CDNN secondary structure analysis software.

2.4.2. Fluorescence spectroscopy

The structural change phenomenon of *Chromobacterium viscosum* lipase tertiary structure was analyzed by observing aromatic amino acids using a fluorescence spectrophotometer (Spectra004Dax M2^e, Molecular Devices Corp., CA, USA) (Elmnasser, et al., 2008). After IPL treatment of the lipase, the emission spectra of the substance excited at 280 nm was measured. The emission slit was 5 nm and 1 nm for conformational change of protein and λ_{max} shift analysis, respectively. The treatment conditions were 10, 20, and 30 min at 14.86 mJ/cm² and 150, 180, and 240 min at 2.66 mJ/cm².

2.5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

analysis was conducted with Laemmli method using 12% polyacrylamide (Laemmli, Beguin, & Gujer-Kellenberger, 1970) to run *Chromobacterium viscosum* lipase after IPL treatment at 14.86 mJ/cm². The IPL-treated lipase was diluted in sample buffer containing SDS and 2-mercaptoethanol. The diluted sample was boiled for 3 min before loading onto the gels. Each sample was loaded in a well along with the marker proteins. Electrophoresis was performed at constant current of 20 mA per gel for 110 min at 25°C and Hoefer SE 250 mini-gel system (Hoefer Inc., Holliston, MA, USA). For protein staining, Coomassie Brilliant Blue R-250 was used. Gels were destained overnight using destaining solution composed of 6:3:1(v/v/v) ratio of water:methanol:acetic acid. Also, densitometry analysis was performed using ImageJ (NIH, Bethesda, Md., USA) software.

2.6. Matrix assisted laser desorption/ionization-time of flight-mass spectrometry (MALDI-TOF)

The fragments of the lipase were analyzed by matrix-assisted laser desorption/ionization time-of-flight-mass spectrometry (MALDI-TOF). The MALDI-TOF analysis was performed at the Korea Basic Science Institute (Seoul, Korea), using MALDI-TOF/TOF 5,800 of AB Sciex (Foster city, CA,

USA) in reflector mode and positive polarity. The matrix employed was a solution containing 5.0 mg of α -cyano-4-hydroxy-cinnamic acid (CHCA, Sigma-Aldrich) and 0.1% trifluoroacetic acid (v/v) (TFA, Sigma-Aldrich) in 1.0 mL of acetonitrile/water (1:1, v/v). Before plating the samples on MALDI plate, they were desalted and concentrated using ZipTip (Millipore). ZipTip was washed by 100% and 50% acetonitrile each and then equilibrated using 0.1% TFA. The samples (30 μ L) were loaded onto the ZipTip and washed again using 0.1% TFA. Then the samples were mixed with prepared matrix (1:1) and deposited on a MALDI plate, where 1.0 μ L sample solution was loaded and dried. The analysis range was 800-4,000 Da. Prior to operation, the instrument was calibrated using a mixture of standard peptides (Mass Standards Kit for Calibration, AB SCIEX TOF/TOFTM instruments) with a maximum error tolerance of 50 ppm. The analyses data were processed using Data Explorer software (AB Sciex).

2.7. FindPept[®] tool

For further analysis, fragment mass data from MALDI-TOF were analyzed using FindPept[®] tool (ExPASy, Swiss Institute of Bioinformatics, <http://web.expasy.org/findpept>). The tool was set to cysteine in reduced form,

[M+H]⁺ for peptide masses, ± 50 ppm for error tolerance, and monoisotopic mode. Then, the sequence of *Chromobacterium viscosum* lipase (The European Bioinformatics Institute, <http://www.ebi.ac.uk>) was compared with the data from MALDI-TOF analysis.

3. Results & Discussion

3.1. Lipase deactivation by IPL treatment

3.1.1. Deactivation phenomenon of lipase

Figure 3 shows the deactivation profile of *Chomobacterium viscosum* lipase after IPL treatment (from 8.79 mJ/cm² to 14.86 mJ/cm²) at 4°C. Deactivation of the lipase was achieved in proportion to exposure time and pulse fluence of IPL. For instance, residual activity of 14.86 mJ/cm² treated lipase was decreased most quickly among IPL-treated samples, at the same exposure time. By contrast, the lipase solution treated at 8.79 mJ/cm² showed a most progressive activity decrease in the entire exposure time range among the samples. Consequently, the longer time treated lipase showed lower residual activity compared to shortly treated one. It was observed that the half-life of IPL-treated *Chromobacterium viscosum* lipase in 50 mM Tris-HCl buffer (pH 8.0) is 7.90, 12.65, 20.84, and 37.63 min at 14.86, 13.87, 11.89, and 8.79 mJ/cm², respectively. When taking the reciprocal plot, the deactivation data gave a good correlation ($R^2 \geq 0.976$) in linear plotting. This means that residual activity of the lipase according to the IPL processing time could be obtained with simple calculations.

Fig. 3. Deactivation profile of *Chromobacterium viscosum* lipase after IPL treatment against exposure time.

Fig. 4. Reciprocal plotting of the *Chromobacterium viscosum* lipase deactivation profile.

3.1.2. Deactivation rate constant determination

To quantitatively assess the deactivation phenomenon of the lipase by IPL treatment, a mathematical technique of statistics was applied. In many previous research, deactivation of lipase was known to follow first-order kinetics (Tajchakavit & Ramaswamy, 1997; Soliva-Fortuny, Bendicho-Porta, & Martín-Belloso, 2006; Xu, et al., 2015), however, that was not proper to explain deactivation of the enzyme in some publications (Park, et al., 2013; Hong, et al., 2015). These reports indicated that the kinetic behavior of enzyme deactivation could be related to many factors, such as enzyme structure, energy source for deactivation, and etc. In the case of IPL-treated lipase deactivation profile, single-step first order kinetics was not appropriate, indicating the fractional rate of deactivation was different because of more than one type of bond that must be broken before a total loss of activity (Henley, et al., 1985). Therefore, a two-step series-type enzyme deactivation model was employed for analysis of enzyme deactivation kinetics:

where k_1 and k_2 are first-order deactivation rate coefficients (min^{-1}) for each step, E , E_1 and E_2 are homogeneous states with several specific activities

(initial, intermediate, and final state, respectively), and α_1 and α_2 are the ratios of specific activities (E_1/E and E_2/E , respectively). This kinetic model of enzyme deactivation was reported in literature (Henley, et al., 1985), and is expressed in Eq. 2 assuming that α_2 is equal to 0, which means that the final lipase activity (α_2) is totally deactivated by IPL treatment.

$$a = \left(1 + \frac{\alpha_1 k_1}{k_2 - k_1}\right) e^{-k_1 t} - \left(\frac{\alpha_1 k_1}{k_2 - k_1}\right) e^{-k_2 t} \quad [2]$$

In this equation, fractional remaining activity (a in Eq. 2) can be derived as a function of time. The parameters (k_1 , k_2 , and α_1) were calculated from the experimental data and the values are shown in Table 2.

In this case, initial enzyme state is more active than that of the intermediate ($\alpha_1 < 1$), and the enzyme was totally deactivated at the final state ($\alpha_2 = 0$). This biphasic behavior has been extensively discussed in various publications and may be exhibited by both soluble and immobilized enzymes. The convexity (toward the origin) exhibited by these curves is a function of the k_1 , k_2 , and α_1 values. Relatively higher values together with moderate values of k_2 and α_1 yield a higher degree of convexity. A higher degree of convexity implies more sensitivity to the rate of enzyme deactivation (Park, et al., 2013).

As shown in the Table 2, k_1 was greatly larger than k_2 . For instance, k_1 (9.05

h^{-1}) and k_2 (1.64 h^{-1}) at 14.86 mJ/cm^2 were 3.04, 3.88-fold higher than those at 8.79 mJ/cm^2 (2.98 h^{-1} and 0.42 h^{-1}), respectively. From the results in Table 2, it can be concluded that the lipase activity decreases more rapidly in both phases (from E to E_1 and from E_1 to E_2) at higher treatment pulse fluence.

Table 2. Deactivation kinetic parameters of the lipase after IPL treatment

mJ/cm²	α_1	k_1 (h⁻¹)	k_2 (h⁻¹)
14.86	0.2878	9.0535	1.6424
13.87	0.4245	7.1534	1.1145
11.89	0.5878	5.6453	0.7454
8.79	0.7945	2.9770	0.4234

3.1.3. Effect of total fluence on lipase deactivation

It was observed that the deactivation of lipase was affected by two factors (exposure time and pulse fluence). According to Bunsen-Roscoe reciprocity law, radiation to a subject shows same efficiency when the total fluence is reached with high pulse fluence and short exposure time or low pulse fluence and long exposure time. This principle of equi-effectivity is a general rule in photochemical reactions (Gómez-López, Ragaert, Debevere, & Devlieghere, 2007). Therefore, it was postulated that if the same total fluence is delivered to lipase, the activity will decrease in the same extent ($[\text{total fluence}] \propto [\text{exposure time}] \times [\text{pulse fluence}]$). In previous studies, Bunsen-Roscoe law was used for the investigation of the effect of IPL on microorganisms. Although there were several cases that obeyed the law (Otaki, Okuda, Tajima, Iwasaki, Kinoshita, & Ohgaki, 2003; Rice & Ewell, 2001; Wang, MacGregor, Anderson, & Woolsey, 2005), the adverse cases, following the peak power effect, were also introduced (Sommer, Haider, Cabaj, Heidenreich, & Kundi, 1996; Takeshita, et al., 2003). Thus, this law is quite controversial in the case of IPL.

As in the case of lipase (Fig. 5), the degree of deactivation was different despite of the same total fluence. From the result, more deactivation of lipase

was observed at higher pulse fluence at each input energy state. When the total fluence reached 50 J/cm², the remained fractional activity was 26.0, 38.1, 46.2, and 56.2% at pulse fluence of 14.86, 13.87, 11.89, and 8.79 mJ/cm², respectively. The result indicated that deactivation of lipase is hard to be predicted by the Bunsen-Roscoe law, therefore, two variables (exposure time and pulse fluence) may not have equivalent effect on enzyme deactivation. The reciprocal plot between residual activity and total fluence also showed a good correlation ($R^2 \geq 0.985$), and this indicates that same amount of total fluence has contrasting impact on the lipase activity because of the different effect of IPL treatment on the lipase according to pulse fluence. And through the linearized plot, it could be possible to calculate residual activity of lipase by exposure time and pulse fluence.

Fig. 5. Deactivation profile of *Chromobacterium viscosum* lipase according to IPL total fluence increase.

Fig. 6. Reciprocal plotting of the *Chromobacterium viscosum* lipase deactivation profile according to total fluence increase.

3.2. Evaluation of critical point for deactivation

In order to confirm the unequal influence of deactivation factors, pulse fluence was lowered below 8.79 mJ/cm^2 to evaluate the aspects of deactivation. In the researches of other fields, it was studied that small molecules, such as HNCO, have the dissociation threshold (Brown, Berghout, & Crim, 1996). From the information, it was assumed that proteins would also have a certain energy point for degradation. Thus, it was analyzed using possible lowest energy of the IPL system in terms of residual activity.

When $\pm 10\%$ change was set to no significance, the activity was reduced above 2.77 mJ/cm^2 as expected. However, the activity was not decreased below 2.66 mJ/cm^2 (Fig. 7), although the exposure time and total fluence were 1.50-fold longer and 1.44-fold higher compared to those at 2.77 mJ/cm^2 , which indicated that the activity is not affected by exposure time below a certain pulse fluence value. This implied the existence of a critical point, so that two different phases were thought to exist for deactivation (susceptible and resistant to IPL). To conclude from the data, pulse fluence is thought to be superior to exposure time because the activity of lipase was decreased more rapidly at higher pulse fluence with short exposure time, which indicated the peak power dependence of the lipase, and the activity was not affected by

exposure time below the critical point.

Fig. 7. Residual activity (%) of *Chromobacterium viscosum* lipase after IPL treatment below 2.91 mJ/cm².

3.3. Structure modification by IPL treatment

3.3.1. Secondary structure analysis of lipase

The secondary structure of the *Chromobacterium viscosum* lipase was studied using CD spectroscopy in Far-UV region (190-260 nm) to assess the effect of IPL on secondary structure of the lipase. In theory of CD spectroscopy, α -helix proteins have negative bands at 208 nm and 222 nm and a positive band at 193 nm and proteins with β -sheet have a negative band at 218 nm and a positive band at 195 nm, whereas disordered proteins such as β -turn and random coil have negative bands at 189 nm and 198 nm and positive bands at 210 nm and 212 nm, respectively (Greenfield, 2006).

To analyze the effect of IPL on *Chromobacterium viscosum* lipase by time flow, Far-UV CD spectra were obtained after IPL treatment of 10, 20, and 30 min. The results of CD spectra were shown in Figure 8. There were no significant fluctuations of CD spectra in secondary structure analysis. Moreover, the secondary structure composition of *Chromobacterium viscosum* lipase was analyzed from CD spectra, the number of amino acids in sequence, and molecular weight of the lipase (Table 3). Although the activity of *Chromobacterium viscosum* lipase was lost after IPL treatment, the composition of lipase was not changed dramatically. A small change was

detected in the α -helix and antiparallel β -sheet (both maximally 1.2%), but no constant changing trend was found according to the exposure time increase.

Fig. 8. Far-UV CD spectra of IPL-treated *Chromobacterium viscosum* lipase.

Table 3. Content (%) of *Chromobacterium viscosum* lipase secondary structure elements at different IPL exposure time

Secondary structure	Control	10 min	20 min	30 min
α-Helix	22.2	22.7	21.9	21.0
Antiparallel β-sheet	17.7	18.4	18.1	18.9
Parallel β-sheet	9.4	9.5	9.4	9.6
Random coil	33.8	33.8	33.7	33.9
β-Turn	16.8	16.7	16.8	16.6

3.3.2. Assessment of tertiary structural changes by IPL treatment

As no distinct change was found in CD analysis, fluorescence spectroscopy was applied for tertiary structural change of the lipase. A fluorescence spectroscopy has been widely employed for aromatic amino acid analysis in a protein. Typically, excitation and emission of the amino acids occurs at 280 nm and 280-340 nm in fluorescence analysis, respectively. Tryptophan is a crucial fundamental fluorescent probe because the amino acid arrangement can affect the microenvironment and the quantum yields of the tryptophan. Tryptophan usually locates in the hydrophobic core of a protein, however, when the protein structure is denatured, a red-shift and decrease of fluorescence intensity in emission spectrum appear due to the exposure of the residue to an hydrophilic environment. In contrast, micro-environmental change from hydrophilic to hydrophobic will cause blue-shift and increased intensity of emission spectrum (Chen & Barkley, 1998; Vivian & Callis, 2001).

For the analysis of fluorescence emission spectra, IPL was treated on *Chromobacterium viscosum* lipase for 10, 20, and 30 min at 14.86 mJ/cm². As shown in Figure 9a, fluorescence emission was decreased in proportion to the exposure time, and λ_{max} shift was observed from 332 nm to 336 nm (Fig. 10). By contrast, in the case of 2.66 mJ/cm² treated lipase in Figure 9b,

fluorescence emission spectra was not significantly changed and no λ_{max} shift was observed.

From the CD and the fluorescence spectroscopy data, it was evident that the deactivation of *Chromobacterium viscosum* lipase by IPL treatment occurs along with the tertiary structural changes. And decreased fluorescence of the protein after IPL treatment is in accordance with another literature (Elmnasser, et al., 2008).

Fig. 9. Fluorescence emission spectra of IPL-treated *Chromobacterium viscosum* lipase after 280 nm excitation at (a) 14.86 mJ/cm² and (b) 2.66 mJ/cm².

Fig. 10. Variation of fluorescence maximum emission wavelength of *Chromobacterium viscosum* lipase after IPL treatment.

3.4. SDS-PAGE

Protein structural change by IPL treatment is known as degradation of protein into peptide fragments in literature. SDS-PAGE was conducted to compare this information with the case of lipase. An illustration of control and IPL-treated lipase SDS-PAGE profile is in Figure 11. By SDS-PAGE result showing a single band with approximately 32 kDa throughout the experiments (Taipa, Aires-Barros, & Cabral, 1992), it was confirmed that *Chromobacterium viscosum* lipase protein has high purity without other bands.

SDS-PAGE profiles showed that the amount of intact lipase was reduced in a time dependent manner and uncertain trace bands appeared below the main band. A significant reduction in band intensity was observed after 30 min of treatment at 14.86 mJ/cm². However, no distinct bands were detected in repeated tests above the control protein, in which demonstrates aggregation such as formation of dimers. The SDS-PAGE gel densitometry analysis verified the pattern of degradation (Fig. 12). The data were normalized with respect to the control value. These findings indicated that IPL treatment on the enzyme plays a role in decreasing the activity of lipase by fragmentation. Similar results of decrease in protein band intensity on SDS-PAGE were found in literature using the soy allergen proteins glycinin and β -conglycinin (Yang,

Chung, Ajayi, Krishnamurthy, Konan, & Goodrich-Schneider, 2010), and tropomyosin in shrimp (Shriver, Yang, Chung, & Percival, 2011). While, the SDS-PAGE profile of the IPL-treated lipase at 2.66 mJ/cm^2 showed no difference in band intensity compared to the control band (Fig. 13). This explained that the reason for maintenance of lipase activity is due to the unharmed protein structure.

The major possibilities of fragmentation by IPL treatment has been attributed to photo-chemical, photo-thermal, and photo-physical reactions of protein due to UV light irradiation (Krishnamurthy, Demirci, & Irudayaraj, 2007). It was reported in publications that UV light induces cross-linking, agglomeration, polymerization, and fragmentation (Greenberg, 1979; Friso, Barbato, Giacometti, & Barber, 1994; Curley & Lawrence, 1999). Currently, the mechanism for IPL induced structural change is not exactly revealed, therefore, further research is needed to clarify the reason for the effect of IPL treatment on protein.

Fig. 11. SDS-PAGE profile of untreated and IPL-treated *Chromobacterium viscosum* lipase with different exposure time (10, 20, 30, 40, and 60 min) at 14.86 mJ/cm².

Fig. 12. Quantitative densitometry analysis of the *Chromobacterium viscosum* lipase band using ImageJ software.

Fig. 13. SDS-PAGE profile of untreated and IPL-treated *Chromobacterium viscosum* lipase with different exposure time (150, 180, and 240 min) at 2.66 mJ/cm².

3.5. MALDI-TOF

3.5.1. Fragmentation pattern analysis

As the SDS-PAGE profile showed the degradation of lipase structure, further analysis was needed to confirm the fragmentation pattern. Although there were reports that proposed the irregular fragmentation by IPL treatment (Janve, et al., 2014), they did not show any distinct results. Thus, the fragments in the IPL-treated samples were evaluated to obtain the evidence. To identify the fragmentation pattern, molecular weights of the fragments were directly analyzed using MALDI-TOF. As the fragments were mainly observed below m/z 4,000 from the preliminary tests, the analysis was conducted in this region.

Based on the proposed mechanism in the previous literature, it was postulated that if the fragmentation occurs irregularly while exposed to IPL, the result spectra would not show a tendency over increasing IPL exposure time. Therefore, samples (10, 30, 50, and 60 min) were prepared to verify the assumption. However, the result spectra showed similar peak patterns (Fig. 14). Particularly, the molecular weights of the fragments (S/N ratio ≥ 100) displayed remarkable consistency within error range through the time points, although the IPL treatment was conducted separately for each sample. Only 8 out of total 50 fragments had individual molecular weights within the data

(Table 4). In conclusion, the result demonstrated that site-selective fragmentation was induced by IPL treatment. Furthermore, it could be inferred from the data that the internal sensitive bondings of the lipase were cleaved preferentially by IPL, thus specific fragments were formed.

Fig. 14. MALDI-TOF spectra of fragments from *Chromobacterium viscosum* lipase after 10, 30, 50, and 60 min IPL treatment.

3.5.2. Cleavage site estimation using FindPept[®] tool

Meanwhile, although the fragmentation pattern by IPL treatment was identified, the cleavage site was not verified from MALDI-TOF analysis. There were two basic possibilities of cleavage: peptide bond cleavage or covalent bond within the amino acid (including peptide bond). Because the sequences of the fragments could not be analyzed directly due to the limitations such as too low concentration and purity, indirect way was employed. As a tool for fragment analysis, FindPept[®] was chosen, which is used to find the site of unspecific proteolytic cleavage, missed cleavage, or protease autolysis (Gattiker, Bienvenut, Bairoch, & Gasteiger, 2002). The measured molecular weights of the fragments were compared to that of the theoretical peptides from the lipase sequence and cleavage site was determined from the results. Because FindPept[®] tool considers peptide bond cleavage, unmatched data are evidences for random-bond cleavage.

As shown in Table 4, the theoretical candidate peptides existed in more than 80% of identified fragments ($S/N \text{ ratio} \geq 100$) generated from IPL treatment, though exact cleavage site could not be identified from our data. Also, when the amino acids adjacent to cleavage sites were analyzed, top 5 amino acids accounted for 54.4% composed of alanine (12.4%), threonine (11.6%), glycine

(11.4%), serine (10.1%), and leucine (8.8%), which showed same tendency in each treatment time point. Among the 319 amino acids in the sequence of *Chromobacterium viscosum* lipase, the 5 residues consists of alanine (11.6%), threonine (11.0%), glycine (10.6%), leucine (9.7%), and serine (8.2%), respectively, which accounts for 51.1% of the sequence. Moreover, the ratio of rest 15 amino acids in the sequence showed similar trends with the top 5 residues. Thus from the results of fragment analysis, it was found that the more number of particular amino acids exists in the sequence, the more cleavage occurs by IPL. However, as the result was obtained from the prediction using FindPept[®], the cleavage sites should be investigated by more precise and direct way to prove this conclusion in the future work.

Table 4. Identified molecular weights and theoretical positions of the fragments in the sequence of *Chromobacterium viscosum* lipase

Exposure time	Analyzed mass (Da)	Number of fragments	Position in protein*
10 min	1126.622	1	97(A)-108(T)
	1154.616	4	3(T)-12(I), 7(T)-16(G), 289(I)-299(N), 298(A)-308(R)
	1187.603	1	155(N)-165(R)
	1463.799	1	285(H)-297(G)
	1569.738	2	191(Q)-206(L), 259(S)-273(F)
	1576.849	2	101(P)-115(R), 300(A)-313(R)
	1763.919	3	255(I)-271(S), 256(N)-272(L), 283(W)-297(G)
	1898.958	5	148(T)-165(R), 149(L)-166(T), 150(V)-167(L), 153(S)-170(A), 274(G)-289(I)
	1900.977	6	151(S)-168(T), 238(N)-256(N), 241(D)-259(S), 244(T)-262(N), 247(L)-265(L), 282(H)-297(G)
	1926.958	-	-
	2007.019	1	281(Y)-296(R)
	2064.042	4	137(T)-156(T), 205(L)-224(T), 216(P)-237(A), 281(Y)-297(G)
	2252.121	3	82(N)-103(L), 166(T)-187(P), 279(T)-297(G)
	2415.225	1	143(V)-165(R)
	2593.263	3	2(D)-25(N), 48(N)-71(Q), 263(D)-285(H)
	2679.344	6	123(V)-148(T), 160(A)-185(G), 161(L)-186(A), 267(S)-289(I), 274(G)-296(R), 275(Q)-297(G)
	2736.375	4	150(V)-175(Y), 163(A)-189(S), 274(G)-297(G), 276(V)-299(N)

Table 4. (Continued)

	2758.387	2	117(S)-142(F), 197(E)-223(V)
	2766.358	1	133(G)-159(D)
	2902.466	7	108(T)-134(L), 143(V)-170(A), 193(G)-221(L), 194(A)-222(G), 213(A)-242(P), 229(T)-257(R), 242(P)-270(S)
	1098.622	1	288(E)-297(G)
	1126.614	1	97(A)-108(T)
	1154.614	4	3(T)-12(I), 7(T)-16(G), 289(I)-299(N), 298(A)-308(R)
	1187.599	1	155(N)-165(R)
	1207.638	1	300(A)-310(H)
	1397.708	3	117(S)-128(K), 119(F)-130(D), 286(L)-298(A)
	1463.793	1	285(H)-297(G)
	1498.728	3	42(A)-55(D), 117(S)-129(T), 152(S)-165(R)
30 min	1577.837	5	38(Q)-52(F), 39(S)-53(Q), 40(H)-54(S), 196(T)-209(W), 284(N)-297(G)
	1763.908	3	255(I)-271(S), 256(N)-272(L), 283(W)-297(G)
	1900.972	6	151(S)-168(T), 238(N)-256(N), 241(D)-259(S), 244(T)-262(N), 247(L)-265(L), 282(H)-297(G)
	1926.950	-	-
	2064.033	4	137(T)-156(T), 205(L)-224(T), 216(P)-237(A), 281(Y)-297(G)
	2069.009	5	117(S)-135(S), 118(E)-136(S), 173(A)-193(G), 174(T)-194(A), 276(V)-292(L)

Table 4. (Continued)

	2252.111	3	82(N)-103(L), 166(T)-187(P), 279(T)-297(G)
	2593.252	3	2(D)-25(N), 48(N)-71(Q), 263(D)-285(H)
	2758.376	2	117(S)-142(F), 197(E)-223(G)
	2766.348	1	133(G)-159(D)
	864.390	1	229(T)-237(A)
	1098.597	4	288(E)-297(G), 293(L)-303(P), 295(V)-305(A), 296(R)-306(V)
	1126.589	1	168(T)-177(R)
	1154.580	2	3(T)-12(I), 298(A)-308(R)
	1187.567	1	155(N)-165(R)
	1267.646	2	3(T)-13(L), 207(Y)-218(S)
	1463.754	1	285(H)-297(G)
	1498.688	3	42(A)-55(D), 117(S)-129(T), 152(S)-165(R)
50 min	1533.692	3	189(S)-204(H), 194(A)-208(S), 197(E)-210(G)
	1535.705	2	49(L)-63(E), 173(A)-187(P)
	1577.798	7	19(G)-31(Y), 20(T)-32(G), 38(Q)-52(F), 39(S)-53(Q), 40(H)-54(S), 196(T)-209(W), 284(N)-297(G)
	1601.793	7	32(G)-46(V), 35(S)-49(L), (36(D)-50(S), 81(V)-95(Y), 82(N)-96(V), 166(T)-179(F), 251(G)-266(V)
	1760.770	1	51(G)-67(A)
	1763.858	3	255(I)-271(S), 256(N)-272(L), 283(W)-297(G)

Table 4. (Continued)

	1900.919	4	151(S)-168(T), 238(N)-256(N), 247(L)-265(L), 282(H)-297(G)
	1912.915	-	-
	1923.836	2	25(N)-40(H), 51(G)-68(Y)
	1926.894	-	-
	2063.976	3	137(T)-156(T), 216(P)-237(A), 281(Y)-297(G)
50 min	2068.954	5	117(S)-135(S), 118(E)-136(S), 173(A)-193(G), 174(T)-194(A), 276(V)-292(L)
	2074.920	4	47(A)-66(L), 48(N)-67(A), 53(Q)-71(Q), 143(V)-162(A)
	2150.983	4	51(G)-70(K), 142(F)-161(L), 167(L)-187(P), 280(S)-297(G)
	2252.050	2	166(T)-187(P), 279(T)-297(G)
	2279.034	2	25(N)-44(V), 51(G)-71(Q)
	2286.959	-	-
	2465.122	2	48(N)-70(K), 50(S)-72(V)
	2593.180	2	48(N)-71(Q), 263(D)-285(H)
	2766.268	1	133(G)-159(D)
	2778.267	3	216(P)-244(T), 265(L)-288(E), 266(V)-289(I)
	868.393	4	1(A)-8(R), 30(W)-36(D), 49(L)-56(D), 285(H)-291(Q)
	951.433	-	-
	1023.575	1	308(R)-315(K)

Table 4. (Continued)

	1069.571	1	83(L)-93(S)
	1081.605	1	9(Y)-18(A)
	1098.604	4	288(E)-297(G), 293(L)-303(P), 295(V)-305(A), 296(R)-306(V)
	1126.605	2	97(A)-108(T), 168(T)-177(R)
	1154.595	2	3(T)-12(I), 298(A)-308(R)
	1187.579	1	155(N)-165(R)
	1267.653	2	3(T)-13(L), 207(Y)-218(S)
	1325.686	2	76(T)-88(Q), 136(S)-148(T)
	1535.732	3	49(L)-63(E), 140(A)-154(H), 173(A)-187(P)
	1569.720	2	191(Q)-206(L), 259(S)-273(F)
60 min	1577.828	5	38(Q)-52(F), 39(S)-53(Q), 40(H)-54(S), 196(T)-209(W), 284(N)-297(G)
	1601.821	8	32(G)-46(V), 35(S)-49(L), 36(D)-50(S), 81(V)-95(Y), 82(N)-96(V), 89(G)-104(V), 166(T)-179(F), 251(G)-266(V)
	1763.903	3	255(I)-271(S), 256(N)-272(L), 283(W)-297(G)
	1898.942	5	148(T)-165(R), 149(L)-166(T), 150(V)-167(L), 153(S)-170(A), 274(G)-289(I)
	1900.961	6	151(S)-168(T), 238(N)-256(N), 241(D)-259(S), 244(T)-262(N), 247(L)-265(L), 282(H)-297(G)
	1926.933	-	-
	2064.027	4	137(T)-156(T), 205(L)-224(T), 216(P)- 237(A), 281(Y)-297(G)

Table 4. (Continued)

2252.111	3	82(N)-103(L), 166(T)-187(P), 279(T)-297(G)
2279.101	2	25(N)-44(V), 51(G)-71(Q)
2287.019	-	-
2415.216	1	143(V)-165(R)
2593.253	3	2(D)-25(N), 48(N)-71(Q), 263(D)-285(H)
2736.341	4	150(V)-175(Y), 163(A)-189(S), 274(G)-297(G), 276(V)-299(N)
2758.350	2	117(S)-142(F), 197(E)-223(V)
2902.453	7	108(T)-134(L), 143(V)-170(A), 193(G)-221(L), 194(A)-222(G), 213(A)-242(P), 229(T)-257(R), 242(P)-270(S)

* Sequence number (amino acid) - Sequence number (amino acid)

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국문초록

비가열 처리의 한 종류인 광펄스는 최근 미국 등 일부 선진국에서 식품·약품 표면 살균 및 포장재 살균에 소규모로 적용되고 있고, 산업 적용을 위한 대형화 과정을 거치며 새로이 주목받고 있는 기술이다. 이 기술을 *Chromobacterium viscosum* lipase (triacylglycerol hydrolase, EC 3.1.1.3)에 적용할 경우 효소의 활성과 구조가 받는 영향을 살피는 연구를 수행하였고, 연구 결과를 응용하여 실제 산업에서 식품 내부의 효소 활성 제어에 광펄스를 응용할 수 있는 근거를 마련하고자 하였다.

Lipase에 에너지를 달리한 광펄스를 처리한 후 reverse micelle system에서 활성의 변화를 살핀 결과, 활성이 감소하는 것이 확인되었으며 이는 에너지 밀도와 처리 시간이 증가함에 따라 감소량이 증가 하는 것이 확인되었다. Lipase의 활성 감소현상을 보다 구체적인 응용에 적용하기 위해, two-step series type deactivation kinetic model을 이용하여 활성 감소를 정량적으로 해석하였다. 그 결과, 14.86 mJ/cm²에서의 k_1 과 k_2 값(9.05 h⁻¹, 1.64 h⁻¹)은 8.79 mJ/cm²에서의 상수 값(각 2.98 h⁻¹, 0.42 h⁻¹)보다 각각 3.04배, 3.88배가 높게 확인되었다. 한편, lipase의 활성은 투입된 에너지 총량에 의해서도 감소 경향을 보였고, 동일한 총 에너지 투입 시 처리하는 에너지 밀도가 클수록 급격한 활성 감소를 나타내었다. 이러한 경향을 통해, 에너지 밀도에 따른 활성 변화 경향을 살피고자 에너지 밀도를 낮추며 활성 변화를 살폈다. 그 결과, 2.77 mJ/cm² 이상의 에너지에서 활성 감소 현상을 보였으나, 2.66 mJ/cm² 이하를 처리하였을 때는 활성이 감소되지 않았다.

이러한 현상은 광펄스가 lipase에 특정 에너지를 기준으로 두 가지 다른 방식으로 영향을 주기 때문으로 추측된다. 이어서 광펄스에 의한 효소의 활성 감소 현상에 대한 원인을 밝히기 위해 구조적 분석을 진행하였다. Circular dichroism 기법을 통한 2차 구조 분석에서는 변화가 확인되지 않았으나, fluorescence spectroscopy 분석을 진행한 결과 3차 구조의 변화가 나타났다. 광펄스에 의해 유발되는 lipase의 구조 변화 양상을 살피기 위해 SDS-PAGE를 진행한 결과, 32 kDa 부근의 lipase 밴드는 광펄스 처리 시간에 비례하여 감소 하였으며, 단편화에 의해 3차 구조가 변화됨이 확인되었다. 광펄스에 의해 단편화가 단백질 서열 상 특정 위치에서 발생하는지 확인하기 위해 MALDI-TOF 분석을 진행하였고, 그 결과 특정 위치에서 단편화가 일어났다. 또한, FindPept tool[®]을 이용하여 생성된 조각들의 분자량과 이론상 펩타이드의 관계를 살핀 결과, 펩타이드 결합의 절단 가능성을 확인했다.

본 연구를 통해 광펄스가 lipase의 활성에 미치는 영향을 규명 하였으며, 구조적인 분석을 통해 광펄스에 의한 단백질의 단편화 현상을 살폈다. 이를 통해 광펄스에 의한 효소의 활성 감소를 정량적으로 분석할 수 있는 기반을 마련했고, 차후에는 이 기술을 효소의 제어가 필요한 산업에 실질적으로 적용할 수 있을 것으로 기대된다.

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A THESIS FOR THE DEGREE OF MASTER OF SCIENCE

**Effect of intense pulsed light on the deactivation of
lipase: enzyme-deactivation kinetics and tertiary
structural changes by fragmentation**

광펄스가 라이페이스 활성 감소에 미치는 영향:

효소-불활성화 동역학적 해석 및 단편화에 의한 3 차구조 변화

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Seoul National University

Jeon, Min Sik

석사학위논문

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지도교수 장 판 식

이 논문을 석사학위 논문으로 제출함

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서울대학교 대학원 농생명공학부

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위원장 최 상 호 (인)

부위원장 장 판 식 (인)

위원 강 동 현 (인)

Abstract

Intense pulsed light (IPL), a novel non-thermal technology, was applied to investigate the effect on activity and structure of *Chromobacterium viscosum* lipase (triacylglycerol hydrolase, EC 3.1.1.3) by employing hydrolysis of triacylglycerol in reverse micelle system. After IPL treatment, the lipase deactivation was observed, and the lipase activity was decreased with increase of pulse fluence and exposure time. The half-life of *Chromobacterium viscosum* lipase at 8.79 mJ/cm² (37.63 min) was 4.76-fold longer than that at 14.86 mJ/cm² (7.90 min). In order to determine deactivation constants (k_1 and k_2), two-step series type deactivation kinetic model was employed. As a result, k_1 (9.05 h⁻¹) and k_2 (1.64 h⁻¹) at 14.86 mJ/cm² were 3.04, 3.88-fold higher than those at 8.79 mJ/cm² (2.98 h⁻¹, 0.42 h⁻¹), respectively. Meanwhile, pulse fluence showed a dominant impact on deactivation, in that higher pulse fluence treatment with short exposure time showed more decreased activity at the same degree of total fluence. Also, no activity reduction phenomenon appeared below 2.66 mJ/cm², demonstrating that the IPL treatment to the lipase was composed of susceptible phase and resistant phase. Based on the experiments in lipase hydrolysis, structural analysis was conducted in order to elucidate the cause of deactivation. From secondary structure analysis using

circular dichroism spectroscopy, the result spectra were not fluctuated significantly. However, changes in its tertiary structure were detected through fluorescence spectroscopy. SDS-PAGE results showed that the amount of intact lipase (32 kDa) was reduced in a time dependent manner and uncertain fragmented peptides were increased below the main protein band. For validation of fragmentation sites in the sequence of lipase, the peptides were analyzed by MALDI-TOF analysis. It was revealed that the lipase was site-selectively fragmented (<5 kDa) by IPL treatment. Furthermore, evidences for peptide bond cleavage was identified using Findpept[®] tool. From these findings, it is evident that the lipase deactivation was occurred quantitatively in molecular level.

***Keywords:* *Chromobacterium viscosum* lipase; intense pulsed light; deactivation kinetics; site-selective fragmentation; peptide bond cleavage**

***Student number:* 2013-23277**

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1. Introduction

Recently, increasing food-borne illness is being major concern for the food industry because food-borne outbreaks are frequent nowadays. Several technologies have significant impact to food products, including heating, freezing, dehydration, and the use of preservatives. However, these methods can increase side effects, such as degradation of certain components which can impair food quality (Elmnasser, Guillou, Leroi, Orange, Bakhrouf, & Federighi, 2007). To prevent undesirable thermal effects on foods, major efforts have been made to develop non-thermal technologies, which can produce safe but minimally processed foods with satisfactory nutritional and organoleptic qualities (Barbosa-Canovas, Gongora-Nieto, & Swanson, 1998). Thus non-thermal technologies enhance the food safety while maintaining their nutrient content and appearance as well as lengthening their shelf life (Cheigh, Park, Chung, Shin, & Park, 2012).

Intense pulsed light (IPL) is a technique performed to decontaminate food surfaces by killing micro-organisms, and employs a flash lamp filled with inert gas, such as xenon, which emits high-frequency pulses of broad spectrum radiation containing wavelengths from 180 nm (UV) to 1100 nm (near infrared) region (Cheigh, et al., 2012; Elmnasser, et al., 2008). IPL technique has been

suggested to decontaminate the surface of shell eggs, fresh-cut vegetables, and ready-to-eat meat products as well as to kill microorganisms in milk, fruit juices, and infant food (Marquenie, Michiels, Van Impe, Schrevens, & Nicolai, 2003; Hierro, Manzano, Ordonez, de la Hoz, & Fernandez, 2009; Choi, Cheigh, Jeong, Shin, & Chung, 2010; Caminiti, et al., 2011; Hierro, Barroso, de la Hoz, Ordonez, Manzano, & Fernandez, 2011; Pataro, Munoz, Palgan, Noci, Ferrari, & Lyng, 2011; Ramos-Villarroel, Aron-Maftei, Martin-Belloso, & Soliva-Fortuny, 2012).

Enzymes are the major targets for photo-induced modifications due to the abundance of internal chromophores within their structure. Broad radiation spectrum of IPL may affect enzymes, because proteins have strong UV absorption at 280 nm due to aromatic amino acids (Moriyama, Ohta, Hachiya, Mitsui, & Takeda, 1996; Hollosy, 2002). And peptide bonds in proteins exhibit a strong and weak absorption in the Far-UV region at of 190 nm (Aitken & Learmonth, 1996) and 210 to 220 nm (Davies & Truscott, 2001), respectively (Janve, Yang, Marshall, Reyes-De-Corcuera, & Rababah, 2014). Typically, proteins absorb the light in the range of 250 to 300 nm, originated from tryptophan and tyrosine residues while phenylalanine absorbs weakly below 275 nm and disulfide bond has a broad absorbance in the near-UV region

(Wetlaufer, 1958; Mach, Middaugh, & Lewis, 1992). Both amino acid side-chains and bound prosthetic groups (for example, flavins and heme) may act as efficient chromophores (Manzocco, Panozzo, & Nicoli, 2013). Enzymes have additional ability to bind exogenous chromophores, and quickly react with other excited state species (Davies, et al., 2001). The result would thus be a modification in the protein properties because of side-chain oxidation, backbone fragmentation, and/or formation of cross-links and aggregates (Davies, 2003). It has been reported that IPL treatment reduced the amount of intact proteins, especially in case of an enzyme, decrease in activity was observed compared to untreated one (Manzocco, et al., 2013; Janve, et al., 2014). On the other hand, some inconsistent reports are found in literature. Oms-Oliu et al. (Oms-Oliu, Aguiló-Aguayo, Martín-Belloso, & Soliva-Fortuny, 2010) showed that pulsed light stimulated an increase in polyphenol oxidase (PPO) activity in fresh-cut mushrooms. And Charles et al. (Charles, Vidal, Olive, Filgueiras, & Sallanon, 2013) demonstrated the increase in PPO activity in fresh-cut mango submitted to IPL treatment.

Lipase (triacylglycerol hydrolase, EC 3.1.1.3) is one of the most important enzymes in food industry. It has been widely applied to the industrial bioconversions such as synthesizing emulsifiers and functional esters through

hydrolysis, glycerolysis, and esterification (Houde, Kademi, & Leblanc, 2004; Park, Lee, Sung, Lee, & Chang, 2011). However, the lipase is known to cause hydrolytic rancidification by lipolysis in foods such as milk, rice bran, oil products, and etc. (Ramezanzadeh, Rao, Windhauser, Prinyawiwatkul, Tulley, & Marshall, 1999; Deeth, 2006; Ngando-Ebongue, Dhouib, Carrière, Amvam Zollo, & Arondel, 2006). General treatment for deactivating lipase in foods is heat treatment, which can induce unwanted results. Therefore, IPL system could be applied to reduce the activity for food preservation without serious damages.

Although there are reports about IPL deactivation of enzymes, it is desired to confirm the possibility of quantitative analysis and visualized expression of IPL induced lipase fragmentation. Thus, the objective of this research was to investigate the effect of IPL on lipase activity and express the deactivation phenomenon quantitatively using kinetics model. By this numerical interpretations, a possibility of practical application of IPL was evaluated. After that, the reason for lipase deactivation was assessed by evaluating secondary and tertiary structure changes with various techniques.

2. Materials & Methods

2.1. Intense pulsed light (IPL) treatment system

2.1.1. IPL apparatus

The IPL treatment system used to generate pulsed light was a slight modification of Choi's method (Choi, et al., 2010). The instruments were composed of a power supply, control, chamber, and lamp (Fig.1). The treatment chamber was made of wood and acrylic. The lamp was a xenon flash lamp (Heraeus Noblelight XAP series, type NL 4006, Cambridge, UK) comprised of a quartz tube. The size of the cylindrical lamp was 145×7.14 mm [(length) × (outer diameter)], and its emission wavelength spectrum ranged from UV to infrared. The light source was placed at the top of the chamber lamp, and the chamber had a cooling fan to remove the heat generated from the lamp during IPL treatment. Moreover, a light-blocking black quartz sheet with a small hole in the center was placed under the xenon flash lamp in order to prevent any heat associated with the light generation and to control the amount of light extend to the sample.

Fig. 1. Schematic representation of IPL instrument.

2.1.2. Measurement of IPL pulse fluence

Pulse fluence of IPL was controlled in voltage at the controller, and the input energy was applied by 200 V interval. The instrument was set at the distance of 9 cm from the light source, and the measurements were conducted at least 15 times for each pulse fluence. As the pulse fluence was controlled in voltage and converted to pulse fluence by integration, it was not expressed in an integral number.

The measurement of IPL pulse fluence was conducted using the spectroradiometer (ILT-900, InternationalLight Technologies, Peabody, MA, USA). The IPL pulse fluence was displayed on a spectrum through the software (SpectrilightTM, InternationalLight Technologies). The pulse fluence of the flash lamp in chamber was measured at different input voltages of IPL treatment system (Fig. 2). Table 1 shows total energy density of IPL from 300 nm to 1,100 nm. The values in Table 1 were pulse fluence which increased along with the input voltage.

Fig. 2. Energy distribution spectrum of IPL system at 3 Hz and 0.2 ms.

Table 1. Pulse fluence of IPL system from 300 nm to 1,100 nm

Voltage (V)	Pulse fluence (mJ/cm²)
500	4.64
600	5.03
800	6.00
1,000	6.98
1,200	8.97
1,400	11.89
1,600	13.87
1,800	14.86

2.1.3. IPL treatment on lipase

Purified *Chromobacterium viscosum* lipase was purchased from Millipore (Billerica, MA, USA) and reported to have catalytic activity of 3,400 units/mg solid. The lipase solution of pre-determined concentration in 50 mM Tris-HCl buffer (pH 8.0) was loaded in a transparent vial and set in a center of water jacket connected to water circulator in the constant temperature condition (4 °C) at the distance of 9 cm. The water jacket was placed under the xenon flash lamp in the IPL chamber. The energy density was controlled depending on the experiment and 0.2 ms pulses of IPL treatment were emitted at a frequency of 3 Hz.

2.2. Lipase assay in reverse micelle system

2.2.1. Chemicals and reagents

Bis (2-ethylhexyl) sulfosuccinate sodium salt (AOT) was purchased from Sigma-Aldrich (St. Louis, MO, USA) to be used as a surfactant for the formation of reverse micelles, and purified according to the method of Tamamushi and Watanabe (Tamamushi & Watanabe, 1980). Isooctane was purchased from Daejung Chemicals (Siheung-si, Gyeonggi-do, Korea) in

spectrophotometric grade and dehydrated over a 4 Å molecular sieve (Sigma-Aldrich) with filtration through a 0.5 µm membrane filter (J050A047A, Advantec, Tokyo, Japan), prior to use as a reaction medium in the reversed micellar system. The highly refined and low acidity olive oil (Sigma-Aldrich) was used as substrates in lipase-catalyzed hydrolysis. Cupric acetate reagent was prepared according to the method of Lowry and Tinsley (Lowry & Tinsley, 1976). The cupric acetate solution was prepared by dissolving 5 g of cupric acetate (98%, Sigma-Aldrich) in 100.0 mL of distilled water. The prepared cupric acetate solution was then filtered through Whatman No. 1 filter paper, and the pH was adjusted to 6.1 using pyridine (Showa Chemical Industry Co., Ltd, Tokyo, Japan). All of other chemicals were of extra pure grade and used without further purification.

2.2.2. Preparation of reverse micelles

Reverse micelles containing the lipase were formed by adding lipase solution to a solution with 100 mM of AOT in isooctane followed by vigorous mixing for 1 min to produce a transparent micellar solution. The desired water content was defined as the *R*-value, which means the molar ratio of water to surfactant ($[H_2O]/[AOT]$) (Banerjee, Ghosh, & Datta, 2011; Kwon, Park, Choi,

& Chang, 2015). In this study, experiments were conducted with the most optimized condition using the *R*-value at 10.0 (Prazeres, Garcia, & Cabral, 1992).

2.2.3. Determination of lipase activity

Hydrolysis activity was determined according to copper-soap colorimetric method (Hong, et al., 2015). A screw-cap vial (10.0 mL) was filled with AOT/isooctane reversed micellar solution to a 4.5 mL that was pre-incubated in a water bath at 37°C for 10 min with magnetic stirring at 800 rpm. Lipase-catalyzed hydrolysis was initiated by adding 0.5 mL of olive oil as a substrate to the reversed micellar solution. Each aliquot (200 µL) of reactant was collected at predetermined intervals and diluted with 2.3 mL of isooctane. Subsequently, 0.5 mL of cupric acetate reagent and 1.0 mL of acetonitrile (used to eliminate turbidity caused by AOT) were added and mixed vigorously using a vortex mixer for 1 min. The absorbance of the upper layer (colorized isooctane layer) was measured at 715 nm to quantitatively determine the free fatty acids produced (Prazeres, Garcia, & Cabral, 1993) using UV-vis spectrophotometer (UV-2450, Shimadzu Co. Ltd., Japan).

One unit of enzyme was defined as the amount of lipase that hydrolyzed with 1 μmol of fatty acids/min under the assay condition. And the calibration curve was obtained by calculating the regression equation of the molar concentration of oleic acid and the absorbance at 715 nm.

2.3. Analysis of IPL-treated lipase activity

2.3.1. Deactivation of lipase

The lipase deactivation was conducted at different pulse fluence of IPL and different exposure time. Samples were illuminated from 1,800 V to 1,200 V (14.86, 13.87, 11.89, and 8.79 mJ/cm^2 , respectively) at predetermined time intervals. The lipase residual activity was assessed by hydrolytic activity of IPL-treated lipase to that of untreated lipase.

2.3.2. Two-step series type deactivation kinetics

The data for deactivation by the IPL treatment (14.86, 13.87, 11.89, and 8.79 mJ/cm^2 , respectively) on *Chromobacterium viscosum* lipase were fitted to a two-step series-type deactivation kinetic model (Henley & Sadana, 1985; Knezevic, Siler-Marinkovic, & Mojovic, 1998; Longo & Combes, 1999;

Moquin & Temelli, 2008), and the kinetic parameters were determined using a non-linear regression procedure based on the Marquardt-Levenberg method of iterative convergence included in a solver tool of Microsoft Office Excel 2013 software (Valerio, et al., 2009).

2.3.3. Critical point for deactivation

IPL flash lamp was set to low pulse fluence state (minimally 2.58 mJ/cm²) for the assessment of a critical point. IPL treatment on *Chromobacterium viscosum* lipase was performed with lowering the IPL pulse fluence. Lipase assay was conducted as described above. The activity change of $\pm 10\%$ was considered to be no effect of IPL on the lipase.

2.4. Structural analysis of IPL-treated lipase

2.4.1. Circular dichroism (CD)

Measurements were performed on homogeneous *Chromobacterium viscosum* lipase at a concentration of 1.33 mg/mL (10,000 units/mL). CD spectroscopy (ChirascanTM-plus, Applied Photophysics, Ltd., Leatherhead, Surrey, UK) was performed using 10, 20, and 30 min IPL-treated (14.86

mJ/cm²) *Chromobacterium viscosum* lipase in the Far-UV regions (190-260 nm), path length (0.5 mm), and bandwidth (1.0 mm), and the temperature was set to 37°C. The structural changes of lipase were analyzed from the CD spectra using the CDNN secondary structure analysis software.

2.4.2. Fluorescence spectroscopy

The structural change phenomenon of *Chromobacterium viscosum* lipase tertiary structure was analyzed by observing aromatic amino acids using a fluorescence spectrophotometer (Spectra004Dax M2^e, Molecular Devices Corp., CA, USA) (Elmnasser, et al., 2008). After IPL treatment of the lipase, the emission spectra of the substance excited at 280 nm was measured. The emission slit was 5 nm and 1 nm for conformational change of protein and λ_{max} shift analysis, respectively. The treatment conditions were 10, 20, and 30 min at 14.86 mJ/cm² and 150, 180, and 240 min at 2.66 mJ/cm².

2.5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

analysis was conducted with Laemmli method using 12% polyacrylamide (Laemmli, Beguin, & Gujer-Kellenberger, 1970) to run *Chromobacterium viscosum* lipase after IPL treatment at 14.86 mJ/cm². The IPL-treated lipase was diluted in sample buffer containing SDS and 2-mercaptoethanol. The diluted sample was boiled for 3 min before loading onto the gels. Each sample was loaded in a well along with the marker proteins. Electrophoresis was performed at constant current of 20 mA per gel for 110 min at 25°C and Hoefer SE 250 mini-gel system (Hoefer Inc., Holliston, MA, USA). For protein staining, Coomassie Brilliant Blue R-250 was used. Gels were destained overnight using destaining solution composed of 6:3:1(v/v/v) ratio of water:methanol:acetic acid. Also, densitometry analysis was performed using ImageJ (NIH, Bethesda, Md., USA) software.

2.6. Matrix assisted laser desorption/ionization-time of flight-mass spectrometry (MALDI-TOF)

The fragments of the lipase were analyzed by matrix-assisted laser desorption/ionization time-of-flight-mass spectrometry (MALDI-TOF). The MALDI-TOF analysis was performed at the Korea Basic Science Institute (Seoul, Korea), using MALDI-TOF/TOF 5,800 of AB Sciex (Foster city, CA,

USA) in reflector mode and positive polarity. The matrix employed was a solution containing 5.0 mg of α -cyano-4-hydroxy-cinnamic acid (CHCA, Sigma-Aldrich) and 0.1% trifluoroacetic acid (v/v) (TFA, Sigma-Aldrich) in 1.0 mL of acetonitrile/water (1:1, v/v). Before plating the samples on MALDI plate, they were desalted and concentrated using ZipTip (Millipore). ZipTip was washed by 100% and 50% acetonitrile each and then equilibrated using 0.1% TFA. The samples (30 μ L) were loaded onto the ZipTip and washed again using 0.1% TFA. Then the samples were mixed with prepared matrix (1:1) and deposited on a MALDI plate, where 1.0 μ L sample solution was loaded and dried. The analysis range was 800-4,000 Da. Prior to operation, the instrument was calibrated using a mixture of standard peptides (Mass Standards Kit for Calibration, AB SCIEX TOF/TOFTM instruments) with a maximum error tolerance of 50 ppm. The analyses data were processed using Data Explorer software (AB Sciex).

2.7. FindPept[®] tool

For further analysis, fragment mass data from MALDI-TOF were analyzed using FindPept[®] tool (ExPASy, Swiss Institute of Bioinformatics, <http://web.expasy.org/findpept>). The tool was set to cysteine in reduced form,

$[M+H]^+$ for peptide masses, ± 50 ppm for error tolerance, and monoisotopic mode. Then, the sequence of *Chromobacterium viscosum* lipase (The European Bioinformatics Institute, <http://www.ebi.ac.uk>) was compared with the data from MALDI-TOF analysis.

3. Results & Discussion

3.1. Lipase deactivation by IPL treatment

3.1.1. Deactivation phenomenon of lipase

Figure 3 shows the deactivation profile of *Chomobacterium viscosum* lipase after IPL treatment (from 8.79 mJ/cm² to 14.86 mJ/cm²) at 4°C. Deactivation of the lipase was achieved in proportion to exposure time and pulse fluence of IPL. For instance, residual activity of 14.86 mJ/cm² treated lipase was decreased most quickly among IPL-treated samples, at the same exposure time. By contrast, the lipase solution treated at 8.79 mJ/cm² showed a most progressive activity decrease in the entire exposure time range among the samples. Consequently, the longer time treated lipase showed lower residual activity compared to shortly treated one. It was observed that the half-life of IPL-treated *Chromobacterium viscosum* lipase in 50 mM Tris-HCl buffer (pH 8.0) is 7.90, 12.65, 20.84, and 37.63 min at 14.86, 13.87, 11.89, and 8.79 mJ/cm², respectively. When taking the reciprocal plot, the deactivation data gave a good correlation ($R^2 \geq 0.976$) in linear plotting. This means that residual activity of the lipase according to the IPL processing time could be obtained with simple calculations.

Fig. 3. Deactivation profile of *Chromobacterium viscosum* lipase after IPL treatment against exposure time.

Fig. 4. Reciprocal plotting of the *Chromobacterium viscosum* lipase deactivation profile.

3.1.2. Deactivation rate constant determination

To quantitatively assess the deactivation phenomenon of the lipase by IPL treatment, a mathematical technique of statistics was applied. In many previous research, deactivation of lipase was known to follow first-order kinetics (Tajchakavit & Ramaswamy, 1997; Soliva-Fortuny, Bendicho-Porta, & Martín-Belloso, 2006; Xu, et al., 2015), however, that was not proper to explain deactivation of the enzyme in some publications (Park, et al., 2013; Hong, et al., 2015). These reports indicated that the kinetic behavior of enzyme deactivation could be related to many factors, such as enzyme structure, energy source for deactivation, and etc. In the case of IPL-treated lipase deactivation profile, single-step first order kinetics was not appropriate, indicating the fractional rate of deactivation was different because of more than one type of bond that must be broken before a total loss of activity (Henley, et al., 1985). Therefore, a two-step series-type enzyme deactivation model was employed for analysis of enzyme deactivation kinetics:

where k_1 and k_2 are first-order deactivation rate coefficients (min^{-1}) for each step, E , E_1 and E_2 are homogeneous states with several specific activities

(initial, intermediate, and final state, respectively), and α_1 and α_2 are the ratios of specific activities (E_1/E and E_2/E , respectively). This kinetic model of enzyme deactivation was reported in literature (Henley, et al., 1985), and is expressed in Eq. 2 assuming that α_2 is equal to 0, which means that the final lipase activity (α_2) is totally deactivated by IPL treatment.

$$a = \left(1 + \frac{\alpha_1 k_1}{k_2 - k_1}\right) e^{-k_1 t} - \left(\frac{\alpha_1 k_1}{k_2 - k_1}\right) e^{-k_2 t} \quad [2]$$

In this equation, fractional remaining activity (a in Eq. 2) can be derived as a function of time. The parameters (k_1 , k_2 , and α_1) were calculated from the experimental data and the values are shown in Table 2.

In this case, initial enzyme state is more active than that of the intermediate ($\alpha_1 < 1$), and the enzyme was totally deactivated at the final state ($\alpha_2 = 0$). This biphasic behavior has been extensively discussed in various publications and may be exhibited by both soluble and immobilized enzymes. The convexity (toward the origin) exhibited by these curves is a function of the k_1 , k_2 , and α_1 values. Relatively higher values together with moderate values of k_2 and α_1 yield a higher degree of convexity. A higher degree of convexity implies more sensitivity to the rate of enzyme deactivation (Park, et al., 2013).

As shown in the Table 2, k_1 was greatly larger than k_2 . For instance, k_1 (9.05

h^{-1}) and k_2 (1.64 h^{-1}) at 14.86 mJ/cm^2 were 3.04, 3.88-fold higher than those at 8.79 mJ/cm^2 (2.98 h^{-1} and 0.42 h^{-1}), respectively. From the results in Table 2, it can be concluded that the lipase activity decreases more rapidly in both phases (from E to E_1 and from E_1 to E_2) at higher treatment pulse fluence.

Table 2. Deactivation kinetic parameters of the lipase after IPL treatment

mJ/cm²	α_1	k_1 (h⁻¹)	k_2 (h⁻¹)
14.86	0.2878	9.0535	1.6424
13.87	0.4245	7.1534	1.1145
11.89	0.5878	5.6453	0.7454
8.79	0.7945	2.9770	0.4234

3.1.3. Effect of total fluence on lipase deactivation

It was observed that the deactivation of lipase was affected by two factors (exposure time and pulse fluence). According to Bunsen-Roscoe reciprocity law, radiation to a subject shows same efficiency when the total fluence is reached with high pulse fluence and short exposure time or low pulse fluence and long exposure time. This principle of equi-effectivity is a general rule in photochemical reactions (Gómez-López, Ragaert, Debevere, & Devlieghere, 2007). Therefore, it was postulated that if the same total fluence is delivered to lipase, the activity will decrease in the same extent ($[\text{total fluence}] \propto [\text{exposure time}] \times [\text{pulse fluence}]$). In previous studies, Bunsen-Roscoe law was used for the investigation of the effect of IPL on microorganisms. Although there were several cases that obeyed the law (Otaki, Okuda, Tajima, Iwasaki, Kinoshita, & Ohgaki, 2003; Rice & Ewell, 2001; Wang, MacGregor, Anderson, & Woolsey, 2005), the adverse cases, following the peak power effect, were also introduced (Sommer, Haider, Cabaj, Heidenreich, & Kundi, 1996; Takeshita, et al., 2003). Thus, this law is quite controversial in the case of IPL.

As in the case of lipase (Fig. 5), the degree of deactivation was different despite of the same total fluence. From the result, more deactivation of lipase

was observed at higher pulse fluence at each input energy state. When the total fluence reached 50 J/cm², the remained fractional activity was 26.0, 38.1, 46.2, and 56.2% at pulse fluence of 14.86, 13.87, 11.89, and 8.79 mJ/cm², respectively. The result indicated that deactivation of lipase is hard to be predicted by the Bunsen-Roscoe law, therefore, two variables (exposure time and pulse fluence) may not have equivalent effect on enzyme deactivation. The reciprocal plot between residual activity and total fluence also showed a good correlation ($R^2 \geq 0.985$), and this indicates that same amount of total fluence has contrasting impact on the lipase activity because of the different effect of IPL treatment on the lipase according to pulse fluence. And through the linearized plot, it could be possible to calculate residual activity of lipase by exposure time and pulse fluence.

Fig. 5. Deactivation profile of *Chromobacterium viscosum* lipase according to IPL total fluence increase.

Fig. 6. Reciprocal plotting of the *Chromobacterium viscosum* lipase deactivation profile according to total fluence increase.

3.2. Evaluation of critical point for deactivation

In order to confirm the unequal influence of deactivation factors, pulse fluence was lowered below 8.79 mJ/cm^2 to evaluate the aspects of deactivation. In the researches of other fields, it was studied that small molecules, such as HNCO, have the dissociation threshold (Brown, Berghout, & Crim, 1996). From the information, it was assumed that proteins would also have a certain energy point for degradation. Thus, it was analyzed using possible lowest energy of the IPL system in terms of residual activity.

When $\pm 10\%$ change was set to no significance, the activity was reduced above 2.77 mJ/cm^2 as expected. However, the activity was not decreased below 2.66 mJ/cm^2 (Fig. 7), although the exposure time and total fluence were 1.50-fold longer and 1.44-fold higher compared to those at 2.77 mJ/cm^2 , which indicated that the activity is not affected by exposure time below a certain pulse fluence value. This implied the existence of a critical point, so that two different phases were thought to exist for deactivation (susceptible and resistant to IPL). To conclude from the data, pulse fluence is thought to be superior to exposure time because the activity of lipase was decreased more rapidly at higher pulse fluence with short exposure time, which indicated the peak power dependence of the lipase, and the activity was not affected by

exposure time below the critical point.

Fig. 7. Residual activity (%) of *Chromobacterium viscosum* lipase after IPL treatment below 2.91 mJ/cm².

3.3. Structure modification by IPL treatment

3.3.1. Secondary structure analysis of lipase

The secondary structure of the *Chromobacterium viscosum* lipase was studied using CD spectroscopy in Far-UV region (190-260 nm) to assess the effect of IPL on secondary structure of the lipase. In theory of CD spectroscopy, α -helix proteins have negative bands at 208 nm and 222 nm and a positive band at 193 nm and proteins with β -sheet have a negative band at 218 nm and a positive band at 195 nm, whereas disordered proteins such as β -turn and random coil have negative bands at 189 nm and 198 nm and positive bands at 210 nm and 212 nm, respectively (Greenfield, 2006).

To analyze the effect of IPL on *Chromobacterium viscosum* lipase by time flow, Far-UV CD spectra were obtained after IPL treatment of 10, 20, and 30 min. The results of CD spectra were shown in Figure 8. There were no significant fluctuations of CD spectra in secondary structure analysis. Moreover, the secondary structure composition of *Chromobacterium viscosum* lipase was analyzed from CD spectra, the number of amino acids in sequence, and molecular weight of the lipase (Table 3). Although the activity of *Chromobacterium viscosum* lipase was lost after IPL treatment, the composition of lipase was not changed dramatically. A small change was

detected in the α -helix and antiparallel β -sheet (both maximally 1.2%), but no constant changing trend was found according to the exposure time increase.

Fig. 8. Far-UV CD spectra of IPL-treated *Chromobacterium viscosum* lipase.

Table 3. Content (%) of *Chromobacterium viscosum* lipase secondary structure elements at different IPL exposure time

Secondary structure	Control	10 min	20 min	30 min
α-Helix	22.2	22.7	21.9	21.0
Antiparallel β-sheet	17.7	18.4	18.1	18.9
Parallel β-sheet	9.4	9.5	9.4	9.6
Random coil	33.8	33.8	33.7	33.9
β-Turn	16.8	16.7	16.8	16.6

3.3.2. Assessment of tertiary structural changes by IPL treatment

As no distinct change was found in CD analysis, fluorescence spectroscopy was applied for tertiary structural change of the lipase. A fluorescence spectroscopy has been widely employed for aromatic amino acid analysis in a protein. Typically, excitation and emission of the amino acids occurs at 280 nm and 280-340 nm in fluorescence analysis, respectively. Tryptophan is a crucial fundamental fluorescent probe because the amino acid arrangement can affect the microenvironment and the quantum yields of the tryptophan. Tryptophan usually locates in the hydrophobic core of a protein, however, when the protein structure is denatured, a red-shift and decrease of fluorescence intensity in emission spectrum appear due to the exposure of the residue to an hydrophilic environment. In contrast, micro-environmental change from hydrophilic to hydrophobic will cause blue-shift and increased intensity of emission spectrum (Chen & Barkley, 1998; Vivian & Callis, 2001).

For the analysis of fluorescence emission spectra, IPL was treated on *Chromobacterium viscosum* lipase for 10, 20, and 30 min at 14.86 mJ/cm². As shown in Figure 9a, fluorescence emission was decreased in proportion to the exposure time, and λ_{max} shift was observed from 332 nm to 336 nm (Fig. 10). By contrast, in the case of 2.66 mJ/cm² treated lipase in Figure 9b,

fluorescence emission spectra was not significantly changed and no λ_{max} shift was observed.

From the CD and the fluorescence spectroscopy data, it was evident that the deactivation of *Chromobacterium viscosum* lipase by IPL treatment occurs along with the tertiary structural changes. And decreased fluorescence of the protein after IPL treatment is in accordance with another literature (Elmnasser, et al., 2008).

Fig. 9. Fluorescence emission spectra of IPL-treated *Chromobacterium viscosum* lipase after 280 nm excitation at (a) 14.86 mJ/cm² and (b) 2.66 mJ/cm².

Fig. 10. Variation of fluorescence maximum emission wavelength of *Chromobacterium viscosum* lipase after IPL treatment.

3.4. SDS-PAGE

Protein structural change by IPL treatment is known as degradation of protein into peptide fragments in literature. SDS-PAGE was conducted to compare this information with the case of lipase. An illustration of control and IPL-treated lipase SDS-PAGE profile is in Figure 11. By SDS-PAGE result showing a single band with approximately 32 kDa throughout the experiments (Taipa, Aires-Barros, & Cabral, 1992), it was confirmed that *Chromobacterium viscosum* lipase protein has high purity without other bands.

SDS-PAGE profiles showed that the amount of intact lipase was reduced in a time dependent manner and uncertain trace bands appeared below the main band. A significant reduction in band intensity was observed after 30 min of treatment at 14.86 mJ/cm². However, no distinct bands were detected in repeated tests above the control protein, in which demonstrates aggregation such as formation of dimers. The SDS-PAGE gel densitometry analysis verified the pattern of degradation (Fig. 12). The data were normalized with respect to the control value. These findings indicated that IPL treatment on the enzyme plays a role in decreasing the activity of lipase by fragmentation. Similar results of decrease in protein band intensity on SDS-PAGE were found in literature using the soy allergen proteins glycinin and β -conglycinin (Yang,

Chung, Ajayi, Krishnamurthy, Konan, & Goodrich-Schneider, 2010), and tropomyosin in shrimp (Shriver, Yang, Chung, & Percival, 2011). While, the SDS-PAGE profile of the IPL-treated lipase at 2.66 mJ/cm² showed no difference in band intensity compared to the control band (Fig. 13). This explained that the reason for maintenance of lipase activity is due to the unharmed protein structure.

The major possibilities of fragmentation by IPL treatment has been attributed to photo-chemical, photo-thermal, and photo-physical reactions of protein due to UV light irradiation (Krishnamurthy, Demirci, & Irudayaraj, 2007). It was reported in publications that UV light induces cross-linking, agglomeration, polymerization, and fragmentation (Greenberg, 1979; Friso, Barbato, Giacometti, & Barber, 1994; Curley & Lawrence, 1999). Currently, the mechanism for IPL induced structural change is not exactly revealed, therefore, further research is needed to clarify the reason for the effect of IPL treatment on protein.

Fig. 11. SDS-PAGE profile of untreated and IPL-treated *Chromobacterium viscosum* lipase with different exposure time (10, 20, 30, 40, and 60 min) at 14.86 mJ/cm².

Fig. 12. Quantitative densitometry analysis of the *Chromobacterium viscosum* lipase band using ImageJ software.

Fig. 13. SDS-PAGE profile of untreated and IPL-treated *Chromobacterium viscosum* lipase with different exposure time (150, 180, and 240 min) at 2.66 mJ/cm².

3.5. MALDI-TOF

3.5.1. Fragmentation pattern analysis

As the SDS-PAGE profile showed the degradation of lipase structure, further analysis was needed to confirm the fragmentation pattern. Although there were reports that proposed the irregular fragmentation by IPL treatment (Janve, et al., 2014), they did not show any distinct results. Thus, the fragments in the IPL-treated samples were evaluated to obtain the evidence. To identify the fragmentation pattern, molecular weights of the fragments were directly analyzed using MALDI-TOF. As the fragments were mainly observed below m/z 4,000 from the preliminary tests, the analysis was conducted in this region.

Based on the proposed mechanism in the previous literature, it was postulated that if the fragmentation occurs irregularly while exposed to IPL, the result spectra would not show a tendency over increasing IPL exposure time. Therefore, samples (10, 30, 50, and 60 min) were prepared to verify the assumption. However, the result spectra showed similar peak patterns (Fig. 14). Particularly, the molecular weights of the fragments (S/N ratio ≥ 100) displayed remarkable consistency within error range through the time points, although the IPL treatment was conducted separately for each sample. Only 8 out of total 50 fragments had individual molecular weights within the data

(Table 4). In conclusion, the result demonstrated that site-selective fragmentation was induced by IPL treatment. Furthermore, it could be inferred from the data that the internal sensitive bondings of the lipase were cleaved preferentially by IPL, thus specific fragments were formed.

Fig. 14. MALDI-TOF spectra of fragments from *Chromobacterium viscosum* lipase after 10, 30, 50, and 60 min IPL treatment.

3.5.2. Cleavage site estimation using FindPept[®] tool

Meanwhile, although the fragmentation pattern by IPL treatment was identified, the cleavage site was not verified from MALDI-TOF analysis. There were two basic possibilities of cleavage: peptide bond cleavage or covalent bond within the amino acid (including peptide bond). Because the sequences of the fragments could not be analyzed directly due to the limitations such as too low concentration and purity, indirect way was employed. As a tool for fragment analysis, FindPept[®] was chosen, which is used to find the site of unspecific proteolytic cleavage, missed cleavage, or protease autolysis (Gattiker, Bienvenut, Bairoch, & Gasteiger, 2002). The measured molecular weights of the fragments were compared to that of the theoretical peptides from the lipase sequence and cleavage site was determined from the results. Because FindPept[®] tool considers peptide bond cleavage, unmatched data are evidences for random-bond cleavage.

As shown in Table 4, the theoretical candidate peptides existed in more than 80% of identified fragments ($S/N \text{ ratio} \geq 100$) generated from IPL treatment, though exact cleavage site could not be identified from our data. Also, when the amino acids adjacent to cleavage sites were analyzed, top 5 amino acids accounted for 54.4% composed of alanine (12.4%), threonine (11.6%), glycine

(11.4%), serine (10.1%), and leucine (8.8%), which showed same tendency in each treatment time point. Among the 319 amino acids in the sequence of *Chromobacterium viscosum* lipase, the 5 residues consists of alanine (11.6%), threonine (11.0%), glycine (10.6%), leucine (9.7%), and serine (8.2%), respectively, which accounts for 51.1% of the sequence. Moreover, the ratio of rest 15 amino acids in the sequence showed similar trends with the top 5 residues. Thus from the results of fragment analysis, it was found that the more number of particular amino acids exists in the sequence, the more cleavage occurs by IPL. However, as the result was obtained from the prediction using FindPept[®], the cleavage sites should be investigated by more precise and direct way to prove this conclusion in the future work.

Table 4. Identified molecular weights and theoretical positions of the fragments in the sequence of *Chromobacterium viscosum* lipase

Exposure time	Analyzed mass (Da)	Number of fragments	Position in protein*
10 min	1126.622	1	97(A)-108(T)
	1154.616	4	3(T)-12(I), 7(T)-16(G), 289(I)-299(N), 298(A)-308(R)
	1187.603	1	155(N)-165(R)
	1463.799	1	285(H)-297(G)
	1569.738	2	191(Q)-206(L), 259(S)-273(F)
	1576.849	2	101(P)-115(R), 300(A)-313(R)
	1763.919	3	255(I)-271(S), 256(N)-272(L), 283(W)-297(G)
	1898.958	5	148(T)-165(R), 149(L)-166(T), 150(V)-167(L), 153(S)-170(A), 274(G)-289(I)
	1900.977	6	151(S)-168(T), 238(N)-256(N), 241(D)-259(S), 244(T)-262(N), 247(L)-265(L), 282(H)-297(G)
	1926.958	-	-
	2007.019	1	281(Y)-296(R)
	2064.042	4	137(T)-156(T), 205(L)-224(T), 216(P)-237(A), 281(Y)-297(G)
	2252.121	3	82(N)-103(L), 166(T)-187(P), 279(T)-297(G)
	2415.225	1	143(V)-165(R)
	2593.263	3	2(D)-25(N), 48(N)-71(Q), 263(D)-285(H)
	2679.344	6	123(V)-148(T), 160(A)-185(G), 161(L)-186(A), 267(S)-289(I), 274(G)-296(R), 275(Q)-297(G)
	2736.375	4	150(V)-175(Y), 163(A)-189(S), 274(G)-297(G), 276(V)-299(N)

Table 4. (Continued)

	2758.387	2	117(S)-142(F), 197(E)-223(V)
	2766.358	1	133(G)-159(D)
	2902.466	7	108(T)-134(L), 143(V)-170(A), 193(G)-221(L), 194(A)-222(G), 213(A)-242(P), 229(T)-257(R), 242(P)-270(S)
	1098.622	1	288(E)-297(G)
	1126.614	1	97(A)-108(T)
	1154.614	4	3(T)-12(I), 7(T)-16(G), 289(I)-299(N), 298(A)-308(R)
	1187.599	1	155(N)-165(R)
	1207.638	1	300(A)-310(H)
	1397.708	3	117(S)-128(K), 119(F)-130(D), 286(L)-298(A)
	1463.793	1	285(H)-297(G)
	1498.728	3	42(A)-55(D), 117(S)-129(T), 152(S)-165(R)
30 min	1577.837	5	38(Q)-52(F), 39(S)-53(Q), 40(H)-54(S), 196(T)-209(W), 284(N)-297(G)
	1763.908	3	255(I)-271(S), 256(N)-272(L), 283(W)-297(G)
	1900.972	6	151(S)-168(T), 238(N)-256(N), 241(D)-259(S), 244(T)-262(N), 247(L)-265(L), 282(H)-297(G)
	1926.950	-	-
	2064.033	4	137(T)-156(T), 205(L)-224(T), 216(P)-237(A), 281(Y)-297(G)
	2069.009	5	117(S)-135(S), 118(E)-136(S), 173(A)-193(G), 174(T)-194(A), 276(V)-292(L)

Table 4. (Continued)

	2252.111	3	82(N)-103(L), 166(T)-187(P), 279(T)-297(G)
	2593.252	3	2(D)-25(N), 48(N)-71(Q), 263(D)-285(H)
	2758.376	2	117(S)-142(F), 197(E)-223(G)
	2766.348	1	133(G)-159(D)
	864.390	1	229(T)-237(A)
	1098.597	4	288(E)-297(G), 293(L)-303(P), 295(V)-305(A), 296(R)-306(V)
	1126.589	1	168(T)-177(R)
	1154.580	2	3(T)-12(I), 298(A)-308(R)
	1187.567	1	155(N)-165(R)
	1267.646	2	3(T)-13(L), 207(Y)-218(S)
	1463.754	1	285(H)-297(G)
	1498.688	3	42(A)-55(D), 117(S)-129(T), 152(S)-165(R)
50 min	1533.692	3	189(S)-204(H), 194(A)-208(S), 197(E)-210(G)
	1535.705	2	49(L)-63(E), 173(A)-187(P)
	1577.798	7	19(G)-31(Y), 20(T)-32(G), 38(Q)-52(F), 39(S)-53(Q), 40(H)-54(S), 196(T)-209(W), 284(N)-297(G)
	1601.793	7	32(G)-46(V), 35(S)-49(L), (36(D)-50(S), 81(V)-95(Y), 82(N)-96(V), 166(T)-179(F), 251(G)-266(V)
	1760.770	1	51(G)-67(A)
	1763.858	3	255(I)-271(S), 256(N)-272(L), 283(W)-297(G)

Table 4. (Continued)

	1900.919	4	151(S)-168(T), 238(N)-256(N), 247(L)-265(L), 282(H)-297(G)
	1912.915	-	-
	1923.836	2	25(N)-40(H), 51(G)-68(Y)
	1926.894	-	-
	2063.976	3	137(T)-156(T), 216(P)-237(A), 281(Y)-297(G)
50 min	2068.954	5	117(S)-135(S), 118(E)-136(S), 173(A)-193(G), 174(T)-194(A), 276(V)-292(L)
	2074.920	4	47(A)-66(L), 48(N)-67(A), 53(Q)-71(Q), 143(V)-162(A)
	2150.983	4	51(G)-70(K), 142(F)-161(L), 167(L)-187(P), 280(S)-297(G)
	2252.050	2	166(T)-187(P), 279(T)-297(G)
	2279.034	2	25(N)-44(V), 51(G)-71(Q)
	2286.959	-	-
	2465.122	2	48(N)-70(K), 50(S)-72(V)
	2593.180	2	48(N)-71(Q), 263(D)-285(H)
	2766.268	1	133(G)-159(D)
	2778.267	3	216(P)-244(T), 265(L)-288(E), 266(V)-289(I)
	868.393	4	1(A)-8(R), 30(W)-36(D), 49(L)-56(D), 285(H)-291(Q)
	951.433	-	-
	1023.575	1	308(R)-315(K)

Table 4. (Continued)

	1069.571	1	83(L)-93(S)
	1081.605	1	9(Y)-18(A)
	1098.604	4	288(E)-297(G), 293(L)-303(P), 295(V)-305(A), 296(R)-306(V)
	1126.605	2	97(A)-108(T), 168(T)-177(R)
	1154.595	2	3(T)-12(I), 298(A)-308(R)
	1187.579	1	155(N)-165(R)
	1267.653	2	3(T)-13(L), 207(Y)-218(S)
	1325.686	2	76(T)-88(Q), 136(S)-148(T)
	1535.732	3	49(L)-63(E), 140(A)-154(H), 173(A)-187(P)
	1569.720	2	191(Q)-206(L), 259(S)-273(F)
60 min	1577.828	5	38(Q)-52(F), 39(S)-53(Q), 40(H)-54(S), 196(T)-209(W), 284(N)-297(G)
	1601.821	8	32(G)-46(V), 35(S)-49(L), 36(D)-50(S), 81(V)-95(Y), 82(N)-96(V), 89(G)-104(V), 166(T)-179(F), 251(G)-266(V)
	1763.903	3	255(I)-271(S), 256(N)-272(L), 283(W)-297(G)
	1898.942	5	148(T)-165(R), 149(L)-166(T), 150(V)-167(L), 153(S)-170(A), 274(G)-289(I)
	1900.961	6	151(S)-168(T), 238(N)-256(N), 241(D)-259(S), 244(T)-262(N), 247(L)-265(L), 282(H)-297(G)
	1926.933	-	-
	2064.027	4	137(T)-156(T), 205(L)-224(T), 216(P)- 237(A), 281(Y)-297(G)

Table 4. (Continued)

2252.111	3	82(N)-103(L), 166(T)-187(P), 279(T)-297(G)
2279.101	2	25(N)-44(V), 51(G)-71(Q)
2287.019	-	-
2415.216	1	143(V)-165(R)
2593.253	3	2(D)-25(N), 48(N)-71(Q), 263(D)-285(H)
2736.341	4	150(V)-175(Y), 163(A)-189(S), 274(G)-297(G), 276(V)-299(N)
2758.350	2	117(S)-142(F), 197(E)-223(V)
2902.453	7	108(T)-134(L), 143(V)-170(A), 193(G)-221(L), 194(A)-222(G), 213(A)-242(P), 229(T)-257(R), 242(P)-270(S)

* Sequence number (amino acid) - Sequence number (amino acid)

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국문초록

비가열 처리의 한 종류인 광펄스는 최근 미국 등 일부 선진국에서 식품·약품 표면 살균 및 포장재 살균에 소규모로 적용되고 있고, 산업 적용을 위한 대형화 과정을 거치며 새로이 주목받고 있는 기술이다. 이 기술을 *Chromobacterium viscosum* lipase (triacylglycerol hydrolase, EC 3.1.1.3)에 적용할 경우 효소의 활성과 구조가 받는 영향을 살피는 연구를 수행하였고, 연구 결과를 응용하여 실제 산업에서 식품 내부의 효소 활성 제어에 광펄스를 응용할 수 있는 근거를 마련하고자 하였다.

Lipase에 에너지를 달리한 광펄스를 처리한 후 reverse micelle system에서 활성의 변화를 살핀 결과, 활성이 감소하는 것이 확인되었으며 이는 에너지 밀도와 처리 시간이 증가함에 따라 감소량이 증가 하는 것이 확인되었다. Lipase의 활성 감소현상을 보다 구체적인 응용에 적용하기 위해, two-step series type deactivation kinetic model을 이용하여 활성 감소를 정량적으로 해석하였다. 그 결과, 14.86 mJ/cm²에서의 k_1 과 k_2 값(9.05 h⁻¹, 1.64 h⁻¹)은 8.79 mJ/cm²에서의 상수 값(각 2.98 h⁻¹, 0.42 h⁻¹)보다 각각 3.04배, 3.88배가 높게 확인되었다. 한편, lipase의 활성은 투입된 에너지 총량에 의해서도 감소 경향을 보였고, 동일한 총 에너지 투입 시 처리하는 에너지 밀도가 클수록 급격한 활성 감소를 나타내었다. 이러한 경향을 통해, 에너지 밀도에 따른 활성 변화 경향을 살피고자 에너지 밀도를 낮추며 활성 변화를 살폈다. 그 결과, 2.77 mJ/cm² 이상의 에너지에서 활성 감소 현상을 보였으나, 2.66 mJ/cm² 이하를 처리하였을 때는 활성이 감소되지 않았다.

이러한 현상은 광펄스가 lipase에 특정 에너지를 기준으로 두 가지 다른 방식으로 영향을 주기 때문으로 추측된다. 이어서 광펄스에 의한 효소의 활성 감소 현상에 대한 원인을 밝히기 위해 구조적 분석을 진행하였다. Circular dichroism 기법을 통한 2차 구조 분석에서는 변화가 확인되지 않았으나, fluorescence spectroscopy 분석을 진행한 결과 3차 구조의 변화가 나타났다. 광펄스에 의해 유발되는 lipase의 구조 변화 양상을 살피기 위해 SDS-PAGE를 진행한 결과, 32 kDa 부근의 lipase 밴드는 광펄스 처리 시간에 비례하여 감소 하였으며, 단편화에 의해 3차 구조가 변화됨이 확인되었다. 광펄스에 의해 단편화가 단백질 서열 상 특정 위치에서 발생하는지 확인하기 위해 MALDI-TOF 분석을 진행하였고, 그 결과 특정 위치에서 단편화가 일어났다. 또한, FindPept tool[®]을 이용하여 생성된 조각들의 분자량과 이론상 펩타이드의 관계를 살핀 결과, 펩타이드 결합의 절단 가능성을 확인했다.

본 연구를 통해 광펄스가 lipase의 활성에 미치는 영향을 규명 하였으며, 구조적인 분석을 통해 광펄스에 의한 단백질의 단편화 현상을 살폈다. 이를 통해 광펄스에 의한 효소의 활성 감소를 정량적으로 분석할 수 있는 기반을 마련했고, 차후에는 이 기술을 효소의 제어가 필요한 산업에 실질적으로 적용할 수 있을 것으로 기대된다.