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Evaluation of Various Methodologies Used in Purification of Biologically Active Carbohydrates Derived from Marine Life

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Abstract

Although various biologically active drugs derived from natural products, especially marine life such as antiproliferation and antioxidant drugs have been launched. Biologically active carbohydrates purification field involves different purification methods. There are major issues with extraction and purification of glycosaminoglycan (GAG)-derived from marine life. Lack of standard method for GAGs purification from marine life has led to incomplete purification and it made the characterization step very challenging. There are three main purification methods used to purify GAGs from marine life, which are de-fat, proteolysis and chromatographic techniques. The main issue is that in each method there are several techniques used, thus, it would lead to poor GAGs purification as well as the biological activity could be lost, thus It would make GAG purification methods not reliable and it might be not reproduceable. This review concluded several methods used in GAGs purification in order to allow better understanding of different methods used in GAG purification.

Abbreviations: PG: Proteoglycans; DS: Dermatan Sulfate; KS: Keratan Sulfate; HA: Hyaluronan RER: Rough Endoplasmic Reticulum; EMA: European Medicines Agency; USFDA: United State Food and Drug Administration; SEC: Size-exclusion Chromatography

Introduction

Carbohydrates are molecules consisting mainly of three basic elements of carbon, oxygen and hydrogen with the empirical formula (CH_2O). Carbohydrates can be classified according to its length into monosaccharides, oligosaccharides, and polysaccharides. Monosaccharides comprise the basic building blocks of carbohydrates. This type of carbohydrates is known as simple sugars; therefore, it cannot be hydrolysed into simpler forms of carbohydrates. Monosaccharides structure is based on the number of carbon atoms, for instance, three carbons monosaccharide is known as triose, four carbons (tetrose), five carbons (pentose) and six carbons (hexose). Monosaccharides can be classified into aldehydes or ketones, according to the carbonyl group. In addition, carbohydrates have a chiral carbon that can give monosaccharides their structural configuration. Chiral carbon is represented via dextro-rotary(D) or levo-rotary (L) isomers [1,2]. Oligosaccharides are shorter sugar polymers, typically consisting of two to about hundred monosaccharides, attached together via glyosidic linkages. Oligosaccharides can be found freely in the blood or it can be attached to proteins or lipids forming a special type of carbohydrates [2].

Polysaccharides consist of long chain of monosaccharides attached together via glyosidic linkages. It can be classified according to its chain length, basic building blocks, branching points and types of glycosidic linkages. According to the polysaccharides basic building blocks, it can be divided into homo- polysaccharides and hetero-polysaccharides. Homo-polysaccharides consists of one type of monosaccharides along the chain. However, hetero polysaccharide consists of more than one monosaccharide along the chain, for example, glycoconjugates such as heparan sulphate which consists of repeating glucosamine and uronic acid units [1].

Glycoconjugate

Glycoconjugate is a carbohydrate moiety that is covalently attached to a protein or lipid molecule. Carbohydrate which linked protein residues known as glycoproteins. There are subtype of glycoproteins known as proteoglycans (PG), which is composed of a particular amino-sugars known as glycosaminoglycan (GAG) attached to core protein residues [3]. GAGs are a group of long, unbranched hetero-polysaccharides consisting of repeated disaccharide units of uronic acid covalently attached to amino-sugar via glycosidic bonds. There are six types of GAGs in the literature, which are heparan sulfate (HS), heparin, chondroitin sulfate (CS), dermatan sulfate (DS), keratan sulfate (KS) and hyaluronan (HA) [1]. HS is composed of repeating disaccharide building block that is thought to be [GlcA (1→4) $GlcNH_2$]. Heparin is an intracellular type, which is composed of repeating disaccharide formula as [IdoA (1→4) GlcNH2]. CS common repeating disaccharide units are composed of [GlcA (1→3) $GalNH_2$]. DS basic disaccharide building block is composed of [IdoA (1→3) $GalNH_2$]. KS consists of a linear polymer of repeated disaccharides [→3) β-D-galactose (1→4) β-D-GlcNAc (1→].

Finally, HA is considered as simple GAG-type, which is unbranched and unaltered GAG. Its basic repeating disaccharides is composed of GlcA (1→3) GlcNAc [4]. GAGs biosynthesis starts from the rough endoplasmic reticulum (RER), where core protein in synthesised, afterwards, thus core protein is transferred to Golgi, where protein glycosylation occurs. In Golgi, several monosaccharides catalysed from their precursor and attached to the core protein residues in a process known as protein glycosylation, resulting in N-linked or O-linked glycan. N-linked glycans occur when GAG monosaccharides attached to asparagine (Asn) residue withing core protein residues. On the other hand, O-linked glycans occur when GAGs monosaccharides attach to serine (Ser)or threonine (Thr) residues of the core protein [1,5-7]. GAGs structures are very diverse, and their synthesis is not template driven, like that of other biomolecules. All these structural modifications give GAGs their structural heterogeneity and wide range of biological functions. GAGs structural heterogeneity can lead to several cellular and extracellular functions, which depend on GAG types and the cell type that would vary from simple cellular function to cellular morphogenesis [8].

GAGs Derived from Marine Life as A Potential Pharmaceutical Agent

Recently, there has been increase demand for development of new drugs from natural products such as bacteria, animals including marine life. Marine life has derived several pharmaceutical utilities as therapeutic agent [9]. One of the largest phylain the animal kingdom known as molluscs, including snails, bivalves, squid and others. Various molluscs were used for dietary intake, as it has excellent dietary benefits. Over the past several years, there has been an increased demand for developing new anti-cancer agents derived from natural products such as plants, bacteria and animals, including marine life. The main advantage of natural product–derived anticancer drugs that it would have acceptable side-effects than of other chemically synthetic drugs [10]. There are several drugs either approved by the United State Food and Drug Administration (US-FDA), European Medicines Agency (EMA) orin clinical trial that have been derived from marine sources. For instance, Cytarabin that was the first marine-derived chemotherapeutic drug isolated from a sponge (*Tethya crypta*) [11]. Dolastatin which is a polypeptide product from marine mollusc (*Dolabellaauricularia*) can inhibit microtubule assembly as well astubulin polymerization, resulting in cell apoptosis. Although it was not successfully approved, this drug progressed to advanced phases in clinical trials, and its derivatives are still being considered as anticancer candidate [12,13].

Halichondrin is a tubulin inhibitor agent, which was isolated from Japanese sponge (*Halichondria okadai*), is synthetic macrocyclic ketone with enhanced antitumor activities against breast cancer. The US-FDA has approved its synthetic analogue since 2010 [14]. Cyclic-peptide drug Kahalalide-F was isolated from the mollusc (*Elysiarubefescens*) has anti-lysosomal properties. Ithasentered phase-I clinicalt rials against prostate cancer and phase-II against hepatocellular carcinoma, non-small cell lung cancer and melanoma [15,16]. Didemnin-B isolated from (*Carribean tunicate*) shows inhibition of DNA-synthase that leads to cell cycle arrest [17]. Many molluscs have been used for dietary intake, as they have great nutritional benefits. Traditionally, molluscs have been used as natural treatments in Chinese traditional medicine; therefore, there has been great interest in investigating their valuable elements to be used as pharmaceutical resources [18]. The high content of uncommon polysaccharides structures in marine organisms make them a favourable candidate of natural products with various pharmaceutical benefits, including in cancer therapy. Generally, mollusc soft-body structural investigations have revealed high amounts of carbohydrates with some proteins and lipids, which makes carbohydrates a major area of interest from a pharmaceutical perspective [19].

Polysaccharides from vertebrates and invertebrates have emerged as powerful platforms for medical utilities, as they have various biological activities such as immunostimulatory, antioxidant, and antitumor activities [9,20,21]. Polysaccharides isolated from invertebrates have shown very different structures to common mammalian polysaccharides in terms of monosaccharide composition, molecular weight, degree of sulphation, type of glycosidic linkages, and branching points. For instance, a novel polysaccharide isolated from the head of ashrimph as a novel combination structure between heparin and HS known as a hybrid hep/HS structure [22]. This structural diversity in polysaccharides has generated additional features for the senovel molecules that could make them potent tool singlecobiology, especially as pharmaceutical agents [9]. Although there are several studies of

marine life as source of promising biologically active substances that can be used as pharmaceutical target for various disorders such as cancer, however, various purification techniques were used to purify biologically active GAG and using different structural analysis techniques to characterize this active compound made this field of carbohydrates challenging. These major issues in biologically active GAGs from marine life including, extraction procedure, purification step and characterisation technique, which has created a wide gap on the final structure for GAGs to be used as pharmaceutical agent.

There is no standard method to extract GAGs from in marine life to purified active compounds. GAGs extraction and purification methods from marine source mainly follow three consecutive steps, namely, de-shell (shell removal), de-fat (lipid removal) and proteolysis. However, there are inconsistency in GAG extraction techniques. Indeed, the nature of the GAG polysaccharide derived from marine source, which has an unknown structure and lacks any chromophore that affects adopting an appropriate purification step. For instance, size-exclusion chromatography requires a well-known molecular weight sample, and lectin affinity chromatography requires a particular ligand to allow the complex to interact. This lack of standardization has assigned as major issues in GAGs purification from marine life. Therefore, GAGs with potent biological activity, for instance, antiproliferative or anticoagulant activity were not assigned as pure polysaccharide, but it could be identified as proteoglycan, as other biomolecules could play a role in the biological function. The aim of this review is to evaluate different methodologies used to extract and purify GAG-derived from marine life with potent biological activities.

Purification Methods of GAG-Derived from Marine Life

The literature has demonstrated several purification techniques to obtain purified GAGs from different marine life species. Firstly, de-fat technique, which considered as the first step in GAGs purification. Next, proteolysis technique which is used to ensure removing on N-linked or O-linked proteins that is attached to the polysaccharides chain. Finally, using various chromatographic methods in order to obtain GAG purified chain.

GAG-Defat Techniques

The Defat process is the first step of the purification, which ensure removing the lipid-soluble molecules by using different solvents such as acetone. The procedure begins to remove the shell and submerge the soft body in de-fat solvents. The literature has demonstrated several types of solvents used, different incubation period and temperature used to remove lipid-soluble molecules.

De-Fat Method Using Acetone: Acetone was used as dehydrating agent, which used to achieve complete lipid removal from polysaccharide chain. Although acetone was used, different techniques were observed to remove lipids including different incubation time and temperatures (Table 1) (23-35).

Table 1: Shows different techniques used to remove lipid-soluble molecules using acetone.

Species	Defat Process Using Acetone	Reference
Mollusc Gastropods (*Achatina fulica*)	The whole soft body was merged in fresh acetoneevery 24 hours over three days at 4 °C.	[23]
Bivalve mollusc (*Cerastoderma edule*)		[9]
Bivalve Whelk (*Buccinum undatum*)		[21]
Chordata Tunicata (*Styela plicata*)	The whole soft body was cut into small pieces then merged in acetone for 24 h at 4 °C.	[24]
Bivalve Mollusc (*Nodipecten nodosus*)	Whole-body tissue defatted using acetone for 24 h at 60 °C.	[25]
Bivalve Mollusc (*Amusium pleuronectes*)		[26]
Chordata Chondrichthyes (*Aetobatus narinari*)		[27]
Bivalve Mollusc (*Scapharca inaequivalvis*)		[28]
Bivalve Mollusc (*Mytilus galloprovincialis*)		[29]
Fish (Monkfish, codfish, spiny dogfish, salmon and tuna)		[30]
Fish (*Salmo salar*)	Fish skin was defatted in acetone for 48 h.	[31]
Chordata Chondrichthyes (*Isurus oxyrinchus* and *Prionace glauca*)	Sample was homogenized with acetone and incubated overnight	[32]
Mollusc(*Enteroctopus dofleini*)		[33]
Bivalve Molluscs (*Ruditapes philippinarum, Scapharca broughtonii, Mizuhopecten yessoensis, Turbo cornutus, Crassostrea nippona, Corbicula japonica, Mytilus galloprovincialis, Neptunea intersculpta, Pseudocardium sachalinense* and *Crassostrea gigas*)		[34]
Bivalve Mollusc (*Mactra chinensis*)	Sample was homogenised in acetone X3 times, each time acetone was removed using centrifugation at room temperature.	[35]
Bivalve Mollusc (*Tapes phylippinarum*)	Soft body was merged in acetone, then filtered and dried for 24 h.	[18]

De-Fat Method Using Sodium Sulfate (Na_2SO_4): The second def-at method was performed using $Na2SO_4$ as a dehydrating agent (Table 2) (36,37).

Table 2: Shows method used Na_2SO_4 as dehydrating agent, which used as a potential step in removing lipid-soluble molecules from sample.

Species	De-fat Process Using Na2SO4	Reference
Bivalve Mollusc (Katelysia opima)	Body tissue was homogenized with Na_2SO_4 pH 11.5, at 55 °C for 90 min.	[36]
Bivalve Mollusc (*B. spirata* and *p.glaucum*)		[37]

De-Fat Method Using Chloroform/Methanol: One of the most versatile and effective lipid extraction procedures. Briefly, this procedure uses a one-phase alcoholic solvent system, namely chloroform-methanol-water (1:2:0.8 v/v) that rapidly and efficiently extracts the lipids. The extract is then diluted with one volume each of chloroform and water to form the two phased system, chloroform and methanol-water. Any water-soluble contents are thus readily partitioned into the methanol-water, leaving the lipids relatively free of contaminants in the chloroform phase [38,39] (Table 3).

Table 3: Shows method used Chloroform/Methanol as dehydrating agent, which used as a potential step in removing lipid-soluble molecules.

Species	De-Fat Process Using Chloroform/Methanol	Reference
Sea Cucumber (Stichopus Japonicus)	The soft body was homogenized with chloroform/methanol, then, filtered to ensure fat removal	[39]

De-Fat Method Using Acetone and Ethanol: According to this method, lipid-soluble molecules were removed by the action of acetone and ethanol. Acetone will remove lipid-soluble molecules; while ethanol was used to enhance the dehydration process (Table 4) [40].

Table 4: Shows GAG d-fat technique using acetone and ethanol as solvents.

Species	De-Fat Using Chloroform/Methanol	Reference
Seagrass (*Phanerogam Zostera marina*)	The sample was submerged in acetone overnight, then, washed with acetone and ethanol, until the supernatant become clear.	[40]

De-fat Method Using Ethanol (Table 5)[41]

Table 5: Shows GAG d-fat technique using ethanol as dehydrating solvents.

Species	De-Fat Using Ethanol	Reference
Chordata Chondrichthyes (Dasyatis kuhlii)	The sample was cut into small pieces and soaked in ethanol for 7-days to ensure full removal of lipids.	[41]

GAG-Proteolysis Techniques

This is the second step in GAG purification process, after de-fat, which lead to cleave GAG chain from the attached protein chain. This process mainly concerns about cleaving the polysaccharide chain, thaerefore, protein residues were hydrolysed either via enzymatic or chemical techniques. Enzymatic extraction, which mean removing of proteins by using enzymes, for instance, alcalase, papain or pronase [42]. On the other hand, chemical extraction by using different chemical such as Aluminium sulfate [36]. The following literature distinguished different techniques to remove protein residues using different concentrations, centrifugation, incubation period and temperatures.

Proteolysis Technique Using Papain Enzyme: Papain is a proteolytic enzyme derived from Papaya (Carica papaya) It was used to remove protein residues. Different proteolysis techniques were applied using papain as proteolytic enzyme (Table 6) [43,44].

Table 6: Demonstrate various methodologies used papain as proteolytic agent. Different sample, enzyme concentration, buffers concentration and incubation periods were noticed.

Species	Proteolysis Techniques Using Papain Enzyme	Reference
Bivalve Mollusc (*Mytilus galloprovincialis*)	2.4 g of dried materials were dissolved in 45 ml of 100 mM sodium acetate buffer pH 5.5 containing (5 mM EDTA and 5 mM cysteine). 240 mg of papain was added to the buffer containing dried materials and incubated for 24 h at 60°C.	[29]
Bivalve Mollusc (*Tapes phylippinarum*)	14 g was dissolved in 280 mL in 100 mM Sodium acetate, 5 mM ethylenediamine acetic acid and 5 mM cysteine pH 5.5. 1 mg papain/1 g of the dried materials was mixed and incubated for 24 h at 60°C	[18]

Bivalve Mollusc (*Scapharca inaequivalvis*)	The dried body was dissolved in (1g/20 mL) 0.1 M sodium acetate buffer pH 5.5, then, 1 mg papain/1 g of the dried materials were mixed and incubated for 24 h at 60 °C	[28]
Bivalve Mollusc (*Nodipecten nodosus*)	25 g of dried materials were dissolved in 500 mL (0.1 M sodium acetate) buffer (pH 5.5) with 5 g of papain in 5 mM EDTA, and 5 mM cysteine. Mixture was incubated overnight at 60°C. After incubation period, the mixture was centrifuged 2000 × g for 10 min. This proteolysis method was been repeated three times	[25]
Chordata Chondrichthyes (*Raja clavate*)	Proteolysis method were dependent on dissolving the 5 g of *Raja clavata* on alcohol, then added 250 mL of (0.1M sodium acetate buffer, EDTA 5 mM, cystein 5 mM pH 6). Afterwards, immediately added 510 mg of papain. The mixture was incubated for 24 hours at 60 °C, then filtered with 138 mL of distilled water.	[44]
Bivalve Mollusc (*Amussium pleuronectus*)	5 g of defatted tissue in 100 ml (100 mM sodium acetate, 5 mM EDTA and cysteine pH 5.5) and 100 mg of papain/1g of defatted tissues was added and the buffer. The mixture was incubated at 60°C for 24 h.	[26]
Chordata Chondrichthyes (*Aetobatus narinari*)		[27]
Fish (*Salmo salar*)		[31]
Chordata Tunicata (*Styela plicata*)		[24]
Fish (Monkfish, codfish, spiny dogfish, salmon and tuna)	1 g of defatted tissue was dissolved in 10 mL (100 mM sodium acetate containing 5 mM EDTA and 5 mM cysteine buffer pH 5.5). Then, added 60 mg of papain to the mixture. The mixture was incubated at 60°C for 24 h	[30]

Proteolysis Technique Using Alcalase Enzyme: Is a serine endopeptidase that is suitable for the hydrolysis of proteins. It has previously been used to produce soluble hydrolysates that digest the protein in the defatted tissues (Table 7) [45,46,47].

Table 7: Shows various proteolysis techniques using alcalase enzyme to cleave GAGs that's attached via O-linages to protein residues via serine/threonine.

Species	Proteolysis Technique Using Alcalase Enzyme	Reference
Mollusc Gastropods (*Achatina fulica*)	The sample was suspended in 40 ml of 0.05 M sodium carbonate buffer (pH 9.2), then, incubated for 48 h at 200 rpm at 60°C after adding 2 ml of alcalase enzyme (2.4 Anson units/g).	[23]
Mollusc (*Cerastoderma edule*)		[9]
Mollusc (*Buccinum undatum*)		[21]
Chordata Actinopterygii (*Cyclopterus lumpus*)	Alcalase enzyme (1:100) was added to the dried tissue, which dissolved in water, then, and the mixture was incubated overnight at 60°C.	[47]

Proteolysis Technique Using Actinase-E Enzyme: Actinase-E is a protease that is derived from Streptomyces griseus, it is has mixture of three proteolytic activities containing serine protease (Table 8) [48].

Table 8: Shows different techniques used actinase-E enzyme to cleave protein residues from GAG chain.

Species	Proteolysis Techniques Using Actinase-E	Reference
Bivalve Mollusc (*Mactra chinensis*)	30 g of dried tissue was dissolved in 120 mL of Tris-acetatebuffer (pH 8.0) containing actinase-E, then, the mixture was incubated for 18 h at 45°C. Afterwards, 600 mL of NaOH buffer was added and the mixture was cooled at 4°C for 18 h.	[35]
Fish maw	2 g of dried fish was homogenized in 100 mL distilled water,then, 10 mg/mL actinase-E was added. The mixture was incubated at 55°C for 48 h, then, the mixture was centrifuged at 4000 x g for 30 min.	[48]
Bivalve Mollusc (*Ruditapes philippinarum, Scapharca broughtonii, Mizuhopecten yessoensis, Turbo cornutus, Crassostrea nippona, Corbicula japonica, Mytilus galloprovincialis, Neptunea intersculpta, Pseudocardium sachalinense* and *Crassostrea gigas*)	10 mg of actinase-E g of dry powder was dissolved in 50 mM Tris-acetate (pH 8.0), then, the mixture was incubated for 48h at 45°C. Afterwards, the sample was further treated with a solution containing 0.5 M NaOH and 0.3M sodium borohydride (20 mL/g of dried powder), the mixture was incubated at 4 °C for overnight. Finally, proteins were removed by centrifugation at 1000 x g for 30 min.	[34]

Proteolysis Technique Using Pectinase Enzyme: Pectinase enzyme derived from Aspergillus niger, which is used to purify pectins molecules from the sample (Table 9).

Table 9: Proactinase enzyme was used to cleave pectin polysaccharides chain from residual proteins.

Species	Proteolysis Technique Using Pectinase Enzyme	Reference
Seagrass (*Zostera marina*)	1 g of defatted tissue was dissolved in distilled water, then, pectinase enzymes (0.2 % v/v) was added. The mixture was incubated for 1 h at 37 °C. Proteins were removed by centrifugation (5000 × g for 15 min).	[40]

Aluminium Sulfate [$Al_2(SO_4)_3$]: In this method, chemical hydrolysis of proteins residues from GAG chain (Table 10).

Table 10: Demonstrated the use of [$Al_2(SO_4)_3$] as proteolysis technique.

Species	Proteolysis Technique Using [$Al_2(SO_4)_3$]	Reference
Bivalve Mollusc (*Katelysia opima*)	For proteolysis, 80 mg of [$Al_2(SO_4)_3$] was added per kg of tissues and the mixture was incubated for 1 h at 95°C	[36]
Mollusc Gastropods (*Babylonia spirata* and *Phalium glaucum*)		[37]

Urea and Sodium hydroxide (NaOH): Urea was used aid protein denaturing process, in addition, NaOH was used to hydrolyse GAGs polysaccharides, which result in monosaccharides or oligosaccharides (Table 11).

Table 11: Sows the use of urea and NaOH in GAG purification.

Species	Proteolysis Technique Using Urea and NaOH	Reference
Chordata Chondrichthyes (*Dasyatis kuhlii*)	Sample wad defatted, then, mixed with urea and sodium hydroxide in order to denature protein as well as to degrade GAG chain.	[41]

GAG Purification Using Various Chromatographic Techniques

The aim of this process is to purify the biologically active components that is derived from polysaccharides, especially GAGs. The purification techniques were mostly used chromatographic techniques to purify GAGs according to their charge or size structure.

GAG Purification Using Anion-Exchange Chromatography: Anion-exchange chromatography was one of the most common methods for GAGs purification and fractionation. As GAGs known to carry highly negatively charged molecules such as heparin Penta saccharide structure [49]. Therefore, various purification techniques were particularly based on this feature. Anion-exchange resins were used with various solvents such as high molar NaCl solution in order to exchange the biologically active molecules.

Quaternary Aminoethyl (QAE)-Sephadex: QAE functional group is used as strong anion-exchanger. In this method, QAE functional group is attached to the sephadex resin, which is composed of cross-linked dextran with epichlorohydrin, to allow separation of negatively charged molecules (Table 12) [50].

Table 12: Shows different chromatographic techniques using QAE as anion-exchange resin, which purify GAGs according to their negatively charge.

Species	Chromatographic Techniques Using QAE- Sephadex Resin	Reference
Monkfish, cod, spiny dogfish, salmon and tuna	The process used a column (2×40 cm) packed with QAE Sephadex (A-25) anion-exchange resin. GAGs were eluted with a linear gradient of NaCl from 0.05-1.2 M over 150 min at flow rate 1 mL/min	[30]
Clam (Tapes phylippinarum)		[18]
Scapharca inaequivalvis		[28]
Mytilus galloprovincialis	The sample was applied to QAD Sephadex A-25 (1 cm× 20 cm) column. GAGs were eluted using a linear gradient of NaCl from 0.05-1.2 M over 150 min at flow rate 1 mL/min	[29]

Diethylaminoethyl (DEAE)-Sepharose: DEAE is used as weak anion exchanger. In this method, DEAE is attached to Sepharose resin, which is composed of 6% cross-linked agarose (Table 13) [42].

Table 13: Shows different chromatographic techniques using DEAE-Sepharose as anion-exchange resin.

Species	Chromatographic Techniques Using DEAE-Sephrose Resin	Reference
Mactra chinensis	Crude GAG sample was dissolved in distilled water, then applied to DEAE-sepharose fast flow column. GAGs were eluted using gradient elution of two buffers, which are 50 mM sodium phosphate (pH 6) as buffer (A), and 2.0 M NaCl in 50 mM sodium phosphate (pH 6) as buffer (B) at flow rate 2 mL/min over 180 min.	[35]
Marine mollusc (*Cerastoderma edule*)	Crude GAG sample was dissolved in distilled water. Then applied to anion exchange column (16 X 200 mm), which packed with 10 mL of DEAE-Sepharose fast flow. GAGs were eluted using a linear gradient (0–1.5 M) NaCl over 70 min. Elution was monitored using UV 280 nm	[9]

DEAE-Cellulose: In this methods, DEAE anion exchanger is attached to the cellulose resin (Table 14) [51].

Table 14: Shows different chromatographic techniques using DEAE-cellulose as anion-exchange resin.

Species	Chromatographic Techniques Using DEAE- Cellulose Resin	References
Bivalve (*Katelysia opima*)	Crude GAG sample was purified using DEAE cellulose resin. GAGs elution was achieved using 1 M and 2 M NaCl buffer at flow rate 12 mL/h.	[36]
Marine bivalve mollusc (*Amussium pleuronectus*)	Crude GAG sample was purified using DEAE cellulose A- 25 resin. GAGs were eluted using a linear gradient of NaCl buffer (0.05-1.2 M) at the flow rate of 1 mL/min.	[26]
Liver of marine stingray (*Aetobatus narinari*)		[27]
Marine shrimp (*litopenaeus vannamei*)	Crude GAG sample was purified using DEAE–Sephacel. GAGs were eluted using a linear gradient of NaCl buffer of 0.5-1 M. Elution was monitored using UV/VIS 525 nm	[51]

Size-exclusion Chromatography (SEC)

This method depends on molecules size, as SEC purifies molecules according to their molecular size. Sephadex is a gel that contains dextran with epichlorohydrin, where G-100 refer to a particular property of Sephadex as G-100 has fractionation range 4000-15000 kDa (Table 15).

Table 15: Shows different chromatographic techniques using SEC to purify GAGs according to chain size.

Species	Chromatographic Techniques Using SEC Column	Reference
Raja radula Raja clavata	GAGs sample was applied to sephadex G-100 column (2.5 X 70 cm) using (0.05 M) sodium acetate as elution buffer at flow rate 10 mL/h. Elution was monitored using UV 215 nm.	[44]

Conclusion

In conclusion, GAGs have wide range of structural diversity as well as biological activity, therefore, setting up a standard method in GAG purification would be result in highly purified biologically active compound from GAGs, which can be used as pharmaceutical agent. As conducted in this review, there are three main steps are used in purification of GAG-derived from marine life, which are de-fat, proteolysisand polysaccharide chain purification, however, various techniques were used in each method that resulted in lack of standard method in GAG purification. Hence, it is highly recommended to adapt versatile standard method to allow better purification of biologically active molecules derived from GAGs. Thus, it would be possible by evaluating the current purification techniques used in GAGs analysis and assign a gold standard method on purification techniques.

Conflict of Interest

The authors declare that there is no conflict of interest statement.

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