MINISTRY OF EDUCATION AND SCIENCE OF UKRAINE
KYIV NATIONAL UNIVERSITY OF TECHNOLOGIES AND DESIGN
Faculty of Chemical and Biopharmaceutical Technologies
Department of Biotechnology, Leather and Fur

QUALIFICATION THESIS

on the topic **Construction of metalloenzyme mimics based on self-assembled peptides**
First (Bachelor's) level of higher education
Specialty 162 "Biotechnology and Bioengineering"
Educational and professional program "Biotechnology"

Completed: student of group BEBT-21
Haoran Peng

Scientific
supervisor Iryna VOLOSHYNA,
Ph.D., As. prof.

Reviewer Olga IUNGIN
Ph.D., As. prof.

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Educational and professional program Biotechnology

APPROVE
Head of Biotechnology, Leather and Fur Department, Professor,
Dr. Sc., Prof.
_____Olena MOKROUSOVA
«___»_____2025

ASSIGNMENTS
FOR THE QUALIFICATION THESIS
Peng Haoran

1. Thesis topic **Construction of metalloenzyme mimics based on self-assembled peptides**
Scientific supervisor Ph.D., Assoc. Prof. Iryna Voloshyna

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2. Initial data for work: assignments for qualification thesis, scientific literature on the topic of qualification thesis, materials of Pre-graduation practice

3. Content of the thesis (list of questions to be developed): literature review; object, purpose, and methods of the study; experimental part; conclusions

4. Date of issuance of the assignments 05.03.2025

WORK CALENDAR

№	The name of the stages of the qualification thesis	Terms of performance of stage	Note on performance
1	Introduction	until 11 April 2025	
2	Chapter 1. Literature review	until 20 April 2025	
3	Chapter 2. Object, purpose, and methods of the study	until 30 April 2025	
4	Chapter 3. Experimental part	until 11 May 2025	
5	Conclusions	until 15 May 2025	
6	Draw up a bachelor's thesis (final version)	until 25 May 2025	
7	Submission of qualification work to the supervisor for feedback	until 27 May 2025	
8	Submission of bachelor's thesis to the department for review (14 days before the defense)	28 May 2025	
9	Checking the bachelor's thesis for signs of plagiarism (10 days before the defense)	01 June 2025	Similarity coefficient ____% Citation rate ____%
10	Submission of bachelor's thesis for approval by the head of the department (from 7 days before the defense)	04 June 2025	

I am familiar with the task:

Student _____ Peng Haoran

Scientific supervisor _____ Iryna VOLOSHYNA

SUMMARY

Peng Haoran. Construction of metalloenzyme mimics based on self-assembled peptides – Manuscript.

Qualification thesis on the specialty 162 «Biotechnology and Bioengineering». – Kyiv National University of Technologies and Design, Kyiv, 2025.

Laccase is a natural enzyme with high catalytic activity capable of catalyzing the oxidation of many different substrates. Laccase is of great importance in lignin degradation, bioremediation, environmental protection and biocatalysis. However, in practical application scenarios, natural laccase suffers from poor stability, high preparation cost, and difficulty in large-scale production, which seriously limits the large-scale application of laccase in various industries. In this study, in order to solve the problems of poor stability and high production cost of natural laccase, a peptide sequence Ac-ICVHLHLHVHI-$CONH_2$ was rationally designed according to the active center and structural characteristics of natural laccase, and a metal mimetic enzyme with peptide as material was successfully constructed. By examining the effects of different metal ions and different metal ion concentrations on the catalytic activity of the mimic enzyme, the results showed that Cu^{2+} was the most suitable metal ion for binding with the mimic enzyme, and the catalytic activity of Cu^{2+} was the highest for the substrate 2,4 dichlorophenol (2,4-DP) after combining with the peptide mimic enzyme when the concentration of Cu^{2+} was 8 mM at the pH 6. The experiments were further performed by using Circle II (CD) chromatography, Fourier (FTIR) infrared spectroscopy, and transmission electron microscopy (TEM) to characterize the structure of the mimetic enzyme, and the

results proved that Cu^{2+} could induce the polypeptide to increase the degree of assembly, become more structurally ordered, and form an obvious β-folded secondary structure with tightly assembled aggregates. The metal-mimetic enzymes constructed in this study with peptides as materials provide a new design direction and experimental basis for applications in fields such as industrial catalysis and green chemistry.

Key words: Laccase mimetic enzymes, Peptides, Metal ions, Catalytic activity, Structural characterization

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INTRODUCTION

Enzyme is a common biomolecule with catalytic properties produced by cells in living organisms, consisting of proteins and, to a lesser extent, RNA[0]. Enzymes can efficiently catalyze the occurrence of various biochemical reactions under mild conditions and promote metabolism in organisms [2]. Enzyme activity refers to the magnitude of an enzyme's ability to catalyze a biochemical reaction, which can be regulated and altered by a variety of factors, thus allowing the organism to adapt to changes in the external environment as much as possible. With the increasing need for enzymes and the continuous in-depth study of enzymes, enzymes are widely used in various fields such as biochemistry and food industry[3]. Natural enzymes can specifically catalyze a certain biochemical reaction, which is a kind of biocatalyst with high catalytic efficiency and specificity [4]. However, natural enzymes usually have the disadvantages of poor stability, narrow source pathway and high extraction cost, resulting in very limited reaction scenarios catalyzed by natural enzymes[56]. This situation has led to the increasing inability of natural enzymes to meet the requirements of specific reaction scenarios, prompting the need to construct and develop a variety of new types of simulated enzymes based on the catalytic mechanism and structural characteristics of natural enzymes to meet the requirements, thereby replacing the smaller functions played by natural enzymes.

As the catalytic mechanism and structural characteristics of natural enzymes are gradually studied in depth, more and more people want to simulate the characteristics of natural enzymes to prepare a variety of simulated enzymes to meet the needs in various aspects, so as to realize the greenness, high efficiency, and low cost of chemical processes in applications. Substances prepared by mimicking the catalytic activity and structural features of natural enzymes based on the above are called mimetic enzymes or artificial enzymes[78]. These simulated enzymes have highly similar catalytic activity to natural enzymes and also have the advantages of high catalytic efficiency, low cost and simple and convenient preparation methods. There is another type of enzyme that has metal ions

incorporated, known as metalloenzymes, which are also involved in numerous catalytic reaction processes. The metal ions will be closely bound to the active site of the enzyme, and the combination will jointly participate in the catalytic reaction, thus realizing the efficient catalytic characteristics of the enzyme[9]. Metal ions enable the enzyme to form an active center and stabilize the structure of the enzyme[1011]. This metal-mimetic enzyme also has the same catalytic activity and structural characteristics of natural enzymes, and metal-mimetic enzymes made of peptides are being intensively investigated and have the potential to replace natural enzymes in various chemical reaction processes.

CHAPTER 1
LITERATURE REVIEW

1.1 Reasons for choosing the topic

Laccase is an important copper-containing metalloenzyme found widely in nature in bacteria fungi, plants and animals, and it is included in the superfamily of polycopper oxidases. Laccase is a natural enzyme with high catalytic activity, capable of catalyzing the oxidation of a wide range of substrates. It has very important applications in the fields of lignin degradation, bioremediation, environmental protection and biocatalysis[12]. Enzymes are characterized by high substrate suitability (can catalyze the oxidation of most organic and inorganic substrates), good catalytic activity, and high regeneration capacity, and because laccase can catalyze over a wide range of pH and temperature, they are suitable for a variety of biotechnological applications (e.g., wastewater treatment, food industry, and decoloration of dyes, etc.). Laccase is also known as a "green catalyst"[13] because it requires only oxygen molecules for the reaction, oxidizes single electrons in the substrate and produces only water molecules as by-products, with few other toxic products[1415]. Although laccase has been intensively developed and utilized, in practical application scenarios, natural laccase suffers from poor stability, high preparation cost[16], and difficulty in large-scale production[17], which severely limits the large-scale industrial application of laccase. Compared with other types of materials, metal-mimetic enzymes made of peptides show obvious advantages: the sequence of peptides is highly designable, and the design of a few key amino acids into the peptide sequence can preserve the catalytic activity characteristics of the enzyme. Its structure and chemical properties are closer to those of natural laccase, the molecule itself of the mimetic enzyme is easier to be modified, and it exhibits a high level of functionality and biocompatibility[18]. Therefore, it is hoped to construct metal-mimetic enzymes made of peptides with laccase-like catalytic activity[19].

1.2 Status of research

In recent years, researchers have carried out a lot of research work around laccase-mimicking enzymes and achieved certain results. Like a laccase-mimicking enzyme made of a novel copper-containing layered silicate (ACP) [20], which has a great role in environmental remediation. Although ACP has the advantage of good stability, it has a low affinity for substrates and may not be able to efficiently bind to the substrate and catalyze the reaction. Moreover, the synthesis of ACP requires relatively complicated processes and conditions, which can lead to the high cost of synthesis and low efficiency of synthesis, which is not conducive to the large-scale production and application of ACP and other shortcomings. Immediately after being inspired by the active center and electron transfer of natural laccase, researchers constructed a copper-based bi-ligand mimetic enzyme (Cu-BH) with good laccase-like activity and excellent fluorescence properties[21], although Cu-BH can oxidize many kinds of substrates, there are still times when the selectivity is not high enough, that is to say, it may play a certain degree of catalytic effect on some substances that we don't expect to have a reaction, thus reducing the catalytic effect of these substances. catalytic effect, thus reducing the accuracy of these reactions. Carboxymethyl cellulose (CMC) was used as the material to construct platinum nanoparticles (CMC-PtNPs)[22] as laccase mimetic enzyme, and the prepared CMC-PtNPs had small particle size and good dispersion. The disadvantage is that the cost price of platinum is very high, which leads to the high cost of laccase-mimicking enzyme constructed with PtNPs as the material.PtNPs may also cause undesirable risks such as toxic effects.

In order to solve these problems, we started to investigate how to perform the construction of metal-mimicking enzymes based on peptides. By designing different amino acid sequences to regulate the activity and selectivity of the mimicking enzyme, the structure and characteristics of the mimicking enzyme are closer to those of the natural enzyme. Most importantly, the bioavailability of this simulated enzyme is good, and the peptide material used has the advantages of low

cytotoxicity and low threat to the environment. Moreover, the polypeptide is composed of amino acids, which are composed of the same elements as most of the natural enzymes, so the biocompatibility is also better. There are many types of amino acids, and the chemical properties of each amino acid have great differences, and different simulated enzyme characteristics can also be designed by adjusting the type and order of amino acids[2324]. Therefore, this idea of designing metal-mimetic enzymes using peptides as materials shows great potential for application in the field of green chemistry[2526].

1.3 Problem solved and research methodology

1.3.1 Problems to be solved

The problems that need to be solved in this study are the lack of stability of natural laccase, the high production cost as well as the difficulty to be used for large-scale production and application. By using peptides as materials to prepare metal mimetic enzymes, we actively try to improve the catalytic efficiency of the mimetic enzymes in different aspects. It is also necessary to design experiments on the effect of different metal ions and the effect of different concentrations of metal ions to study the optimum metal ion and optimum metal ion concentration. The structural features and structural characterization changes of the peptide-based metal mimetic enzyme were analyzed using Circular Dichroism (CD) chromatography, Fourier Infrared Spectroscopy (FTIR) and Transmission Electron Microscopy (TEM) analyses, and finally, the optimal pH was found by optimizing the reaction conditions.

1.3.2 Research method

Firstly, according to the active center and catalytic characteristics of natural laccase, an amino acid sequence containing histidine and cysteine was selected to rationally design a peptide sequence. The structural characterization features of the simulated enzyme were investigated by Fourier Transform Infrared Spectroscopy (FTIR) technique, Circular Dichroism (CD) analysis, and Transmission Electron

Microscopy (TEM) analysis. Assessment of the catalytic activity of the simulated enzymes utilized a UV-visible spectrophotometer to determine the change in absorbance of the substrate or product at 510 nm. The catalytic effect of the simulated enzyme was optimized by changing the reaction conditions (e.g. pH, temperature, etc.).

1.4 Results and significance

In this study, laccase was used as a simulated object, and according to the structural characteristics and catalytic features of its catalytic activity center, a peptide simulated enzyme with the same catalytic activity as laccase was constructed by introducing active amino acid sequences into the peptide through rational design. The purpose is to provide theoretical basis and technical support for the development of efficient and stable peptide mimetic enzyme, and at the same time to lay a solid foundation for the application of peptide mimetic enzyme in various fields. It is hoped that through the study of a series of reactions after the combination of metal ions and peptides, we can construct a metal mimetic enzyme using peptides as materials and study its catalytic activity and structural characteristics. The requirement of similarity to natural laccase was followed to a great extent, on the basis of which the shortcomings such as poor stability and high cost of natural laccase were overcome. The effects of different metal ions and the effects of different concentrations of metal ions were also investigated by designing experiments to find out the optimum metal ion and the optimum metal ion concentration. The structural features and structural characterization changes of the peptide-based metal mimetic enzyme were analyzed using circular dichroism (CD) chromatography, Fourier infrared spectroscopy (FTIR) and transmission electron microscopy (TEM) analysis, and finally, the optimal pH was found by optimizing the reaction conditions. This research result will pave the way for the application of peptide-based metal-mimetic enzymes in biochemistry, and provide experimental basis and technical support for their large-scale use.

Summary of the chapter I

1. Reasons for choosing the topic
2. Status of research
3. Problem solved and research methodology
4. Results and significance

CHAPTER 2
OBJECT, PURPOSE, AND METHODS OF THE STUDY

2.1 Experimental materials and apparatus

2.1.1 Experimental reagents

Peptide sequence Ac-ICVHLHLHVHI-$CONH_2$; lyophilized peptide powder; DMSO; acetonitrile, analytically pure, Tianjin Concord Science and Technology Co. Ltd; ultrapure water; PB buffer; copper chloride; manganese chloride; magnesium chloride; barium chloride; zinc chloride.

2.1.2 Experimental apparatus

Shaker; vortex shaker; low temperature refrigerator; balance; UV-Vis spectrophotometer.

2.2 Experimental methods

2.2.1 Peptide sequence design

According to the active center and catalytic mechanism of natural laccase, the sequence containing histidine, cysteine and other metal-ligand amino acids was selected, with the aim of rationally designing the polypeptide sequence Ac-ICVHLHLHVHI-$CONH_2$ containing active amino acids.

2.2.2 Effects of different metal ions

Metal ions play an extremely important role as a cofactor in simulated enzymes, which can participate in the catalytic process by transferring electrons and changing the directed reaction. In order to investigate whether different metal ions have different effects on the simulated enzyme, it is necessary to add different metal ions to react with the peptide to find out a most suitable metal ion. The specific experimental method was as follows: produce the lyophilized peptide powder from the refrigerator and leave it at room temperature for 1 h. After the peptide temperature returned to room temperature, take the dried peptide powder and dissolve it in 20 μL of DMSO, shake it sufficiently, then add a certain volume of buffer containing different metal ions (Zn^{2+}, Ca^{2+}, Mg^{2+}, Ba^{2+}, Mn^{2+}, Cu^{2+} and so on)[27], mix it and sonicate it for 90 s, and then leave it at 25℃, protected from light for 24h. After completion of the above operations the absorbance values were

measured at 510nm using the photometric mode of UV-Vis spectrophotometer. In the process of the experiment, it is necessary to strictly control the irrelevant variables, so as to exclude the influence of other irrelevant factors in the background on the final results of the experiment, and each group needs to be set up three times in parallel samples, in order to ensure the accuracy of the experimental results.

2.2.3 Effect of metal ion concentration

The above experiments have proved that Cu^{2+} is the best metal ion, and now we need to find out the optimal concentration of Cu^{2+} in order to combine with the peptide. The specific experimental steps were as follows: take the lyophilized peptide powder out of the refrigerator, leave it at room temperature for 1 hour, wait for the peptide temperature to return to room temperature, take the dried peptide powder and dissolve it in 20μL of DMSO, then add the optimal metal ions (Cu^{2+}) derived from the above experiments after sufficient shaking, adjust the concentration of the metal ions (0mM, 4mM, 6mM, 8mM, and 10mM respectively) in order to study the effect of different metal concentrations on the metal mimicking enzyme. metal-mimicking enzymes, and finally select the optimal concentration.

2.3 Results and analysis

2.3.1 Analysis of the results of the effects of different metal ions

Since laccase belongs to the family of hyperpolycopper oxidases, it is hypothesized that the presence of metal ions may cause changes in the activity of the simulated enzyme. The absorbance properties of different metal ions (Cu^{2+}, Ba^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}) in the wavelength range of 400-650nm can be very different as can be seen from the figure. The curves of Ba^{2+}, Mg^{2+}, Mn^{2+}, and Zn^{2+} are relatively flat from the figure, and the trend of absorption change is relatively simple. Only the curve of Cu^{2+} has obvious highest and lowest values in the figure, which indicates the large variation of Cu^{2+} absorption of light, proving the uniqueness and strong liganding ability shown by Cu^{2+} as well as its irreplaceability in the construction of peptide mimetic enzymes.

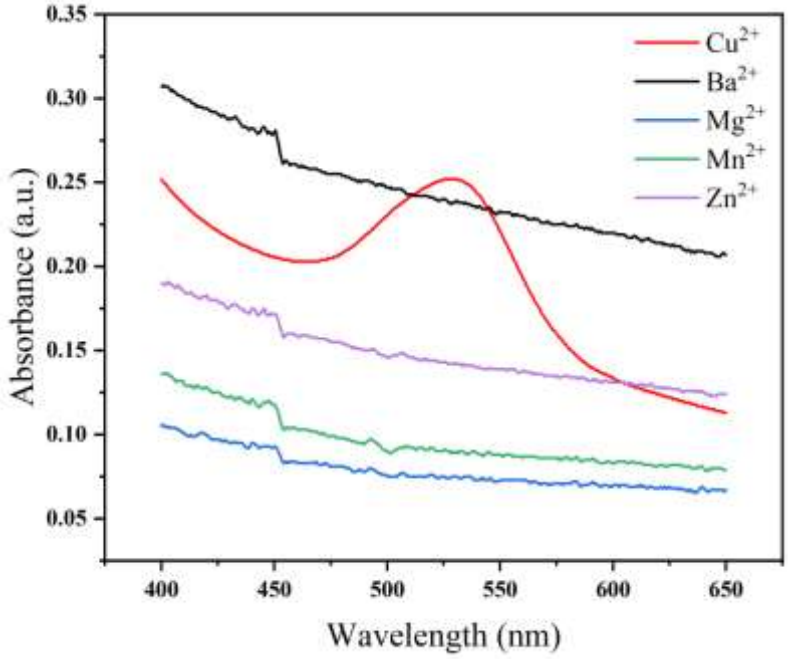

Figure 2.1 Different metal ions

The reason for this result is that Cu^{2+} orders the polypeptide structure through a strong coordination pathway[34] to form an electron transfer pathway necessary for catalytic activity, which is functionally identical to that of the copper active center of the natural laccase.Cu^{2+} forms a stably coordinated complex with the polypeptide, and is the only metal ion capable of significantly changing the metal ion that significantly alters the light-absorbing properties of the peptide. In contrast, Ba^{2+}, Mg^{2+}, Mn^{2+}, and Zn^{2+} were not able to induce functional structural changes because of their poor interaction force with the polypeptide and insufficient coordination ability, and thus could not effectively mimic laccase activity. Based on the significant enhancement of the activity of the mimic enzyme by the addition of Cu^{2+}, it was decided to choose Cu^{2+} as the most suitable metal ion for the subsequent experiments, and then the peptide-Cu^{2+} complex was the laccase mimic enzyme needed for this study.Various metal ions have great influence on the practical application of the mimic enzyme, and the addition of metal ions such as Cu^{2+}, Ba^{2+}, Mg^{2+}, Mn^{2+}, and Zn^{2+} can significantly enhance the catalytic activity of the peptide mimic enzyme, which can improve the application of the peptide mimic enzyme in the bleaching of paper and wastewater treatment. Two other

extremely important advantages of adding metal ions with peptide-mimicking enzymes are that they can reduce the production cost[35] and increase the processing speed in industrial applications.

2.3.2 Analysis of the results of the effects of different concentrations of metal ions

It has been known from the above experiments that Cu^{2+} is the optimum metal ion and its concentration may have an effect on the final catalytic activity. As can be seen from the bar graphs, the presence of Cu^{2+} (4mM, 6mM, 8mM, 10mM, and 12mM) all showed higher activities compared to the absence of Cu^{2+} (0mM). Especially at 8mM, 10mM, and 12mM, the activity of the metal-mimetic enzyme was surprisingly almost 100%. In order to measure the optimal Cu^{2+} concentration, it is necessary to continue to increase the Cu^{2+} concentration after 8 mM to observe whether the activity of the metal-mimicking enzyme increases with the increase of Cu^{2+} concentration. According to the graph, it was observed that this was clearly not the case, and the activity of the metal-mimicking enzyme reached its highest value at 8 mM, which was almost close to 100%. Through a series of operations it can be deduced that the Cu^{2+} concentration that gives the highest activity to the metal mimic enzyme is 8mM.

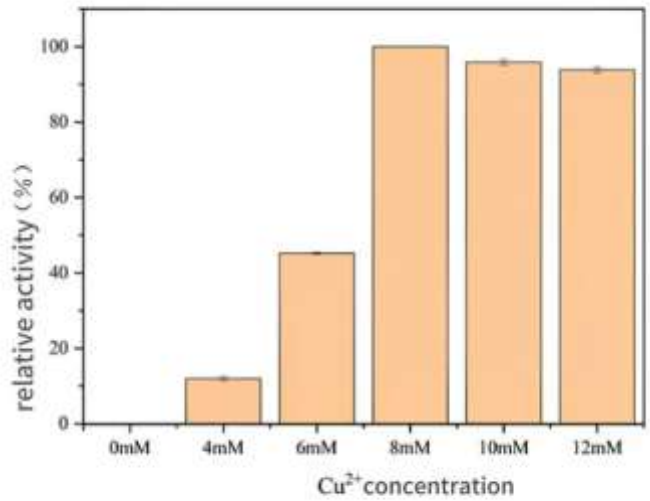

Figure 2.2 Different concentrations of metal ions

2.4 Summary of this chapter

In this chapter, we first constructed an artificial metal mimic enzyme based on peptide material (peptide sequence Ac-ICVHLHLHVHI-$CONH_2$) according to the active center and catalytic characteristics of natural laccase. Firstly, the best metal ion needs to be determined, and several metal ions (Cu^{2+}, Ba^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}) were selected to design experiments to compare the effects of different metal ions on the activity of the peptide, and the experimental data proved out that Cu^{2+} is the most suitable metal ion for binding with the peptide. The peptide-Cu^{2+} complex will be chosen as the laccase mimetic enzyme for subsequent experiments. Next, the optimal concentration of Cu^{2+} will be demonstrated, and the highest activity of the peptide mimic enzyme can be achieved by adding 8 mM Cu^{2+} through setting a concentration gradient.

Summary of chapter II

1. Peptide sequence design
2. Effects of different metal ions
3. Effect of metal ion concentration
4. Results and analysis

CHAPTER 3
EXPERIMENTAL PART

3.1 Experimental materials and apparatus

3.1.1 Experimental reagents

Double-distilled water; copper chloride; potassium bromide; 10% v/v phosphotungstic acid staining solution.

3.1.2 Experimental apparatus

Circular dichroism (CD) spectrometer; Fourier transform (FTIR) infrared spectrometer; transmission electron microscope (TEM).

3.2 Experimental Methods

3.2.1 Circular dichroism (CD) analysis

Circular dichroism is a common and simple method for rapid and accurate determination of the secondary structure of peptides and proteins, and different peptide molecular structures have different circular dichroism characteristics. The principle of circular dichroism is that due to the existence of two structures with different chirality in different peptide molecules which leads to the different absorption of plane polarized light when it is decomposed into right-rotated and left-rotated circularly polarized light, different circularly dichroic qualities are produced when peptides with rotational properties are irradiated by plane polarized light. The basic secondary structures in proteins include random curls, α-helices, β-folds, β-turns, etc., which will exhibit absorption bands with different characteristics in the ultraviolet region of the spectrum. Whether Cu^{2+} affects the structure of the peptide can be detected and analyzed more accurately by circular dichroism, the specific experimental method is as follows: the background of solvent is deducted on the circular dichroism before preparing for the test, the scanning range is set to 185 nm-260 nm, the scanning speed is 120 nm/min-1, the bandwidth is 2 nm[28], the optical range difference is 0.5 nm, the resolution is 0.5 nm, and the testing temperature was room temperature. After setting up the

experimental conditions, 2 groups of samples (copper ion free and 2.5 times copper ion, respectively) of 80uL2mM were taken and diluted with double-distilled water, ultrasonically dispersed for 15 min, and then dropped in a 1mm quartz cuvette cuvette sample cell, respectively, and scanned for circular dichroic spectroscopy under the protection of nitrogen gas. Each group of samples need to be collected at least 3 times, and the final data need to be averaged.

3.2.2 Infrared spectroscopy (FTIR) analysis

Because FTIR spectroscopy is based on the different intensities and frequencies of infrared light absorbed by different molecules, it is widely used in the study of the chemical composition of substances and molecular structure, so as to determine the spatial configuration and structural state of the molecules under study. In order to further investigate whether the presence of Cu^{2+} affects the structural characterization of peptides, infrared spectroscopy was applied to conduct experiments by uniformly grinding potassium bromide crystals, which had already had their moisture removed with an infrared lamp, into powder, then pressing the obtained powder into ingot tablets in a milling machine, and then deducting the air background after scanning through the spectrum. After the above preexperimental preparations were completed, two groups of samples (with copper ions and without copper ions, respectively) were subjected to liquid nitrogen snap-cooling treatment, frozen at -40℃ and dried for 24 hours to obtain powdered solids, and a small portion of the lyophilized solid powder was taken to be co-milled with potassium bromide and then pressed into a rounded ingot tablet. The scanning resolution was set to 4 cm^{-1}, the number of scans was set to 20, and the scanning range was set to 400-4000 cm^{-1}. Finally, the Fourier transform infrared (FTIR) spectra of the samples were tested using an infrared spectrophotometer[29], and the data were organized and plotted.

3.2.3 Transmission Electron Microscopy (TEM) Analysis

Transmission electron microscopy (TEM) requires a very thin sample and involves the emission of a number of assembled and accelerated electron clusters into the sample, which causes the clusters to collide with atoms in the sample. After collisions and interactions, the electrons change direction to produce different images. It is an extremely high-resolution microscopy technique that allows in-depth observation of the internal details of nanoscale structures.

Before the transmission electron microscopy test to bake the copper mesh under the infrared light for 6min, respectively, take two groups of samples (with Cu^{2+}, without Cu^{2+}) drop to the top of the copper mesh, let it stand for 20s, and the existing solution on the copper mesh will be sucked away with dust-free paper. Wait for its natural drying and then drop 10% volume ratio of phosphotungstic acid staining solution[30], wait for 20 min and then the excess staining solution was sucked away with dust-free paper. Finally, the micro-morphology of the two groups of samples was analyzed and characterized using transmission electron microscopy TEM by observing and taking images at a voltage of 100-200 kV.

3.3 Results and Analysis

3.3.1 Analysis of circular two (CD) chromatography results

In order to study the secondary structure characterization of the complex formed by the self-assembly of Cu^{2+} and peptide, the following circular two chromatographic data were obtained after testing, which were organized and then processed with special software to make a graph after processing the data. The respective peak positions of the single peptide and the peptide-Cu^{2+} complex can be clearly seen from the figure below.

As can be seen from the figure, there are obvious differences between the curves of the single polypeptide and those of the polypeptide-Cu^{2+} complex, which indicates that the structure of the polypeptide has changed significantly after binding with Cu^{2+}. In the entire wavelength range of 190-260 nm, the peptide curve is a relatively smooth state as a whole, with only a small maximum value at about 200 nm, followed by a continuous decline to 210 nm and a small minimum

value. On the other hand, the curve of the peptide-Cu^{2+} complex showed a very different situation from that of the single peptide curve, in which the overall change of the peptide-Cu^{2+} complex curve was drastic in the entire wavelength range of 190 nm-260 nm. An extremely strong maximum value appeared at about 200 nm, followed by a large and rapid decrease to reach the minimum value at 215 nm, and the overall change was very drastic. The reason for the above results was analyzed according to the principle of circular dichroism: the peptide interacted with Cu^{2+} after binding and Cu^{2+} interacted with some of the amino acids (histidine and cysteine) in the peptide, which caused the overall structure of the peptide to change, and finally formed a completely new structural state based on the peptide-Cu^{2+} complex[3637]. This brand new structural change may affect the biological characteristics and physicochemical properties in the presence of only a single peptide, i.e., it may affect the stability of the peptide and the interaction of the peptide with other molecules. This experiment can demonstrate that the addition of metal ions (Cu^{2+}) can cause significant changes in the structure of the peptide.

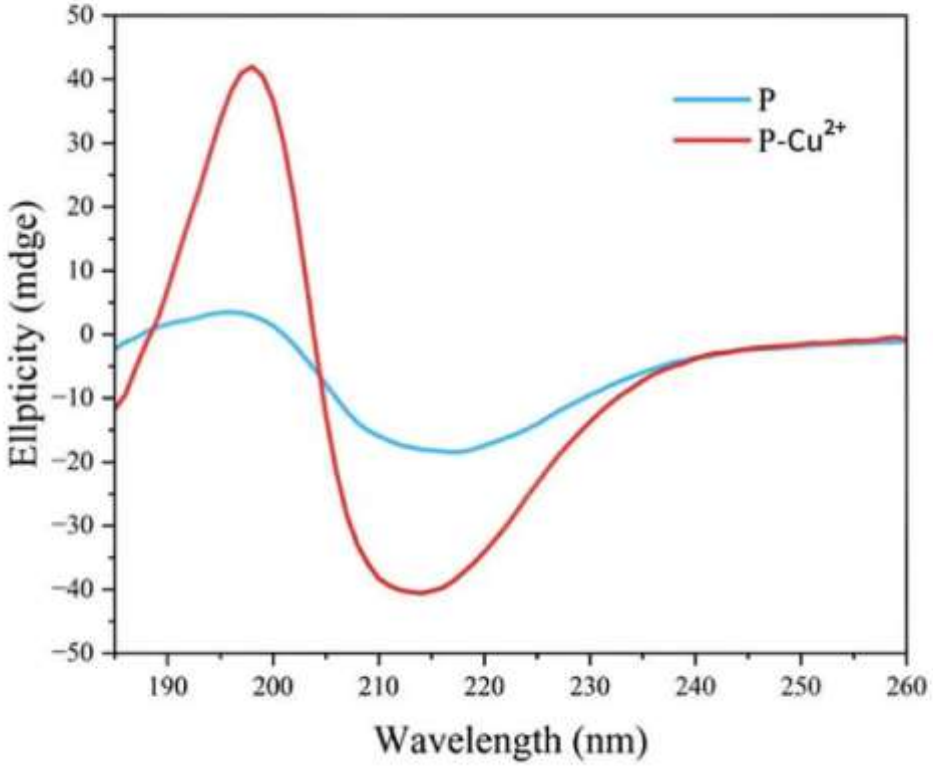

Figure 3.1 Circle II (CD) Chromatography

3.3.2 Analysis of infrared spectroscopy (FTIR) results

In order to further investigate the effect of the presence of Cu^{2+} on the structure of the peptide, the following infrared spectral data were obtained after testing and the data were processed with Origin software and then plotted. The characteristics of the curves of single peptide and peptide-Cu^{2+} complexes can be clearly seen in the figure below, thus proving that Cu^{2+} has a considerable effect on the structure of the peptide.

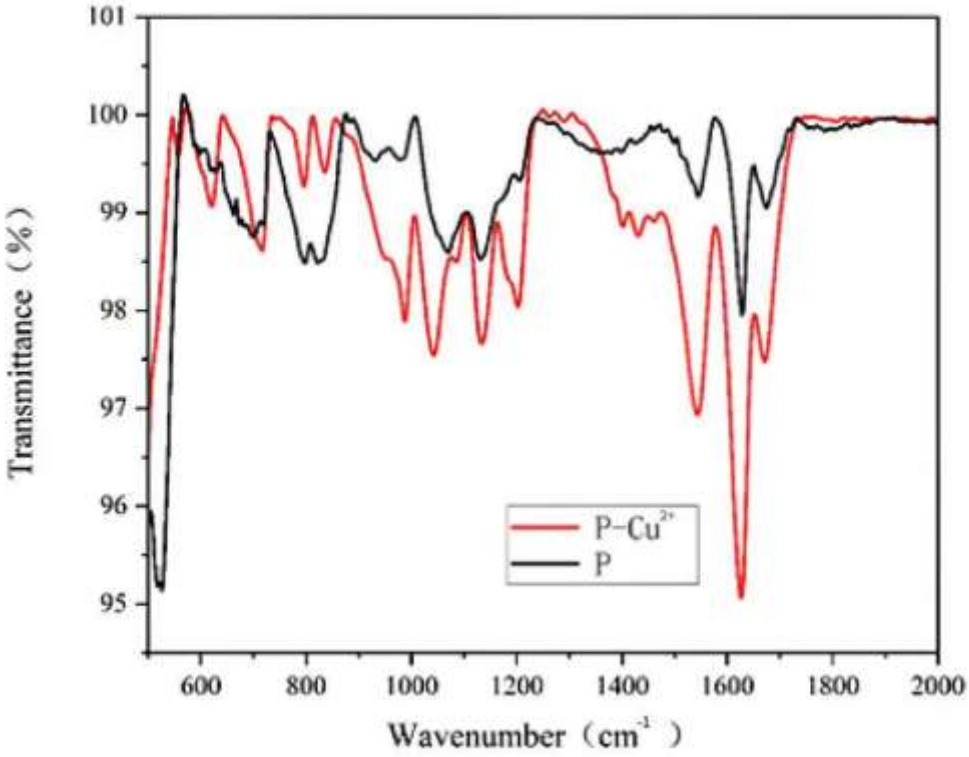

Figure 3.2Infrared spectroscopy (FTIR)

As can be seen in the figure, the graph for the single peptide (Cu^{2+} free) sample fluctuates approximately only in the 98%-100% range and produces only small amplitudes of change. These magnitudes of change, although small, indicate that the single polypeptide produces specific chemical bond vibrations and absorption within this range interval. In detail, this refers to the occurrence of telescopic vibrations of N-H and C-N chemical bonds at about 1530 cm^{-1}, the production of disordered structures or α-helical C=O telescopic vibrations at about

1640 cm^{-1}, etc.[38]. Although the overall transmittance of the peptide-Cu^{2+} samples is also relatively smooth in the range, the magnitude of the curve is much more drastic than that of the single peptide (without Cu^{2+}) samples. In particular, there are significant minimums at several locations[43], which indicate that extremely high absorption occurs at these locations. In detail, this refers to the apparent sharp lows at 1000 cm^{-1} and at 1600 cm^{-1}, where changes in secondary structure from disorder to order (e.g., β-folding or α-helices) may occur[39]. This suggests that Cu^{2+} binds and interacts with some nitrogen- and oxygen-containing functional groups in the polypeptide molecules, resulting in large changes in some of the structures and vibrational modes of the polypeptide molecules, which in turn change the vibrational level of the chemical bond. In this experiment, by comparing the absorption differences embodied in the infrared spectra of a single polypeptide (without Cu^{2+}) sample and a polypeptide-Cu^{2+} sample, it was demonstrated that Cu^{2+} is responsible for the transformation of the secondary structure of the polypeptide from disordered to ordered by coordinating with some of the polypeptide moieties[40]. It provides a spectroscopic data base for the whole experiment in the study of the interaction characteristics of Cu^{2+} with peptides[44].

3.3.3 Analysis of transmission electron microscopy (TEM) results

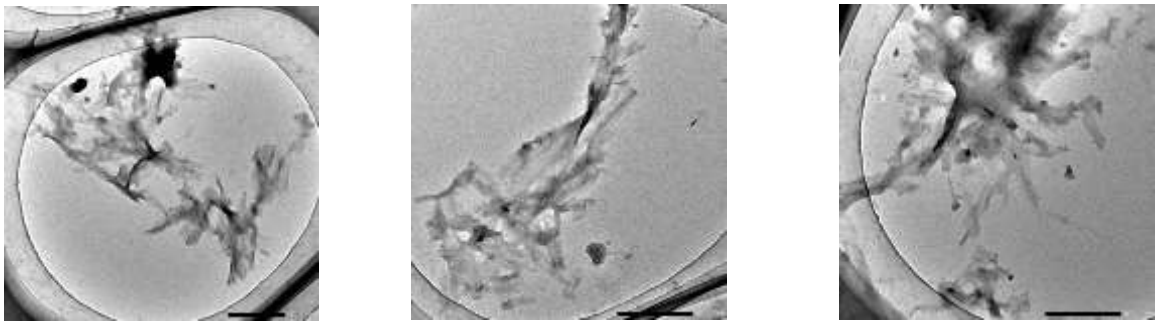

Figure 3.3 Transmission electron microscopy (TEM) without Cu^{2+}

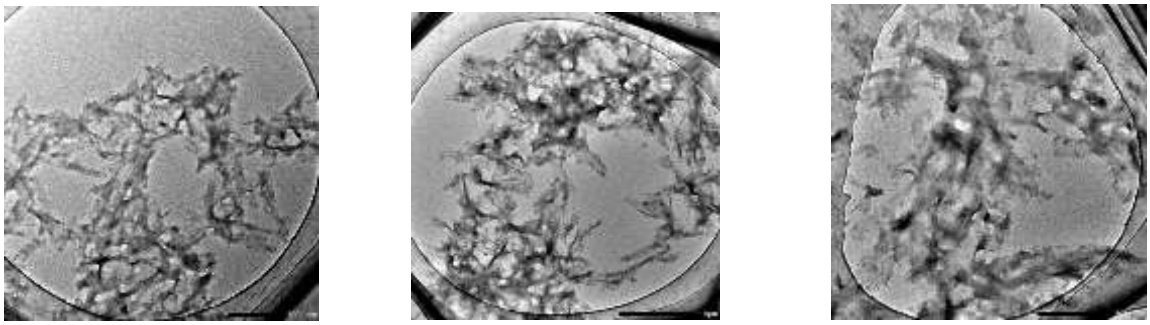

Figure 3.4 Transmission electron microscopy (TEM) with Cu^{2+}

After analyzing and characterizing the micro-morphology of the peptide samples as well as the peptide-Cu^{2+} samples using transmission electron microscopy TEM, a series of images were taken, and three images of each group were selected for comparison. Comparison of the images of the two groups of samples revealed that the distribution of substances in the field of view of the single peptide (without Cu^{2+}) sample was sparse, with most of the structures being slender and disordered, with fewer filamentous and fibrous structures, and with no obvious aggregation of large particles. The overall morphology is relatively simple and occupies a relatively small proportion in the whole field of view, which indicates that the nanostructures in the single polypeptide (Cu^{2+}-free) sample are basically unformed, and the overall distribution is inclined to be dispersed. On the other hand, the peptide-Cu^{2+} samples showed a significant increase in the amount of material in the field of view and a lot of aggregation phenomena, with a large increase in filamentous and fibrous structures, and the overall distribution is inclined to be aggregated. The aggregated structures were also found to be highly interwoven, with complex aggregation states, and the shapes and sizes of these aggregated structures were relatively irregular. The presence of uniform nanoparticles and fibrous structures with diameters of 20-40 nm[45] can be observed in the carefully enlarged images, which suggests that the addition of Cu2+ has had a very obvious effect on the microstructure of the original single polypeptide, promoting the aggregation of its nanostructures. This may be due to the chemical reaction of Cu^{2+} with some components in the single polypeptide samples, which changed the surface properties of the single polypeptide and affected its dispersion.

In detail, Cu^{2+}, as an inducing factor, induced the molecular groups in the single peptide samples to undergo intertwining and aggregation reactions, which resulted in the production of a large number of filamentous and fibrous structures and their intertwining and aggregation in the single peptide samples. This reaction resulted in the transformation of the peptide from a sparse microscopic feature to a complex and dense structural state. It can be concluded that Cu^{2+} is a kind of important metal ion that can make the peptide structure orderly and functional, and it can form a state similar to the active center of natural laccase by non-stop stabilizing the structure of the peptide, which provides a real and reliable experimental basis and support for the construction of efficient metal mimetic enzymes.

3.4 Experimental materials and apparatus

3.4.1 Experimental reagents

2,4 dichlorophenol (2,4-DP); 4-aminoantipyrine coupling (4-AP); ultrapure water; PB buffer with different pH values; copper chloride; manganese chloride; magnesium chloride; barium chloride; zinc chloride; peptide sequences Ac-ICVHLHLHVHI-$CONH_2$

3.4.2 Experimental apparatus

UV-visible spectrophotometer; vortex oscillator.

3.5 Experimental Methods

3.5.1 Catalytic activity test of metal-mimicking enzyme

There are many methods for detecting the catalytic activity of simulated enzymes, the common ones include electrochemical method, colorimetric method and spectrophotometric method and so on. In this experiment, the spectrophotometric method is still used to determine the catalytic activity of the mimetic enzyme, the principle is to use the mimetic enzyme catalytic oxidation of the substrate 2,4 dichlorophenol (2,4-DP) and 4-aminoantipyrine coupling (4-AP) to produce a red chromogenic product at a specific wavelength of 510 nm in the ultraviolet absorbance characteristics of the mimetic enzyme catalytic activity is indirectly determined[3132]. The specific experimental methodology was as follows:

the catalytic activity of the simulated enzyme was assessed by measuring the change in absorbance at 510 nm by UV-visible[46]spectrophotometer containing Ba^{2+}, Mn^{2+}, Mg^{2+}, and Zn^{2+}, as well as their blank controls (three parallel samples for each), using 2,4-DP as the substrate. The control group (blank group) was added 10uL of peptide, 10uL of 2,4-DP and 10uL of 4-AP, and 170uL of buffer in the last step. The experimental group (Ba^{2+}, Mn^{2+}, Mg^{2+}, Zn^{2+}) was added 8uL of metal ions, 10uL of 2,4-DP and 4-AP, and 172uL of buffer was added in the last step.

3.5.2 Determination of optimum pH of metal-mimetic enzymes

To determine the optimum pH for the catalytic activity reaction of metal-mimetic enzymes[33], the pH of the buffer was set to 5, 6, 7, 8, 9, and 10, respectively, and all other adjustments were kept the same. The samples of each group after adding different pH buffers were placed on a circular sponge sheet and oscillated at 25°C for half an hour. After completion the absorbance values were measured at 510 nm using photometric mode of UV-Vis spectrophotometer[5152]. Strict control of extraneous variables is needed in the process of conducting the experiments, so as to exclude the influence of other extraneous factors in the background on the final experimental results, and three parallel samples were set for each group of pH values to ensure the accuracy of the experimental results.

3.6 Results and Discussion

3.6.1 Catalytic activity test

Based on the measured data, it can be calculated that the average value of absorbance of Ba^{2+} is 0.092, Mn^{2+} is 0.065, Mg^{2+} is 0.060, and Zn^{2+} is 0.093. The above data were analyzed by using the Origin software to make a bar chart and then observed and analyzed, which shows that different metal ions (Ba^{2+}, Mn^{2+}, Mg^{2+}, Zn^{2+}) have different effects on the catalytic activity of the simulated enzyme using 2,4 - DP as the substrate. Mg^{2+}, Zn^{2+}) had different effects on the catalytic activity of the simulated enzyme using 2,4 - DP as substrate. Among them, the catalytic activities of Ba^{2+} and Zn^{2+} systems were higher, and the catalytic activities of Mn^{2+} and Mg^{2+} systems were relatively lower. The absorbance of each blank group was generally low and the values were close to each other[47], basically

in the range of 0.039-0.053, basically there is no catalytic activity, which indicates that the catalytic activity of the simulated enzyme is weak in the absence of metal ions. The purpose of setting the blank group is to exclude the interference of other irrelevant factors on the absorbance during the experiment. The data of the blank group indicates that the interference generated by the background of the experimental process is small, and the experimental data are real and credible, and it can also be confirmed that the measured absorbance changes can effectively reflect the catalytic activity of the simulated enzyme is different under the action of different metal ions[4849]. It can be concluded that, except for Cu^{2+}, Ba^{2+} and Zn^{2+} have higher catalytic activity when combined with peptides[50].

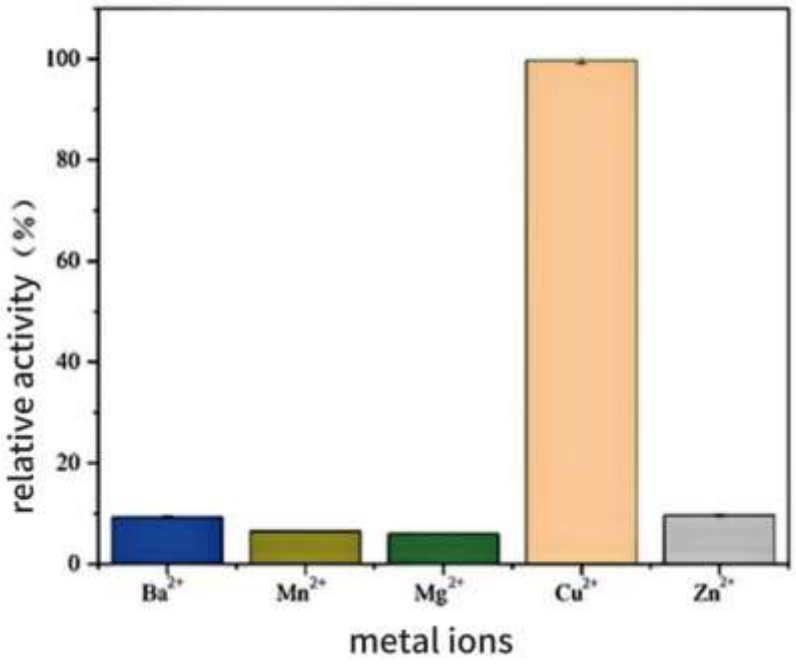

Figure 4.1 Catalytic Activity Test

3.6.2 Analysis of the results of determining the optimum pH value

In addition to the type and concentration of metal ions, the difference in pH also affects the activity of the simulated enzyme. Higher or lower pH affects the spatial structure of the mimetic enzyme, which hinders the binding of the mimetic enzyme to the substrate[41], making the activity of the mimetic enzyme affected[42].

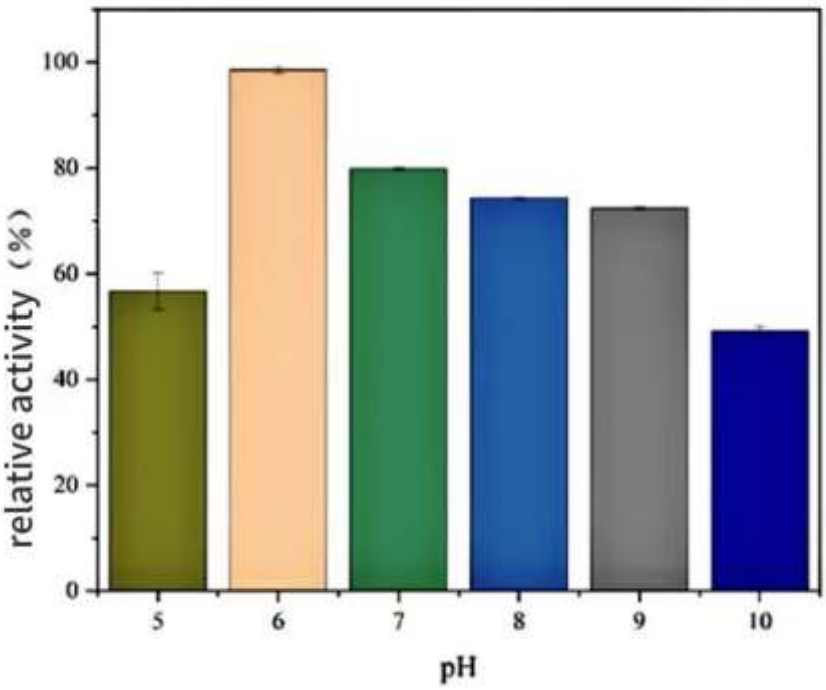

Figure 4.2 Optimum pH Test

By setting the pH of different buffers in the reaction system, the results of absorbance values read at 510 nm by UV-visible spectrophotometer at different pH values could be determined, and the optimum pH value for the catalytic activity of metal mimetic enzyme of laccase-like activity was determined. After making a bar graph of the measured data using Origin software, it was observed that the absorbance reached its highest value at pH 6, indicating that the relative vigor of laccase-like activity was highest at pH=6. The decrease in absorbance at extreme pH values (e.g., pH = 5 or 10) may be due to the denaturation reaction of the metal mimic enzyme or a decrease in substrate stability that results in the inhibition of the entire extent of the reaction.

Summary of chapter III

In this chapter, the structures of metal-mimicking enzymes that have been prepared with peptides as materials are observed and analyzed. The internal structures of single peptide samples and peptide-Cu^{2+} samples were explored using

circular dichroism (CD) chromatography, Fourier infrared spectroscopy (FTIR), and transmission electron microscopy (TEM), and the results all indicate that with the addition of Cu^{2+}, it will make the structure of the peptide undergo a great change, specifically, it will make the secondary structure of the peptide change from disordered to ordered.

In this chapter the enzymatic properties of peptide-based metal mimetic enzymes were explored. Firstly, the catalytic activity of the metal mimic enzyme was tested by using 2,4-DP as substrate and measured by UV-visible spectrophotometer, the change in absorbance reflects that the catalytic activity of the peptide mimic enzyme increases with the addition of metal ions. Finally, by setting different pH gradients, the experiment concluded that the activity of peptide-mimicking enzyme reached the highest value at pH=6.

1. Circular dichroism (CD) analysis
2. Infrared spectroscopy (FTIR) analysis
3. Transmission electron microscopy (TEM) analysis
4. Catalytic activity testing of metal-mimicking enzymes
5. Determination of optimum pH for metal-mimicking enzymes
6. Results and discussion

CONCLUSION

1. In this study, based on the active center and structural features of natural laccase, the peptide sequence Ac-ICVHLHLHVHI-$CONH_2$ was rationally designed and a metal mimetic enzyme using the peptide as material was successfully constructed.

2. The effects of different metal ions (Cu^{2+}, Ba^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}) on the activity of the peptide were experimentally investigated, and it was found that Cu^{2+} was the most suitable metal ion for binding with the peptide, and the constructed peptide-Cu^{2+} complex could be used as a laccase mimicking enzyme.

3. The optimal binding concentration of Cu^{2+} to the peptide was further analyzed, and it was found that the peptide-mimicking enzyme could reach the highest activity when 8 mM of Cu^{2+} was added.

4. An in-depth study of the metal mimetic enzyme using circular dichroism (CD) chromatography, Fourier infrared spectroscopy (FTIR), and transmission electron microscopy (TEM) revealed that the internal structures of the single polypeptide and the polypeptide-Cu^{2+} complex were significantly different, and that the secondary structure of the polypeptide was transformed from disordered to ordered with the addition of Cu^{2+}. This ordered structure results in the formation of a catalytic activity similar to or even higher than that of natural laccase.

5.Finally, the catalytic activity of the metal mimic enzyme was tested. By setting different pH gradients, the experiment concluded that the activity of the peptide mimic enzyme reached the highest value at pH=6.

6. In conclusion, this experiment used a simple and environmentally friendly method to construct a peptide as the material of the metal mimic enzyme, and carried out a more in-depth study and analysis of its structure and catalytic activity and other aspects. It shows great applicability and provides a new direction and experimental basis for the development of low-cost and high-stability mimetic enzymes. In the future, we can further optimize the culture conditions of the peptide-based metal mimic enzyme, which can greatly reduce the production cost

and the threat to the environment, and hope to realize the mass production and wide application of the peptide-based metal mimic enzyme.

7. There are still some limitations in this study, the storage stability and catalytic activity of peptide-based metallo-mimetic enzymes under long time and complex environment have not been proved, and the applicability of the substrate in practical application needs to be further examined. The relationship between the coordination of metal ions and catalytic activity can be explained by molecular dynamics simulation, and the peptide-based metal mimetic enzyme can be applied to paper wastewater or biosensor for more in-depth application research.

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