EXTRACTION OF SULFATED GLYCOSAMINOGLYCANS FROM MACKEREL AND HERRING FISH WASTE

by

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TO:

My parents

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ABSTRACT

Marine capture fisheries contribute over 50% of total world fish production and more than 70% of this production is utilized for processing. The Canadian commercial fishing industry is one of the world's most valued industries but generates large quantities of solid waste and wastewater. The increasing growth of the fish processing industry, the need for reduction of pollutants and the need to increase returns on raw material has led fish processors to adopt new ways of utilizing the wastes. In particular, efforts have focused on converting the biological substance in solid fish processing waste to various valuable compounds including both nutritional and non-nutritional products. Sulfated glycosaminoglycans (sGAGs) are heteropolysaccharide molecules with potential therapeutic applications and anticoagulant properties. Anticoagulants are responsible for curing major death-causing diseases such as strokes and cardiovascular diseases. The aim of this study was to develop an economically feasible technique to extract sulfated glycosaminoglycans (sGAGs) from fish processing waste. Two different fish (mackerel and herring) were used to optimize the extraction of sGAG. The effects of hydrolysis time (3, 6, 12 and 24 hrs) and papain concentration (15 and 20u/ml) on the extraction of sGAGs from different fish parts (whole fish, flesh, head, gut, fins and tails, skin and bones) were evaluated. The highest concentration of sGAGs (206.7 mg/g) was obtained from the mackerel head sample at 6 hrs of hydrolysis time and 20 u/ml of enzyme concentration while the highest concentration of sGAGs (236.3 mg/g) was obtained from herring gut at 12 hrs of hydrolysis time and 20 u/ml of enzyme concentration. The concentration of sGAG obtained from other part of mackerel were flesh (23.96 mg/g), waste (163.23 mg/g), fins and tail (86.63 mg/g), gut (203.52 mg/g), skin (105.45 mg/g) and bones (97.2 mg/g). However, the concentration of sGAG obtained from other parts of herring were flesh (39.34 mg/g), waste (130.15 mg/g), head (162.76 mg/g), fins and tail (148.53 mg/g), skin (65.89 mg/g) and bones (75.57 mg/g). Comparing the overall concentration of sGAG in waste samples of the fish, the mackerel produced higher sGAG than the herring.

LIST OF ABBREVIATIONS USED

ACS:	Acute coronary syndrome
AD:	Alzheimer disease
ADP:	Adenosine diphosphate
ANOVA:	Analysis of variance
APTT:	Activated partial thromboplastin time
BSE:	Bovine spongiform encephalopthy
CPC:	Cetyl pyridinium chloride
CS:	Chondroitin sulfate
DEAE:	Diethylaminoethyl cellulose
DMMB:	Dimethylmethylene blue
DS:	Dermatan sulfate
EDTA:	Ethylenediaminetetra acetic acid
FPC:	Fish protein concentrate
FPH:	Fish protein hydrolysate
GAG:	Glycosaminoglycans
GC-MS:	Gas chromatography- Mass spectrometry
GP:	Glycoprotein
HPCE:	High performance capillary electrophoresis
HPLC:	High performance liquid chromatography
HS:	Heparan sulfate
KS:	Keratan sulfate
LMWH:	Low molecular weight heparin

MS:	Mass spectrometry
NMR:	Nuclear magnetic resonance
OD:	Optical density
PT:	Prothrombin time
PUFA:	Polyunsaturated fatty acid
SAX:	Strong anion exchange
sGAG:	Sulfated Glycosaminoglycans
SP:	Sulfated polysaccharides
TFPI:	Tissue factor pathway inhibitor
TLC:	Thin layer chromatography
TT:	Thromin time
UFH:	Unfractionated heparin
VLMWH:	Very low molecular weight heparin
vWF:	von Willebrand factor

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CHAPTER 1. INTRODUCTION

The fish capture and processing industries provide employment for millions of people around the world and billions of foreign exchange earnings to many countries (Opara and Al- Jufaili, 2006). Marine capture fisheries contribute over 50% of total world fish production and more than 70% of this production is processed. The current discards from the world's fisheries exceeds 20 million tonnes which is about 25% of the total production of marine capture fisheries (FAO, 2001). Canadian commercial fishing industries are among of the world's most valued industries. Canada exported 703,000 tonnes of fish products in 2005, which was valued at $4.3 billion (Lalonde et al., 2007).

The commercial fish-processing industry generates large quantities of solid waste and wastewater. Solid waste includes whole spoiled fish and offal that contains viscera, fins, head, skin and fish scrap (Hwang and Hansen 1998). Wastewater originates from cleaning and washing raw materials and contains low levels of soluble protein and fat. Some of the waste is rendered while the majority of the waste is dumped in landfills (Knuckey et al., 2004). The increasing growth of the fish-processing industry, the necessity for reduction of pollutants and the need to maximize returns on raw material has encouraged producers to seek new ways for converting the organics in the solid waste to various marketable products such as bait, fertilizers, fish oil and organic acids (Hwang and Hansen 1998; Mathur et al., 1988).

Fish waste is rich in potentially valuable oils, minerals, enzymes, pigments and flavors which have enormous applications in food, pharmaceuticals, agricultural and aquacultural industries. The fish processing waste can be used for production of nutritional and non-nutritional products. Nutritional products include fishmeal, fish oil, fish protein concentrate, fish protein hydrolysate and organic fertilizer. Non-nutritional uses include chitin and chitosan, carotenoid pigments, enzyme, leather, glue, pharmaceuticals, cosmetics, fine chemicals, collagen, gelatin and pearl essence (Archer et al., 2001).

Sulfated glycosaminoglycans like heparin sulphate and chondroitin sulphate possess high anticoagulant properties. Anticoagulants are in high demand as a medication, since the major death-causing diseases like strokes and cardiovascular diseases can be treated with anticoagulants. Anticoagulants also have applications in cancer treatments. Anticoagulants are widely used as an injecting anticoagulant and are also used to form an anticoagulant layer in experimental devices and dialysis machines (Desai et al., 2000). Currently, anticoagulants are derived from an animal source (porcine). Hence, there is always a threat of various animal diseases which can possibly affect human health such as Mad Cow disease (Linhardt and Gunay, 1999; Marcum et al., 1986). In order to develop and extract anticoagulants from a safer source, fish processing waste, which is a major environmental concern in Atlantic Canada, is considered a good source. The aim of this study was to develop an extraction technique for the recovery of sulfated glycosaminoglycans from fish processing waste.

CHAPTER 2. OBJECTIVES

The aim of the study was to extract sulfated glycosaminoglycans (sGAGs) from fish processing waste. The specific objectives were to:

1. Quantify the different components of wastes from the processing of mackerel and herring fish.
2. Study the effect of enzyme hydrolysis time (3, 6, 12 and 24 hrs) and papain concentration (15 and 20 u/ml) on the extraction of sGAGs from different parts (whole fish, flesh, head, gut, fins and tails, skin and bones) of mackerel and herring fish.
3. Compare the concentration of sGAGs isolated from different parts of mackerel and herring.

CHAPTER 3. LITERATURE REVIEW

3.1. Fish Production

Both captured fisheries and aquaculture operations supplied the world with about 142 million tonnes of fish in 2004. Global capture fisheries production reached 95 million tonnes in 2004, with an estimated first-sale value of US$84.9 billion. China, Peru and the United States of America remained the top producing countries. The production in the Eastern Indian Ocean and Western Central Pacific continued their long-term increasing trends. In the highly regulated Northwest Atlantic and Northwest Pacific areas, recent increases were observed following troughs in production (FAO, 2006).

Canadian commercial fishing industries are among the world's most valued industries. Canada exported 703,000 tonnes of fish products in the year 2005, valued at $4.3 billion. The value recorded in 2005 was up 2.6 percent from year 2004 (Lalonde et al., 2007). There are over 1400 fish processing plants in Canada (Novatec, 1994). Tables 3.1 and 3.2 show Canadian commercial fish catches and value by province.

Atlantic mackerel, *Scomber scombrus L*., belong to the scombrids which have compact, sleek and streamlined bodies with 23 to 33 dark, vertical bands across their upper bodies (CSAR, 1999). Mackerel is mostly found in the Northwest Atlantic and the east coast of Newfoundland. This area is divided in two spawning zones: (a) Canadian waters where mackerel spawn primarily in the southern Gulf of St. Lawrence in June and July followed by long migration in early spring on Georges Bank, and (b) US water where mackerel spawn in March and April along the New Jersey coast. In the year 2002, approximately 15,000 commercial fishers participated in the mackerel fishery in Eastern Canada. Fishing for commercial purpose was performed inshore, using gillnets, jiggers, purse seines and traps (Desjardins, 2005).

Table 3.1. Canada's commercial sea fisheries landings by province (DFO, 2009).

	2008		2009		2010	
	Quantity (tonnes)	Value (10 $)	Quantity (tonnes)	Value (10 $)	Quantity (tonnes)	Value (10 $)
Sea-fisheries Landings						
Nova Scotia	255,490	677,059	292,220	600,702	277,087	487,031
New Brunswick	96,037	169,738	96,408	151,463	96,358	121,072
PEI	34,276	124,102	36,100	96,585	35,110	99,031
Quebec	58,212	141,537	61,091	125,093	55,434	115,856
Newfoundland	337,756	530,647	315,625	460,981	324,610	510,692
Atlantic-Total	761,601	1,625,62	755,408	1,421,762	748,238	1,320,72
Pacific -Total	155,341	262,309	158,787	267,574	150,861	294,212
Seafisheries Total	916,942	1,887,93	914,194	1,689,336	899,100	1,614,93

Table 3.2. Canada's commercial freshwater landings by province (DFO, 2009).

	2008		2009		2010	
	Quantity (tonnes)	Value (10 $)	Quantity (tonnes)	Value (10 $)	Quantity (tonnes)	Value (10 $)
Freshwater Landings						
New Brunswick	477	447	173	137	32	76
Quebec	590	1,392	645	1,627	613	1,355
Ontario	14,027	27,315	13,174	28,225	11,298	31,611
Manitoba	11,428	24,746	11,182	22,474	10,934	21,733
Saskatchewan	2,451	3,039	3,002	3,790	2,731	3,192
Alberta	1,030	1,240	1,049	1,504	1,205	1,565
NWT	327	413	318	337	424	450
Freshwater Total	30,330	58,591	29,543	58,094	27,237	59,982

Atlantic herring, *Clupea harengus*, is a pelagic fish from the family Clupeidae. The structure of herring is an elongated, laterally compressed, streamline body, with a deeply forked tail and single dorsal fin. Atlantic herring has a blue-green back, silver shade on its sides and abdomen, which provide camouflage in the ocean (Desjardins, 2005). According to British Columbia seafood production (2009), $23.5x10^3$, $11.8x\ 10^3$ and $11.4x10^3$ tonnes of herring was harvested with landed values of $18.3x\ 10^6$, $20.2x10^6$ and $15.5x10^6$ dollar for the year 2006, 2007 and 2008, respectively.

3.2. Fish Processing

The fish industry generates a significant amount of waste. Fish processing wastes not only affect the surrounding area directly, but can also spoil a wider coastal zone and different ecosystems. These wastes reduce the biomass density and diversity of the benthos, plankton and nekton, thus modify natural food webs (Gowen, 1991; Pillay, 1991). As the aquaculture industry is growing every year, efficient, cost-effective and environmentally friendly bioremediation methods for improving effluent water quality are necessary prior to discharging waste water into the environment (Jones et al., 2001).

Table 3.3 shows the edible portion of demersal and shellfish species, respectively. Most demersal fish are processed to some extent and the resulting waste consists of guts, liver and other viscera which are removed during the gutting operation. The amount of gutting waste varies according to the species, fishing grounds and season. During most of the fish processing procedures, 66% of the fish is discarded whereas the fillets were retained (Knuckey et al., 2004). For cod, it varies between 8-22% of the whole weight of the fish. However, a typical value is about 16%. Edible flesh portions of Indian mackerel, Atlantic mackerel and Atlantic herring were 61%, 62% and 61%, respectively (FAO, 1989).

Generally, wastes are dumped at sea where they are produced, but if these wastes were retained for utilization, they would be landed with the fish. The majority of fish and

Table 3.3. Edible portions of some fish species (Archer et al., 2001)

Species	Edible Portions (%)
Demersal Species	
Catfish	35%
Demersal (general)	43%
Haddock (Ave.)	43%
Hake	50%
Lemon sole	42%
Ling	48%
Plaice	35%
Redfish	30%
Whiting	38%
Shellfish Species	
Crab	32
Lobster	44
Nephrops (whole)	24
Nephrops (unshelled tails)	58
Shrimp, brown	35
Prawn	40
Crustacea (Ave.)	39
Oyster	14
Cockle	12
Winkle	23
Scallop	14
Mussel	14
Whelk	42
Mollusc (Ave.)	20

shellfish processing operations were carried out in shore-based processing facilities. Fish parts like lungs, fillet, flaps, cheeks, tongue are sold out as by-products, whereas the remaining fish waste parts (including the fins, head, skin and viscera) are discarded as waste (Archer et al., 2001). Waste from the most commonly canned fishery products (salmon, sardines, tuna and catfish) are also processed in a similar manner (Nair, 1990; Veiga et al., 1994).

The processing operations produce large amount of wastewater, which results from various processing steps involved in the industry. The concentration of the contaminants present in the wastewater depends upon the origin of the wastewater. The concentration of wastewater produced while washing is less than the wastewater produced from filleting and storing (Corkum et al., 2003). Table 3.4 shows the water consumption and wastewater generation in fish processing plants.

However, there is considerable potential for gaining more value from fish wastes. The wastes are rich in valuable minerals, enzymes, pigments and flavours that are required by many industries including food, agriculture, aquaculture and pharmaceuticals (Archer et al., 2001).

3.3. Utilization and Disposal of Fish Processing Waste

Marine capture fisheries contributed over 50% of total world fish production and more than 70% of this production is processed. The discards from the world's fisheries exceeded 20 million tonnes, equivalent to 25% of the total production of marine capture Fisheries (FAOSTAT, 2001). In 2000, more than 60% of the total world fisheries production underwent some form of processing (FAO, 2002). The worldwide expansion of marine fish farming caused growing concern regarding its environmental impact (Lupatsch et al., 2003). World fisheries, aquaculture production and utilization are shown in Table 3.5.

Table.3.4. Water consumption and wastewater discharge in fish processing plants (Chowdhury et al., 2009).

Fish Processing Plants	Amount
Gulf Shrimp Canning	184.36 L/kg (22 gal/lb)
Peelers	58.1%
Washers	8.8%
Separators	6.9%
Blancher	1.6%
De-icing	4.2%
Cooling	12.1%
Wash down	8.3%
Salmon	
Large processing plant	3.12 L/kg (374 gal/1000 lb)
Small processing plant	9.898 L/kg (1186 gal/1000 lb)
Others	
Tuna processing plant	13627.4 m^3 /d (3600, 000 gpd)
Bottom fish	22.71-1514.2 m^3/d (6000 - 400,000 gpd)
Fish meal plants	37.85-348.26 m^3/d (10,000 - 92,000 gpd)
Finfish	0.9179 L/kg (110 gal/1000 lb)
Canning of tuna and sardine	14-22 m^3/tonne

Table 3.5. World fisheries, aquaculture production and utilization for the period of 2002-2006 (FAO 2008)

Type	Production (Million tonnes)				
	2002	2003	2004	2005	2006
Total inland	32.7	34.4	36.7	39.3	41.7
Inland Capture	8.7	9.0	8.9	9.7	10.1
Aquaculture	24.0	25.5	27.8	29.6	31.6
Total marine	100.9	98.7	103.8	103.4	102.0
Marine Capture	84.5	81.5	85.7	84.5	81.9
Aquaculture	16.4	17.2	18.1	18.9	20.1
Total world fisheries	133.6	133.2	140.5	142.7	143.6
Inland capture	93.2	90.5	94.6	94.2	92.0
Inland aquaculture	40.4	42.7	45.9	48.5	51.7
Utilization					
Human consumption	100.7	103.4	104.5	107.1	110.4
Non-food uses	32.9	29.8	36.0	35.6	33.3
Per capita food fish	16.0	16.3	16.2	16.4	16.7

3.3.1. Nutritional Utilization of Fish Processing Waste

3.3.1.1. Fishmeal. Fish waste is often converted to fish meal (Hall, 1992; Keller, 1990). Fishmeal is a highly nutritious powder produced by the drying and grinding of whole fish or fish processing waste. Generally, fish meal is mixed with other ingredients and used as animal feed. The animal feed produced from fish meal has a superior quality. Fish meal production involves the cooking and pressing of the raw materials (whole fish or fish waste). Fish meal is produced in various grades based on composition and quality. Fish meal is rich in highly digestible protein and essential vitamins and has high demand in agricultural and aquacultural industries (Archer et al., 2001).

According to Yamamoto et al. (2005) production of fish meal is an expensive process because it requires heat for drying. Yano et al. (2008) studied a fermentation technique which helps in improving the quality of fish meal obtained from fish waste. Fast fermentation of squid by-products was also reported for low salt fish sauce production by Xu et al. (2008).

3.3.1.2. Fish Oil. There are two types of fish oil: body oil contained within the muscle of the fish and liver oil obtained from the liver and viscera. Pelagic fish that are rich in muscle oil are mackerel, herring, pilchard and certain types of sharks. Anchovy and horse mackerel are particularly high in omega-3 fatty acid. Other fish such as cod, haddock, hake, skate, ray and sharks contain high quantities of oil in their livers. Significant amounts of the fish oil produced are used for aquaculture (70-80% of all fish oil produced). The oil extracted from whole fish or processing waste is a mixture of both oil from the muscle and liver. Omega-3 fatty acids are found in relatively large amounts in all types of fish oil (Barlow and Young, 1988).

Fish oils are a source of long-chain polyunsaturated fatty acids (PUFAs), especially cis-5,8,11,14,17-eicosapentaenoic acid (EPA; C20:5) and cis-4,7,10,13,16,19-docosahexaenoic acid (DHA; C20:6) (Simopoulos, 1991). However, EPA and DHA cannot be synthesized by the human body and must be obtained from the diet (Linko and

Hayakawa, 1996). Omega-3 fatty acids reduce the risk of coronary heart disease and appear to reduce susceptibility to inflammatory, allergic and immune disease (Pike, 1999).

3.3.1.3. Silage. Fish silage is produced from a large variety of fish species and used as animal feed. It is a liquid product with a high nutritional value equal to that of fish. Silage is an effective alternative for fish meal and the production process for silage is simple, fast and economical when compared to fish meal production (Kompiang, 1981; Beerli et al., 2004). Silage is produced either by natural enzymatic and microbial liquefaction or by the addition of acid (Archer et al., 2001). Acid is added to minced or chopped fish (Acid silage), which helps lowering the pH adequately to avoid microbial spoilage. Due to the degradation of fish tissue structures by enzymes, the fish becomes liquid. In bacterial fermentation, minced or chopped fish is mixed with a fermentable sugar, which helps in the growth of lactic acid bacteria (Opara and Al-Jufiaili, 2006). Bio-ensiling production has advantage over chemical ensiling production because it is a simpler and fast process, environmentally friendly and cost effective (Opara and Al-Jufiaili, 2006).

Alwan et al. (1993) studied the production of fish silage from fresh fish waste using formic acid to reduce the pH to 3.5 and maintain it for up to 30 days. The results showed that the ensiling process caused an initial decrease in the total number of bacteria in the first 48 h followed by a gradual decrease. The silage viscosity showed greater decrease at pH 3.5. Raghunath and Gopakumar (2002) reported that higher value organic acids such as acetic, lactic and propionic acid stimulated the bioconversion of fish performed by lactic acid bacteria.

3.3.1.4. Fertilizer. Fish meal based fertilizers are used along with balanced sources of nitrogen, phosphorous and potassium. Liquid fish fertilisers are also used and they are produced by mixing whole fish or fish processing waste with sulphuric acid which releases sulphates and phosphates, thereby reducing fishy odours. Alternatively, the addition of urea solubilises the fish proteins, which become available nitrogen to plants after being broken down by bacteria in the soil (Chitralekha et al., 2000). The advantage of using liquid fish fertilizer over modern fertilizers is that it does not leach out readily to

the soil and releases the nutrients slowly to the plant (Dao and Kim, 2011). Another source of fertiliser comes from the anaerobic digestion of fish waste where methane and sludge are produced. The sludge is used as an organic fertilizer whereas the methane is used to generate electricity or heat (Archer et al., 2001).

Dao and Kim (2011) studied the scale-up conversion process of fish waste to liquid fertilizer in a 5 L ribbon-type reactor and the biodegradation was performed by inoculating autoclaved fish waste with mixed microorganisms for 96 h. The results showed that after 96 hours, the culture of the inoculated fish waste possessed equivalent fertilizing ability to commercial fertilizers in hydroponic culture and that the biodegraded broth of fish waste did not undergo putrefaction for 6 months at room temperature due to the addition of 1% lactate.

Zhuang et al. (1996) stated that long term preservation of liquid fertilizers is required to obtain higher value. They suggested that maintaining the quality of liquid fertilizers during circulation period can be achieved by lowering the pH which helps in lowering the growth of microbes and spoilage. Kim et al. (2010) reported on the potential conversion of fish waste into plant fertilizer using proteolytic bacteria.

3.3.1.5. Fish Proteins. Fish protein hydrolysate (FPH) is a powdered product, typically cream in colour. It is produced by breaking down fish proteins into amino acids by enzymes (proteolysis). Whole demersal fish or frames are the favoured raw material for FPH production (Shahidi and Venugopal, 1993). Although the technology is available to utilise pelagic fish, the species have a high content of oil, which must be removed in order to prevent strong flavours forming in the product. Fish protein concentrate (FPC) is a highly nutritious powdered product with a protein concentration higher than that of the original fish. FPC are categorised into three grades: type A, type B, type C. Type A is a tasteless, odourless white powder, type B retains a fishy flavour and odour and type C is essentially fishmeal (Mackie, 1982).

Batista (1999) studied the recovery of proteins from hake and monkfish by chemical extraction and precipitation with HCl and sodium hexametaphosphate. He evaluated the effects of pH, type of alkaline solution used [NaOH or $Ca(OH)_2$], concentration of NaCl, ratio of extracting media to raw material, and temperature on the percentage of protein solubilised. The results indicate that high protein isolates can be obtained from hake-filleting. Faid et al. (1997) used fermentation to produce fish protein for use in animal feeds. Nurdiyana et al. (2008) studied the optimization of protein extraction from freeze dried fish waste from sardines (*Sardina pilchardus*). The optimal protein yield was 83.51 mg/ml.

3.3.1.6. Compost. Composting uses micro-organisms to convert fish waste and plant material into useful soil enhancers. Highly nitrogenous fish material such as aquaculture mortalities, viscera, frames, whole oily fish and shellfish waste can be used. These materials are relatively rich in protein and putrefy rapidly. Composting takes about 4-6 weeks during which the waste is converted by micro-organisms into rich humus. The final compost product is generally rich in organic matter (40-70%) and contains 1-4% nitrogen.

Fish based compost can be commercially produced, particularly in areas with large quantities of fish and forestry waste and the heat produced during the process used to heat commercial greenhouses (Archer et al., 2001). According to Liao et al. (1997), the utilization of fish waste in composting is a viable environmentally friendly solution.

Liao and Fetcho (2008) studied the effects of two bulking agents (alder and fir) and two amendments (peat moss and vermiculite) on the in-vessel composting process. The temperature rise which occurred as composting progressed was accompanied by an increase in ammonia and volatile fatty acid production in the composting bay. The results showed that fir was a very good bulking agent because its compost became stable earlier than the others. Peat moss was found to be a good bulking agent and vermiculite was a good amendment.

Murthy et al. (2009) studied the preparation of fish compost from waste of deep sea fish, *pricanthus hamrur*, along with coffee husk as the bulk material. The effect of fish compost on the growth and survival of Indian major carp (*Labeo rohita*) and in the production of phytoplankton and zooplankton was evaluated. The results showed that there was significantly higher growth of *Labeo rohita* and also higher and sustained plankton production.

3.3.2. Non-Nutritional Utilization of Fish Processing Waste

3.3.2.1. Enzymes. Biological catalysts that speed up the biochemical reactions are known as enzymes. A range of protease (protein splitting) enzymes including pepsin, trypsin, chymotrypsin, collagenases and calpains are found in the gut and viscera of demersal and pelagic fish, cephalopods and shellfish. Fish enzymes work at low temperatures and a range of neutral to alkaline conditions. Fish enzymes are currently used commercially in fish processing applications to remove skin, scales and membranes that are otherwise difficult to remove. Cod pepsin is used to prevent off flavours developing during the ageing of cheddar cheese. Fish enzymes are used in baking, meat tenderisation, milk and leather production, caviar, fish sauce and production of fish flavours (Archer et al., 2001).

Fish waste can be used to recover crude fish pepsin and a low molecular weight peptone fraction using ultrafiltration. Atlantic cod possesses a high stomach pepsin content all year around (Gildberg, 1992). According to Gildberg (1988) pepsin from cold water fish are active at low temperatures and are used for gentle enzymatic processing of some fishery products. Castillo-Yanez et al. (2005) studied the isolation and characterization of trypsin from *pyloric caeca* of Monterey sardine *Sardinops sagax caerulea*. Purification was carried out by fractionation, gel filtration, affinity and ionic exchange chromatography. From the result it was shown that the optimum pH was 8.0 and the molecular mass of the isolated trypsin was 25kDa. The trypsin from the Monterey sardine had similar characteristics to that of other fish, particularly trypsins from the anchovy *Engraulis japonica* and the sardine *Sardinops melanostica*.

Esakkiraj et al. (2009) produced extracellular protease using *Bacillus cereus* obtained from fish guts. Different preparations of tuna processing waste such as defatted fish meat, raw fish meat, alkali hydrolysate and acid hydrolysate were used as the nitrogen source. Among these preparations, defatted fish meat supported the maximum protease production. Linnaeus (2011) studied the extraction and characterization of protease from the viscera of skip jack tuna fish using potassium phosphate 20 mM and precipitated using cold acetone (using ratios of 1:1 and 1:2 protease extract to cold acetone) and ammonium sulfate. The enzyme showed highest activity when precipitated with cold acetone in a ratio of 1:2. This enzyme was stable at pH 7.0 and less stable at pH 10.

Daboor et al. (2012) studied the isolation and activation of collagenase from fish processing waste using a Tris buffer system. The results showed that collagenase enzyme was produced as a latent enzyme and it could be activated with bovine trypsin and potassium thiocyanate.

3.3.2.2. Chitin and Chitosan. Chitin is a naturally occurring non-toxic, biodegradable polymer which is found in abundance in nature and can be industrially manufactured from shellfish waste. The chitin source should be processed as soon as possible to prevent microbial and enzymatic spoilage. After extraction, chitin is converted into a more readily usable form called chitosan which is used in many industries including: water treatment, cosmetics, food and pharmaceuticals. The majority of industrially produced chitosan is used in wastewater treatment to remove heavy metals, pesticides and dyes from contaminated water. In effluent treatment, chitosan is used as a coagulating agent to remove proteins and aid in clarification. In the cosmetics industry, it is used in the development of hair and skin-care products. In paper production, chitosan is added to wet pulp in order to give high strength to the finished paper. In the food industry, chitosan is used to clarify fruit juices. Chitosan is incorporated into bandages to reduce inflammation and promote wound healing and purified chitosan is used in hypocholesterolemic treatment to reduce cholesterol levels in the blood (Archer et al., 2001).

Shrimp waste ensilation was carried out by Cira et al. (2002) for the recovery of value added by-products such as chitin. Palpandi et al. (2009) studied the extraction of chitin from the shell and operculum of *Nerita crepidularia* and conversion of chitin into chitosan through deactylation. The yields of chitin and chitosan were observed to be 23.91 and 35.43% and 31.14 and 44.29% for the shell and operculum, respectively. Das and Ganesh (2010) studied the extraction of chitin from trash crab (*Podophthalmus vigil*) and a demineralization step for the purification of chitin was carried out. The chemical demineralization method was replaced with organic acid produced from using *Lactobacillus plantarum*. The results showed that demineralization with lactic acid had the same effect as chemical demineralization. Percot et al. (2003) optimized chitin extraction process from shrimp shells and found that the demineralization could be achieved in 15 min at ambient temperature in an excess of 0.25 M HCl. Viarsagh et al. (2009) studied the preparation of chitosan from Persian Gulf shrimp shells and the effect of time on the degree of deacetylation. It was noted that deacetylation for 90 and 180 minutes resulted in 69.75% and 77.63%, respectively.

3.3.2.3. Carotenoid Pigments. Carotenoids are a group of pigments that give fish and shellfish their red and pink colouring. These pigments cannot be synthesised by the fish and shellfish themselves but are taken up in the diet. Carotenoid pigments are commercially extracted from shellfish waste during chitin and chitosan production. The main use of these pigments is to provide colour and to ensure maximum growth and development of fish and shellfish (Archer et al., 2001). The pricing of salmon, shrimp, rockfish and snapper is mostly related to the intensity of the red hue (Sacton, 1986). Astaxanthin from crustacean meal is known as an effective pigmenting agent when incorporated into formulated diets for the coloration of salmonids and crustaceans (D'Abramo et al., 1983).

Omara-Alwala et al. (1985) demonstrated that mono and diester astaxanthins were primary carotenoids in pigmented oil from heat processed crawfish waste using commercial soy, menhaden and herring oils. The acid ensilage treatment did not affect the quality of pigment enriched oil. It was noted that the fatty acid composition of oil

extractant was slightly modified by the pigment extraction process. There was a 2% decrease in the total saturated fatty acid and 4% decrease in total PUFA of concentrated crawfish pigments in soy oil.

According to Shahidi et al. (1998), shrimp waste is the most important natural source of carotenoids. Shrimp wastes can be used in the extraction of carotenoids using various organic solvents (ethanol, methanol, hexane and petroleum ether). The carotenoids obtained after extraction can be used in aquaculture feed formulation and the residue available after extraction can be used for the preparation of chitin and chitosan (Sachindra et al., 2006).

3.3.2.4. Biodiesel. Biodiesel fuel produced from the fats and oils of vegetables and animals is a substitute or an additive to petroleum derived diesel (Alcantara et al., 2000). Since the vegetable oils are higher in viscosity than petroleum derived diesel, it can lead to excessive deposition of carbon and the thickening of lubricating oil. This is a major drawback of diesel derived from vegetable oil, especially in cold regions and during cold seasons (Dunn and Bagby, 2000; Clark et al., 1983).

Kato et al. (2004) found that ozone treated fish waste oil is more efficient than the methyl esterificated vegetable oil and more suitable for diesel engines, especially at lower temperature during cold seasons. The diesel produced from fish waste shows significant changes in the emission of polluting gases like sulphur oxides, polyaromatic and carbon dioxide.

Refaat et al. (2008) studied the feasibility of producing biodiesel from waste oils to help reduce the cost of biodiesel and reduce waste and pollution coming from waste oils. It was observed that the best yield was obtained using a methanol/oil molar ratio of 6:1, potassium hydroxide as catalyst and temperature of 65°C for 1 hour.

Vijayaraghavan and Hemanathan (2009) studied the production of biodiesel from freshwater algae. Transesterification of algal oil was performed using ethanol in the

presence of potassium hydroxide and GC-MS was used to analyze the compounds present in crude biodiesel. The results showed that the lipid yield was determined to be 45% and the copper strip corrosion was less than that of class 1.

3.3.2.5. Pharmaceuticals and Chemicals. A range of high value chemicals can be produced from fish and shellfish. Antifreeze proteins are extracted from the blood of cold water fish. Deoxyribonucleic acid (DNA) is extracted and purified from cod, herring and salmon milt for pharmaceutical use. Squalene is a naturally occurring hydrocarbon found in some fish oils and is commercially extracted from shark livers and used to treat diabetes, tuberculosis and cancer and possesses anti-fungal and antioxidative properties, providing scope for other pharmaceutical uses (Anon, 1995).

Chun et al. (2011) studied the extraction of trypsin from defatted powder of mackerel viscera obtained using supercritical carbon dioxide and purification by series of chromatography. It was observed that the obtained trypsin was unstable below pH 5.0 and above 40°C but could be stabilized by calcium ion.

3.3.2.6. Collagen. Collagen is the most common protein in animal kingdom. The protein composition depends upon the uniqueness of the amino acids present in the fish. Most of the amino acids (80%) responsible for collagen are non polar amino acids like valine, glycine, alanine and proline (Gomez-Gillen et al., 2002; Kim et al., 1994). Collagen is isolated from the skin, bone and fins of fish. The collagens derived from fish are more in demand than bovine-derived collagen because of "mad cow disease", bovine spongiform encephalopthy (BSE). Collagens from fish sources are free from pathogens such as BSE (Djabourov et al., 1993).

Collagen, extracted from the swim bladders of sturgeon is used as the source of isinglass, which is used as a clarification agent in beer production. Other uses include gelatin production, nutritional supplements, sausage casings and in cosmetic products because of its anti-ageing properties (Archer et al., 2001).

Nagai and Suzuki (2000) studied the effective use of under-utilized resources and extracted type I collagen from fish skin, bone and fins. The results indicated that the yield of skin collagen for production of collagen was 51.4% for Japanese sea-bass, 49.8% for chub mackerel and 50.1% for bullhead shark. The yield for bone collagen was 42.3% for skipjack, 40.7% for Japanese sea-bass and 43.5% for horse mackerel. The yield for fin collagen was 5.2% for Japanese sea-bass acid-soluble collagen and 36.4% for Japanese sea-bass acid-insoluble collagen.

Collagens are widely used as carriers in pharmaceutical and medicinal industries for delivering drug, protein and genes (Lee et al., 2001). Micro-fibrous collagen sheets are used to deliver drugs more efficiently in cancer treatment. Recent studies showed that collagens can be used to deliver genes with high accuracy for bone and cartilage formation (Sato et al., 1996; Nakagawa and Tagawa, 2000).

Collagen plays a vital role in the formation of tissues, organs and expression of cells. Medical investigations found that the ingestion of collagen hydrolysates reduced the pain of patients suffering from osteoarthritis and collagen hydrolysates are also used in cartilage matrix synthesis (Moskowitz, 2000). Collagen is advised as a supplement for facilitating bone integrity, nourishing hair scalp and for treating brittle nails (Kim and Mendis, 2006).

3.3.2.7. Gelatin. Fish collagen is a complex structural protein, which helps maintain the flexibility and strength of skin, bone, joints, muscle gum and teeth (Birk and Silver, 1984). According to Friess (1998), gelatin is derived from fish skin and bone but much less studied than the conventional gelatin from animals. Fish gelatin is a clear sweet solution with the capability to form gels and is produced by the hydrolysis of collagen at a much lower temperature and over a shorter time compared to collagen extraction to ensure the removal of impurities. Fish gelatin is used in food products, photographic processing and coating applications and in the chemical etching of metals (Archer et al., 2001).

Aberoumand (2010) studied the isolation of gelatin from limed bovine split (unused animal waste) wastes as a high value added product for use in the food and pharmaceutical industries. The concentrations of sodium hydroxide, sulphuric and acetic acid were investigated. It was observed that the highest yield of gelatin was obtained when a concentration of 3.5% (w/v) of both sulphuric acid and sodium hydroxide was applied to the fish skin, followed by treatment with 4% (w/v) acetic acid. Gomez-Guillen et al. (2005) studied the extraction of gelatin from fish skin using high pressure applied to the process at 250 and 400 MPa, for 10 or 20 min. The results showed that the use of high pressure to extract gelatin from fish skin was a useful alternative to the conventional procedure and the treatment time can be shortened by producing gelatin of high gelling quality in only a few minutes.

Sukkwai et al. (2011) studied the extraction of gelatin from bigeye snapper (*Priacanthus tayenus*) skin for gelatin hydrolysate production. From the result it was observed that protein content and hydroxyproline (Hyp) yield of skin gelatin increased with increases in extraction temperature and time. Degradation of the fish gelatin showed low bloom strength of gelatin gel and the solubility of gelatin was greater than 85% at all pHs investigated (1-10).

3.3.2.8. Anticoagulants. Heparin is chemically heterogeneous and exhibits poly-dispersion in its molecular weight ranging from 4,000 to 30,000 daltons and higher. Commercial heparin mucopolysaccharides are extremely soluble in water and insoluble in most organic solvents. Some proteins like protamines can interfere with its anticoagulant activity (Hollick et al., 1985). Anticoagulant (heparin) is used to prevent clotting and thrombus formation in cardiovascular diagnostic and surgical procedures, cardiopulmonary catheters, surgery of the heart and vessels, metal and plastic prostheses extra corporeal circulation, artificial organs and transplants (Bradshaw and Wessler, 1975). For decades, heparins have been used in the treatment and prophylaxis of thrombotic disease. Heparin is a potent anticoagulant and antithrombotic agent. Prophylactic agents such as heparin and heparin-like compounds are used widely against post-operative deep vein thrombosis since the introduction of heparins with low

molecular weight (LMW) which have greater bioavailability over the parent material (Crafoord and Jorpes, 1941).

Chondroitin sulphuric acid is a heparin-like compound, however it cannot be extracted easily from the horse liver source. Naturally acquired compounds including heparin result in a positive color reaction when analyzed for uronic acids (Erik, 1959). For the conformational studies of heparin, an NMR spectroscopy method is adapted for use, as the polysaccharide does not crystallize. Heparin has a well-defined solution conformation (Mulloy et al., 1993).

3.4. Glycosaminoglycans

The glycosaminoglycans (GAGs) are long unbranched polysaccharides, which contain repeated disaccharide units. The disaccharide units may contain *N*-acetylgalactosamine or *N*-acetylglucosamine and uronic acids like iduronate or glucuronate. The glycosaminoglycans are highly negatively charged. They are mostly present in cell surfaces or in extracellular matrix. GAGs are highly viscous, low in compressibility and are ideal for lubricating the joints. They include heparin, heparin sulfate, hyaluronic acid, dermatan sulfate, chondroitin sulfate and keratan sulfate (Jaques et al., 1966).

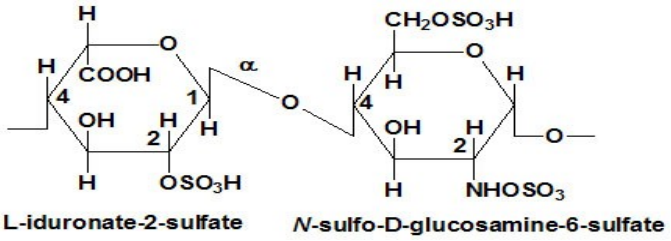
Glycosaminoglycans (GAGs) have been implicated in the regulation and maintenance of cell adhesion, motility, proliferation, differentiation, tissue morphogenesis and embryogenesis. The biological activities of GAGs depend on their ability to interact with a variety of proteins including growth factors, cytokines, enzymes, serine protease inhibitors and extracellular matrix proteins. For saccharide sequences embedded in GAG chains, such interactions are highly specific as the physiological functions of these proteins are regulated. GAGs are linear polymers with a polysaccharide backbone composed of alternating hexuronic acid residues and amino sugars. The repeat structure and extensive modifications of GAGs results in a considerable degree of variability involving sulfations and uronate epimerization. The basis for structural variability of GAGs and the wide variety of domain structures with biological activities are generated

by the elaborate concerted actions of biosynthetic enzymes (Kazuyuki and Hiroshi, 2000). Galactosaminoglycan chains are linked in different connective tissues such as tetrasaccharide β-D glucopyranosyluronate- β-D galactopyranosyl- β-D galactopyranosyl- β-D xylopyranose to the hydroxyl group of serine in the core protein (Roden, 1980). Also, very few chains of proteoglycans are attached to the same core protein (as in the cornea and skin). Large numbers of proteoglycan chains are attached as in cartilage. Cartilaginous proteoglycans are attached to hyaluronate with the support of non-covalent linkages forming large aggregates (Muir and Hardingham, 1975).

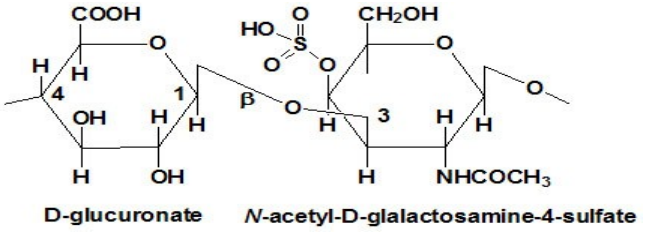
The GAG's are divided into four distinct classes of sulphated polysaccharides (heparin, dermatan sulphate and keratan sulphate) based on their interaction with the enzymes and inhibitors of the coagulation system (Figure 3.1) (Mourano et al., 1996; Pereira et al., 1999).

Heparin is a linear polysaccharide consisting of α-D-glucosamine with alternating uronic acid, its biosynthetic precursor is either β-D-glucuronic acid or α-L-iduronic acid. A more complex structure can be made with variations in its pattern by substituting with N- and O-sulphate and N-acetyl groups. By 1966 the first report of an NMR spectrum of heparin appeared (Jaques et al., 1966). Heparin consists of 1-4 linked pyranosyluronic acid (uronic acid) and 2-amino-2-deoxyglucopyranose (D-glucosamine, GlcN) repeating units (Casu, 1990). Heparin has a molecular weight range of 5-40 kDa with an average molecular weight of 12 kDa, and an average negative charge of about -75 (Linhardt and Toida, 1997).

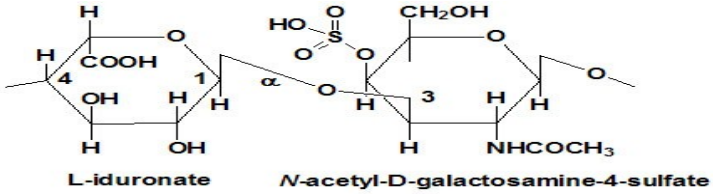
Chondroitin sulphates and dermatan sulphates possess polysaccharide chains with various types of repeated disaccharide units such as chondroitin sulphate chain of cornea. The dominant-repeating units in the cornea are chondroitin 4-sulphates that are polycondensed with a small proportion of chondroitin 6-sulphate, dermatan sulphate and chondroitin disaccharide units (Davidson and Meyer, 1954).



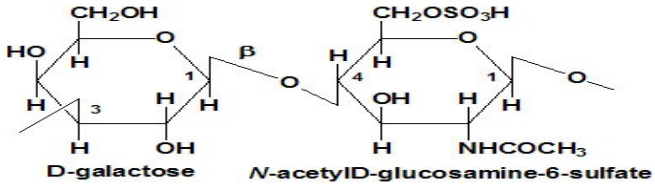
(a) Heparin



(b) chondroitin sulfate



(c) dermatan sulfate



(d) keratan sulfate

Figure 3.1. Structure of sulfated glycosaminoglycans (GAGs) (King, 2010).

Keratan sulphate I also known as keratosulphate, found in the cornea, cartilage, bone and the horns of animals (Manjusha, 2011). According to Meyer et al. (1953), keratin sulphate is a linear polymer of galactose and N-acteylglucosamine sulphated at the C6 of both hexose moieties. Unlike many other GAGs, keratin sulphate has variable sulphate content.

3.5. Application of Sulfated Glycosaminoglycans as Anticoagulants

Anticoagulants are widely used in the medicinal field for treating major death causing diseases like strokes and myocardial infarction. The major effect of hyper coagulation or the malfunction of coagulation cascade leads to various medical complication in humans such as thrombosis acute coronary syndrome and pulmonary embolism (Furie and Furie, 2008).

3.5.1. Thrombosis

Thrombosis is the formation of a thrombus (fibrin clot) inside blood vessel. The clot formed inside the blood vessel affects the proper circulation of blood in the body. Fibrin clots are formed when there is injury in the blood vessel to prevent excessive loss of blood and if it leads to extended formations of clots, the fibrins break free to form an embolus (Handin, 2005). Thus, the thrombus blocks 75% of the surface area of the lumen in the artery and as a result the blood flow to the tissues is reduced enormously, leading to loss of oxygen which in turn leads to cell death (Webster et al., 2005). There are two different forms of thrombosis based on the blood vessel where it forms: venous thrombosis and arterial thrombosis. These two forms of thrombosis have different subtypes based on the way of formation and severity of the formation. Anticoagulants can effectively prevent thrombosis and facilitate proper flow of blood in the body (Bruijn and Stam, 1999).

Rosen and Gelfand (2009) achieved antithrombin activity by using low molecular weight heparin (LMWH), which inactivates factor Xa. Factor Xa inhibitor (fondaparinux) inactivates factor Xa which prevented the conversion of prothrombin to thrombin.

3.5.2. Acute Coronary Syndrome (ACS)

Acute coronary syndrome is largely caused by thrombus formation on pre-existing plaque and has been shown in both autopsies and coronary angiography. There are two major mechanisms using either platelets or the plasma coagulation system, through which a thrombus is formed (Silva et al., 1998).

Rosen and Gelfand (2009) reported that in an ACS setting, the normal balance between thrombosis and endogenous fibrinolysis is disrupted in favor of thrombosis. And in addition to medications aimed at inhibiting formation of a platelet plug, anticoagulants such as unfractionated heparin, LMWH, direct thrombin inhibitors, and factor Xa inhibitors were beneficial in the treatment of ACS. Figure 3.2 shows main sites of action of antithrombin therapies.

Direct thrombin inhibitors are an important group of anticoagulants (Weitz and Buller, 2002). Heparin or its derivate, antiplatelet agents (aspirin and thienopyridine), are the most frequently used anticoagulant. Clinical studies have demonstrated that a combination of these agents can be used to treat acute or chronic thromboembolic complications (Hirsh et al., 2008). The important complication of haemorrhage, which may be serious and even life threatening is treated with anticoagulant (Mannucci and Levi, 2007). Wahlander et al. (2002) reported that melagatran, a synthetic thrombin inhibitor, which possesses pharmacokinetic properties, can be used in a fixed dose to delay blood clotting.

Clopidogrel and prasugreal belong to the thienopyridine derivatives and act by blocking adenosine diphosphate (ADP) receptors on the platelet (Yusuf et al., 2001). According to Fischer (2007), anticoagulation in hemodialysis is targeted to prevent activation of coagulation.

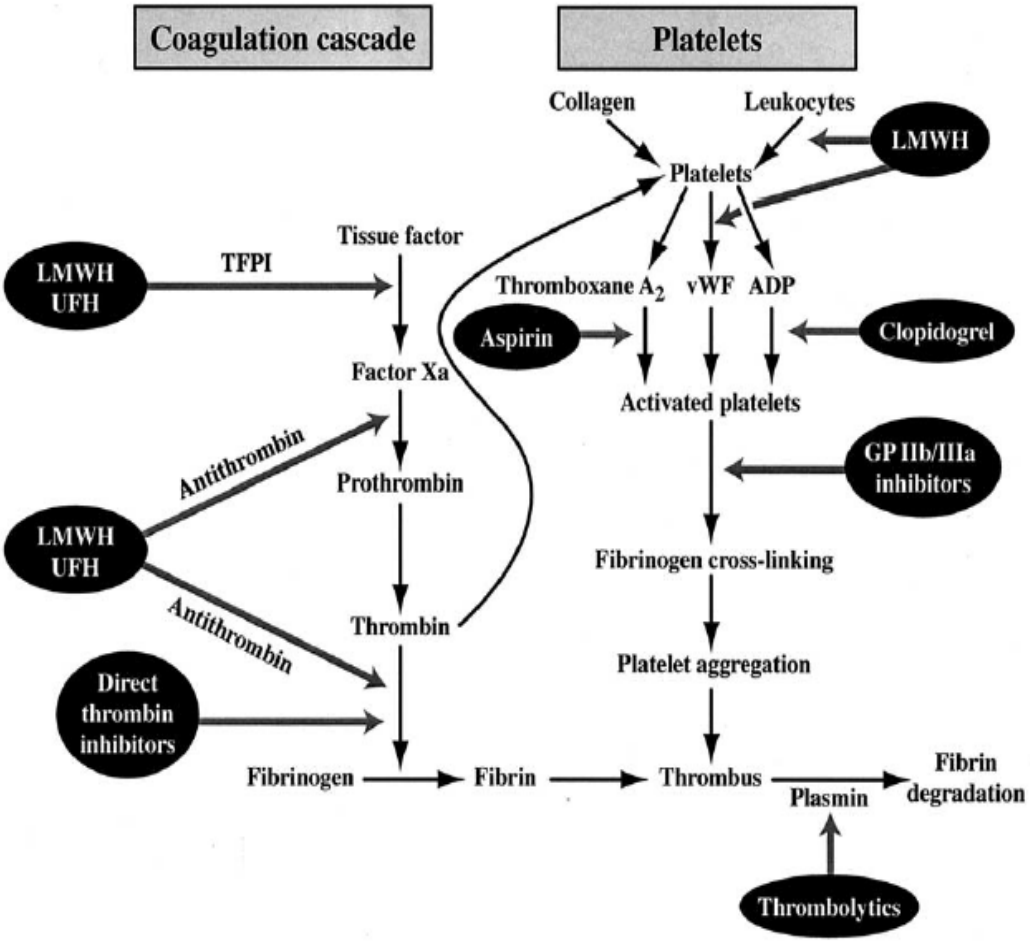


Figure 3.2. Main sites of action of antithrombin therapies (Rosen and Gelfand, 2009).

ADP - adenosine diphosphate.

GP - glycoprotein.

LMWH - low molecular weight heparin.

TFPI - tissue factor pathway inhibitor.

UFH - unfractionated heparin.

vWF - von Willebrand factor

3.6. Sources of Anticoagulant

The isolation of sulfated glycosaminoglycans from 23 species of invertebrate (from 13 phyla) has been reported by Guilherme et al. (2000). Bianchini et al. (1980) reported that heparan sulfate compound is present in all 23 species whereas chondroitin sulfate is present in 20 species. Glycosaminoglycans (GAG) have been largely extracted and characterized from terrestrial vertebrates compared to aquatic and marine vertebrates (Manjusha, 2011)

3.6.1. Vertebrate

Sulfated glycoaminoglycans have been extracted from vertebrates such as cow, pig, monkey, toad and frog, chicken and fish.

3.6.1.1. Cow: Achur et al. (2004) studied the identification of proteoglycans in bovine corneal tissue. From the result it was observed that structurally diverse chondroitin sulfate chains were present in bovine cornea and were mainly linked to decorin core protein. The corneal chondroitin sulfate proteoglycans differ in their chondroitin sulfate and dermatan sulfate contents. In chondroitin sulfate structure, the overall sulfate content is 4- to 6- position of sulfate pattern ratio.

Nakano et al. (2001) extracted chondroitin sulfate-peptide from bovine nasal cartilage. A procedure was used to extract chondroitin sulfate (CS) from the nasal cartilage without the addition of any chemicals except acetic acid which was used for pH adjustment. From the result it was observed that more than 70% of total was extracted by this endogenous enzymatic method. The purity of CS can be improved approximately 1.4 fold by anion-exchange chromatography.

Betty et al. (2000) studied the developmental expression of dermatan sulfate proteoglycans in the elastic bovine nuchal ligament. The changes in GAG's profile were investigated during nuchal ligament development in-order to identify proteoglycans.

Dermatan sulfate proteoglycans isolated from 230-day nuchal ligament were investigated and showed that glucuronate-rich copolymer was predominant.

3.6.1.2. Pig: Cigliano et al. (2011) studied the fine structure of glycosaminoglycans from fresh and decellularized porcine cardiac valves and pericardium. The fine structural characteristics of galactosaminoglycans chondroitin sulfate and dermatan sulfate were examined by FACE. From the results it is observed that galactosaminoglycans and hyaluronan were differently distributed between pericardium and valves and within the heart valves themselves before and after decellularization. The distribution of glycosaminoglycans was dependent from the vascular district and topographic localization.

Lovekamp et al. (2006) studied the stability and function of glycosaminoglycans in porcine bioprosthetic heart valves (BHV). To gain insight into the role of GAGs, properties of fresh and Glut-fixed porcine heart valve cusps before and after complete GAG removal were compared. Removal of GAG showed significant morphological and functional tissue alterations including decreases in cuspal thickness, reduction of water content and diminution of rehydration capacity. However, removal of GAGs did not alter calcification potential of BHV cusps.

Thornton et al. (1983) isolated glycosaminoglycans from pig colonic wall connective tissue. Enzymatic hydrolysis was carried out using papain followed by ethanol precipitation in the presence of Ca^{2+}. From the result it was observed that the content of glycosaminoglycan in the tissue was low (0.05% dry weight) which comprised of dermatan sulphate (38%), heparan sulphate (18%), heparin (34%) and chondroitin sulphates (10%) as a percentage of total glycosaminoglycan content.

3.6.1.3. Monkey: Stoeckelhuber et al. (2004) investigated the distribution of proteoglycans (PG) in the intervertebral disc of the rhesus monkey (*Macaca mulatta*). It was observed that the PG's were found free in the matrix in all regions of the intervertebral disc.

Acott et al. (1985) extracted glycosaminoglycans from cynomolgus monkey eye by sequential enzymatic degradation and cellulose acetate electrophoresis. From the result it was observed that the cynomolgus monkey eye GAGs included: 14.3% chondroitin- 4-sulfate, 15.2% dermatan sulfate, 42.1% keratan sulfate and 15.6% heparan sulfate.

Fujita and Okazaki (1992) studied the isolation of glycosaminoglycans in the lamina propria and submucosal layer of the monkey palatal mucosa. From the results it was showed that after chemical analysis, the GAG contents of the lamina propria and glandular zone were higher than that of the fatty zone. Dermatan sulfate, chondroitin sulfate and heparan sulfate were observed.

3.6.1.4. Toad and Frog: Pelli et al. (2007) studied the characterization of glycosaminoglycans from the ventral and dorsal integuments of the anuran *Bufo ictericus* (toad). The dermatan sulfate was the major metachromatic glycosaminoglycan found in these tissues, but a small amount of heparin sulfate was also detected. The isolated dermatan sulfate with an average molecular mass of 20 kDa was similar to the glycosaminoglycan isolated from mammalian skin.

Marcia et al. (1968) studied the isolation of glycosaminoglycans from adult frog back skin (*Rana catesbeiana)*. From the result it was observed that about 45% of the glycosaminoglycan content was dermatan sulfate (chondroitin sulfate B), 15% chondroitin 4 - 5 % sulfate (chondroitin sulfate A and/or C) and 15% may be a dermatan-like substance.

Otilia et al. (1997) studied the isolation of chondroitin sulfate in the oocytes of frog (*Rana ridibunda*). Distribution of chondroitin sulfate in cytoplasm of these oocytes was polarized. The microscopic findings indicated that chondroitin sulfate fluorescein isothiocyanate (FITC) was internalized by all developing oocytes of frog (*Rana ridibunda*).

3.6.1.5. Chicken: Nakano et al. (2001) extracted glycoaminoglycans from chicken eggshell. Eggshells were decalcified with acetic acid, followed by digestion with papain. The eggshell GAG consisted of approximately 48% hyaluronic acid and 52% galactosaminoglycan. Chondroitin sulfate and dermatan sulfate copolymers were the major galactosaminoglycans with dermatan sulfate disaccharide as a relatively minor component.

Harrisson et al. (1984) studied the presence of glycosaminoglycans in chicken embryo. The method of microinjection with glycosaminoglycan-degrading enzymes in the chicken embryo culture was used. From the results it was concluded that GAGs play the role in the maintenance of tissue spaces in the early chicken embryo.

Pane and Wegelin (1996) studied the qualitative and quantitative pattern of GAGs by electrophoresis in aging chicken brain. Four main GAGs have been identified: hyaluronate, condroitin sulfate, heparan sulfate and dermatan sulfate. It was concluded that in chicken brain the GAG percentage undergoes age-related changes.

3.6.1.6. Fish: Sakai et al. (2003) reported on the isolation of glycosaminoglycans from eel skin (*Anguilla japonica*) by actinase and endonuclease digestions, which were followed by an elimination reaction and DEAE-Sephacel chromatography. The major glycosaminoglycan in the eel skin was dermatan sulfate with 88% of the total uronic acid. The sequence in eel skin was IdoA2Sα1→4GalNAc4S content, which showed that the anticoagulant activity (through binding to heparin cofactor II) was two times higher than that of dermatan sulfate from porcine skin. The activity dermatan sulfate of anti-IIa activity of eel skin was 2.4 units/mg and that of the dermatan sulfate from porcine skin is 23.2 units/mg.

Mansour et al. (2009) studied the isolation of sGAG from skin of the ray (*Raja radula*) by papain digestion followed by cetylpyridinium chloride and ethanol precipitation and then subjected to gel chromatography and anion exchange chromatography. From these chromatography analyses, it was found that two negatively charged polysaccharides were

different in both molecular weight and charge. The two negatively charged polysaccharides isolated from skin of ray were dermatan sulfate and non-sulfated hyaluronic acid.

Uchisawa et al. (2001) studied the isolation of chondroitin sulfate from salmon nasal cartilage. The structure was identified by unsaturated disaccharide analysis. It was observed that the chondroitin sulfate was shown to be composed of ΔDi-0S/ΔDi-4S/ΔDi-6S = 8:31:61 disaccharides. The NMR showed that the binding between salmon nasal cartilage chondroitin sulfate and calcium ions that chondroitin sulfate bound selectively to calcium ions in the presence of both calcium and sodium ions.

Bernhardt and Schachner (2000) studied the presence of chondroitin sulfate in zebrafish embryo and observed that chondroitin sulfate was present at the interface of the somites and the notochord where spinal motor axons extend ventrally to establish the mid-segmental ventral motor nerves.

3.6.2. Invertebrate

Sulfated glycoaminoglycans were extracted from invertebrates like nematode, cockroaches, shrimp, molluscs and algae.

3.6.2.1. Nematode: Beebera and Fred (2002) isolated and characterized the GAGs from the wild type nematode *Caenorhabditis elegans*. It is used in the preparation for the characterization of the transgenic form of GAGs constructed by Link, which expresses various forms of b-peptide (or A4 peptide). Deposition formed by this peptide is very similar to the ones found in the neuritic plaques and neurofibrillary tangles in Alzheimer disease (AD).

Guerardel et al. (2001) studied the synthesis of unusual O-linked glycans from the nematode *Caenorhabditis elegans* using nuclear magnetic resonance and mass spectrometry. From the result it was noted that most of the glycans were characterized by

type I core substitute on Gal and/or Gal NAc which led to the isolation of GAG like components and oligomannosyl-type N-glycans.

Schimpf et al. (1999) studied the distribution pattern of glycosaminoglycans during aging in the nematode *Caenorhabditis elegans*. From the results it was observed that chondroitin-4-sulphate was present in the epicuticula, chondroitin-4-sulphate and dermatan sulphate in the mesocuticula and heparan sulphate in the terminal web of intestinal cells.

3.6.2.2. Cockroaches: Andre et al. (2006) isolated and characterized the sulfated glycosaminoglycans (GAGs) in thoracic muscle, fat body, whole digestive tract (stomach and intestine) and reproductive tract of adult male cockroaches (*Periplaneta Americana*). From the tissues analyzed, 90% of the total sulfated GAG content was heparan sulfate (HS). Heparan sulfate was predominantly detected in both the thoracic muscle and fat body.

Ashhurst (1984) studied the isolation of glycosaminoglycans of the thoracic ganglia of the nymphal stages of the cockroach, *Periplaneta americana*, and the locust, *Locusta migratoria.* From the result it was observed that chondroitin sulphate was present only in neural lamella in the early nymphs, but keratan sulphate accumulated slowly in the later nymphs.

Francois (1978) studied the presence of glycosaminoglycans from mesenteric connective tissue of the cockroach *Periplaneta americana L*. (*Insecta, Dictyoptera*). From the result, glycosaminoglycans were observed by various histochemical reactions, in particular alcian blue staining, metachromasia and enzymatic digestion.

3.6.2.3. Shrimp: A heparin-like compound with anti-inflammatory properties was isolated from the shrimp *Litopenaeus vannamei* by Brito et al. (2008). In addition to significantly reducing the influx of inflammatory cells to the injury site in a model of acute inflammation, the heparin-like shrimp compound was able to reduce the matrix

metalloproteinase (MMPs) activity in inflamed animals. The compound reduced almost 90% of the activity of MMP-9 secreted by human activated leukocytes and exhibited negligible anti-coagulant activities in APPT assay. A poor bleeding potential makes this compound a better alternative than mammalian heparin as a possible anti-inflammatory drug.

Regatieri et al. (2009) studied the isolation of heparin-like glycosaminoglycans from marine shrimp and evaluated the effects of this new compound on a laser-induced choroidal neovascularization (CNV). From the results it was observed that there was a significant reduction in CNV lesion area of all groups treated with heparin-like glycosaminoglycan obtained from marine shrimp.

Cahu et al. (2012) recovered glycosaminoglycans from Pacific white shrimp (*Litopenaeus vannamei*) processing waste. From the result it was observed that the sulfated glycosaminoglycans recovered were similar to mammalian standards and the degradation products observed the presence of C6- sulfated heparan sulfate. Isolating highly bioactive molecules, such as sulfated- and amino-polysaccharides, with a broad spectrum of applications from shrimp processing waste were possible.

3.6.2.4. Molluscs: Guoyun et al. (2011) purified glycosaminoglycan-like sulfated polysaccharide (AAP) from molluscs (pleopods of Haliotis discus hannai Ino) by DEAE ion exchange chromatography followed with S-300 HR geltrion chromatography. It was observed that the chemical composition of AAP was composed of galactosamine, glucuronic acid, fructose and galactose, the content of sulfate was 15.5%.

Volpi (2006) studied the presence and structural characterization of glycosaminoglycans from molluscs. Variable amounts of sulfated and non-sulfated glycosaminoglycans were detected and measured. The results showed that heparin can be extracted from different clam species.

Qingman et al. (2012) optimized glycosaminoglycan extraction from scallops (*Patinopecten yessoensis*) waste using response surface methodology. From the results the order affecting glycosaminoglycan extraction rate was determined as the enzymatic pH > solid-liquid ratio > enzymatic time > enzymatic temperature. Also, the optimal conditions of extraction were obtained with the pH of enzymatic hydrolysis at 8.0, enzymolysis temperature of 40ºC, enzymatic time of 3.5 h and solid-liquid ratio of 1:2.

A high anticoagulant activity heparin (activated partial thromboplastin time of 347±56.4 sec and anti-Xa activity of 317±48.3 sec) was isolated from a marine clam species by Cesaretti et al. (2004). Agarose-gel electrophoresis resulted in a high content of the slow-moving heparin component (22±6.8%) and 78±5.4% of the fast-moving species. Using PAGE analysis, an average molecular mass of 13,600 Da was calculated. Depolymerizing heparin samples with heparinase (EC 4.2.2.7) for structural analysis followed by separating the resulting unsaturated oligosaccharides by strong anion exchange–HPLC revealed the presence of large amounts (130% more than standard pharmaceutical heparin obtained from bovine intestine) of the oligosaccharide sequence bearing part of the ATIII-binding region, DUA2S (1→4)-a-D-GlcN2S6S (1→4)-a-L-IdoA (1→4)-a-D-GlcNAc6S (1→4)-b-D-GlcA (1→4)-a-D-GlcN2S3S6S in the *T. phylippinarum* heparin.

3.6.2.5. Algae: Mohsen et al. (2007) isolated crude water-soluble sulfated polysaccharides (SP) from brown algae *Surgassium latifolium* by hot water extraction followed by ethanol precipitation. Approximately 4.75, 4.39, 5.11 and 3.96% of dried *Surgassium latifolium* was obtained from the extraction of crude water-soluble polysaccharides. Fractionation of polysaccharide by anion exchange chromatography and gel filtration chromatography, gave rise to three fractions termed SP-I, SP-II and SP-III. It consisted of glucouronic acid, mannose, glucose, xylose and fructose with molar ratios of 3.2: 1.0: 6.0: 2.0: 2.0; 4.0: 1.0: 5.6: 1.4: 1.8 and 5.1: 1.0: 4.7: 1.7: 2.2, respectively.

Zhang et al. (2008) extracted a sulfated polysaccharide from the green algae *Monostroma latissimum* in hot water and purified it by ion exchange and size-exclusion

chromatography. Five sulfated polysaccharide fragments were prepared from the sulphated polysaccharide with different molecular weights by H_2O_2 degradation. The parent sulfated polysaccharide and its fragments had molecular weights of 725.4, 216.4, 123.7, 61.9, 26.0 and 10.6 kDa, respectively. These sulfated polysaccharide preparations contained rhamnose and had anticoagulant activities similar to the parent sulfated polysaccharide. The molecular weights of the sulfated polysaccharide fragments were in the range 216.4–61.9 kDa.

WF1 and WF3 were two sulfated polysaccharides that were isolated from marine green algae *Monostroma nitidum* by Mao et al. (2008). Using assays of the activated partial thromboplastin time (APTT), thrombin time (TT), prothrombin time (PT), antithrombin and anticoagulation factor Xa activities, the anticoagulant activities of WF1 and WF3 were determined. The results show WF1 and WF3 have similar high contents of rhamnose, whereas their sulfation positions, sulfate contents, linkage patterns and molecular sizes of rhamnose residues were different. WF1 and WF3 have high anticoagulant activities, and are potent thrombin inhibitors mediated by heparin cofactor II. They also hasten coagulation factor Xa inhibition and thrombin using potentiating antithrombin III.

Mao et al. (2009) isolated polysaccharides from the marine green algae *Monostroma latissimum*. It was mainly composed of 1, 2-linked l-rhamnose residues with sulfate groups substituted at positions C-3 and/or C-4. High anticoagulant activities were exhibited by the sulfated polysaccharide using the activated partial thromboplastin time (APTT) and thrombin time (TT) assays.

3.6.3. Comparative Analyses

Gomes and Dietrich (1982), studied the distribution of heparin and sulphated glycosaminoglycans in vertebrates (chicken, snake, lizard, frog, fish and shark) and observed that same tissues from different vertebrates had similar types of sulfated

glycosaminoglycans but with different molecular weight. However, heparin was observed in only few tissues of six different vertebrates.

Yamada et al. (2011) stated that sulfated glycosaminoglycans were distributed widely from very primitive organisms to humans. Varied sulfation structures were revealed from lower animals such as *H. magnipapillata* and *C. Elegans*. Their studies showed that the structural complexity of chondroitin sulfate/Dermatan sulfate (sGAG) from lower organisms is limited and chondroitin sulfate might have complex functions in higher organisms.

Yamada and Sugahara (2008), compared the structural complexity of GAGs and found that higher animals do not necessarily produce more highly sulfated GAGs. Vieira et al. (1991) observed that GAGs from sharks, squid, mollusks, hagfish, king crabs, sea cucumbers and ascidians were considered as over-sulfated complex structures.

Silva et al. (2001) stated that generally aquatic species contains more structural distribution in their GAGs than terrestrial animals. The GAGs obtained from mollusks have complicated structures with a variety of modifications which includes high sulfation, sulfation at unusual positions, fucosylation, uronate epimerizaton, and glucosidation. In vertebrates, sGAG were majorly found in skin, lung and intestine.

3.7. Glycosaminoglycans Extraction Procedures

3.7.1. Enzymatic Extraction

Exogenous enzymes like papain and pronase can be used to liberate whole glycosaminoglycan (GAG) from the tissues. Hydrolysis of tissues with papain or pronase produces single chain GAG attached with small peptide containing several amino acids (Silva, 2006).

Nakano et al. (1996) decalcified porcine longissimus dorsi epimysium muscles by incubating in 8 ml of 25% glacial acetic acid at 21 °C for 24 h. The decalcified sample

was digested with crystallized papain twice in 1:250 ratios (enzyme:sample ratio) in a 0.1M sodium acetate buffer, 0.005 M disodium EDTA, 0.005 M cystein hydrochloride and 0.02% sodium azide at pH 5.5 and 60 °C overnight. After proteolysis, cold tricholoroacetic acid was added and the sample was kept for 2 h at 4 °C. Precipitated protein was removed by centrifugation and the supernatant was dialyzed in running tap water for 24 h and for another 24 h in deionised water at 4 °C. GAG's were precipitated as a cetylpyridinium-GAG complex and washed with 0.4 M and 2.1 M NaCl to obtain two fractions.

Digestions with chondroitinase-ABC, chondroitinase-ACI and chondroitinase-ACII were carried out with 0.005 unit of each enzyme per mg of uronic acid in 0.01 M sodium acetate buffer containing 0.02% sodium azide for 1 h at 37°C. The pH for buffer was 6.0 for chondroitinase-ACII, 7.3 for chondroitinase-ACI and 8.0 for chondroitinase-ABC (Nakano et al. 2001). Figure 3.3 shows a schematic representation of enzymatic extraction for GAG isolation.

Mansour et al. (2009) extracted crude polysaccharide using enzymatic extraction from fish skin. About 5 g of sample was dissolved in 250 ml sodium acetate 0.1 M, 5 mM EDTA, 5 mM cystein. 510 mg papain was added and incubated for 24 hrs at 60°C. The mixture was filtered and the residue was washed with 138 ml distilled water and filtered again. The filtrates were combined and precipitated with 20 ml cetylpyridinium chloride (CPC) 10%. The mixture was incubated for 24 hrs at room temperature and centrifuged for 30 min at 5000 rpm at 4°C. The pellet was washed with 610 ml CPC and then dissolved in 172 ml NaCl solution in ethanol. The polysaccharide containing solution was incubated for 24 hrs at 4°C and then centrifuged at 5000 rpm for 30 min. The pellet collected was washed twice with ethanol and then redissolved in desionised water and lyophilised.

Oguri et al. (1987) extracted GAG from rabbit bone marrow by using enzymatic extraction using pronase. The defatted tissue sample was digested with preincubated pronase for 1 hr at 50°C. To this mixture, 2 mmol/L of $CaCl_2$ and 0.1 mol/L of Tris-HCI

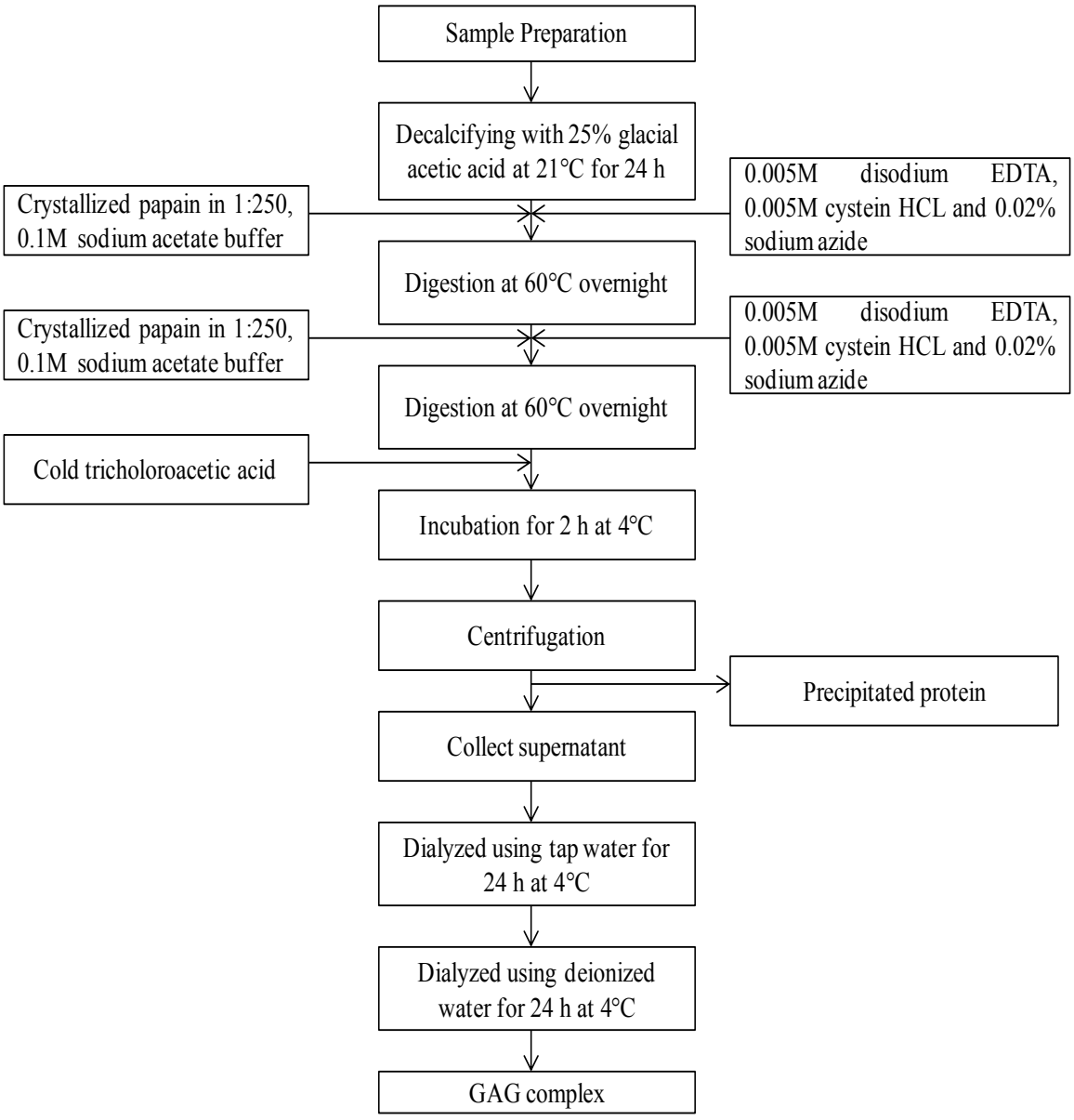


Figure 3.3. Schematic representation of enzymatic extraction for GAG isolation (Nakano et al. 2001).

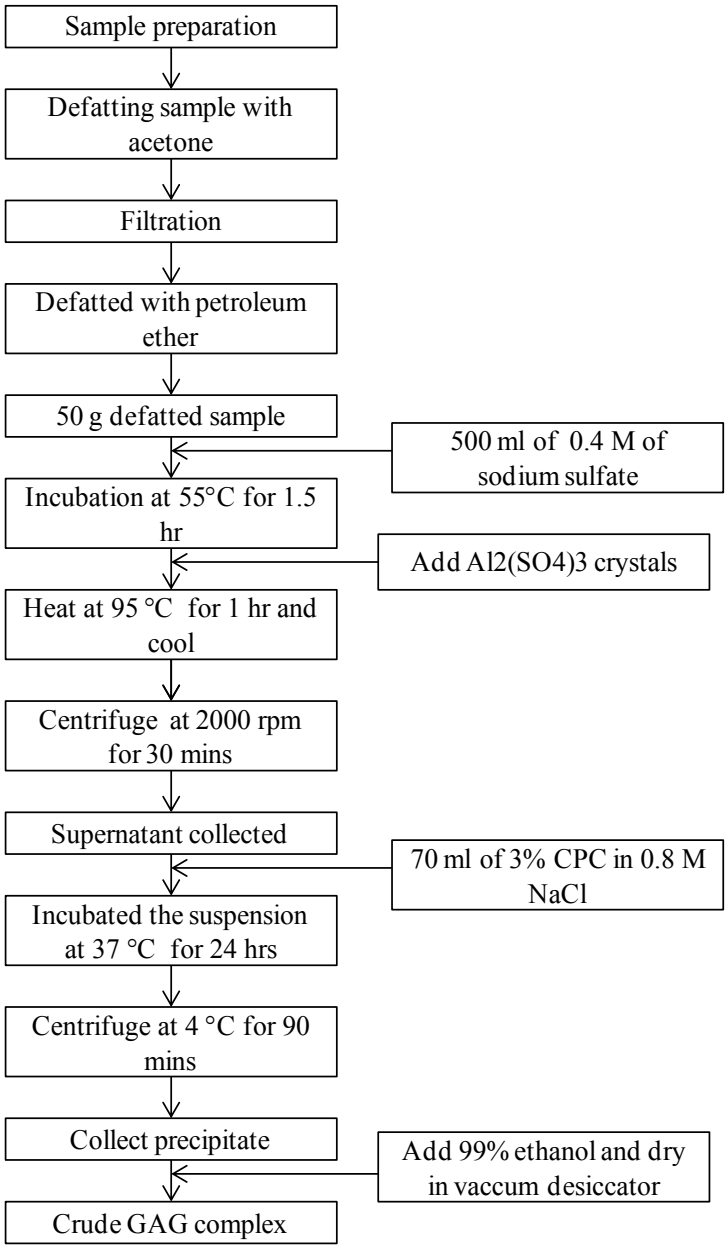
(pH 8.0) was added and incubated at 50°C for 70 hours with two further additions of the same amount of the enzyme pronase at 24-hour intervals. From the result it was observed that chondroitin sulfate isomer in the bone marrow is chondroitin 6-sulfate.

3.7.2. Chemical Extraction

GAGs are highly negatively charged in nature and are precipitated using positively charged chemicals. GAGs have been precipitated using quarternary ammonium compounds like cetylpyridinium chloride (CPC) or cetyltrimethylammonium bromide (CTAB) and ethanol in the presence of sodium or potassium acetate (Taniguchi, 1982).

Arumugam et al. (2009) extracted glycosaminoglycans from bivalve molluscs (*Tridacna maxima* and *Perna viridis)* by defatting the whole sample with acetone, filtering and further defatting with petroleum ether and then air drying at room temperature. About 50 g of defatted sample were mixed with 500 ml of 0.4 M sodium sulphate (Na_2SO_4) and incubated at 55°C for 1 hr and 30 mins (pH was maintained at 11.5). After incubation, aluminium-di-sulphate $Al_2(SO_4)_3$ crystals were added to bring down the pH to 7.7 and heated to 95°C for 1 hr, cooled and centrifuged at 2000 rpm. After centrifugation the supernatant was collected and 70 ml of 3 % CPC in 0.8 M sodium chloride (NaCl) was added and the mixture was stirred well. This suspension was incubated at 37°C for 24 h and centrifuged at 4°C for 90 mins in a refrigerated centrifuge to collect the crude GAG complex. The precipitate was also washed with 99.9% ethanol and then dried in vacuum desiccators to obtain the crude GAGs complex. Figure 3.4 shows schematic representation of chemical extraction of glycosaminoglycans.

Yoon et al. (2007) extracted glycosaminoglycan from brown alga (*laminaria cichorioides*) by immersing the tissue sample in MeOH and incubating at room temperature for 24 hrs. The mixture was filtered and the residual material was extracted with 0.4% HCL at room temperature for 4 h, after which the supernatant was collected. The second extraction was carried out with 0.4% HCL to 50ºC for 5 hrs, filtered and combined. The supernatant



3.4. Schematic representation of chemical extraction of glycosaminoglycans.

containing water-soluble polysaccharide was precipitate with 3:1 ratio MeOH:1-butanol for 24 hrs and centrifuged for 20 min at 6500 rpm. After drying, the precipitate was dissolved in water and acidic polysaccharide was precipitated with 0.5M cetyltrimethylammonium bromide. The resulting precipitate was collected and re-dissolved in 3M $CaCl_2$ at 37 ºC for 48 hr. To the solution four volumes of ethanol was added and the resulting precipitate was dissolved in water followed by dialysis against running water for two days. The non-dialyzable portion was centrifuged at 6500 rpm and the supernatant was lyophilized giving rise to the crude polysaccharide.

3.8. Parameters Affecting Enzymatic Extraction

3.8.1. Enzyme Concentration

Garnjanagoonchorn et al. (2007) used papain at the concentration of 4 mg/g to study the enzymatic extraction of chondroitin sulfate from different sources of cartilage. Results indicated that chicken keel, crocodile hyoid and sternum cartilage yielded higher chondroitin sulfate with values ranging from 11.55 to 14.84 g/100 g of dried cartilage. Shark cartilage and crocodile trachea cartilage, yielded lower value ranges from 9.5 to 9.6 g/100 g of dried cartilage.

Manjusha (2011) used papain at the concentration of 20 U/g dry weights to isolate GAG from species of cephalopods and reported that the yield of crude GAG from cranial cartilage was 8.09 g% and the gladius of cephalopods species gave very low yield.

Alicia et al. (2006) studied the optimization of extraction of glycosaminoglycans from normal and osteoarthritic cartilage. The enzyme concentration used was about a 1% papain suspension which resulted in the isolation of chondroitin sulfate A, B and C-like isomers at 8.5%, 0.9% and 90.4%, respectively from human osteoarthritic cartilage samples.

3.8.2. Digestion Time

Manjusha (2011) studied the isolation of GAGs from species of cephalopods, *Loligo duvauceli* and *Sepia pharaonis* for 72 h in which fresh papain was added every 24 h. From the results it was observed that a pale yellow solution was obtained after 72 h of digestion.

Garnjanagoonchorn et al. (2007) studied the isolation of chondroitin sulfate from different source of cartilage for 48 h and obtained a clear solution after 48 h.
Farias et al. (2000) studied the structure and activity of anticoagulant sulfated galactans from the red algae *Botryocladia occidentalis* and compared them with those of invertebrates. The digestion time used was 24 h.

Mansour et al. (2009) extracted GAG from skins of ray *Raja radula* using papain with digestion time of 24 h. It was observed that the whole polysaccharide from dried skin was approximately 1%.

3.8.3. Temperature

Cesaretti et al. (2004) studied the isolation of heparin from clams (*Tapes phylippinarum*) at the incubation temperature of 60°C and observed that the yield was approximately 2.1 mg heparin/g of dry animal. Pereira et al. (2005) studied the structure and anticoagulant activity of sulfated galactan from red algae (*Gelidium crinale*) using an incubation temperature of 60°C. From the results it was observed that the structure of the polysaccharide was composed of repeating structure-4-α-Galp-(1-3)-β-Galp1- but with a variable sulfation pattern. It was also observed that 15% of the total α-units were 2,3-disulfated and another 55% are 2-sulfated.

Mitropoulou et al. (2001) studied the identification, quantification and structural characterization of glycosaminoglycans from uterine leiomyoma and normal myometrium at the incubation temperature of 60°C. A significant difference in

glycosaminoglycans content (HA -27.7%, DS 158.5%, KS 116.4% and HS 52.5%) was detected between uterine leiomyoma and normal myometrium.

Himonides et al. (2011) studied the enzymatic hydrolysis of fish frames using a pilot plant scale system at an incubation temperature of 40°C and it was observed that more than 84% of protein was hydrolysed in 60 min but when the temperature was further increased and held at 78°C, the mixture was not only hydrolysed completely but also pasteurised.

Heaney-Kieras et al. (1977) reported that 0.3-0.5 mg/ml of polysaccharide were obtained from red alga (*Porphyridium cruentum*) at the incubation temperature of 65°C.

3.8.4. pH

Santos et al. (2007) studied the isolation and characterization of heparin with low antithrombin activity from *Styela plicata* at the pH of 5.5 and observed that heparin was composed of 47.5% of di-sulfated disaccharide, 38.3% of tri-sulfated disaccharide and 8% of smaller 3-O –sulfated disaccharide. Nakano et al. (2001) studied the extraction of GAG from chicken eggshell at pH of 5.5 and observed that 0.024% of the dry weight was uronic acid. It was also observed that the eggshell GAG contained approximately 48% of hyaluronic acid and 52% of galactoaminoglycan (chondroitin sulfate-dermatan sulfate copolymers).

Luo et al. (2002) studied chicken keel cartilage as a source of chrondroitin sulfate at the pH of 6.5 and observed that 32.9 ± 4.8 mg (dry weight) of glycosaminoglycans was obtained after magnesium chloride extraction and it was also known that 75.5 ± 4.2 % of the glycosaminoglycans were chondroitin sulfate.

Himonides et al. (2011) reported that the rate of hydrolysis for papain was the highest at a pH of 6.5 and decreased with an increase in pH. According to Wasswa et al. (2007) papain is a neutral protease and the pH should be adjusted to 7.

3.9. Glycosaminoglycans Purification Procedure

Crude glycosaminoglycans can be purified by ion exchange chromatography and/or gel permeation chromatography.

3.9.1. Anion Exchange Chromatography

Ion exchange chromatography is the method for the purification of proteins and other charged molecules. In anion exchange chromatography, the negatively charged molecules attract to the positively charged solid support (Mohsen et al., 2007).

Vieira et al. (1991) described a chromatography purification procedure in which 200 mg crude extracts of GAG were applied to a Diethylaminoethyl cellulose (DEAE) –anion exchange chromatography column (7 x 2 cm) equilibrated with a 0.05 M sodium acetate buffer. 0.05 M sodium acetate buffer was used first to elute the column followed by 0.05 M sodium acetate buffer (containing 1:1 v/v 1.2 M NaCl and finally up to 2.0 M NaCl). The column flow rate was 12 ml/h and fractions of 3 ml were collected. GAG content was analysed by a 1,9-dimethylmethylene blue (DMMB) dye binding assay and finally the column was washed with a four bed volume of 0.2 M NaCl. Falshaw et al. (2000) reported that identification of constitutive disaccharides obtained from digestion using chondroitinase ABC could be determined by analytical strong anion exchange (SAX) HPLC. SAX-HPLC was performed using continuous gradient elution with solvent A (NaH_2PO_4 buffer 0.001 M, pH 6 containing 0.15 M NaCl) and solvent B (NaH_2PO_4 buffer 0.001 M, pH 6 containing 2.0 M NaCl). From 0 to 4 min, the column was eluted with NaH_2PO_4 buffer. Then, from 4 to 15 min, NaCl concentration was increased to 0.47 M. Then, from 15 to 18 min NaCl concentration was increased to 2.0 M. The column flow rate was 1 ml/min and disaccharides were identified using retention time. The obtained peak areas of all samples were compared with standard disaccharides as described by Vieira et al. (1991). Figure 3.5 shows the schematic representation of the chromatography method of GAG purification and analysis used by Vieira et al. (1991) and Falshaw et al. (2000).

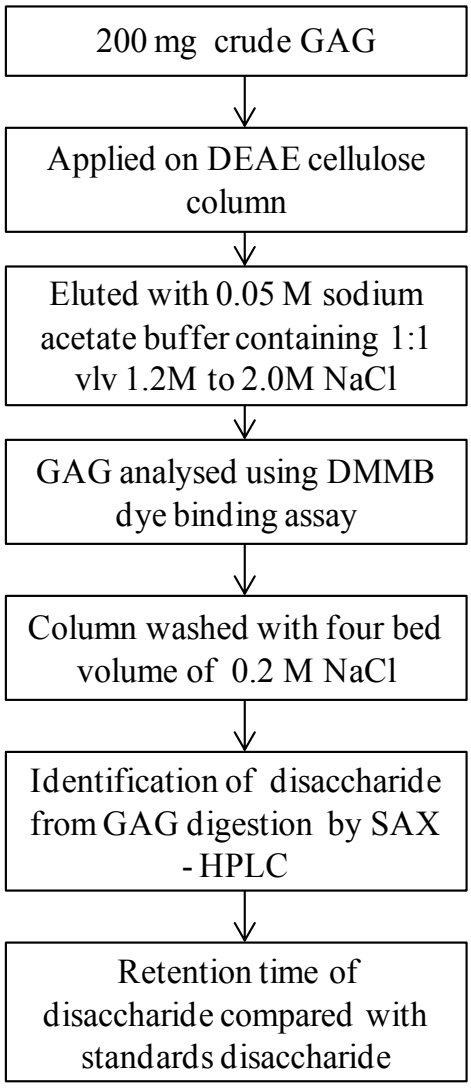


Figure 3.5. Schematic representation of purification of sulfated GAG using ion exchange chromatography (Vieira et al., 1991 and Falshaw et al., 2000).

Mohsen et al. (2007) studied the chemical structure and antiviral activity of crude water-soluble sulfated polysaccharides (SP) which were isolated from brown algae (*Surgassium latifolium*) by hot water extraction followed by ethanol precipitation. Purification of the polysaccharides was carried out using anion exchange chromatography and gel filtration. It was concluded that the antiviral activities were dependent on both the degree of sulfation and molecular weight.

Yoon et al. (2007) studied the purification of polysaccharides using anion exchange chromatography. About 60 mg crude acidic polysaccharide was applied to a Mono Q-FPLC column, equilibrated with a linear gradient of 0-3.0 M NaCl in the same buffer. The column flow rate was 0.5 mL/min and fractions of 0.5 mL were collected. The obtained fractions were assayed by phenol-sulphuric acid for hexose by metachromasia and by carbazole reaction. The fractions collected contained dermatan sulfate and condroitin sulfate.

3.9.2. Gel permeation Chromatography

Gel permeation chromatography is a technique which separates dissolved molecules on the basis of their size through specialized microporous packing material column (Mohsen et al., 2007).

Mitropoulou et al. (2001) investigated the types, amount and fine chemical composition of GAGs present both in human normal myometrium and uterine leimyoma. GAGs were fractionated using ion exchange chromatography on DEAE-Sephacel, purified by gel-permeation chromatography and characterized using electrophoresis in cellulose acetate membranes. Specific enzymic treatments and analysis by high performance capillary electrophoresis (HPCE) were carried out. The results showed that there was no statistical difference in total GAG content in both tissues or in chemical composition. Chondroitin sulphate (CS), dermatan sulphate (DS), hyaluonan (HA), heparan sulphate (HS), and keratan sulphate (KS) were identified in both tissues.

Cho et al. (2010) studied the purification of sulfated polysaccharides from *Enteromorpha prolifera* gel permeation chromatography. Gel permeation was performed to determine the molecular characteristics and biological activities. It was observed that the crude and fractionated polysaccharides (F1, F2, and F3) consisted mostly of carbohydrates, sulfates and uronic acid with different levels of monosaccharides such as glucose, xylose and rhamnose. F2 sample had the most protein content.

Pushpamali et al. (2008) purified polysaccharide by using gel permeation chromatography on sepharose-4B column equilibrated with distilled water. The column was eluted with distilled water with the column flow rate of 15 mL/h. Total polysaccharide content was monitored by 1,9-dimethylmethylene blue assay.

Mansour et al. (2009) purified the polysaccharide using gel permeation with sodium acetate 0.05 M and pH 6. 1 mg/ml polysaccharide was applied to sephadex G-100 column at the flow rate 10 ml/h.

CHAPTER 4. EXPERIMENTAL MATERIALS

4.1. Glassware

The glassware used in the experiments included test tubes, beakers, conical flasks, reagent bottles, measuring cylinders, pipettes and separating funnels. All glassware were washed with soap and tap water, rinsed with distilled water and then dried in an oven (Model 655F, Isotemp Oven, Fisher Scientific, Toronto, Ontario, Canada).

4.2. Fish Samples

Fifteen fishes each of mackerel and herring were obtained from Clearwater Seafoods Ltd., Halifax, Nova Scotia, Canada. Samples were collected in sealed plastic bags and transported to the Biotechnology Laboratory, Department of Process Engineering and Applied Science, Dalhousie University, Halifax, Nova Scotia, Canada. 10 fishes were used to dissect to separate various parts and 5 fishes were used for whole fish sample preparation. The samples were stored at -6˚C.

4.3. Chemicals and Enzyme

Papain, cetyl pyridinium chloride (CPC), acetone, petroleum ether and trichloroacetic acid were obtained from Sigma-Aldrich, Oakville, Ontario, Canada. The sGAGs assay kit was purchased from Biocolor Ltd., Northern Ireland, U.K.

4.4. Reagents

The main reagent used was papain extraction reagent consisting of 0.1M sodium acetate, 0.01M EDTA and 0.005M cysteine in 0.2M sodium phosphate buffer of pH 6.4. The papain extraction reagent was made by adding 0.1M sodium acetate (8.2 g), 0.005 M cysteine (790 mg) and 0.01m EDTA (3.7 g) in 1 litre of 0.2 m sodium phosphate buffer (pH 6.4). Then, 6 g of papain was added and the pH was adjusted to 6.4 using pH meter (Orion 5 star 1119001, Thermo Scientific, Ottawa, Ontario, Canada). 7% (w/v)

trichloroacetic acid was prepared by dissolving 7 grams of trichloroacetic acid in 100 ml distilled water. 10% (w/v) cetylpyridinium chloride (CPC) was prepared by dissolving 10 grams of CPC in 100 ml of distilled water.

4.5. Equipment

Equipment used in the experiments included: food blender (Model-53257C, Hamilton Beach, Halifax, Canada), centrifuge (Sorvall RT1, Thermo Scientific, Ottawa, Ontario, Canada), water bath (Microprocessor Controlled 280 Series, Precision, Ottawa, Ontario, Canada), water bath shaker (Thermo model 2870, Thermo Scientific, Ottawa, Ontario, Canada) and pH meter (Orion 5 star 1119001, Thermo Scientific, Ottawa, Ontario, Canada). In addition, a magnetic stirrer (Barnstead thermolyne), mettler weight bridges (PM4600 and AE200s), microcentrifuge (Accu spin 40427797, Fisher Scientific, Toronto, Ontario, Canada), μ Quant plate reader (MQX200, BIO-TEK instruments, Inc., Vermont, USA), and automatic pipettes (3608089, Eppendorf research plus, Fisher Scientific, Toronto, Ontario, Canada) were used.

CHAPTER 5. EXPERIMENTAL PROCEDURE

5.1. Experimental Design

Experiments were carried out to extract sulfated glycosaminoglycans (sGAGs) from various parts of two fish: herring and mackerel. The parts considered for extraction were: the flesh, head, fins and tail, gut, skin, bones and whole fish. Extraction of sulfated glycosaminoglycans involved enzymatic hydrolysis using papain followed by dialysis for purification. Four hydrolysis times (3, 6, 12 and 24 hours) and two papain concentrations (15 and 20 units/ml) were evaluated. After extraction and purification, the amount of sGAGs presents in various parts of herring and mackerel were determined using a sulfated glycosaminoglycan assay. Figure 5.1 shows a schematic representation of the experimental plan for investigating the enzymatic extraction of sGAG. Three replicates were carried out resulting in a total of 336 experimental runs.

5.2. Determination of Wastes from Herring and Mackerel

To determine the amount of waste (head, fins and tail, gut, skin and bones) produced from herring and mackerel fish, 10 fish of each kind were dissected carefully to separate the various parts. Each fish was filleted and the head, skin, fins and tail, gut and bones were separated. Each fish was weighed before dissection and each part was weighed after dissection. All parts were stored at -20°C till further use.

5.3. Sample Preparation

The herring and mackerel parts (flesh, head, gut, fins and tail, skin and bones) and a few whole fish were used for sample preparation. Each part was blended separately and defatted twice using acetone and once with petroleum ether. A few of each whole fish (mackerel and herring) were ground separately and defatted using the same procedure. All parts and whole fish samples of both fish types were dried at room temperature and stored at -20˚C till further use.

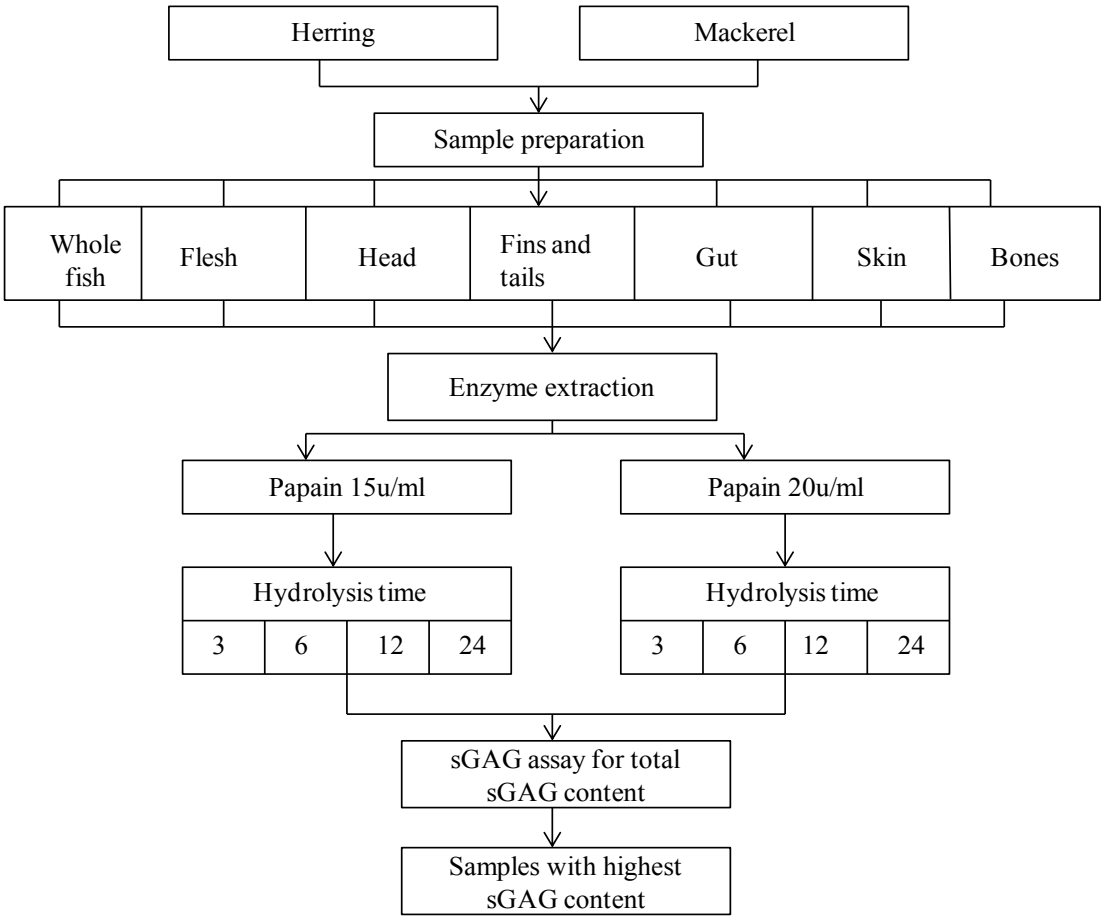


Figure 5.1. Schematic representation of the experimental plan for optimization of enzymatic extraction of sGAG.

5.4. Enzymatic Extraction of Sulfated Glycosaminoglycans

The enzymatic extraction was carried out using a modified procedure from the work reported by Farias et al. (2000), Maccari et al. (2003) and Garnjanagoonchorn et al. (2007). The flowchart for the enzyme extraction process used is shown in Figure 5.2. Five grams of defatted sample were dissolved in 250 ml of 0.2 M sodium phosphate buffer (pH 6.4) containing 0.1M sodium acetate, cysteine 0.005M and EDTA 0.01M mixture. Papain (15 u/ml or 20 u/ml) was added to the mixture and the mixture was left to hydrolyze (for 3, 6, 12 or 24 hours) at 65°C (water bath, Microprocessor Controlled 280 Series, Precision, Ottawa, Ontario, Canada). The mixture was allowed to cool down to room temperature until a clear solution was obtained. 7% (w/v) trichloroacetic acid was added to the solution to stop the reaction and the mixture was incubated at 4°C for 24 hrs. In order to remove precipitated proteins, the mixture was centrifuged at 5000 rpm (Sorvall RT1, Thermo Scientific, Ottawa, Ontario, Canada) for 30 mins at 4°C. The supernatant containing liberated GAGs was collected and 20ml of 10% cetylpyridinium chloride (w/v) was added to the solution to precipitate sGAG. The mixture was centrifuged for 30 mins at 5000 rpm at 4°C. The pellets that were obtained were then washed with 610 ml of 0.05 % (w/v) cetylpyridinium chloride and dissolved in 172 ml NaCl ethanol solution (100:15, v/v). The mixture was then dialyzed in chilled distilled water for 24 hours. The solution containing crude sulfated glycosaminoglycans was collected and stored at -6°C for further analysis.

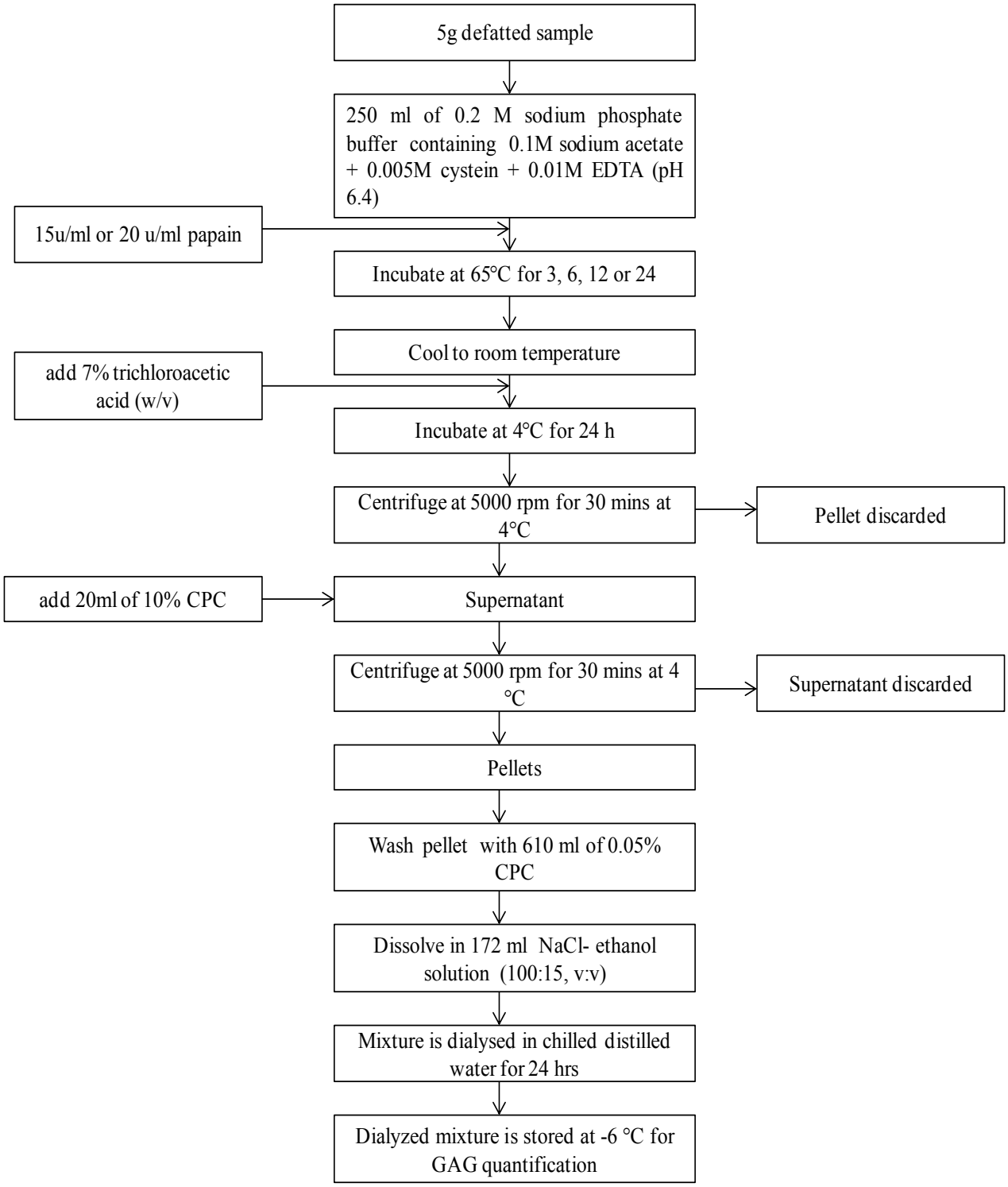


Figure 5.2. The enzymatic extraction procedure used for sulfated glycosaminoglycans from various parts of herring and mackerel.

CHAPTER 6. EXPERIMENTAL ANALYSES

6.1. Determination of the Amount of Sulfated Glycosaminoglycans (sGAG)

The total sGAG content in the samples were measured using a quantitative dye-binding method (Gandra et al., 2000). The dye label used in the assay was 1,9-dimethylmethylene blue. The dye was employed under specific conditions to provide a specific label for the sulfated polysaccharide component of proteoglycans or the chains of sulfated glycosaminglycans that are free from other proteins. The dye binding occurs at both sulfate and carboxyl groups on the GAG molecules. Chondroitin 4-sulfate was used as a standard to determine total sGAG content in the samples.

6.1.1. Standard Curve

A standard curve was first constructed using aliquots containing 1.0, 2.0, 3.0, 4.0 and 5.0 µg of chondroitin 4- sulfate in micro centrifuge tubes (Table 6.1).The micro centrifuge tubes were made up to 100 µl by adding distilled water. The absorbance was measured at 656 nm using a plate reader (MQX200, BIO-TEK instruments, Inc., Vermont, USA) along with the samples after addition of dye reagent. Table 6.1 shows absorbance for standard sGAG at 656 nm and Figure 6.1 shows the standard curve for sGAG concentration.

6.1.2. Fish Sample

The test samples were thawed out in room temperature for an hour and approximately 30 µl placed in labelled micro centrifuge tubes. The micro centrifuge tubes containing test samples were made up to 100 µl using automatic pipettes (3608089, eppendorf research plus, Fisher Scientific, Toronto, Ontario, Canada). To each tube, 1 ml of dye reagent was added, mixed by inverting the tubes and then the tubes were placed on a mechanical shaker (Thermo Model 2870, Thermo Scientific, Ottawa, Ontario, Canada) for 30 minutes. A sGAG-dye complex formed during 30 minutes of shaking. All the tubes were then transferred to a micro centrifuge (Model 40427797, Fisher Scientific, Toronto,

Table 6.1. Standard sGAG measured at 656 nm.

Chondroitin-4-sulfate Concentration (μg/ml)	OD (656 nm)		Average OD (656 nm)
	Sample 1	Sample 2	
0	0.000	0.000	0.000
10	0.284	0.286	0.285
20	0.532	0.518	0.525
30	0.742	0.740	0.741
40	0.959	0.928	0.944
50	1.127	1.129	1.128

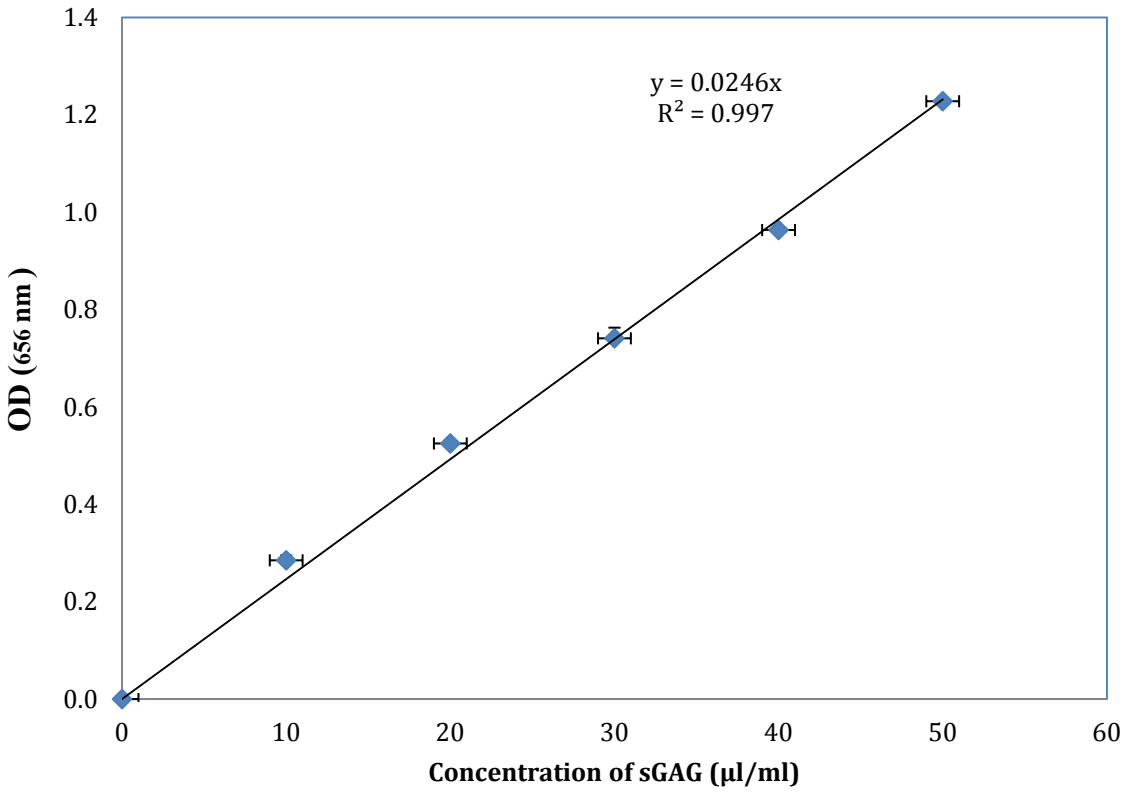


Figure 6.1. Standard curve for sGAG concentration (mean±std, n=3).

Ontario, Canada) and centrifuged at 12000 rpm for 10 minutes. The sGAG -dye complex was formed as pellet and the unbound dye was carefully drained. 500µl of dissociation reagent containing sodium salt of anionic surfactant was added to all the tubes to release the sGAG-dye complex. After the addition of the dissociation reagent the mixture in the tubes was mixed using a vortex mixer (M16715, Type 16700 Mixer, Barnstead Thermolyne, Iowa, USA). After 10 mins, 200 µl of each sample was transferred to a 96 micro well plate (Model 2593 EIA/RIA Strip Well Plate, Corning Incorporation, NY, USA) and the absorbance read at 656 nm using a plate reader (MQX200, BIO-TEK instruments, Inc., Vermont, USA) against water for the reagent blanks, standards and test samples (Papy Garcia et al., 2002). The absorbance read at 656 nm was used to compare with the standard curve to determine sGAG concentration in the test samples.

6.2. Statistical Analysis of Data

The sGAG concentration of all the samples from different parts of herring and mackerel were determined and the standard errors were calculated using Minitab software (Version 16, Minitab Inc. State College, Pennsylvania, USA). The effects of various parameters on the sGAG concentration were determined using analysis of variance (ANOVA). The significance of the parameters levels used in the experiment was determined using Tukey's grouping method.

CHAPTER 7. RESULTS

7.1. Weight Distribution

Tables 7.1 and 7.2 show the average weight of the various parts of mackerel and herring fish. The weight distribution and weight percentage of mackerel and herring fish are shown in Figures 7.1-7.4. The average weight of the whole fish was 466.97 and 267.20 g for mackerel and herring, respectively. The weight percentage of the flesh, head, fins and tail, gut, skin and bones were 62.65, 19.79, 0.76, 5.29, 4.08 and 6.40% for mackerel and 64.55, 13.36, 1.33, 6.64, 5.28 and 6.99% for herring, respectively. About 4.83% and 4.96% of the fish weight were lost during dissecting mackerel and herring, respectively. The total fish waste was 37.35% and 35.45% of the body weight for mackerel and herring, respectively.

7.2. Sulfated Glycosaminoglycan Concentration

The enzymatic extraction was conducted on 5 g test samples prepared from the various parts (whole fish, head, flesh, fins and tail, gut and bone) of both mackerel and herring fish. The parameters investigated were hydrolysis time (3, 6, 12 and 24 hours) and papain (enzyme) concentration (15 and 20 U/ml). Post extraction, aliquots of 30-µl extract containing suspended sulfated glycosaminoglycans (sGAG) was used to perform the sulfated glycosaminoglycan assay. Tables 7.3 and 7.4 show the sGAG concentrations in different parts of mackerel and herring treated with papain concentrations of 15 and 20 U/ml for 3, 6, 12 and 24 hrs.

The analysis of variance was performed on the sGAG data using MINITAB (Minitab Inc., State College PA, USA). The results are shown in Table 7.5. The results showed that the effects of fish type, fish parts, hydrolysis time and enzyme concentration were highly significant at the 0.001 level. Also, the one way, two way, three way and four way interactions of these parameters were significant at the 0.001 level.

Table 7.1. Average weight of mackerel parts

Sample	Whole Fish (g)	Flesh (g)	Total Waste (g)	Waste					Others* (g)
				Head (g)	Fins and tail (g)	Gut (g)	Skin (g)	Bones (g)	
1	483.80	310.00	173.80	92.70	3.20	25.95	17.00	29.55	5.40
2	393.00	231.00	162.00	87.30	3.90	24.10	18.10	24.40	4.20
3	512.20	312.00	200.20	104.53	3.60	23.63	23.90	38.80	5.74
4	461.10	298.30	162.80	84.40	3.20	23.51	17.10	28.03	6.56
5	483.20	312.02	171.18	91.80	3.30	24.62	19.05	29.62	2.79
6	436.00	270.00	166.00	89.90	3.25	23.90	18.92	28.20	1.83
7	522.91	322.33	200.58	102.41	3.94	26.93	24.30	36.34	6.66
8	489.20	317.62	171.58	91.30	3.39	24.84	16.90	29.98	5.17
9	409.00	243.05	165.95	88.55	4.12	25.05	18.65	25.02	4.56
10	479.33	308.37	170.96	91.30	3.70	24.80	16.80	28.96	5.40
Avg	466.9±42.0	292.4±32.6	174.4±13.5	92.4±6.3	3.56±0.34	24.7±1.0	19.0±2.7	29.8±4.5	4.83±1.5
Percent	(100%)	(62.65%)	(37.35%)	(19.79%)	(0.76%)	(5.29%)	(4.08%)	(6.40%)	(1.03%)

*lost material during dissection

Table 7.2. Average weight of herring parts

Sample	Whole Fish (g)	Flesh (g)	Total Waste (g)	Waste					Others* (g)
				Head (g)	Fins and tail (g)	Gut (g)	Skin (g)	Bones (g)	
1	261.00	168.00	93.00	35.52	3.25	17.52	13.70	18.40	4.61
2	248.00	166.09	81.91	28.50	2.81	14.28	13.50	18.80	4.02
3	272.00	171.21	100.79	38.41	3.82	17.76	13.90	18.90	8.00
4	251.30	168.20	83.10	29.80	2.96	16.31	13.30	16.70	4.03
5	287.00	182.03	104.9	38.68	3.96	19.80	15.01	20.04	7.48
6	248.90	164.40	84.50	33.41	3.12	16.66	12.80	16.70	1.81
7	274.04	175.07	98.97	37.31	3.92	18.24	14.02	18.88	6.60
8	288.61	184.07	104.5	39.02	4.01	20.01	15.72	20.88	4.90
9	276.40	175.89	100.5	38.90	3.98	18.92	14.77	19.21	4.73
10	264.84	169.08	95.76	37.55	3.84	17.96	14.43	18.49	3.49
Avg	267.2±14.9	172.4±6.6	94.79±7.6	35.71±3.8	3.57±0.47	17.75±1.7	14.11±0.8	18.70±1.2	4.96±1.8
Percent	(100%)	(64.55%)	(35.45%)	(13.36%)	(1.33%)	(6.64%)	(5.28%)	(6.99%)	(1.85%)

*lost material during dissection

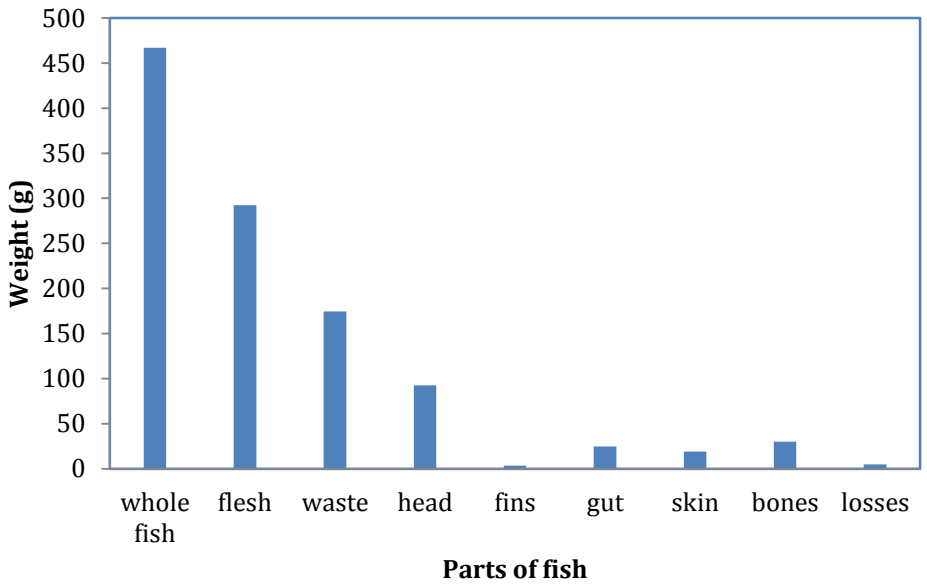


Figure 7.1.Weight distribution of different parts of mackerel fish.

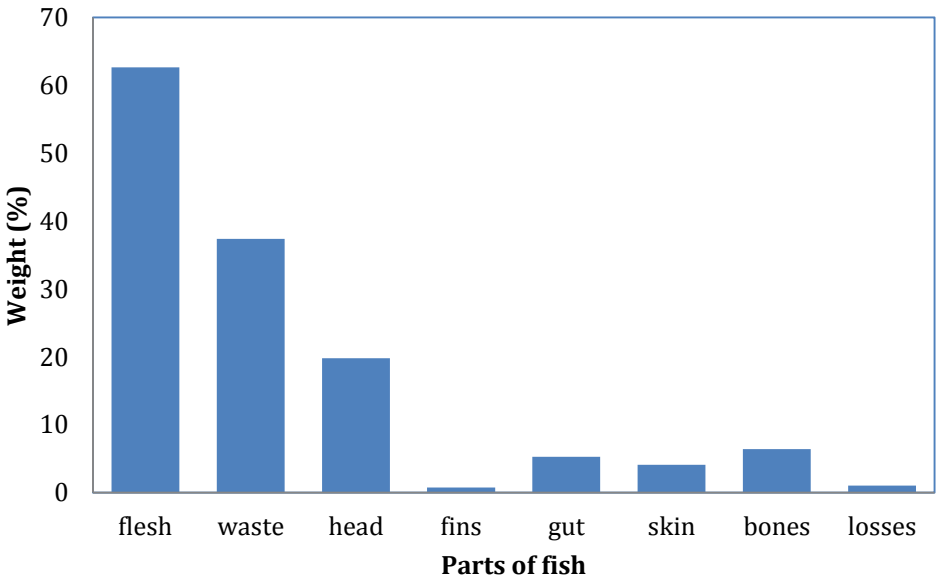


Figure 7.2.Weight percentage of different parts of mackerel fish.

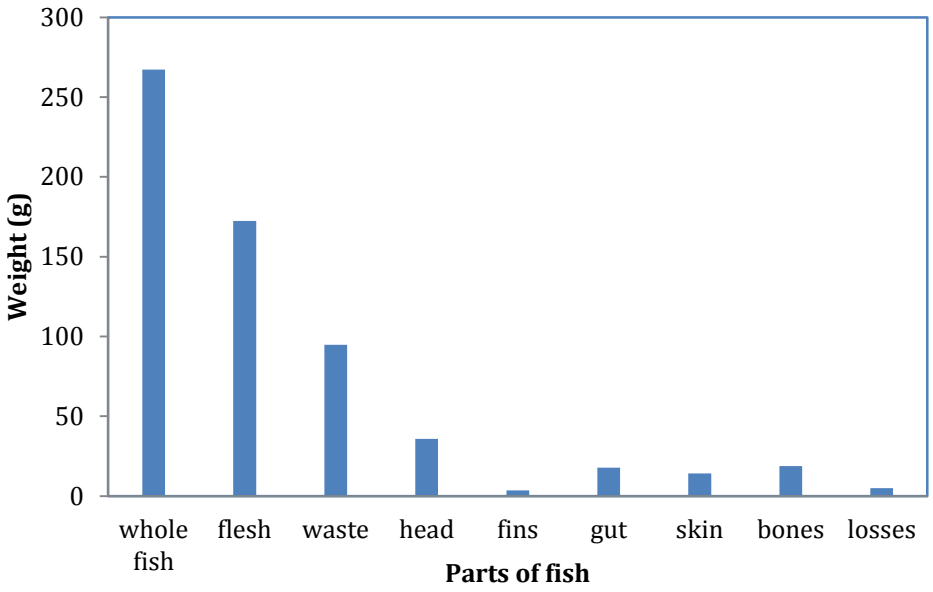


Figure 7.3. Weight distribution of different parts of herring fish.

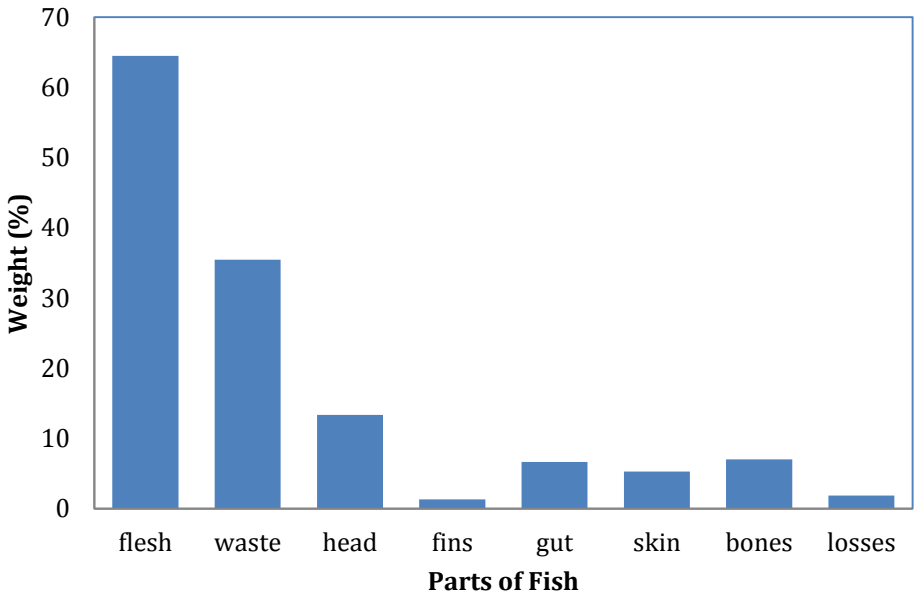


Figure 7.4. Weight percentage of different parts of herring fish.

Table 7.3. Sulfated Glycosaminoglycan concentrations in different parts of mackerel fish treated with different concentrations of papain for different hydrolysis times

Fish Parts	Enzyme Concentration (units/ml)	sGAG Concentration (mg/g)			
		3 h	6 h	12 h	24 h
whole fish	15	30.85±2.0	33.86±0.8	29.99±0.2	25.95±0.2
	20	34.73±1.6	34.85±1.1	33.95±0.1	30.00±0.1
flesh	15	4.63±0.9	16.51±1.5	13.86±0.3	9.91±1.3
	20	8.17±0.8	23.95±1.3	12.47±1.3	10.24±1.5
waste	15	40.86±1.6	110.59±0.4	97.72±1.2	73.89±0.9
	20	89.02±0.4	163.23±0.5	113.03±0.5	96.34±0.7
head	15	29.76±0.4	117.22±1.1	97.88±0.3	60.04±1.8
	20	116.01±1.7	206.70±0.8	120.52±0.4	100.52±1.1
fins and tail	15	24.51±0.5	74.65±2.3	71.42±0.4	49.10±0.4
	20	31.70±2.0	86.63±1.1	63.21±0.9	52.39±1.2
gut	15	119.26±1.9	185.04±0.6	176.17±1.5	132.03±0.2
	20	112.47±0.6	203.52±0.7	160.20±0.7	109.10±0.5
skin	15	38.94±1.3	105.45±0.7	67.03±0.8	60.20±0.7
	20	37.23±1.9	102.60±1.1	91.21±1.0	66.34±0.3
bones	15	13.49±2.0	36.00±2.0	55.00±1.7	80.28±1.9
	20	25.97±1.2	43.24±0.9	70.69±0.5	97.19±1.4

Table 7.4. Sulfated Glycosaminoglycan concentrations in different parts of herring fish treated with different concentrations of papain for different hydrolysis times

Fish Parts	Enzyme Concentration (units/ml)	sGAG Concentration (mg/g)			
		3 h	6 h	12 h	24 h
whole fish	15	22.36±1.5	26.01±0.2	24.57±0.4	25.31±0.8
	20	26.38±2.0	25.69±1.7	27.01±0.9	25.89±2.0
flesh	15	12.23±0.7	19.55±1.3	18.13±0.7	12.56±1.4
	20	37.31±1.5	39.34±0.6	23.29±0.5	8.37±0.2
waste	15	88.88±1.1	106.36±0.7	130.15±1.2	94.91±0.4
	20	74.49±0.9	91.55±0.4	100.60±1.2	73.64±0.9
head	15	99.71±1.5	115.97±0.8	162.76±0.5	104.26±1.1
	20	55.44±2.0	76.38±0.9	73.33±0.7	60.97±1.7
fins and tail	15	53.17±1.3	81.62±0.2	48.98±0.6	48.00±0.6
	20	39.59±1.3	55.85±1.3	101.09±1.6	148.53±1.6
gut	15	202.92±1.0	205.77±1.6	235.77±1.2	181.01±1.2
	20	221.58±3.3	229.71±0.2	236.30±0.1	173.37±0.8
skin	15	17.52±1.5	33.78±0.1	63.69±0.8	65.89±1.5
	20	14.43±1.4	23.41±1.2	31.99±0.6	23.37±0.9
bones	15	20.60±0.8	53.13±1.1	33.29±1.4	26.17±0.8
	20	23.25±1.6	47.64±0.9	75.56±0.2	26.82±0.7

Table 7.5. Analysis of variance of sGAG extracted from different parts of mackerel and herring fish

Source	DF	SS	MS	F	P
Total	335	1170345			
Model					
F	1	397	397	273	0.001
P	6	888583	148097	101654	0.001
E	1	1866	1866	1281	0.001
T	3	47444	15815	10855	0.001
F*P	6	69326	11554	7931	0.001
F*E	1	4066	4066	2791	0.001
F*T	3	11866	3955	2715	0.001
P*E	6	4265	711	488	0.001
P*T	18	41019	2279	1564	0.001
E*T	3	400	133	92	0.001
F*P*E	6	40091	6682	4586	0.001
F*P*T	18	29752	1653	1135	0.001
F*E*T	3	1066	355	244	0.001
P*E*T	18	17441	969	665	0.001
F*P*E*T	18	12437	691	474	0.001
Error	224	326	1		

F- Fish type
P- Parts
T- Hydrolysis time
E- Enzyme concentration
R^2 = 99.97
CV = 0.84%

Tukey's grouping was also performed on the data to test the differences among the levels of each parameter. The results are shown in Table 7.6. The two fish types were significantly different from one another at the 0.05 level. The highest mean value of sGAG concentration of 70.83 mg/g was extracted from mackerel. The whole fish and flesh were not significantly different from one another but were significantly different from other fish parts (head, fins and tail, gut, skin and bones) at the 0.05 level. The head, fins and tail, gut and bones were significantly different from one other at the 0.05 level. The skin was not significantly different from bones at the 0.05 level. The highest mean value of sGAG concentration of 180.26 mg/g was extracted from gut samples. The 6 and 12 h reaction times were not significantly different from each other but were significantly different from the 3 and 24 h reaction times at the 0.05 level. The highest sGAG concentration of 82.29 mg/g was extracted with the 6 h reaction time. The two enzyme concentrations were significantly different from one another at the 0.05 level. The highest mean value of sGAG concentration of 72.10 mg/g was achieved with the enzyme concentration of 20 u/ml.

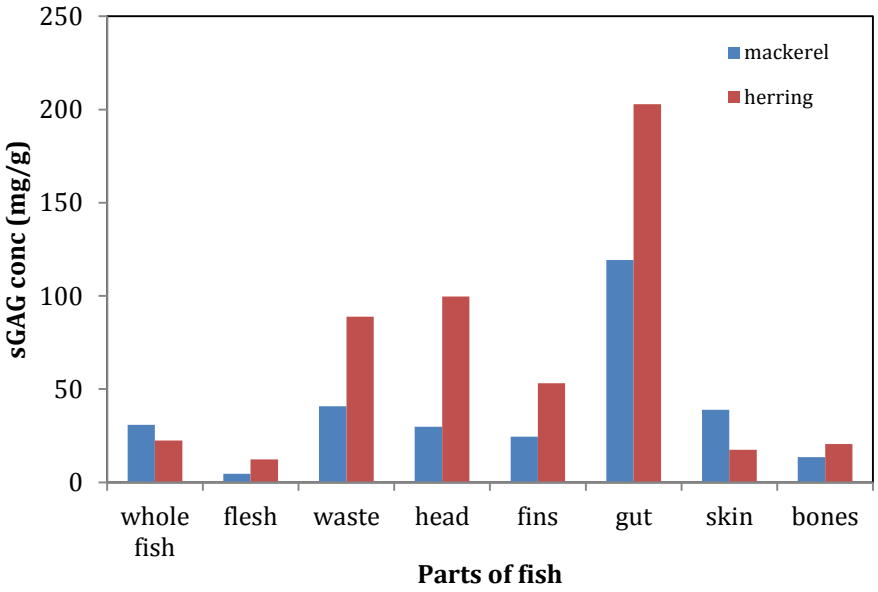
7.2.1. Effect of Fish Type and Parts

Figures 7.5-7.8 show the sulfated glycosaminoglycan concentration in different parts of mackerel and herring fish treated with different concentrations of papain (15 and 20 u/ml) for 3, 6, 12, 24 hours. For the 3 h hydrolysis time and the 15 u/ml enzyme concentration (Figure 7.5 a), the whole fish and skin samples of mackerel had higher sGAG concentration than those of the herring fish. On the other hand, the flesh, waste, head, fins and tail, gut and bones samples of herring fish had higher sGAG concentrations than those of mackerel fish. The highest sGAG concentrations of 202.9 and 119.6 mg/g were obtained from guts of herring and mackerel samples, respectively.

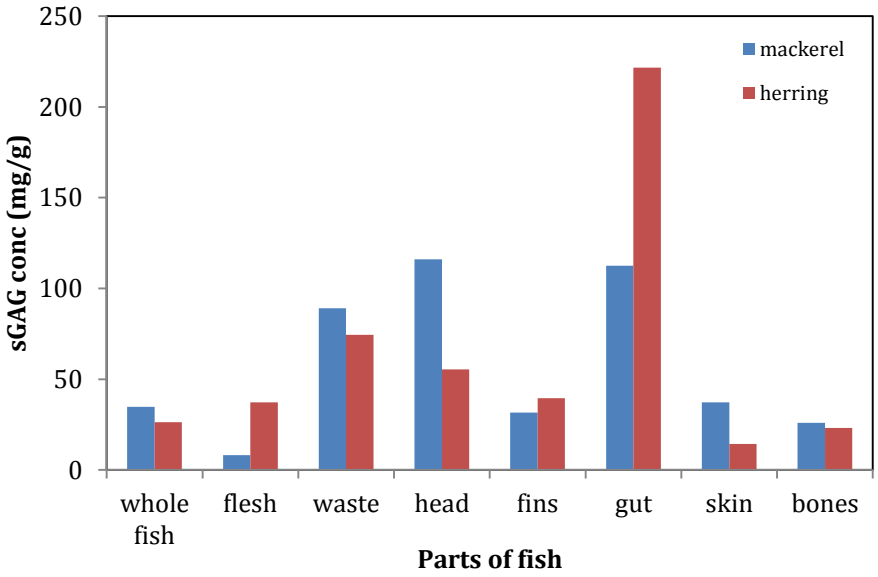
For the 3 h hydrolysis the time and the 20 u/ml enzyme concentration (Figure 7.5 b), the whole fish, waste, head, skin, bones samples of mackerel fish had higher sGAG concentrations than those of herring fish. However, the flesh, fins and tail and gut samples of herring fish had higher sGAG concentrations than those of mackerel fish. The

Table 7.6. Tukey's grouping of various parameters affecting sGAG concentration

Parameter	Number of Observation	Mean (mg/g)	Tukey Grouping
Fish			
Mackerel	168	70.83	A
Herring	168	68.66	B
Parts			
Whole fish	48	28.59	E
Flesh	48	16.91	E
Head	48	99.84	B
Fins and tail	48	64.40	C
Gut	48	180.26	A
Skin	48	52.69	CD
Bones	48	45.52	D
Hydrolysis Time			
3	84	52.65	D
6	84	82.29	A
12	84	79.26	A
24	84	64.78	C
Enzyme Concentration			
20u/ml	168	72.10	A
15u/ml	168	67.39	B

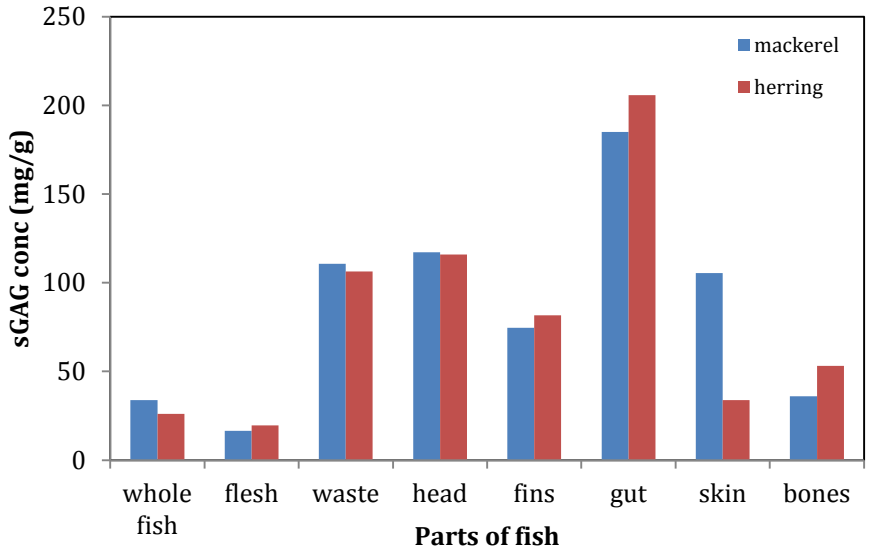


(a) 15 µ/ml

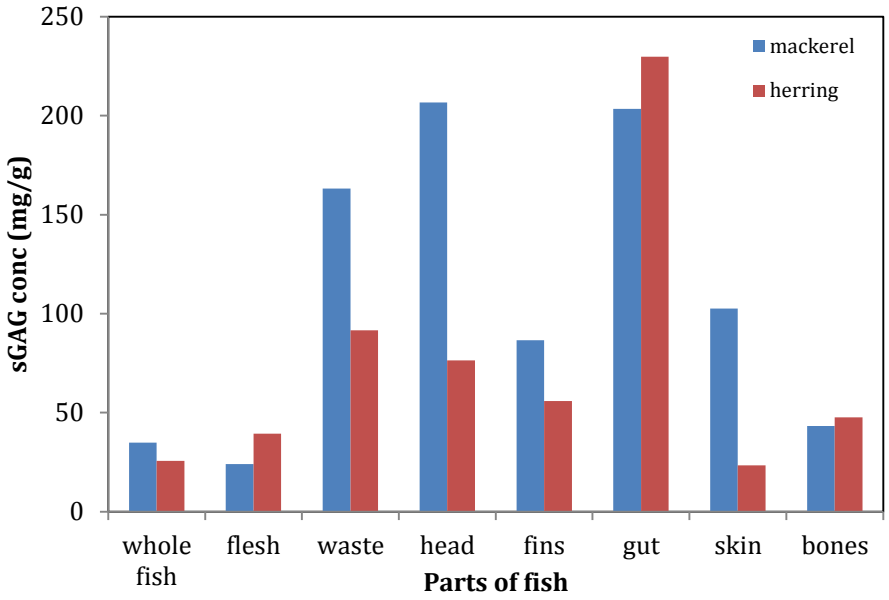


(b) 20 µ/ml

Figure 7.5. Sulfated Glycosaminoglycan concentration in different parts of mackerel and herring fish treated with different concentration of papain for 3 hours.

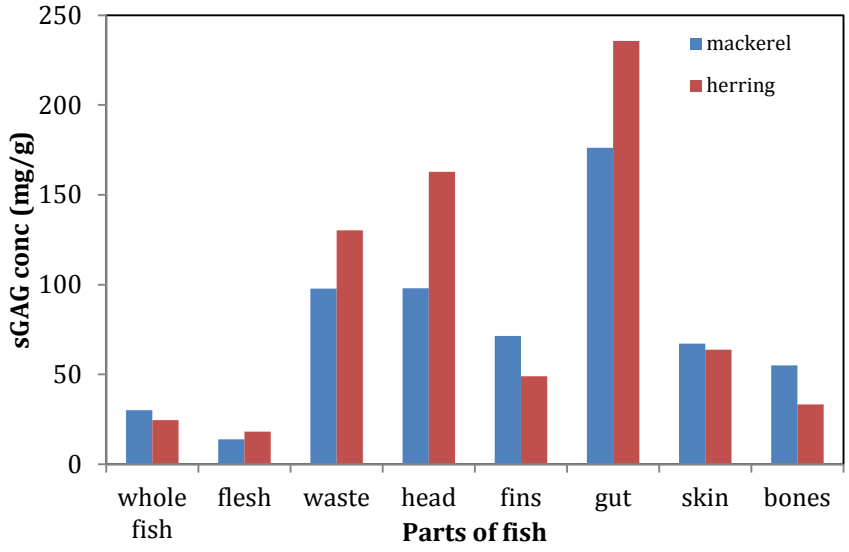


(a) 15 µ/ml

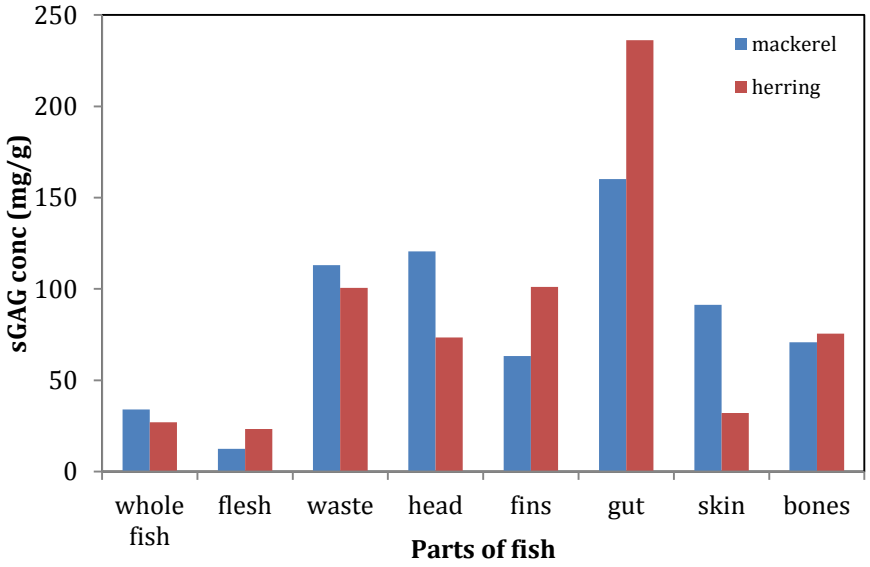


(b) 20 µ/ml

Figure 7.6. Sulfated Glycosaminoglycan concentration in different parts mackerel and herring fish treated with different concentration of papain for 6 hours.

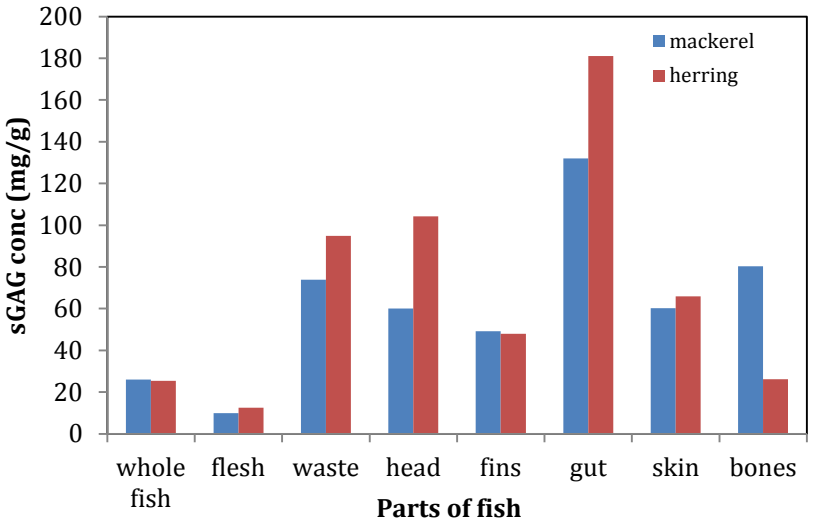


(a) 15 µ/ml

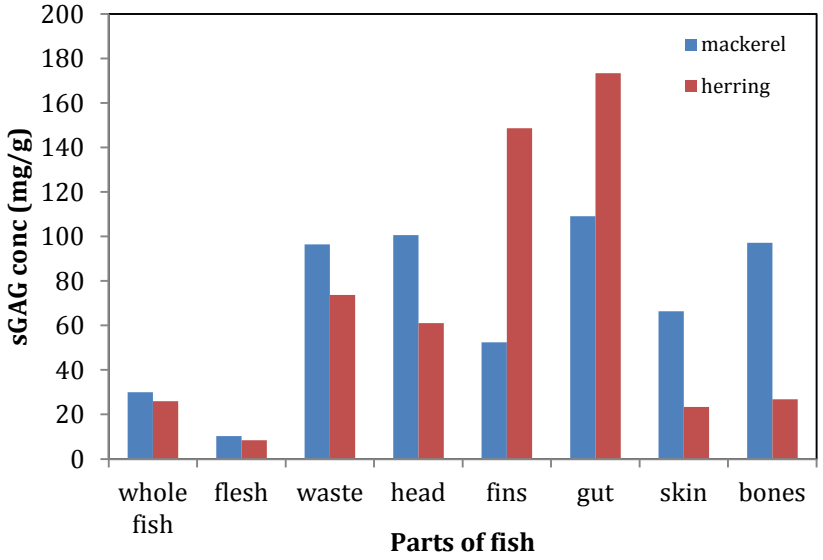


(b) 20 µ/ml

Figure 7.7. Sulfated Glycosaminoglycan concentration in different parts of mackerel and herring fish treated with different concentration of papain for 12 hours.



(a) 15 µ/ml



(b) 20 µ/ml

Figure 7.8. Sulfated Glycosaminoglycan concentration in different parts of mackerel and herring fish treated with different concentration of papain for 24 hours.

highest sGAG concentrations of 221.5 and 116.01 mg/g were obtained with herring gut and mackerel head, respectively.

For the 6 h hydrolysis time and the 15 u/ml enzyme concentration (Figure 7.6 a), the whole fish, waste, head, skin samples of mackerel fish had higher sGAG concentrations than those of herring fish. On the other hand, the flesh, fins and tail, gut and bones samples of herring fish had higher sGAG concentrations than those of mackerel fish. The highest sGAG concentrations of 205.7 and 185.04 mg/g were obtained from herring gut and mackerel gut, respectively.

For the 6 h hydrolysis time and the 20 u/ml enzyme concentration (Figure 7.6 b), the whole fish, waste, head, fins and tail, skin samples of mackerel had higher sGAG concentrations than those of herring fish. However, the flesh, gut and bones samples of herring had higher sGAG concentration than those of mackerel fish. The highest sGAG concentrations of 229.7 and 206.52 mg/g were obtained from herring gut and mackerel head, respectively.

For the 12 h hydrolysis time and the 15 u/ml enzyme concentration (Figure 7.7 a), the whole fish, fins and tail, skin and bones samples of mackerel had higher sGAG concentrations than those of herring fish. On the other hand, the flesh, waste, head and gut samples of herring had higher sGAG concentrations than those of mackerel fish. The highest sGAG concentrations of 235.7 and 176.17 mg/g were obtained from the herring gut and mackerel gut, respectively.

For the 12 h hydrolysis time and the 20 u/ml enzyme concentration (Figure 7.7 b), the whole fish, waste, head, skin samples of mackerel had higher sGAG concentrations than those of herring fish. However, the flesh, fins and tail, gut and bones samples of herring had higher sGAG concentrations than those of mackerel fish. The highest sGAG concentration of 236.3 and 160.20 mg/g were obtained from herring gut and mackerel gut, respectively.

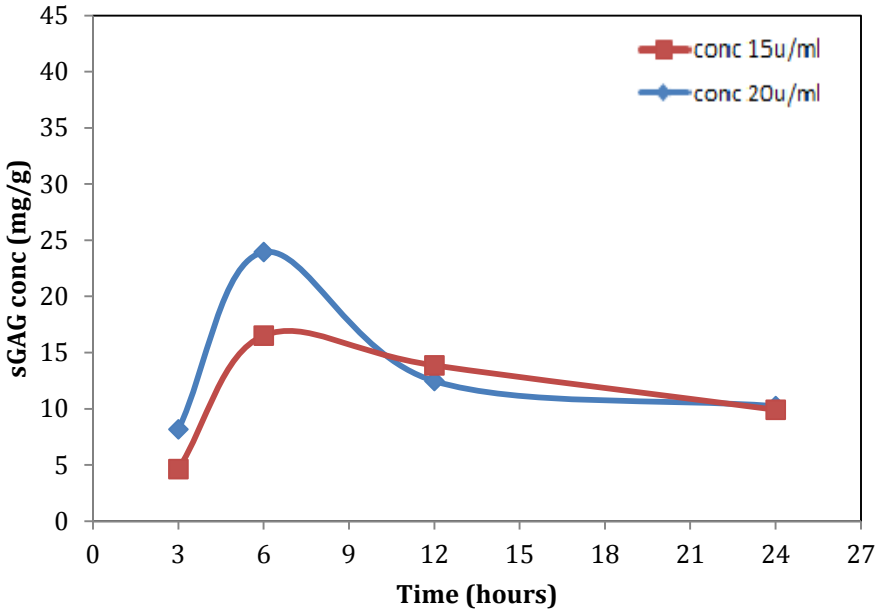
For the 24 h hydrolysis time and the 15 u/ml enzyme concentration (Figure 7.8 a), the whole fish, fins and tail and bones samples of mackerel had higher sGAG concentrations than those of herring fish. However, the flesh, waste, head, gut and skin samples of herring had higher sGAG concentrations than those of mackerel fish. The highest sGAG concentrations of 181.0 and 132.02 mg/g were obtained from herring gut and mackerel gut, respectively.

For the 24 h hydrolysis time and the 20 u/ml enzyme concentration (Figure 7.8 b), whole fish, flesh, waste, head, skin and bones samples of mackerel had higher sGAG concentrations than those of herring fish. However, the fins and tail and gut samples of herring had higher sGAG concentrations than those of mackerel fish. The highest sGAG concentrations of 173.3 and 109.10 mg/g were obtained from herring gut and mackerel gut, respectively.

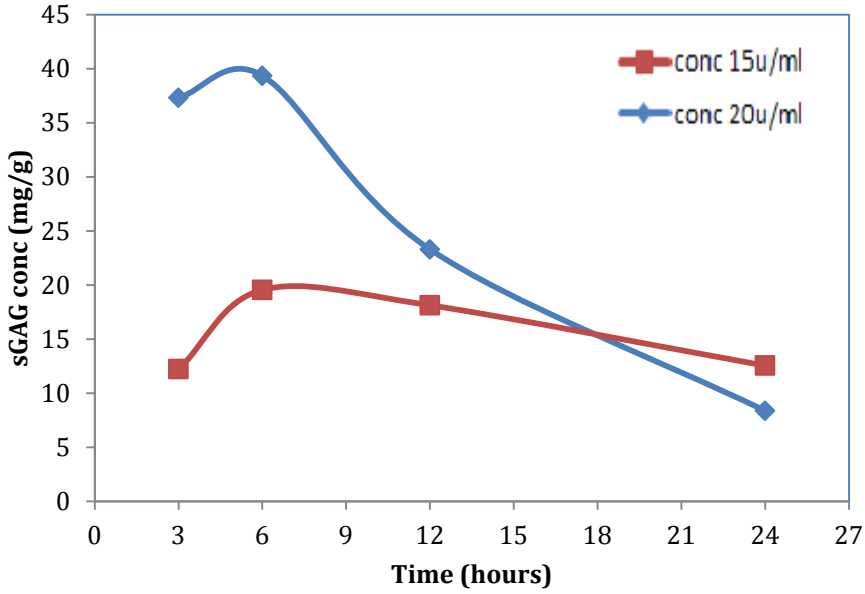
7.2.2. Effect of Hydrolysis Time

Figures 7.9-7.15 show the effect of the hydrolysis time on the concentration of sulfated glycosaminoglycans extracted from the flesh, waste, head, fins and tail, gut, skin and bones of herring and mackerel fish using different enzyme concentrations.

For the flesh samples (Figure 7.9), the highest sGAG concentrations were obtained with the 6 h hydrolysis time for both mackerel (16.51 and 23.95 mg/g with the 15 and 20 u/ml enzyme concentrations, respectively) and herring (19.55 and 39.34 mg/g with the 15 and 20 u/ml enzyme concentrations, respectively). Increasing the hydrolysis time from 3 to 6 h, increased the sGAG concentration from 4.63 to 16.51 mg/g (256.59 %) with the 15 u/ml enzyme concentration and from 8.17 to 23.95 mg/g (193.15 %) with the 20 u/ml enzyme concentration for mackerel and from 12.23 to 19.55 mg/g (59.85%) with the 15 u/ml enzyme concentration and from 37.31 and 39.34 mg/g (5.4 %) with the 20 u/ml enzyme concentration for herring. However, when the hydrolysis time was further increased from 6 h to 24 h, the sGAG concentration decreased from 16.51 to 9.91 mg/g (39.97 %) with the 15 u/ml enzyme concentration and from 23.95 to 10.24 mg/g

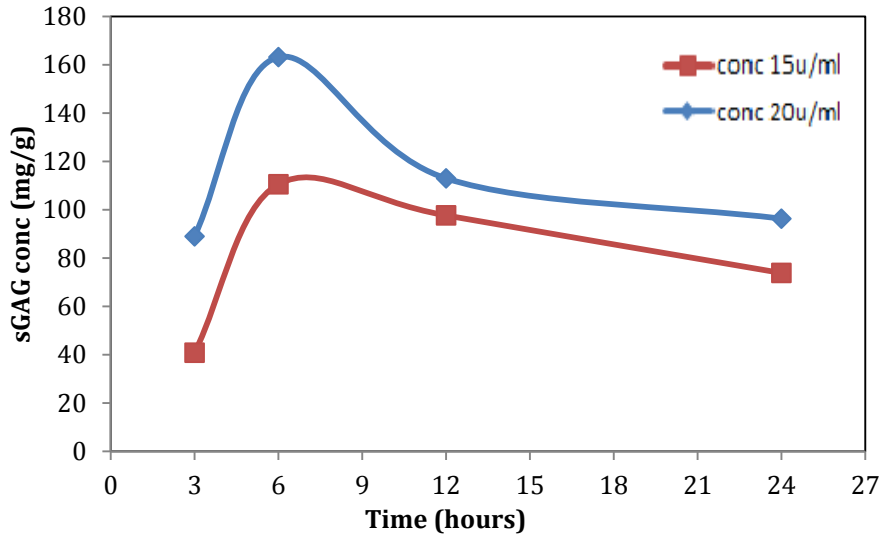


(a) Mackerel flesh

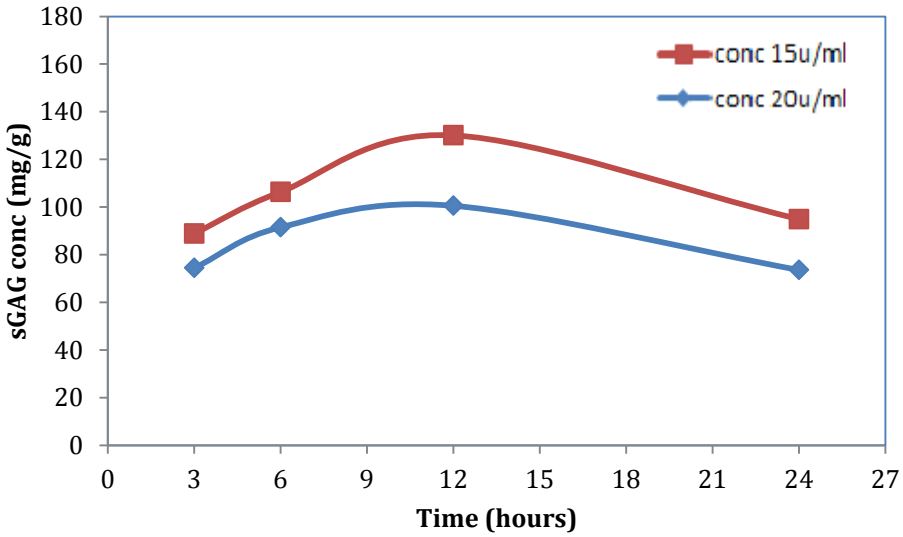


(b)Herring flesh

Figure 7.9. Effect of hydrolysis time on the concentration of sGAG extracted from the fish flesh with different enzyme concentrations.

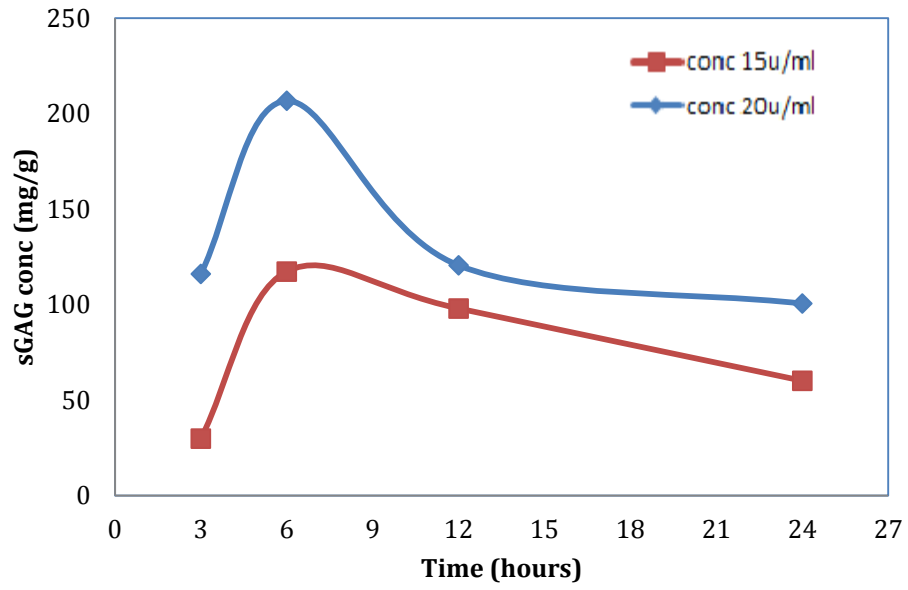


(a) Mackerel waste

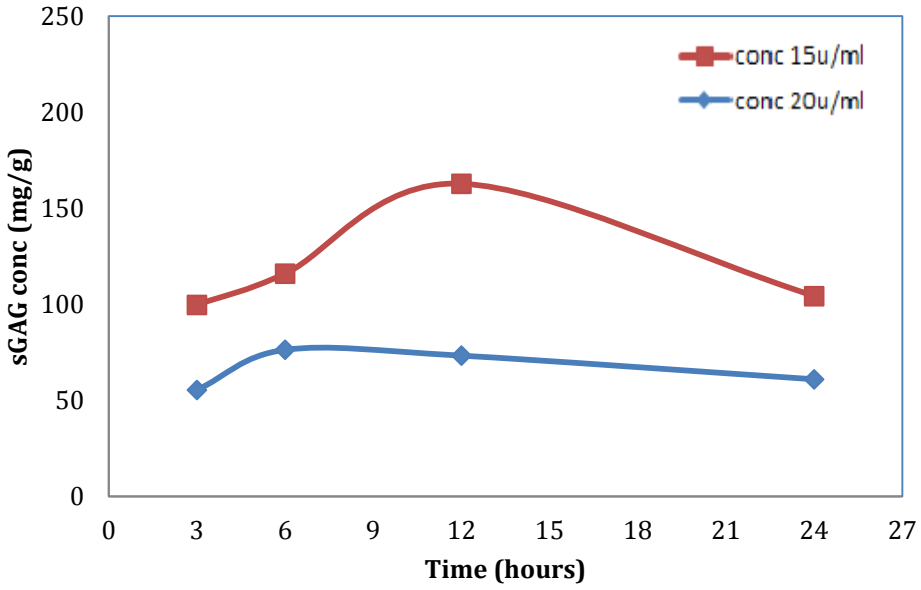


(b)Herring waste

Figure 7.10. Effect of hydrolysis time on the concentration of sGAG extracted from the fish waste with different enzyme concentrations.

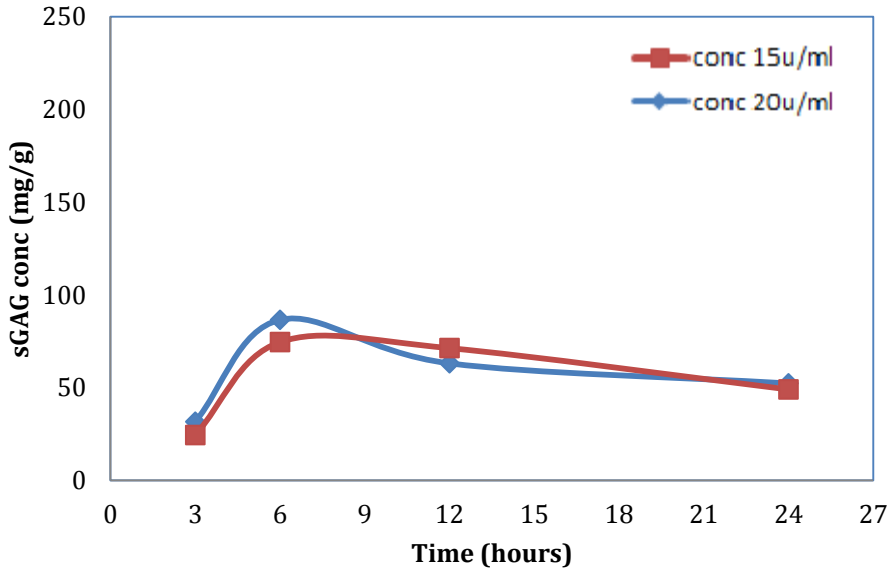


(a) Mackerel head

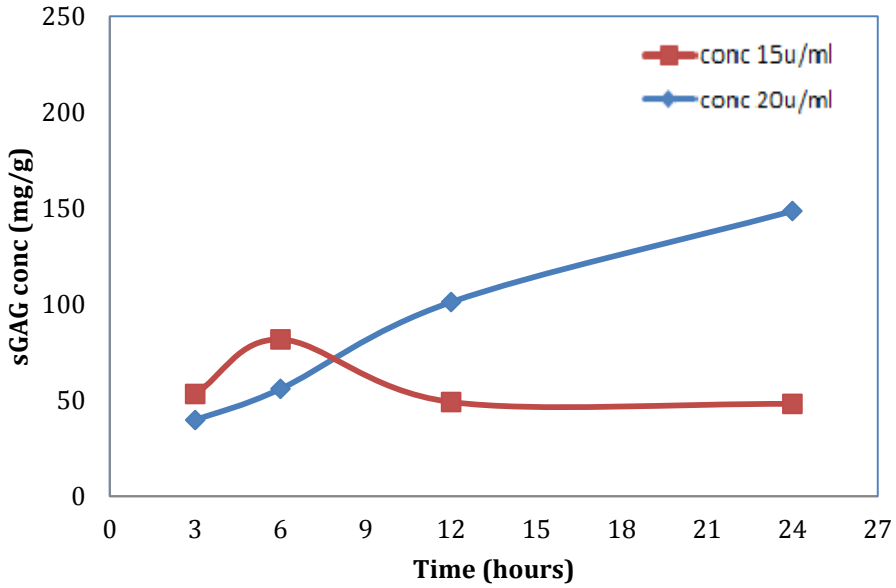


(b) Herring head

Figure 7.11. Effect of hydrolysis time on the concentration of sGAG extracted from the fish head with different enzyme concentrations.

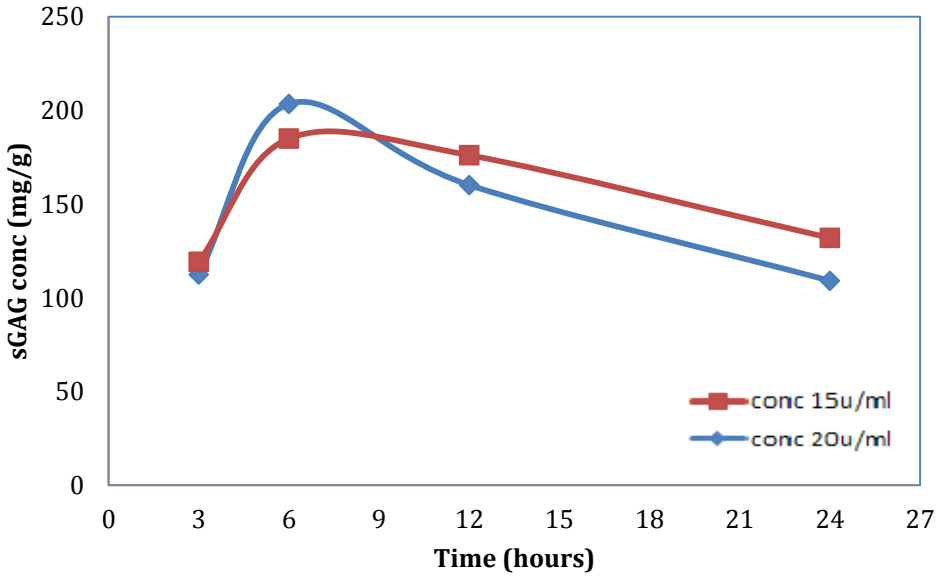


(a) Mackerel fins and tail

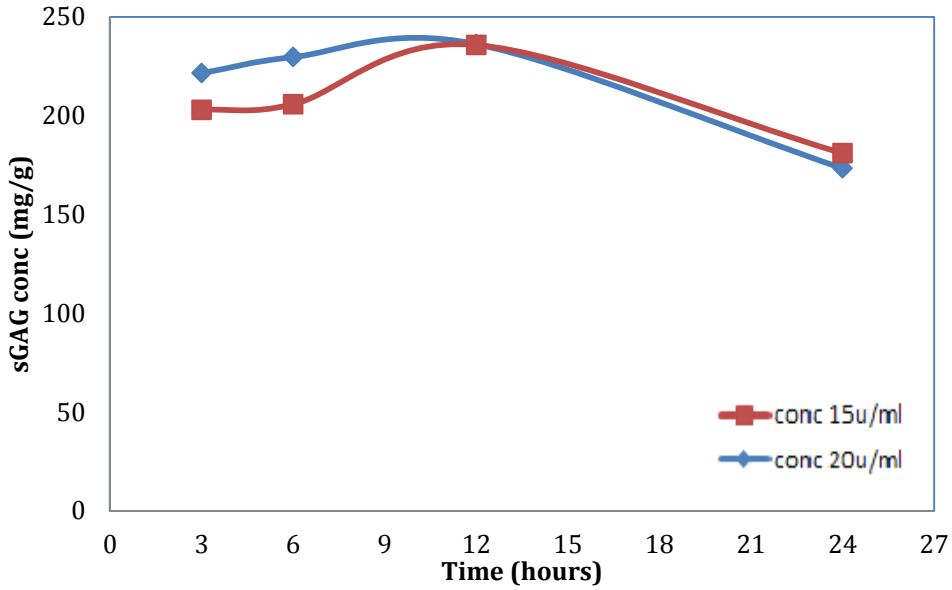


(b) Herring fins and tail

Figure 7.12. Effect of hydrolysis time on the concentration of sGAG extracted from the fish fins and tail with different enzyme concentrations.

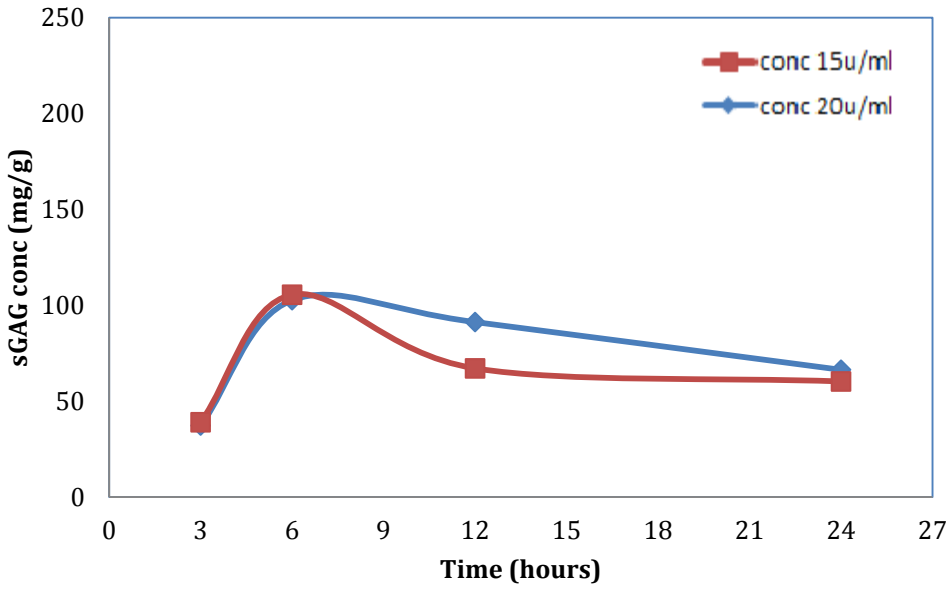


(a) Mackerel gut

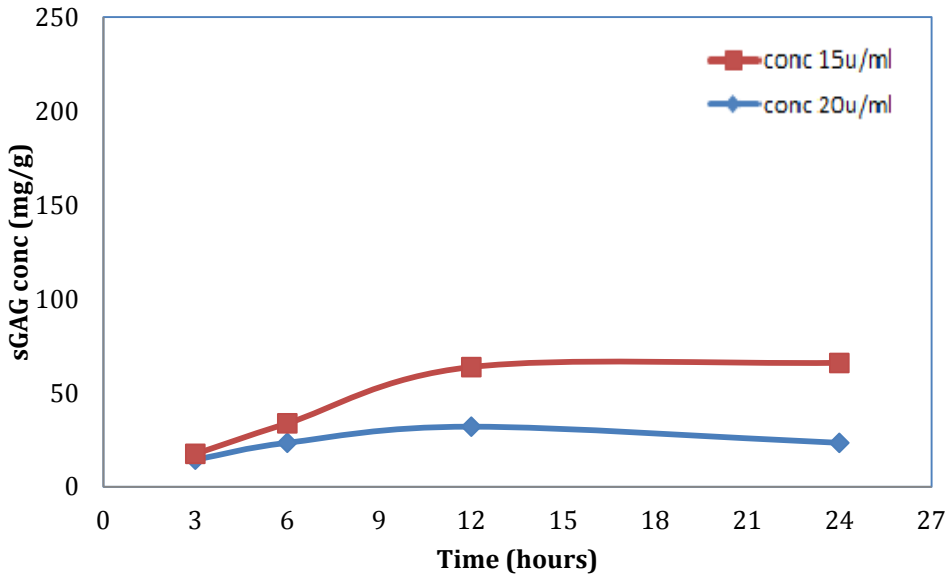


(b) Herring gut

Figure 7.13. Effect of hydrolysis time on the concentration of sGAG extracted from the fish gut with different enzyme concentrations.

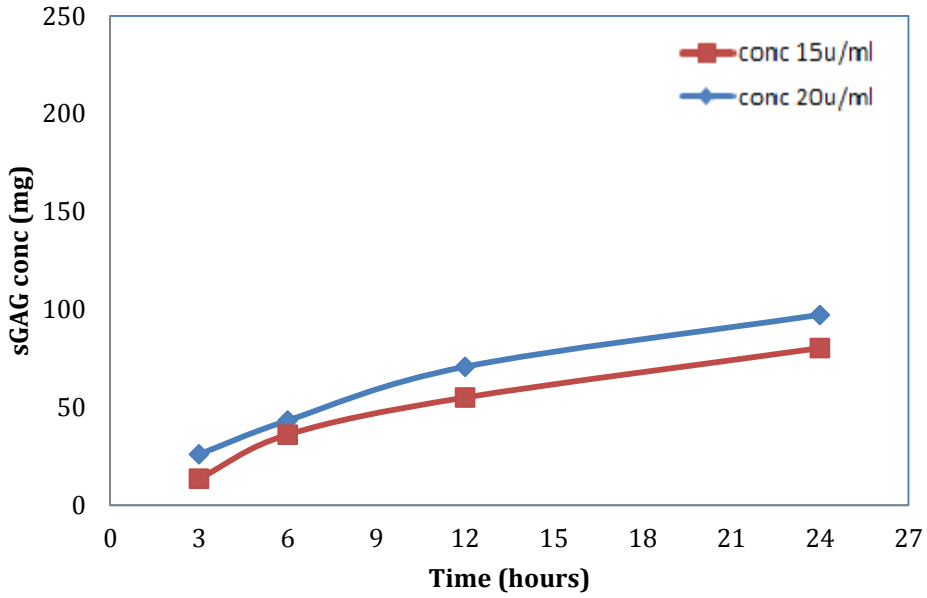


(a) Mackerel skin

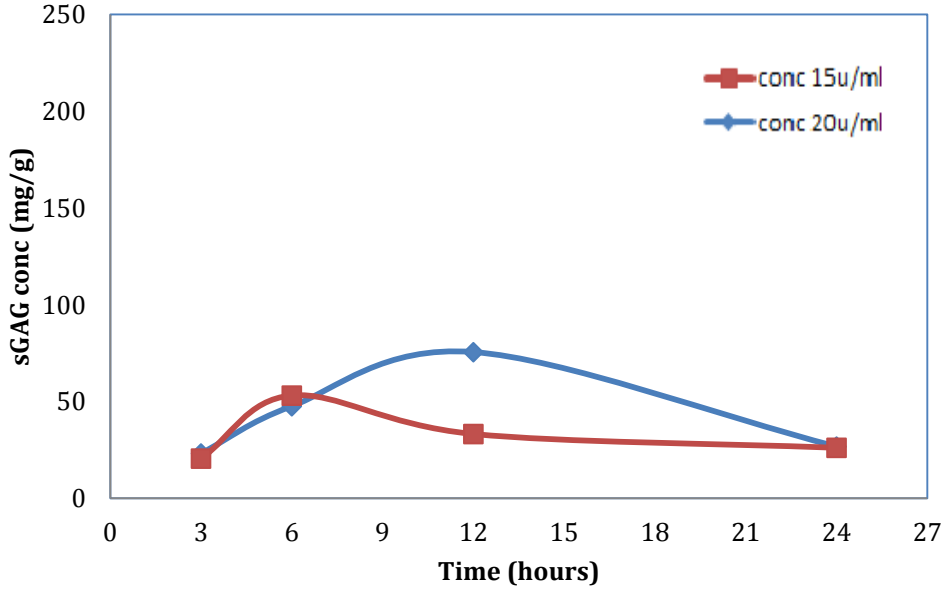


(b) Herring skin

Figure 7.14. Effect of hydrolysis time on the concentration of sGAG extracted from the fish skin with different enzyme concentrations.



(a) Mackerel bones



(b) Herring bones

Figure 7.15. Effect of hydrolysis time on the concentration of sGAG extracted from the fish bones with different enzyme concentrations.

(57.24 %) with the 20 u/ml enzyme concentration for mackerel and from 19.55 to 12.56 mg/g (35.75 %) with the 15 u/ml enzyme concentration and from 39.34 to 8.37 mg/g (78.72 %) with the 20 u/ml enzyme concentration for herring. The 20 u/ml enzyme concentration produced the highest yield of sGAG (39.34 mg/g) from the flesh of both fish at the 6 h hydrolysis time.

For the mackerel waste samples (Figure 7.10 a), the highest sGAG concentrations (110.59 and 163.23 mg/g with the 15 and 20 u/ml enzyme concentrations, respectively) were obtained with the 6 h hydrolysis time. Increasing the hydrolysis time from 3 to 6 h, increased the sGAG concentration from 40.86 to 110.59 mg/g (170.65 %) with the 15 u/ml enzyme concentration and from 89.02 to 163.23 mg/g (83.36 %) with the 20 u/ml enzyme concentration. When the hydrolysis time was further increased from 6 to 24 h, the sGAG concentration decreased from 110.59 to 73.89 mg/g (33.18 %) with the 15 u/ml enzyme concentration and from 163.23 to 96.34 mg/g (40.97 %) with the 20u/ml enzyme concentration. The 20 u/ml enzyme concentration produced the highest yield of sGAG (163.23 mg/g) from the waste of mackerel fish at the 6 h hydrolysis time. However, for the herring waste samples (Figure 7.10 b), the highest sGAG concentrations (130.15 and 100.60 mg/g with the 15 and 20 u/ml enzyme concentrations, respectively) were obtained with the 12 h hydrolysis time. Increasing the hydrolysis time from 3 to 12 h, increased the sGAG concentration from 88.88 to 130.15 mg/g (46.43 %) with the 15 u/ml enzyme concentration and from 74.49 to 100.60 mg/g (35.05 %) with the 20u/ml enzyme concentration. When the hydrolysis time was further increased from 12 to 24 h, the sGAG concentration decreased from 130.15 to 94.91 mg/g (27.07 %) with the 15 u/ml enzyme concentration and from 100.60 to 73.64 mg/g (26.79 %) with the 20u/ml enzyme concentration. The 15 u/ml enzyme concentration produced the highest yield of sGAG (130.15 mg/g) from the waste of herring fish at the 12 h hydrolysis time.

For mackerel head samples (Figure 7.11 a), the highest sGAG concentrations (117.22 and 206.70 mg/g with the 15 and 20 u/ml enzyme concentrations, respectively) were obtained with the 6 h hydrolysis time. Increasing the hydrolysis time from 3 to 6 h, increased the sGAG concentration from 29.76 to 117.22 mg/g (293.88 %) with the 15 u/ml enzyme

concentration and from 116.01 to 206.70 mg/g (78.17 %) with the 20 u/ml enzyme concentration. When the hydrolysis time was further increased from 6 to 24 h, the sGAG concentration decreased from 117.22 to 60.04 mg/g (48.78 %) with the 15 u/ml enzyme concentration and from 206.70 to 100.52 mg/g (51.37 %) with the 20 u/ml enzyme concentration. The 20 u/ml enzyme concentration produced the highest yield of sGAG (206.70 mg/g) from the head of mackerel fish at the 6 h hydrolysis time. However, for herring head samples (Figure 7.11 b), the highest sGAG concentration of 162.76 mg/g was obtained at the 12 h hydrolysis time with the 15 u/ml enzyme concentration whereas the highest sGAG concentration of 76.38 mg/g was obtained at the 6 h hydrolysis time with 20 u/ml enzyme concentration. Increasing the hydrolysis time from 3 to 12 h, increased the sGAG concentration from 99.71 to 162.76 mg/g (63.23 %) with the 15 u/ml enzyme concentration and from 55.44 to 76.38 mg/g (37.77 %) with the 20 u/ml enzyme concentration. When the hydrolysis time was further increased from 12 to 24 h, the sGAG concentration decreased from 162.76 to 104.26 mg/g (35.94 %) and from 76.38 to 60.97 mg/g (20.17 %) with the 15 u/ml and the 20 u/ml enzyme concentration respectively. The 15 u/ml enzyme concentrations produced the highest yield of sGAG (162.76 mg/g) from the head of herring fish at 12 h hydrolysis time.

For mackerel fins and tail samples (Figure 7.12), the highest sGAG concentrations (74.65 and 86.63 mg/g with the 15 and 20 u/ml enzyme concentrations, respectively) were obtained at the 6 h hydrolysis time. Increasing the hydrolysis time from 3 to 6 h, increased the sGAG concentration from 24.51 to 74.65 mg/g (204.56 %) with the 15 u/ml enzyme concentration and from 31.70 to 86.63 mg/g (173.28 %) with the 20 u/ml enzyme concentration. When the hydrolysis time was further increased from 6 to 24 h, the sGAG concentration decreased from 74.65 to 49.10 mg/g (34.22 %) with the 15 u/ml enzyme concentration and from 86.63 to 52.39 mg/g (39.52 %) with the 20 u/ml enzyme concentrations. The 20 u/ml enzyme concentration produced the highest yield of sGAG (86.63 mg/g) from the head of mackerel fish at the 6 h hydrolysis time. However, for herring fins and tail samples (Figure 7.12 b), the highest sGAG concentration (81.62 mg/g) was obtained at the 6 h with the 15 u/ml enzyme concentration and the highest sGAG concentration (148.53 mg/g) was obtained at the 24 h with the 20 u/ml enzyme

concentration. Increasing the hydrolysis time from 3 to 6 h, increased the sGAG concentration from 53.17 to 81.62 mg/g (53.50 %) with the 15 u/ml enzyme concentration but when the hydrolysis time was further increased from 6 to 24 h, the sGAG concentration decreased from 81.62 to 48.00 mg/g (41.19 %). Also, when the hydrolysis time was further increased from 3 to 24 h with the 20 u/ml enzyme concentration, the sGAG concentration increased gradually from 39.59 to 148.53 mg/g (275.17 %). The 20 u/ml enzyme concentration produced highest yield of sGAG (148.53 mg/g) from the fins and tail of herring fish at the 24 h hydrolysis time.

For mackerel gut samples (Figure 7.13 a), the highest sGAG concentrations (185.04 and 203.52 mg/g with the 15 and 20 u/ml enzyme concentration, respectively) were obtained at the 6 h hydrolysis time. Increasing the hydrolysis time from 3 to 6 h, increased the sGAG concentration from 119.26 to 185.04 mg/g (55.15 %) with the 15 u/ml enzyme concentration and from 112.47 to 203.52 mg/g (80.95 %) with the 20 u/ml enzyme concentration. When the hydrolysis time was further increased from 6 to 24 h, the sGAG concentration decreased from 185.04 to 132.03 mg/g (28.67 %) with the 15 u/ml enzyme concentration and from 203.52 to 109.10 mg/g (46.39 %) with the 20 u/ml enzyme concentration. The 20 u/ml enzyme concentration produced the highest yield of sGAG (203.52 mg/g) from the gut of mackerel fish at 6 h hydrolysis time. However, for herring gut sample (Figure 7.13 b), the highest sGAG concentrations (235.77 and 236.30 mg/g with the 15 and 20 u/ml, enzyme concentration, respectively) were obtained at the 12 h hydrolysis time. Increasing the hydrolysis time from 3 to 12 h, increased the sGAG concentration from 202.92 to 235.77 mg/g (16.19 %) with the 15 u/ml enzyme concentration and from 221.58 to 236.30 mg/g (6.64%) with the 20 u/ml enzyme concentration. When the hydrolysis time was further increased from 12 to 24 h, the sGAG concentration decreased from 235.77 to 181.01 mg/g (23.22 %) with the 15 u/ml enzyme concentration and from 236.30 to 173.37 mg/g (26.63 %) with the 20 u/ml enzyme concentration. The 20 u/ml enzyme concentration produced the highest yield of sGAG (236.30 mg/g) from the gut of herring fish at the 12 h hydrolysis time.

For mackerel skin samples (Figure 7.14 a), the highest sGAG concentrations (105.45 and 102.60 mg/g with the 15 and 20 u/ml, enzyme concentration, respectively) were obtained at the 6 h hydrolysis time. Increasing the hydrolysis time from 3 to 6 h, increased the sGAG concentration from 38.94 to 105.45 mg/g (170.80%) with the 15 u/ml enzyme concentration and from 37.23 to 102.60 mg/g (175.58 %) with the 20 u/ml enzyme concentration. When the hydrolysis time was further increased from 6 to 24 h, the sGAG concentration decreased from 105.45 to 60.20 mg/g (42.91 %) with the 15 u/ml enzyme concentration and from 102.60 to 66.34 mg/g (35.34 %) with the 20 u/ml enzyme concentration. The 15 u/ml enzyme concentration produced the highest yield of sGAG (105.45 mg/g) from the skin of mackerel fish at the 6 h hydrolysis time. However, for herring skin samples (Figure 7.14 b), the highest sGAG concentration (65.89 mg/g) was obtained at the 24 h with 15 u/ml enzyme concentration and the highest sGAG (31.99 mg/g) was obtained at the 12 h with 20 u/ml enzyme concentration. Increasing the hydrolysis time from 3 to 24 h, increased the sGAG concentration from 17.52 to 65.89 mg/g (276.08 %) with the 15 u/ml enzyme concentration, whereas increasing the hydrolysis time from 3 to 12 h, increased the sGAG concentration from 14.43 to 31.99 mg/g (121.69 %) with the 20 u/ml enzyme concentration. When the hydrolysis time was further increased from 12 to 24 h, the sGAG concentration decreased from 31.99 to 23.37 mg/g (26.94 %) with the 20 u/ml enzyme concentration. The 15 u/ml enzyme concentration produced the highest yield of sGAG (65.89 mg/g) from the skin of herring fish at the 24 h hydrolysis time.

For mackerel bone samples (Figure 7.15 a), the highest sGAG concentrations (80.28 and 97.19 mg/g with the 15 and 20 u/ml, enzyme concentrations, respectively) were obtained at the 24 h hydrolysis time. Increasing the hydrolysis time from 3 to 24 h, increased the sGAG concentration gradually from 13.49 to 80.28 mg/g (495.11 %) with the 15 u/ml enzyme concentration and from 25.97 to 97.19 mg/g (274.23 %) with the 20 u/ml enzyme concentration. The 20 u/ml enzyme concentration produced the highest yield of sGAG (97.19 mg/g) from the bones of mackerel fish at the 24 h hydrolysis time. For herring bone samples (Figure 7.15 b), the highest sGAG concentration (53.13 mg/g) was obtained with 15 u/ml enzyme concentration at the 6 h hydrolysis time and the highest

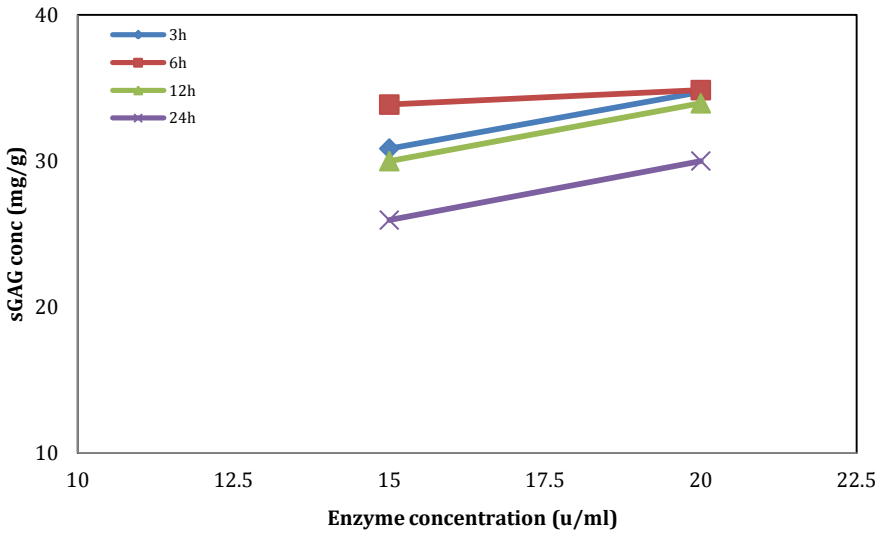
sGAG concentration (75.56 mg/g) with the 20 u/ml enzyme concentration at the 12 h hydrolysis time. Increasing the hydrolysis time from 3 to 6 h with the 15 u/ml enzyme concentration, increased the sGAG concentration from 20.60 to 53.13 mg/g (157.91 %) and when the hydrolysis time was further increased from 6 to 24 h, the sGAG concentration decreased from 53.13 to 26.17 mg/g (50.74 %). Also, increasing the hydrolysis time from 3 to 12 h with the 20 u/ml enzyme concentration, increased the sGAG concentration from 23.25 to 75.56 mg/g (224.99 %) and when the hydrolysis time was increased from 12 to 24 h, the sGAG concentration decreased from 75.56 to 26.82 mg/g (64.50 %). The 20 u/ml enzyme concentration produced the highest yield of sGAG (75.56 mg/g) from the bones of herring fish at the 12 h hydrolysis time.

7.2.3. Effect of Enzyme Concentration

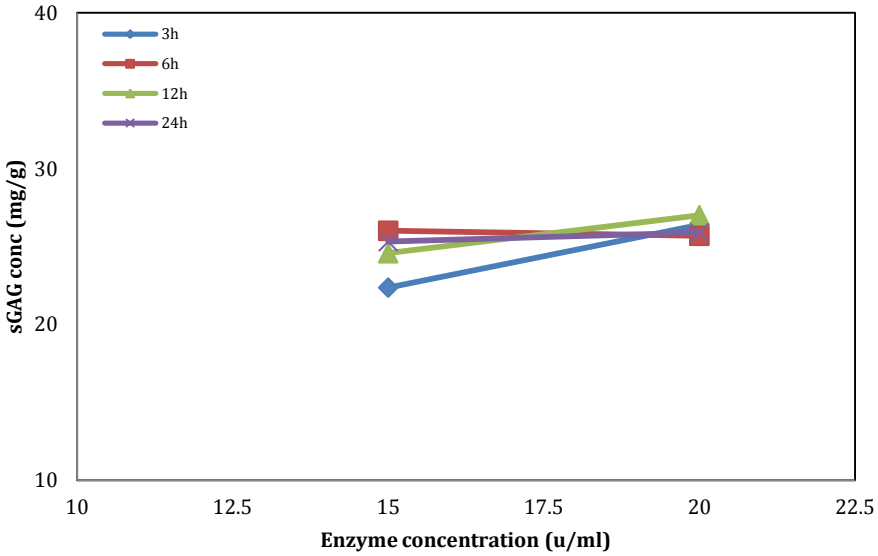
Figures 7.16 and 7.23 show the effect of enzyme concentration on the sGAG concentration in the whole fish, flesh, waste, head, fins and tail, gut, skin and bones of mackerel and herring fish at different hydrolysis times and temperatures.

The sGAG concentration in whole mackerel fish (Figure 7.16) increased from 30.85 to 34.73 mg/g (12.57 %), from 33.86 to 34.85 mg/g (2.92 %), from 29.99 to 33.95 mg/g (13.20 %) and from 25.95 to 30.00 mg/g (15.60 %) when the enzyme concentration was increased from 15 to 20 u/ml for the 3, 6, 12 and 24 h hydrolysis time, respectively. Similarly, the sGAG concentration in the whole herring fish increased from 22.36 to 26.38 mg/g (17.98 %), from 24.57 to 27.01 mg/g (9.93 %) and from 25.31 to 25.89 mg/g (2.29 %) when the enzyme concentration was increased from 15 to 20 u/ml for the 3, 6, 12 and 24 h hydrolysis time.

The sGAG concentration in mackerel flesh (Figure 7.17) increased from 4.63 to 8.17 mg/g (76.45 %), from 16.51 to 23.95 mg/g (45.06 %) and from 9.91 to 10.24 mg/g (3.32 %) when the enzyme concentration was increased from 15 to 20 u/ml for the 3, 6 and 24 h hydrolysis times, respectively. It, however, decreased from 13.86 to 12.47 mg/g

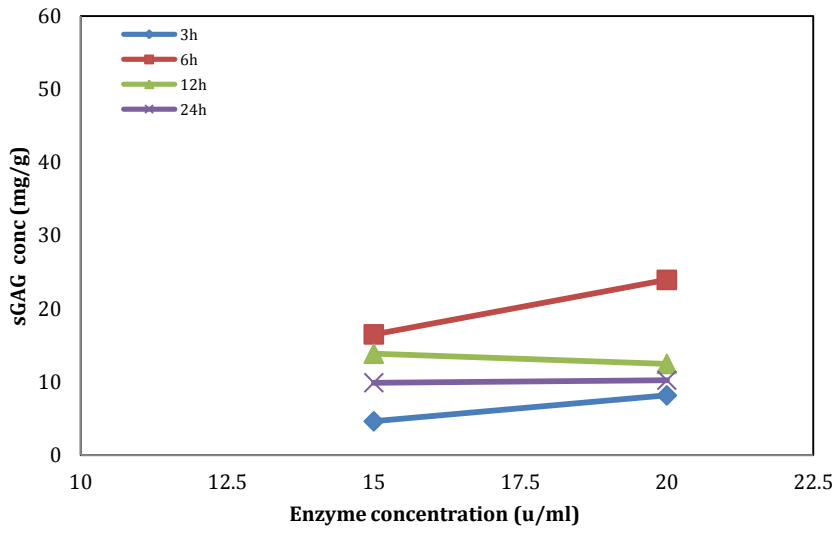


(a) Mackerel

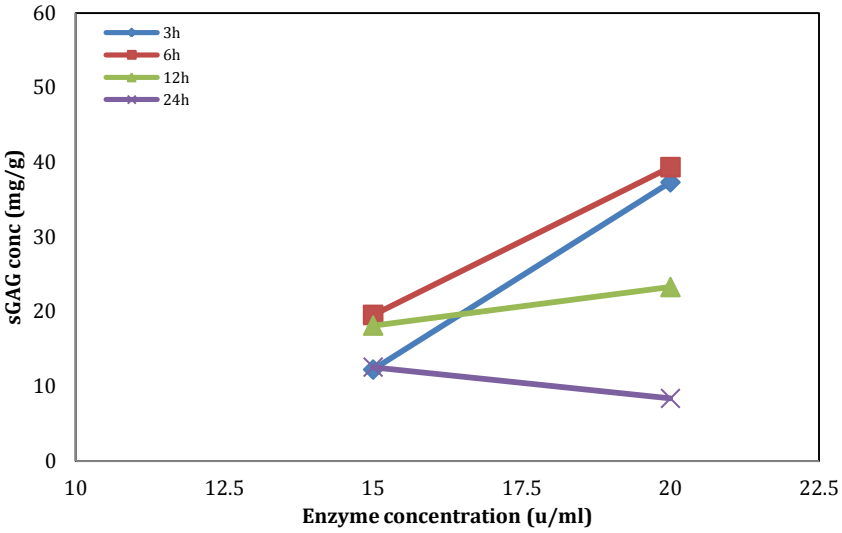


(b) Herring

Figure 7.16. Effect of enzyme concentration on sGAG concentration in the whole fish of mackerel and herring fish at different hydrolysis times.

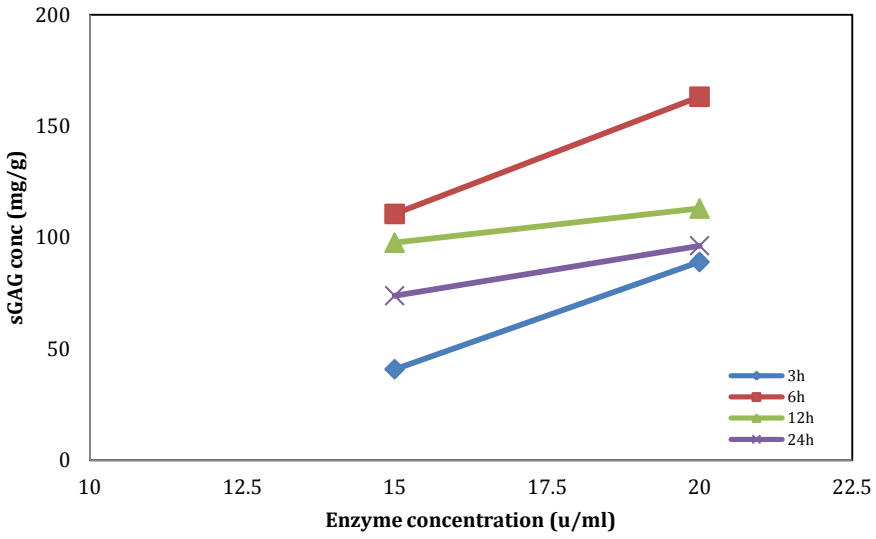


(a) Mackerel

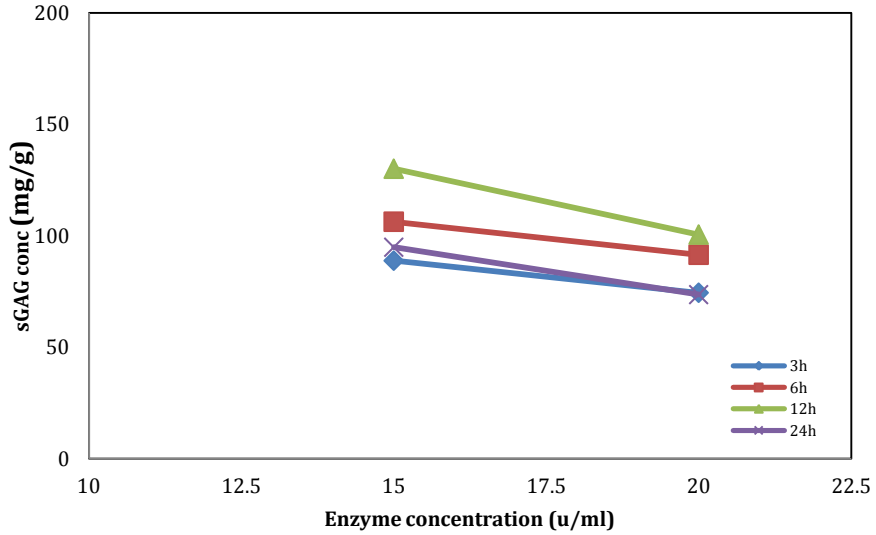


(b) Herring

Figure 7.17. Effect of enzyme concentration on sGAG concentration in the flesh of mackerel and herring fish at different hydrolysis times.

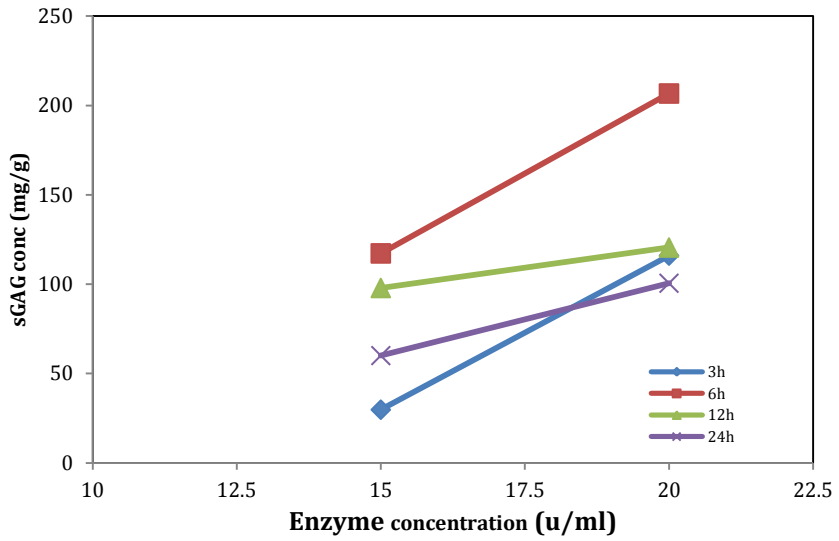


(a) Mackerel

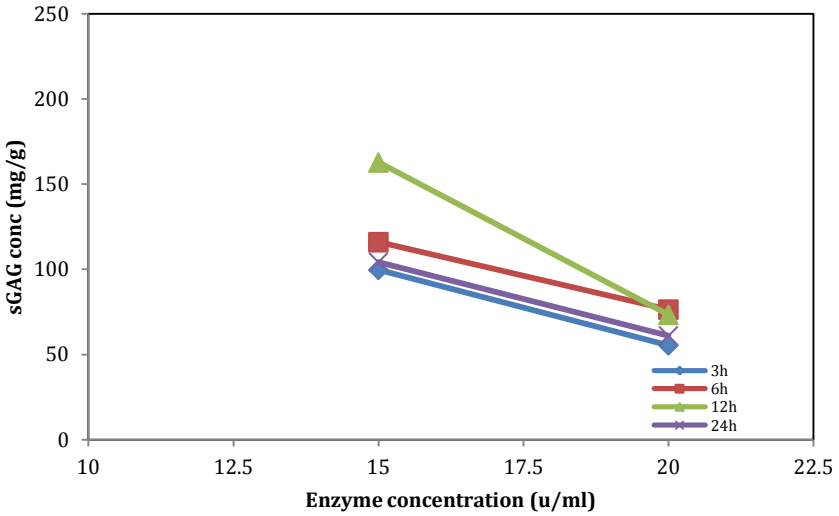


(b) Herring

Figure 7.18. Effect of enzyme concentration on the sGAG concentration in the waste of mackerel and herring fish at different hydrolysis times

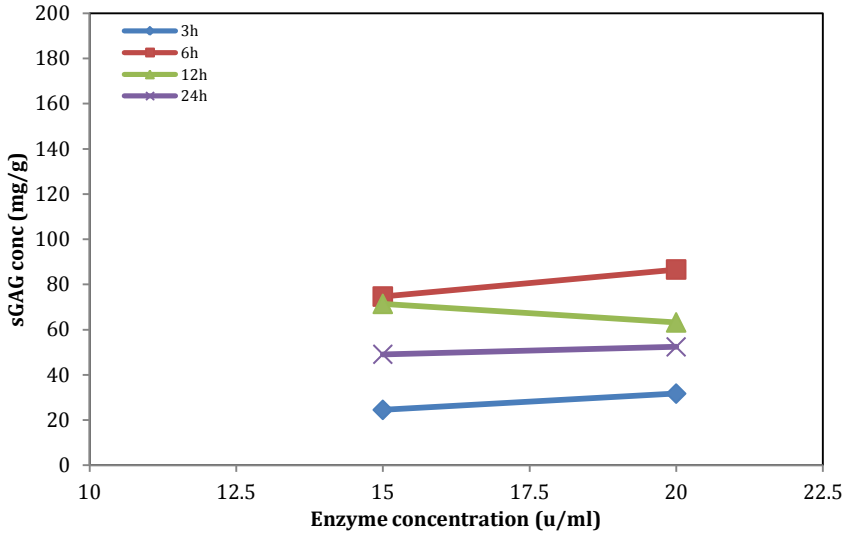


(a) Mackerel

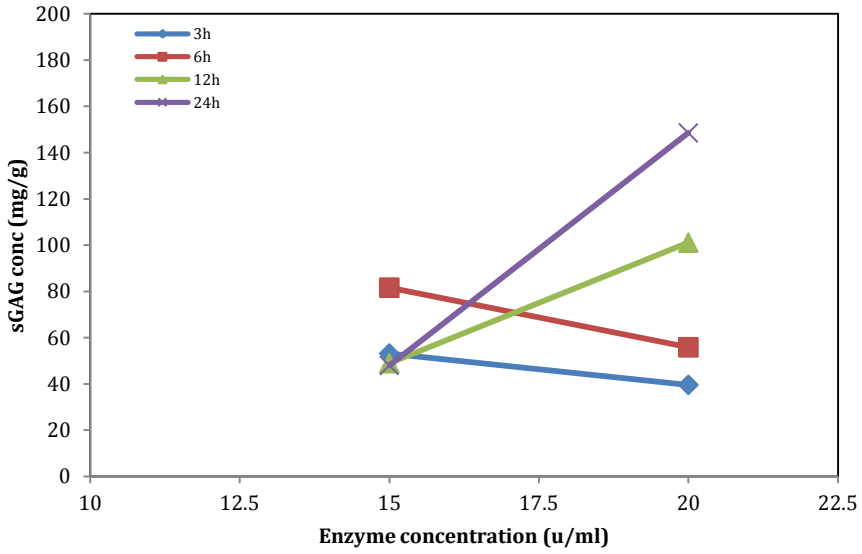


(b) Herring

Figure 7.19. Effect of enzyme concentration on the sGAG concentration in the heads of mackerel and herring fish at different hydrolysis times

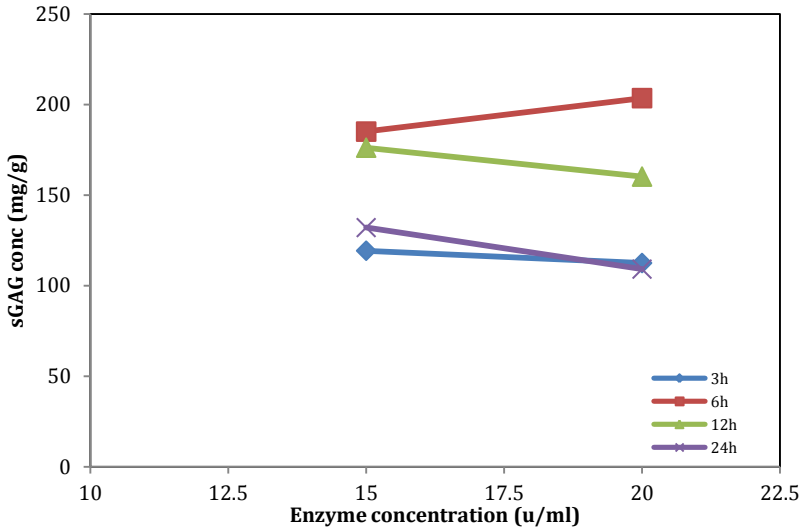


(a) Mackerel

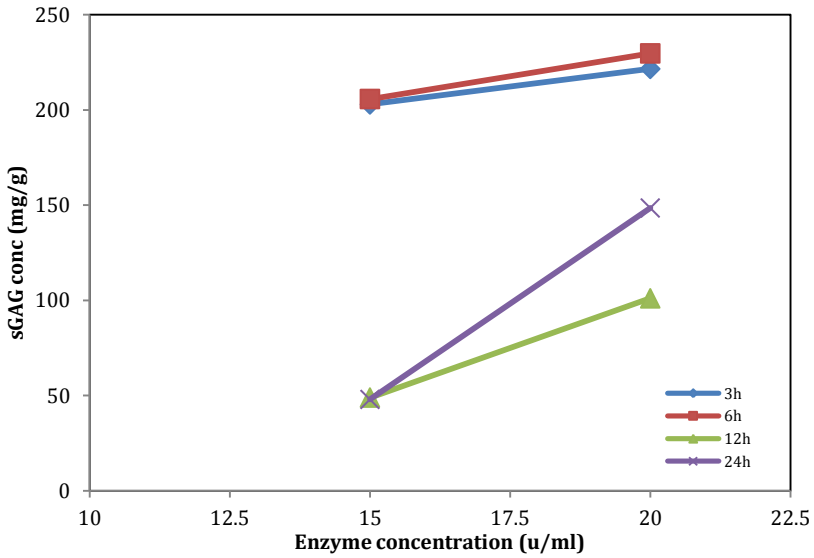


(b) Herring

Figure 7.20. Effect of enzyme concentration on the sGAG concentration in the fins and tail of mackerel and herring fish at different hydrolysis times.

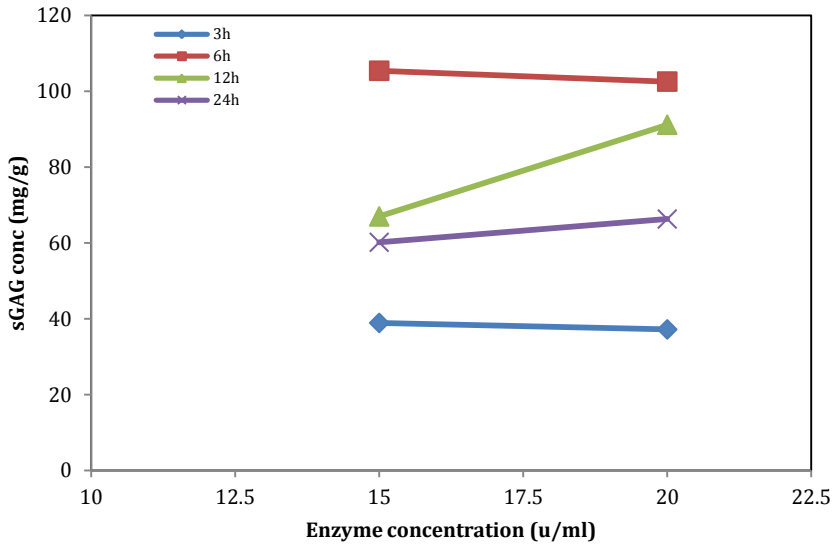


(a) Mackerel

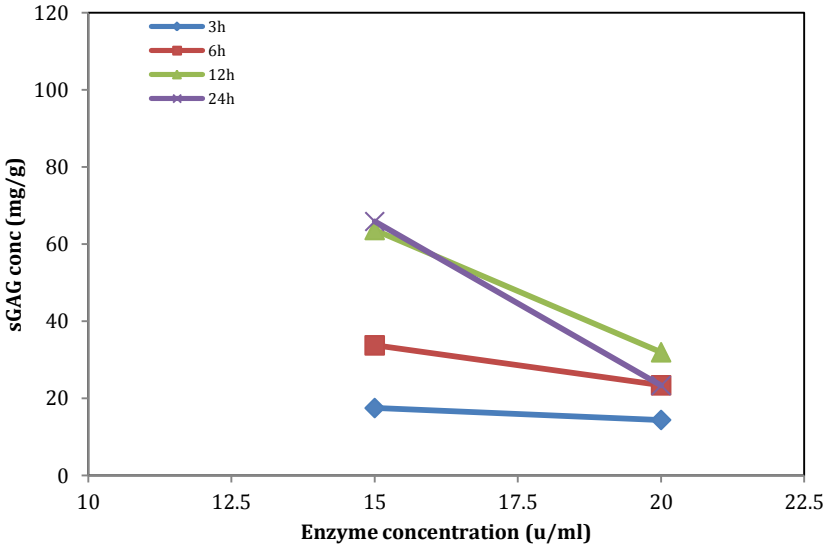


(b) Herring

Figure 7.21. Effect of enzyme concentration on the sGAG concentration in the gut of mackerel and herring fish at different hydrolysis times

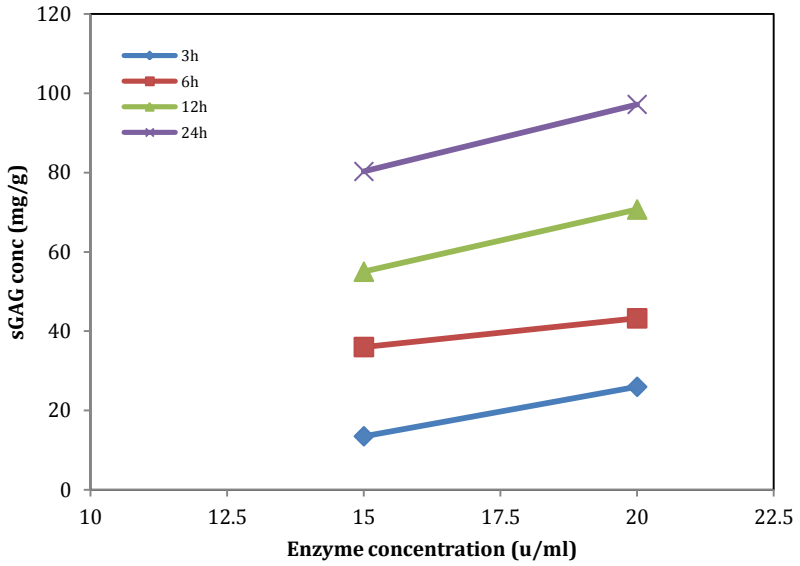


(a) Mackerel

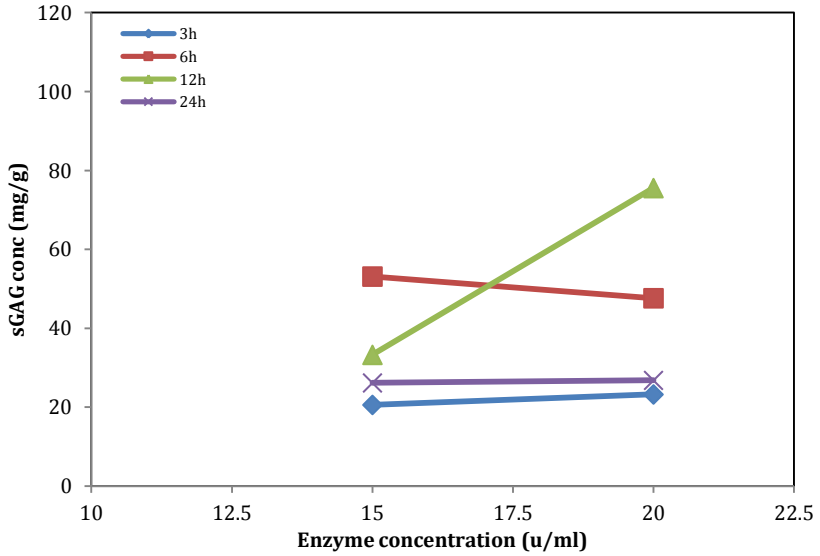


(b) Herring

Figure 7.22. Effect of enzyme concentration on the sGAG concentration in the skin of mackerel and herring fish at different hydrolysis times



(a) Mackerel



(b) Herring

Figure 7.23. Effect of enzyme concentration on the sGAG concentration in the bones of mackerel and herring fish at different hydrolysis times

(10.02 %) for the 12 h hydrolysis time. Also, the sGAG concentration in the herring flesh increased from 12.23 to 37.31 mg/g (205.06 %), from 19.55 to 39.34 mg/g (101.22 %) and from 18.13 to 23.29 mg/g (28.46 %) when the enzyme concentration was increased from 15 to 20 u/ml for the 3, 6 and 12 h hydrolysis times, respectively. However, it decrease from 12.56 to 8.37 mg/g (33.35 %) for the 24 h hydrolysis time.

The sGAG concentration in mackerel waste (Figure 7.18) increased from 40.86 to 89.02 mg/g (117.86 %), from 110.59 to 163.23 mg/g (47.59 %), from 97.72 to 113.03 mg/g (15.67 %) and from 73.89 to 96.34 mg/g (30.38 %) when the enzyme concentration was increased from 15 to 20 u/ml for the 3, 6, 12 and 24 h hydrolysis times, respectively. The sGAG concentrations in herring waste decreased from 88.88 to 74.49 mg/g (16.19 %), from 106.36 to 91.55 mg/g (13.92 %), from 130.15 to 100.60 mg/g (22.70 %) and from 94.91 to 73.64 mg/g (22.41 %) when the enzyme concentration was increased from 15 to 20 u/ml for the 3, 6, 12 and 24 h hydrolysis times, respectively.

The sGAG concentrations in mackerel head (Figure 7.19) increased from 29.76 to 116.01 mg/g (289.82 %), from 117.22 to 206.70 mg/g (76.33 %), from 97.88 to 120.52 mg/g (23.13 %) and from 60.04 to 100.52 mg/g (67.42 %) when the enzyme concentration was increased from 15 to 20 u/ml for the 3, 6, 12 and 24 h hydrolysis times, respectively. However, the sGAG concentration in herring head decreased from 99.71 to 55.44 mg/g (42.39 %), from 115.97 to 96.38 mg/g (16.89 %), from 162.76 to 73.33 mg/g (54.94 %) and from 104.26 to 60.97 mg/g (41.52 %) when the enzyme concentration was increased from 15 to 20 u/ml for the 3, 6, 12 and 24 h hydrolysis times, respectively.

The sGAG concentration in mackerel fins and tail (Figure 7.20) increased from 24.51 to 31.70 mg/g (29.33 %), from 74.65 to 86.63 mg/g (16.04 %) and from 49.10 to 52.39 mg/g (6.70 %) when the enzyme concentration was increased from 15 to 20 u/ml for the 3, 6 and 24 h hydrolysis times respectively. It, however, decreased from 71.42 to 63.21 mg/g (11 %) for the 12 h hydrolysis time. The sGAG concentration in herring fins and tail decreased from 53.17 to 39.59 mg/g (25.54 %) and from 81.62 to 55.85 mg/g (25.77 %) when the enzyme concentration was increased from 15 to 20 u/ml for the 3 and

6 h hydrolysis time, and increased from 48.98 to 101.09 mg/g (106.3 %) and from 48 to 148.53 mg/g (209.4 %) for the 12 and 24 h hydrolysis times, respectively.

The sGAG concentration in mackerel gut (Figure 7.21) decreased from 119.26 to 112.47 mg/g (5.69 %), from 176.17 to 160.20 mg/g (9.06 %) and from 132.03 to 109.10 mg/g (17.36 %) when the enzyme concentration was increased from 15 to 20 u/ml for the 3, 12 and 24 h hydrolysis times, respectively. It increased from 185.04 to 203.52 mg/g (9.9 %) for the 6 h hydrolysis time. However, the sGAG concentration in herring gut increased from 202.92 to 221.58 mg/g (9.19 %), from 205.77 to 229.71 mg/g (11.63 %) and from 235.77 to 236.30 mg/g (0.22 %) when the enzyme concentration was increased from 15 to 20 u/ml for the 3, 6 and 12 h hydrolysis time, respectively. It, however, decreased from 181.01 to 173.37 mg/g (4.2 %) for the 24 h hydrolysis time.

The sGAG concentration in mackerel skin (Figure 7.22) decreased from 38.94 to 37.23 mg/g (4.39 %) and from 105.45 to 102.60 mg/g (2.70 %) when the enzyme concentration was increased from 15 to 20 u/ml for the 3 and 6 h hydrolysis times and increased from 67.03 to 91.21 mg/g (36 %) and from 60.20 to 66.37 mg/g (10.2 %) for the 12 and 24 h hydrolysis time, respectively. The sGAG concentration in herring skin decreased from 17.52 to 14.43 mg/g (17.63 %), from 33.78 to 23.41 mg/g (30.69 %), from 63.69 to 31.99 mg/g (49.77 %) and from 65.89 to 23.37 mg/g (64.53 %) when the enzyme concentration was increased from 15 to 20 u/ml for the 3, 6, 12 and 24 h hydrolysis time, respectively.

The sGAG concentration in mackerel bone (Figure 7.23) increased from 13.49 to 25.97 mg/g (92.51 %), from 36.00 to 43.24 mg/g (20.11 %), from 55.00 to 70.69 mg/g (28.52 %) and from 80.28 to 97.19 mg/g (21.06 %) when the enzyme concentration was increased from 15 to 20 u/ml for the 3, 6, 12 and 24 h hydrolysis times, respectively. The sGAG concentration in herring bone increased from 20.60 to 23.24 mg/g (12.81 %), from 33.29 to 75.56 mg/g (126.97 %) and from 26.17 to 26.82 mg/g (2.48 %) when the enzyme concentration was increased from 15 to 20 u/ml for the 3, 12 and 24 h hydrolysis time, respectively. It decreased from 53.13 to 47.64 mg/g (10.3 %) for the 6 h hydrolysis time.

7.3. Optimum Extraction Condition

Table 7.7 shows the optimum conditions for extracting the highest amount of sGAG from different parts of herring and mackerel fish. The highest sulfated glycosaminoglycans concentration in herring (236.3 mg/g) was obtained from the gut after 12 h incubation with 20u/ml of papain, whereas the highest concentration of sGAG in mackerel (206.7 mg/g) was obtained from the head after 6 h incubation with 20u/ml papain. The optimum conditions for highest recovery of sulfated glycosaminoglycans from the whole waste samples were 6 h of incubation with 20u/ml of papain for mackerel and 12 h of incubation with papain for herring.

7.4. Sulfated Glycosaminoglycans Yield from Different Fish Parts

Tables 7.8 and 7.9 show the amount of sGAG extracted from different parts of mackerel and herring fish treated with papain (15 and 20 u/ml) for different hydrolysis times (3, 6, 12 and 24 hours). The data were calculated by multiplying the average weight of each part by the sGAG concentration in this part. Table 7.10 shows the yield of sGAG extracted at optimum conditions from various parts of mackerel and herring fish. Table 7.11 shows the percent yield of sGAG from various parts of mackerel and herring fish.

About 79.8 % of sGAG was obtained from the mackerel waste samples whereas, 20.2% of sGAG was extracted from the flesh samples. The sGAG obtained from the total waste was distributed as follows: 55.05 % from head, 0.89 % from fins and tail, 14.49 % from gut, 5.64 % from skin, 3.73 % from bones.

About 78.9 % of sGAG was obtained from the herring waste samples whereas, 21.1% of sGAG was extracted from the flesh samples. The sGAG obtained from the total waste was distributed as follows: 39.22 % from head, 1.17 % from fins and tail, 28.24 % from gut, 6.07 % from skin and 4.2 % from bones.

Table 7.7. Optimum conditions to extract the highest sulfated glycoaminoglycans concentration from different parts of herring and mackerel fish

Parts	Mackerel		Herring	
	Highest Concentration (mg/g)	Optimum Condition	Highest Concentration (mg/g)	Optimum Condition
Whole fish	34.86	20u/ml for 6h	27.01	20u/ml for 12h
Flesh	23.96	20u/ml for 6h	39.34	20 u/ml for 6h
Waste	163.23	20u/ml for 6h	130.15	15u/ml for 12h
Head	206.70	20u/ml for 6h	162.76	15u/ml for 12h
Fins and tail	86.63	20u/ml for 6h	148.53	20u/ml for 24h
Gut	203.52	20u/ml for 6h	236.30	20u/ml for 12h
Skin	105.45	15u/ml for 6h	65.89	15u/ml for 24h
Bones	97.20	20u/ml for 24h	75.57	20u/ml for 12h

Table 7.8. Amount of sGAG extracted from different parts of mackerel fish treated with different concentration of papain for different hydrolysis times

Fish Parts	Enzyme Concentration (units/ml)	sGAG Yield* (mg)			
		3 h	6 h	12 h	24 h
whole fish	15	8288.20±933.94	23593.6±373.57	20634.46±93.39	15437.64±93.39
	20	17494.9±747.15	34702.04±513.66	22828.54±46.69	19341.89±46.69
flesh	15	1355.34±263.22	4830.03±438.70	4054.15±87.74	2900.92±380.21
	20	2390.88±233.97	7007.51±380.21	3649.92±380.21	2996.03±438.70
waste	15	6932.84±271.47	18763.23±67.86	16580.31±203.60	12536.72±152.70
	20	15103.22±67.86	27694.53±84.83	19178.62±84.83	16345.86±118.76
head	15	2750.05±36.96	10834.1±101.66	9046.64±27.72	5548.95±166.35
	20	10722.23±157.11	19103.56±73.93	11139.24±36.96	9290.84±101.66
fins and tail	15	87.26±1.78	265.76±8.18	254.26±1.42	174.81±1.42
	20	112.87±7.26	308.41±4.23	225.03±3.20	186.53±4.27
gut	15	2949.50±46.99	4576.16±14.83	4356.90±37.09	3265.16±4.96
	20	2781.62±14.83	5033.11±17.31	3961.82±17.31	2698.18±12.36
skin	15	742.64±24.79	2010.93±13.34	1278.31±15.25	1148.07±13.34
	20	710.08±36.23	1956.72±20.97	1739.55±19.07	1265.13±5.72
bones	15	403.39±59.78	1076.25±60.97	1644.19±50.81	2399.70±56.79
	20	776.41±35.86	1292.72±26.90	2112.95±14.94	2905.16±41.84

*sGAG yield = sGAG concentration *average weight of parts

Table 7.9. Amount of sGAG extracted from different parts of herring fish treated with different concentration of papain for different hydrolysis time

Fish Parts	Enzyme Concentration (units/ml)	sGAG Yield* (mg)			
		3 h	6 h	12 h	24 h
whole fish	15	10094.66±400.80	12926.43±53.44	14819.13±106.88	10692.67±235.13
	20	13126.40±534.40	15009.60±454.24	13054.18±240.48	8060.28±537.07
flesh	15	2109.44±120.68	3370.91±224.12	3125.62±120.68	2165.51±248.25
	20	6433.46±260.32	6783.86±103.44	4015.65±86.20	1443.67±48.27
waste	15	7985.22±98.82	9555.52±62.88	11693.51±107.80	8527.16±41.32
	20	6692.94±80.85	8225.74±35.93	9038.53±107.80	6616.61±80.85
head	15	3560.83±53.56	4141.48±28.56	5812.31±17.85	3723.42±39.28
	20	1980.01±73.20	2727.60±32.13	2618.73±24.99	2177.43±63.20
fins and tail	15	189.81±4.64	291.40±0.71	174.87±2.14	171.38±2.35
	20	141.34±4.64	199.39±4.64	360.91±5.71	530.27±5.89
gut	15	3601.95±18.46	3652.46±28.40	4184.96±21.3	3213.03±22.01
	20	3933.14±58.57	4077.44±3.55	4194.33±1.77	3077.38±14.91
skin	15	247.21±21.16	476.64±1.41	898.79±11.28	929.76±22.01
	20	203.61±20.31	330.38±16.93	451.40±8.46	329.80±13.26
bones	15	385.40±14.96	993.53±20.57	622.57±26.18	489.54±16.45
	20	434.81±31.41	890.91±16.83	1413.14±3.74	501.70±14.58

*sGAG yield = sGAG concentration *average weight of parts

Table 7.10. sGAG yield from various parts of mackerel and herring fish at optimum conditions

Parts	Mackerel		Herring	
	Yield (mg)	Optimum Condition	Yield (mg)	Optimum Condition
Whole fish	34702.04	20u/ml for 6h	14819.13	15u/ml for 12h
Flesh	7007.51	20u/ml for 6h	3125.62	15u/ml for 12h
Waste	27694.53	20u/ml for 6h	11693.51	15u/ml for 12h
Head	19103.56	20u/ml for 6h	5812.31	15u/ml for 12h
Fins and tail	308.41	20u/ml for 6h	174.87	15u/ml for 12h
Gut	5033.11	20u/ml for 6h	4184.96	15u/ml for 12h
Skin	1956.72	20u/ml for 6h	898.79	15u/ml for 12h
Bones	1292.72	20u/ml for 6h	622.57	15u/ml for 12h

Table 7.11. Distribution of sGAG in various parts of mackerel and herring fish

Parts	Percent (%)	
	Mackerel	Herring
Flesh	20.20	21.10
Waste	79.80	78.90
Head	55.05	39.22
Fins and tail	0.89	1.17
Gut	14.49	28.24
Skin	5.64	6.07
Bones	3.73	4.20
Total	100.00	100.00

CHAPTER 8. DISCUSSION

In this study, the effects of fish type (mackerel and herring) and fish parts (whole fish, flesh, head, fins and tail, gut, skin and bone), papain enzyme concentration (15 and 20 u/ml) and hydrolysis time (3, 6, 12 and 24 h) on the yield of sGAG were evaluated.

8.1. Effect of Fish Types and Parts

Seven different samples (whole fish, head, flesh, skin, fins and tail, gut and bones) from both mackerel and herring were investigated. The edible portion of both mackerel and herring was found to be around 60% and the remaining 40% was inedible or waste. The inedible portion included the head, fins and tail, gut, skin and bones. It was observed that the herring gut sample and mackerel head sample produced the highest concentrations of sGAG of 236.3 mg/g and 206.7 mg/g, respectively. The higher sGAG concentration in the head sample of mackerel is due to the presence of dense cranial cartilages (bound sGAG) while the higher sGAG concentration in the herring gut sample is likely due to the presence of eggs (free sGAG) in almost all samples of gut.

Arima et al. (2013) extracted GAGs (heparin sulfate and keratin sulfate were not analyzed) from the head and skin of Japanese jack mackerel, the head of Atlantic mackerel and the head of squid. The head and skin of Japanese mackerel yielded 187 mg/100g and 609 mg/100g of pure GAG respectively. The head of Atlantic mackerel yielded 289 mg/100g and the head of squid yielded 838 mg/100g of pure GAG.

Mansour et al. (2009) studied the extraction of sGAG from the skin of the ray *Raja radula* (fish) and obtained approximately 50 mg/g of sGAG. Garnjanagoonchorn et al. (2007) isolated 108 mg/g (per dry weight of cartilage) of sGAG from 10 g of dry cartilage of ray. Manjusha (2011) isolated glycoaminoglycans from the fin, skin and head of squid and cuttle fish. The head of squid and cuttle fish yielded 78.15 mg/g and 69.19 mg/g of sGAG, the fin of squid and cuttle fish yielded 12.73 mg/g and 8.49 mg/g of

sGAG and the skin of squid and cuttle fish yielded 5.28 mg/g and 1.47 mg/g of GAG, respectively. The result obtained from these studies emphasizes the variation in the concentration of sGAG among fish parts and fish types.

The yield of GAGs obtained in this study is much higher than the GAGs reported by other studies. The GAGs obtained from mackerel head (206.7 mg/g) and herring head (162.76 mg/g) in this study were three times higher than the GAGs content in the head of squid (78.15 mg/g) and cuttle fish (69.19 mg/g). The GAGs obtained from the skin of mackerel (105.54 mg/g) and herring (65.89 mg/g) were higher than the GAG obtained from skin of ray fish (50 mg/g). The variations in GAG content were due to difference in source, extraction procedures, purity and type of GAGs extracted. In this study crude GAG was extracted and total sulfated GAG content was determined.

The factors that may affect sGAG content in fish include the differences in matrix and muscle requirements in fish species due to swimming behavior as well as the differences in body shape and flexibility of the bone matrix and environmental conditions (Wardle et al., 1995; Altringham and Ellerby, 1999). Kazuya (2013) studied the amount of sGAG in fish tissue which mainly consisted of chondroitin sulphate-O, A and C. Different sulfation patterns were observed with closely related species such as those of *perciformes*. The fishes living in the bottom of the sea showed lower ratios of A/C sGAG and the sGAG composition was influenced by environmental factors such as temperature, diet, pressure, depth and also, motility might closely relate to the sulfation patterns. According to Alberto et al. (1969), young animals possess higher levels of free sGAG compared to bound sGAG. These authors observed similar rates of synthesis of the free and bound GAG and reported that due to the action of tissue proteases, part of the bound sGAG may be altered into free sGAG.

According to Stacy and Barker (1965), any tissues of natural origin containing proteoglycans (cornea, cartilage, bone, trachea, liver and small intestine) can be used as the raw material for sGAG. Nakano et al. (2010) stated that cartilaginous tissues are a source of chrondroitin sulfate whereas galactoaminoglycans are in non-cartilaginous

connective tissue such as skin, skeletal muscle epimysium and tendon. Cartilage tissues with low fat contents can be used to extract GAG without defatting. However, it is important to defat tissues (with high fat contents) to reduce turbidity during GAG analysis. In this study, since mackerel and herring fish had considerable fat content, all the samples were defatted before the enzymatic extraction.

Falshaw et al. (2000) observed differences in chondroitin sulfate chain structure between mammals and other organisms. Mammalian chondroitin sulfate (CS) is comprised of a mixture of the monosulfated species chondroitin 4-sulfate and chondroitin 6-sulfate with small amounts of other disaccharide units. However, in lower organisms such as fish, squid, sharks and sea cucumbers, the chondroitin sulfate has different substitution pattern consisting of neutral carbohydrate and sulfate substituents. Gomes and Dietrich (1982) investigated the amount of sGAG in the tissues of 7 vertebrates. It was observed that different tissues produced different amounts types, and molecular sizes of sGAG. However, the tissue from similar parts of different vertebrates had the same types of sGAG but different molecular weights.

8.2. Effect of Hydrolysis Time

Four different hydrolysis times (3, 6, 12 and 24 h) were investigated. When the hydrolysis time was increased from 3 to 6 h, the sGAG concentration increased for all parts of mackerel and herring fish (flesh, waste, head, fins and tail, gut, skin and bones) with both enzyme concentrations (15 and 20 u/ml). However, when the hydrolysis time was increased from 6 to 12 h, the sGAG concentration decreased for all mackerel parts (flesh, waste, head, fins and tail, gut and skin) and only for the flesh of herring with both enzyme concentrations (15 and 20 u/ml). Additional increase in the hydrolysis time (from 12 to 24 h) decreased the sGAG concentration for the mackerel flesh, waste, head, fins and tail, gut and skin and for the herring flesh, waste, head, gut and bones. Studies on the extraction of glycosaminoglycans from abalone *Haliotis discus hannai* Ino reported by Li et al. (2011) showed that increasing the hydrolysis time increased the amount of glycosaminoglycans till an optimum time of 10 h and further increases in hydrolysis time

decreased the amount of glycosaminoglycans. The decrease in sGAG concentration with increase in hydrolysis time might be due to the breakage of core peptide of sGAGs which is attached to the disaccharides as reported by Gandhi and Mancera (2008).

It was observed that the highest recovery of sGAG of 236.3 (mg/g) was obtained after 12 h of hydrolysis for herring and 206.7 (mg/g) was obtained after 6 h of hydrolysis for mackerel. It is likely that the presence of eggs and various amino acids in the herring intestine required longer hydrolysis time (12 h) to isolate the highest concentration of sGAG while the presence of soft cranial cartilages in the head of mackerel required only 6 h of hydrolysis to isolate the highest concentration of sGAG.

Although 6 - 12 h were generally the optimum hydrolysis times observed for most parts of mackerel and herring fish at both enzyme concentrations in this study, there were some unusual trends observed with the fins and tail of herring fish at the 20 U/ml enzyme concentration and with the bones of mackerel at both enzyme concentrations. The sGAG content from these samples increased continuously with increases in hydrolysis time (from 3 h to 24 h). The longer hydrolysis time required for fins and tail of herring at the 20 U/ml and the bones of mackerel at both enzyme concentrations were due to the presence of bound sGAG content in these tissues which required longer hydrolysis time for release into the system. Alberto et al. (1969) reported that optimum hydrolysis time for hard tissues (bones, fins and tail) is longer than that required for soft tissues (skin, flesh and gut). The effect of hydrolysis times on sGAG yield from various tissues has not been reported widely in the literature.

The results showed that the required hydrolysis time was affected by the type of fish and fish part. To achieve a high yield of glycosaminoglycan, a high degree of protein breakdown is required (Ashie, 2005). Aurelia et al. (2008) reported that increases in hydrolysis time increases the extraction yield. Guerard et al. (2002) reported that the hydrolysis rate decreases with time due to the decrease in the concentration of peptide bonds available for hydrolysis, enzyme deactivation and enzyme inhibition.

The concentrations of sGAG extracted from mackerel and herring samples observed in this study were much higher than other values reported in the literature. Mansour et al. (2009) studied the extraction of GAG from the skin of ray *Raja radula* using a hydrolysis time of 24 h and obtained approximately 50 mg/g of GAG. Pereira et al. (2005) obtained 24 mg/g of sGAG from red alga with papain after a hydrolysis time of 24 h. Mitropoulou et al. (2001) extracted glycosaminoglycans from uterine leiomyoma and normal myometrium using a hydrolysis time of 20 h and reported 2.06 mg/g and 1.59 mg/g of sGAG, respectively. These are much smaller than the sGAG concentration of 105.45 mg/g obtained after only 6 h of hydrolysis for mackerel skin and the sGAG concentration of 65.89 mg/g obtained after 24 h of hydrolysis for herring skin.

Garnjanagoonchorn et al. (2007) isolated sGAG from ray cartilage at a hydrolysis time of 48 h and obtained 108 mg/g (per dry weight of cartilage) of sGAG. In this study, 206.7 mg/g and 162.76 mg/g sGAG were obtained after much shorter hydrolysis times from the cartilaginous-rich head samples of mackerel and herring respectively.

8.3. Effect of Enzyme Concentration

The papain enzyme used in this study is one of the sulfhydryl proteases isolated from the latex of Carica papaya. This latex contains several enzymes in which papain is present as a minor part of the total proteolytic activity. Papain is very stable and causes minimal damage to the tissues. It is known to be the archetype of cysteine proteinase (Arnon, 1970). It has been reported that incubation with papain increases the affinity of the enzyme for the sulfated glycoaminoglycans (Almeida et al. 1999). According to Pinto (2007), hydrolysis by the papain enzyme specifically occurs at the peptide bonds of hydrophobic amino acids at P2 position and the basic amino acids at P1 position. Scott (1969) stated that crude papain is a highly efficient lipase in digesting cartilaginous tissues.

To increase the activity of papain and to enhance the hydrolysis process, all assays in this study were carried out in presence of activators comprising of cysteine (0.005M) and

(0.01M) EDTA as recommended by Arnon (1970). Papain activity was terminated in the presence of air and low concentration of cysteine. However, it was impossible to differentiate between the inactivated enzyme and the native crystalline papain. Brubacher and Bender (1966) reported that the inactivated enzyme can be reactivated in the presence of higher concentration of cysteine.

Papain has been known to produce macro and microscopic changes in cartilage. According to Nakano (2001), most of the galactoaminoglycans have copolymeric structures consisting of repeating disaccharide units with glucuronosyl and iduronosyl residues in different proportions with the exception of cartilage chondroitin sulfate. In the study by Nakano (2001), sGAGs were extracted from cartilage with activation of endogenous enzymes without using exogenous proteinase.

Silva (2006) reported that glycoaminoglycans can be liberated directly from tissues by hydrolysis in presence of exogenous enzymes such as papain. Proteolysis with papain yields a single sGAG chain to which a small peptide (consisting of several amino acids) is covalently attached (Roden et al., 1972).

According to Himonides et al. (2001) and Wasswa et al. (2007), papain is a neutral protease. Its rate of hydrolysis is the highest at a pH of 6.5 and further increases in pH decreases the rate of hydrolysis. Kilara et al. (1977) stated that the optimum temperature for highest papain activity is at 65ºC. However, exposure to high temperature, organic solvents and reagents do not affect the stability of papain. For example, papain powder can resist dry heat at 100ºC for 3 h and it showed remarkable temperature stability even in solution (Hwang and Ivy, 1951). In this study, the experiments were conducted at a pH of 6.4 and a temperature of 65ºC.

In this study, when the enzyme concentration was increased from 15 to 20 u/ml, the sGAG concentrations increased for most parts of mackerel and herring at all hydrolysis times (3, 6, 12 and 24 h). The highest recovery yields of sGAG 236.3 and 206.7 (mg/g) were obtained with the enzyme concentration of 20 u/ml for both herring and mackerel

fish, respectively. Aurelia et al. (2008) reported that increases in enzyme concentration increased the degree of hydrolysis and resulted in higher sGAG concentrations.

In this study, although the sGAG concentration increased with increase in enzyme concentration (from 15 U/ml to 20 U/ml) for most parts of mackerel and herring fish for all hydrolysis times (3, 6, 12 and 24 h), there were some opposite trends observed with the flesh of herring with the 24 h hydrolysis time, the waste of herring with all hydrolysis times, the head of herring with all hydrolysis time, the fins and tail of herring with the 3 h and 6 h hydrolysis times, the skin of mackerel with the 3 h hydrolysis time, the skin of herring with all hydrolysis times and the bones of herring with the 6 h hydrolysis time. The sGAG content from these samples decreased when the enzyme concentration increased from 15 U/ml to 20 U/ml. These decreases in sGAG content with increase in the enzyme concentration may be due to the extensive breakdown of core peptides which in turn reduced the sGAG available for analysis (Lindahl et al. 1998). Since papain binding to sGAG is mediated mainly by electrostatic interactions and papain exhibits high affinity in binding to sGAG, longer hydrolysis time (higher than optimum time) with higher enzyme concentrations and lesser sGAG content in the sample leads to inhibition of sGAG hydrolysis (Li et al., 2006).

Pereira et al. (2005) studied the extraction of sulfated galactans from red alga with 1 g papain and obtained approximately 24 mg/g of sGAG. Mansour et al. (2009) studied the extraction of sGAG from the ray *Raja radula* using 510 mg papain and obtained approximately 50 mg/g of sGAG. Monica et al. (2005) identified sulfated glycoaminoglycans from the muscle tissue of the Atlantic cod and spotted wolf-fish using 14 units/mg of papain and observed sGAG 3-4 times more from wolf-fish than that of the Atlantic cod. One of the limitations in comparing the extraction results obtained in this study with those of other studies reported in the literature is that the activity of papain can vary. Although, the mass of enzyme added to an extraction process may be given, the level of enzyme activity was not reported.

There is a consistent two-step mechanism that is proposed in different kinetics studies of papain action. The mechanism involves acylation and deacylation which determine the turnover rate constant (K_{cat}) for various substrates (Cohen and Petra, 1967). Kimmel and Smith (1960) showed that the hydrolysis of substrate with papain leads to the formation of an acyl-enzyme intermediate as shown in equation (1).

$$E + S \underset{}{\overset{k_a}{\rightleftharpoons}} ES \xrightarrow{k_2} ES' + P1 \xrightarrow[H_2O]{k_3} E + P2 \quad (1)$$

Where,

ES = Enzyme substrate complex

k_a = Equilibrium constant

ES' = Acyl enzyme

P1 and P2 = Alcohol and acid portions of an ester substrate

8.4. Optimum Extraction Condition for Sulfated Glycosaminoglycans

From the results of this study, it is evident that mackerel and herring fish waste samples produced significant amounts of sGAG compared to the edible portions of the two fishes. The results showed that 79.8% and 78.9% of sGAG were achieved from waste samples of mackerel and herring fish and only 20.2% of the total mackerel sGAG yield and 21.1% of total herring sGAG yield were obtained from the flesh samples. The sGAG yield obtained from the head, fins and tail, gut, skin and bones were 55.05, 0.89, 14.49, 5.64 and 3.37 % for mackerel and 39.22, 1.17, 28.24, 6.07 and 4.20% for herring, respectively. The results indicate that herring and mackerel fish processing waste can be a very good source for the extraction of sulfated glycosaminoglycans. The recommended parameters for extracting sGAG from mackerel and herring fish are shown in Table 8.1.

Table 8.1. Recommended parameters for sGAG extraction from mackerel and herring fish waste

Factors	Parameters	
	Mackerel	Herring
Enzyme	Papain	Papain
Enzyme concentration	20 units/ml	15 units/ml
Hydrolysis Time	6 h	12 h
pH	6.4	6.4
Temperature	65ºC	65ºC

CHAPTER 9. RECOMMENDATIONS

1. In this study, a measurement technique was used to determine the overall concentration of extracted GAGs. However, the details of the individual sGAG molecules are not known for more information about the sGAG structure, other analytical techniques should be used such as NMR, MS, LC-MS, enzymatic post column fluorescence HPLC, electrophoretic methods (Guerrini et al. 2005; Volpi and Maccari, 2006). There is also a need to develop a robust, simple quantification method for routine GAG quantification.

2. sGAG are used as an anticoagulant drug which also possesses anti-sepsis, anti-selecting mediated inflammation and anti-tumor metastasis properties. sGAG are currently extracted from discards from porcine/cattle slaughterhouses and also from cartilages of whale and ray. sGAG from porcine/cattle are questionable due to risks of interspecies infections. Since whale and ray are potentially endangered, fish wastes are are viable and safer sources of GAG production.

3. To assess the commercial viability of the sGAG obtained from the present study, the anti-coagulant and anti-inflammatory activities of the extract should be characterized.

4. The extraction procedure would need to be scaled up for industrial purposes. Successful scaling up of this procedure can help in aiding the economics of fish processing industries and also address the environmental risks associated with disposal of processing waste.

CHAPTER 10. CONCLUSIONS

Sulfated Glycosaminoglycans were isolated from various parts (whole fish, head, flesh, fins and tail, gut and bone) of mackerel and herring fish. The effects of enzyme concentration (15 and 20 u/ml) and hydrolysis time (3, 6, 12 and 24 h) on sGAG yield were studied. The amount of sGAG produced from both mackerel and herring wastes were determined. The sGAG concentrations in various parts (whole fish, head, flesh, fins and tail, gut and bones) of mackerel and herring fish were compared. The following are the conclusions drawn from the study.

1. The sGAG concentration increased with increases in the enzyme concentration (from 15 to 20 u/ml) for the mackerel and herring parts (whole, waste, head and bones).
   (a) The enzyme concentration had significant effect on the sGAG yield at 0.001 levels.
   (b) The increase in enzyme concentration increases the degree of hydrolysis which in turn resulted in increases in the sGAG concentration.
   (c) The highest sGAG extracted was observed at the enzyme concentration of 20 u/ml for the mackerel samples and 15 u/ml for the herring samples.
   (d) Among the fish wastes, mackerel head and herring gut produced the highest sGAG concentration.

2. The sGAG concentration increased with increases in the hydrolysis time (from 3 to 6 h) for all parts of mackerel and herring (flesh, head, fins and tail, gut, skin and bones) with further increase in hydrolysis time (from 6 to 24 h), the sGAG concentration decreased for several mackerel parts (flesh, waste, head, fins and tail, gut and skin) and for the herring flesh.
   (a) The hydrolysis time had significant effect on the sGAG yield at 0.001 levels.
   (b) Longer time resulted in high degree of protein breakdown which helped achieving higher yield of sGAG.

(c) The highest sGAG extracted was observed at the hydrolysis time of 6 h for the mackerel samples and the 12 h hydrolysis time for the herring samples.
(d) Among the waste, mackerel head and herring gut obtained the highest sGAG concentration.

3. The edible and inedible portions (head, fins and tail, gut, skin and bones) of mackerel and herring fish possess sGAG.
   (a) The fish type and parts had significant effect on the sGAG yield at 0.001 levels.
   (b) The sGAG obtained from the mackerel samples were 34.85, 23.96, 163.23, 86.63, 203.52, 105.45, 97.2 mg/g for the whole fish, flesh, waste, fins and tails, gut, skin and bones, respectively.
   (c) The sGAG obtained from the herring samples were 27.01, 39.34, 130.15, 162.76, 148.53, 65.89, 75.57 mg/g for the whole fish, flesh, waste, head, fins and tails, skin and bones, respectively.
   (d) Higher sGAG was extracted from mackerel fish compared to herring.
   (e) The highest sGAG was extracted from head (206.7 mg/g) sample of mackerel and gut (236.3 mg/g) sample of herring fish.

4. The two-way, three-way and four-way interactions between the enzyme concentration, hydrolysis time, fish type and parts appeared to be significant for both mackerel and herring at the 0.001 levels.

5. Different parts of mackerel and herring had different optimum conditions for the isolation of sGAG. Bones and cartilages (hard tissues) samples needed higher hydrolysis time and enzyme concentration compared to flesh samples (soft tissues).

6. The sGAG yield obtained from various parts of both mackerel and herring showed that around 80% of sGAG were extracted from waste/inedible parts of the fish and only 20% of sGAG were extracted from edible portion of the fish. This

indicates that mackerel and herring processing wastes can be potential source of viable and safer sGAG.

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APPENDIX A: Enzymatic Extraction Data

Table A1: sGAG measured at 656nm for herring fish at 15 μ/ml

Herring Parts	Time	OD 1	OD 2	OD 3	Average
Skin	3	0.222	0.209	0.216	0.215
Head	3	1.229	1.224	1.227	1.226
Bones	3	0.250	0.257	0.254	0.253
Fins and tail	3	0.692	0.616	0.654	0.654
Gut	3	2.524	2.468	2.496	2.496
Flesh	3	0.154	0.147	0.151	0.150
Skin	6	0.422	0.409	0.416	0.415
Head	6	1.429	1.424	1.427	1.426
Bones	6	0.650	0.657	0.654	0.653
Fins and tail	6	0.992	1.016	1.004	1.004
Gut	6	2.494	2.568	2.531	2.531
Flesh	6	0.234	0.247	0.241	0.240
Skin	12	0.787	0.780	0.784	0.783
Head	12	2.036	1.968	2.002	2.002
Bones	12	0.415	0.404	0.410	0.409
Fins and tail	12	0.610	0.595	0.603	0.602
Gut	12	2.944	2.856	2.900	2.900
Flesh	12	0.224	0.222	0.223	0.223
Skin	24	0.828	0.793	0.811	0.810
Head	24	1.262	1.303	1.283	1.282
Bones	24	0.327	0.317	0.322	0.322
Fins and tail	24	0.589	0.592	0.591	0.590
Gut	24	2.223	2.230	2.227	2.226
Flesh	24	0.154	0.155	0.155	0.154

Table A2: sGAG measured at 656nm for herring fish at 20 μ/ml

Herring Parts	Time	OD 1	OD 2	OD 3	Average
Skin	3	0.170	0.185	0.178	0.177
Head	3	0.714	0.650	0.682	0.682
Bones	3	0.288	0.284	0.286	0.286
Fins and tail	3	0.487	0.487	0.487	0.487
Gut	3	2.623	2.828	2.726	2.725
Flesh	3	0.474	0.444	0.459	0.459
Skin	6	0.291	0.285	0.288	0.288
Head	6	0.914	0.965	0.940	0.939
Bones	6	0.588	0.584	0.586	0.586
Fins and tail	6	0.687	0.687	0.687	0.687
Gut	6	2.823	2.828	2.826	2.825
Flesh	6	0.484	0.484	0.484	0.484
Skin	12	0.391	0.396	0.394	0.393
Head	12	0.911	0.893	0.902	0.902
Bones	12	0.919	0.940	0.930	0.929
Fins and tail	12	1.234	1.253	1.244	1.243
Gut	12	2.879	2.934	2.907	2.906
Flesh	12	0.291	0.282	0.287	0.286
Skin	24	0.289	0.286	0.288	0.287
Head	24	0.752	0.748	0.750	0.750
Bones	24	0.328	0.332	0.330	0.330
Fins and tail	24	1.774	1.880	1.827	1.827
Gut	24	2.125	2.140	2.133	2.132
Flesh	24	0.109	0.097	0.103	0.103

Table A3: sGAG measured at 656nm for mackerel fish at 15 μ/ml

Mackerel Parts	Time	OD 1	OD 2	OD 3	Average
Skin	3	0.499	0.459	0.479	0.479
Head	3	0.369	0.363	0.366	0.366
Bones	3	0.168	0.164	0.166	0.166
Fins and tail	3	0.305	0.298	0.302	0.301
Gut	3	1.447	1.487	1.467	1.467
Flesh	3	0.053	0.061	0.057	0.057
Skin	6	0.799	0.759	0.779	0.779
Head	6	0.869	0.863	0.866	0.866
Bones	6	0.268	0.264	0.266	0.266
Fins and tail	6	0.505	0.598	0.552	0.551
Gut	6	1.347	1.387	1.367	1.367
Flesh	6	0.123	0.121	0.122	0.122
Skin	12	0.844	0.805	0.825	0.824
Head	12	1.227	1.181	1.204	1.204
Bones	12	0.695	0.658	0.677	0.676
Fins and tail	12	0.887	0.870	0.879	0.878
Gut	12	2.164	2.170	2.167	2.167
Flesh	12	0.162	0.179	0.171	0.170
Skin	24	0.756	0.725	0.741	0.740
Head	24	0.747	0.730	0.739	0.738
Bones	24	0.989	0.986	0.988	0.987
Fins and tail	24	0.613	0.595	0.604	0.604
Gut	24	1.686	1.562	1.624	1.624
Flesh	24	0.120	0.124	0.122	0.122

Table A4: sGAG measured at 656nm for mackerel fish at 20 μ/ml

Mackerel Parts	Time	OD 1	OD 2	OD 3	Average
Skin	3	0.456	0.460	0.458	0.458
Head	3	1.461	1.393	1.427	1.427
Bones	3	0.323	0.316	0.320	0.319
Fins and tail	3	0.406	0.374	0.390	0.390
Gut	3	1.298	1.469	1.384	1.383
Flesh	3	0.103	0.098	0.101	0.100
Skin	6	0.756	0.760	0.758	0.758
Head	6	1.561	1.493	1.527	1.527
Bones	6	0.323	0.316	0.320	0.319
Fins and tail	6	0.606	0.674	0.640	0.640
Gut	6	1.498	1.509	1.504	1.503
Flesh	6	0.173	0.181	0.177	0.177
Skin	12	1.013	1.231	1.122	1.122
Head	12	1.486	1.479	1.483	1.482
Bones	12	0.867	0.872	0.870	0.869
Fins and tail	12	0.782	0.773	0.778	0.777
Gut	12	1.970	1.971	1.971	1.970
Flesh	12	0.146	0.161	0.154	0.153
Skin	24	0.825	0.807	0.816	0.816
Head	24	1.252	1.221	1.237	1.236
Bones	24	1.195	1.196	1.196	1.195
Fins and tail	24	0.642	0.647	0.645	0.644
Gut	24	1.332	1.352	1.342	1.342
Flesh	24	0.126	0.126	0.126	0.126