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Effect of Laser and LED on Enzymatic Production of Ceramide

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ABSTRACT

An enzyme (Phospholipase C Type I from *Clostridium perfringens*) was exposed to 0–810 J cm^{-2} of energy using laser light at wavelengths 808, 532, 1064 and 1342 nm and two LED light sources at wavelengths 810 and 640 nm. Enzyme responses were evaluated by measuring ceramide concentration using high performance thin-layer chromatography (HPTLC) at 0.5, 1, 2, 3, 4, 6, 17, 24 h after irradiation. The duration of effect was evaluated from the experimental data. The results show that enzyme activity can be increased by using both laser and LED sources whose wavelength is located within a certain range. The effect depends on the energy and wavelength of the light. The increase in enzyme activity continued for about 4 h after irradiation. This study shows that the duration of irradiation should be included as one of the main laser parameters when reporting on the effects of laser irradiation on enzymes. We also find that laser sources and LED sources have the same effect on enzyme activity if the wavelength and absorbed energy are equal.

INTRODUCTION

Laser radiation has been comprehensively explored in the context of cell diagnostics, clinical therapy, and microbiological studies (1,2). Laser technology has also been widely used to treat various skin diseases, neonatal jaundice (hyperbilirubinemia) and cancer (3,4). The field of enzyme application has developed at a fast pace in the last few decades. The number of enzyme-based products is on the rise, having increasingly important roles in food, environmental, pharmaceutical and cosmetic industry applications. Consequently, the application of laser technology in enzymology and biocatalysis has emerged as an interesting topic. If laser irradiation within a certain range of intensity and frequency has a positive effect on enzymatic reactions, then laser technology can be used to increase the reaction rate or yield in many enzyme applications. In terms of biological research, information on the effects of lasers on specific enzymatic reactions could also improve our understanding of their effects on entire biological systems. Unfortunately, only a limited number of publications have described the effects of lasers on specific enzymatic reactions. Satoshi *et al*. showed that thermal treatment using a 1547 nm laser (30–300 mW, spot size 50 μm, pulse duration 1 ns, exposure time 60 s) had a fatal influence on enzyme activity, while a femtosecond laser (0.4 mW, spot size 5 μm, pulse duration 200 fs, exposure time 1–10 s) and a pulsed UV laser (0.3 mW, spot size 25 μm, pulse duration 1 ns, exposure time 60 s) did not affect the protein integrity (5). Chen *et al*. also studied the effect of ultraviolet laser power on the enzyme activity of lactate dehydrogenase (LDH). In that study, LDH from bovine heart was exposed to 3.6–18 kJ m^{-2} of UV radiation in the 300 nm wavelength region and the degree of inactivity was found to be proportional to the amount of ultraviolet radiation power (6). However, the mechanism of these effects remains unclear.

Ceramide (Fig. 1A) is the crucial intermediate in the metabolism of all complex sphingolipids. Because of its major role in maintaining the water-retaining properties of the epidermis, ceramide has great commercial potential in cosmetic and pharmaceutical industries for use in hair and skin care products (7,8). However, chemical synthesis of ceramide is a costly and time-consuming process (9). Therefore, the development of alternative cost-efficient and high yield production methods is of great interest. Sphingomyelin (SM) is a ubiquitous component of animal cell membranes and is one of the major phospholipids in bovine milk. In SM, the ceramide part of the molecule is bound by a phosphodiester bridge to a choline moiety (Fig. 1B). Systematic investigation and optimization have been conducted for the enzymatic production of ceramide from SM hydrolysis (10) and phospholipase C (PLC) from *Clostridium perfringens,* and high activity towards the hydrolysis reaction has been demonstrated.

In the present study, the aim is to evaluate the potential of laser technology in enzymatic reactions, specifically the

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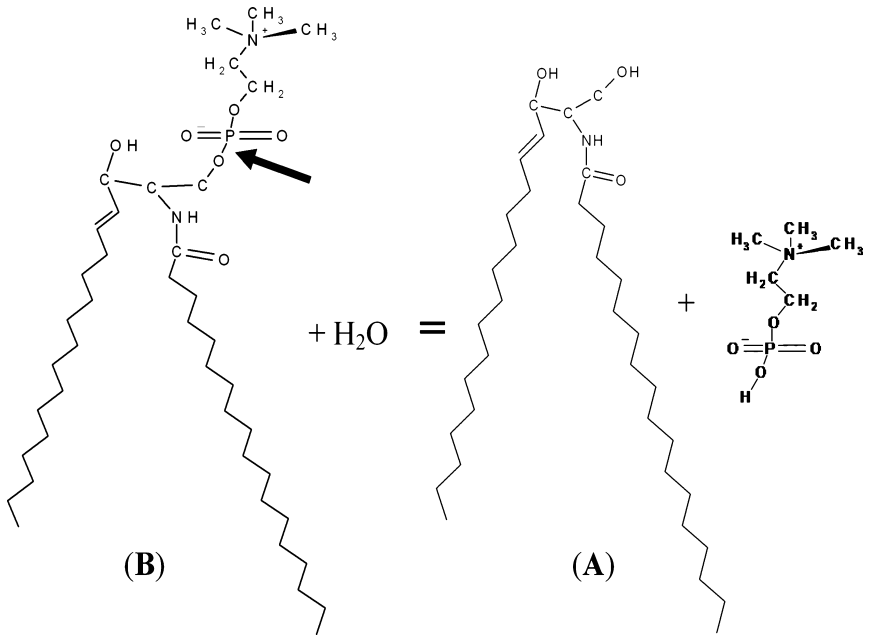

Figure 1. Hydrolysis reaction including the structures of ceramide (A) and sphingomyelin (SM) (B). The arrow indicates the bond to be cleaved in SM for producing ceramide.

enzymatic hydrolysis of SM to produce ceramide. The effect of laser or LED irradiation on the catalytic activity towards SM hydrolysis was examined using different wavelengths. Several factors such as power and energy were also investigated to optimize the conditions for ceramide production.

MATERIALS AND METHODS

Reference SM and ceramide from bovine brain, *C. perfringens* PLC (Type I) were purchased from Sigma-Aldrich Denmark A/S (Copenhagen, Denmark). Substrate SM was purified from Lacprodan PL-75 (phospholipids extract from milk fat globule membranes; Arla Foods Amba, Viby, Denmark) using mild alkaline hydrolysis (11). Standards and other chemicals were of analytic and chromatographic purity.

Experimental procedure. Reaction conditions for the enzymatic reaction have been previously described (12). SM solution (0.4 mg mL^{-1}) in ethylacetate:hexane (50:50 by vol.) was prepared and placed in an ultrasonic machine (Bie & Berntsen, Rødovre, Denmark) for 5 min at 37°C. Substrate solution (200 μL) was introduced into the reactor *via* a small glass tube (diameter, 0.9 cm; height, 7.4 cm). The reaction was initiated by adding 12 μL enzyme solution (0.026 mg protein per mL 0.01 M Tris(hydroxymethyl)aminomethane (Tris)-HCl buffer [pH 8.6] containing 25% ethanol). The reactor was sealed with a cap, and horizontal shaking was performed (200 rpm) at room temperature. After 10 min, 100 μL of the reaction mixture from the upper phase (organic solvent phase) was separated and stored at −20°C until further analysis. To study the time development of the hydrolysis reaction, the experiments were performed at various reaction time points (from 0 to 480 min). We also measured the temperature change of the reactant before and after irradiation, the temperature change was very small and so we think the reaction is in a static environment.

Irradiation process. We transferred a certain amount of enzyme and substrate, respectively, to small glass tubes (diameter, 0.9 cm; height, 7.4 cm), which were then irradiated with different light sources (640 nm LED, 810 nm LED, 808 nm laser, 532 nm laser, 1064 nm laser, 1342 nm laser, which were kindly provided by the Department of Physics, Technical University of Denmark, Lyngby, Denmark) at room temperature (Fig. 2). To ensure even irradiation of samples, a shaker (200 rpm) was used during the irradiation procedure.

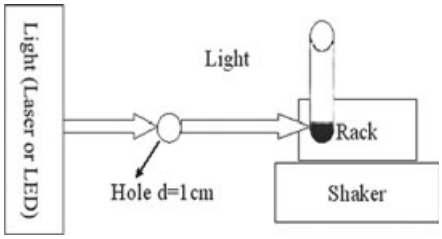

Figure 2. Reaction schematic figure. The samples in the reaction vessel were put on the rack which was fixed on the shaker and were irradiated by laser or LED through the wall of reaction vessel.

The four selected conditions of the laser irradiation were laser irradiation of the substrate solution before initiating the enzymatic reaction, laser irradiation of the enzyme solution before the reaction, laser irradiation during the reaction, and laser irradiation of both the substrate and the enzyme solutions before the reaction.

Ceramide analysis. Ceramide concentrations were quantified using high performance thin-layer chromatography (HPTLC) and *in situ* densitometry, as described previously (13). The HPTLC plate (Silica gel 60; E. Merck, Darmstadt, Germany) was prewashed in a chloroform/methanol mixture (2:1) in a horizontal developing chamber (Camag, Muttenz, Switzerland), and the plate was activated at 120°C for 30 min. Standards and samples were applied using DESAGA AS30 HPTLC Applicator (Sarstedt Ag & Co, Nümbrecht, Germany). Following equilibration and development with heptane:isopropanol:acetic acid (85:15:1) in the chamber, the plate was dried and sprayed with charring reagent (10% $CuSO_4$ in 8% H_3PO_4) until soaked. Lipids were visualized by heating the plate at 160°C for 6 min. The intensity of the spots was determined using DESAGA CD60 HPTLC densitometer (Sarstedt Ag & Co) at 390 nm in the absorbance/reflection mode. The lipid mass of each component was calculated with the software DESAGA ProQuant (1.03.200; Sarstedt Ag & Co) using a standard curve run on the same plate (Fig. 3). Because the reaction time (10 min) was controlled to ensure that the detected reaction rate was that of the initial reaction, the catalytic activity of the enzyme is expressed as the reaction rate in the present study and is shown as ceramide concentrations (μg mL^{-1}) after 10 min. The initial reaction rate was calculated as ceramide concentration divided by the reaction time.

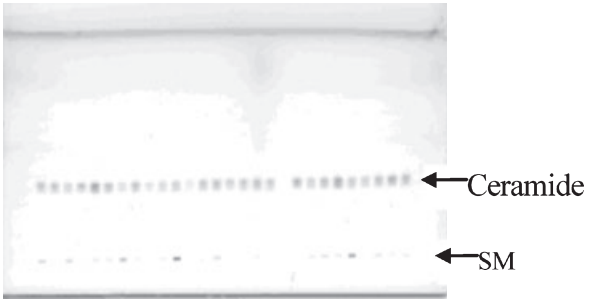

Figure 3. HPTLC plate from scanning. The lipids in the plate have been visualized by spraying with the charring reagent and heating. Ceramide shows double bands.

Statistical analysis. Data from the experiments performed in this study are presented as mean ± SD from triplicate samples ($n = 3$). Chi-square test was used to assess the statistical significance with a significance level of $P < 0.05$.

RESULTS

Laser irradiation effectively enhances enzyme activity

Laser irradiation influences the enzymatic reaction mainly through its effects on the enzyme. From the results (Fig. 4), laser irradiation on the enzyme solution only before the reaction had the most significant positive effect compared with laser irradiation of the substrate solution and laser irradiation during the reaction and no laser irradiation ($P < 0.01$, $P < 0.05$ and $P < 0.05$, respectively). However, there was no statistically significant effect ($P > 0.05$) when compared with laser irradiation for both substrate and enzyme solutions before the reaction. Laser irradiation of only the substrate (SM) solution had almost identical effect as laser treatment of the reaction mixture ($P > 0.05$). These results indicated that laser irradiation is able to increase not only the catalytic activity of the enzyme, but also the reaction activity of the substrates. However, laser treatment during the reaction had a negative effect because both the irradiated enzyme and the substrate received energy from the light, which in turn excited the molecules and rendered them unstable; hence, successful reactions were hindered.

The level of energy is crucial in determining the enhancement of enzyme activity. Based on the results, prelaser irradiation of the enzyme solution had the most significant effect on the enzymatic reaction. Thus, we applied laser irradiation on the enzyme solution before the reaction in the subsequent steps. This positive effect is probably due to the fact that laser irradiation enables the enzyme molecule to reach an activated state where the enzyme can catalyze the reaction at a higher rate. To identify this effect, the energy used in laser irradiation of the enzyme solution was kept constant, but the time duration and power parameters were altered. The energy was then calculated as power multiplied by irradiation levels. We can transfer a constant level of energy to the enzyme by altering both power and irradiation time. When the enzyme solution is irradiated by an 808 nm laser with constant energy (540 J), regardless of the power and time duration within a certain range, enzyme catalytic activity remained constant (Fig. 5). There was no statistically significant difference between any two of five irradiation conditions of varying time and power components ($P > 0.05$). Another experiment was also performed using different energy levels (0–900 J, 500 mW) while all the other variables remained constant (Fig. 6). Results showed that an increase in the catalytic activity of the enzyme is proportional to irradiation time within a certain energy range. Further prolonged irradiation did not increase catalytic activity.

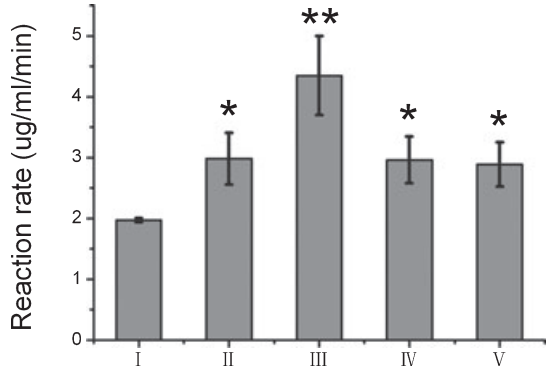

Figure 4. Laser irradiation effect on the enzymatic reaction. The catalytic activity of the enzyme and the reaction rate were shown as ceramide concentrations ($\mu g\ mL^{-1}$) after 10 min reaction time under various irradiation treatments: I. Control (without laser irradiation); II. Substrate solution irradiated with an 808 nm laser at 900 mW for 10 min; III. Enzyme solution irradiated with an 808 nm laser at 900 mW for 10 min; IV. Mixture of enzyme and substrate solution irradiated with an 808 nm laser at 900 mW for 10 min; V. Enzyme and substrate solution irradiated individually, and then mixed together. The data listed are the mean ± SD from triplicate samples, $n = 3$. *Statistical significance with a significance level of $P < 0.05$, II, IV, V (three different treatment group) compared with I, respectively. **Statistical significance with a significance level of $P < 0.01$, III (treatment group) compared with I.

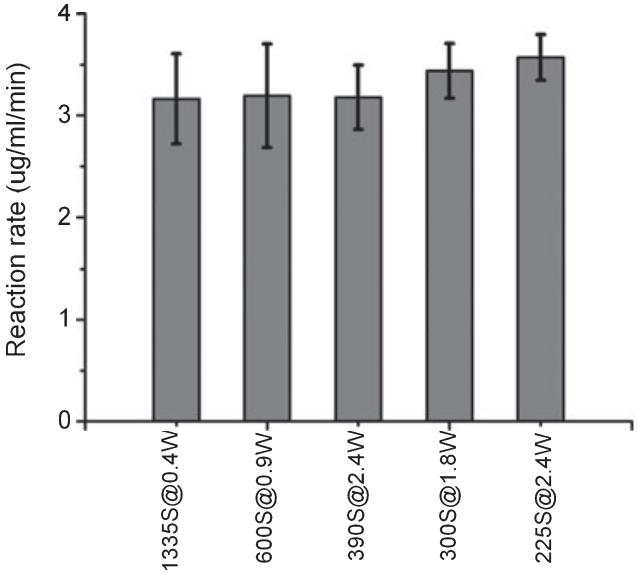

Figure 5. The reaction rate with the enzyme solution pretreated by 808 nm laser irradiation at the same energy under different power intensities. *X*S@*Y*W means laser irradiation time is *X* seconds at power *Y* W. The data listed are the mean ± SD from the triplicate samples, $n = 3$.

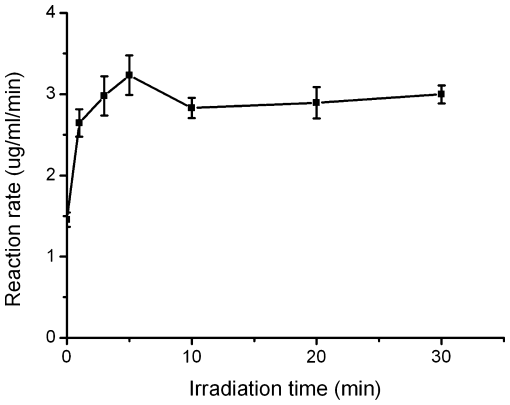

Figure 6. Reaction rate at different irradiation times using an 808 nm laser. Laser power is 500 mW. The data listed are the mean ± SD from the triplicate samples, $n = 3$.

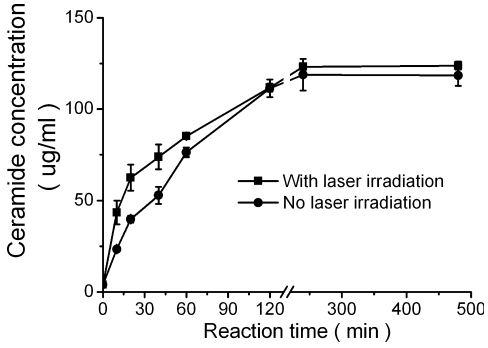

Figure 7. Time course of the hydrolysis reactions catalyzed by the enzyme with laser irradiation with and without pretreatment. Before the hydrolysis reaction, enzyme solution was irradiated using an 808 nm laser (power 900 mW and irradiation time 10 min). The data listed are the mean ± SD from the triplicate samples, $n = 3$.

Time development of hydrolysis catalyzed by enzyme pretreated by laser irradiation

It is expected that laser technology can be applied to improve reaction times in many enzyme applications. Consequently, we investigated the time development of laser irradiation on the SM hydrolysis reaction. The enzyme solution was irradiated using an 808 nm laser (900 mW, irradiation time 10 min), and the results (Fig. 7) show that laser irradiation on enzyme solution increased reaction rate significantly at the beginning of the reaction, compared with a process with no laser irradiation ($P < 0.05$). With a prolonged reaction time, the difference between these two experiments was reduced. Between 4 and 8 h of reaction time, the ceramide concentrations showed no statistically significant difference ($P > 0.05$). With laser irradiation of the enzyme solution before the reaction, the effect on the reaction was indirect by activating the enzyme. Theoretically, the addition of the enzyme is not able to alter the thermodynamic equilibrium of the reaction, *i.e.* the enzyme catalytic activity should have no effect on the final reaction yield. This explains why the results have no significant difference in ceramide concentrations from the above two reactions at the end of the hydrolysis.

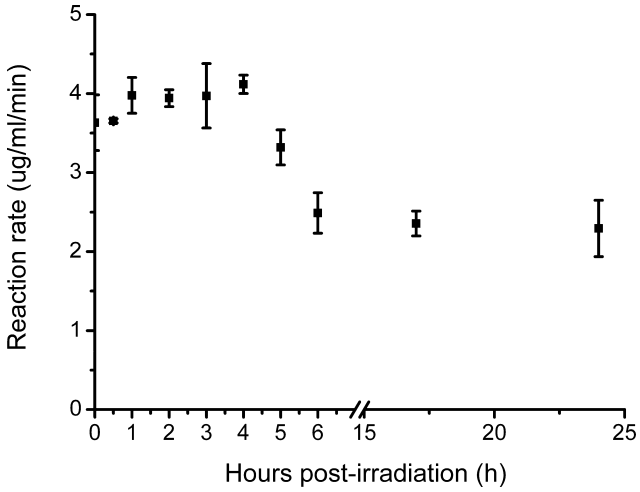

Figure 8. Effect of storage time of the enzyme activated by laser irradiation. Enzyme solution was irradiated with an 808 nm laser (power 900 mW and irradiation time 10 min). The data listed are the mean ± SD from the triplicate samples, $n = 3$.

Prolonged duration of enzyme activation by laser irradiation

One problem with most of the published data on laser irradiation is that they failed to investigate the duration of enzyme activation in the period after the irradiation (postirradiation). Undoubtedly, activity duration is a crucial factor in determining how the enzyme should be used if laser technology is to be applied on an industrial scale. Hence, in this study the effect of laser-activated enzyme storage time was further evaluated. Our experimental results indicate that, when the enzyme is treated with an 808 nm laser for 10 min at 900 mW, the enzymatic activity is higher than in other treatments (Fig. 4). After irradiation by the 808 nm laser at 900 mW for 10 min, the enzyme solution was further mixed with the substrate at the following time points: 0, 0.5, 1.0, 2.0, 3.0, 4.0, 6.0, 17.0, 24.0 h. The purpose was to detect the duration of the enzyme activation. From Fig. 8 we can see that the enzyme activation peaked at 4 h, proceeded to decline thereafter, and resumed a background level after 6 h.

Comparison between the laser and LED irradiations

If energy is constant, the effect of an 810 nm LED and an 808 nm laser on the enzyme reaction is basically the same. Because LED irradiation is more economical with less energy consumption than the laser counterpart, the application of LED might be more attractive in the large-scale enzymatic applications. However, only a few groups have endeavored to elucidate the effects of LED irradiation on enzymatic activity. Therefore, the present study provided a deeper understanding as to whether laser and LED irradiation have similar effects on the enzymatic reaction, and how the level of energy influences the catalytic activity of the enzyme irradiated with LED. The results (Table 1) show that when the irradiation energy was 0, 270, 540 and 810 J, the influence of the 808 nm laser was similar to that of the 810 nm LED ($P > 0.05$).

Furthermore, Fig. 5 illustrates that the effect of laser and LED irradiation on the catalytic activity of the enzyme depends on the irradiation energy. This result supports the hypothesis that enzymatic activity can only be enhanced when

Table 1. Reaction rate with enzyme irradiated by laser and LED with the same energy ($\bar{X} \pm SD$) ($P > 0.05$).

Energy (J)		0	270	540	810
Reaction rate ($\mu g\ mL^{-1}\ min^{-1}$)	808 nm laser	2.47 ± 0.06	3.36 ± 0.23	4.99 ± 0.48	5.00 ± 0.48
	810 nm LED	2.47 ± 0.06	3.43 ± 0.19	4.62 ± 0.37	4.62 ± 0.26

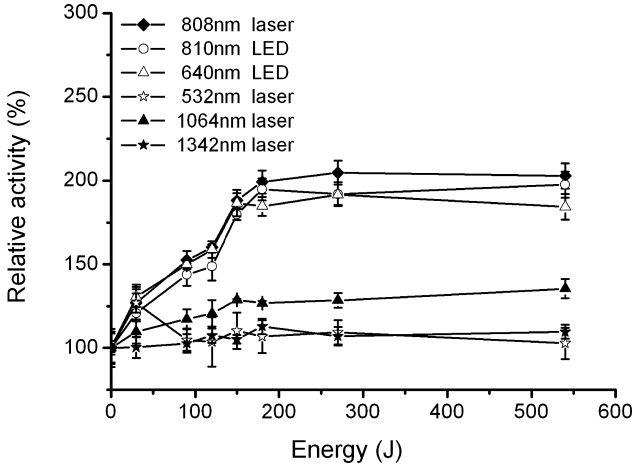

Figure 9. Effect of laser and LED irradiation energy under different wavelengths. Relative activity (Y-axis) is calculated using the following equation: $\beta = \frac{C1}{C0} \times 100\%$, where $C1$ is the reaction rate from the reaction catalyzed by light irradiation-activated enzyme and $C0$ the reaction rate from the reaction without laser irradiation. The data listed are the mean ± SD from the triplicate samples, $n = 3$.

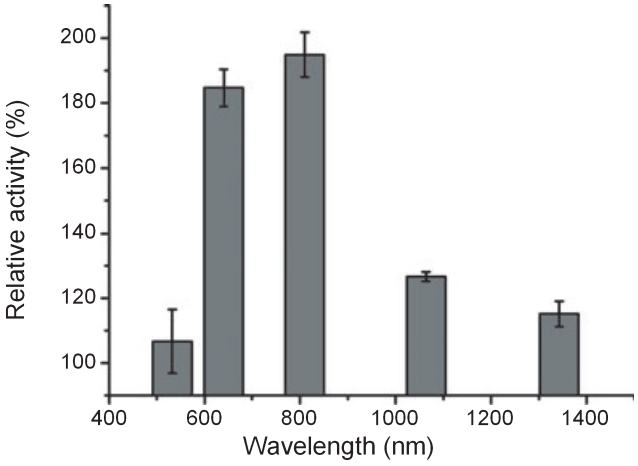

Figure 10. Comparison of laser irradiation under different wavelengths at 540 J energy. For relative activity see Fig. 9. The data listed are the mean ± SD from the triplicate samples, $n = 3$.

the enzyme obtains a certain level of energy (around 810 nm) in the form of laser irradiation or LED irradiation. We define this energy value as the threshold value. We assume that the change in enzyme activity in a macroscopic electromagnetic field can be evaluated by considering the state of a single enzyme molecule in the microscopic field. When the enzyme molecule obtains energy from a laser or LED, it changes its energy state from the ground state to an excited state. However, as the excited state does not appear to be stable, the enzyme molecule retreats back to the ground state. When the number of excited state enzyme molecules that fall to the ground state equals the number of ground state molecules elevated to the excited state, a dynamic equilibrium called the saturated state is achieved. When the energy obtained from the laser or LED irradiation is lower than the threshold value, the concentration of enzyme molecules in the excited state is small; the activity will not be enhanced significantly. On the other hand, when the energy level is equal or higher than that of the threshold value, enzyme molecules are excited into a saturated state, and the enzyme reaches its maximum activation level. When the enzyme receives more energy than the threshold value, enzyme activity cannot be further altered because enzyme molecules are already in the saturated state. This theoretical hypothesis is in accordance with the experimental results obtained in this study. To further validate the hypothesis, 532 nm laser, 640 nm LED instead of a 640 nm laser, 808 nm laser and 810 nm LED, 1064 nm laser and 1342 nm laser were all applied in the subsequent experiments. Figure 9 demonstrates that enzymatic activity was increased at high energy levels for light sources of wavelengths 808, 810 and 640 nm, but was not increased significantly with high energy for light sources at 532, 1064 and 1342 nm, suggesting that lights at these wavelengths could not influence the enzyme activity. No statistically significant difference was observed for the influence of the 808 nm laser and 810 nm LED ($P > 0.05$).

Obviously, the influence of light on enzyme PLC, which catalyzes SM hydrolysis to produce ceramide, depends on the wavelength. In accordance with this interesting finding, laser irradiation with an energy of 540 J was compared at different wavelengths. In Fig. 10, the profile of the light influence with different wavelengths is provided. The results suggest possible mechanisms by which the wavelength may potentially influence the irradiation effects of light on enzymatic activity.

DISCUSSION

Experimental results indicate that when PLC is irradiated by a certain wavelength laser or LED before and during the hydrolysis reaction, the reaction rate is enhanced significantly. The resulting data supports the hypothesis that the enzyme only is activated when it is exposed to adequate levels of energy from laser radiation in a certain wavelength range.

Effects of low-level laser irradiation on serum concentration of nitric oxide (NO), β-endorphin (β-end) and nitric oxide synthase (NOS) activity of mice were investigated by Chen *et al*. (14). Their results showed that laser irradiations using 532, 632.8 and 650 nm wavelengths (3 mW, 60 min irradiation time) evidently raise serum NOS activity and that β-end concentration increased significantly after irradiation at a laser wavelength of 532 nm. However, at wavelengths of 842 and 1300 nm, laser treatment had no significant influence on NO, NOS and β-end concentrations (14). This supports the conclusion made in this work on a PLC enzyme. Da Silva *et al*. also studied the effect of gallium–aluminum–arsenide (GaAlAs) 904 nm laser irradiation on enzyme activity and

they concluded that an optimum laser dose exists (in a range of 0.1–0.5 J cm^{-2}) at which the enzyme activity is maximal (15). In another study made by Kujawa, human red blood cells or isolated cell membranes were irradiated with low-intensity laser lights (810 nm, 10–400 mW, 3.75–25 J cm^{-2}) at 37°C (10). Near-infrared low-intensity laser irradiation was found to change the ATPase activities of the membrane ion pumps in the dose (16). Kilańczyk *et al*. (17) applied 670 nm laser with energy dose of 19.1, 38.2, 57.3, 76.4 and 95.5 J cm^{-2} to study the effect of laser lights on ATPase activity and found that irradiation with all energy doses applied caused a rise in enzymatic activity. However, in the presence of ZnPc, a significant decrease of Na+, K(+)-ATPase activity was observed. This finding is consistent with our results, especially in terms of the energy dose applied, which was larger than the threshold value of ATPase activity. Therefore all the energy doses are able to influence ATPase activity although only 670 nm laser was used. More work has been done on ATPase (18). Irradiations were carried out using a 685 nm laser (35 mW, 4–50 J cm^{-2}). The ATPase activity was not altered with exposition to irradiation doses between 4 and 24 J cm^{-2}. However, with irradiation doses ranging from 32 to 40 J cm^{-2}, a 28% increase on the ATPase activity was observed. When the energy increased further to 50 J cm^{-2}, there was no additional enhancement for the ATPase activity at the end. This means that an irradiation dose within certain energy range (32–40 J cm^{-2}) could influence ATPase activity, while out of this range it would have no effect on enzyme activity. This is partially consistent with the results in the present study.

The influence of lights on the PLC enzyme was studied using different wavelengths. We found the optimal wavelength on enzyme PLC to be 808 nm. Because the photon energy of green light (532 nm laser) is larger than its red counterpart (640 and 808 nm), it might be expected that green light should have a greater effect on the catalytic activity of PLC than red light. This, however, has not been supported by the experiments. The red light has an improved effect on the enzyme activity while the green light does not. We therefore conclude that the laser wavelength should be within a certain range. Outside this range, the laser irradiation could not influence the enzyme activity. The precise range may depend on the type of enzymes, as indicated by the above literature discussion.

CONCLUSION

In conclusion, the present study made a systematic investigation on the influence of light with different wavelengths on the catalytic activity of PLC. Light irradiation was shown to provide a feasible approach to enhance the enzymatic production of ceramide by SM hydrolysis. The results indicate that the enzyme activity was enhanced significantly by light in certain wavelength range, roughly within 532 and 1064 nm. The effect depends on the energy and wavelength of the light. The increase in PLC activity can continue for about 4 h after irradiation. We believe the results obtained from this study can provide valuable information for further developments and possible applications in industry.

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