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 **Preparation and spectroscopic determination of C phycocyanin, R phycoglobin and B phycoglobin by salting out**

First (Bachelor's) level of higher education
Specialty 162 "Biotechnology and Bioengineering"
Educational and professional program "Biotechnology"

Completed: student of group BEBT-20
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Doctor of Technical Science
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ASSIGNMENTS
FOR THE QUALIFICATION THESIS
Ruiying Zheng

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

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I am familiar with the task:

Student _____ Ruiying ZHENG

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SUMMARY

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Phycobilin is an important pigment protein in photosynthesis, which mainly exists in blue-green algae. According to their maximum absorption peaks in the spectrum, they can be divided into C-phycocyanin, R-phycoglobin and B-phycoglobin. These three phycobilin proteins each have specific functions in living organisms, and their existence and mechanism of action in nature are also different.

In this paper, we describe in detail the preparation process of C-phycocyanin, R-phycoglobin and B-phycoglobin, and how to determine them by spectroscopic method to understand their spectral characteristics. First, the cells are broken and then coarse extracted using a salt-out extraction method. The physical, chemical and spectral characteristics of three phycobilins were studied by various analytical methods, including UV-VIS spectroscopy, fluorescence spectroscopy and circular binary chromatography.

Key words: phycobilin,biosynthesis, spectral, analysis

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INTRODUCTION

Phycobilin is a light-trapping pigment protein peculiar to some algal cells, which is mainly found in cyanobacteria and green algae. The known phycobilin can be divided into phycocyanin, phycoglobin, phycocyanin and isophycocyanin. Three phycobilin proteins, C-phycocyanin, R-phycocyanin and B-phycocyanin, were studied in this paper. They each have specific functions in living organisms, and their existence and mechanism of action in nature are also different.

The cells were broken by repeated freeze-thaw method, and then salted out with 65% ammonium sulfate to obtain the crude extract of phycocyanin. The highest purity of phycocyanin obtained by two-step salting out method was 3.95, and the recovery was 9.56%. The concentration of phycocyanin obtained by chromatographic chromatography is about 3, and the purity of phycocyanin obtained by chromatographic chromatography is about 3.5. Extraction and salting out purification are important steps in the preparation of high purity phycobilin, which can improve the yield and purity of phycobilin and provide high quality raw materials for subsequent experimental operations. After obtaining high purity phycobilin, the spectral characteristics of three kinds of phycobilin were studied by various analysis methods, including UV-VIS spectrum and fluorescence spectrum, and the absorption spectrum and fluorescence spectrum of three kinds of phycobilin were obtained. In addition to the absorption peak of 497 nm, R-phycoglobin also has two absorption peaks of 537 nm and 565 nm, and a fluorescence emission peak at 576 nm. C-phycocyanin has characteristic absorption peaks near 278 nm, 360 nm, 620 nm in the UV-visible region, and fluorescence emission peaks near 620~640 nm. B-phycoglobin has two main absorption peaks at 562 and 545 nm, a acromial peak at 498 nm, and a strong fluorescence emission peak at 574 nm.

The relevance of the topic is related to the extraction and purification of phycobilin.

The purpose of the study is salt-out extraction and spectroscopic determination of three phycobilinins, including their absorption spectra and fluorescence spectra.

The objectives of the study is three kinds of phycobilin with high purity were obtained, their absorption spectra and fluorescence spectra were measured, and they were used in life production and biomedical research. Three phycobilin proteins are of interest to humans, C-phycocyanin has anti-cancer, anti-apoptosis and anti-oxidation effects, R-phycoglobin can be used to make fluorescent probes, and B-phycoglobin can be used as an additive in the food industry.

The object of the study are three types of phycobilin.

The subject of the study is Efficient preparation and spectroscopic determination of phycobilin.

Research methods:

1. Extraction of three phycobilin by two-step salting-out method

2. UV-550 ultraviolet spectrophotometer, JascoFP-560 fluorescence spectrometer (Absorption spectra and fluorescence spectra were detected respectively)

The scientific novelty: The spectral properties of phycobilin were studied on the basis of its high efficiency preparation.

The practical significance of the results obtained is helpful for human to better understand and use phycobilin, fight against diseases, and help human to better understand the spectral characteristics of three phycobilin.

CHAPTER 1
LITERATURE REVIEW

1.1 Structure of phycobilin

Phycobilin is a kind of protein, which mainly exists in cyanobacteria, red algae and crypto-algae, and has the function of absorbing and transferring light energy. In addition to having properties common to other proteins, phycobilin is an excellent natural food coloring, making it an ideal health food. Phycobilin is a complex protein composed of one or more polypeptide chains combined with one or more pigment molecules. Phycobilins can be classified according to the way they bind to pigment molecules, including phycocyanin, phycoglobin and allophycocyanin.

Phycocyanin has a complex structure, which is dominated by $\alpha_3\beta_3$ ring trimers and $(\alpha_3\beta_3)_2$ hexamers. The size of the α subunit is about 13-20 kDa, and the size of the β subunit is about 14-24 kDa. The protein contains 160-180 amino acids, which form the basic structure of phycocyanin, allowing them to efficiently absorb outside light and thus perform important biological functions. Phycocyanin derives its color from linear tetrapyrrole compounds, which can be attached to proteins in the form of thioether bonds. C-phycocyanin has A light blue color, while A-phycocyanin has a distinct blue color[1].

R-phycorubinin binds to phycorubine-like pigment molecules and usually appears red. Its structure is similar to C-phycocyanin in that the polypeptide chain interacts with the pigment molecule through non-covalent bonds. Phycoglobin has a unique structure, it is composed of α, β and γ three subunits, of which the molecular weight of the α subunit is generally between 13-20 kDa, the β subunit is larger, up to 14-24 kDa, and the γ subunit is higher, up to 30-34 kDa, thereby improving its stability on the surface of phycobilisome.

B-phycohemin binds to another phycohemin-like pigment molecule, which also usually appears red. Its structural characteristics are similar to R-phycoglobin, but the specific details of the interaction between the polypeptide chain and the pigment molecule may be different.

1.2 Characteristics of phycobilin

C-phycocyanin has a unique structure and exhibits different properties in different environments. The visible absorption peak of purified spirulina C-phycocyanin was 620 nm, and the fluorescence emission peak was about 657 nm at room temperature. By measuring their characteristic peaks and protein absorption rates, they can be classified into different purity levels. If A620/A280≥0.7, it is considered edible protein. If A620/A280 is in the range of 0.7 to 3.9, it belongs to the experimental protein; If A620/A280≥ 4.0, it is a molecular protein. With the improvement of C-phycocyanin quality, its market competitiveness has become more and more powerful. The price of C-phycocyanin in food grade is only 0.9 yuan per gram, and that of analytical grade is 105 yuan per gram[2].

According to the characteristic absorption spectrum, R-phycoglobin can be divided into two peaks and one shoulder type (only 2 absorption peaks at 498 and 565 nm, and 535 nm is the absorption shoulder) and three peaks (3 complete absorption peaks at 498, 535 and 565 nm). It was found that the total molecular weight of R-phycoglobin was up to 240 kDa, and the α subunit contained the characteristic structure of phycoglobin, and the β subunit contained the characteristic structure of phycocyanin, with a total of 6 subunits. The correlation between R-phycoglobin and them makes R-phycoglobin a phycobilic protein with extremely high fluorescence properties, the quantum efficiency of R-phycoglobin even reaches more than 90%, and the orange-red color of R-phycoglobin is clearly visible even at lower concentrations.

The three absorption bands of B-phycoglobin show its unique characteristics, with the highest absorption value at 545 nm, and its subunit structure is similar to R-

phycoglobin, but the α and β subunits have more chromophores, making its absorption peak more pronounced. In addition, the gamma subunit also contains a certain amount of phycoglobin and phycocyanin, which affect its absorption capacity. B-phycoglobin is a special protein commonly found in cyanobacteria and red algae, and their color is dominated by bright pinks and oranges, which makes them fundamentally different from R-phycoglobin, which is difficult to recognize with the human eye.

1.3 Function and research significance of phycobilin

1.3.1 Function of phycobilin

Phycobilin has many functions, including anticancer, anti-apoptotic, and antioxidant abilities. Phycoglobin can be used as a fluorescent probe with high sensitivity, and can also be used as a food additive with good coloring ability. Higher purity phycocyanin can resist inflammation, antioxidant, inhibit cancer and promote immunity, so it can be widely used to treat and prevent diseases[3][4]. Therefore, more high-quality phycocyanin has been developed to meet the urgent need for its efficient utilization.

At present, the domestic and foreign research is very in-depth. The study found that when phycocyanin was added to rats with liver cancer, the survival rate was significantly higher than that of the control group without the addition, and after 5 weeks, the survival rate in the treatment group reached 90%. This indicates that phycocyanin protein can inhibit the expansion of cancer cells and enhance the body's immunity[5].

The most widespread effects of phycocyanin are its anticancer and apoptotic effects and its anticancer antioxidant capacity. Phycocyanin can induce apoptosis of cancer cells and is a natural antioxidant. Subhashini et al. found that the proliferation rate of chronic myeloid leukemia cell line (K562) was significantly reduced after treatment with 50 μmol phycocyanin protein for 48 h. After flow cytometry test, when 25 μmol phycocyanin protein was applied to K562 cells,

14.11% of the cells showed G_0/G_1 phase within 48 h, indicating that phycocyanin protein could promote the synthesis of cytochrome C and lead to the death of K562 cells[6]. Dolastatin 10, an 18-membered cyclohexapeptide originally isolated from Dolabella auricularia, is weakly cytotoxic to tumor cells. Luesch et al. found that Dolastatin-10 is actually derived from Symploca sp, one of the food sources of sea rabbits[7]. These results suggest that algae could be used to develop drugs to treat cancer.

Gao et al. used R-phycoglobin as a fluorescent probe directly bound to lgG protein to establish a method for rapid detection of HEV antibody in animal serum, which greatly reduced the detection time compared with enzyme-linked immunosorbent and fluorescence-based Luminex analysis[8]. R-phycoglobin can be used as a fluorescent probe for the detection of heavy metal ions. Later, Xu et al. used R-PE-AgNPS as a strong red fluorescent probe to establish a method for the detection of Cu^{2+} without special equipment[9].

The amino acid composition of B-phycohemoglobin is very complex, its color stability is strong, and excellent safety, so it is widely used in various industrial production, such as food, cosmetics, fiber manufacturing, dye manufacturing. The high quality protein combination of B-phycoglobin, along with a variety of unique nutrients, makes them one of the highest quality proteins in nature and has been unanimously recognized by consumers[10].

1.3.2 Research significance of phycobilin

Phycobilin proteins can be regarded as valuable substances in plants, which not only convert solar radiation into heat, but also play their vital role in promoting photosynthesis in plants. C-phycocyanin, R-phycoglobin and B-phycoglobin are three main phycobilic proteins, and their preparation and spectroscopic determination are of great significance in the fields of biology, bioengineering and Marine science.

The preparation of phycobilin is the prerequisite for further study and application of phycobilin. Although there have been some reports on phycobilin, there are still some challenges in its preparation process. The extraction and purification of C-phycocyanin, R-phycocyanin and B-phycocyanin require sophisticated experimental conditions and techniques, and their yield is low, which limits their application. Therefore, it is of great practical significance to study their preparation methods and improve their yield and purity.

Spectroscopic determination is an important means to study the structure and function of phycobilin. The molecular mechanism of phycobilin in the process of absorption, transfer and conversion of light energy can be deeply understood by spectroscopic determination, which will help us to understand the process and mechanism of photosynthesis more deeply. In addition, through the spectral determination of phycobilin in different states, it can also reveal their changes under different environmental conditions, and provide important theoretical basis and practical guidance for bioengineering and Marine science.

The preparation and spectroscopic determination of C-phycocyanin, R-phycocyanin and B-phycocyanin have important research background and significance. This study not only provides a valuable reference for us to better understand the structure, function and mechanism of phycobilin, but also brings substantial application value for the development of new biological materials, improve the efficiency of photosynthesis, and protect the Marine ecological environment. Through continuous research and innovation, we are expected to achieve more breakthrough results in future photosynthesis research.

1.4 Preparation of phycobilin

Including cell crushing and extraction, separation and purification of ammonium sulfate.

1. Cell fragmentation

Using biochemical wall breaking technology, phycobilin can be effectively isolated and purified [11], thereby improving its structure and promoting its intermolecular interaction. The method mainly relies on a variety of biochemical agents, such as acids, bases, organic synthetic solutions[12], surfactants[13], etc., which can change the structure of phycobilis to a certain extent, so as to achieve the interaction between molecules, so as to achieve the efficient separation and purification of phycobilis, and can be recovered twice in the process of AGAR preparation. Although this technique can greatly improve the speed and effectiveness of the extraction of phycobilin, the final purity is reduced due to the use of chemicals that may inhibit phycobilin biosynthesis.

By using a variety of different technical means, such as multiple freeze-thawing methods[14], osmotic pressure[15], high-pressure crushing, grinding[16] and crushing crushing[17], the morphology of the cell can be greatly changed, and its shell and surface can be destroyed, thereby transferring its internal material to the extraction solution. Through continuous cooling and cooling, ice can be condensed on the cell wall, which reduces the water content of the cell and increases the osmotic pressure inside it, eventually causing the cell to rupture and release phycobilin. Because of the simplicity of this technology, it is widely used in industrial production. In this paper, freeze-thaw method was used to break cells.

2. Separation and purification of protein

Through different technical means, the efficient and accurate purification of proteins can be effectively achieved. Among them, common techniques include salt-out method, ultrafiltration method[18], chromatography method[19], isoelectric point precipitation method[20]and chromatography method[21], which can effectively separate specific components of proteins from other components to achieve efficient protein purification. Through ammonium sulfate salting out technology, we can effectively obtain the required information from phycobilin.

With the further study of phycobilin, the separation and purification methods of phycobilin have been further optimized. It was found that the extracted liquid was

purified by dextranan gel and DEAE ion exchange chromatography. The purity of phycobilin was higher and the quality of phycobilin was improved greatly. The chromatographic column method has many advantages, such as temperature environment conditions can ensure the biological activity of phycobilin protein, but the chromatographic column method is mostly used for small-scale extraction, and does not involve large-scale extraction, which needs further research. The diaqueous phase extraction is based on the diversity of proteins in the middle of the two phases, such as static, hydrophobic and other functions. Common dual systems are polyethylene glycol (PEG) and dextran. Nascimento et al. compared the extraction efficiency of systems with different proportions of PEG, potassium phosphate, ammonium sulfate and sodium citrate, and believed that the purification efficiency of 13% PEG and 14% potassium phosphate system was better, and continuous extraction efficiency was better than intermittent extraction[22]. Rochak et al. used PEG1450-potassium phosphate system for continuous extraction. Compared with PEG3350-potassium phosphate system, the former achieved better purification efficiency after purification, improved quality by 11 times, and increased yield by 57%[23]. The two-phase aqueous extraction method can be used under low pressure to preserve the activity of phycobilin, and can also be combined with chromatography to obtain higher purity phycobilin. The electrostatic attraction and hydrophobic power of different proportion systems are different, so the purification effect will be different, and the selection of appropriate separation system for the target protein is the primary problem to be considered in this method. High performance liquid chromatography (HPLC) is a method for separating and purifying components from complex mixtures. The purity and molecular weight of phycobilin can be analyzed by HPLC to ensure the high purity and consistency of the prepared samples. In general, extraction and purification are important links in the preparation of high-purity phycobilin. By optimizing extraction and purification methods, the yield and purity of phycobilin can be improved, and high-quality raw materials can be provided for subsequent experimental operations.

1.5 Fluorescence determination of phycobilin

1. Detection of spectral characteristics

By using ultraviolet spectrophotometer, we can accurately detect the light-absorbing properties of phycobilin. In addition, we can also use the Hitachi 850 fluorescence spectrophotometer to detect its fluorescence luminescence properties, and use 50 mmol/L phosphate buffer (PBS buffer) as a blank. Finally, we can draw the phycobilin elution curve and accurately detect the light absorption at different time points.

2. Fluorescence spectrometry

By using the Is55 spectrophotometer, we can accurately detect the presence of phycobilin in the wavelength range Ex=498 nm, and in the wavelength range Em=615 nm.

Conclusions to chapter 1

This chapter mainly introduces the following problems:

1. Introduces the classification and structure of phycobilin, as well as the characteristics
2. The research significance and progress of phycobilin were introduced.
3. The research methods of phycobilin were summarized

CHAPTER 2
OBJECT, PURPOSE AND METHODS OF THE STUDY

2.1 Experimental equipment and reagents

2.1.1 Experimental materials

The experimental materials for extracting C-phycocyanin include spirulina dried algae powder.

The materials for extraction of R-phycoglobin include polysiphonium powder.

The extraction of B-phycoglobin was made from laver.

2.1.2 Experimental reagents

Ammonium sulfate, sodium chloride, barium chloride, 50 mmol/L phosphate buffer with PH 7.0, ultrapure water, ammonium sulfate and Sephadex G-25 glucan gel.

2.1.3 Experimental instruments

Dialysis bag (800-14000 kDa); Dextran gel column G-75; Electrophoresis kit; Ultraviolet visible spectrophotometer (UV-9000); Centrifuge (H1850R); Computer UV chromatograph (HD-3001); High pressure homogenizer (GJJ-0.06/100); Ultrasonic cell shredder; Electrophoretic system; Gel imaging analyzer; Color difference meter. Ultraviolet visible spectrophotometer (UV-2401PC); Chromatographic column; High speed centrifuge; Analytical balance (BS210S, Sartorius, Germany); Ultrafiltration centrifugal tubes (Vivaspin-PES MWCO 100 kDa, 300 kDa, 500 kDa, Sartorius, Germany); Liquid chromatography system (KTA prime plus, GE, USA); Superose12 10/300 GL column (10 μ m, GE, USA).

Preparation of biurea reagent: Weigh 0.30 g copper sulfate ($CuSO_4 \cdot 5H_2O$), 1.20 g potassium sodium tartrate ($KNaC_4H_4O_6 \cdot 4H_2O$), dissolve in 100 ml water, add 60 ml 10% NaOH solution under agitation, and dilute with water to 200 ml.

Phosphate buffer (pH 7.2) : Take 0.2mol /L potassium dihydrogen phosphate solution 50 ml and 0.2 mol /L sodium hydroxide solution 35 ml, add newly boiled cold water to dilute to 200 ml, shake well, and get.

1 g/L of casetyrosine solution.

Weigh 0.100 g, add 100 ml distilled water, boil over low heat, dissolve and store at low temperature.

2.2 Cell Fragmentation

Repeated freeze-thaw is a good method, through continuous freeze-thaw, can solidify the cell fluid into ice, which will reduce its content, and strengthen its internal osmotic pressure, and eventually cause the cell to rupture, thereby releasing phycobilin.

(1) Crude extraction of R-phycoglobin.

The polysiphon powder was poured into the container, and 1:20 (w/v) PBS buffer (0.1 mol/L PH 7.0) was added, and it was stirred to fully integrate, then it was moved to the refrigerator at -20℃ for 5h cooling, and finally solidified, and then it was moved to a water bath at 37℃ for 4 thawing treatments. The obtained phycoglobin crude extract was centrifuged at 4℃ and 6000 rpm for 20 min to remove cell debris and obtain cell-free red crude extract.

(2) Crude extraction of C-phycocyanin

Weigh 50 g spirulina powder, add 200 ml 0.1M phosphate buffer (PH 6.8), freeze and melt for 2 to 3 times, filter through gauze, centrifuge, and collect supernatant to obtain C-phycocyanin crude extract.

(3) Crude extraction of B-phycoglobin

In order to extract B-phycoglobin, 4 g laver was immersed in deionized water for 4 h. Then, the laver was put into the ice bath, ultrasonic crushing technology was used to prevent the protein denature caused by high temperature, each work lasted 5s, the interval time was 10s, every 10 times for a cycle, and finally put the laver into the refrigerator for a night. The centrifuge was run at 6000 rpm at 4℃ for 20

min to remove the algal residue, and the purplish red supernatant obtained was the initial extract of B-phycoglobin.

After the cell is broken, the protein will ooze, the use of biuret reagent method, the protein content in the solution is determined, the following is the determination of protein content:

(1) Take 1 ml of freeze-thaw extraction solution at each freeze-thaw interval, dissolve in 4 ml of distilled water, and prepare 4 times thinner solution.

(2) The 4 times diluent is centrifuged in a low temperature centrifuge to remove insoluble impurities.

(3) Add 0.2 ml of biuret reagent to 0.8 ml and mix well.

(4) Static color development for 30 min, the absorbance was measured at 540 nm by ultraviolet spectrophotometer.

2.3 Salt-out purification of phycobilin

The salt-out method is mainly through the addition of high concentration of electrolytes, so that the solubility of the protein is significantly reduced, and finally the purification of the protein is realized. Phycobilin is usually precipitated by sulfate. The purification of phycobilin can be achieved by adding appropriate amount of ammonium sulfate. At smaller concentrations, protein molecules can be well absorbed and improve their adsorption capacity. However, when the concentration increases to a certain extent, the protein in the protein can no longer be directly adsorb on the protein, which causes the solute of the protein to be affected, and ultimately leads to the solidification of the protein[24].

At present, the commonly used method is ammonium sulfate precipitation. In this case, a concentration of 20% to 65% ammonium sulfate is used to achieve the best settling effect. When the saturation reaches a certain degree, the quality and recovery of phycobilin will also be significantly improved, and when the saturation reaches 65%, the quality and recovery of phycoglobin will also be significantly improved. After a thorough evaluation, we decided to use 20% ammonium sulfate

as a two-step treatment agent to ensure a final saturation of 65%. This step consists of two parts: one is a one-step treatment: adding 20% saturated ammonium sulfate from the freeze-thaw broken crude extract, and then mixing at a slow rate to prevent the addition of too much at one time to lead to the deterioration of phycobilin; The other part needs to be completely mixed and then placed in an environment of 4℃ until the end of coagulation. In the centrifuge, we centrifuge at a speed of 6000 rpm/min for 20 minutes and dilute it with a phosphate buffer (20 mol/L, PH 7.0). Then, ammonium sulfate was used for secondary salting out to obtain the supernatant with higher recovery rate. The concentration of ammonium sulfate was added to 65% and stored at 4 ℃ overnight. It is then centrifuged at 6000 rpm/min and the obtained precipitate is added to the phosphate buffer and adjusted to PH 7.0 to dissolve it completely. Through the complete spectral analysis, we can determine the purity of the sample and the adsorption effect.

The process of ammonium sulfate experiment is as follows:

1. Put the protein solution in an ice bath at 0℃, and then stir it in a magnetic stirrer;

2. While stirring, add the corresponding gram of ammonium sulfate to saturation, the step is completed within 5 to 10 minutes;

3. Continue stirring for 10 ~ 30 min;

4. Let it stand overnight and then centrifuge at 6000 rpm for 20 min at 4℃.

5. Pour off the upper liquid and add PBS buffer with a volume ratio of 1:20 to observe the solution, some of which may be polysaccharides.

6. Ammonium sulfate can be effectively removed by a variety of methods, such as dialysis, ultrafiltration and desalination columns.

Precautions during the experiment:

1. Mixing should follow the rules and be gentle. If the speed is too fast, the protein will denature and appear as foaming. It is also important that the magnetic stirrer does not produce significant heat.

2. Most proteins precipitate in the first 20 minutes after salt dissolution, and some proteins can be completely precipitated after several hours.

3. The buffer should contain an chelating agent such as EDTA to remove trace heavy metal ions in ammonium sulfate, because these ions are harmful to the target protein.

4. To ensure maximum precipitation, use an initial protein concentration of at least 1 mg/ml.

5. Ammonium sulfate precipitation is an effective technology for protein preservation and stabilization, and its effect is remarkable.

6. In the concentration range of ammonium sulfate, a few proteins can precipitate below 24%, while most require more than 65% to form a precipitate. Therefore, the difference in solubility of protein in ammonium sulfate can be used to achieve the separation and purification of protein. RNA and DNA can be removed by ammonium sulfate precipitation.

2.4 Purification of phycobilin by ion chromatography

In order to further purify the protein, the ion exchange chromatography column such as DEAE-cellulose DE-52 ion exchange chromatography is often used to purify phycobilis protein, and hydroxyapatite chromatography is used to achieve linear ion intensity gradient method. There are two main methods to characterize the purity of phycobilin, which are comparing the ratio of maximum visible light absorbance to absorbance at 280 nm and polyacrylamide gel electrophoresis.

The 65% saturated precipitate was dissolved in a 20 mM phosphate buffer (PH 7.0) and dialysed overnight in the same buffer.

Phosphate buffer (20 mM, PH 6.5) : accurately weigh 2.26 g NaH2PO4, dissolve 1L of deionized water with 2.14 g Na2HPO4, and store at 4℃.

NaCl eluent (0.3 M, 20 mM PBS) : 8.7 g of NaCl was accurately weighed and dissolved in 500 mL 20mM phosphate buffer and stored at 4℃.

NaCl column regeneration solution (1.5 M, 20 mM PBS) : 43.5 g NaCl was weighed and dissolved in 500 mL 20 mM phosphate buffer and stored at 4℃.

The R-phycohemoglobin isolated by DEAE-sephaRose Fast Flow column was pretreated to PH 5.6 at PH 5.6 in 20 mm phosphate buffer containing 0.05 M NaCl to ensure the accuracy and reliability of separation. Before cleaning with the same buffer, a phosphate buffer with a 0.05 M NaCl content, PH 5.6-4.0 value, volume of 2×50 ml, 1 ml per minute is used. The eluent was detected at 280 nm and collected in 2 ml. As the elution PH changes, different proteins can be elated with different eluents according to different pI (PE is 3.7, PC is 4.6).

(1) DEAE Sepharose Fast Flow gel was slowly added to the chromatographic column (length 20 cm, diameter 1.5 cm) on the chromatographic platform along the edge and left for one hour. Phosphate buffers were added slowly (20 mM, PH 6.5) and applied to DEAE gels to ensure maximum column volume and up to 5 times column balance.

(2) After column balance, an appropriate amount of phycobilin crude extract was added to the gel column, and then 3 times the column volume was washed with phosphate buffer (PH 6.5, 20 mM) to remove the impurity protein in the crude extract.

(3) Elution with 0.3M and 0.5M NaCl eluents (PH 6.5), respectively, and collection is determined according to the color of the effluent.

(4) The phycobilin solution purified by DEAE was centrifugated at 6000 rpm for 20 min at 4℃, the precipitation was removed, and the absorbance at 562 nm, 615 nm and 652 nm was determined after dilution. The concentration and purity of the purified phycobilin were calculated according to the absorbance values at specific wavelengths.

2.5 Determination of phycobilin absorbance

By applying optical methods such as ultraviolet spectroscopy and infrared spectroscopy, we are able to measure the absorption luminance of phycobilin.

Among them, the spectral range of ultraviolet spectroscopy is between 200 and 780 nm, where the measurement range of ultraviolet spectroscopy is between 200 and 400 nm, and the measurement range of infrared spectroscopy is between 400 and 780 nm. Infrared spectroscopy is 2.5~1000 um. The light absorption coefficient of phycobilin at the characteristic absorption peak was obtained according to Lambert-Beer Law. A linear equation set with the light absorption value (A) of specific wavelength as variable and the mass concentration of phycobilin as function was established for the analysis of the absorbance of phycobilin extract.

The light absorption characteristics of phycobilin were studied with Hitachi U-3400 ultraviolet spectrophotometer, and its fluorescence discharge characteristics were studied with Hitachi 850 fluorescence spectrophotometer, and 50 mmoL/L PBS phosphate buffer was used as a reference to draw the elute curve of phycobilin, so as to determine the optical characteristics of samples in different periods. With the Is55 fluorescence spectrophotometer, we can measure the Ex=498 nm fluorescence emission and Em=615 nm excitation spectrum of the phycobilin in the sample, so as to obtain accurate information about phycobilin.

By measuring the absorbance of phycobilin at different wavelengths, the ultraviolet-visible spectrum can be obtained. The spectra can be used to determine the content of various phycobilis, to analyze the properties of their chromophores, and to study their biological activities related to light absorption. Various phycobilin have different fluorescence properties and can be characterized by fluorescence spectroscopy. Fluorescence spectra can provide information about the fluorescence emission and fluorescence quenching of phycobilin, which is helpful to understand its fluorescence mechanism and the influence of environment on its fluorescence properties. Circular dichroism is also a method to study molecular structure and can be used to characterize the secondary structure of phycobilin. By measuring the circular dichroism of phycobilin at different wavelengths, information about its secondary structure can be obtained, which helps to understand its conformational change and biological activity. Afm analysis: AFM can directly observe the

morphology and structure of the sample surface. By observing the surface morphology and structure of phycobilin through atomic force microscopy, the microstructure and biological activity of phycobilin can be deeply understood.

Conclusions to chapter 2

This chapter mainly introduces the following problems:

1.Introduces the materials needed for the extraction of phycobilin, including reagents and instrument.

2.The experimental method of extraction of phycobilin was introduced.

3. The method of spectral determination of phycobilin was introduced.

CHAPTER 3
EXPERIMENTAL PART

3.1 Cell fragmentation

By visual observation, we found that after the first freeze and thaw, the algae began to break up. The freeze-thaw solution began to change color. The color of the polysiphonium solution was a lighter red, while the color of the spirulina solution was a lighter blue. After the second freeze-thaw, we found that the color of the freeze-thaw solution was further deepened, and after the third and fourth times, the color of the solution was more obvious, and there was no significant change in color after the increase of freeze-thaw times.

FIG. 1 shows the determination results of protein content in freeze-thaw solution after each freeze thaw. In FIG. 1, it is found that the color of the protein deepens with the increase of protein content. Before 4 times of freeze thaw, the more times of freeze thaw, the higher the protein content and the darker the color. With the increase of freeze-thaw times, the protein content of freeze-thaw solution did not increase significantly, and the amount of freeze-thaw protein dissolved decreased slightly. The protein content was not affected when the number of freezing and thawing cycles was small. With the increase of freezing and thawing cycles, the probability of protein denaturation also increased, and the loss of soluble protein also increased with the loss of water in the cell during this process, which affected the protein content. Considering the freezing and thawing times 4 times, the best number of cell breakage was determined. The darkest phycobilin solutions were obtained, in which the solutions of Polysiphonium and porphyra were red, and the solutions of spirulina were blue-green.

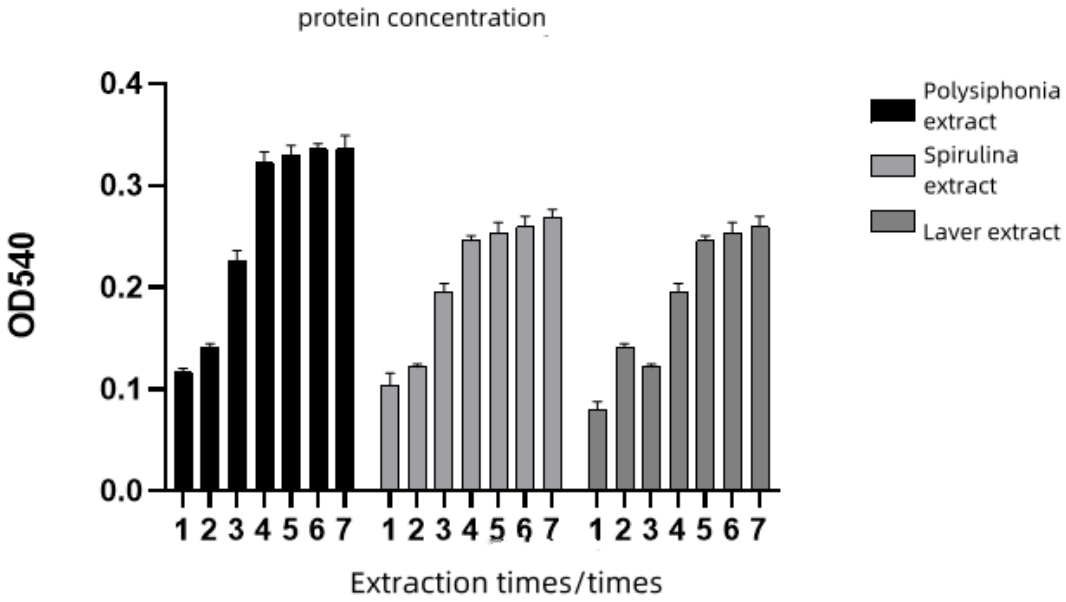

Figure 3-1 Determination of protein content in freeze-thaw solution after each freeze-thaw

3.2 Purification of phycobilin

Using two-step salting out method and DEAE-Sepharose Fast Flow column technology, we have successfully purified three phycobilinins, most of which have excellent activity. The highest purity of phycocyanin obtained by two-step salting-out method was 3.95, and the recovery was 9.56%. The concentration of phycocyanin obtained by chromatographic chromatography is about 3, and the purity of phycocyanin obtained by chromatographic chromatography is about 3.5.

3.3 Analysis of spectral characteristics of phycobilin

Set the appropriate wavelength range (such as the visible light region) on the spectrometer and ensure that the instrument is calibrated. C-phycocyanin, R-phycoglobin and B-phycoglobin were dissolved in the experimental water using a colorimetric dish, and the solution with appropriate concentration was configured. The absorbance values of three kinds of phycobilin solutions at different wavelengths were recorded in spectrometers.

The spectral data were processed and analyzed to compare the spectral characteristics of the three phycobilis, such as the position of absorption peak and absorbance value. The structure and properties of different phycobilins were studied by spectroscopic data.

The spectrograms of three phycobilinins were drawn to show their spectral characteristics. According to the data analysis results, the similarities and differences of the three phycobilins were summarized and compared. Combined with the experimental results, the possible roles and functions of different phycobilin in vivo were analyzed.

3.3.1 Spectral characteristics analysis of R-phycoglobin

The fluorescence intensity of R-phycoglobin is generally around 570 nm, but its fluorescence duration is longer than that of other proteins. This indicates that its fluorescence properties are different from other phycobilin proteins, which may be related to its special chromophore structure. The absorption spectra of R-phycothrin purified by ion exchange chromatography (0.10 mg/ml) or hydroxyapatite chromatography (0.14 mg/ml) are shown in Figure 3-2. In addition to the absorption peak at 620 nm, there are three absorption peaks in the visible spectral region in the natural state. In addition to the absorption peak of 490-500 nm, there are two absorption peaks of 540 nm and 560 nm. The A565/A280 ratio is also greater than 3.2.

The maximum emission wavelength is 580 nm and the excitation wavelength is 498 nm. This result is consistent with the known fluorescence spectra of phycoglobin. The dissociation of the hemper R-phycoglobin results in a loss of the absorption peak at 565 nm and a decrease in the ratio of A565/A498. Therefore, the above purification method provides R-phycoglobin solution in the hexamer aggregation state[25].

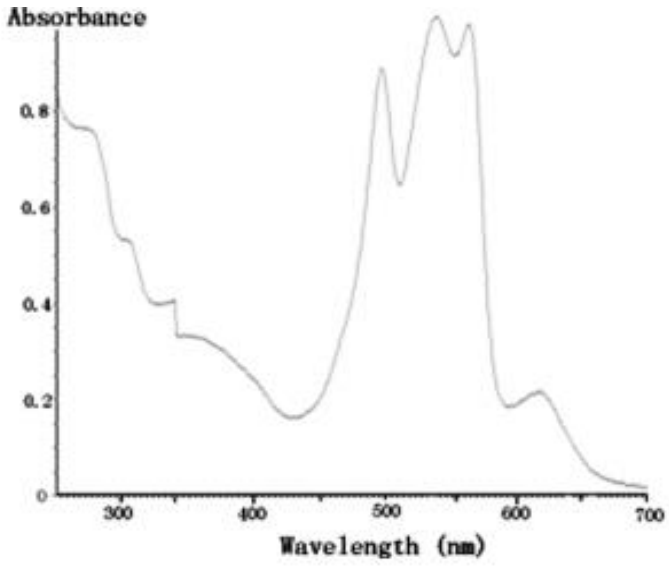

Figure 3-2 Absorption spectra of R-phycothrin purified by ion exchange chromatography (0.10 mg/ml) or hydroxyapatite chromatography (0.14 mg/ml)

3.3.2 Spectral characteristics analysis of C-phycocyanin

C-phycocyanin: When excited at a wavelength of about 620 nm, C-phycocyanin will show a strong fluorescence peak between 635-650 nm. Its fluorescence spectral characteristics show that it has an obvious fluorescence emission peak, which indicates that it has a relatively high fluorescence quantum efficiency, which may play a certain role in photosynthesis.

It has characteristic absorption peaks near 280 nm, 360 nm and 620 nm in the UV-visible region, and the fluorescence excitation wavelength is 610~620 nm, and the fluorescence emission peak is 620~640 nm.

3.3.3 Analysis of spectral characteristics of B-phycoglobin

B-phycoglobin: The fluorescence emission peak position of B-phycoglobin is similar to that of R-phycoglobin, also located at about 570 nm, but it has a relatively long fluorescence lifetime. This suggests that its fluorescence properties may be different from R-phycoglobin, possibly due to its molecular structure or environmental factors.

B-phycoglobin has an absorption peak of 498 nm.

3.3.4 Summary

In general, the fluorescence spectra of C-phycocyanin, R-phycocyanin and B-phycocyanin are different, which may be related to their chromophore structure, molecular conformation and environmental factors. Further study of the fluorescence properties of these phycobilis proteins will help to understand their mechanism of action and regulation in photosynthesis.

3.4 Analysis and characterization of phycobilin

The analysis and characterization of phycobilin is a complex process involving multiple steps and experimental techniques. Here is a general process:

1. Molecular weight determination: Gel Filtration Chromatography or Mass Spectrometry was used to determine the molecular weight of three phycobilins.

2. Structural analysis: Through X-ray crystallography, nuclear magnetic resonance (NMR) and other technologies, the structure of phycobilin was analyzed to understand its three-dimensional structural characteristics.

3. Functional analysis: Study the spectral properties of phycobilin such as light absorption and fluorescence emission, as well as its interaction with other proteins or small molecules, so as to understand its functional properties.

6. Kinetic analysis: Using fluorescence quenching, enzyme kinetics and other methods to study the kinetic properties of phycobilin, such as stability, reaction rate, etc.

7. Bioinformatic analysis: The amino acid sequence of phycobilis protein is compared with the known protein database to predict its possible biological function and evolutionary relationship.

8. Cell or animal model analysis: phycobilin is applied in cell culture or animal model to observe its influence on biological activity and further reveal its physiological function.

9. Stability assessment: Through heating, acid-base treatment and other ways to evaluate the stability of phycobilin, to understand its performance under different environmental conditions.

10. Application research: Based on the above analysis results, explore the application potential of phycobilin in bioengineering, biomedicine, environmental protection and other fields.

Through the above steps, the three phycobilinins can be comprehensively analyzed and characterized to provide basic data for their further application and research.

Conclusions to chapter 3

This chapter mainly introduces the following problems:

1. The results of phycobilin cell fragmentation, solution color, etc. 2. The results of salt-out extraction of phycobilin were introduced. 3. The results of spectroscopic determination of phycobilin were introduced

CONCLUSIONS

Summary of research results

C-phycocyanin, R-phycocyanin and B-phycocyanin are important pigment proteins in photosynthesis and have unique spectral characteristics. After years of research, researchers have achieved some important results in the preparation and spectral determination.

First, in terms of preparation, researchers have successfully developed a series of effective extraction methods, including organic solvent extraction, ionic liquid extraction and ultrasonic assisted extraction. These methods can improve the extraction efficiency of phycobilin and provide sufficient materials for subsequent spectroscopic determination and functional study.

Through the use of advanced analytical methods, such as UV-VIS spectroscopy, fluorescence spectroscopy, and circular dichroism, the scientists conducted a comprehensive exploration of the structure, characteristics, and functions of the three phycobilins. Through these studies, it has been found that phycobilis have rich spectral characteristics, which are closely related to their conformation, conjugate system and environmental factors. These findings provide important clues for further exploring the mechanism of phycobilin in photosynthesis.

In addition, the fluorescence properties of phycobilin were further studied. They found that the fluorescence emission peak position, fluorescence lifetime and fluorescence quantum efficiency of the three phycobilis were different, which was related to the chromophore structure, molecular conformation and environmental factors. These results help to understand the function and mechanism of phycobilin in photosynthesis, and provide theoretical support and practical guidance for optimizing photosynthesis and improving light energy use efficiency.

In summary, a series of important results have been obtained in the preparation and spectroscopic determination of C-phycocyanin, R-phycoglobin and B-phycoglobin. These results not only contribute to the in-depth understanding of the

structure and properties of these three phycobilis proteins, but also provide new ideas and methods for the study of photosynthesis. With the continuous progress of science and technology, it is believed that more research results will emerge in the future to promote the in-depth development of photosynthesis research.

Suggestions and prospects for future research

Although there have been some improvements in the existing extraction methods, it is still an important research direction to find new preparation technologies that are more efficient, environmentally friendly and suitable for large-scale production. For example, the use of genetic engineering and cell culture techniques to produce phycobilin could be a promising route. The structural and functional relationships of these three phycobilis proteins have been understood, but there are still many unknown areas that need to be further explored. For example, how their conformational changes affect their spectral properties, and how these changes relate to photosynthetic efficiency. Studies of interactions with other proteins Phycobilin proteins do not work alone in photosynthesis, they interact with other proteins such as photosynthetic reaction centers. The study of these interactions can help us better understand the role of phycobilin in photosynthesis. C-phycocyanin, R-phycoglobin and B-phycored opal have great potential applications in biosensors, fluorescent labeling and bioimaging, and these proteins can not only play an important role in basic research. Therefore, it is also an important direction to carry out research in these application fields in the future. Photosynthesis is a complex process involving multiple disciplines, so future research needs interdisciplinary cooperation. For example, chemists can provide new fluorescent probes and dyes, biologists can provide information at the cellular and molecular levels, and physicists can provide insights into spectroscopy and quantum mechanics. The application of artificial intelligence and machine learning in spectral analysis, with the development of artificial intelligence and machine learning technology, these technologies can also be applied to the spectral analysis of phycobilin. For example, machine learning

models can be trained to predict the structural and spectral properties of phycobilin, or used to analyze and interpret spectral data more quickly.

There are still many interesting problems in the preparation and spectroscopic determination of C-phycocyanin, R-phycoglobin and B-phycoglobin, and it is believed that more innovative research results will emerge in the future.

This chapter mainly introduces the following problems: the present research results and the future research direction are introduced

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Appendix

Phycobilins are fluorescent proteins of various colors, including purple-red, purple-blue, and cyan, that can capture light energy in an auxiliary photosynthetic complex called phycobilisome. Phycobilin has several highly preserved structural and physicochemical characteristics. In the PBS environment, phycobilin's function is to capture light energy in the 450-650 nm range and transfer it to the photosystem for photosynthesis. In addition to its energy-harvesting function, phycobilin has a variety of biological activities, including antioxidant, antibacterial, and anti-tumor, making it an interesting focus for different biotechnology applications in areas such as biomedicine, bioenergy, and scientific research. Today, the main sources of phycobilin are cyanobacteria and microalgae and macroalgae from the phylum rhodophyllum. Because of their diverse biological activities, phycobilin proteins have attracted the attention of different industries such as food, biomedicine and cosmetics. This is why a large number of patents related to the production, extraction, purification of phycobilin and its use as a cosmetic, biopharmaceutical or diagnostic application have been generated. Cyanobacteria, which appear to have less ecological impact, shorter culture time and higher productivity in macroalgae, are widely used to meet a large number of market needs. In this paper, we summarize the main structural characteristics of phycobilis, their biosynthesis and biotechnology applications, and their respective spectral properties. We also discuss the current trends and future prospects of the phycobilin market. They have attracted the attention of different industries such as food, biomedicine and cosmetics, and have broad application prospects.