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Non-thermal effects of 0.1 THz radiation on intestinal alkaline phosphatase activity and conformation*

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Alkaline phosphatase (ALP) plays an integral role in the metabolism of liver and development of the skeleton in humans. To date, the interactions between different-duration terahertz (THz) radiation and ALP activities, as well as the influence mechanism are still unclear. In this study, using the para-nitro-phenyl-phosphate (pNPP) method, we detect changes in ALP activities during 40-minute THz radiation (0.1 THz, 13 mW/cm^2). It is found that the activity of ALP decreases in the first 25 min, and subsequently increases in the later 15 min. Compared with the activity of ALP being heated, the results suggest that short-term terahertz radiation induces a decrease in enzyme activity through the non-thermal mechanism. In order to explore the non-thermal effects of THz radiation on ALP, we focus on the impacts of 0.1 THz radiation for 20 min on the activity of ALP in different concentrations. The results reveal that the activity of ALP decreases significantly after exposure to THz radiation. In addition, it could be deduced from fluorescence, ultraviolet-visible (UV-vis), and THz spectra results that THz radiation has induced changes in ALP structures. Our study unlocks non-thermal interactions between THz radiation and ALP, as well as suggests that THz spectroscopy is a promising technique to distinguish ALP structures.

Keywords: THz radiation, alkaline phosphatase, non-thermal effects, spectroscopy, conformations

PACS: 87.50.uj, 87.14.ej, 87.15.hp **DOI:** 10.1088/1674-1056/ab50fd

1. Introduction

Alkaline phosphatase (ALP) is a homodimeric enzyme, which exists across a multitude of organisms, prokaryotes and eukaryotes alike. It can catalyze the hydrolysis and transphosphorylation of phosphomonoesters.[1] In the human body, intestinal alkaline phosphatase plays a pivotal role in preserving gut homeostasis, regulation of intestinal surface pH, absorption of lipids, detoxification of free nucleotides and bacterial lipopolysaccharide, and attenuation of intestinal inflammation.[2–4] In addition, the decrease of intestinal ALP activity is related to many chronic inflammatory diseases such as inflammatory bowel disease (IBD).[5] Thus, it is crucial to have a better understanding of the factors that affect the alkaline phosphatase activity. One external factor that can significantly affects this biological function is the electromagnetic wave (EMW) radiation. It has been proved that EMW could activate changes in protein-energy states, resulting in modulation of various biological processes.[6] To date, many research efforts have been devoted to the interactions between EWM radiation and enzyme activity. It was reported that microwave radiation (at 968 MHz) for two hours could inhibit the L-lactate dehydrogenase (LDH) activity.[7] Infrared radiation (at 362 THz) for 15 min could increase the LDH activity.[8] Ultraviolet (UV) radiation for 1–2 days has been shown to be detrimental to the activity of ALP.[9]

The terahertz (THz) region is typically defined as an electromagnetic wave with frequencies ranging from 0.1 THz to 10 THz, which is located between the infrared (IR) and microwave (MW) regions. In the past 10 years, due to the development in applications of THz technology in security check, communication, and substance detection, considerable attention has been paid to the biosafety of terahertz radiation to human body.[10] It is important to note that THz waves have low photon energies (1 THz = 4.1 meV), one million times weaker than x-rays, and do not cause harmful photoionization in biological tissues. The biological influence mechanisms of THz radiation include thermal and non-thermal effects. On the one hand, since THz energy is strongly absorbed by solutions, higher doses of THz power are likely to induce thermal effects in biological materials, causing conformation changes and/or denature in biological molecules, morphological changes in cellular organelles, apoptosis in cells, etc.[10] On the other hand, several researchers have proposed that THz radiation can also induce nonthermal effects. It was initially hypothesized by Frohlich *et al.* in 1971,[11] and then more studies proposed that nonthermal effects are mediated through the direct coherent excitation of biomolecules[11] or linear/nonlinear resonance mechanisms.[12,13] Moreover, THz

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waves overlap the vibration and rotational levels with biological macromolecules.[14]

Considering the great matching of THz frequencies to collective vibrational modes of proteins,[15] it is interesting to explore the interactions between THz radiation and ALP activity. Much work so far has focused on the impacts of THz radiation on protein activity. A certain dose of pulsed radiation at a frequency of 3.2 THz (energy of 0–1.4 J) has been described to decrease the activity of lyophilized albumin, alcohol dehydrogenase, peroxidase and trypsin.[16] The activity of α-amylase could be affected by 0.87 THz (intensity of 7 mW/cm^2) radiation for 30 min.[17] It has been reported that 0.1 THz radiation with a low radiation power of 0.008 mW/cm^2 for two hours could decrease the activity of ALP in both soluble and immobilized states.[19]

In this contribution, ALP was radiated by high-power (13 mW/cm^2) 0.1 THz light-source, it was found that the ALP activity was depressed initially but gradually restored with further radiation. The collective, low frequency and high amplitude vibrational modes of biomolecules notably accord to frequencies in THz regions. In proteins, these modes are highly related to their biological activities.[20–22] Moreover, this was demonstrated in a number of studies that THz radiation could cause conformation changes of protein molecules in α-amylase,[17] bovine serum albumin,[23,24] and trypsin.[25] Accordingly, we deduced that THz radiation could risk the ALP activities by altering the structures of ALP. Therefore, to trace the influence mechanism of THz radiation to the enzyme, fluorescent, UV-vis, and terahertz time-domain spectroscopy techniques were carried out to investigate the conformational alterations of ALP.

2. Materials and methods

2.1. Samples and reagents

The alkaline phosphatase (from bovine intestinal mucosa) and *para*-nitrophenyl-phosphate (pNPP) were purchased from Sigma-Aldrich (Shanghai, China). The ALP solutions were prepared in Tris-HCl buffer (0.05 M, pH 7.4), and stored at 4 ℃ till used; pNPP was dissolved in the Tris-HCl buffer to 1 M NaOH for requirement.

2.2. Radiation experiments

ALP solution (150 μL) was added to one well of a 48-well transparent polystyrene cell culture plate (Corning, Shanghai). The well was exposed to 0.1 THz radiation using the radiation setup described in Fig. 1. The THz generator (Tera Sense Group Inc.) produced THz beam (continuous wave) with a frequency of 0.1 THz, power of 77.8 mW/cm^2 (88 mW), and a beam diameter of 1.2 cm. A terahertz polarizer was applied to decay the power to 13 mW/cm^2. The experiments were carried out at room temperature (about 28 ℃).

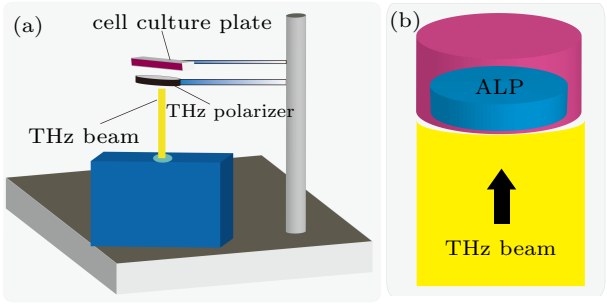


Fig. 1. Experimental setup for exposure of ALPs to 0.1 THz radiation. (a) Schematic representation of the THz exposure system. (b) Magnification of THz transmission and delivery optics.

2.3. Assays for ALP activity

The ALP activity was measured as previously recommended.[26] Briefly, 150 μL ALP solution was loaded into a 1.35 mL of pNPP solution (1 mM). Then the reaction mixture was incubated in a water bath (37 ℃) for 10 min. Finally, 750 μL NaOH (1 M) solution was added to terminate the reaction. The next step was to monitor the UV absorption of *p*-nitrophenol (pNP), generated in enzyme-catalyzed hydrolysis of pNPP. Shimadzu UV-240 spectrophotometer (Japan) was used at room temperature, scanning from 200 nm to 600 nm. The spectra were blankly corrected. A quartz cell with a 1.0 cm path length was assembled.

2.4. Fluorescence spectroscopy measurements

Among various optical spectroscopic techniques, fluorescence spectroscopy has been established as a credible tool to probe the biochemical conditions of proteins.[17] The fluorescence spectra of enzymes were contributed by their aromatic amino acid residues. Protein consists of several endogenous fluorophores, such as tryptophan (Trp), tyrosine (Tyr) and phenylalanine (Phe). In particular, Trp is sensitive to conformational changes in proteins.[27] It could be excited by UV light at 295 nm. Therefore, we measured the fluorescence of ALP at an excitation wavelength of 295 nm. All fluorescence measurements were performed on a Hitachi F-4010 fluorescence spectrophotometer at room temperature. Excitation was selected by a single monochromator at 295 nm respectively, and emission was scanned from 290 nm to 500 nm. Excitation and emission slit width was fixed at 5 nm. A 1.0-cm path length quartz cuvette was assembled. The spectra were corrected for the buffer background.

2.5. Ultraviolet-visible spectroscopy measurements

To further verify the alterations of protein structures, UV-vis spectroscopy as a powerful technique for probing protein structure was also employed. UV-vis absorption spectra of ALP were measured with a Shimadzu UV-240 spectrophotometer over the range of 150–350 nm at room temperature. A quartz cell with a 1.0-cm path length was used. The spectra were blankly corrected.

2.6. THz absorption measurements

The spectra of ALP solutions were collected at room temperature using a THz time-domain spectroscopy (THz-TDS) system, TAS7400 (Advantest Corporation, Japan; Fig. S1 in the supporting information). It worked with a dynamic frequency range of 0.1–4.0 THz, a frequency resolution of 1.9 GHz and an SNR of 60 dB. The electrically controlled dual lasers magnified the time evolution of the ultrafast transient signal arbitrarily in the time scale, based on the asynchronous optical sampling technique. The measurement time was substantially shortened to hundreds of milliseconds for one scan in this system. For each sample, three consecutive measurements were carried out and each measurement was an average 4096 scans. A quartz cell with a 0.1 cm path length was used. The spectra were blankly corrected.

3. Results and discussion

3.1. Effects of 0.1 THz radiation on ALP activity

The hydrolysis reaction of pNPP was adopted to compare the catalytic activity of ALP. We measured the ALP (5 U/mL) activity between 28 ℃ and 45 ℃ (Fig. S2 in the supporting information). It was found that increasing temperature in the range of 28–45 ℃ would enhance the catalytic activity of ALP, coinciding with many previous reports.[28,29] Since ALP is an important enzyme for the human being, the following measurements for the catalytic activity of ALP were all performed at the temperature of human bodies, 37 ℃.

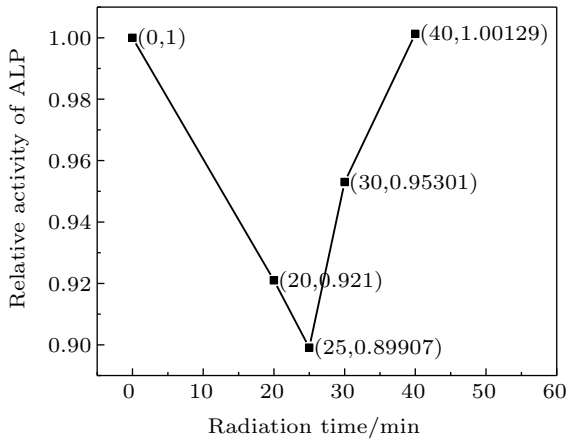


Fig. 2. The relative activities of ALP solutions (5 U/mL) in 40-min THz radiation, defined as the activity ratio of the different-duration radiated ALP to the non-radiated ALP.

We then examined the effects of 0.1 THz radiation with a power of 13 mW/cm^2 within 40 min on ALP activity for once. The relative activities of ALP solutions (5 U/mL), defined as the activity ratio of the radiated ALP to the non-radiated ALP, in different-duration THz radiation were illustrated in Fig. 2. When exposed to THz radiation, the activity of ALP declines initially and reaches the lowest point after about 25-min radiation. Further radiation leads to the gradual restoration of ALP activity and restores to the similar value of the natural enzyme after 40-min radiation.

Thus, using a thermocouple, we investigated the temperature changes of enzyme solutions after exposed to radiation for 40 min. The results showed that the average temperature increment induced by radiation was about 3–4 ℃. As can be seen from Fig. S1, enzyme activity rose as temperature increased. By contrast, our results (Fig. 2) show that the activity of ALP decreased during the first 25 min of radiation. This implies that the denaturation of ALP function during this period was mainly induced by THz non-thermal effects. In addition, the enzyme activity began to rise in the later 15 min. It might be mainly induced by THz thermal effects or a combination of thermal and non-thermal effects. However, we aimed at exploring the non-thermal effects of THz radiation on ALP activity. Hence, we focused on interactions between 20-min THz radiation and ALP in different concentrations. All the radiation experiments in different concentrations were repeated three times on different days to minimize the measurement error. Figure 3 shows the relative activities of ALP solutions in concentrations of 5 U/mL, 7 U/mL, and 9 U/mL with 20-min radiation. The average values of three independent experiments were placed on the columns (absorption spectra of pNP in different ALP concentrations were given in Fig. S3 in the supporting information).

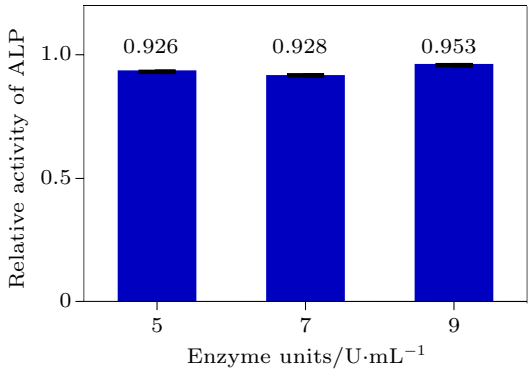


Fig. 3. The relative activities of ALP solutions in concentrations of 5 U/mL, 7 U/mL, and 9 U/mL with 20-min radiation, defined as the activity ratio of the radiated ALP to the non-radiated ALP.

As shown in Fig. 3, the ALP activity in radiation-treated groups was lower than the control groups. No significant concentration-related effects were found. The three experimental results have good consistency. Intestinal ALP is mainly distributed on the cell membrane, cytoplasm, microvilli and micro-villi surface of intestinal villus epithelial cells. It is reported that the activity of intestinal ALP increases by 10%–15% when the mouse of two days grows to three days. The reporter deduced that the lower ALP activity may affect intestinal absorption function.[30] In this experiment, alkaline

phosphatase decreased by about 10% after being radiated by the THz wave. We speculate that it may affect some intestinal functions, such as absorption function. Moreover, further *in vivo* experiments could be conducted to explore more about it.

In order to explore how short-duration (20 min) THz radiation affects the activity of ALP, we conducted three spectroscopy methods to measure changes of the protein conformations as will be described in the subsequent experiments.

3.2. Fluorescence spectra of ALPs

Fluorescence spectral profiles of ALP (5 U/mL, 7 U/mL, and 9 U/mL) in the absence and presence of radiation are shown in Fig. 4. When excited by UV light at 295 nm, the fluorescence intensities of Trp declined after exposed to 0.1 THz (13 mW/cm^2) radiation for 20 min. In addition, the fluorescence intensities showed a good positive correlation with the enzyme concentrations.

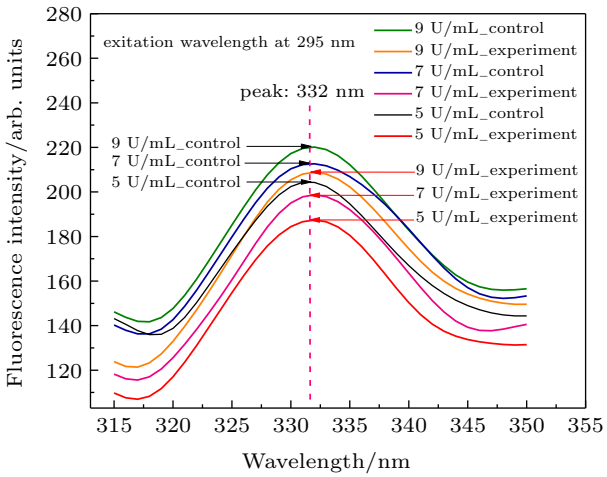


Fig. 4. Fluorescence emission spectra of ALP with excitation wavelength at 295 nm.

A decrease in fluorescence emission of residues may be induced by changes in disulfide, hydrogen bonds, and the metallic active centers of the enzymes (e.g., heme group).[31] Thus, changes in fluorescence emission of Trp may reflect the structural alterations in proteins.[32,33] In addition, many researches have reported that the modification of tryptophan residues in lysozyme,[34] α-amylase,[1] glucoamylase,[35] and Taka-amylase[36] was greatly related to the decrease of enzymes' catalytic activities. Accordingly, we inferred from the fluorescence results that the structures of ALP exposed to THz radiation have been modified, and the results demonstrated a good agreement with the enzyme activity results.

3.3. UV spectra of ALPs

We found that the concentrations of 5 U/mL, 7 U/mL, and 9 U/mL were too low to be detected distinctly by a UV spectrometer. Hence, we expanded the enzyme concentrations by 100 times. As displayed in Fig. 5, the UV absorbance spectra of ALP in 500 U/mL, 700 U/mL, and 900 U/mL were obtained. There were two absorbance peaks of ALP, one was at about 214 nm, and the other was at about 279 nm. All UV spectra of the three concentrations shared the same feature that the absorbances of radiation groups were lower. Again, the UV absorption was positively correlated to the enzyme concentration.

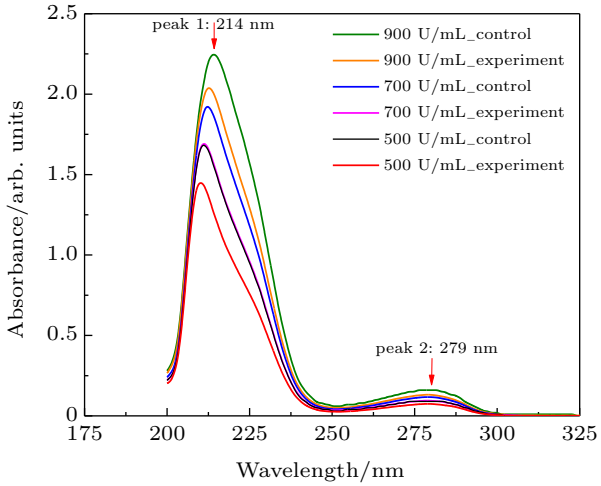


Fig. 5. UV absorption spectra of ALP in 500 U/mL, 700 U/mL, and 900 U/mL. There are two peaks in the spectrum, peak 1 is at about 214 nm, and peak 2 is at about 279 nm.

The features in the UV-vis absorption spectra of molecules are governed by collective oscillations of valence electrons. Additionally, UV-vis spectra could reflect the changes in characteristic structures of proteins upon binding with small molecules.[37] Generally, there are two main UV absorption peaks of enzymes. One is at 200–235 nm, dominated by peptide bonds, representing the framework conformation of protein.[38] The results showed that the absorbance at 214 nm in radiated groups was lower than that in control groups. The absorption of protein at 214 is contributed by n–π^* electron transition. In general, the reduce of peptide bonds absorption reflects its fold and denaturation, such as fragmentation, polymerization and cross-linking.[39] The absorption at 214 nm of the radiation groups and lower-concentration groups show a slight blue shift. We deduced that the terahertz radiation reduced the hydrophobicity of the microenvironment of protein molecules, resulting in an increase in n–π^* transition energy.[40] Another absorption peak is at 279–290 nm, the characteristic absorption peak of aromatic amino acid residues, mainly tryptophan (Trp) and tyrosine (Tyr).[41] These two residues are sensitive to their surrounding microenvironmental changes. Thus, they can reflect the flexible structural changes of proteins.[1] From characteristic peaks at 279 nm, we could observe that the absorption of radiation groups was also weaker. Accordingly, we inferred from these two UV results that the conformation of ALP has been modified by THz radiation (0.1 THz, 13 mW/cm^2, 20 min). The results corresponded with the observation from fluorescence spectra.

3.4. THz spectra of ALPs

Apart from these two traditional methods, the THz absorption spectra of ALP solutions collected from 0.5 THz to 2.5 THz (Fig. 6(a)) were carried out to further detect changes of protein conformations (the spectra of THz absorption coefficient of ALP solutions in concentrations of 5 U/mL, 7U/mL, and 9 U/mL are shown in Fig. S4 in the supporting information). The curves of control and experimental groups (radiation at 0.1 THz, 13 mW/cm^2, 20 min) are generally intertwined with each other. This is because water can make up the vast majority of the aqueous sample, largely covering the THz absorption difference between proteins. Therefore, the absorption coefficients were averaged in frequency bands to magnify the difference between groups. The average THz absorption α in four frequency bands were displayed in Figs. 6(b)–6(e)). Each point represented a single measurement. The error bar was drawn according to the standard deviation.

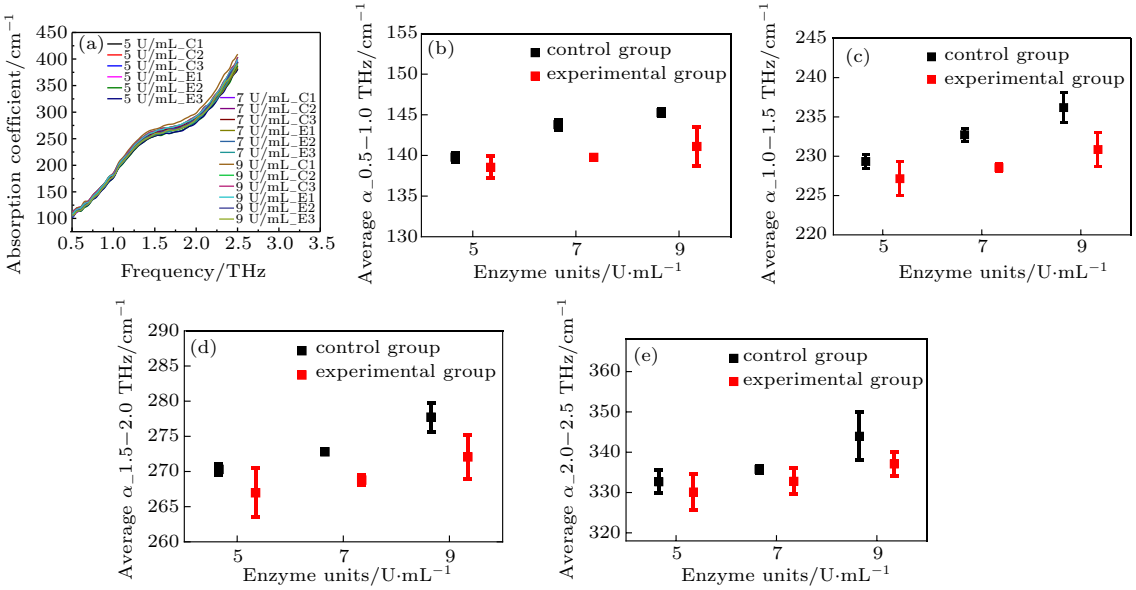


Fig. 6. (a) The absorption coefficient of ALP solutions between 0.5 THz and 2.5 THz, in concentrations of all the groups (C: control, E: experiment). The average THz absorption of ALP solutions in four frequency bands: (b) 0.5–1.0 THz, (c) 1–1.5 THz, (d) 1.5–2.0 THz, (e) 2.0–2.5 THz.

As shown in Figs. 4(b)–4(e), for all the three concentrations of ALP, the results shared the same trend that the absorption coefficient of control groups was higher. What's more, the absorption coefficient was positively correlated with enzyme concentrations.

Our previous work has verified that THz absorption spectra could distinguish the conformational alterations in insulin amyloid.[42] It has also been reported to successfully determine the different conformations of bovine serum albumin (BSA),[43,44] ubiquitin mutants, hen egg-white lysozyme, and horse heart myoglobin.[45,46] Proteins are composed of polar and polarized molecular units, and the low-frequency collective vibrations of the molecular are at THz frequencies. Thus, THz waves could interact with protein molecules, and the parameters of THz spectra, such as absorption coefficient and refractive index, could reflect the structural difference in proteins.[47,48] Furthermore, some researches have conducted experiments and simulations to verify that THz absorption spectra could distinguish aqueous solutions of enzymes in different conformations by detecting the changed hydrate shell and water structure.[49,50] THz-TDS results suggested that the absorption of ALP solution in radiated groups was lower than that of control groups, thus we deduced that there are changes in ALP structure or the hydrate shell in ALP solutions.

4. Conclusion and perspectives

Although it is found that 0.1 THz radiation for 20 min reduced the activity of alkaline phosphatase by changing its conformation, further experiments are still needed to study the influence and mechanism of THz radiation in different frequencies, power, and duration to proteins, in order to have a comprehensive understanding of interactions between THz radiation and ALP.

In conclusion, when it comes to the interactions between terahertz radiation (0.1 THz, 13 mW/cm^2) in 40 min, the ALP activity dropped during the first 25 min, and then rose to the intrinsic value at 40 min. Our study indicated that the THz radiation decreased the activity of alkaline phosphatase by non-thermal effects. Moreover, the fluorescence, UV-vis, and THz spectroscopy results implied that the THz wave has caused conformational changes in ALP solutions. Taken together, these results presented here show that 0.1 THz radiation (power of 13 mW/cm^2) for 20 min has a negative impact on enzyme activities and we inferred the influence mechanism

is the conformational changes in enzymes. In addition, THz-TDS as a non-destructive, fast testing technology could be applied to detect ALP structures in future.

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