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Temperature- and pH-dependent protein conformational changes investigated by terahertz dielectric spectroscopy

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ABSTRACT

Polypeptides and protein drugs have received enormous attention from pharmaceutical industry, medical sector and consumer groups because of a favourable combination of their bioactivity, specificity and overall success rate for the treatment of a variety of diseases. The efficacy and safety of drugs may be degraded due to fluctuating environmental factors, accompanied by changes of the natural conformation of protein. Thus, it is necessary and meaningful to evaluate drug status before use. To that end, the evolving new and pre-existing protein activity/conformation technologies have proven to ensure the safety of drug use consistently. Recently, terahertz time-domain spectroscopy (THz-TDS) has demonstrated suitability for label-free and non-destructive detection of polypeptide and protein conformational changes. In this paper, THz optical parameters of pepsin solutions under different temperatures and with varying pH are measured to demonstrate the feasibility and the considerable potential of THz spectroscopic method in detecting protein drugs. In cases where temperature or pH change is apparent, the THz absorption coefficient, the refractive index, and the dielectric loss tangent change noticeably, independently verified by enzyme activity testing. These findings strongly support the conclusion that THz spectroscopy of pepsin solutions can be used for qualitative analysis to identify the folding or unfolding of protein drugs caused by changes of environmental factors, laying the foundation of a new label-free method for quality control of protein drugs.

1. Introduction

With the rapid advent of gene engineering and biotechnology, an increasing number of protein drugs have been developed and applied to clinical trials. They are significant in the treatment of tumors, infections, autoimmune diseases, and metabolic diseases, among many others, because of their high activity, excellent specificity, low toxicity, and clear biological function [1]. However, the drug bioactivity endued by protein natural conformation is easily affected by environmental factors such as pH, temperature, and ion concentration etc. [2,3] during the whole process including production, transportation and storage. Thus, using protein drugs without proper preservation would pose a potential health risk. Therefore, monitoring and evaluation of protein bioactivity or structure associated with biological function became the primary condition for ensuring the safety and efficacy of protein drugs. Especially, most of these drugs including universally used vaccines and expensive cytokines are solutions for injection, and it is a great challenge to keep protein activity in the aqueous state due to its low stability [4]. There is a direct link between the protein activity and its folding & unfolding status [3,5], and detecting and determining the conformation of protein formulation appear particularly useful in gauging its bioactivity [6,7].

Commonly adopted methods to detect the activity of protein drugs include the techniques based on immunology [8,9], cell biology [10], and biochemistry [11]. The immunological methods usually involve corresponding reagent according to the different kinds of protein samples due to the specific antigen-antibody reaction with the advantages of accuracy and reliability. Generally, cell viability evaluation

entails much time, labor, and cost. In addition, biochemical methods also need a variety of reagents because of the diversity and complexity of proteins. The lack of universality of the reagents inevitably fuels the complexities and tediousness of such approaches. Protein structural information can be utilized to estimate protein activity as well, and such information can usually be characterized and detected by NMR spectroscopy [12], X-ray diffraction [13], fluorescence spectroscopy [14], circular dichroism spectroscopy [15], infrared spectroscopy [16], Cryo-SEM [17], and the like. Individual or combined use of these techniques can obtain some useful knowledge regarding sequence of amino acid, chemical bonds, and three-dimensional structures. But most of these techniques are unsuitable for nondestructive detection, because of the elaborate arrangements of protein sample preprocessing required, such as crystallization, mark and frozen, which would influence the performance of the protein drug and cause damage to the drug product containers and closures. Therefore, it is desirable to develop an effective and economical protein analysis method with universality, label-free and nondestructive properties for quality control of protein drugs.

Recently, terahertz (THz) spectroscopy has emerged as a powerful tool for studying solvated biomolecules with its purported bio-specificity and rich spectral information. THz spectroscopy has been proven to be capable of probing conformational changes and associated hydration dynamics of bio-macromolecules in solution through precise measurements of optical parameters of solvated solutes [18–20]. Castro-Camus and Johnston observed that the partial unfolding of photoactive yellow protein in Tris-HCl solvent led to a clear increase in absorption at THz frequencies [21]. Oleksandr et al. pointed out that the minimum value of THz absorption corresponded to the most stable protein conformation in solution [22]. And Nikita et al. observed BSA conformational changes in aqueous phase through dielectric permittivity in the THz spectral region [23]. The solvent water dynamics are connected with the protein structural changes and plays a crucial role in the enzymatic activity and protein recognition, which could lead to spectral changes in THz band [24]. Moreover, the THz spectral changes are also related to the changes of thermodynamic parameters [25], that will occur during the folding or unfolding process of protein [26]. Thus the changes of optical parameters in the THz spectral region could be used as indicators of the conformation or even activity changes of the protein suspended or resolved in solution. THz-TDS could provide complementary global dynamics information to current techniques [27].

Pepsin, a drug for the treatment of dyspepsia [28], was one of the first discovered enzymes. There has been a great deal of research works on its physical, chemical and biological properties, including structure, activity, catalytic principle, temperature stability, and pH stability. It is a bi-lobal monomeric enzyme belonging to the aspartic proteinase family that contains a large percentage of acidic amino acid [29], which is most active in acidic environments between 37 °C and 42 °C, while denaturation will occur at high temperature, in neutral or alkaline environments because of conformational changes. These important basic data will help study and analyze the relationship between conformation and THz spectrum of pepsin with different conformations. Therefore, pepsin had been chosen and tested as a representative of protein drugs in this paper.

Protein unfolding at high temperature or inappropriate pH could happen in solution [3]. Studying the influence of environmental changes on the parameters of THz spectrum is the key to understand and use the relationship between the status and the testing results of protein formulation. We found that the absorption coefficient of pepsin solution in the THz band decreased with increasing temperature, while tended to increase with increasing pH. At the same time, the THz refractive index increased while the THz dielectric loss tangent decreased under both conditions. Corroborating the results of THz dielectric spectroscopy, enzyme activity testing indicated that the activity of unfolded pepsin was slashed or even completely missing.
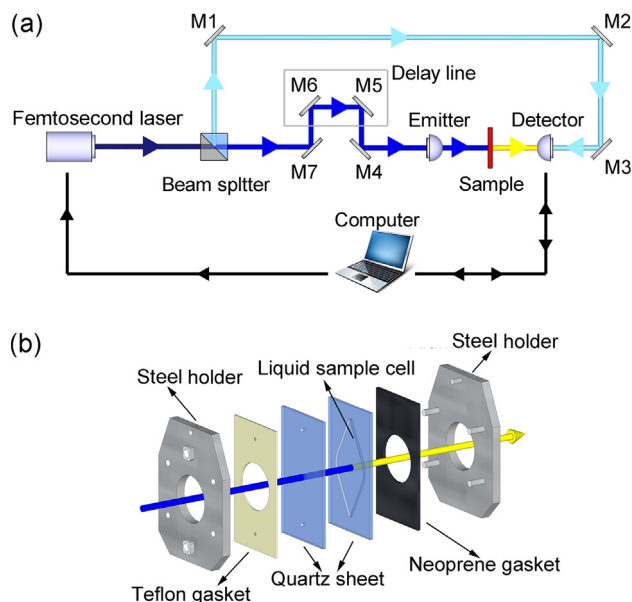

Fig. 1. Schematic diagrams of the THz time-domain spectroscopy setup (a), and stereo diagram showing the liquid sample cell (b).

2. Materials and methods

2.1. Experiment equipment

The THz-TDS system deployed in our research was the T-Gauge 5000 model from Advanced Photonix, Inc., with a spectral bandwidth ranging from 0.1 to 3.5 THz, a signal to noise ratio better than 60 dB at the frequency of analysis. The schematic diagram of the whole system in the transmission mode is displayed in Fig. 1a, and the main specifications of the system meet the test requirements. The tailor-made microfluidic chip shown in Fig. 1b, having a 70 µm depth and about 2 cm^2 area, was used as liquid sample cell, to save agents and standardize the process of experiment. More detailed descriptions can be found in our previous reports [30,31].

2.2. Sample preparation

Pepsin A (CAS No.: 9001-75-6) from porcine gastric mucosa was purchased from Sigma-Aldrich LLC (St. Louis, MO, US). The pepsin, according to the product specification, is white lyophilized powder of molecular weight 35 kDa with an optimum pH of 2–4 (it will be irreversibly inactivated at pH 8.0–8.5), and stable under 60 °C. The optimum temperature is about 38.8 °C [32].

In order to explore the influence of temperature, an acetate buffer containing 1 mM acetic acid and 1 mM sodium acetate, pH 4, located in the optimum range, was chosen as solvent for 1 mM pepsin solutions to provide a constant pH. Pepsin solutions were heated in a water bath to 40 °C, 55 °C, and 70 °C for 10 min respectively, and then naturally cooled down to room temperature (RT) of 22 °C. Moreover, the gradual process of temperature cooling from 55 °C to 46 °C, 37 °C and RT respectively was measured to monitor the dynamic changes of THz spectrum. Pepsin solution staying at RT served as control. A variable temperature cell holder (Specac Inc., Orpington, UK) was used to control the temperature for variable temperature measurements and the testing temperature range was from 55 °C to RT with an accuracy of ± 0.2 °C. At the same time, in order to provide a pH-adjustable environment, a solution of 0.90% w/v of NaCl was chosen as solvent for 1 mM pepsin solutions to analyze and investigate the influence of pH change. The pH was adjusted to 3, 5, 7, 8.5, and 11 with 1 mM HCl or 1 mM NaOH.

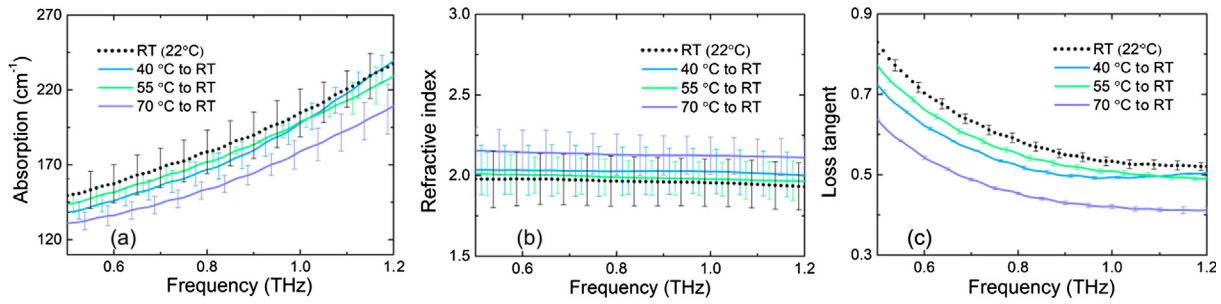The tested ambient temperature was about 22 ± 0.4 °C through air

Fig. 2. Absorption coefficient (a), refractive index (b), and loss tangent (c) of pepsin at 40 °C to RT, 55 °C to RT, 70 °C to RT, and staying at RT (control).

conditioning and the humidity during measurement was under 2% by purging with dry nitrogen (N_2). The THz waveforms of the sample and the reference without sample (air) were measured under the same experimental conditions. Before each measurement, the sample cell was cleaned three times with deionized water, and then with alcohol flush, and at last dried off with a N_2 injector. Each sample was measured at least five times by the THz-TDS system to minimize errors that could result from changes in the location of the sample and the heterogeneity of the solution.

2.3. Data analysis

The absorption coefficient of solution, characterizing quantitatively energy loss by the THz beam propagating in the medium, is derived according to an improved theoretical formula based on the Beer-Lambert Law, and the influence of the reflection between the quartz window of the holder and the sample is taken into account in Eq. (1).

$$\alpha(\omega) = \frac{2}{d} \ln\left(\frac{4n(\omega)n_q(\omega)}{[n(\omega) + n_q(\omega)]^2} \cdot \frac{[n_q(\omega) + 1]^2}{4n_q(\omega)} \cdot \frac{1}{A(\omega)} \right) \tag{1}$$

where d is the thickness of the sample; $A(\omega)$ is the amplitude ratio of the Fourier transforms of the power transmission of the solution sample I_s and the reference (the blank sample cell) I_{ref}; $n_q = 1.95$ is the refractive index of quartz [33]. And $n(\omega)$ is the refractive index of the sample, determining how much the path of the THz beam is bent or refracted when entering a material, can be obtained by [34]

$$n(\omega) = [\varphi(\omega)c]/(\omega d) + 1 \tag{2}$$

where $\varphi(\omega)$ is the phase difference between I_s and I_{ref}, and c is the velocity of light. The loss tangent, quantifying a material's inherent dissipation of electromagnetic energy, is defined as [35]

$$tan\delta = \varepsilon''/\varepsilon' \tag{3}$$

where $\varepsilon' = [n(\omega)]^2 - [k(\omega)]^2$ and $\varepsilon'' = 2n(\omega)k(\omega)$ are the real and imaginary parts of the complex dielectric constant respectively, and $k = c\alpha(\omega)/(2\omega)$ is the extinction coefficient. The relative absorption coefficient [18]

$$\alpha_r = (\alpha_{pepsin} - \alpha_{solvent})/\alpha_{solvent} \tag{4}$$

is used to describe the optical properties of solvent at different temperature. Where α_{pepsin} and $\alpha_{solvent}$ are the absorption coefficient of protein solution and solvent respectively.

2.4. Enzyme activity testing

Micro pepsase assay kit (BC2325) was purchased from Solarbio (BJ, CN) for enzymatic reaction to test the enzyme activity under standard protocol. The principle is detecting the difference of absorbance cause by characteristic absorption peak of hydrolysates of pepsin-catalyzed hemoglobin hydrolysis. The absorbance was measured by the microplate reader (Infinite M 200 PRO) from Tecan Group, Ltd. (Mannedorf, CH).

In this paper, one unit (U) is defined as that pepsin catalyzes the production of 1 μmol tyrosine per minute at 37 °C using hemoglobin as substrate. Pepsin activity (U/mg prot) is obtained as

$$Acticity = \Delta_A V_{sum}/(C_{pep}\varepsilon V_{samp}dT) \tag{5}$$

where Δ_A is the difference of absorbance at 275 nm caused by the product, ε is the absorption coefficient of tyrosine, C_{pep} is the pepsin solution concentration (mg/mL), d is the optical path, T is reaction time, V_{sum} and V_{samp} are the reaction volume and the sample volume respectively.

3. Results and discussion

Temperature fluctuates at every moment from manufacture to usage may result in protein drugs' loss of effectiveness, which mandates that protein drugs need to be stored at low temperature and transported by cold chain. Nevertheless, it is very difficult to avoid all potential risks stemming from temperature change in these steps, such as loading and unloading, changing the mode of transportation, replacing the packing materials and so on. It remains necessary to make sampling inspections before use. In order to simulate a possible scenario of sudden temperature fluctuations in practical processes, pepsin solutions treated with heating to 40 °C, 55 °C, 70 °C and cooling to RT successively were detected. The pepsin solution staying at RT was used as control. In Fig. 2, the distinction of α_{pepsin} (the absorption coefficient of pepsin solution), n_{pepsin} (the refractive index of pepsin solution) and $tan\delta_{pepsin}$ (the loss tangent of pepsin solution) between the control group (RT) and the treatment group of 70 °C to RT is shown. The optical parameters of the treatment groups of 40 °C to RT and 55 °C to RT are close, and fall in between the corresponding data of control and of the treatment group of 70 °C to RT. The irreversible thermal unfolding of porcine pepsin appears above 66.2 °C [32], that is to say, the pepsin unfolding at temperature above 66.2 °C will stay in the unfolded state after cooling. While reversible conformational changes at 40 °C or 55 °C lead to refolding of pepsin after cooling, i.e. all or part of the thermally unfolded protein will refold into the correct native conformation and restore enzymatic activity during the cooling process. The THz dielectric spectroscopic properties as represented by α_{pepsin}, n_{pepsin}, and $tan\delta_{pepsin}$, among different groups confirm these trends.

Further, the pepsin solution was detected during the cooling process to verify the spectral difference caused by reversible conformational changes. The pepsin solution at 55 °C and the solutions cooling from 55 °C to 46 °C, 37 °C and 22 °C were tested and the pepsin solution staying at RT was used as control. The relative absorption coefficient α_r is a factor affected by protein conformation, which has been shown to weaken the effect of temperature on solvents [36]. Fig. 3 shows that α_r of pepsin at 55 °C is lower than that of the control group, and α_r of the treatment group is increasing with step wise decreasing temperature from 55 °C to RT. The two curves, 55 °C to RT and RT (control group), are similar, because the reversible conformational changes at 55 °C lead to refolding of pepsin after cooling. The differences in α_r between different temperatures may be resulting from the dynamic folding
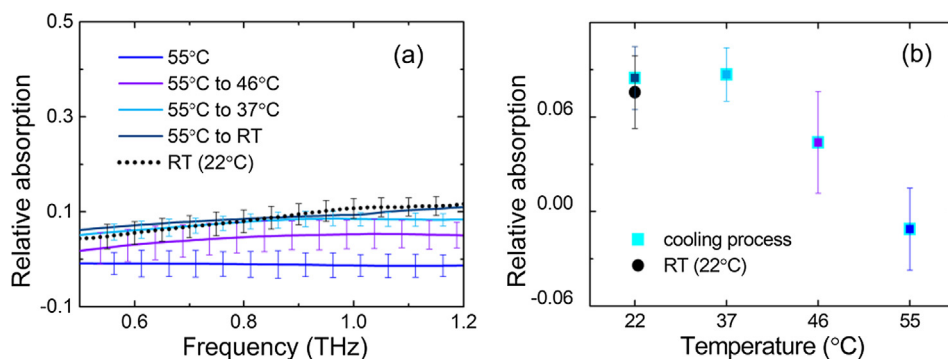
Fig. 3. Relative absorption coefficient (a) and integrated relative absorption coefficient (0.5 − 1.2 THz) (b) of pepsin at 55 °C, 55 °C to 46 °C, 55 °C to 37 °C, 55 °C to RT and RT (control).

intermediates known to exist in the refolding process [37,38], and the thermal motion of whole protein molecules which increases with temperature [39].

Because suitable pH of the solution favors the formation of protein drugs, improper pH would directly affect drug efficacy by changing the protein physico-chemical properties. Detecting the spectral difference of pepsin solutions under different pH helps explore the relationship between protein conformation and environmental factor, which can assist in detecting the protein denaturation due to incorrect pH in the manufacturing process. The ion and H^+ environment influenced by pH may in turn influence the solvents' optical properties in the THz spectral region, so the spectra of solvents (NaCl aq) were prior measured. Taking into account measurement error, there is little difference in $\alpha_{solvent}$ (the absorption coefficient of solvent) between pH 3, 5, 7, 8.5 and 11 (Fig. 4a). The standard deviations of $\alpha_{solvent}$ at pH 3 and 7 are much higher than others, the largest of which is ± 2.72%, but the maximum standard deviation of all solvents is only ± 1.09% (Fig. 4d). It indicated that the difference in $\alpha_{solvent}$ resulted from the random error of measurement, so $\alpha_{solvent}$ at different pH could be considered to be essentially the same in the THz band. The analysis of $n_{solvent}$ (the refractive index of solvent) and $\tan\delta_{solvent}$ (the loss tangent of solvent) at different pH leads to a similar conclusion. Both the curves of $n_{solvent}$ (Fig. 4b) and $\tan\delta_{solvent}$ (Fig. 4c) between pH 3, 5, 7, 8.5 and 11 overlap and interweave, and

the standard deviations of $n_{solvent}$ at pH 3, 5, and 8.5 are higher than that of all other pH (Fig. 4e); meanwhile the standard deviation of $\tan\delta_{solvent}$ at pH 8.5 is also higher than that of all other pH (Fig. 4f). So the random error of measurement was also the main reason for the difference in $n_{solvent}$ and $\tan\delta_{solvent}$. Therefore, the changes of the pepsin solutions' optical parameters are mainly caused by pepsin itself.

The absorption coefficient of pepsin solutions at pH 3, 5, 7, 8.5 and 11 are plotted in Fig. 5a, showing that α_{pepsin} increases in a clustering pattern with the increasing pH: α_{pepsin} at pH 3 is the lowest, α_{pepsin} at pH 8.5 and 11 are the largest without significant difference, and α_{pepsin} at pH 5 and 7 are clustering between them. And the change trend of n_{pepsin} is the same as that of α_{pepsin}, shown in Fig. 5b, while $\tan\delta_{pepsin}$ decreases in the clustering pattern with the increase of pH, shown in Fig. 5c. The values of n_{pepsin} and $\tan\delta_{pepsin}$ at pH 8.5 and 11 are also adjacent to each other. Thus the correlation of optical parameters with conformations can also be observed.

As a protein denaturation process caused by pH, pepsin denaturation experiences three different states, i.e., native, intermediate and unfolded. Pepsin has stable and normal conformation to complete catalytic hydrolysis only under acidic conditions (pH 2–4). Furthermore, the pepsin secondary structure will change with decreasing H^+ concentration, for instance, and the flexibility of the ring structure formed by the first and second β-sheet at n-terminal domain is likely to
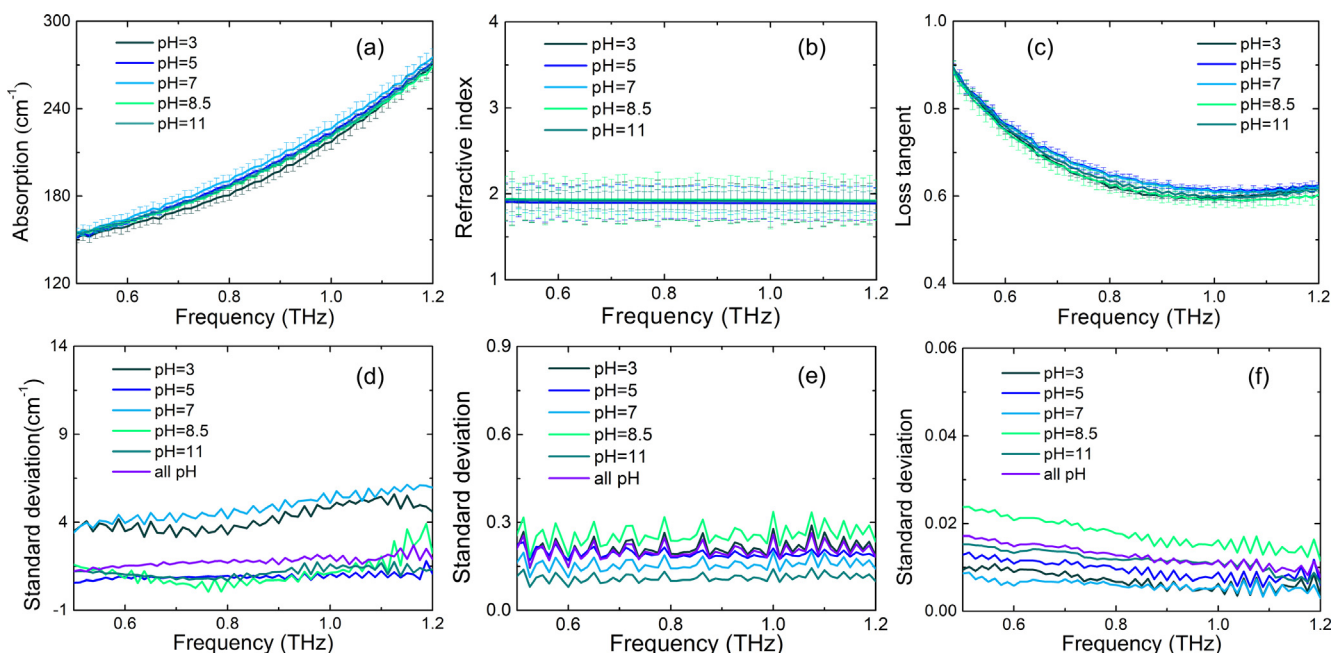

Fig. 4. Absorption coefficient (a), refractive index (b), and loss tangent (c) of solvents with different pH, and the standard deviations of corresponding absorption coefficient (d), refractive index (e) and loss tangent (f).
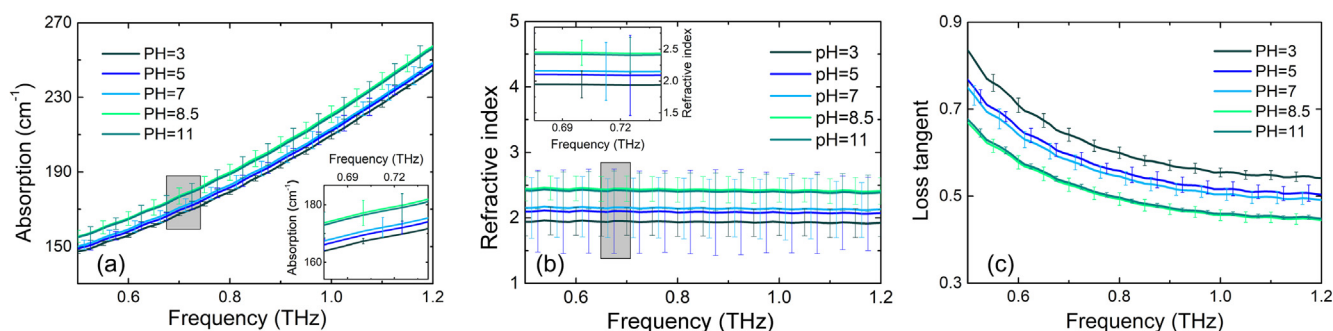
Fig. 5. Absorption coefficient (a), refractive index (b), and loss tangent (c) of pepsin at pH 3, 5, 7, 8.5 and 11. Insets in (a) and (b) show the details of absorption coefficient and refractive index from 0.68 to 0.74 THz, respectively.
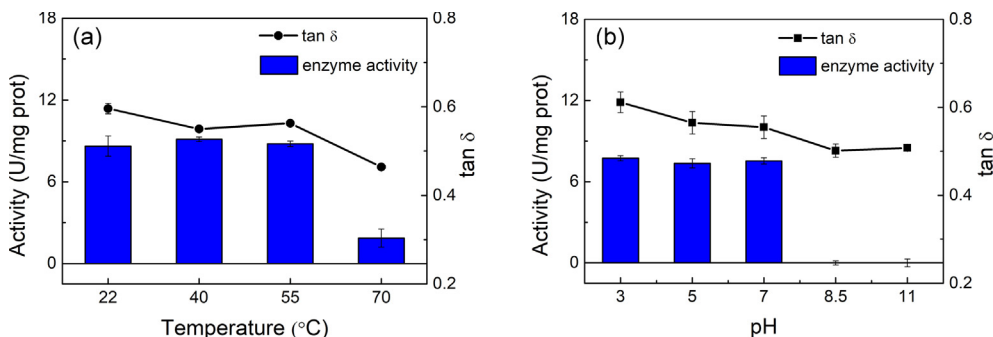

Fig. 6. Integrated loss tangent (0.5–1.2 THz) and enzyme activity measured by enzymatic reaction of pepsin treated with different temperature (a), and different pH (b).

increase. At last when the pH value is above 8.5, an irreversible change will occur in the n-terminal domain, as a result pepsin is permanently inactivated [40].

Proteins composed of polar and polarizable molecular units can be considered as electrical dipoles, whose motion can be thought of as a superposition of many normal modes of vibration [41]. During an unfolding process, protein stretches from a slightly spherical structure to a loose chain without specific spatial structure. The unfolded protein has a higher density of vibration modes between 0 and 2 THz, leading to increased THz absorption [21]. Moreover, hydration shells can be sensitively detected by THz spectroscopy [42,43], because they will induce a blue-shift in the vibrational density of states and reduce the THz spectral amplitude at low frequency [44]. The process of protein unfolding is able to change the absorbance and thickness of the extended hydration shells [18], which may cause the absorption to diminish [44]. In addition, the gradually loosened structure results in increased instability of the residues [21] with the exposing of some side chains [45]. Loss tangent is extremely sensitive to the reorientation of side chains of residues as they are subjected to an oscillating electric field [46]. These may lead to a significant change of loss tangent during protein denaturation. These previous findings strongly support our own conclusion that absorption coefficient, refractive index, and loss tangent in the THz spectral region could be used to distinguish pepsin solutions with different conformations. And it follows THz-TDS has the potential to test whether a liquid protein drug has lost its effectiveness.

Further, the activity of the pepsin solutions treated at corresponding temperature and pH were tested and verified though enzymatic reaction, shown with blue columns in Fig. 6. The activity of pepsin solution treated with 70 °C reduced to a quarter of that in the control group (22 °C), while 40 °C and 55 °C treatment samples had similar enzyme activity level compared with the control group. The pepsin under pH 8.5 and 11 completely lost their catalytic ability. But there was little effect on enzyme ability throughout the changing pH value from 3 to 7. These results were consistent with the differences of the THz spectra between native pepsin solution and the irreversibly degenerated one,

and suggested that THz-TDS could be used to detect pepsin with different enzyme activity. Remarkably, 10 μl sample and a few minutes were all it required for a single measurement by THz-TDS, while the Micro pepsase assay kit used in this paper needed a minimum of 40 μl sample and more than 30 min of processing time due to hydrolysis, centrifugation and other pretreatment. The label-free THz-TDS method raised the efficiency of protein drug detection through decreased test time and sample size.

It has been proven that THz spectroscopy has the ability to determine the heat capacity, enthalpy, and entropy of solvated solute through measurement of THz optical parameters [26,47]. These thermodynamic parameters will change in the protein solution system during the process of protein conformational changes affected by environmental factors [25,48]; also the structure of amino acid sequences and the number of amino acid residues are known to be associated with the changes in enthalpy and entropy [49]. Although the specific and detailed correlation between THz optical parameters and protein structure is yet to be worked out, there were clear differences in the THz spectra of proteins with different activities namely different conformations, which implied that there was a correspondence between the energy state of the protein and its THz spectrum. Therefore, it is reasonable to speculate that the thermodynamics of protein system can be reflected by THz spectroscopy, which means it is potentially possible to analyze the protein conformation state through the thermodynamic parameters measured by THz-TDS. Obviously, a thorough understanding of the relationship between protein conformation and THz calorimetry would facility the calculation of the specific thermodynamic parameters corresponding to each state. And it is also probable to analyze the activity of protein through THz calorimetry, thanks to the link between protein conformation and activity established in this study.

4. Conclusions

THz dielectric spectroscopy was employed to detect the spectra of

pepsin solutions under different temperature and pH. Absorption coefficient, refractive index, and loss tangent all reflected the unfolding and refolding of pepsin under different temperature or pH conditions. These results were consistent with the pepsin activity obtained by enzymatic reaction and indicated that THz-TDS could be used to detect pepsin conformational changes, and as such can be employed for quality inspection of protein drugs. Essentially, the sensitivity of THz spectrum to pepsin treated by different temperature or pH derives from the pepsin conformational changes, which influences the thermodynamic properties of pepsin and brings about THz spectral amplitude variation and phase shift. The experiment also provides a convincing example of detection of biological macromolecular structures using THz-TDS.

Conflict of interest

There are no conflicts to declare.

Acknowledgements

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