MINISTRY OF EDUCATION AND TRAINING
NHA TRANG UNIVERSITY

DINH HUU DONG

REARCHING ON PRODUCTION OF HYDROLYZED PROTEIN POWDER CONTAINING CHRONDROITIN SULFATE FROM WHITECHECK SHARK CARTILAGE (*CARCHARHINUS DUSSUMIERI*) BY PROTEASES

SUMMARY OF DOCTORAL THESIS
Major: Aquatic Products Technology
Code: 9540105

KHANH HOA – 2021

The research has been done at Nha Trang University

The scientific advisors: Assoc.Prof.Dr. Vu Ngoc Boi
Assoc.Prof.Dr. Nguyen Anh Tuan

Reviewer 1: Prof.Dr. Nguyen Anh Dung
Reviewer 2: Assoc.Prof.Dr. Nguyen Thi My Huong
Reviewer 3: Assoc.Prof.Dr. Ngo Dai Nghiep

The dissertation will be defended at the Board of Examiners of Nha Trang University at 8AM on 26th November, 2021

The dissertation can be found at National Library of Vietnam
Library of Nha Trang University

SUMMARY OF NEW CONTRIBUTIONS OF THE THESIS

Title: Researching on production of hydrolyzed protein powder containing chondroitin sulfate from whitecheck shark cartilage (*Carcharhinus dussumieri*) by proteases

Major: Aquatic Products Technology **Code:** 9540105

PhD. Student: Dinh Huu Dong **Year:** 2014

The scientific advisors: 1**.** Assoc.Prof.Dr. Vu Ngoc Boi

2. Assoc.Prof.Dr. Nguyen Anh Tuan

School: Nha Trang University

Contents:

The dissertation has obtained some new results added to the field of researching the hydrolysis and making the hydrolyzed protein powder containing chondroitin sulfate from whitecheck shark cartilage *(C. Dussumieri)* oriented for application in dietary supplements:

1) The optimal parameters for the hydrolysis of shark cartilage were determined: the combination of the Alcalase: Papain with the ratio of 60/40, the concentration of 0.3%; The ratio of raw materials: water is 1/1; suitable pH is 6.8; The hydrolysis temperature was 50^oC for 20 hours. The recovery efficiency of chondroitin sulfate was 96.97%. Hydrolyzed protein solution has nutritional energy: 20.42 Kcal/100 ml, chondroitin sulfate content: 40.5 mg/ml, total nitrogen: 7.46 g/l, total mineral: 3.02 g/ l, Zn: 7.63 mg/l, Mg: 205 mg/l, Fe: 4.78 mg/l, the solution contains 18 types of amino acids and meets food hygiene and safety standards according to current regulations of the Ministry of Health.

2) Determined the characteristic structure that preserves both C4 and C6 sulfate radicals of chondroitin sulfate products. The product is a mixture of 2 isomers GlcA-GalNAc-4SO_3^- and GlcA-GalNAc-6SO_3^-.

3) The optimal parameters for spray drying to create chondroitin sulfate-containing protein powder from shark cartilage hydrolyzate were determined: 12% maltodextrin carrier, drying chamber temperature 80^oC, flow rate 12 ml/min and pressure. drying

chamber capacity 2.5bar. The recovery efficiency of chondroitin sulfate was 87.81%. The product has chondroitin sulfate content of 203mg/g, total nitrogen 5.04g/100g, total ash 3.95g/100g, moisture 4.27% and meets microbiological standards for food use as prescribed of the Ministry of Health.

4) Determining the production process of hydrolyzed protein powder containing chondroitin sulfate from fresh shark cartilage hydrolyzate by protease enzyme at experimental scale. Protein powder products containing chondroitin sulfate meet food hygiene and safety standards according to current regulations of the Ministry of Health.

THE SCIENTIFIC ADVISORS 1 **PhD. STUDENT**

Assoc.Prof. Dr. Vu Ngoc Boi

THE SCIENTIFIC ADVISORS 2

Assoc.Prof. Dr. Nguyen Anh Tuan **Dinh Huu Dong**

PREAMBLE

1. The urgency of the thesis

The chondroitin sulfate (CS) is the basic component that makes cartilage and joints and makes up the elastic fibers (tendons, muscles, ligaments ...) to help in flexible movement and elasticity in joint activities, creating durability when pressed. The chondroitin sulfate increases mucus production and lubricity of joint fluid, ensuring nutritional function and flexible movement of joints. Therefore, the chondroitin sulfate is used to support the treatment of bone and joint diseases, and to reduce the degenerative joint process. The chondroitin sulfate also plays a role in protecting joint cartilage by inhibiting enzymes that destroy joint cartilage such as collagenase, phospholipase A2, N-acetylglucosamindase. In addition, the chondroitin sulfate also contributes to the growth and regeneration of the cells of the eye cornea.

The chondroitin sulfate is obtained mainly from natural sources, of which, fish cartilage, especially the shark cartilage, usually has a higher chondroitin sulfate content than other animals. That is why the cartilage is often used in chondroitin sulfate extracts to produce functional foods that aid in the treatment of osteoarthritis. Chondroitin sulfate often binds to protein by o-glycoside links to form a proteoglycan (PG) (glucoprotein) located in the structure of cartilage tissue, so it is difficult for humans to absorb. Currently there are some studies using chemical agents or enzymes to hydrolyze dried or fresh fish cartilage and precipitate the chondroitin sulfate used in functional food production. However, only intake of the chondroitin sulfate leads to wastage of natural substances from the fish cartilage, ineffective use as well as limited production efficiency and expensive product cost. To deal with these problems, we have conducted research using enzymes in the fresh shark cartilage hydrolysis with a desire to create a hydrolysis solution containing substances from the fish cartilage and the chondroitin sulfate to be used as food providing essential ingredients to help support osteoarthritis. Therefore, the thesis conducted: "*Researching on production of hydrolyzed protein powder containing chondroitin sulfate from whitecheck shark cartilage (C. dussumieri) by proteases*".

2. The purposes of the dissertation

Utilizing the remaining raw materials - fresh shark cartilage in hydrolysate processing and protein powder containing chondroitin sulfate and natural substances from shark cartilage (*C. dussumieri*) with environmentally friendly enzyme protease technology Directed to use as functional foods to support the treatment of osteoarthritis.

3. Objects and scope of the study

3.1. *Objects of the study*

Whitecheck shark cartilages

3.2. *Scope of the study*

1) Research on hydrolysis of shark cartilage (C. dussumieri) by protease.

2) Evaluation of shark cartilage hydrolyzate quality and determination of the structure of chondroitin sulfate.

3) Study on spray drying to create hydrolyzed protein powder containing chondroitin sulfate.

4. Methodology

The thesis uses basic research methods of biochemistry and microorganisms with the support of mathematical theories to discover new properties, relationships between quantities and verify the hypothesis. The experimental results were statistically processed, tested and compared the average values between groups by software Box-Behnken, ANOVA, Minitab 20 and JMP 10. In addition, the thesis also uses normal methods. Used in structural analysis to analyze the structure of chondroitin sulfate

5. Novelty to science

- The thesis conducted the first comprehensive research from the hydrolysis of fresh shark cartilage, ... to the drying study to create hydrolyzed protein powder containing chondroitin sulfate. The research results of the thesis have scientific significance and enrich the understanding of the possibility of using protease in shark cartilage hydrolysis.

- The thesis has identified alcalase - papain enzyme mixture as a suitable agent for shark cartilage hydrolysis (*C. dussumieri*) and determined optimal parameters for shark cartilage hydrolysis by

mixture. the enzyme alcalase-papain. On the other hand, the thesis also identified the optimal parameters for the spray drying process to create protein powder containing chondroitin sulfate from shark cartilage hydrolysis. The research results of the thesis, when applied in practice, will contribute to creating food products containing chondroitin sulfate and being used as functional foods to support the treatment of osteoarthritis and anti-degenerative joint diseases.

- The thesis also identified the structure of chondroitin sulfate from shark cartilage (*C. dussumieri*) which is a mixture of 2 isomers, including chondroitin - 4 - sulfate with characteristic structural fragment GlcA-GalNAc-4SO3- and chondroitin - 6 - sulfate with its characteristic structural fragment, GlcA-GalNAc-6 SO3-.

6. Structure of the dissertation

The dissertation includes 150 pages, of which 17 pages of overview, 23 pages of research methods, 100 pages of research results, 2 pages of conclusions, 17 tables of data, 101 figures, 93 references (33 Vietnamese references and 59 English references), and 38 pages of appendices.

CHAPTER 1. OVERVIEW OF RESEARCH PROBLEMS

1.1. INTRODUCTION TO CHONDROITIN SULFATE AND THE APP

*** Structure of chondroitin sulfate**

Chondroitin sulfate (CS) is a polysaccharide, belonging to a heteropolysaccharide group called glycosaminoglycan (GAG), found naturally in the form of a straight-chain polymer consisting of the basic units composed of two sugars, N-acetyl-D. - galactosamine (GalNAc) and D-glucuronic acid (GlcA) alternate. These sugars can be sulphated or not sulphated, some GlcA radicals are epimeized to L-iduronic acid (IdoA). Chondroitin sulfate is a natural polymer containing 15 ÷ 150 structural units of the basic unit consisting of 2 sugars, GlcA and

GalNAc. The molecular mass of CS usually fluctuates between 10,000 ÷ 100,000 Dalton.

CS often binds proteins by binding o-glycosides to form a proteoglycan (PG) complex. CS is a hydrophilic polymer so cartilage tissue has a high water content. Sugar molecules in the structure of CS may not be sulphated, sulphated once or twice, most of the OH group at carbon 4 and 6 positions of GalNAc is sulphated, for some GAG chains may be sulphate at position 2 of GlcA. GAG is a major component of cartilage tissue. PG together with protein and hyaluronic acid form a hydrodynamic complex with reversible compression to help cartilage tissue resist compression with minimal deformation.

*** Technology for inclusion of chondroitin sulfate**

The process of obtaining raw CS from fish cartilage can be done by chemical methods or biological methods (using enzymes) to hydrolyze fish cartilage. In the chemical method, cartilage tissue is treated with hot water or salt or alkaline solution (NaOH) or acid (HCl, CH3COOH, ...) to separate GAG from molecules (protein, hyaluronic acid, ...). This method has been applied to collect CS from chicken cartilage and bovine cartilage. However, the disadvantage of the chemical method is the breakdown of the glycoside bonds of CS upon CS intake.

The biological method uses enzymes to hydrolyze cartilage tissue, which is an effective method to absorb CS. Because CS is not structurally modified, it retains biological activity and this method does not cause environmental pollution caused by chemicals (salts, alkalis, acids, ...). Some studies have suggested that the protease enzyme can be used to hydrolyze cartilage, releasing GAG from binding proteins. Then, if protein is removed, CS is separated and purified. The long chain hydrophilic GAG polymers are insoluble in ethanol, acetone and methanol. Therefore, organic solvents can be used to precipitate raw CS and separate CS from other polysaccharides.

*** Application of chondroitin sulfate**

CS has been widely used as a dietary supplement for human nutrition and is increasingly being applied in different fields, especially in the field of medicine, especially in the treatment of diseases. Osteoarthritis. CS is the physiology of the cornea, helps maintain ophthalmic transparency, creates proper viscosity and nourishes the corneal endothelium, nourishes the eye corneal cells, regenerates the tear key layers before the cornea, enhance the elasticity of the lens, prevent dry eyes. CS works to reduce the damage to the eye lenses. CS is used in eye drops to prevent and treat dry eyes, eye fatigue, and retinal degeneration. In addition, CS also has the ability to inhibit the formation of neoplastic micro-capillaries that cancer tissues need to grow, so it can limit the growth of malignancies. Some studies show that shark cartilage also has the ability to help strengthen the immune system, so it helps to ease the immune system such as rheumatism, bone pain, psoriasis, eczema, lupus erythematosus, ... It has been shown that CS has the ability to enhance anti-inflammatory, inhibit the secretion of proinflammatory cytokines and free radicals.

1.2. SITUATION EXPLOITATION AND USE SHARK CARTILAGE

According to scientists' statistics, there are currently more than 420 species of sharks in the world with different sizes. The exploitation and fishing of sharks has become an outstanding profession, attracting the majority of fishermen in countries bordering the world. People catch sharks mainly for finfish, because shark fin is very valuable in the world market. Currently, the countries that catch and consume the world's largest shark fin are the United States and China. In Vietnam, there are also shark fishing areas such as Phu Yen, Nha Trang, Binh Dinh, ...

China's Hong Kong Special Administrative Region, one of the largest commercial centers for shark fin trade in the world. It is thought that Hong Kong is the import market for a substantial amount of

imported shark fin and shark fin for domestic consumption or re-export. The decline in shark populations around the World is attributed to the overfishing of shark species around the world that has been associated with the sale of their fins for use in shark fin soup. Very few fishing countries currently keep accurate data on shark catches by species.

There are currently no official data on shark exploitation in Vietnam. Currently in Vietnam there is only one report on the investigation of the exploitation of shark resources by Nguyen Van Long and Vo Si Tuan - Nha Trang Institute of Oceanography implemented in the period 2010 ÷ 2011. Results Investigation of Nguyen Van Long and Vo Si Tuan shows that there are about 530 ships and boats participating in the exploitation of shark/shark. The survey results of the above authors also showed a sharp decrease in the number of means of fishing and catches. Investigation results of authors Nguyen Van Long and Vo Si Tuan also show that marine shark resources have been overexploited, especially in coastal areas of Vietnam.

*** Some products from shark cartilage**

Shark cartilage is believed to have many rare natural nutrients such as collagen, protein, and essential minerals essential for the human body, especially chondroitin sulfate, so it is used as a dietary supplement. treatment of bone and joint diseases and improve health. Currently, there are many shark cartilage products on the market from different countries such as the US, Canada, Australia, ... Shark cartilage products are advertised as a source of nutritional and mineral supplements good for human health.

1.3. SOME RESEARCH HYDROLYSIS SHARK CARTILAGE

In the world, there have been some research on hydrolysis of animal cartilage such as tilapia cartilage, chicken cartilage, shark cartilage ... by hydrolysis method using chemical agent or combination between

chemical agent and protease enzyme. However, studies around the world only focus on chondroitin sulfate alone but with low efficiency and chondroitin sulfate is often lost its sulfate base at position 4 or No. 6. Moreover, chemical use such as NaOH in hydrolysis will make the product not "green" and "clean". Therefore, the research and search for methods of hydrolysis of animal cartilage by protease enzymes friendly with environment is a necessary research direction.

In Vietnam, there is only one work of Tran Canh Dinh, Vo Hoai Bac, Do Ngoc Tu - Hai Phong Seafood Institute (2010) published a study on collecting CS from stingray and dry shark cartilage by the method of Using protease enzyme preparation from B. subtilis B26 for hydrolysis. However, the hydrolysis time is long and the structure of CS has not been determined. On the other hand, this study only stopped at the precipitation to obtain chondroitin sulphate and had no specific application.

1.4. INTRODUCTION TO SPRAY DRYING TECHNOLOGY

Drying is the process of evaporating water out of a liquid material under the effect of temperature. During the drying process, water is separated from the material by diffusion due to the difference in moisture between the surface and inside the material, the difference in the partial vapor pressure of water at the surface of the material and the surrounding environment.

The raw material of the spray-drying process is usually a pre-concentrated liquid, emulsion or suspension (40 ÷ 60% moisture) sprayed into fine droplets, falling into the hot air stream in the same direction or in the opposite direction. creating a drying chamber temperature (70 ÷ 300^0C) to dry the raw materials. The resulting steam is rapidly evaporated. The product particles are separated from the drying agent as a fine powder by a separate recovery system.

In the world and Vietnam, there are many publications on spray drying research of tomato, vegetable, and carotene extracts from plants due to the spray-drying technique to conserve bioactive substances in the epidemic. extract. Spray-drying is a drying technique that best preserves substances in food and is suitable for drying food liquids. Therefore, the thesis oriented to use spray drying technique to dry hydrolyzed fluid from shark cartilage.

CHAPTER 2. MATERIALS AND METHODOLOGY

2.1. MATERIALS OF THE STUDY

*** Shark cartilage:** Sharks (*C. dussumieri*) are purchased whole at Vinh Luong fishing port - Nha Trang - Khanh Hoa. Fresh fish has an average weight of 40 ÷ 60kg/fish. Sharks are exploited in the period from February to October every year**.** After purchasing, all fins and cartilages are collected and transported to the laboratory, where they are processed to remove meat and connective tissues, cleaned, and frozen at -20°C for use throughout the study.

Figure 2.1. Image of shark cartilage before and after treatment

* Protease enzymes: protease enzymes: neutrases are commercial protease preparations produced and supplied in Vietnam by Advanced enzyme Technologies Ltd - India. Alcalase and flavourzyme are commercial protease preparations supplied by Novozyme - Denmark.

Commercial Papain is produced by Merck - Germany.

* Carriers: maltodextrin and saccharose made in Japan. Gum arabic food produced by China.

2.2. METHODS OF THE STUDY

*** Research methods for protease and chondroitin sulfate**

- Determination of the protease activity according to Anson method.

- Determination of the chondroitin content by the method using blue methylene by Farndale et al.

- The method for determining the recovery efficiency of CS during hydrolysis and protein powder production is calculated as the percentage (%) of the total amount of CS contained in shark cartilage hydrolyzate or protein powder to the total amount of chondroitin. Sulfate is present in shark cartilage material or hydrolyzate.

*** Chemical analysis methods**

- Determination of soluble protein content by the Lowry method.

- Quantifying N_{aa} according to Sorensen method.

- Quantifying N_{NH3} by steam entrapment distillation method.

- Quantifying lipid according to Soxhlet method.

- Methods of determination of moisture: According to the drying method to constant weight at 105^0C to TCVN 1867:2001.

- Determination of ash content: ash content is determined by the heating method to constant mass 600^0C to TCVN 5611 - 1991.

- Determination of metal ions composition by atomic absorption spectroscopy AAS.

- Quantifying peptide follows a method based on the standard tyrosine.

- Determination of the composition and content of amino acids by liquid chromatography according to AOAC 994.12 (2012).

- Determination of nutritional energy: according to CAC/GL-2/1985 (Rev.1-1993) FAO.

*** Structural analysis methods of chondroitin sulfate**

Analysis of functional groups in CS by infrared spectroscopy (IR) and structure of chondroitin sulfate analyzed by ^{1}H-NMR, ^{13}C-NMR, HSQC, COSY and HMBC spectrum, determined thermal properties of CS by DSC-60 energy compensation differential scan thermal analysis.

*** Quantitative method of microorganisms**

Determination of microbial content: total aerobic bacteria, total number of yeast spores, molds, Coliforms, Salmonella by colony count method.

2.3. DATA ANALYSIS

Processing research data according to the biological statistical method. Each experiment was conducted 3 times, each time 3 samples and the results were the average of the experiments. Data are processed and presented as mean ± SD. Experimental design of shark cartilage hydrolysis using JMP 10 software. Box - Benkhen model is used to optimize hydrolysates to obtain chondroitin sulfate with high yield. Compare the differences between the variables processed by Minitab 18.0 software. ANOVA analysis to evaluate the difference of the value at the significant level ($p < 0.05$).

CHAPTER III. RESULTS AND DISCUSSION

3.1. RESEARCHING HYDROLYZED SHARK CARTILAGE (*C. DUSSUMIERI*) BY PROTEASES ENZYME

3.1.1. Analysis of the basic composition of whitecheck shark cartilage

Sampling whitecheck shark cartilage to analyze some chemical parameters at the National Institute of Food Safety and Hygiene. The results presented in Table 3.1 showed that the whitecheck shark cartilage mixture had a rather high content of chondroitin sulfate up to 41.77mg/g. Therefore, whitecheck shark cartilage is very suitable as a

raw material for hydrolysis to obtain hydrolyzate containing chondroitin sulfate with high content.

Table 3.1. The results of analysis of some basic chemical parameters of whitecheck shark cartilage

No	Chemical properties	Unit	Results
1	Total amino acid	g/kg	20.13
2	Total nitrogen	g/100g	2.64
3	Total crude protein	g/100g	16.50
4	Total canxi	%	4.15
5	Total sugar	mg/kg	583.33
6	Total ash	g/100g	4.22
7	Humidity	%	80.71
8	Total chondroitin sulfate	mg/g	41.77

3.1.2. The study has selected the protease enzyme to hydrolyze whitecheck shark cartilage

Conducted experiments on hydrolysis of shark cartilage mixtures with different protease enzymes: neutrase, alcalase, flavourzyme, papain and mixture of enzymes alcalase and papain in the ratio 1/1. After a period of time: 0 hours, 2 hours, 4 hours, 6 hours, 8 hours and 10 hours, samples of hydrolysates are taken to assess soluble protein, peptide, Naa, and content. chondroitin sulfate and N_{NH3} content showed that using a mixture of alcalase + papain enzyme to hydrolyze shark cartilage would be better than other enzymes. Therefore, the alcalase + papain enzyme mixture is selected for hydrolysis of shark cartilage for further research.

3.1.3. The study has determined the ratio between the enzyme alcalase and papain for the shark cartilage hydrolysis

Conducting experiments on hydrolysis of shark cartilage with a mixture of alcalase - papain enzymes with different alcalase/papain ratios and hydrolyzate samples to assess soluble protein, peptide, Naa, and chondroitin content. sulfate and N_{NH3} content formed by hydrolysis time

showed that a mixture ratio of alcalase/papain enzyme equal to 60/40 is suitable for the hydrolysis of shark cartilage.

3.1.4. The study has determined mixed concentration of enzymes alcalase and papain for the shark cartilage hydrolysis.

The research process of hydrolysis of shark cartilage by alcalase - papain enzyme mixture with concentration of enzyme mixture in the range of 0.1% ÷ 0.5%, step is 0.1%. The analytical results showed that the appropriate concentration of alcalase - papain enzyme mixture was 0.3%

3.1.5. The study has determined the proportion of water added for the shark cartilage hydrolysis

Carrying out hydrolysis of shark cartilage with a mixture of alcalase-papain enzyme with different amounts of water added that it is appropriate to use a cartilage/water ratio of 50/50.

3.1.6. The study has determined the appropriate pH for the shark cartilage hydrolysis

Hydrolysis of shark cartilage with alcalase-papain enzyme mixture at different pH shows that nature (pH = 6.8) is suitable.

3.1.7. The study has determined the appropriate temperature for the shark cartilage hydrolysis

Results of hydrolysis of shark cartilage with a mixture of alcalase - papain enzymes at different hydrolysis temperatures showed that using a mixture of alcalase - papain enzyme with shark cartilage hydrolysis temperature at 50^0C is appropriate.

3.1.8. The study has determined the appropriate time for the shark cartilage hydrolysis

Shark cartilage hydrolysis experiment was conducted with alcalase-papain enzyme mixture and assessed soluble protein, peptide, Naa, N_{NH3} and chondroitin sulfate content according to hydrolysis time. suitable time for shark cartilage hydrolysis in about 20 ÷ 22 hours.

3.1.9. Optimization of hydrolysis

Using orthogonal experimental planning level I to optimize shark cartilage hydrolysis with a mixture of alcalase + papain enzymes (Figure 3.2) shows optimal conditions for shark cartilage hydrolysis by alcalase + papain enzyme mixture to obtain chondroitin sulfate: concentration of enzyme alcalase - papain is 0.3%, hydrolysis temperature is 50^0C, hydrolysis time is 20 hours. At that time, the hydrolysis efficiency was 96.97% and the chondroitin sulfate content, the total nitrogen content of the hydrolysis solution was 40.5 mg/mL and 7.46 g/liter of hydrolyzate respectively.

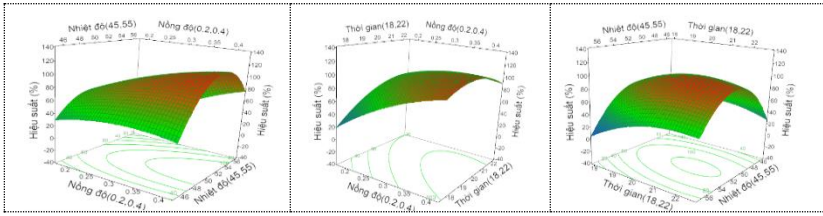

Figure 3.2. The response surface shows the correlation between enzyme concentration (X1), temperature (X2) and time (X3) to the high hydrolysis efficiency of chondroitin sulfate

3.1.10. Proposed shark cartilage hydrolysis with a mixture of enzyme alcalase-papain

From the above studies, it allows to propose the process of hydrolysis of shark cartilage with alcalase - papain enzyme mixture presented in Figure 3.3.

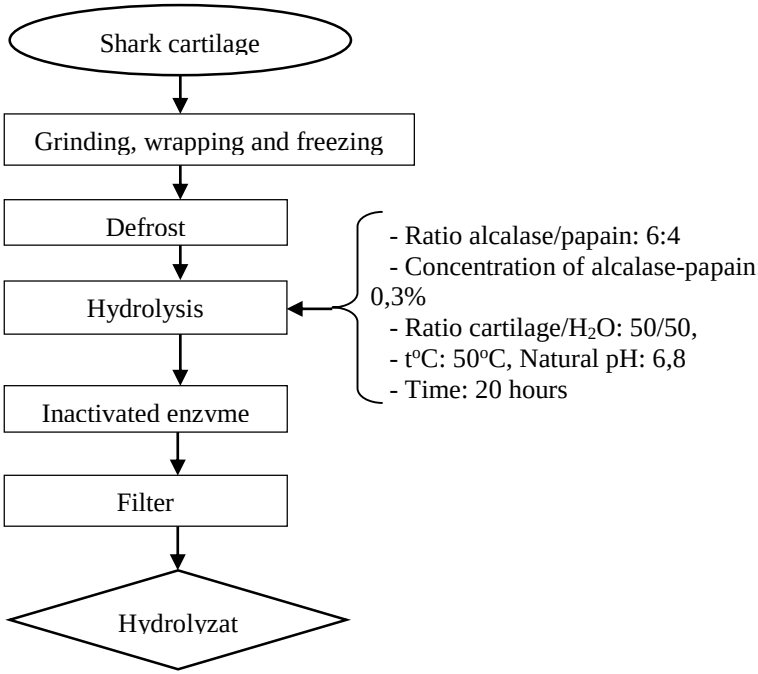

Figure 3.3. Diagram of hydrolysis of shark cartilage with alcalase - papain enzyme mixture

*** Process description**

+ Shark cartilage: shark cartilage with: Content of chondroitin sulfate: 41,77 ± 0,21mg/g, total ash content: 4,2 ± 0,11g/100g, total nitrogen content: 2,64 ± 0,02g/100g, humidity 80,7±1,12%.

+ Grind, pack and freeze: shred shark cartilage by a meat grinder to homogenize the sample and pack with PA 2kg/bag, vacuum 100%, freeze and freeze at -20^{0}C to used throughout the research process.

+ Thawing: Before hydrolysis, conduct quick thawing in 2 minutes in the microwave.

+ **Hydrolysis**: Carry out hydrolysis of shark cartilage by a mixture of enzymes alcalase - papain in hydrolysis tanks according to the following specifications: The ratio of enzymes alcalase/papain is 6:4;

The concentration of alcalase-papain enzyme mixture is 0.3%, cartilage/water ratio is 50/50, hydrolysis temperature is 50^{o}C, hydrolysis at natural pH (6.8) for 20 hours.

+ **Enzyme Inactivation**: Finish the hydrolysis process, conduct enzyme inactivation by boiling hydrolyzate for 10 minutes

+ **Filtering:** pre-filter the hydrolyzed mixture through 2 layers of filter cloth to collect the crude filtrate. Then, continue to filter the crude filtrate through a vacuum filtration system with ceramic core size allowing substances to pass through with sizes less than 5μm to collect clear filtrate. The filtrate is boiled for 10 minutes and stored in the refrigerator cooler (4 ± 1^{0}C) for use in the analysis and research of spray drying (Figure 3.4).

Figure 3.4. Image of shark cartilage hydrolysis

3.2. QUALITY ASSESSMENT OF CHONDROITIN SULFATE COMPLEX - ENZYME ALCALASE - PAPAIN AND STRUCTURE ANALYSIS OF CHONDROITIN SULFATE

3.2.1. Shark cartilage hydrolysis quality assessment

The results of assessment of chemical and energy composition of hydrolyzate at Nha Trang Pasteur Institute and National Food Hygiene and Safety Testing Institute - Ministry of Health presented in table 3.2 ÷ 3.4 show fish cartilage hydrolysis Fat in liquid, viscous, light yellow color, characteristic taste and very energetic 20.42 Kcal/100ml, rich in minerals, especially rich in Magnesium: 205.49mg/liter and zinc 7.63mg/liter These are minerals that are essential for human health. In addition, hydrolyzate contains 18 amino acids and especially

chondroitin sulfate content up to 40.5mg/ml - this is an essential ingredient for regenerating cartilage tissue, anti-aging cartilage in human. The analysis results also showed that shark cartilage hydrolysates met the microbiological requirements according to the current regulations of the Ministry of Health. Thus, shark cartilage hydrolyzate has been produced to meet the quality standards for use in food and orientation as functional foods.

Table 3.2. Chemical composition of shark cartilage hydrolyzates

No.	Chemical properties	Unit	Results
1	Total nitrogen	g/l	7.460
2	Nutritional energy	Kcal/100ml	20.420
3	Total mineral	g/l	3.020
4	Magnesium	mg/l	205.490
5	Zinc	mg/l	7.630
6	Iron	mg/l	4.780
7	Mercury	mg/l	Not detected
8	Arsen	mg/l	Not detected
9	Raw protein	g/l	46.630
10	Alanine	g/l	2.617
11	Arginine	g/l	7.921
12	Aspartic	g/l	2.164
13	Cysteine	g/l	0.254
14	Cystine	g/l	0.469
15	Glutamic	g/l	3.102
16	Glycine	g/l	5.202
17	Histidine	g/l	0.221
18	Isoleucine	g/l	0.415
19	Leucine	g/l	3.680
20	Lysine	g/l	1.043
21	Methionine	g/l	0.994
22	Phenylalanine	g/l	0.653
23	Tryptophane	g/l	0.129

24	Serine	g/l	0.836
25	Threonine	g/l	1.009
26	Tyrosine	g/l	0.047
27	Valine	g/l	0.812
28	Chondroitin sulfate	mg/ml	40.500

Table 3.3. Sensory state of hydrolyzate

No	Targets	Result
1	Status	Liquid. match
2	Color	Yellowish color
3	Taste	Characteristics of hydrolyzate

Table 3.4. Results of analysis of some microbiological parameters of shark cartilage hydrolysis

No	Targets	Unit	Result	Regulation QCVN 4 - 16: 2010/BYT
1	Total number of aerobic microorganisms	CFU/g	8	< 5000
2	*Salmonella*	CFU/g	Neg	Negative
3	*Coliforms*	CFU/g	Neg	Negative
4	Total number of spores of yeasts. molds	CFU/g	Neg	< 500

(Neg: Not detected)

3.2.2. Structure analysis of chondroitin sulfate contained in shark cartilage hydrolysates

Precipitation of chondroitin sulfate present in shark cartilage was carried out with ethanol at a concentration of 80%. Then purified chondroitin sulfate through DEAE-cellulose ion exchange chromatography system. sephadex G25 gel filter chromatography and structural analysis of chondroitin sulfate.

*** Prediction of functional groups in the structure of chondroitin sulfate by FTIR spectroscopy**

The results of the analysis of the FTIR spectrum at the Center for Radiation Technology - Da Lat Nuclear Institute are presented in Figure 3.5.

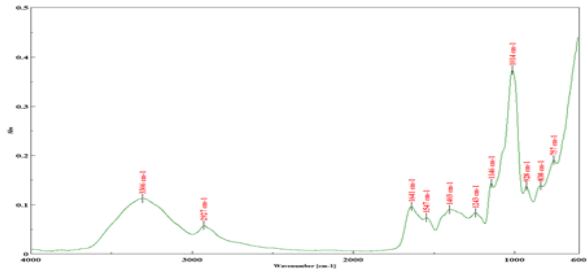

Figure 3.5. Results of determination of functional groups of chondroitin sulfate contained in hydrolyzed shark cartilage based on FTIR spectrum

From the above results. it is possible to predict functional groups in the structure of chondroitin sulfate of shark cartilage hydrolysis based on Figure 3.6

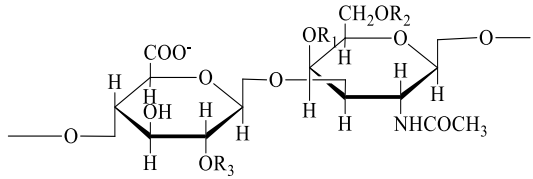

Figure 3.6. Prediction of functional groups in the structure of chondroitin sulfate from shark cartilage

D - glucuronic acid (GlcA) N-acetyl-D-galactosamine (GalNAc)

$R_1 = SO_3^-$. $R_2 = R_3 = H$: Chondroitin - 4 - sulfate (CSA)

$R_2 = SO_3^-$. $R_1 = R_3 = H$: Chondroitin - 6 - sulfate (CSC) acetyl-D-galactosamine (GalNAc) qua liên kết glucozit (1-3).

To determine the structure of chondroitin sulfate in shark cartilage hydrolysates. the thesis analyzed 1H-NMR spectrum. 13C-NMR spectrum. HSQC spectrum. COSY spectrum. HMBC spectrum. Analysis results in figures 3.7 ÷ 3.12.

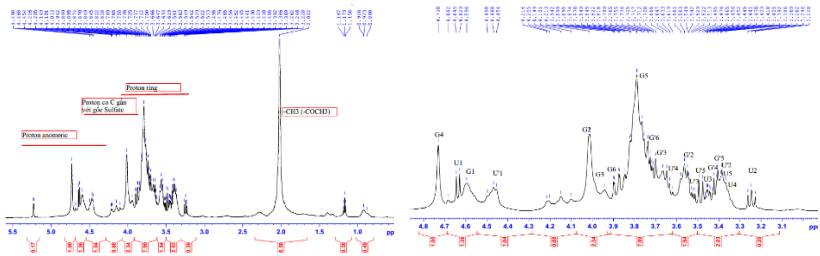

Figure 3.7. Structural signal chondroitin sulfate obtained by ^{1}H-NMR spectrum with proton chemical shift from 0.0ppm ÷ 5.2ppm

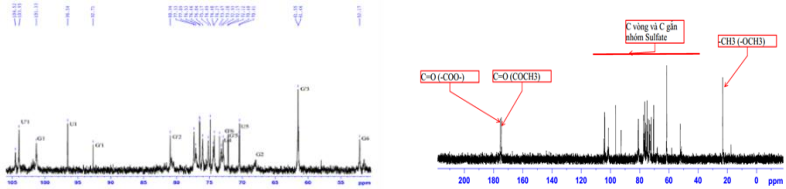

Figure 3.8. Structural signal chondroitin sulfate obtained by ^{13}C-NMR spectrum with proton chemical shift from 0.0ppm ÷ 200ppm

Table 3.5. Table of mutant signals of H/C linked on HSQC spectrum

H/C	GlcA (U)		GlcA (U')		GalNAc-6S (G)		GalNAc-4S (G')	
H1/C1	4.63/96.54	**U1**	4.46/103.95	**U'1**	4.59/101.33	**G1**	5.23/92.71	**G'1**
H2/C2	3.24/74.40	**U2**	3.41/76.46	**U'2**	4.01/68.23	**G2**	3.78/80.94	**G'2**
H3/C3	3.45/77.33	**U3**	3.65/77.33	**U'3**	3.94/76.04	**G3**	3.78/61.45	**G'3**
H4/C4	3.38/72.93	**U4**	3.89/74.89	**U'4**	4.73/77.09	**G4**	3.61/74.27	**G'4**
H5/C5	3.39/70.41	**U5**	3.71/75.17	**U'5**	3.78/73.20	**G5**	3.56/72.12	**G'5**
H6/C6					4.01/52.17	**G6**	3.87/72.17	**G'6**
H/CH3					2.02/23.18	**G7**	2.02/23.18	**G'7**

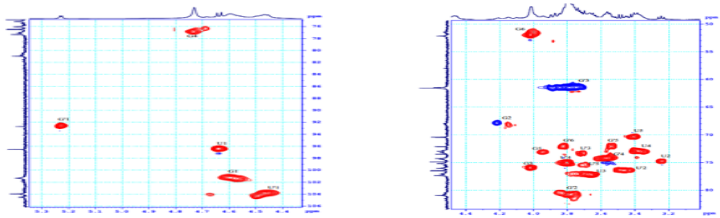

Figure 3.9. Signals of chondroitin sulfate structure by HSQC spectrum based on proton chemical shift of H from 3.1 ÷ 4.5ppm and C from 50 ÷ 85ppm

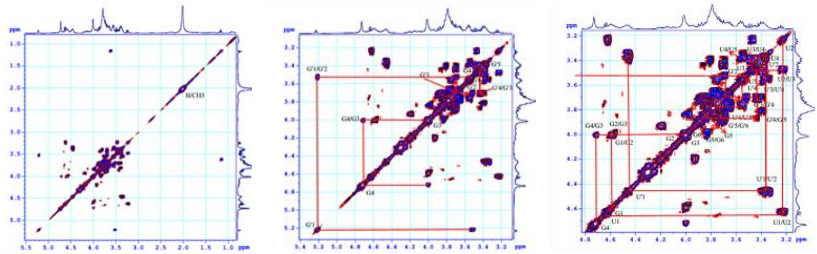

Figure 3.10. Signals of chondroitin sulfate structure by COSY spectrum based on proton chemical shift of H from 3.1 ÷ 4.5ppm and C from 50 ÷ 85ppm

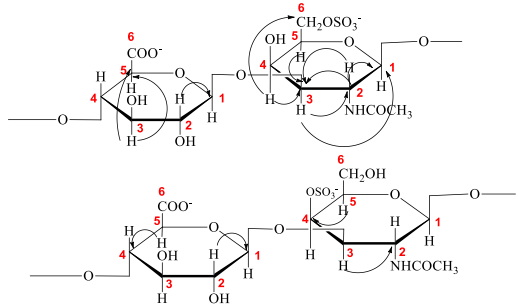

Figure 3.11. The structure of chondroitin sulfate was obtained by HMBC spectroscopy

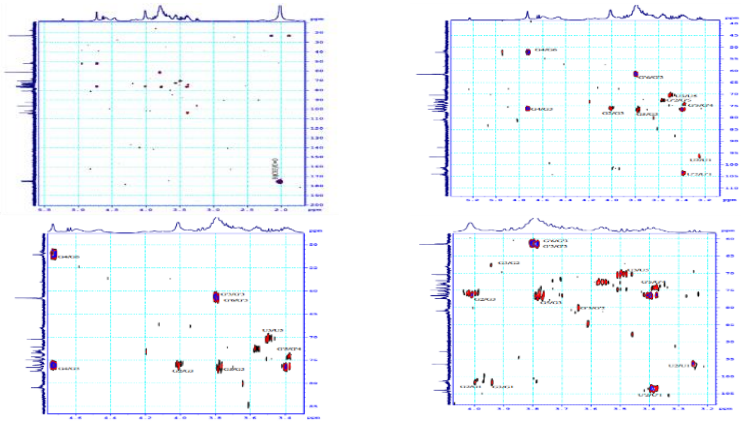

Figure 3.12. The structure of chondroitin sulfate was obtained by HMBC spectroscopy

From analysis results of 1H-NMR spectrum. 13C-NMR spectrum. HSQC spectrum. COSY spectrum. HMBC spectrum can confirm the structure of chondroitin sulfate from shark cartilage (*C. dussumieri*) is a mixture of 2 isomers. consists of chondroitin - 4 - sulphate (CSA) (GlcA-GalNAc-4S) with characteristic structural fragment GlcA-GalNAc-4SO3- and chondroitin - 6 - sulphate (CSC) (GlcA-GalNAc-6S) with structural piece Typically GlcA-GalNAc-6 SO3- (Figure 3.11)

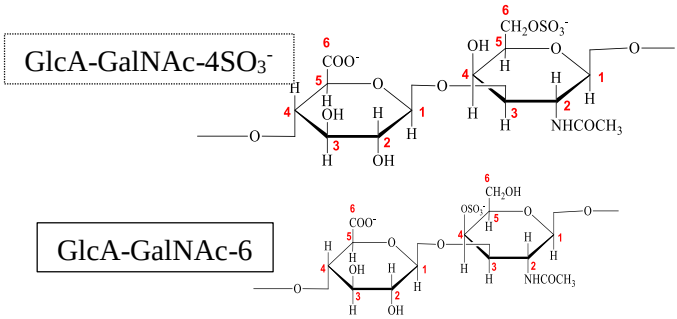

Figure 3.13. The structure of chondroitin sulfate is present in the hydrolyzed protein powder from shark cartilage

3.3. STUDY ON CHONDROITIN SULFATE HYDRAULIC SPRAY POWDER

3.3.1. Determine the suitable carrier for the spray drying process

Spray-drying to create hydrolyzed protein powder from shark cartilage hydrolyzate with different gills: maltodextrin. gum arabic. saccharose. After spray-drying. protein powder product was collected and samples were taken to determine total nitrogen content. chondroitin sulfate content. product recovery performance. The analysis results showed that maltodextrin is a suitable carrier for the spray drying to create protein powder containing chondroitin sulfate and other ingredients in shark cartilage hydrolysis.

3.3.2. Determination of the proportion of maltodextrin added to performance of chondroitin and protein powder recovery

The results of spray drying with shark cartilage solution at

temperature 80°C. drying chamber pressure 2.5bar. input speed 12ml/min. with different rate of used maltodextrin showed the rate of additional maltodextrin 12% is appropriate.

3.3.2. Determine the appropriate carrier ratio for the spray drying process

The shark cartilage hydrolysate was spray-dried at the temperature of the drying chamber at 80°C. the drying chamber pressure was 2.5bar. the input speed was 12ml/min. the rate of maltodextrin used was different: 8%. 10%. 12%. 14%. 16%. After spray drying. sampling for the recovery efficiency of chondroitin sulfate and protein powder showed that the appropriate supplemental maltodextrin ratio was 12%.

3.3.3. Determine the appropriate drying chamber air temperature

Results of spray drying hydrolyzed protein solution from shark cartilage at the pressure of the drying chamber 2.5bar. input speed 12ml/min. the rate of additional maltodextrin carrier 12% and the temperature of the drying chamber changed in the range: 70^0C ÷ 90^0C shows that the 80^0C drying chamber temperature is suitable for spray drying to create a protein powder containing chondroitin sulfate from shark cartilage hydrolysis.

3.3.4. Determine the input pump speed

Conducting the spray drying of hydrolyzed protein solution from shark cartilage according to the selected appropriate parameters with the input speed changing in the range of 10ml/min ÷ 14ml/min shows that the input pump speed of 12 ml/min is appropriate.

3.3.5. Determine the pressure of the drying chamber

From the analysis of the spray drying process to create protein powder from shark cartilage hydrolysis according to selected appropriate parameters with the drying chamber pressure changing in the range of 2.1bar ÷ 2.9 bar shows the drying chamber pressure 2.5 bar is suitable.

3.3.6. Optimized spray drying to produce protein powder containing chondroitin sulfate from shark cartilage hydrolysis

Optimizing the spray drying process to create protein powder containing chondroitin sulfate from shark cartilage hydrolysis according to the rotation center planning method (Figure 3.12) determines the optimal parameters: drying chamber temperature is 80^0C. ratio additional maltodextrin was 12%. infusion rate 12 ml/min. From these optimal parameters. the thesis has conducted a trial production of hydrolyzed protein powder and determined the spray-drying efficiency calculated according to the chondroitin content of 87.81% compared with the chondroitin sulfate content in the hydrolyzate. The corresponding chondroitin sulfate content in protein powder is 203 mg/g and total nitrogen content is. 50 mg/g powder.

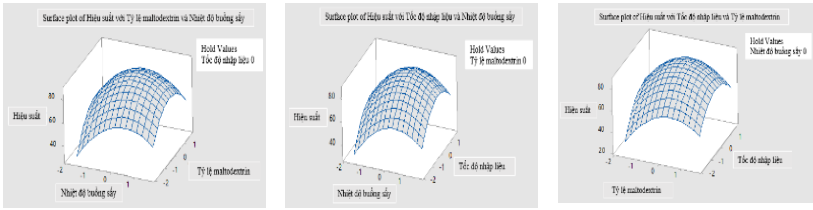

Figure 3.14. The response surface showed the correlation between drying chamber temperature (X4). maltodextrin ratio (X5) and input speed (X6) to high chondroitin sulfate collection efficiency

3.3.7. Proposing the production process of hydrolyzed protein powder containing chondroitin sulfate from the shark cartilage

*** Proposal for the production of protein powder containing chondroitin sulfate from shark cartilage**

From the above research results. it allows to propose the production process of protein powder containing chondroitin sulfate from shark cartilage presented in Figure 3.13.

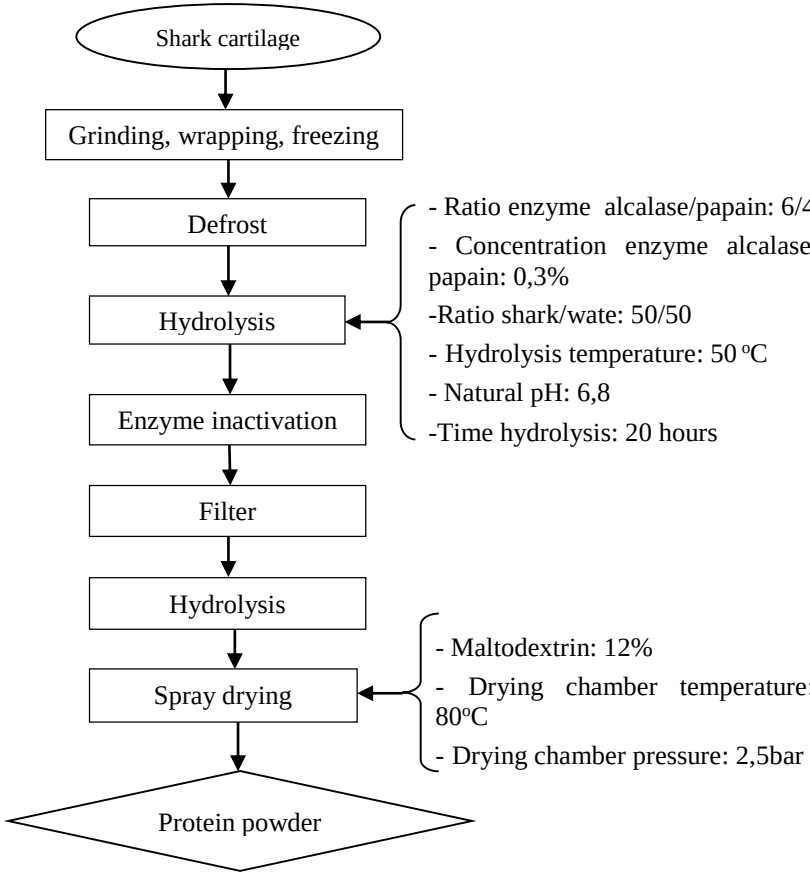

Figure 3.15. Diagram of production process for hydrolyzed protein powder containing chondroitin sulfate from shark cartilage

*** Descripting the process**

+ Shark cartilage: shark cartilage with: Content of chondroitin sulfate: 41.77 ± 0.21mg / g. total ash content: 4.2 ± 0.11g / 100g. total nitrogen content: 2.64 ± 0.02g / 100g. humidity 80.7 ± 1.12%

+ Grind. pack and freeze: shred shark cartilage by a meat grinder to homogenize the sample and pack with PA 2kg/bag. vacuum 100%. freeze and freeze at -20^{0}C to used throughout the research process.

+ Thawing: Before hydrolysis. conduct quick thawing in 2 minutes in the microwave.

+ **Hydrolysis**: Carry out hydrolysis of shark cartilage by a mixture of enzymes alcalase - papain in hydrolysis tanks according to the following specifications: The ratio of enzymes alcalase/papain is 6: 4; The concentration of alcalase-papain enzyme mixture is 0.3%. cartilage/water ratio is 50/50. hydrolysis temperature 50^{o}C. hydrolysis at natural pH (6.8) for 20 hours.

+ **Enzyme Inactivation:** Finish the hydrolysis process. conduct enzyme inactivation by boiling hydrolyzate for 10 minutes**.**

+ **Filtering:** pre-filter the hydrolyzed mixture through 2 layers of filter cloth to collect the crude filtrate. Then. continue to filter the crude filtrate through a vacuum filtration system with ceramic core size allowing substances to pass through with sizes less than 5μm to collect clear filtrate. The filtrate is boiled for 10 minutes and stored in the refrigerator cooler (4 ± 1^{0}C) for use in the analysis and research of spray drying.

+ **Spray-drying**: spray drying to create protein powder containing chondroitin sulfate with additional maltodextrin ratio of 12%. drying chamber temperature 12^{0}C. input speed of 12ml/min. The resulting protein powder has a bright yellow color.

*** Evaluation of the quality and thermal properties of protein powder containing chondroitin sulfate**

Test production of protein powder containing chondroitin sulfate according to the process proposed above and evaluate the quality and thermal properties of protein powder. The results presented in table 3.6 ÷ 3.7 and figure 3.16.

Table 3.6. Results of some chemical parameters of hydrolyzed protein powder

No	Chemical properties	Unit	Results
1	Chondroitin sulfate	mg/g	203
2	Total nitrogen	g/100g	5.04
3	Total ash	g/100g	3.95
4	Humidity	%	4.27

Table 3.7. Results of analysis of some microbiological parameters of protein powder containing chondroitin sulfate

No	Chemical properties	Unit	Results	Regulations QCVN 4 - 16: 2010/BYT
1	Total number of aerobic microorganisms	CFU/g	10	< 5000
2	*Salmonella*	CFU/g	Neg	Negative
3	*Coliforms*	CFU/g	Neg	Negative
4	Total number of spores of yeasts. molds	CFU/g	Neg	< 500

(Neg: not detected)

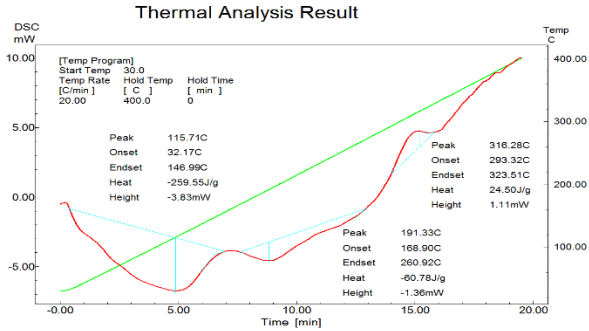

Figure 3.16. Schematic diagram of differential thermal analysis of protein powder containing chondroitin sulfate

From the above analysis. it shows that the protein powder containing chondroitin sulfate. produced according to the process of the thesis. is of good quality and meets the microbiological requirements according to current regulations of the Ministry of Health. Thus. protein powder containing chondroitin sulfate has been produced to meet the quality standards for use in the production of functional foods.

CONCLUSION AND RECOMMENDATION

1. CONCLUSION

From the above research results. it shows that the dissertation has completed all the set contents and objectives. The conclusions drawn from the research results are presented as follows:

1) The optimal parameters for the hydrolysis of shark cartilage were determined: a mixture of the enzyme alcalase-papain in the ratio 60/40. with a mixed concentration of 0.3%; The ratio of raw materials: water is 1/1; suitable pH is 6.8; The hydrolysis temperature was 50oC for 20 hours. The recovery efficiency of chondritin sulfate was 96.97%. Hydrolyzed protein solution has nutritional energy: 20.42 Kcal/100 ml. chondroitin sulfate content: 40.5 mg/ml. total N: 7.46 g/l. total mineral: 3.02 g/ l. Zn: 7.63 mg/l. Mg: 205 mg/l. Fe: 4.78 mg/l. 18 amino acids and meeting food safety standards according to current regulations of the Ministry of Health.

2) Determined the characteristic structure that preserves both C4 and C6 sulfate radicals of chondroitin sulfate products. The product is a mixture of 2 isomers GlcA-GalNAc-4SO_3^- and GlcA-GalNAc-6SO_3.

3) The optimal parameters for spray drying to create chondroitin sulfate-containing protein powder from shark cartilage hydrolysate were determined: 12% maltodextrin carrier. drying chamber temperature 80^{o}C. flow rate 12 ml/min and pressure. drying chamber capacity 2.5bar. The recovery efficiency of chondroitin sulfate was 87.81%. The product has chondroitin sulfate content of 203mg/g. total nitrogen 5.04g/100g. total ash 3.95g/100g. moisture 4.27% and meets microbiological standards for food use as prescribed. of the Ministry of Health.

4) Determining the production process of hydrolyzed protein powder containing chondroitin sulfate from fresh shark cartilage hydrolyzate by protease at experimental scale. Protein powder products containing chondroitin sulfate meet food hygiene and safety standards according to current regulations of the Ministry of Health.

2. RECOMMENDATION

From the research results above. the following recommendations are suggested:

1) Researching on completing the production of hydrolyzed fluid and hydrolyzed protein powder containing chondroitin sulfate from shark cartilage by hydrolysis using alcalase-papain enzyme mixture on a large scale needs to be continued to calculate costs for product commercialization.

2) It is necessary to continue to study on evaluate the peptides in shark cartilage hydrolysis to know their biological activity on human health.

3) Application of hydrolyzate and protein powder containing chondroitin sulfate needs to be deployed in the production of functional foods to support osteoarthritis treatment

LIST OF AUTHOR'S PUBLISHED WORKS RELATED TO THE THESIS

1. Dinh Huu Dong. Vu Ngoc Boi. Nguyen Thi My Trang (2020). "Effects of hydrolysis time and enzyme type on the hydrolysis of shark cartilage (*Carcharhinus dussumieri*) by protease". *Journal of Agriculture and Rural Development*. Ministry of Agriculture and Rural Development. No. 382. Issue 7 (4.2020). pp. 96-102 (ISSN 1859-4581).

2. Dinh Huu Dong. Vu Ngoc Boi. Nguyen Thi My Trang (2020). "Effect of the ratio between enzymes alcalase and papain on the hydrolysis of shark cartilage (*Carcharhinus dussumieri*)". *Journal of Agriculture and Rural Development*. Ministry of Agriculture and Rural Development. No. 387. Issue 12 (6.2020). pp. 60-66 (ISSN 1859-4581).

3. Dinh Huu Dong. Vu Ngoc Boi. Nguyen Thi My Trang (2020). "Effects of enzyme concentration and time on the hydrolysis of shark

cartilage (*Carcharhinus dussumieri*)". *Journal of Fishery Science and Technology*. Nha Trang University. No. 2.2020. pp. 10-19 (ISSN 1859-2252).

4. Dinh Huu Dong. Vu Ngoc Boi. Nguyen Thi My Trang (2020). "The effect of some parameters on the drying process of spraying aquatic protein solution from shark cartilage (*Carcharhinus dussumieri)". Journal of Fishery Science and Technology*. Nha Trang University. No. 3.2020. pp. 10-22 (ISSN 1859-2252).

5. Dinh Huu Dong. Vu Ngoc Boi. Nguyen Thi My Trang. Nguyen Anh Tuan (2020). "Optimization of the shark cartilage hydrolysis spray drying process (*Carcharhinus dussumieri)". Journal of Fishery Science and Technology*. Nha Trang University. No. 3.2020. pp. 112-122 (ISSN 1859-2252).

6. Dinh Huu Dong. Vu Ngoc Boi. Nguyen Thi My Trang (2021). "Optimization of shark cartilage hydrolysis (*Carcharhinus dussumieri) with alcalase-papain enzyme mixture according to responsive face method". Journal of Agriculture and Rural Development*. Ministry of Agriculture and Rural Development. No. 5. Issue 1 (3.2021). (ISSN 1859-4581).