Extraction of chondroitin sulfate rich powder from chicken trachea

Wipaporn Seena
Farrin Sanmuang

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Wipaporn Seena Student ID 59080047

Farrin Sanmuang Student ID 59080198

Approved by

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(Asst. Prof. Dr. Sitthipong Nalinanon)

Advisor

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Student name Wipaporn Seena Student ID 59080047

Farrin Sanmuang Student ID 59080198

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Advisor Asst. Prof. Dr. Sitthipong Nalinanon

Abstract

This research was aimed to isolate the chondroitin sulfate (CS) from chicken trachea, a byproduct from chicken processing, by 3 extraction methods including Enzymatic hydrolysis method, Simplified enzymatic hydrolysis method and Alkali extraction method. The results showed that the use of papain for CS extraction resulted in the degradation proteins in the sample effectively. The best condition for CS extraction was the Simplified enzymatic hydrolysis method, using 0.5 % papain to gain the yield of 17.19%. The chondroitin sulfate rich powder (CSP) were successfully obtained by those 3 CS extraction methods, but somehow high amount of protein still remained. In addition, the higher amount of papain used resulted in higher yield of CSP ($p<0.05\%$). Alkaline extraction method could also use to prepare the CSP the but lower yield of extraction was obtained, compared to those papain hydrolysis methods. Therefore, CSP could be effectively prepare by papain hydrolysis. However, the purification and application of the prepared chondroitin sulfate should be further studied.

Keywords: Chondroitin sulfate, Chicken trachea, Extraction, papain

หัวข้อปัญหาพิเศษ	การสกัดผงที่อุดมไปด้วยคอนดรอยตินซัลเฟตจากหลอดลมไก่
ชื่อนักศึกษา	วิภาพร สีนา รหัสนักศึกษา 59080047
	ฟ้ารินทร์ แสนเมือง รหัสนักศึกษา 59080198
หลักสูตร	วิทยาศาสตร์บัณฑิต สาขาวิทยาศาสตร์และเทคโนโลยีการอาหาร
พ.ศ.	2563
อาจารย์ที่ปรึกษา	ผศ.ดร. สิทธิพงศ์ นลินานนท์

บทคัดย่อ

งานวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาการสกัดคอนดรอยตินซัลเฟต (Chondroitin sulfate) จากหลอดลมไก่ ซึ่งเป็นวัสดุเศษเหลือจากการแปรรูปไก่ โดยใช้วิธีการสกัด 3 วิธี คือ วิธีการย่อยสลายด้วยเอนไซม์ (Enzymatic hydrolysis method) วิธีการย่อยสลายด้วยเอนไซม์อย่างง่าย (Simplified enzymatic hydrolysis method) และวิธีการสกัดในสภาวะด่าง (Alkali extraction method) ผลการทดลองพบว่า การใช้ปาเปนในการสกัดคอนดรอยตินซัลเฟต มีผลทำให้เกิดการย่อยสลายโปรตีนที่เป็นองค์ประกอบอยู่ในตัวอย่างได้อย่างมีประสิทธิภาพ โดยสภาวะที่ดีที่สุดในการสกัดคอนดรอยตินซัลเฟต คือ การใช้วิธีการย่อยสลายด้วยเอนไซม์อย่างง่าย โดยใช้ปริมาณปาเปน 0.5 เปอร์เซ็นต์ ซึ่งจะทำให้ได้ผลผลิตสูงถึง 17.19 เปอร์เซ็นต์ การสกัดคอนดรอยตินซัลเฟตด้วยวิธีทั้ง 3 วิธี จะทำให้ได้ผงที่อุดมไปด้วยคอนดรอยตินซัลเฟต (Chondroitin sulfate rich powder; CSP) แม้ยังคงมีปริมาณโปรตีนปะปนอยู่ค่อนข้างมาก นอกจากนี้การใช้ปริมาณปาเปนที่สูงขึ้นจะส่งผลให้ได้ปริมาณผลผลิตการสกัดคอนดรอยตินซัลเฟตที่เพิ่มสูงขึ้นด้วย($p \leq 0.05$) ส่วนการใช้วิธีการสกัดด้วยสารละลายด่าง สามารถทำให้เกิดการสกัดผงที่อุดมไปด้วยคอนดรอยตินซัลเฟตได้เช่นเดียวกัน แต่ทำให้ได้ปริมาณผลผลิตที่ต่ำกว่า เมื่อเทียบกับการใช้ปาเปน ดังนั้นวิธีการการใช้ปาเปนย่อยสลายจึงมีประสิทธิภาพในการเตรียมผงที่อุดมไปด้วยคอนดรอยตินซัลเฟต อย่างไรก็ตามควรมีการศึกษาการทำบริสุทธิ์และประยุกต์ใช้คอนดรอยตินซัลเฟตเพิ่มเติม

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Wipaporn Seena

Farrin Sanmuang

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Chapter 1
Introduction

1.1 Introduction

Thailand is one of the world's top ten exporters for poultry meat products (The World Factbook, 2019). Office of Agricultural Economic (OAE) reported the fleet growing of the poultry meat industry, in which broiler are the major products (Office of Agricultural Economic, 2019). Chicken trachea is a by-product discarded from chicken processing containing connective tissue, particularly cartilage as a main composition (Ihyc, 2001). Cartilage is composed of collagen, else proteins and glycosaminoglycans, mostly chondroitin sulfate (Garnjanagoonchorn et al., 2007; Vazquez et al., 2013). The glycosaminoglycan provenance is composed of three purviews of chondroitin sulfate, glycoproteins and proteoglycans. Chondroitin sulfate composed of chains of disaccharide unit of N-acetyl D-galactosamine and D-glucuronic acid (Iozzo, 1998). Chondroitin sulfate has been categorizing to chondroitin 4-sulfate and chondroitin 6-sulfate (Schiller and Huster, 2012). Chondroitin sulfate is a connective and fundamental in biological processes such as elasticity, structural, maintenance in the bone. In addition, chondroitin sulfate is also in gaily desire for its applications in tissue engineering, pharmaceutical, cosmetic and food industries (Rani et al., 2017). Moreover, tale documented areas of interest are chondroitin sulfate potential use as antiviral and anti-infective, and in tissue incarnation, biological function in cancerous cells and tissues is habitual (Bianchera et al., 2014) so chondroitin sulfate are appealing materials for biomedical and engineering applications. As one of the most rapidly growing nutraceutical supplements in many countries, chondroitin sulfate is used extensively as a treatment for Osteoarthritis in humans and companion animals (Gottlieb, 1997). Although common sources of chondroitin sulfate include broiler chicken carcasses (Nakano et al., 2012; Srichamroen et al., 2013), duck trachea (Vittayanont and Jaroenviriyapap, 2013) and chicken keel cartilage (Luo et al., 2002; Khan et al., 2013) It is also gainful to explore other sources of chondroitin sulfate, i.e. chicken trachea as a potential source. The Extraction of chondroitin sulfate by digestion with proteolytic enzymes is the most ordinary procedure for releasing chondroitin sulfate from tissues. Commonly, extensive proteolysis with a protease of large specificity is advisable, and realization with papain or pronase yields sole of CS chains with only small residual peptide (Papain enzyme is generally very practical in attain complete solubilization of various tissues). (Medeiros et al., 2000; Rocha et al., 2000). This study, therefore, was conducted to investigate the simple techniques for isolation of glycosaminoglycans containing chondroitin sulfate from chicken trachea in broiler chicken by-product.

1.2 Purpose of Research

1. To prepare chondroitin sulfate rich powder (CSP) from chicken trachea.

Chapter 2

Literature review

2. Literature Review

2.1 Poultry industry

Over the past 50 years, in the modern era of the poultry meat industry, the production of poultry meat for global consumption has been steadily growing, especially chicken and duck meat (Mead, 2004).

Production is estimated at 85-90 million tons per year for global chicken products. Thailand is one of top ten world exporting countries for poultry products, accounting for about 2% or amount 2.0-2.1 million tons of exports worldwide. Between 2018 and 2020 It is expected that the demand for Thai frozen and processed chicken from foreign customers will increase (Figure 2.1). Most domestic consumption is raw chicken, which accounts for 60% of the total (approximately 1.2-1.3 million tons). The rest 40% of Thai products are mainly for export and some for local stores (Chuasuwan., 2018).

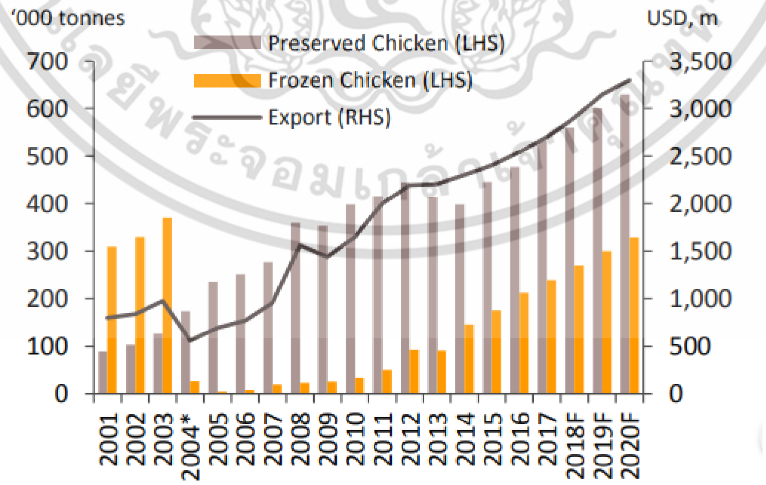

Figure 2.1 Forecast Thai chicken exports

Source: Chuasuwan (2018).

Chicken meat is a good source of nutrition. The research reported that raw chicken meat had the average components of water, protein, fat, and ash as follows 71.1, 19.8, 7.5 and 1.6%, respectively. However, the breed, raising system and sex of meat are factors that cause nutritional differences. Significant variation in the amount of fat may be caused by different sampling sites. Different fat sources, such as muscle position or fat under the skin (Demby et al., 1980).

2.2 Chondroitin sulfate

2.2.1 Structure of Chondroitin sulfate

Chondroitin sulfate (CS) is one of the Glycosaminoglycans (GAG). It is an important component extracellular matrix (ECM) of cartilage, skin, bone, tendons, ligaments and connective tissues. It contains N-acetyl-D-galactosamine and disaccharide units of sulfated D-glucuronic acid (Figure 2.2). It is attached covalently to core proteins in a form of proteoglycan (Figure 2.3). The important biochemical properties of chondroitin sulfate are resistance and elasticity of cartilage (Henrotin et al., 2010).

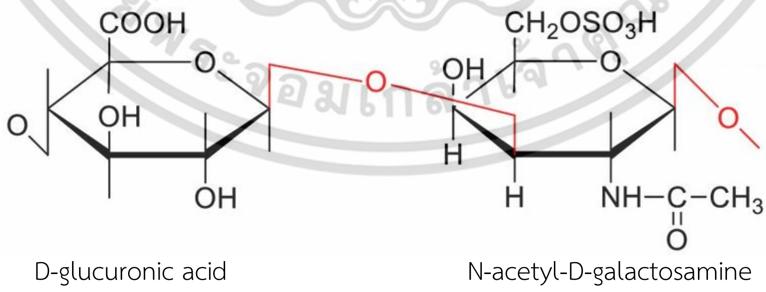

Figure 2.2 Chemical structures of units of sulfated D-glucuronic acid and N-acetyl-D-galactosamine.

Source: Beldowski (2017).

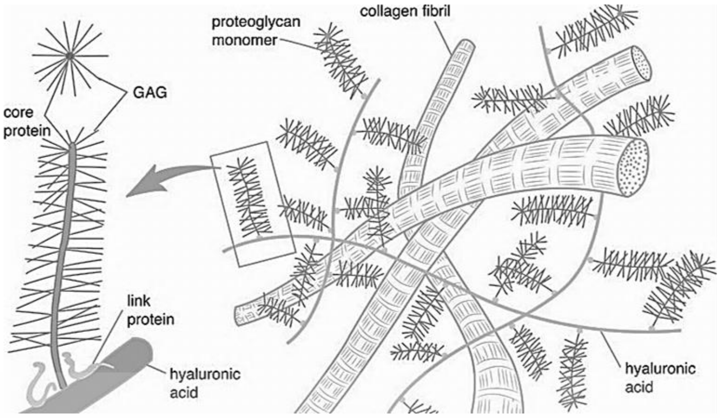

Figure 2.3 Proteoglycan monomer and aggregate.

Source: Ross and Pawlina (2007)

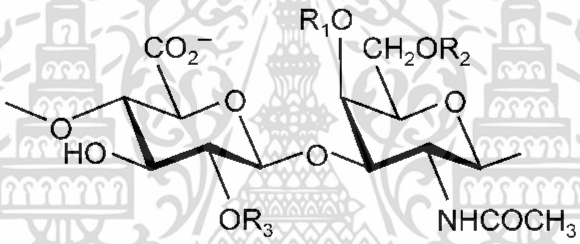

Figure 2.4 Structures of disaccharides forming chondroitin sulfate. R1 = R2 = R3 = H: non-sulfated chondroitin. R1 = SO3−; R2 = R3 = H: chondroitin-4-sulfate; R1 = R3 = SO3−; R2 = H: chondroitin-2,4-disulfate; R2 = SO3−; R1 = R3 = H: chondroitin-6-sulfate; R2 = R3 = SO3−; R1 = H: chondroitin-2,6-disulfate; R1 = R2 = SO3−; R3 = H: chondroitin-4,6-disulfate; R1 = R2 = R3 = SO3−: tribulated chondroitin.

Source: Henrotin (2010).

The best arrangement with aggrecan, the major load-bearing proteoglycan of cartilaginous tissues. The ~300 kDa core protein is represented with ~ 100 chondroitin sulfate and in some species, keratan sulfate glycosaminoglycans chains. It's linked non-covalently with hyaluronan and the ~ 45 kDa associates glycoprotein to form high molecular weight aggregates (> 200 MDa) (Wierzcholski, 2016). In cartilage, these are densely collected. It is used in dietary supplements as an alternative medicine to treat osteoarthritis (Michael, 2008).

2.2.2 Chemical composition of chondroitin sulfate

The composition of chondroitin sulfate from porcine, bovine, chicken, shark and skate cartilage were analyzed by Volpi et al. (2007). The reported that molecular weights of chondroitin sulfates (14–26 kDa) from porcine, bovine and chicken were similar to the reference standard (21.4 kDa), but the molecular weight of chondroitin sulfates from shark and skate (50–70 kDa) were substantially higher (Volpi, et al., 2010). Not astonishingly, The proportion of chondroitin-6-sulfate (33%) and chondroitin-4-sulfate (61%) from cows had approximately same as standard (34% and 61%, respectively), but chondroitin-4-sulfate from porcine and chicken had higher than those (80% and 72%, respectively), while chondroitin-6-sulfate from shark and skate samples had higher than those (50% and 39% respectively). In addition, chondroitin sulfates from skate and shark had 15% and 18%, respectively, compared with 0% of chondroitin sulfates from other samples and the standard (Volpi et al., 2007). Tat et al. (2010) paralleled three formulations of chondroitin sulfate from different source. As shown in the table 2.1, chondroitin sulfate molecular weight, protein content, content and sulfation from different products. In this experiment, they found that the CS1 had the lowest molecular weight and the freest sulfates (0.75%), which indicates desulfation and depolymerization during processing. Sakai et al. (2003) were studied similar discrepancies in 12 chondroitin sulfate components. They found that the element it has different amounts and patterns of sulfation and a wide range of molecular weights : chondroitin-4,6- disulfate 0%–3%, chondroitin-2,6-disulfate 0%–13%, chondroitin-6-sulfate 23%–63%, chondroitin-4-sulfate 26%–70% and non-sulfated chondroitin 1%–9% (Sakai et al., 2003). Malaviki et al. (2008) were studied quantify chondroitin sulfate concentrations by capillary electrophoresis. They found that the disaccharide content of 11 commercially available formulations;

hyaluronic acid 0%–1.5%, chondroitin-4,6-disulfate 0%–13%, chondroitin-2,6-disulfate 0%–5%, chondroitin-2,4-disulfate 0%–8%, chondroitin-6-sulfate 11%–58%, chondroitin-4-sulfate 23%–85% and non-sulfated chondroitin 2%–6%. Those have a density of 0.95 to 1.07.

Table 2.1 Chondroitin sulfate characteristics.

Characteristic	CS1	CS2	CS3
Species	Porcine	Bovine	Bovine
Chondroitin sulfate content (%)	90.4	96.2	99.9
Molecular weight [a] (kDa)	12.9	13.8	15.1
Protein (%)	7.4	3.3	ND
Intrinsic viscosity	0.034	0.036	0.040
Chlorides (%)	0.70	0.02	0.34
Free sulfates (%)	0.75	0.05	0.14
Oxalate (%)	0.021	ND	0.01
Sodium (%)	7.10	6.75	7.05
Non-sulfated chondroitin (%)	5.9	5.1	5.7
Chondroitin-4-sulfate (%)	78.3	72.7	62.8
Chondroitin-6-sulfate (%)	15.8	21.3	31.5
Chondroitin-2,6-disulfate (%)	ND	ND	ND
Chondroitin-4,6-disulfate (%)	ND	ND	ND

[a]: Indicates average molecular weight. CS, chondroitin sulfate; ND, not detected.

Source: Tat et al. (2010).

2.2.3 Applications of chondroitin sulfate

The use of chondroitin sulfate, such as the maintenance of knee osteoarthritis and helping to alleviate anti-inflammatory symptoms. On this topic, there are interesting studies, including chondroitin sulfate has also been used as an antiviral drug. Infection resistant and used to build tissue In addition to interesting applications, biological work in cancer cells and tissues is created. Therefore, the application of chondroitin sulfate is a biomarker foreseen (Schiraldi et al., 2010).

Chondroitin Sulfate has anti-inflammatory effect shown inhibition in vitro synthesis of various inflammatory mediators such as nitric oxide cyclooxygenase (COX), (NO) synthase, microsomal prostaglandin synthase (mPGES) and prostaglandin (PG). Chondroitin sulphate's anti-inflammatory effects suggest that chondroitin sulfate bodies are reduced in synovitis in Rheumatoid arthritis caused by mouse (Henrotin et al., 2010).

The method of preparation Chondroitin Sulfate has the effect of reducing the healing process. In particular, Gilbert and his group (2004) based on the results of animal models at chondroitin sulfate-hydrogels (CS-ADH / poliethilene glycol dialdehyde) increase the efficiency of wound healing in the sinonasal lining of the agent's function. Extracellular Matrix in which the hydrogel is also able to provide storage space in cytokines and growth factors which is produced during the creation of new tissues. Other research trials that applied hyaluronic acid (gelatin - C6S - HA) bilayer chondrointin-6-sulfate gelatin (Wang et al., 2006) which show that saw the ability to use this form to overcome skin scarcity by raising In vitro keratinocytes and dermal fibroblasts (Wang et al., 2006). This substituted gelatin bilayer - C6S - HA is insufficient. But increases wound healing there is still a high rate of bribes. From the proposed examples that apply to medical treatment which was burned widely and deep as a result of severe CS-C defects found in this study to improve the healing of palate ulcers. By controlling cell adhesion by cell multiplication and cell movement (Zou et al., 2004). The introduction of chondroitin-6-sulfate Used to help with cell adhesion increases and increased amounts, which are intertwined with the use of the enzyme chondroitin sulfate (Schaller, 2001). In the study of bio-chondroitin sulfate found that the effect Will depend on the status and position of the structure of the sulfate group. However, inhibiting GAG Sulfation from chlorate treatment results in reduced cell adhesion and increased cell numbers, which reduces the rate of wound closure in the experiment. The researchers suggest that the combination of sulfate types in chondroitin different sulfates may improve the results from wound healing compared to successful with chondroitin-6-sulfate (Volpi, 2009). Another research by Zou et al. (2009) shows that CS-C-rich polysaccharides help increase cell numbers and adhesion while at CS-A duplicate distribution promotion units but inhibiting adhesion. Another interesting research is the application of chondroitin sulfate in the repair mechanisms of the central nervous system. It was found that monosodium supplied and disaccharide acted as an effective inhibitor of the formation of the amyloid fibers caused by GAG (Fraser et al., 2001). In addition, recent research has found that chondroitin-derived proteolytic degradation products promote the growth of axons increase microbial activation and control of T-cell functions (Rolls et al., 2004) CS / DS. High sulfate It is found that it affects the growth of hepatic cells bound to heparin which is important for the development of liver cells in liver regeneration (Mitchell et al., 2005). The domain may be useful for development. Therapy for regeneration of the liver from the regulation of liver tissue physiology (Toyoki et al., 1997, Yamada and Sugahara, 2008).

2.2.4 Chondroitin sulfate production in past, present and future

The main issues relating to the success and suitability of chondroitin sulfate and oligosaccharides Received because the principles of medication used in the treatment are unique and quality Potential contaminants include cell proteins used as the source of organic solutions used in downstream processes and the use of small organic molecules extracted from tissues. In addition, it is used as an ingredient in the purification process (Schiraldi et al., 2010). For example, using only 10% less chondroitin sulfate in some categories (dietary supplements) causes poor quality products. Experiments may find a low-cost type of chondroitin bronchial replacement for extraction from relatively expensive sharks. From the structure of chondroitin sulfate, depending on the source of the extracted sample and its function, which may vary according to the structure type of the stem. Quality and low quality identities should be improved. By determining the dosage and source of chondroitin sulfate in the product The lack of this important information may lead to poor interpretation of experimental studies, and therefore the development of new treatment applications. Today, chondroitin sulfate, similar to heparin, is extracted and purified from animal tissues (Sumi et al. Therefore, the source material, the production process, the presence of contaminants and other factors related to the biological and pharmacological activities of all living things Vol Vol (2010) reports that the development of chondroitin is of high quality and meets standards. It is in line with production goals, where strict regulations from the production process are in good practice (GMP). On the other hand, the quality of chondroitin sulfate used in dietary supplements is poor (Volpi, 2009). In natural products is the heart and is the factor used to guarantee the quality and safety of the application. Especially for chondroitin sulfate derived from a variety of sources such as sharks, ducks, birds and cows, resulting in relatively low quality chondroitin sulfate products (Schiraldi et al., 2010). Luo et. L (2002) reports that chondroitin sulfate can be recovered and Purification from cartilage of chicken keel, yielding 30% by dry weight They may also find that chondroitin sulfate receptors from fish cartilage (Tadashi, et al., 2005). Figure 2.5 shows the extraction process from this experiment, explaining the process that can reduce costs in terms of time and price. For the production of chondroitin sulfate from the use of an improved process compared to the original process using special filtration and purification methods (Schiraldi, in particular, the extraction is carried out by alkaline and enzyme pretreatment, after which it is precipitated with alcohol and is continuously restored to solid state by centrifuge in the production of chondroitin sulfate. Is the best quality raw material in the market (From the purity level Type of sulfate) and problems with the use of shark fin cartilage as the main source of chondroitin sulfate that has an ecological effect and is resistant to current use (such as Bioiberica, SABarcelona, Spain; Ching Kangyuan, Chinese medicine) (Schiraldi et al., 2010). Therefore, the use of sharks may result in the extinction of sharks claimed by animal rights activists and the use of shark fins as components that Important used in the traditional way In addition, the use of animal biopolymers is human. Treatment is hindered by the risk of spreading the virus between strains and / or prionic contamination (such as Bovine Spongiform Encephalopathy (BSE) and

Epizootic Aphtha) from the above problems along with the demand for chondroitin sulfate in the market of the industry. Food, cosmetics, dietary supplements and additions have led to more research. Towards the discovery of new sources for achieving the production of this molecular biotechnology, Schiraldi et al., 2010) that can be used in the future.

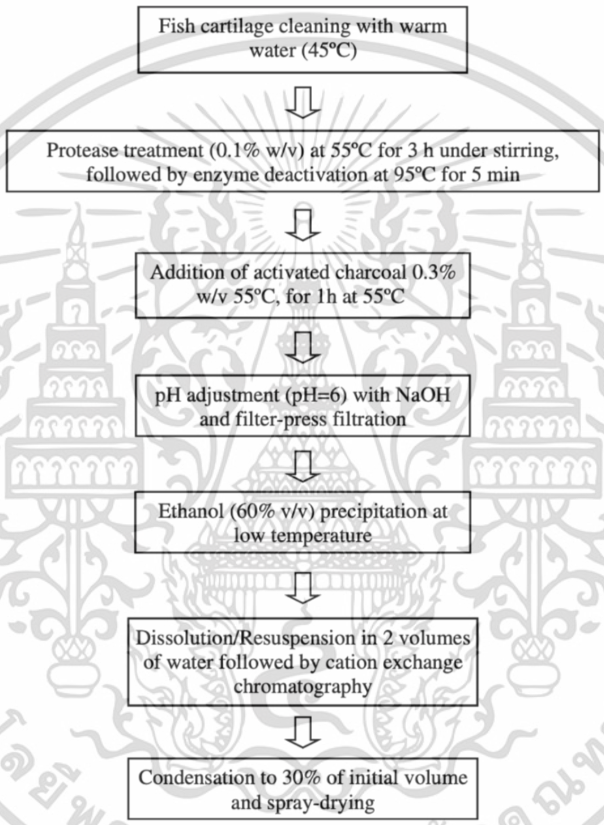

Figure 2.5 Overview of downstream purification process performed to extract chondroitin sulfate

Source: Tadashi (2005).

2.3 Source of chondroitin sulfate

Chondroitin sulfate is a major component of the extracellular matrix of connective tissues. It is a component contained in the skin, bones, cartilage, tendons and ligaments (Tat et al., 2010). Cartilage of various animals can be extracted as a product for use as a dietary supplement.

2.3.1 Cartilages

The matrix of cartilage consists of fibrous tissue that is an important part of the body. It's softer, strong and more flexible than bone. Cartilage is a connective tissue in many parts of the body such as the joints between the bones (Fig. 2.6) including knees, elbows, ankles, rib ends, nose, trachea. Cartilage consist of special cells called chondrocytes that make produce of extracellular for proteoglycans, collagen fibers and elastin fibers. Blood vessels of cartilage do not actively engage in provide nutrients containing chondrocytes but the nutrients extend to the dense connective tissue surrounding the cartilage (called the perichondrium) and into the core of cartilage. Owing to lack of blood vessels, cartilage develop and restore slower than other tissues (Mandal, 2019)

Chondroitin sulfate from shark cartilage is in demand for product widespread. Shark cartilage has a high price. Other CS sources have been suggested for commercial use such as cow bones, pork bones. There are studies of extracting CS from different sources such as fish scales, sea cucumber, keel, squid, Ciona intestinalis, chicken, eggshell, cartilage of skates and crocodiles (Nakano et al., 2001; Gu et al., 1999; Luo et al., 2002; Shin et al., 2006; Park et al., 2001; Jo et al., 2004; Sumi et al., 2002; Choi et al., 2005 and Garnjanagoonchorn, et al., 2007). There is also a study of CS extraction from microorganism (Schiraldi et al., 2010).

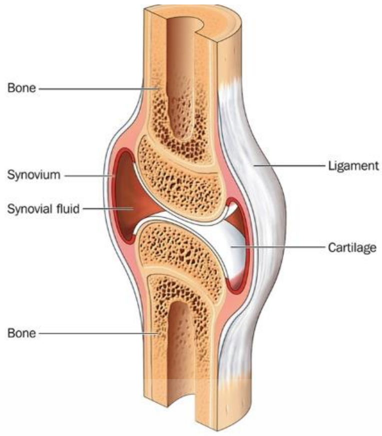

Figure 2.6 Cross section through a typical synovial joint

Source: Mandal (2019)

2.3.2 Trachea

The trachea is surrounded cartilaginous rings stacked that prevent its tumble down from the negative pressure engendered by sophistication of air. Poultry can respire through the nostrils (nares) or the jaws. Air entering these openings (during inspiration) passes through the pharynx & then into the trachea (or windpipe). The trachea is repeatedly as long as the neck (Figure 2.7). The poultry trachea is 1.29 times broad and 2.7 times longerthan that of likewise sized mammals. (Jacob, 2015)

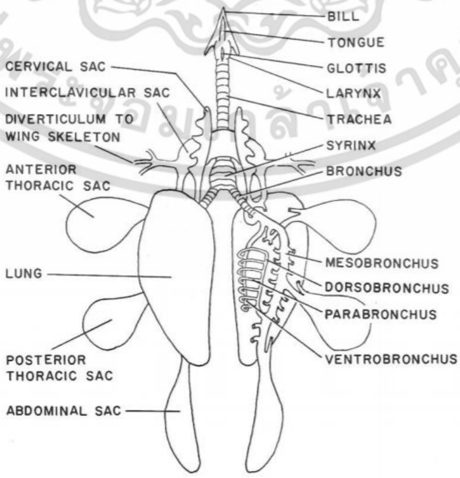

Figure 2.7 Shows the location of the chicken trachea

Source: Welty (1975).

2.4 Chondroitin sulfate extraction and purification

The extraction of CS from cartilage consists of four main steps: the first step, degradation of cartilage by chemicals; next, break-down of the proteoglycan core; then, expulsion of proteins and end with the chondroitin sulfate recovery (Shi et al., 2014).

Most of the chemical degradation and protein core destruction are use the high concentration of alkaline hydrolyzed such as cysteine, NaOH, guanidine HCl, or urea, accordingly cationic quaternary ammonium chemicals was added to the solution to precipitate GAG and removal of protein by trichloroacetic acid and end gel filtration and/or ion-exchange and size exclusion chromatography used to purify substances. Murado, 2010 studied various alternative of chondroitin sulfate isolated methods. The extraction CS from cartilage includes of degradation of cartilage and proteins with enzymes; added alcoholic solutions to precipitated protein; resuspension and neutralization with salt solutions; using ultrafiltration diafiltration technologies (UF-DF) for disconnection of molecular weight (Shi et al., 2014).

Maccari et al. (2015) extracted CS from bony fishes. Fish bones were dissolved with 100 mL of acetone to removal of fat prior to filtration and drying at 60 °C for 24 h. The pellet was resuspended in 100 mM Na-acetate buffer pH 5.5 containing 5 mM cysteine and 5 mM EDTA. Tissue was digested with papain (60 mg/g of tissue) and the solution incubated with constant stirring at 60 °C for 24 h then boiling for 10 min. The solution was centrifuged for 15 min at 5,000 g. The supernatant was precipitated by adding three volumes of ethanol saturated with sodium acetate and stored at 4 °C for 24 h.

The precipitate was centrifuged at 5,000 g for 15 min to recuperate and lyophilized at 60 °C for 6 h. The sear precipitate was resuspended in 50 mL of 50 mM sodium chloride prior to centrifuged at 10,000 g for 10 min. The samples containing CS was treated with chondroitinase ABC (or chondroitinase B) to purification samples. The unsaturated disaccharides were quantified and separated with Strong-Anion-Exchange HPLC. The results showed the extraction yield of CS content was 0.011% for cod up to 0.34% for monkfish, while chondroitin-

4-sulfate and chondroitin-6-sulfate were evaluated to be 25.3–63.2% and 27.3–55.7%, respectively.

Guo et al. (2007) extracted CS from eggshell membrane using both trypsin and both are major digestive enzymes. They reported that the optimum weight of liquid and material was 1 .5 and 1, respectively. The solution was dissolved in 5%alkali at 40 °C and 0.8% pepsin, pH 5.5 and then with 1.5%trypsin, pH 8.2 and incubated for 2 h.

Wang et al. (2019) extracted from chicken leg bone soup by the heat-resin static adsorption extraction (HSAE) method and an enzymatic extraction method. The HSAE method was optimized conditions of resin dosage, assimilation time, appropriately concentration and suitably time with 10%, 4.3 h, 2 M and 1.3 h, respectively. In this experiment, they found that the yields of CS from ends of leg bone by SAE methods and an enzymatic extraction method were 0.14% and 4.25%, respectively.

Shen et al. (2019) isolated CS using steam explosion (SE) by membrane separation from chicken sternal cartilage. Cartilage was liquefied via the SE conditions under different pressure, temperature and time. They found that the optimum conditions were the dosage of papain added, 0.11%; incubate temperature, 56.5 °C and digest time, 10.5 h, with steam at a gauge pressure of 1.4 MPa for 120 s. The highest yield of CS was 18.55%.

In addition, Wang and Tang (2009) extracted CS from shark cartilage. They compared the productivity of traditional solvent extractions with solvent-free mechanochemical extraction (SFMCE) technology. The mechanically pretreated sample and solid alkali reagent (sodium hydroxide and silicon dioxide) were added and finely ground in AGO-2 planetary activator to gain mechanochemical composites (MCs). The extraction yield of CS was increased from 8.50 ± 0.16% to 9.33 ± 0.14%.

2.5 Analytical method for Chondroitin sulfate (CS)

2.5.1 Nuclear Magnetic Resonance (NMR) spectroscopy

Non-Magnetic Resonance (NMR) is a spectroscopy technique that detects energy absorbed by changes in the state of nuclear spin. The application of NMR spectroscopy in the study of proteins and nucleic acids provides specific information about the dynamics and chemical kinetics of these systems (Ehret Henry et al., 1992). One of the key features of NMR is It provides atomic data for protein and nucleic acid changes over a very wide time period from seconds to PSs. In addition, NMR can also provide atomic structure information for proteins and acids. Nuclear solution for nuclear spectrum (NMR) has become an indispensable tool for identifying small molecules and is now widely used to classify the structure of large molecules such as proteins and acids. New nucleic

The high level of diversity of GAG polymers is caused by a variety of different types of chains and sulfates, making structural decisions a very challenging task. Various techniques have been used for this purpose, including nuclear magnetic fields (NMR). The NMR technique has been shown to be an effective tool for detailed structural data (Pomin et al., 2010).

NMR spectroscopic approach to glycosaminoglycan It has been used successfully in the past to characterize keratan sulfate oligosaccharides (such as Brown et al., 1994) and the chondroitin sulfate linkage area (Huckerby et al., 1998) of chondroitin sulfate oligosaccharides. The use of methods of destruction without destroying the analysis. glycosaminoglycans It is considered more important because the characteristic material is necessary for the performance study. Data collected from the studies of keratan sulfate oligosaccharides Is also used to interpret data obtained from chain studies (Huckerby et al., 1999; Huckerby and Lauder, 2000).

2.5.2 Differential scanning calorimeter (DSC)

DSC is a thermodynamic device for summary definition of the absorption of heat energy that arise in a sample in a given temperature increase and decrease. Calorimetry is specially used to monitor phase change (Van Holde et al., 2006). DSC is usually used in explore of biochemistry reactions, known as the transformation of a single molecule of a molecule from one structure to another. Thermal change temperature (Tt; Melting point) of the sample is determined in a solid solution or mixed phase, such as a suspension (Cooper et al., 2000). In basic DSC testing, energy is presented simultaneously in the sample cells. (Which has an interesting molecular solution) and reference cells (Only solvent) The temperature of both cells will increase as well, increasing the time that has passed. The difference in the input energy needed to match the sample temperature and the reference will be the amount of excess heat that will absorb or may be released from the molecules in the sample. (During the endothermic processes or the exothermic processes respectively) (Cooper 2004; Cooper 2000 and Cooper et al., 2001). These changes are due to the presence of more interesting molecules. Energy must be brought to the same temperature as reference for further use.

2.5.3 Fourier transform infrared (FTIR) spectroscopic analysis

The FTIR (Fourier Transform Infrared Spectrometer) is used in chemical analysis in the analysis of pharmaceuticals, food supplements and foods as well. Chromatography used to examine unstable chemical mechanisms and detection (Perkins, 1986)

The Fourier transform infrared (FTIR) spectrometers there will be an interface after the IR source and before the beam splitter. The advantage of the FTIR is the rapid scanning of the infrared spectrum with a movable glass system. The scanning interface is combined to dramatically reduce background noise. The IR instrument currently used in the analysis lab is the FTIR spectrometer alone. (Bryan et al., 2016).

FT-IR spectroscopy was used to determine the functional groups contained in chondroitin sulfate. Chondroitin sulfate is pressed into KBr granules (example: KBr, 1: 100) using the 15 Ton Hydraulic Press FT-IR spectrum of commercialDi-4S. Commercial and ΔDi-6S Commercially showing the peak of the corresponding sulfate at 856.9 cm-1 and 826.1 cm-1 respectively. Spectrum of chondroitin sulphate separation shows the highest peak of the sulfate group at 856.9 cm-1 showing polysaccharide as ΔDi-4S. (Rani et al., 2017).

2.5.4 High-performance liquid chromatography (HPLC)

High Performance Liquid Chromatography (HPLC) is a form of a chromatographic column in which a sample pump or solvent analyzer is used. (Called the mobile phase) at high pressure through the column containing the material containing chromatography The sample is carried out by a gas carrier of a helium or nitrogen carrier. HPLC has the ability to separate and identify any compound contained in the sample that can be dissolved in a liquid at low concentrations per trillion parts. With this wide range of capabilities, HPLC is used in industrial and scientific applications such as pharmaceuticals, environment, forensics and chemicals. (Bryan et al., 2016).

In the experiment, the sampling time will vary, as a result of the phase reaction, the molecule being analyzed and the solvent or solvent used. When the sample passes through the column, there will be interaction between two phases at different rates, most likely due to the different polarities in the analytes. The analysis has the smallest possible rate of interaction with stationary phase or the largest number of Interaction with the mobile phase will leave the column faster (Maccari et al., 2015). Then use HPLC to clear chondroitin sulfate from fish bones. Chondroitin sulfate is extracted from different samples that need to be tested. The most relevant structural properties commonly found with chondroitin sulfates are obtained from different samples (Shi et al., 2014).

2.6 Biocompatibility assessment of chondroitin sulfate

Chondroitin sulfate needs to be examined for application. Including experimental effects for many human uses animal studies and in vitro studies If considering the health benefits application and in the external constituents of the human body. In order to explain the biological role of chondroitin sulfate that can be used safely and appropriately (Hathcock and Shao, 2007).

2.6.1 *In vitro cell* proliferation assay

In vitro cell is a cytotoxicity and cell toxicity test kit that is widely used for chemical toxicity testing and drug screening. The application of these test kits has received increased attention in recent years. (Chrzanowska et al., 1990). Currently, these assays are used in tumor research to assess the toxicity of substances and inhibit the growth of tumor cells during drug development. Because it's fast and not expensive and doesn't want to use animals. It is also useful for testing many samples. The possibility of a cell and its cytotoxicity depends on cell functions, such as cell permeability, enzyme activity, cell attachment, ATP production, coenzyme production, and Absorption of nucleotides (Ishiyama et al., 1996).

2.6.2 Antioxidant activity

In molecules it acts more than one function within the biological system. Many structures have the ability to eliminate free radicals. That acts as antioxidants, although their primary biological functions are very different (Halliwell, 1996). During oxidative stress, the concentration increases These molecules are considered biological responses that occur in combination with other antioxidant systems in order to protect cells from oxidation. In these structures, chondroitin sulfate (CS) is becoming increasingly important for current research. This chapter summarizes the action of short chondroitin sulfate, helping to reduce damage to the molecules caused by free radicals and the associated oxygen. chondroitin-4-sulfate Has high antioxidant activity chondroitin-6-sulfate The formation of specific sulfates, which play an important role in inhibiting the activity of these molecules on free radicals, which are captured by the excretion of metals such as Fe2 + and Cu2 +. In contrast, the Fenton reaction Chondroitin-4- is responsible for the production of reactive oxygen species (ROS) which prevents Chondroitin Sulfate in various molecules (such as fats, proteins, DNA, etc.) and in cells from various organs in the body. Sulfate Demonstrated that it can reduce biological injury and free radicals in various forms of oxidative stress caused by damage in cultured cells (Barahona et al., 2012). Other investigations assess the antioxidant properties. These are free in the experimental form of animal diseases and human pathology, especially rheumatism. The antioxidant activity of chondroitin sulfate can be used in the future as a therapeutic agent with free radicals (Campo., 2006).

2.6.2.1 DPPH radical scavenging activity

DPPH radicals, which have a very stable nitrogen center can be used to determine the ability to eliminate free radicals which are related to antioxidant activities. This method is based on the spectrophotometric measurement of DPPH concentration changes caused by DPPH reactions with antioxidants.

The standard procedure described earlier is used for measurement. 2-diphenyl-1-picrylhydrazyl (DPPH) (Ordoudi et al., 2006) briefly 1 ml of DPPH (100 μM, Sigma Aldrich, USA) in ethanol and 1 ml of antler chondroitin sulfate 1. at Various concentrations of chondroitin sulfate (0.625–10 mg / mL above) in a 100 mM buffer, Tris - HCl (pH 7.4). This mixture will shake and disappear in the dark for 20 minutes at room temperature. The absorbance was measured at 517 nm when compared to the empty control unit (Buffer 100 Tris - HCl Tris). The measurement was tripled in 60 seconds for each sample. Intense free radical removal activity DPPH is the inhibitory ratio calculated using the following equation: Driving activity (%) = (1 - Sample / free) × 100 Free space is absorption of snacks. The lower absorption of the active ingredients will indicate a higher free radical removal activity. Use ascorbic acid and butyl hydroxytolene (BHT) (Sigma Alrich, USA) as a positive regulator for two chondroitin sulfates from bovine cartilage (C4384, Sigma-Aldrich. , USA) is used as a reference to chondroitin sulfate, presenting results as an experimental method conducted in ± triple standard deviation (Ordoudi et al., 2006).

2.6.2.2 ABTS radical scavenging activity

Antioxidant test from 2,2'-Azinobis- (3-ethyl benzthiazoline-6-sulfonic acid) (ABTS) is like DPPH assay. ABTS can react with hydroxyl peroxide, inoxyls and inorganic to form ions. Stable free radicals (ABTS) with a maximum absorption of 734 nanometers. Free radicals added to the mixture in the initial stages of adding ROS or free radicals will help eliminate free radicals and prevent free radicals. ABTS This expulsion activity can Was measured via spectrophotometer and volume by comparing the antioxidant activity of extracts or biological fluids with Trolox (Jakus 2000; Barbaste et al., 2002).

ABTS assay is able to monitor the participation of antioxidants from various components in the system because it can react with hydroxyl cystenes and glutathione in biological systems, so it has many benefits. To study the antioxidant activity of various lipophilic and hydrophobic compounds, food products and biological samples (Jakus., 2000; Barbaste et al., 2002; Block 1992 and Serdula., 1996). For example, peroxidase has been shown to be effective in promoting the formation of free radicals ABTS, therefore supporting potential modifications resulting in adsorption values. Higher because the concentration of hydrogen peroxide is relatively low, this test kit cannot accurately measure the occurrence of free radicals because the antioxidants can respond directly to the positive ions of ABT instead of hydrogen peroxide which can appear as The more antioxidant activity is (Jakus., 2000; Barbaste et al., 2002). The test also measures the antioxidant activity of the specimen at a specified time and does not consider the time for the reaction to be completed, which may not be appropriate because lack of attention to overall antioxidant ability (Block., 1992; Serdula., 1996).

2.6.2.3 Metal ion chelating activity

The ability of GAGs to seize metal ions and others that are alleged to be molecules has been studied for over 50 years. The environment of calcium ions in chondroitin-4-sulfate It was described by Cael et al. (1978) ion carboxylate bridges in isolated chains and carboxylate and sulfate chains in a single chain. Chondroitin sulphate wrapped with metal ions such as Na, reconnoiter using Viscometer and ligand-field spectroscopy (Balt et al., 1983) resonance is prohibited for carboxylate and sulfate groups.

Chapter 3
Research methology

3.1 Materials and Chemicals

3.1.1 Materials

Chicken trachea

3.1.2 Chemicals

Acetic acid	Merck	Germany
Bovine serum albumin (BSA)	Sigma-Alorich	USA
Copper(II) sulfate pentahydrate	Sigma-Alorich	USA
Ethanol	V.S.Chem House	Thailand
Ethylene diaminetetraacetic acid (EDTA)	Sigma-Alorich	USA
Folin-Ciocalteu's phenol reagent	Carlo Erba	France
Papain	Sigma-Alorich	USA
Hydrochloric acid	Merck	Germany
Sodium acetate	Ajax Finechem	Austrelia
Sodium carbonate	Ajax Finechem	Austrelia
Sodium citrate	Ajax Finechem	Austrelia
Sodium hydroxide	Ajax Finechem	Austrelia Soybean
Trichloroacetic acid (TCA)	Merck	Germany
Tris	Vivantis	USA

3.2 Instruments

Analytical Balances	Mettler Toledo	German
Filtered paper	5C 1531110, Advantech	USA
Freeze dryer	LAB CONCO, 7755S01	USA
Magnetic Stirrer	IKA C-MAG HS7	China
pH meter	FiveEasy Plus,	Germany
Refrigerated centrifuge	Sorvall Legend mach 1.6 R centrifuge	USA
UV-vis spectrophotometer	UV1601, Shimadzu	Japan

3.3 Sample preparation

Chicken trachea was obtained from Tyson (Thailand); co., Ltd., Pathum Thani. Fresh chicken trachea sample was kept in ice and transported to the laboratory, KMITL within 1 h. Upon arrival, meat, fat and connective tissue was separate manually. Sample will then be subjected to heat treatment at 80 °C for 1 h prior to clean manually of muscle residues. Cleaned sample was milled with a grinder to have an average size of 1 mm. and was then been stored at -20 °C until used (Carballal et al., 2017).

3.4 Isolation of chondroitin sulfate from chicken trachea

3.4.1 Enzymatic hydrolysis method

Chondroitin sulfate from chicken trachea was isolated following the method of Lauder et al. (2000). Twenty grams of chicken trachea sample were in 250 ml of 0.1 M sodium acetate buffer, pH 6.8 containing 2.4 mM EDTA and 10 mM cysteine HCl mixture. Papain (3.0-33.0 % w/w of dry sample) was added to the solution and incubated at 65 °C for 24 h. (Water bath shaker, Memmert, WNB 29, Precision, Germany). Glycosaminoglycans containing chondroitin sulfate was precipitated from soluble fraction by adding 4 vols of ethanol and the solution was cooled to 4 °C and allowed to stand overnight prior to centrifuge at 10,000 g and 4 °C for 30 mins. and the pellet was resuspended in minimum volume of 50 mM sodium acetate buffer (pH 6.8). The solution was precipitated by addition of 2 vols of ethanol with constant stirring at 25 °C. After that, cooled to 4 °C and allowed to stand overnight. chondroitin sulfate rich powder (CSP) was then been resuspended in 5 ml of 50 mM sodium acetate buffer (pH 6.8) and supernatant was dialyzed overnight in distilled water. The dialyzed chondroitin sulfate was lyophilized and chondroitin sulfate rich powder (CSP) prior to pack in polyethylene bag and stored at 4 °C for further analysis.

3.4.2 Simplified enzymatic extraction method

Chondroitin sulfate from chicken trachea was isolated using a modified the method of Lauder et al. (2000). The flowchart for the enzyme extraction process used is shown in Figure 3.1 Twenty grams of chicken trachea sample were in 250 ml of 0.1 M sodium acetate buffer, pH 6.8 containing 2.4 mM EDTA and 10 mM cysteine HCl mixture. Papain (0.5-3.0 % w/w of dry sample) was added to the solution and incubated at 65 °C for 24 h. (Water bath shaker, Memmert, WNB 29, Precision, Germany). The solution was cooled to 4 °C. In order to remove precipitated proteins, the solution was centrifuged at 10,000 rpm for 30 mins at 4°C. The supernatant containing liberated GAGs was collected and 10 ml of 5 %TCA (w/v) was added to the solution to precipitate GAGs. The solution was centrifuged for 30 mins at 10000 rpm at 4°C. The supernatant was dialyzed overnight in distilled water. The solution containing GAGs was stored at 4°C. The dialyzed solution containing GAGs was lyophilized and chondroitin sulfate rich powder (CSP) prior to pack in polyethylene bag and stored at 4 °C for further analysis.

3.4.3 Alkali extraction method

Chondroitin sulfate from chicken trachea was isolated using a modified the method of Lauder et al. (2000). The flowchart for the enzyme extraction process used is shown in Figure 3.2 Twenty grams of chicken trachea sample were dissolved in 250 ml of 0.1 M NaOH was added to the solution and incubated at 25 °C for 24 h. (Water bath shaker, Memmert, WNB 29, Precision, Germany). The solution was cooled to 4 °C. In order to remove precipitated proteins, the solution was centrifuged at 10,000 rpm for 30 mins at 4°C. The supernatant containing liberated GAGs was collected and 10 ml of 5 %TCA (w/v) was added to the solution to precipitate GAGs. The solution was centrifuged for 30 mins at 10000 rpm at 4°C. The supernatant was dialyzed overnight in distilled water. The solution containing GAGs was stored at 4°C. The dialyzed solution containing GAGs was lyophilized and chondroitin sulfate rich powder (CSP) prior to pack in polyethylene bag and stored at 4 °C for further analysis.

3.5 Determination of yield

Yield of the chondroitin sulfate rich powder (CSP) was calculated based on the percentage (dry basis) of the CSP isolated from chicken trachea with protein subtraction per that of dry weight of chicken trachea sample.

3.6 Statistical Analysis

All experiments was rush in triplicates. Data was subjected to Analysis of Variance (ANOVA) and mean comparisons will be taked out using Duncan’s multiple range test (95% confidence). SPSS statistic program (SPSS 11.0 for Windows, SPSS Inc., Chicago, IL, USA) will be used for data analysis.

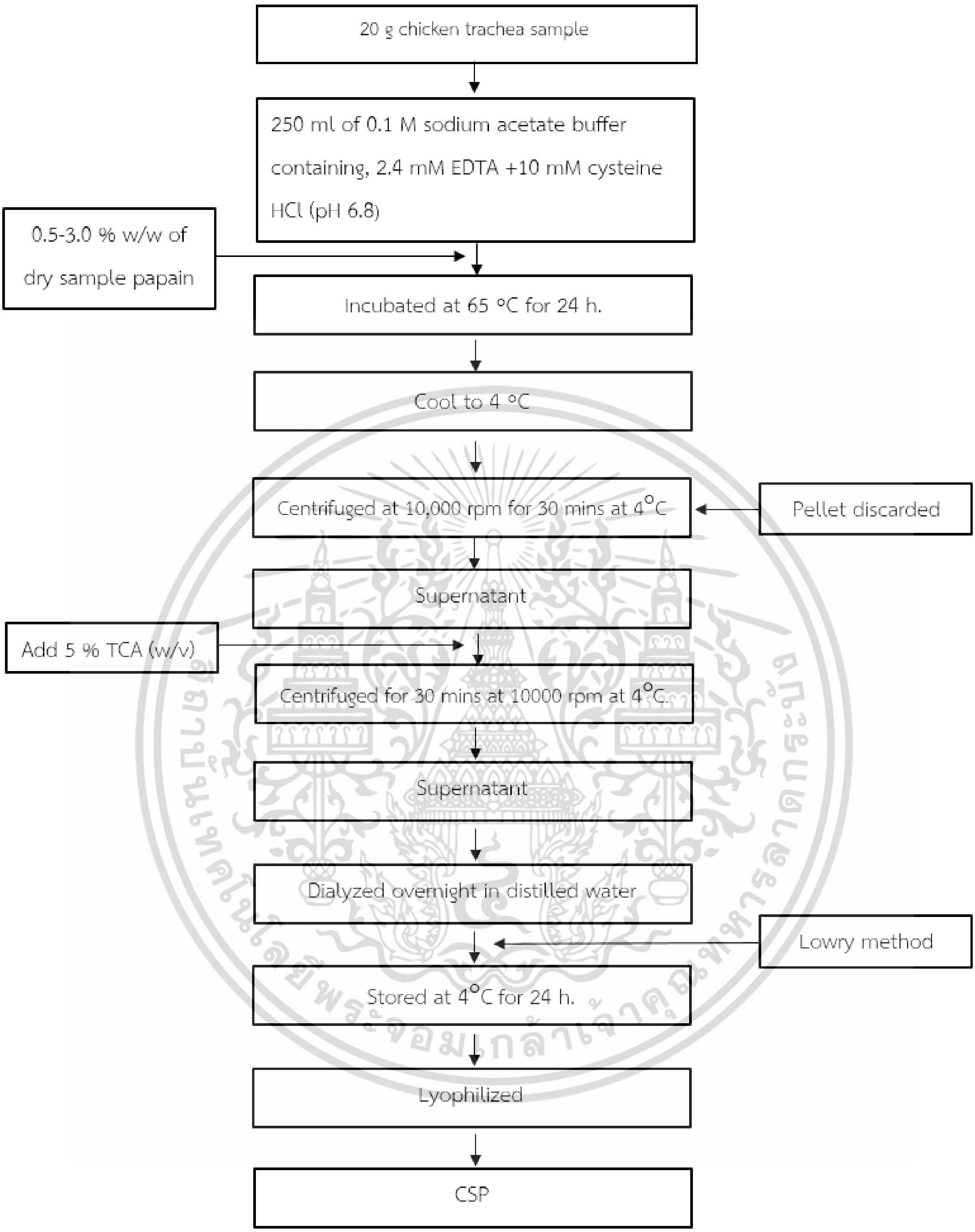

Figure 3.1. Simplified enzymatic extraction method used for chondroitin sulfate from chicken trachea

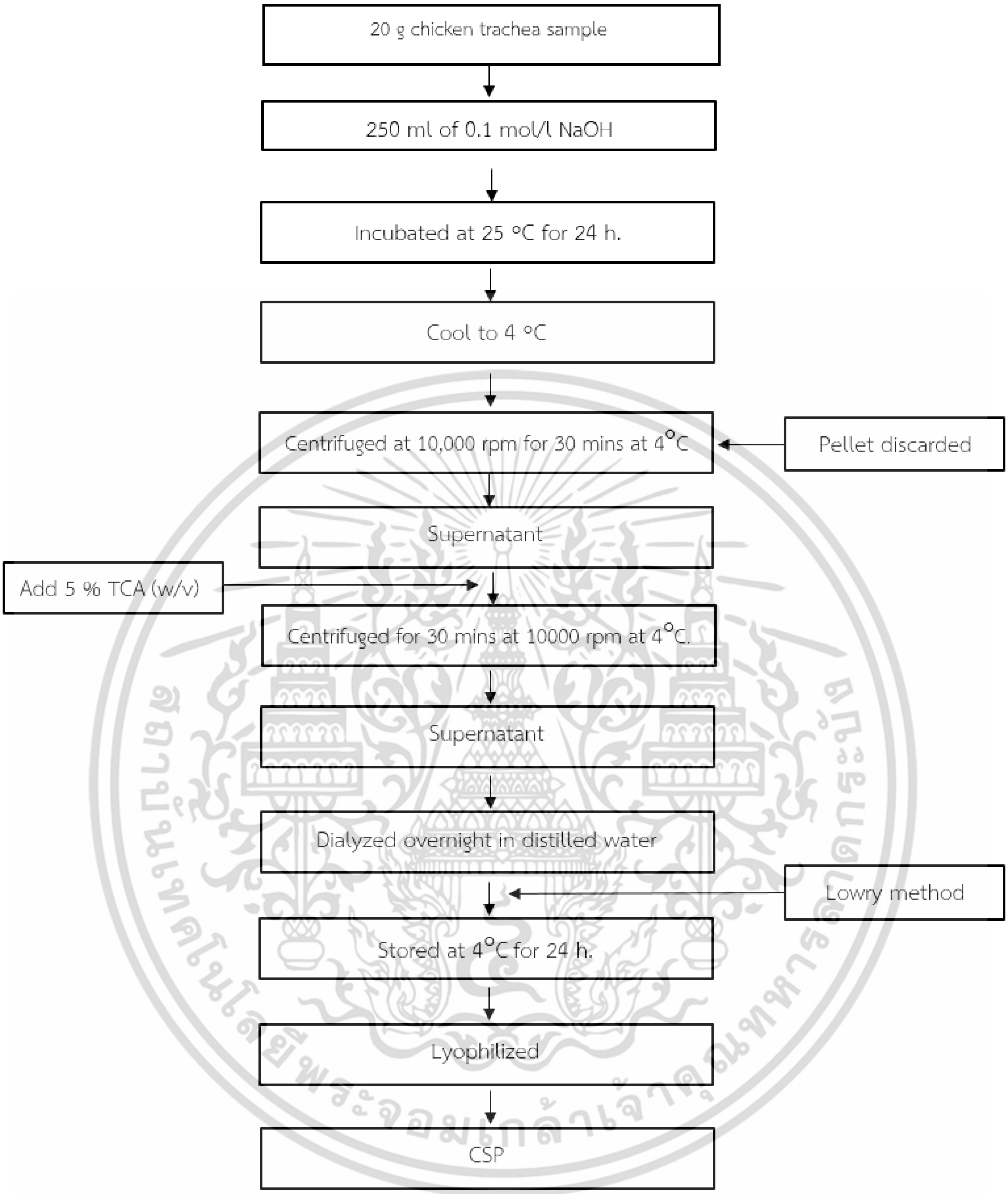

Figure 3.2. Alkali extraction method used for chondroitin sulfate from chicken trachea

Chapter 4
Results and Discussion

4.1 Yield of chondroitin sulfate rich powder (CSP) from chicken trachea

Table 4.1 show the amount of yield of chondroitin sulfate rich powder (CSP) from chicken trachea extracted by enzymatic hydrolysis methods from different concentrations of papain (3 % dry basis, 7 % dry basis, 13 % dry basis and 33 % dry basis). The extraction yield for the treatments experimented. It is observed that the highest yield for papain extraction was obtained with papain 7% dry basis, 3% dry basis, 13% dry basis and 33 % dry basis. Yield were 13.61 ± 0.02, 12.65 ± 0.01, 12.19± 0.0 1 and 10.29 ± 0.03, respectively. Significant differences (P < 0.05). The results of analyzing the general components of the moisture chicken trachea has moisture 84.70%±0.20 (the result not show).

Table 4.1 Yield of chondroitin sulfate rich powder (CSP) from chicken trachea by enzymatic hydrolysis methods.

No	% papain	Sample (g dry weight)	Protein (mg)	CSP (g)	% Yield
1	0	3.0620	1.7966±0.14	0.3056±0.00	9.98±0.00[a]
2	3	3.0634	61.8163±0.35	0.3874±0.00	12.65±0.01[d]
3	7	3.0636	72.5880±0.65	0.4170±0.00	13.61±0.02[e]
4	13	3.0652	114.6133±0.18	0.3738±0.00	12.19±0.01[c]
5	33	3.0605	46.2597±0.94	0.3149±0.00	10.29±0.03[b]

Mean±SD (n=3)

* different superscript letters in the same column indicate significant differences (p≤0.05)

In this study, the papain is type of proteases isolated from papaya fruit. Papain is very stable for extraction and causes least damage to the tissues. (Arnon, 1970). According to Silva (2006), glycosaminoglycan can be isolated directly from tissues by hydrolysis in existence of exogenous enzymes such as papain. Roden et al., (1972) reported that proteolysis with papain yields a GAGs chain to which a small peptide include of various amino acids. Himonides et al. (2011) reported that papain is neutral qualifications protease. In hydrolysis is the highest at a pH of 6.5 and cumulative increases of pH resulting in decreases of hydrolysis. Kilara et al. (1977) indicate that the suitable temperature for highest papain activity is at 65 °C. In this study, the experiments were proceed at a pH of 6.4 and a temperature of 65 °C. In this study, even though the GAGs concentration increased with increase in enzyme concentration (from 3 U/ml to 7 U/ml) but when increase in enzyme concentration (from 7 U/ml to 33 U/ml) there were some opposite trends observed with decreases in GAGs content with increase in the enzyme concentration this may be due to the spacious breakdown of peptides with reduced the GAGs available in solution (Lindahl et al., 1998).Thence papain linking to GAGs is conduct mainly by electrostatic interactions and papain display high affinity in linking to GAGs with higher enzyme concentrations and lesser GAGs content in the sample conduct to inhibition of GAGs hydrolysis (Li et al., 2006).

In this study was followed the method of Lauder et al. (2000) by accelerator comprising of cysteine (0.005M) and (0.01M) EDTA for incubated with papain. Raghuraman (2013) and Brubacher et al., (1966) reported that the inactivated enzyme can be stimulate by cumulative increases of cysteine.

In the process of precipitation with ethanol is general methods for the separation of GAG solution. The method can be practical directly to a trachea. Ethanol is most success implement for remove presence of metal ions. GAGs are precipitate by increasing volumes of ethanol (4.0 volumes) the solution is allowed to equilibrate at 4 C°. After that, precipitate is collected by centrifugation then add minimum volume of 50 mM sodium acetate buffer (pH 6.8) and repeat again by ethanol is added to the supernatants. (Maximum of 2.0 volumes of ethanol) add minimum volume of 50 mM sodium acetate buffer. Finally, supernatants were freezed and freeze dried

Volpi (1996) reported the precipitation with ethanol of GAGs solution. Indicated that the produce of precipitate of the GAGs solution in particular Heparin sulfate (HS) was the first GAG type to precipitate and followed by Dermatan sulfate (DS) and Chondroitin sulfate (CS). Luiz and Silva (2006) reported the tissue GAGs will be precipitated utterly by four to five volumes of ethanol, as long as that the GAGs concentration is high enough and sufficient salt is present.

In addition, purification of tissue GAGs. It is prevalent not to exceed about two volumes thus as to avoid at the same time precipitation of another digestion products. The result (Table 4.) there were some opposite trends observed with decreases in GAGs content may be due to the yield or GAGs concentration that not enough to precipitation with ethanol.

Tables 4.2 show the amount of yield of chondroitin sulfate rich powder (CSP) from chicken trachea extracted by simplified enzymatic extraction method from different concentrations of papain (0.5 % dry basis, 1 % dry basis, and 3 % dry basis). The extraction yield for the treatments experimented. It was observed that the highest yield for papain extraction was obtained with papain 0.5 % dry basis, 1 % dry basis, and 3 % dry basis. Yield were 17.19 ± 0.05, 14.96 ± 0.02 and 14.69 ± 0.07, respectively. Significant differences ($P < 0.05$).

Table 4.2 Yield of chondroitin sulfate rich powder (CSP) from chicken trachea by simplified enzymatic extraction method

% papain	Sample (g dry weight)	Protein (mg)	CSP (g)	% Yield
0	3.0601	52.25±1.95	0.03±0.00	0.86±0.06^{a}
0.5	3.0615	121.49±1.48	0.53±0.00	17.19±0.05^{d}
1	3.0604	94.34±0.75	0.46±0.00	14.96±0.02^{c}
3	3.0606	102.61±2.03	0.45±0.00	14.69±0.07^{b}

Mean±SD (n=3)

* different superscript letters in the same column indicate significant differences ($p \leq 0.05$)

In this method was used papain and buffer for incubated with papain same the first method. The GAGs preparation in this methods and after isolation were precipitate by centrifugation and added TCA to the supernatants for precipitation. After that, precipitate is collected by centrifugation then dialysis remove TCA and supernatants were freezed and freeze dried.

The results showed that the GAGs concentration decreases with increase in enzyme concentration (from 0.5 % dry basis to 3 % dry basis) there were some opposite trends observed with decreases, which the results are consistent with the first method but the process of precipitation with TCA that more yield than precipitation with ethanol. (The yield for papain extraction was obtained with papain 0.5 % dry basis, 1 % dry basis and 3 % dry basis. Yield were 17.19 ± 0.05, 14.96 ± 0.02 and 14.69 ± 0.07, respectively. And the highest yield for papain extraction was obtained with papain 7 % dry basis, 3 % dry basis, 13 % dry basis and 33 % dry basis. Yield were 13.61 ± 0.02, 12.65 ± 0.01, 12.19± 0.0 1and 10.29 ± 0.03, respectively.) Significant differences ($P < 0.05$). On GAGs preparation methods and after isolation, TCA variation is regularly used to precipitate residual proteins and peptides (Nakano and Sim, 1995). Rajalingam et al., (2009) reported that the maximum quantity of precipitated protein is realized when the concentration of TCA is between 5-40% w/v. Rajalingam et al.,(2009) reported that the maximum amount of precipitated protein is achieved when the concentration of TCA is between 5 – 40 % w/v. Trichloroacetic acid (TCA) is the most amply used chemical for protein precipitation, Because of TCA mediated precipitation is as well independent of the physicochemical properties of proteins (Nandakumar et al., 2003; Sivaraman et al., 1997). Moreover, the concentrations of TCA can also degrade the quality of the sample when use of high concentrations of TCA (Rajalingam et al., 2009). So, it is very important to recognize the optimal concentration of TCA.

Tables 4.3 show the amount of yield of chondroitin sulfate rich powder (CSP) from chicken trachea extracted by alkali extraction method from 0.1 mol/l NaOH. The extraction yield for the treatments experimented. The extract yield were 12.42 % ± 0.06.

Table 4.3 Yield of chondroitin sulfate rich powder (CSP) from chicken trachea by alkali extraction method

NaOH (M)	Sample (g dry weight)	Protein (mg)	CSP (g)	% Yield
0.1	3.0607	106.30±1.85	0.38±0.00	12.42±0.06

In this method was alkali extraction method with NaOH. The GAGs preparation in this methods and after isolation were precipitate by centrifugation and added TCA to the supernatants for precipitation. After that, precipitate was collected by centrifugation then dialysis remove TCA and supernatants were freezed and freeze dried.

The results showed that the GAGs concentration has the lowest yield (12.42 % ± 0.06) by alkali extraction method in 0.1 mol/l NaOH .Overview of the results. A comparison result can show the highest yield for papain extraction by simplified enzymatic extraction method papain 0.5 % dry basis yield were 17.19 % ± 0.05 and papain extractions by enzymatic hydrolysis methods with papain 7 % dry basis yield were 13.61% ± 0.02 , respectively. The extraction is implemented with 1.0 mol/l NaOH (Meyer, 1948; Consden and Bird, 1954). In this treatment the protein portion of the proteoglycan is removed by an elimination reaction (Anderson et al., 1963). Extraction at higher temperatures must be avoided because it causes chemical modifications of the glycosaminoglycan (Curtius and Roth, 1974) chondroitin sulfate contain in tissues such as cartilage in a highly polymerized condition. In the chondroitin sulfate as an oligosaccharide of quite low molecular weight (Levene, 1925) and formulated the substance as a non-reducing tetra saccharide composed of residues of glucuronic acid and sulfate of N-acetylglucosamine. The main reason for the failing of the results to recognize the polymerized of the polysaccharide be due to the fact that strongly alkaline reagents were often used to effect the primary extraction from the tissue, and it has been shown that the polysaccharide is rapidly degraded in the strong alkali, especially at temperatures above 0 °C. Jorpes (1929) reported that the extraction of chondroitin sulfate from cartilage remains the important matter of dilute NaOH is still frequently employed. In this method NaOH as high as 0.5 mol/l was imperative to extract the acid polysaccharide from cartilage at 0 °C. From the foregoing it should be seen that the alkaline extraction conditions are important such as concentration of NaOH and temperature. From this research, the effect of temperature may be a factor to the result in lower extraction efficiency due to extraction not used high enough substrate concentrations to extract the acid polysaccharide from cartilage or chicken trachea.

The results. A comparison result can show the highest yield for papain extraction by simplified enzymatic extraction method papain 0.5 % dry basis. Experimental results indicate that showed the greatest extraction efficiency and used low concentrations of papain.

In addition, simplified enzymatic extraction method reduced the amount of time extraction in this method.

4.2 Evaluation of chondroitin sulfate content in the restant chondroitin sulfate rich powder (CSP) from chicken trachea

Tables 4.4 show the total proteins content of chondroitin sulfate rich powder (CSP) solutions were determined by the method of Lowry et al. Chondroitin sulfate rich powder was extracted by enzymatic hydrolysis method, according with the simplified enzymatic extraction method and alkali extraction method. Quantification of Chondroitin sulfate (CS) as proposed does not invalidate the results herein obtained. Chondroitin sulfate content in the restant chondroitin sulfate rich powder, defined as CS (%) = (CS (mg)/CSP freeze dried) x 100. Thus, the results of three method this studies have indicated that has quite a lot of the proteins content remained in chondroitin sulfate rich powder. In the different method involved in the chondroitin sulfate recovery and purification from chicken trachea should been developed. Two experimental designs, simplified enzymatic extraction method and alkali extraction method. From this research, precipitation with ethanol is used as a means of recovering chondroitin sulfate. Ethanol was fairly well reached with recover ability of chondroitin sulfate as well as protein elimination in enzymatic hydrolysis method. Therefore, two experimental of the modified method should be add the precipitation step with ethanol.

Evaluation of chondroitin sulfate content in the restaurant chondroitin sulfate rich powder pointed out that the results. Three method this studies have indicated that has quite a lot of the proteins content remained in chondroitin sulfate rich powder. The utilization of the chondroitin sulfate rich powder for food or food supplementary. Chondroitin sulfate rich powder still can also be used as a protein contamination does not have an impact on utilization. Because protein is a type of nutrient, but utilization of the CSP for ingredient in medicine or medicine it is still considered inappropriate.

Table 4.4 The amount of chondroitin sulfate content in the restant chondroitin sulfate rich powder (CSP) from chicken trachea

Condition	CSP (freeze dried) (mg)	Protein (mg)	CS (mg)	CS (%)
Enzymatic hydrolysis method				
0 % papain	78.20	1.80±0.14	76.4034	97.70
3 % papain	112.30	61.82±0.35	96.8459	86.24
7 % papain	122.40	72.59±0.65	104.2530	85.17
13 % papain	90.30	114.61±0.18	78.7351	87.19
33 % papain	122.10	46.26±0.94	93.4467	76.53
Simplified enzymatic extraction method				
0 % papain	78.70	52.25±1.95	26.4454	33.60
0.5 % papain	647.90	121.49±1.48	526.4135	81.25
1 % papain	552.30	94.34±0.75	449.6938	81.42
3 % papain	552.30	102.61±2.03	457.9593	82.92
Alkali extraction method				
0.1 mol/l NaOH	486.40	106.3021	380.0979	78.15

Chapter 5
Conclusion and Suggestion

5.1 Conclusion

Based on the extraction yield of chondroitin sulfate, the simplified enzymatic extraction method showed the greatest extraction efficiency.

5.2 Suggestions

5.2.1 Purification of chondroitin sulfate from chicken trachea should be studied.

5.2.2 Identification of chondroitin sulfate from chicken trachea should be further investigated.

5.2.3 Characteristics and physicochemical properties of chondroitin sulfate from chicken should be determined.

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APPENDIX

Appendix

1. Lowry's procedure for quantitation of proteins (with slight modified Lowry et al., 1951)

1.1 Reagents

1. A: 2% sodium carbonate in 0.1 N NaOH
2. B: 0.5% $CuSO_4.5H_2O$ in 1% sodium citrate
3. C: 2 N Folin-Ciocalteu's phenol reagent + distilled water (ratio of 1:1)
4. D: 50 mL reagent A + 1 mL reagent B
5. Standard reagent: Bovine serum albumin (BSA) at concentration of 1 mg/mL

1.2 Method

1. To each of eight disposable cuvettes, add the following reagents according to the table.
2. To tubes 8, 200 µL of protein sample were added and mix well by using the vortex mixer.
3. Add 2 mL reagent D to each of the standards and unknown tube and then vortex immediately.
4. Incubate precisely 10 min at room temperature.
5. Add 0.2 mL reagent C (previously dilute 1:1 with distilled water) and vortex immediately.
6. Incubate 30 min at room temperature (sample incubated longer than 60 min should be discarded).

Table: Experimental set up for the Lowry's assay

Tube number	Distilled Water (μL)	1 mg/mL BSA (μL)	Effective BSA Concentration (mg/mL)
1	200	0	0
2	180	20	0.1
3	160	40	0.2
4	140	60	0.3
5	100	100	0.5
6	60	140	0.7
7	0	200	1.0
8	0	0	unknown