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on the topic Preparation and Identification of Antioxidant Peptides from Spirulina platensis

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of specialty 162 «Biotechnology and Bioengineering»
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ABSTRACT

Spirulina is a very economical microalgae, its protein content is high and the content and proportion of essential amino acids required by the human body are up to the proportion specified by FAO or even above. In addition, spirulina also has a variety of biological activities such as antioxidant, blood pressure lowering, blood sugar lowering, and immune regulation.

In this study, spirulina was used as raw material, and the phycobiliprotein protein was extracted by repeated freeze-thaw method and water immersion extraction method, and the extraction rate of the two methods was compared. Antioxidant peptides were prepared by alkaline protease, pepsin, trypsin, papain and enzymatic hydrolysis, which acted on 1h, 2h, 3h, 4h, and 5h, respectively, and the antioxidant capacity of the isolated antioxidant peptides was determined, and the ABTS+ radical scavenging rate was determined by ultrafiltration as three components less than 3 kDa, greater than 3 kDa, less than 10 kDa, and greater than 10 kDa.

The results showed that the extraction rate of phycobiliprotein by water immersion extraction method was about 43.13%, while the extraction rate of phycobiliprotein by repeated freeze-thaw method was about 65.46%. The extraction rate of repeated freeze-thaw method is better than that of water immersion extraction method. Compared with the hydrolysis effect of the four enzymes at the optimal enzymatic hydrolysis time, papain had the best effect on preparing antioxidant peptides, and the DPPH and ABTS+ radical scavenging rates of papain's enzymatic hydrolysate were 61.21±6.58% and 58.12±8.83%, respectively. The ABTS+ radical scavenging rates of alkaline protease digest (CPCAH) and papain digest (CPCPH) less than 3 kDa were relatively higher, 81.29±0.8%, 81.07±1.05%, respectively. It can be seen that the relative molecular weight of antioxidant active peptides in spirulina is mostly concentrated in the range of less than 3 kDa. The obtained CPCAH-F3 and CPCPH-F3 were then identified by mass spectrometry to obtain the relative molecular weight, amino acid sequence and hydrophobicity analysis of

multiple peptides, and the peptides with strong antioxidant capacity were usually rich in hydrophobic amino acids. This experiment further verified the antioxidant capacity of spirulina, and laid an experimental foundation for the development and research of spirulina as a functional food.

The object of the work is spirulina antioxidant peptides.

The subject of the work is antioxidant activity of peptides from spirulina.

The aim of the work is to isolate and study properties of spirulina antioxidant peptides.

The tasks of the work are to extract phycobiliprotein with different methods, provide the study of antioxidant activity with DPPH and ABTS+ radical scavenging rates, identify molecular weight, amino acid sequence and hydrophobicity of spirulina antioxidant peptides.

Key words: Spirulina, Phycobiliprotein, Antioxidant peptide,Preparation of extracting, Optimization of enzymatic hydrolysis conditions,Mass spectrometry identification.

CONTENT

INTRODUCTION

The peptides obtained after enzymatic hydrolysis of proteins are not only easier to be digested, absorbed and utilized by the human body, but also have antioxidant, antibacterial, regulate the immune system, lower blood pressure, lower blood lipids, and other physiological activities, these peptides with physiological activity are collectively referred to as bioactive peptides. The antioxidant peptides obtained by the decomposition of phycobiliprotein have a significant protective effect on ultraviolet organelle damage and lipid peroxidation induced by free radicals, which can not only remove free radicals in the body, but also reduce the damage of free radicals to the body to a certain extent, and is an ideal natural antioxidant. In daily life, the body will inevitably produce oxidants, and the antioxidant defense mechanism will protect the body. Peptides derived from the enzymatic hydrolysis of spirulina algal bile protein, which have strong antioxidant capacity and safety, so they have broad application prospects in the field of biomedicine and health food. This study can provide an experimental basis for spirulina as a functional food with antioxidant capacity, and lay a foundation for the further development of spirulina antioxidant peptides.

The algal bile proteins were extracted by water immersion extraction and repeated freeze-thawing method, and the extraction rate and purity were calculated to compare the best method for extracting algal bile proteins. The extracted algal bile proteins were prepared by enzymatic digestion for the preparation of antioxidant peptides. The products obtained by enzymatic digestion were assayed for antioxidant activity, and the enzymes with the highest antioxidant activity of the peptides and the optimal enzymatic digestion time were screened. The enzymatic digestion products were separated by ultrafiltration to obtain a higher relative molecular mass range of antioxidant activity relative to.

Importance of research question, research methods, research results and structure of the thesis, etc.

CHAPTER I
REVIEW

1.1 Overview of Spirulina

Spirulina belongs to the phylum Cyanobacteria, cyanobacteria, is a plant in the family Tremulaceae, spirulina, is an ancient and low prokaryotic aquatic algae[1], originated 5-3.5 billion years ago, is one of the most ancient plants, usually blue-green. Spirulina filament body length 200 -500 μm, width 5 -10 μm[2], Spirulina is mainly distributed in freshwater and saline lakes in tropical and subtropical regions. In the natural environment, spirulina is mainly distributed in Lake Chad in Central Africa, Lake Tesicoco in Mexico and Yongsheng Chenghai Lake in Yunnan, China, which is suitable for high temperature alkaline environment[4]， under the microscope is loose or tight regular spiral curve, shaped like clockwork and named, in the four major artificial cultivation of microalgae, spirulina is the largest proportion of output and output value of microalgae, in animal husbandry, nutrition and health, medicine field, carbon sequestration and emission reduction, environmental engineering, food engineering and other fields of the role is more and more fully recognized and explored. Cyanobacteria cells have no chromosomes, and the pigments are distributed in the pigment regions outside the protoplasts. The surface of the algae does not have a colloidal sheath, it is not easy to be attached by microorganisms, there are bubbles in the cells, and the floating is good. The apex of the cell or algae filament is often not sharp and thin, the transverse wall is often inconspicuous, the apical cell is rounded, and the outer wall is not thickened. At the same time, spirulina has fast growth rate, strong adaptability, and can be cultivated by seawater, saline water and various wastewater to achieve resource utilization.

1.2 Overview of Spirulina

Spirulina has been diagnosed and identified by many organizations as a "superfood", with nutrients including not only protein but also many vitamins, as well as gold carotenoids, chlorophyll, etc. It has also been found that spirulina can

be used as a dietary supplement for vitamin A and vitamin B2. Spirulina is also extremely rich in vitamins and minerals, the former including vitamins B1, B2, B, B12, E and K; The latter includes zinc, iron, potassium, calcium, magnesium, phosphorus, selenium, iodine and other trace elements, spirulina biological zinc and iron ratio is basically consistent with human physiological needs, the most easily absorbed by the human body. In addition, active ingredients such as phycocyanin (C-PC), phycopolysaccharides (PSP), methyl y-linolenic acid (GLAME), β-carotene, chlorophyll a and so on in spirulina have a regulatory effect on many functions of animals[4] The protein content of Spirulina is 50-78%[5], and The water-soluble protein in Spirulina is different from the alcohol-soluble protein in food and meat in daily life, which is more easily digested and absorbed by human body. The structure, ratio and content of the eight essential amino acids in spirulina are all in line with the needs of human metabolism[6]**.** And the net protein utilization rate, that is, NPU, reaches 53%~92%, and the protein efficiency ratio, that is, PER, reaches 1.8%~2.6%. From the above two sets of data, we can see that the amino acid score of some higher quality spirulina can be greater than 100[7] .

The production of Spirulina powder in China accounts for 80% of the global production, and most of the Spirulina products in the market exist in the form of powder, tablets and capsules, 90% of which are dietary supplements. Other nutrients include 17%-25% of sugars, 30 mg of chlorophyll, 15 mg of carotenoids, 9 mg of zeaxanthin, 6.8 mg of B-carotene, 1080 U of superoxide dismutase, and 30-60 mg of y-linolenic acid[8] .

Spirulina is often considered a nutritious food and health food because it grows in fresh and brackish water and is very rich in protein, minerals and vitamins. Spirulina is also believed to help boost immunity, lower cholesterol, reduce inflammation and enhance cardiovascular health. It is also used in cosmetics and medicine.

1.3 Structure and function of algal bile protein

Phycobiliproteins are an important component of photosynthesis in algae, and are a class of pigment complex proteins found in the bile bodies of some algae such as red algae and cyanobacteria, and in cryptophytes as heterodimers or monomers. Phycobilisomes are supramolecular pigment complexes in red algae and cyanobacteria, which are anchored to the outer surface of the cystoid membrane by anchoring proteins. The phycobilisomes are attached to the surface or inner lumen of the cystoid membrane and have four different phycobilins distributed in them, the algae are phycoerythrin, phycocyanin, phycocyanin violet and phycocyanin. As shown in Figure 1.1 (Appendix A), phycocyanins are a class of linearly open-chain tetrapyrrole ring compounds. Depending on the phycocyanin on the cysteine residues of the thioether bond of the phycocyanin covalently attached to the deacyl carrier protein, phycocyanins are divided into phycoerythrin, phycoerythrocyanin, phycocyanin and allophycocyanin, which can be expressed as PE, PEC, PC and APC respectively[9]. The color of phycocyanin itself can be divided into red, blue and blue, and can emit strong orange-red fluorescence, so it is also sometimes called colored peptide. It can form algal bile body together with linking peptide, which accounts for 80% and 20% of algal bile body respectively[10].

As shown in Figure 1.2 (Appendix A), the phycoglobin is arranged at the outermost part of the bile body, and the six bifurcated structures can promote its light absorption efficiency and expand its capacity, so that the bile body can absorb more light energy, and then the next level of phycocyanin will absorb the light energy from the phycoglobin, and then the phycocyanin will pass to the other phycocyanin in the center of the bile body, and finally the other phycocyanin will pass to the light reaction center. Phycobiliproteins usually have two or three subunits in various forms, but mainly the a-subunit and the β-subunit, which are usually combined into trimers or hexamers in the form of $(a\beta)_3$ or $(a\beta)_6$, respectively[11] .

Algal bile protein has a variety of biological activities. It can induce apoptosis, inhibit tumor angiogenesis, scavenge free radicals, stabilize DNA structure, and thus has anti-tumor activity; it can improve antioxidant capacity and inhibit lipid

peroxidation, improve immunity and thus has certain detoxification effects; it can also improve gene expression of substances such as type II collagen and aggregated proteoglycan, and has anti-arthritic effects; in addition, it can also reduce inflammatory factors, express activation of Insulin signaling pathway, with certain anti-inflammatory and anti-diabetic ability; and improve the level of leukocytes, NK cells, B cells, T cells, macrophages and other cells, thus improving immunity.

One of its most prominent functions is antioxidant and anti-inflammatory, many studies have confirmed that algal bile protein has strong antioxidant activity, scavenging free radicals while also protecting DNA structure from damage[12], it is the only natural product that both scavenges superoxide anions, hydroxyl radicals and peroxides, and can inhibit NADPH oxidase, scavenging oxygen radicals and oxidation factors from the source, playing "It can effectively inhibit and even remove oxidative intermediates produced in the metabolism of macromolecules. In addition, algal bile proteins have certain functions such as antiviral activity, promoting cell proliferation, and acting as fluorescent probes.

Different enzymatic digestions of algal bile proteins can yield a variety of algal bile protein-derived bioactive peptides, including antioxidant peptides, hypoglycemic peptides and hypotensive peptides, as well as antibacterial peptides and anti-inflammatory peptides.

1.4 Research progress on the preparation of algal bile protein

Algal bile protein is a water-soluble intracellular protein. In order to obtain high purity and high recovery rate of algal bile protein, the fragmentation of algal bodies is very important. At the present stage, the methods commonly used to crush algae bodies are mainly physical methods. In practice, a mixture of several methods is often used to maximize fragmentation and to obtain water-soluble algal bile proteins.

The cell structure can be destroyed by physical methods as well as other chemical methods, while physical wall breaking methods are used so that the cell wall and cell membrane are broken and intracellular substances are thus leached out.

Common physical breaking methods include repeated freeze-thawing[13], , water immersion extraction, osmotic pressure, grinding[14], and crushing methods[15]. In repeated freezing and thawing, the cellular fluid will form ice crystals, and the concentration of cellular fluid will decrease, and the osmotic pressure in the cell will increase, which will lead to further cell rupture, and the algal bile protein can be dissolved from the cell, this method is conducive to protein dissolution and easy to enlarge. This method has some obvious advantages, such as simplicity of operation and suitability for industrial applications, but it also has some disadvantages, such as time-consuming and low efficiency. Compared to the repeated freeze-thaw method, the use of the swelling method can shorten the time and improve the extraction rate. Different crushing methods can be used to extract algae hemoglobin from altar nori, and the operation steps and effects are shown below: 0.01 mol/L Tris-HCI was used as the swelling agent for 9 h to obtain algae hemoglobin with a purity of 0.45 and a content of 9.71 mg/g[16], where the purity was higher than that of the chemical reagent treatment method and the churning method. The addition of liquid nitrogen when the algae were ground and crushed, followed by extraction with Tris-HCI buffer, allowed to obtain purity of 4.5 % phycocyanin[17]. The purity of algal bile protein was significantly increased due to the effect of liquid nitrogen, which can reduce the surrounding temperature to avoid the temperature increase caused by grinding and consequent denaturation and inactivation of algal bile protein, as well as the increased cell membrane fragility and easier cell wall fragmentation under low temperature environment. The use of liquid nitrogen can reduce the cost. The liquid nitrogen method is suitable for small-scale laboratory preparation, but if applied to industrial large-scale use, the amount of liquid nitrogen used will be high, and then the cost will increase significantly. The physical breakage method requires mild conditions, but it is time consuming. Further optimization of extraction time, number of times, temperature and other parameters is required in actual production. This method can also be mixed with other methods to further improve the extraction of algal bile protein.

1.5 Research progress of antioxidant peptide preparation

There are two main ways to prepare bioactive peptides: one is through traditional methods, including enzymatic digestion, microbial fermentation, etc. The bioactive peptide in the raw protein is released, and then the peptide with some specific activity is obtained by isolation and purification; the other is by computer-aided simulation of the hydrolysis process with the help of bioinformatics tools, such as databases, so as to screen the suitable protease type and verify the activity by synthesis to obtain bioactive peptides. At present, the only database involving antioxidant peptides is BIOPEP, and only a few sequences are available, thus the preparation of antioxidant peptides by computer-assisted simulated hydrolysis is limited[18] .

(1) Protease hydrolysis method

Antioxidant peptides exist as inactive peptide segments in the parent protein structure and need to be hydrolyzed by proteases to release their activity. Enzymatic hydrolysis is the most commonly used method for the preparation of antioxidant peptides, which has the following characteristics: mild conditions, easy to control ; white enzyme hydrolysis is selective and produces few by-products; high safety, no toxic chemical residues[19] . The proteases usually chosen for the enzymatic method are usually papain, alkaline protease and trypsin. In the process of protease hydrolysis, many factors affect the reaction, such as substrate concentration, enzyme concentration, temperature, activator, inhibitor, hydrolysis time, pH of the solution, metal ions, etc[20]. If the hydrolysis of algal bile protein is to be controlled, these factors must be taken into consideration and further optimized. Alkaline amino acids have strong specificity, so alkaline proteases are often used to prepare antioxidant peptides. The hydrolysate of alkaline protease has in vitro free radical scavenging activity and Caco-2 cell reactive oxygen species (ROS) scavenging activity[21]. For example, sorghum proteolysis can produce active peptides with high antioxidant activity, mainly because alkaline proteases hydrolyze the carboxyl ends of Glu, Met, Leu, Tyr, Lys, and GIn, allowing them to release more antioxidant-active products[22]. neuter Ren Junfeng et al[23] The enzymatic conditions were set at 45.0 ℃, substrate

concentration 33.6 mg/100 mL, enzyme concentration 5 000 Ug, pH 6.5, hydrolysis time 2.0 h ,Under these conditions, the peptides obtained from the enzymatically digested proteins had significant ability to scavenge OH groups and DPPH radicals, and had some ability to scavenge O2 and reduce them. It was further found that the size of the molecular mass of the peptide was closely related to its antioxidant activity, and within a certain range, the OH. and DPPH. scavenging ability and reducing ability of the peptide fragments were enhanced as the relative molecular mass decreased. Zhaoming Liu[24] . he used walnut protein as raw material and set the pH of the reaction system at 8.0, temperature at 40.0 ℃, substrate concentration at 4.0% and enzyme concentration at 1,500 U/g to carry out the reaction. Walnut protein peptides with relative molecular mass less than 3 kDa were obtained by hydrolysis of walnut protein with papain for 4.0 h, followed by multi-step processes such as ultrafiltration and desalination. When the concentration of walnut protein peptide was set at 50 mg/mL, the scavenging rate of OH reached 69.1% and that of 02 reached 85%; when the concentration of walnut protein peptide was 30 mg/mL, its reducing ability could reach 57.1% of that of vitamin C; and when the concentration was 20 mg/mL, the scavenging ability of DPPH free radicals could reach 66%. scavenging capacity can reach 66% of the maximum value. Zhu Yanhua et al[.25] use corn protein as raw material. The process was optimized by using alkaline protease enzymatic hydrolysis, using the deoxyribose-iron system method, taking the hydroxyl radical scavenging rate as the index, and the optimal process parameters for the preparation of peptides with high antioxidant activity were determined, that is, the substrate mass fraction was 4.0%, P H 9.5, the enzyme dosage was 1 000 u/g, the temperature was 55.0 °C, the hydrolysis time was 4.0 h, the hydrolysis degree was 6.9%, and the hydroxyl radical scavenging rate reached 56.8%. Chen Guitang et al.[2627] first selected seven food-grade protease hydrolyzed peanut proteins, and studied the effects of single enzyme action and double enzyme combination on hydrolysate hydrolysis degree and antioxidant effect in vitro, indicating that alkaline protease hydrolysate has the strongest antioxidant capacity. Then, the selected alkaline protease digestion process was further optimized, and the

results showed that the optimal hydrolysis conditions were temperature 55.0°CC, p H 7.5, substrate concentration 8.0%, enzyme addition 3.0%, and hydrolysis time 6.0 h. Nabil Souissi et al. [28]used sardines heads and internal organs as raw materials, using alkaline proteases, and hydrolysis under the conditions of pH 8.0, temperature of 50.0 °C, hydrolysis time of 3.0 h, and enzyme addition amount of 727.3 ulg. It was found that hydrolysates have strong antioxidant properties. It can prevent more than 50% lipid peroxidation of linoleate, and when its hydrolysis degree is 10.16%, the antioxidant activity of proteolysate reaches about 73% of VE[29].

(2) Fermentation method

Fermentation is a method that uses a complex enzyme system produced during microbial metabolism to enzymatically digest substrate proteins to release active peptides. During the fermentation process, conditions such as protein source, pH, strain selection, fermentation temperature and time will affect the degree of protein enzymatic digestion. At this stage, the main microorganisms that can be used for the preparation of antioxidant peptides are Bacillus licheniformis, Bacillus subtilis, Lactobacillus and Aspergillus**Ошибка! Источник ссылки не найден.** . Among them, the rice bran active peptide prepared by fermentation of Bacillus subtilis contains 6 essential amino acids and has a strong ability to scavenge DPPH free radicals, -0, OH[31] The antioxidant peptides LGTFQN, LHALLL and SGYYMH obtained by fermentation of millet with Lactobacillus pasteurii Fn032 have strong DPPH radical scavenging ability [32].

Microbial fermentation method has many advantages: the antioxidant peptide produced by microbial fermentation method can enter human digestive system directly, which is easier to be digested, absorbed and utilized by human body with high safety; microorganisms have the characteristics of wide source and low price; compared with protease hydrolysis method, the peptidase produced in the process of microbial fermentation and metabolism can reduce the bitter taste substances, etc. Of course, microbial fermentation method also has some problems at present: the production technology of microbial fermentation method is not perfect; the non-toxic and harmless enzyme-producing strains that can be used for antioxidant

peptides are limited and the number is small; the mechanism of action of fermentation method to produce complex enzyme system has not been elucidated, etc. In addition, compared with the enzymatic hydrolysis method, microbial fermentation protein can effectively remove the bitter taste of peptides in the fermentation product, and at the same time can modify and recombine the peptide chain, so most of the peptides prepared by microbial fermentation method have no peculiar smell, unique fermentation flavor, and have a wide range of applications in the food and health industry[33]. Xing Meizi et al. [34]Lactobacillus deuteri LB340 LYO fermented skim milk, which showed good ACE inhibitory activity at a concentration of 2ug/mL. Different strains have different enzyme production conditions and enzyme production, and if you want to get high-quality antioxidant peptides, you must have high-quality strains as the premise, which can produce a large number of proteases in a short period of time, control the fermentation conditions, and thus obtain target peptides, so that the antioxidant peptides obtained are odorless, safe, highly targeted, can be better absorbed by the human body, and have great potential in terms of nutritional value[3536].

1.6 Research progress of mass spectrometry identification

Mass spectrometry is an important method that enables accurate determination of protein quality and characterization of proteins[37]. Applications of mass spectrometry to identify proteins include protein identification, identification of post-translational modifications of proteins, protein complex analysis, identification of subunits and functional interactions of proteins, and overall measurement of proteins in proteomics. It can also be used to localize proteins to various organelles, such as mitochondria, chloroplasts, etc. It can also be used to determine the interactions between different proteins or between proteins and membrane lipids[39].

The instruments used for mass spectrometry identification are called mass spectrometers, and there are various types of mass spectrometers, which can also be used in conjunction with other techniques to play different functions, and different instruments have different role characteristics. For mass spectrometry of biological

macromolecules[40], LC-MS and MALDI-TOF analysis can be used in order to obtain relative molecular weight information. In the case of studying protein samples, mass spectrometry analysis can determine their amino acid sequences. In general, if the temperature at which the vaporization of the sample occurs is around 300°C, then the GC-MS mass spectrometer is the most preferred choice, because the C-MS mass spectrometer can use an EI source, and thus the number of mass spectral information is significantly higher, and the obtained results are more accurate, in addition to the better separation of the capillary column. However, if the selected sample does not vaporize at about 300°C, then the most preferred mass spectrometer is LC-MS, which mainly obtains some relative molecular weight information compared to GS-MS. In the experiment, the tandem mass spectrometry is chosen not only to obtain the above information but also to obtain structural information. As an important technical index of the mass spectrometer, the resolution level is crucial to the determination of the structure, and a high-resolution mass spectrometer can provide the composition formula of the compound[41].

With the advancement of mass spectrometry technology, the applications of mass spectrometers are becoming more and more extensive. Due to the numerous advantages of mass spectrometry, such as fast analysis speed and low sample usage. Therefore, mass spectrometry is nowadays widely used in chemistry, sports medicine, chemical industry and other fields.

Summary of the chapter I

1. Spirulina belongs to the phylum Cyanobacteria, cyanobacteria, is a plant in the family Tremulaceae, spirulina, is an ancient and low prokaryotic aquatic algae, originated 5-3.5 billion years ago, is one of the most ancient plants.

2. Spirulina has been diagnosed and identified by many organizations as a "superfood".The protein content of Spirulina is 50-78%[5], and The water-soluble protein in Spirulina is different from the alcohol-soluble protein in food and meat in daily life, which is more easily digested and absorbed by human body.

3. Algal bile protein has a variety of biological activities. It can induce apoptosis, inhibit tumor angiogenesis, scavenge free radicals, stabilize DNA structure, and thus has anti-tumor activity; it can improve antioxidant capacity and inhibit lipid peroxidation, improve immunity and thus has certain detoxification effects

CHAPTER II
PREPARATION OF ANTIOXIDANT PEPTIDES

2.1 Reagents and instruments

Spirulina Powder ; Papain ; Alkaline Protease ; Trypsin ; Pepsin ; Forinol, 1,1-diphenyl-2-picryl radical (DPPH), 95% ethanol, anhydrous ethanol, UV spectrophotometer, electronic balance, centrifuge, electric thermostat water bath, homogenizer, mass spectrometer.

2.2 Extraction method of algal bile protein

Ultrasonic crushing method: Take 20 g of Spirulina powder and suspend it in 150 m L of distilled water.

The beakers were slowly frozen at -18 °C, and then thawed in a water bath at 25 °C ~ 30 °C for 5 times.

placed in the ultrasonic crusher ultrasonic crushing 3 min, so that the algae bile protein fully dissolved.

centrifugation at 6000 g, 4C for 30 min, remove the precipitate after centrifugation, and collect the protein supernatant.

Water immersion extraction method:The appropriate amount of ground Spirulina powder was added to distilled water, mixed well, prepared into a solution of certain concentration and placed at a temperature of 37°C for 1 hour of extraction.

The supernatant was collected after continuous centrifugation at 6000 r/min for 20 min. The precipitate was repeated for three extractions and all supernatants were combined.

Add 1 mol/L HCl to the combined supernatant, adjust its pH to 4.5, and let it stand at room temperature for 30 min.

The precipitate was collected by centrifugation at 6000 r/min for 20 min and washed three times with deionized water to remove the soluble salts attached to the precipitate.

The pH was adjusted to 7.0 with 0.5 mol/L sodium hydroxide solution and freeze-dried to obtain water-soluble protein, which can be stored at -20°C[42].

2.3 Determination of soluble protein content

The amount of soluble protein in the solution can be determined by the Komas Brilliant Blue method

Coomassie brilliant blue G-250 is a dye binding method for the determination of protein content. Coomassie brilliant blue is red in the free state and has the maximum light absorption at 488 nm; when it is combined with protein, it can change to cyan, and the maximum light absorption of protein-pigment binding complex is at 595 nm. And the light absorption value of protein-pigment binding complex is proportional to the protein content, so the protein can be quantitatively determined by this method.

Prepare Bradford's staining solution: dissolve 100mg of Komas Brilliant Blue G-250 in 50ml of 95% ethanol, then add 100ml of 85% phosphoric acid

Replenish the mixture to 200ml with distilled water, and store the dye solution at 4℃ for at least 6 months.

Preparation of standard protein samples: Using proteins with similar properties to the sample to be tested as standard proteins, sample curves for standard proteins were plotted between 20ug-150ug/100ul.

The protein of the sample to be measured is dissolved in a buffer solution that should be the same as the buffer solution used to make the standard curve, and in this experiment PBS is used.

The dye binding solution was then diluted with distilled water in a ratio of 1:4, and if precipitation appeared, it was removed by filtration.

Add 5ml of diluted dye binding solution to each sample and act on the sample for 5 to 30min. when the dye solution binds to the protein, the solution will change from red to blue.

The absorbance was measured at 595 nm. The whole color development reaction needs to be carried out within 30 min. Finally, the concentration of the sample to be measured was calculated from the curve of the standard protein[43].

2.4 Preparation of antioxidant peptides

Weigh 10g of Spirulina powder in a 250mL triangular flask with 100mL of deionized water, mix well and put it at 90℃, keep it warm for 10min, wait for the temperature to drop to 55-60℃, adjust its pH and add the corresponding protease for hydrolysis:0h, 1h, 2h, 3h, 4h, 5h[44] according to the standard of Table 2.1 (Appendix C), respectively, and control by different enzymatic digestion time to determine each The optimum time for enzymatic digestion was determined.

The enzyme in the reaction vessel was inactivated by boiling water bath for 10 minutes after the reaction was finished, and the inactivated enzyme no longer had the ability to hydrolyze the protein, i.e., the enzymatic digestion was terminated. After the enzymatic product was cooled, the enzyme was centrifuged at 4000 r/min for 10 min and the supernatant was collected[45.]

2.5 Measurement of free radical scavenging rate

The enzymatic products were measured for DPPH radical scavenging rate, and the degree of hydrolysis of algal bile protein was measured by the scavenging rate to determine the optimal enzymatic time for different enzymes, to optimize the enzymatic conditions of algal bile protein, and to comprehensively select the enzymatic products with the highest antioxidant activity for subsequent experiments.

Determination of DPPH free radical scavenging capacity by Yama's method[46]**Ошибка! Источник ссылки не найден.**.

Select 2mL of a certain concentration of Spirulina peptide solution and add it to 2mL of 0.2mmol/L DPPH in 95% ethanol solution

The absorbance values at 517 nm were measured after thorough mixing and incubation at room temperature for 30 min. The ability to scavenge DPPH radicals

is SA. The formula of DPPH radical scavenging rate is: DPPH radical scavenging rate (%) $=\left(1-\frac{A_1-A_2}{A_3}\right)\times 100$

In the formula, blank control: 95% ethanol + distilled water; A1: absorbance value measured by DPPH solution + Spirulina peptide solution; A2: absorbance value measured by 95% ethanol + Spirulina peptide solution; A3: absorbance value measured by DPPH solution + distilled water[47].

2.6 Ultrafiltration separation

Ultrafiltration technology is a kind of membrane separation technology, which uses the pressure difference of 0.1~0.5 MPa as the driving force, uses the interception ability of porous membrane, and separates the particulate matter of different sizes in the solution through physical interception, so as to achieve the purpose of purification, concentration and screening of different components in the solution[48].

The alkaline protease enzyme digest product (CPCAH) and papain enzyme digest product (CPCPH) were separated by ultrafiltration centrifuge tubes, and then three groups were set up, numbered 1, 2 and 3[49]. The enzyme digest products were separated into three ultrafiltration fractions with the boundaries of 3 kDa and 10 kDa by ultrafiltration through ultrafiltration membranes with the molecular weight of 3 kDa and 10 kDa, respectively, and the ultrafiltration products were used for The ultrafiltrated products were used for the subsequent determination of antioxidant activity. The ABTS radical scavenging rate was compared with different molecular weight fractions[50].

2.7 Mass spectrometry identification

LC-MS/MS determination of antioxidant peptide molecular mass

The purified antioxidant peptide was dissolved in water to a mass concentration of 0.5 mg/m L.

Chromatographic conditions: 1 uL load; InfinityLab Poroshell 120 SB-C1g column (2.1 mm× 150 mm, 2.7 um); Mobile phase A: ultrapure water + 0.1% trifluoroacetic acid; Mobile phase B: acetonitrile + 0.1% trifluoroacetic acid; Isocratic elution: 90%~~10%A, 10%~90%B, elution time 10 min, flow rate 0.5 mL/min.

Mass spectrometry conditions: ion source: electrospray ion source; Positive ion mode; Injection voltage 3.0 KV; Cone voltage 80V; Ion source temperature 80 °C; Decomposition temperature 200 °C; Cone gas flow rate 10 Lh; Gas desolvation flow rate 60O L/h; Collision energy 10~50eV; Primary mass spectrometry scanning range m/z100~ 3 000; Secondary mass spectrometry scanning range m/z 100 ~ 3 000.

LC-MS/MS determination of antioxidant peptide sequences.The antioxidant peptide was dissolved in water to give it a mass concentration of 0.5 mg/mL. Chromatographic and mass spectrometry conditions are consistent with above[51].

The three groups obtained after the separation of alkaline protease enzyme digestion product (CPCAH) and papain enzyme digestion product (CPCPH) by ultrafiltration centrifuge tubes were numbered CPCAH-F1, CPCAH-F2, CPCAH-F3; CPCPH -F1, CPCPH-F2, CPCPH-F3.

The sequence information of multiple peptides, including the relative molecular weight, amino acid sequence composition, PeptideRanker and hydrophobicity of the peptides, can be obtained by mass spectrometry identification of CPCAH-F3 and CPCPH-F3, respectively.

Summary of chapter II

1. Use Water leaching extraction method and Repeated freeze-thaw method to extract phycobiliprotein

2. The amount of soluble protein in the solution can be determined by the Komas Brilliant Blue method

3. Preparation of antioxidant peptides,and then Measurement of free radical scavenging rate

4. Ultrafiltration technology is a kind of membrane separation technology, which uses the pressure difference of 0.1~0.5 MPa as the driving force, uses the interception ability of porous membrane, and separates the particulate matter of different sizes in the solution through physical interception

5. The sequence information of multiple peptides, including the relative molecular weight, amino acid sequence composition, PeptideRanker and hydrophobicity of the peptides, can be obtained by mass spectrometry identification of CPCAH-F3 and CPCPH-F3, respectively.

CHAPTER III
RESULTS AND DISCUSSION

3.1 Extraction rate of algal bile protein

Three sets of parallel tests were set up for each of the two methods of algal bile protein extraction. The extraction rate of algal bile protein was about 43.13% for the water immersion extraction method and 65.46% for the repeated freeze-thawing method . It was found that the extraction rate and purity of the repeated freeze-thawing method were better than those of the water immersion method.

3.2 Free radical scavenging

For the preparation of antioxidant peptides by protease hydrolysis, the hydrolysis effect of the enzyme was measured by the DPPH radical scavenging rate and ABTS+ radical scavenging rate of the enzymatic hydrolysate. Firstly, the enzymatic hydrolysis effect of papain, alkaline protease, trypsin and pepsin for 6 enzymatic hydrolysis times was compared, and the best enzymatic hydrolysis time was the best enzymatic hydrolysis time of the enzyme. Then, the hydrolysis effect of the four enzymes at the optimal enzymatic hydrolysis time was compared, and the protease with the best hydrolysis effect was screened.

From Figure 3.1 (Appendix B), it can be seen that papain has the strongest DPPH radical scavenging ability at 4 hours, followed by alkaline protease has strong DPPH radical scavenging ability at 5 hours. From Figure 3.2 (Appendix B), it can be seen that papain has the strongest ABTS+ radical scavenging ability at 5h, and alkaline protease has higher ABTS+ radical scavenging ability at 5h. In summary, the preparation of antioxidant peptides by papain was the best, and the DPPH and ABTS+ radical scavenging rates of papain's enzymatic hydrolysate were 61.21±6.58% and 58.12±8.83%, respectively. Compared with the free radical scavenging rates of papain and alkaline protease, although there are some differences in the scavenging capacity and yield of DPPH and ABTS+ free radicals, the cost needs to be

considered. Therefore, in the optimization of enzymatic hydrolysis conditions, alkaline protease can still be selected to hydrolyze spirulina powder to improve the DPPH free radical and ABTS+ free radical scavenging ability of spirulina peptide.

3.3 Determination of antioxidant capacity of enzymatic digestion products

The alkaline protease enzyme digestion product (CPCAH) and papain enzyme digestion product (CPCPH) were separated by ultrafiltration in 3 kDa and 10 kDa centrifuge tubes, and then the enzyme digestion product with the highest antioxidant activity for 5 h was selected and separated by ultrafiltration into three fractions of less than 3 kDa, more than 3 kDa less than 10 kDa, and more than 10 kDa, and their ABTS+ free radical scavenging rates were The free radical scavenging rate was measured. The free radical scavenging rate of ABTS+ was relatively higher for the fractions of alkaline protease (CPCAH) and papain (CPCPH) less than 3 kDa, 81.29±0.8% and 81.07±1.05%, respectively. The free radical scavenging rate of ABTS+ was the next highest for the fractions of 3-10 kDa, 77.74±0.98% and 75.17±1.30%, respectively. 75.17±1.30%, which shows that the relative molecular mass sizes of antioxidant active peptides in Spirulina are mostly concentrated in the range of less than 3 kDa.

Comparative results of ABTS radical scavenging rates for different molecular weight fractions after separation of alkaline protease enzymatic digestion products (CPCAH) by ultrafiltration centrifuge tubes.

Comparative results of ABTS radical scavenging rates of different molecular weight fractions after separation of papain enzymatic digestion products (CPCPH) by ultrafiltration centrifuge tubes.

3.4 Mass spectrometry identification

CPCAH-F3 was identified by mass spectrometry, and the relative molecular weights, sequence information, and hydrophobicity of the multiple peptides obtained by enzymatic digestion are shown in Table 3.6 (Appendix E) The CPCPH-F3 was

identified by mass spectrometry, and the relative molecular weights, sequence information, and hydrophobicity of the multiple peptides were obtained as shown in Table 3.7 (Appendix E).

The analysis of the mass spectrometry identification results showed that the peptides isolated from CPCPH-F3 and CPCAH-F3, which had higher antioxidant capacity, were rich in hydrophobic amino acids, and the relative molecular masses of the peptides were concentrated around 617 Da.

Summary of chapter III

The extraction rate of algal bile protein was about 43.13% for the water immersion extraction method and 65.46% for the repeated freeze-thawing method .

The DPPH and ABTS+ radical scavenging rates of papain's enzymatic hydrolysate were 61.21±6.58% and 58.12±8.83%, respectively.

The free radical scavenging rate of ABTS+ was relatively higher for the fractions of alkaline protease (CPCAH) and papain (CPCPH) less than 3 kDa, 81.29±0.8% and 81.07±1.05%, respectively. The free radical scavenging rate of ABTS+ was the next highest for the fractions of 3-10 kDa, 77.74±0.98% and 75.17±1.30%, respectively. 75.17±1.30%, which shows that the relative molecular mass sizes of antioxidant active peptides in Spirulina are mostly concentrated in the range of less than 3 kDa.

CONCLUSION

1. The extraction of algal bile proteins was performed by repeated freeze-thawing and water immersion methods, respectively. The extraction rate of algal bile proteins by water immersion method was 43.13% , while the extraction rate of algal bile proteins by repeated freeze-thawing method was 65.46% . In comparison, the extraction rate and purity of the repeated freeze-thawing method were better than that of the water immersion method.

2. Comparing the enzymatic effect of papain, alkaline protease, trypsin and pepsin for six enzymatic times respectively, the best results were obtained using papain for the preparation of antioxidant peptides. The DPPH and ABTS+ free radical scavenging rates of their enzymatic products were 61.21±6.58% and 58.12±8.83%, respectively.

3. The enzymatic digestion products (CPCPH) of papain were separated into three fractions of less than 3 kDa, more than 3 kDa less than 10 kDa and more than 10 kDa by ultrafiltration, and their ABTS+ free radical scavenging rates were measured separately. The results showed that the relative molecular mass sizes of antioxidant active peptides in Spirulina were mostly concentrated in the range of less than 3 kDa.

4. Analysis of the mass spectrometry identification results showed that the peptides isolated from CPCPH-F3 and CPCAH-F3, which had higher antioxidant capacity, were rich in hydrophobic amino acids, and the relative molecular masses of the peptides were concentrated around 617 Da.

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Appendix A

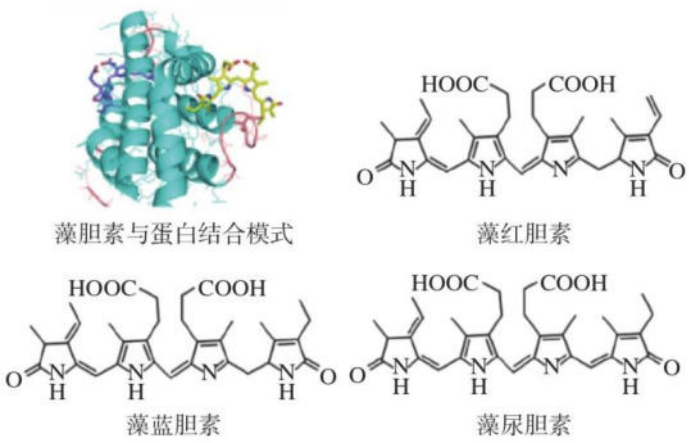

Figure 1.1 Types of phycocyanin

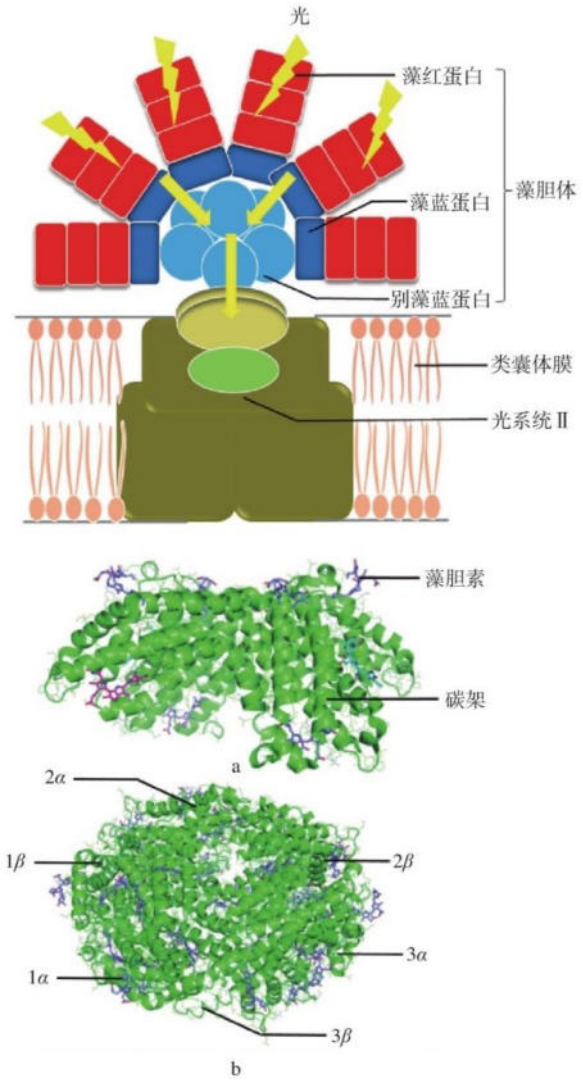

Figure 1.2 Structure of algal bile body

Appendix B

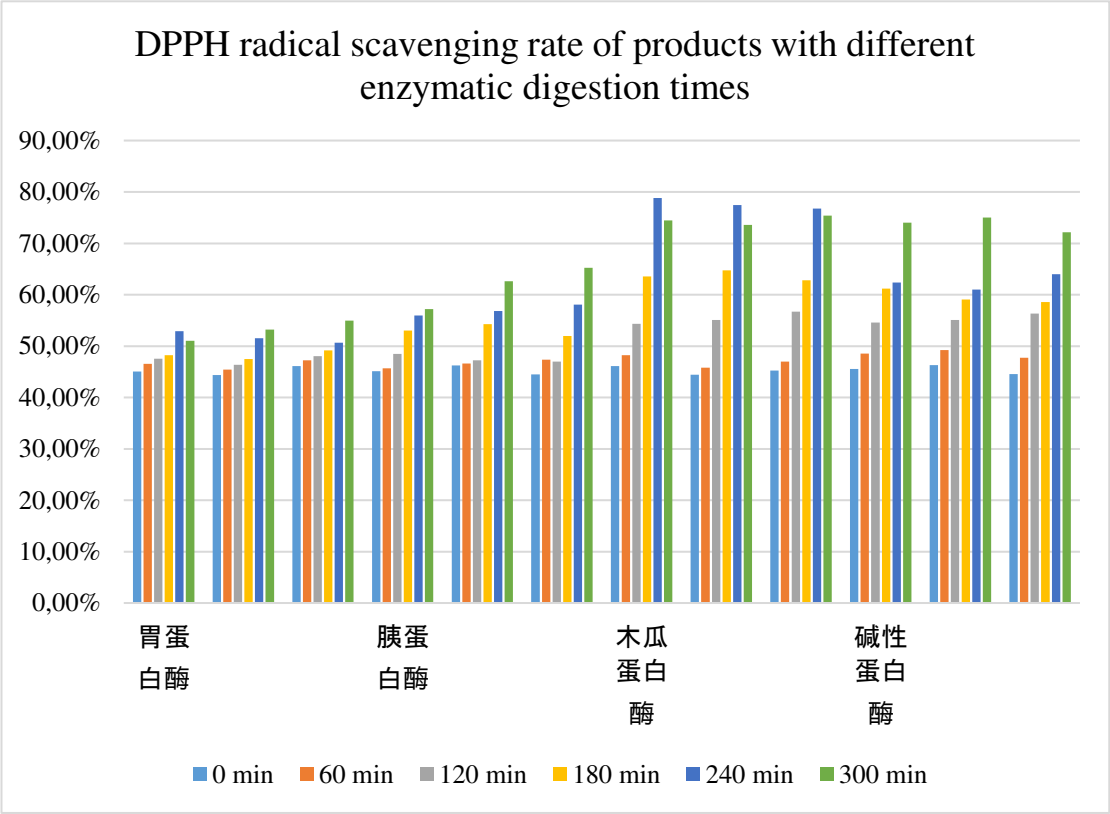

Figure 3.1 DPPH radical scavenging rate of products with different enzymatic digestion time

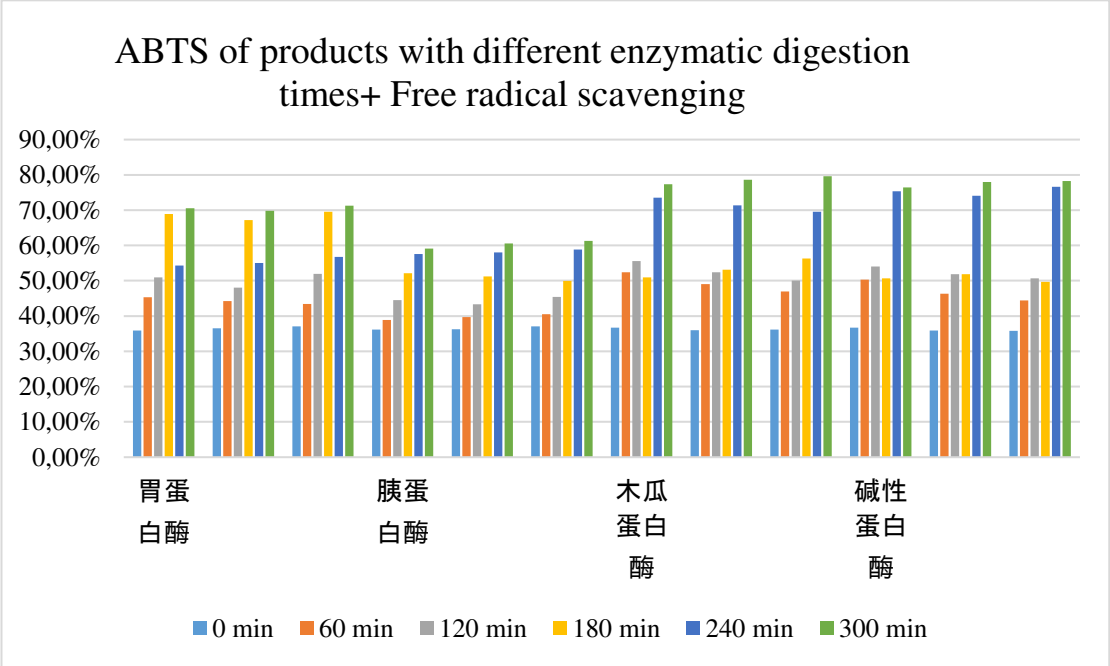

Figure 3.2 ABTS radical scavenging rate of enzymatic products at 1 mg/mL concentration for different enzymatic times

Appendix C

Table 2.1

Standard parameters of each enzyme type

Enzyme type	Protein concentration (feed to liquid ratio)	Temperature/°C	Optimum pH	Time/h	E/S (enzyme substrate ratio)
Alkaline protease	0.05 g/mL	50	10	0/1/2/3/4/5	2%
Trypsin	0.05 g/mL	37	8	0/1/2/3/4/5	2%
Pepsin	0.05 g/mL	37	2	0/1/2/3/4/5	2%
Papaya Protease	0.05 g/mL	50	7	0/1/2/3/4/5	2%

Table 3.1

Extraction rate of algal bile protein from

	1	2	3
Water leaching extraction method	41.12%	43.29%	44.97%
Repeated freeze-thaw method	69.12%	65.33%	61.92%

Table 3.2

Scavenging rate of DPPH radicals at 1 mg/mL for different enzymatic digestion times

	0 min	60 min	120 min	180 min	240 min	300 min
Pepsin	45.02%	46.56%	47.55%	48.23%	52.88%	51.02%
	44.35%	45.45%	46.35%	47.45%	51.56%	53.22%
	46.11%	47.22%	48.01%	49.18%	50.66%	54.96%
Trypsin	45.12%	45.65%	48.45%	53.01%	55.97%	57.21%
	46.23%	46.61%	47.23%	54.24%	56.85%	62.63%
	44.46%	47.33%	46.98%	51.98%	58.09%	65.23%
Papaya Protease	46.12%	48.23%	54.32%	63.56%	78.80%	74.44%
	44.45%	45.82%	55.06%	64.72%	77.42%	73.56%
	45.23%	46.97%	56.71%	62.78%	76.78%	75.39%
Alkaline protease	45.54%	48.56%	54.58%	61.19%	62.38%	73.99%
	46.32%	49.23%	55.06%	59.09%	61.02%	75.03%
	44.56%	47.71%	56.31%	58.55%	63.99%	72.15%

Appendix D

Table 3.3

ABTS radical scavenging rate of enzymatic products at 1 mg/mL concentration for different enzymatic times

	0 min	60 min	120 min	180 min	240 min	300 min
Pepsin	35.88%	45.36%	50.94%	68.91%	54.31%	70.55%
	36.51%	44.23%	48.09%	67.23%	55.03%	69.81%
	37.03%	43.40%	51.95%	69.56%	56.78%	71.29%
Trypsin	36.12%	38.91%	44.52%	52.13%	57.55%	59.13%
	36.29%	39.68%	43.32%	51.23%	58.03%	60.56%
	37.08%	40.52%	45.41%	49.96%	58.81%	61.32%
Papaya Protease	36.74%	52.38%	55.61%	50.98%	73.51%	77.37%
	36.01%	49.03%	52.36%	53.16%	71.32%	78.64%
	36.19%	46.98%	50.05%	56.32%	69.59%	79.61%
Alkaline protease	36.69%	50.33%	54.01%	50.66%	75.33%	76.45%
	35.87%	46.35%	51.89%	51.88%	74.05%	77.99%
	35.79%	44.39%	50.65%	49.67%	76.59%	78.28%

Table 3.4

Comparative results of ABTS radical scavenging rates of different molecular weight components of CPCAH

	Molecular weight	1	2	3
CPCAH-F1	＞10 kDa	76.56%	76.98%	75.88%
CPCAH-F2	3~10 kDa	77.67%	78.79%	76.76%
CPCAH-F3	<3 kDa	80.55%	81.23%	82.09%

Table 3.5

Comparative results of ABTS radical scavenging rates of different molecular weight components of CPCPH

	Molecular weight	1	2	3
CPCPH-F1	＞10 kDa	73.09%	72.23%	71.64%
CPCPH-F2	3~10 kDa	75.06%	76.59%	73.87%
CPCPH-F3	<3 kDa	80.02%	82.22%	80.96%

Appendix E

Table 3.6

Identification results of CPCAH-F3 mass spectrometry

Source	Sequence	Molecular weight(Da)	PeptideRanker	Hydrophobicity
CPCAH-F3	ACSAG	407.49	0.435704	0.09
CPCAH-F3	ADLTR	574.69	0.201399	-0.38
CPCAH-F3	ADSRKPIWI	1085.39	0.646122	-0.2
CPCAH-F3	AHDLP	551.66	0.459042	-0.08
CPCAH-F3	AHSS	400.43	0.113348	-0.17
CPCAH-F3	AKA	288.37	0.109131	-0.2
CPCAH-F3	CNTAEI	649.79	0.112204	-0.07
CPCAH-F3	CSGDS	467.5	0.290361	-0.21
CPCAH-F3	DILRG	572.73	0.391177	-0.21
CPCAH-F3	DRNFLRF	967.18	0.911987	-0.45
CPCAH-F3	DRVYIHPF	1046.3	0.689291	-0.13
CPCAH-F3	DSGDS	479.45	0.157283	-0.36
CPCAH-F3	DTVNAA	589.67	0.0553422	-0.08
CPCAH-F3	DYMGWMDF	1064.3	0.921813	0.03
CPCAH-F3	EDRTY	682.74	0.113197	-0.65
CPCAH-F3	EI	260.31	0.0494608	0.05
CPCAH-F3	EQDRR	702.78	0.156976	-1.11
CPCAH-F3	ER	303.33	0.0704548	-1.19
CPCAH-F3	ESGDS	493.48	0.0874411	-0.34
CPCAH-F3	FAKA	435.56	0.461071	0
CPCAH-F3	FFKA	511.66	0.908544	0.09
CPCAH-F3	FLFVGDL	810.06	0.668347	0.32
CPCAH-F3	FLRF	581.76	0.986411	0
CPCAH-F3	FLRN	548.69	0.779885	-0.31
CPCAH-F3	FMR	452.6	0.980845	-0.3
CPCAH-F3	FMRF	599.79	0.99366	-0.07
CPCAH-F3	FPPWF	692.87	0.997604	0.29
CPCAH-F3	FPPWL	658.86	0.993525	0.27
CPCAH-F3	FPPWVL	758.01	0.977268	0.32
CPCAH-F3	FY	328.38	0.982402	0.32
CPCAH-F3	GCPWDSWC	953.15	0.976943	-0.01
CPCAH-F3	GDNFMRF	886.08	0.968462	-0.21
CPCAH-F3	GEP	301.33	0.326355	-0.18
CPCAH-F3	GFAD	408.45	0.670159	0.08
CPCAH-F3	GFGD	394.43	0.840659	0.05
CPCAH-F3	GHP	309.36	0.718877	-0.1
CPCAH-F3	GKEYND	724.8	0.0828083	-0.48

Continuous appendix E

CPCAH-F3	GNFFRF	786.96	0.987334	-0.07
CPCAH-F3	GNTAEI	603.71	0.0799794	-0.05
CPCAH-F3	GSWD	463.49	0.762472	-0.11
CPCAH-F3	GW	261.3	0.993164	0.27
CPCAH-F3	GYGDR	566.63	0.537767	-0.43
CPCAH-F3	HKEYND	804.89	0.0673446	-0.58
CPCAH-F3	HNTAEI	683.8	0.0681815	-0.14
CPCAH-F3	HTAADLLR	896.12	0.309609	-0.19
CPCAH-F3	IAYKPE	719.91	0.163994	-0.13
CPCAH-F3	IFFEV	653.84	0.477503	0.37
CPCAH-F3	IKEYND	780.91	0.0607918	-0.39
CPCAH-F3	ILME	504.7	0.224846	0.23
CPCAH-F3	IYEPEIA	834.03	0.117426	0.06
CPCAH-F3	KDTE	491.54	0.0333968	-0.66
CPCAH-F3	LPLR	497.69	0.641035	-0.19
CPCAH-F3	LVR	386.53	0.147298	-0.23
CPCAH-F3	LWKT	546.72	0.44299	-0.1
CPCAH-F3	MAHSS	531.64	0.162032	-0.08
CPCAH-F3	MAKA	419.58	0.224433	-0.09
CPCAH-F3	MERQVL	775.02	0.139055	-0.29
CPCAH-F3	MFSPQ	608.77	0.664815	-0.03
CPCAH-F3	MGHP	440.57	0.801456	-0.01
CPCAH-F3	MKEYND	798.94	0.0805879	-0.47
CPCAH-F3	MNIPPGD	742.94	0.474883	-0.05
CPCAH-F3	MNTQL	605.78	0.238631	-0.14
CPCAH-F3	MRTGNAN	762.93	0.126966	-0.36
CPCAH-F3	NIPPGD	611.73	0.488056	-0.1
CPCAH-F3	NPTNLH	694.83	0.249519	-0.23
CPCAH-F3	PAVVL	497.7	0.207088	0.36
CPCAH-F3	PDNFMRF	926.14	0.974119	-0.24
CPCAH-F3	QGLISFPRV	1016.34	0.657318	-0.02
CPCAH-F3	QHP	380.44	0.406295	-0.39
CPCAH-F3	RYLPT	648.82	0.382079	-0.29
CPCAH-F3	SHTVKIYD	962.18	0.0934348	-0.17
CPCAH-F3	SNTAEI	633.73	0.0954573	-0.12
CPCAH-F3	SPEFTV	678.81	0.322427	0
CPCAH-F3	SSGDS	451.44	0.185257	-0.27
CPCAH-F3	STAKSTA	664.79	0.0568368	-0.21
CPCAH-F3	SVTP	402.49	0.128171	0.01
CPCAH-F3	TASMRLK	806.08	0.296879	-0.32
CPCAH-F3	TKPR	500.64	0.157382	-0.78

Continuous appendix E

CPCAH-F3	TSTS	394.42	0.0393518	-0.22
CPCAH-F3	TTDD	450.44	0.0374362	-0.45
CPCAH-F3	WIGRW	716.91	0.973627	-0.03
CPCAH-F3	YAFGL	569.72	0.879082	0.31
CPCAH-F3	YGGTPPFV	837.05	0.69305	0.15
CPCAH-F3	YMFHLMD	956.24	0.700331	0.08
CPCAH-F3	YMRF	615.79	0.964898	-0.22
CPCAH-F3	YSGDS	527.54	0.189037	-0.21

Table 3.7

Identification results of CPCPH-F3 mass spectrometry

Source	Sequence	Molecular weight(Da)	PeptideRanker	Hydrophobicity
CPCPH-F3	AKA	288.37	0.109131	-0.2
CPCPH-F3	CNTAEI	649.79	0.112204	-0.07
CPCPH-F3	DTVNAA	589.67	0.0553422	-0.08
CPCPH-F3	GEP	301.33	0.326355	-0.18
CPCPH-F3	GFAD	408.45	0.670159	0.08
CPCPH-F3	GLLSGLGL	729.02	0.631469	0.29
CPCPH-F3	GQDFMRF	900.11	0.963335	-0.22
CPCPH-F3	GSWD	463.49	0.762472	-0.11
CPCPH-F3	GW	261.3	0.993164	0.27
CPCPH-F3	HNTAEI	683.8	0.0681815	-0.14
CPCPH-F3	IKEYND	780.91	0.0607918	-0.39
CPCPH-F3	LWKT	546.72	0.44299	-0.1
CPCPH-F3	MAKA	419.58	0.224433	-0.09
CPCPH-F3	MGHP	440.57	0.801456	-0.01
CPCPH-F3	MSQSKYRQL	1140.45	0.24954	-0.44
CPCPH-F3	SDMYSFGL	919.12	0.878675	0.04
CPCPH-F3	YMRF	615.79	0.964898	-0.22