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International Journal of Biological Macromolecules 92 (2016) 37–48

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

Bioactivities, isolation and purification methods of polysaccharides from natural products: A review

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ARTICLE INFO

Article history:
Received 1 June 2016
Received in revised form 29 June 2016
Accepted 30 June 2016
Available online 1 July 2016

Keywords:
Polysaccharide
Bioactivity
Isolation
Purification

ABSTRACT

Polysaccharides play multiple roles and have extensive bioactivities in life process and an immense potential in healthcare, food and cosmetic industries, due to their therapeutic effects and relatively low toxicity. This review describes their major functions involved in antitumor, anti-virus, and anti-inflammatory bioactivities. Due to their enormous structural heterogeneity, the approaches for isolation and purification of polysaccharides are distinct from that of the other macromolecules such as proteins, etc. Yet, to achieve the homogeneity is the initial step for studies of polysaccharide structure, pharmacology, and its structure-activity relationships. According to the experiences accumulated by our lab and the published literatures, this review also introduces the methods widely used in isolation and purification of polysaccharides.

Polysaccharides belong to a structurally diverse class of macromolecules, polymers of monosaccharide residues joined to each other by glycosidic linkages. It is noteworthy that, in comparison with other biopolymers such as proteins and nucleic acids, polysaccharides offer the highest capacity for carrying biological information because they have the greatest potential for structural variability. The nucleotides in nucleic acids and the amino acids in proteins can interconnect in only one way whereas the monosaccharide units in polysaccharides can interconnect at several points to form a wide variety of branched or linear structures [1].

The sources of polysaccharides are very diverse. Polysaccharides can originate from higher photosynthetic plants, fungi, algae, bacteria and so on. At cellular level, polysaccharides represent either the reserve compounds in cytoplasm (e.g. starch), or structural components of the membrane or cell wall of organisms (e.g. cellulose). Basically, the isolation, purification and utilization of polysaccharides depend on their structural features. The main structures of polysaccharides from natural products are tremendously complex and diverse, however the basic structures of their backbone chain are often glucan, fructan, xylan, mannan, galactan, etc. or a polymer of two or several monosaccharides (e.g. galactomannan, pectin). The structures of their branched chain are various, representing great diversity. Fig. 1 shows the examples of structures of a few commercialized polysaccharides and several polysaccharides prepared by our lab [2–6].

More and more studies show that polysaccharides possess complex biological activities and a variety of functions, especially effects on organism immune function. It can be said that most of the bioactivities and functions of polysaccharides are related to the immune system [7]. Although the research on polysaccharides started later than three other classes of life macromolecules (i.e. proteins, nucleic acids and lipids), polysaccharides have aroused more and more interests from people because their important physiological functions and wide applications are being continuously explored in life processes. At present, polysaccharides have become an important constituent part in the research and development of natural drugs and healthcare products. According to incomplete statistics, at least 30 polysaccharides are being carried out standard clinical trials involved in antitumor, anti-virus, diabetes therapy and so on in the world, respectively [8]. The global sales of carbohydrate drugs and healthcare products have exceeded US$ 19.3 billion in 2002 [9]. It is clear that the sleeping giant of carbohydrate is awakening. It is said that the research of structure and function of polysaccharides has become the third milestone in exploring the mystery of life after the study of proteins and nucleic acids. Now on one hand, the advanced biochemical instruments and biotechnology are used to search more effective polysaccharides and their derivatives from traditional medicine, microorganism, and marine organism to conquer diseases such as tumor in many countries. On the other hand, the research is

Abbreviations: MW, molecular weight.

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http://dx.doi.org/10.1016/j.ijbiomac.2016.06.100
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focused on the structure-activity relationship and action mechanism of polysaccharides. Up to now, β-glucans from higher fungi (especially mushrooms), pectins from some plants, and sulfated polysaccharides from marine algae have been intensively studied among polysaccharides from natural products.

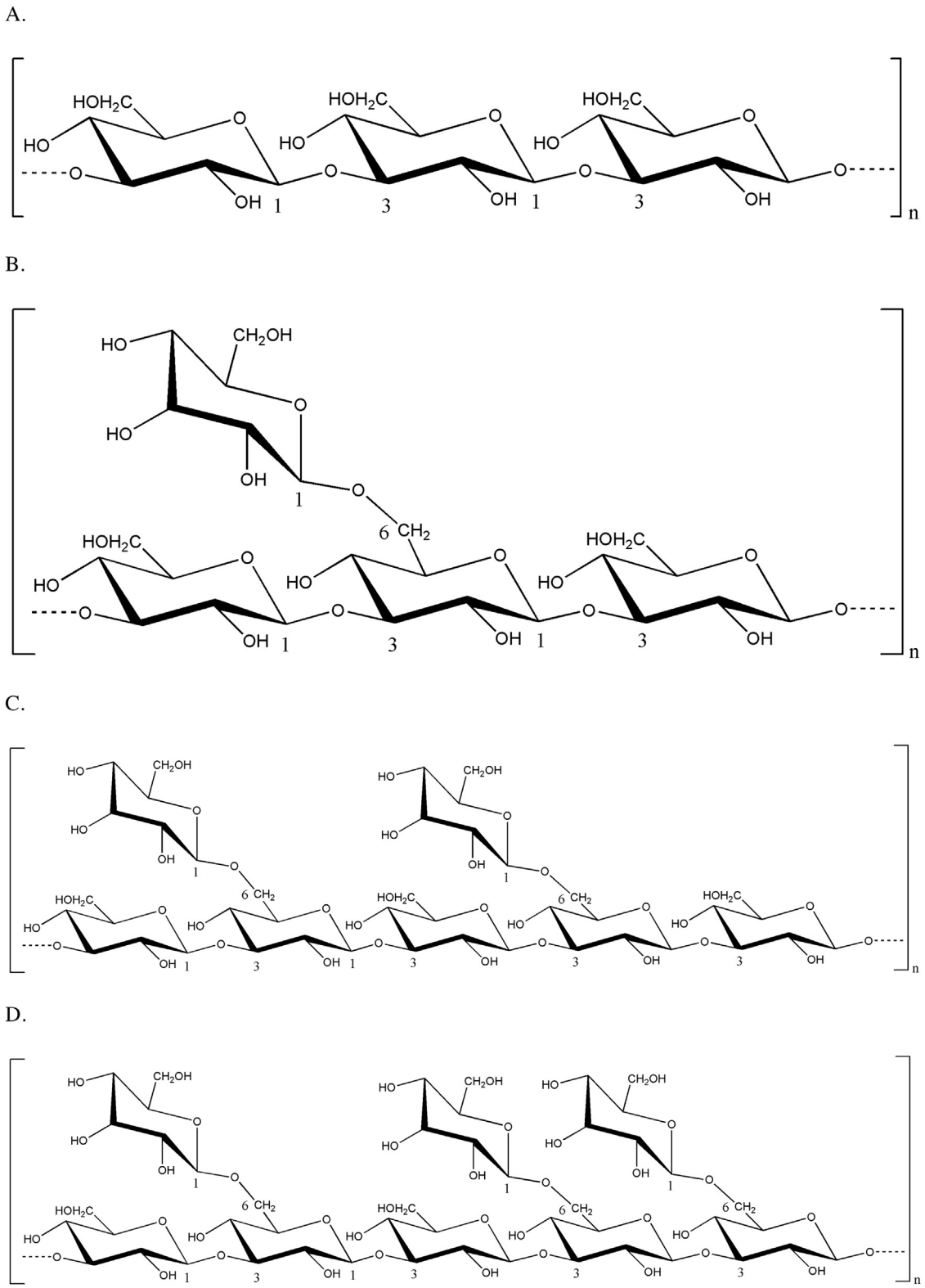

Fig. 1. Examples of structures of some polysaccharides. A. Curdlan; B. Schizophyllan; C. Lentinan; D. Pestolotan; E. Epiglucan. A-E: β-(1 → 3)(1 → 6) glucans [2]. F. a β-(1 → 4)-galactomannan with a branched chain of α-(1 → 6)-galactose [3]. G. a α-(1 → 4)-glucan with a side chain consisting of terminal and α-(1 → 4)-glucopyranosyl residues is attached at position *O*-6 of the branching residues [4]. H. a xylan with the 4-*O*-methyl-D-glucopyranosyluronic acid group linked to *O*-2 of a β-(1 → 4)-xylan [5]. I. an inulin-type fructan [6].

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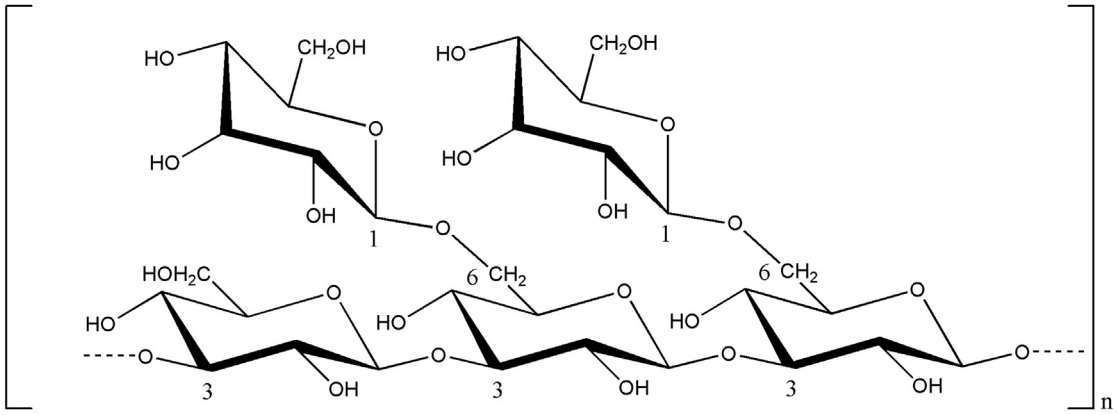

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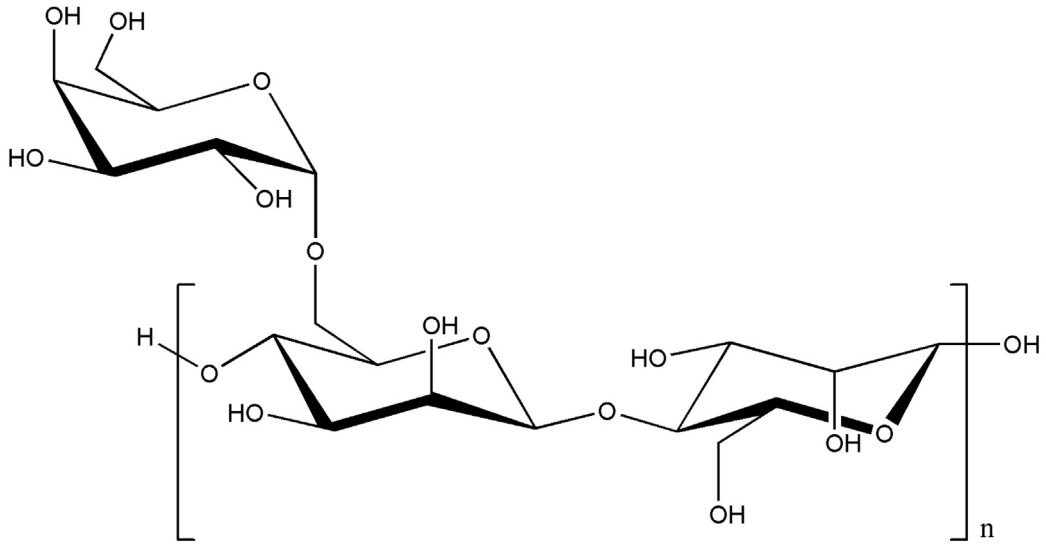

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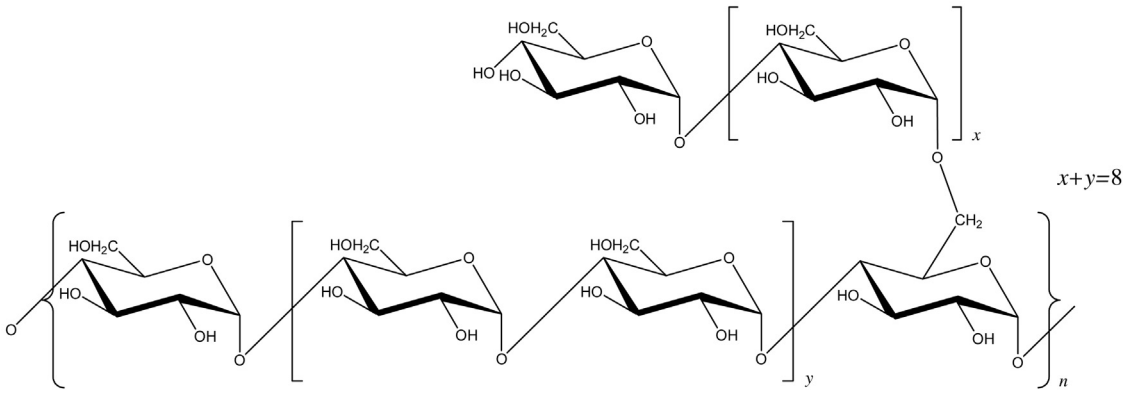


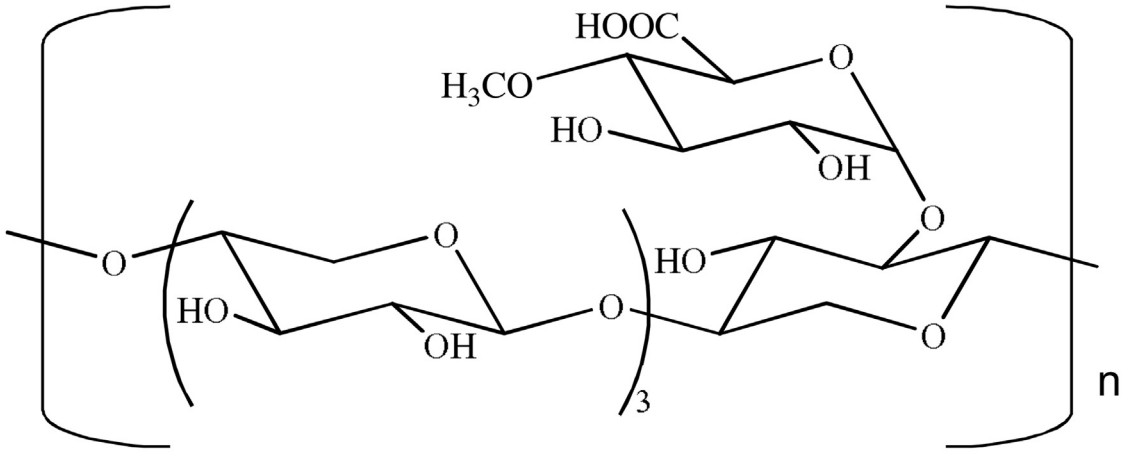
Fig. 1. (*Continued*)

1. Main bioactivities of polysaccharides

1.1. Antitumor activity

Most polysaccharides possessing antitumor activity are not only nontoxic, but also have some other bioactivities, such as inducing cell differentiation, stimulating hematopoiesis, anti-metastasis [10], anti-angiogenesis [11], and inducing production of nitric oxide (NO) [12]. Most of the polysaccharides do not directly act on tumor cells, but play a role by activating the immune system of an organism: i.e. promoting the maturation, differentiation and

H.



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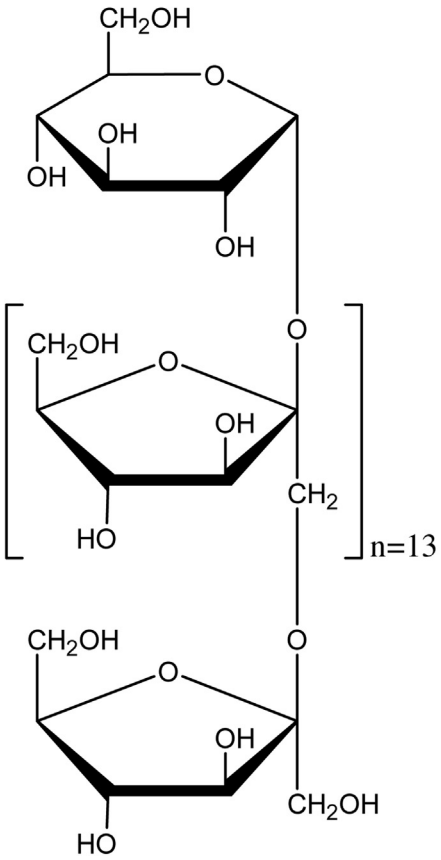

Fig. 1. (*Continued*)

reproduction of three kinds of cells (lymphocytes, macrophages and natural killer cells) [13]; simultaneously activating complements and the endothelial system on the endoplasmic reticulum; promoting the generation of a variety of cytokines [14]; and ultimately inhibiting the growth of tumor cells or causing the apoptosis of tumor cells. A lot of polysaccharides possessing antitumor activity have already been found so far, such as β-glucans which have different planar and three-dimensional structure, pectic polysaccharides, and seaweed polysaccharides. Some polysaccharides, like lentinan [15,16], polysaccharide from *Ganoderma lucidum* [17,18], polysaccharide from *Coriolus versicolor* [19] and so on have been formally used in clinic and favored by doctors. Other polysaccharides which are being researched further include *Bergenia crassifolia* pectin polysaccharide [20], *Phellinus linteus* polysaccharide [21],

Grifola frondosa polysaccharide [22], *Panax ginseng* polysaccharide [23], *Agaricus blazei* polysaccharide [24], *Antrodia cinnamomea* polysaccharide [25], *lycium barbarum* polysaccharide [26], acetobacter polysaccharide [27] and so on [28,29]. It is necessary to note that besides β-glucans, some α-glucans also possess activity [29,30].

Since most polysaccharides perform antitumor activity by activating the body's immune system rather than directly killing tumor cells, the tests in vitro are usually invalid when screening the antitumor polysaccharide. The efficacy of tests in vivo exerted through immune system will emerge slowly only after about 3 weeks [31]. Although for now a few polysaccharides which indeed possess cytotoxic activity have been reported [32,33], i.e. these polysaccharides can directly kill cancer cells not through the immune system mediation, their action mechanism is still not clear and needs further research.

1.2. Antiviral activity

After 1970s, some polysaccharides were found to possess the effects of anti-herpes virus and anti-influenza virus. In particular, in 1980s some polysaccharides were found to possess anti-AIDS virus (HIV) activity [34]. Studies indicated that most of antiviral polysaccharides can interfere the binding of virus surface glycoprotein gp120 to CD4 receptor on the lymphocyte surface [35], and can inhibit the expression of viral antigen, and inhibit the activity of viral reverse transcriptase (RT) [36,37]. The chemical structures of polysaccharides with antiviral activity mostly contain sulfate radical. The existence and amount of sulfate radical in the polysaccharide directly affect its antiviral activity. For example, lentinan from *Lentinus edodes* (Berk) has a remarkable anti-tumor effect, whereas its anti-HIV activity is very low. However, sulfated lentinan possesses a significant anti-HIV activity. Similarly, the polysaccharide from sargassum has anti-tumor effect and the sulfated sargassum polysaccharide possesses an obvious anti-herpes virus activity [38]. Many polysaccharides have been reported to possess antiviral activity so far, such as a sulfated galactan having anti-herpes virus activity isolated from red algae in the sea [39,40], a sulfated fucosan obtained from arabic gum, and a sulfated xylan from green algae etc. They all can selectively inhibit the formation of herpes simplex virus and syncytium [41]. The sulfated K5 *Escherichia coli* polysaccharide possesses the anti-HIV activity and its structure is similar to heparin, but it has no anticoagulant effect like heparin. This polysaccharide is expected to be developed a candidate drug to prevent HIV transmission in developing countries [42,43].

In addition, several antiviral polysaccharides have been found to have no sulfate radical in their structure, such as a polysaccharide from *Nostoc* [14], which is a kind of acidic heteropolysaccharide and has obvious anti-influenza virus activity.

1.3. Hypoglycemic effect

Polysaccharides are polymers of more than 10 monosaccharide residues joined to each other by glycosidic linkages through condensation reaction. Different from the properties of monosaccharide and oligosaccharide, many polysaccharides do not increase blood sugar. On the contrary, they can decrease blood sugar. So polysaccharides are expected to be a new class of hypoglycemic drugs [44]. Studies indicated that although some polysaccharides do not increase the insulin secretion in the body, they can significantly improve the activity of glucokinase, hexokinase, and glucose-6-phosphate dehydrogenase (G6PD) in liver, also reduce the level of triglyceride and cholesterol in plasma [45]. Some polysaccharides are β-receptor agonists. They can pass information to mitochondria through the second messenger, which can speed up the glucose oxidation and finally result in the hypoglycemic effect [46]. At present scientists found a number of polysaccharides having hypoglycemic effect, such as polysaccharides from *Garuga pinnata* [47], *Artemisia sphaerocephala* Krasch [48], *Diospyros Kaki* [49], and so on.

1.4. Anti-inflammatory effect

Polysaccharides have been found to possess anti-inflammatory effect [50–53] since the discovery of oligosaccharides with anti-inflammatory effect. These polysaccharides can selectively adhere to pathogens and block up the adhesion of microbial pathogens to target cells [54], thus exert the anti-inflammatory and anti-infection effect. For example, a zwitterionic polysaccharide produced by a certain bacterium has an obvious preventive and therapeutic effect against abscess [55]. The action mechanism of the polysaccharide is to activate T cells through MHCII pathway thus it can show anti-inflammatory effect.

Up to now, there are some oligosaccharides and polysaccharides which are in clinical trials as oral and topical anti-inflammatory drugs. For example, polyacylated oligosaccharides from a kind of morning glory have obvious effect on killing *staphylococcus aureus* [56]. It is predicted that carbohydrate will be favored as the second generation of anti-inflammatory drugs.

1.5. Anti-complementary effect

The complement system is one of the important human immune defense systems, but excessive activation can cause many diseases, such as rheumatoid arthritis, severe acute respiratory syndrome (SARS) and so on. So far there is no ideal drug for the excessive activation of complement system. The clinic is badly in need of complement inhibitors with high efficacy and low toxicity.

As early as 1989, Yamada et al. [57] obtained a polysaccharide possessing obvious anti-complementary effect from *Bupleurum falcatum*. Xu et al. [58] gained a new polysaccharide D3-S1 which had a remarkable anti-complementary effect from *Bupleurum* as well. The D3-S1 had strong inhibition on both classical complement pathway activation and alternative complement pathway activation, and its anti-complement effect was obviously stronger than heparin and had no anti-coagulation action, indicating that the polysaccharide of D3-S1 has a good prospect in application. In addition, Zhu et al. [59] obtained an acidic polysaccharide, i.e. rhamnogalacturonan which showed the complement-fixing activity from *Panax notoginseng*. Olafsdottir et al. [60] isolated a β-glucan from the lichen, which had certain anti-complementary activity. It has become another research hot spot to look for anti-complement bioactive compounds from natural polysaccharides.

1.6. Other effects

With the deepening understanding of polysaccharide structure and function after 1950s, scientists discovered that polysaccharides with different structures possessed many bioactivities. Besides the above-mentioned bioactivities, new activities of polysaccharides are still continuously found.

Fang et al. [61] isolated and investigated two pure new polysaccharides from the flowers of *Nerium indicum* for their neuroprotective effects on neurons against serum-deprivation and beta-amyloid (Aβ) peptide toxicity in primary rat cortical neuronal cultures. Pretreatment of the polysaccharides significantly reduced the number of apoptotic neurons revealed by DAPI staining when neurons were exposed to serum-free medium. Besides, the polysaccharides could also decrease the activity of caspase-3 triggered by Aβ peptides. Western blot analysis indicated that polysaccharides stimulated the phosphorylation of PDK-1 (Ser-

ine 241) and Akt (Threonine 308). The polysaccharides provide a lead for future development of neuroprotective agent against neuronal death in neurodegenerative diseases and the neuroprotective mechanism may primarily rely on activation of Akt survival signaling pathway [62]. Sun et al. [63] reported an exopolysaccharide from a marine fungus for protection of PC12 cells from hydrogen peroxide-induced injury. Chicoine et al. [64] discovered that the polysaccharide DS-L (500-kDa dextran sulfate) elicited neuroprotection in the brain, including enhanced repair responses through the AMPA (alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) receptor-MAPK (mitogen-activated protein kinase) axis. Polysaccharides are macromolecules. How can they pass through the blood brain barrier (BBB) to act on the nerve cells? This mechanism is worthy of deep investigation.

Xu et al. [65] isolated and purified a novel fructan from *Ophiopogon japonicas*. Animal tests indicated that this polysaccharide can protect myocardial cells, inhibit the generation of free radicals, and scavenge the oxygen free radicals generated from myocardial ischemia, i.e. this fructan possesses good effect of anti-acute myocardial ischemia. This discovery showed that the polysaccharide has a potential to be further studied as a leading compound for the treatment of cardiovascular diseases.

Liao et al. [66] obtained a polysaccharide from black soybean which has anti-radiation effects. Tests in vivo showed this polysaccharide promoted myelopoiesis activity in the bone marrow, stimulated production of various hematopoietic growth factors from spleen cells, and reconstituted bone marrow that has been myelo-suppressed by irradiation and 5-fluorouracil (5-FU).

Toklu et al. [67] found that both systemic and local administration of β-glucan were effective against burn-induced oxidative tissue damage in the rats. β-glucans, besides their immunomodulatory effects, have additional antioxidant properties. Therefore, β-glucans merit consideration as therapeutic agents in the treatment of burn injuries.

Rocha et al. [68] got a sulfated galactofucan from a kind of brown algae. This sulfated galactofucan was 2-fold more potent than heparin in stimulating the synthesis of an antithrombotic heparin sulfate by endothelial cells, suggesting that this galactofucan may have a potential application as an antithrombotic drug.

Tsujita et al. [69] reported that a basic polysaccharide is able to suppress dietary fat absorption from the small intestine by inhibiting pancreatic lipase activity to get the purpose of losing weight.

Ermak et al. [70] studied the protective effect of polysaccharide carrageenan on the damaging effect of endotoxins of gram-negative bacteria in vivo and in vitro, further cluing the advantage of carrageenan as a food additive.

In addition, polysaccharides also have anti-oxidative, complement-activating [71], keratomycosis-treating [72], antihypertensive [73], hair growth promoting [74], lowering cholesterol [75], treating cough [76], enhancing bone density [77], anti-ulcer [78], anti-vomiting, anti-glaucoma, stimulating sexual function bioactivities and so on [79].

2. Research methods of polysaccharides

Polysaccharides have been widely accepted as healthcare products for improving immune functions of body. However, many polysaccharides only stay in the stage of healthcare products and fail to develop into drugs. The main reason is that it is difficult to isolate and purify polysaccharides and the related technological level does not meet the requirement. In general, polysaccharides are hydrophilic macromolecules. The methodologies of isolation and purification of polysaccharides are different from small molecules. Furthermore, different polysaccharides have different properties, hence different methods of isolation and purification will have to be adopted for different polysaccharides. This work requires not only theoretic expertise on polysaccharide but also accumulated work experiences to isolate and purify polysaccharides.

2.1. Isolation and extraction methods of polysaccharides

There are many methods for isolating and purifying polysaccharides. The principle to select isolation method of polysaccharide is to keep the intrinsic properties of polysaccharides unchanged during the process of isolation and purification [80]. Although some polysaccharides exist out of the cell wall of animal/plant (called extracellular polysaccharide or exopolysaccharide), most polysaccharides are still present in the cell wall (called intracellular polysaccharide). So the first step of extracting polysaccharides is to crush materials of animal/plant to make intracellular polysaccharides release easily. Besides traditional crushing methods, ultrasonic gas flow crushing technology is used to rupture the cell wall of animal/plant or fungal spores in recent years, thus greatly increasing the efficiency of polysaccharide extraction. Due to cell walls of animal/plant are largely surrounded by lipid, removal of lipid has to be done after mechanical crushing. The common method for removing lipid is ethanol reflux 6–8 h using Soxhlet extractor [81]. The raw material after removal of lipids can be used to extract polysaccharides. There are a lot of methods of polysaccharide extraction, among which four methods are frequently used.

2.1.1. Extraction method of hot water

This is the most widely used method in polysaccharide extraction currently, which principle is that most polysaccharides have bigger solubility in hot water. Polysaccharide is stable in hot water, so polysaccharide can receive the minimal damage using this extraction method. The usual practice is to extract 2–6 h using hot water [82]. If extract is low in viscosity, the residue in the extract can be easily filtered. If extract is viscous, the residue can be removed using centrifugation.

2.1.2. Extraction method of dilute alkali-water solution

Some acidic polysaccharides, or high MW polysaccharides are not easily dissolved in hot water. Their solubility is generally bigger in dilute alkali solution than in hot water. Therefore, NaOH solution or $NaCO_3$ solution of 5%–15% (w/w) is often used to extract instead of hot water. When using dilute alkali solution to extract, extraction temperature should be kept below 10 °C. Otherwise, polysaccharides are prone to degradation. In practice, usually hot water is first used to extract polysaccharides, and then dilute alkali solution is used to extract the remaining polysaccharides in the obtained residue [83]. So most polysaccharides in animal/plant material will be extracted in this way.

2.1.3. Enzymolysis method

The crushed raw material of plants are suspended in water. According to the optimal reaction condition of composite enzymes to be used, the optimal temperature and pH are set first, and then a certain amount of composite enzymes will be added in the suspension and react for a period of time. The filtrate is the extract solution of polysaccharide after filtering the residue. This method has been applied in the preparation of some polysaccharide healthcare products [81]. However, in practice, the widely used is the combined method of hot water extraction and enzymolysis, i.e., first use hot water to extract, and then use enzymes to extract. Thus the yield of polysaccharide can increase much.

2.1.4. Other methods

The above three methods are commonly used in polysaccharide extraction. Besides these, there are still some methods which are not often used because of high cost and low yield. For example,

DMSO (dimethyl sulfoxide), or some organic solvents of alkali metal salts like 2-methoxyethanol-LiCl, or acidic aqueous solution were once used to extract polysaccharides etc.

The advantages and disadvantages of all the above isolation methods are listed in Table 1.

2.2. Removal of impurities in polysaccharide extracts

The extract solutions of polysaccharide obtained using the above methods contain many impurities, such as inorganic salts, monosaccharides, oligosaccharides, low MW non-polar substances, and high MW organic impurities (e.g. proteins, lignin). Inorganic salts, monosaccharides, oligosaccharides and low MW non-polar substances can be removed using the dialysis method [80,81]. The dialysis duration is not longer than 36 h usually, otherwise the dialysis bag easily gets moldy without preservatives. Since dialysis bags have different specifications of MW cut-off (MWCO), the first step is to select an appropriate dialysis bag of MWCO for dialysis. Dialysis bags need to be pretreated before use. They are usually put in boiling water and boiled for around 0.5 h to remove impurities in them before use. Dialysis bags can be used repeatedly, but they must be washed clean after use, soaked in the water with a little preservative (such as benzoic acid), and stored in the refrigerator (take note to avoid dryness and molding). In general, dialysis bags are stable in the pH of 5–9, hence the extract solution of polysaccharide should be adjusted to pH 5–9 first before dialysis. It should be noted that although dialysis bags have certain exclusion range of MW, some polysaccharide molecules are linear therefore sometimes polysaccharide molecules exceeding exclusion range may also pass through the dialysis bag [84]. In addition, ion exchange resins are also used to remove inorganic salts (charged anions and cations). For removal of salts in a large industrial scale, mixed beds of ion exchange resin can be applied.

There are four basic methods for removal of proteins in extract solutions as below.

2.2.1. Enzyme method

Use related protease to hydrolyze or digest proteins in the extract solution. One of frequently used methods is pronase digestion treatment, i.e. adjust extract solution to pH 7–8, add pronase, and digest 2–4 h at 37 °C [80]. After digestion, the mixed solution is heated in boiling water for 5 min to block the reaction.

2.2.2. Sevag method

According to the feature that proteins can denature in chloroform, chloroform is added to the polysaccharide aqueous solution (pH 4–5) in the ratio of 1:5 (v/v), and then *n*-butanol is added to the mixed solution. The ratio of *n*-butanol to chloroform is 1:5 (v/v). The mixture of polysaccharide solution-chloroform-*n*-butanol is violently shaken for 20 min. Denatured proteins jelly will appear in the interface between aqueous phase and solvent phase. Thus denatured proteins can be removed via centrifugation [81]. This method is mild in terms of reaction conditions but the efficiency is not high. Usually this method is used repeatedly 5 times or more to get rid of proteins. Sevag method is often applied to remove proteins from microbial polysaccharides.

2.2.3. Trichlorotrifluoroethane (CCl_2FCClF_2) method

Trichlorotrifluoroethane, which volume is same as the volume of polysaccharide extract solution, is added to the polysaccharide extract solution under a cooling condition (usually 4 °C). The mixture of polysaccharide solution- trichlorotrifluoroethane is slowly stirred for 10 min and proteins in the solution will appear and become jelly. Tremellose proteins can be removed through centrifugation. The upper aqueous phase is repeatedly treated 2 times using trichlorotrifluoroethane again and the polysaccharide solution without proteins can be obtained. This method is efficient in removing proteins. However, this method must be applied at a low temperature because trichlorotrifluoroethane has a low boiling point (47.7 °C) and is volatile, which restricts this method to be widely used.

2.2.4. Trichloroacetic acid (TCA) method

The extract solution of polysaccharide needs to be precooled in ice bath first. TCA (concentration is 15%–30%, w/w) is slowly added to the cooled extract solution while stirring until no precipitation forms. As TCA has a relatively strong acidity and may cause some polysaccharides to be degraded, the mixture is placed for 4 h at a low temperature (usually 4 °C), and then centrifuged to remove precipitation and obtain protein-free extract solution of polysaccharide [82]. This method is efficient and often used to remove proteins in polysaccharides from plants.

Extract solutions of polysaccharide, especially alkali extract solution, often contain impurities of phenolic compounds which have a darker color. Although this color can be removed in the purification process, the cost is usually very high using complex purification treatments. Some simple and cost-effective methods have been developed. Activated carbon is used to decolorize the extract solution of polysaccharide in practice. However, activated carbon has a strong adsorption for both pigments and polysaccharides. So this point can lead to a big loss for polysaccharide yield. The weakly basic ion exchange resin is also used to decolorize. The resin can remove free pigments in crude polysaccharides, but resin has a strong adsorption to acidic polysaccharides as well. Hydrogen peroxide (H_2O_2) method is more frequently used to decolorize crude polysaccharides. The pH of crude polysaccharide aqueous solution (0.3%–0.8%, w/w) was adjusted to 8.0 using ammonia ($NH_3{\cdot}H_2O$), and then H_2O_2 (final concentration is 10%–30%, v/v) was added gradually at 45 °C under stirring keeping for 2 h. The resulting mixture was adjusted to pH 7.0 using acetic acid, concentrated and precipitated by ethanol to obtain the decolorized polysaccharides [80,81]. It is essential to strictly control decolorizing conditions when using H_2O_2 method, e.g. the concentration of crude polysaccharide solution, temperature, time, pH, etc. Even so, this method still can result in some loss of polysaccharide degradation. Fang et al. [85] invented a novel method of polysaccharide decolorization, which used a kind of reverse micellar solution composed of *n*-hexanol-CTAB (cetyl trimethyl ammonium bromide)-isooctane to decolorize polysaccharide. This method which has been granted a patent is convenient and rapid. Meanwhile the recovery of polysaccharide is high.

2.3. Purification of polysaccharides

The purification of polysaccharides is to purify the isolated mixed polysaccharides to obtain various homogeneous polysaccharides. In essence, it is difficult to clearly distinguish isolation and purification as sometimes isolation already contains purification. For example, during the extraction of polysaccharides, firstly water is used to extract and then alkaline solution is used to extract. So water-soluble polysaccharides and alkali-soluble polysaccharides are isolated according to their respective solubility in water/alkali. On the other hand, sometimes purification also contains isolation. For example, impurities are further isolated in the column chromatography purification.

2.3.1. Graded precipitation method

The principle of this method is that different polysaccharide fractions have different solubility in lower alcohol or ketone (usually ethanol or acetone). Larger MW polysaccharides have less solubility than smaller MW ones in ethanol or acetone, so gradually

Table 1
Advantages and disadvantages of isolation and extraction methods of polysaccharides.

Sr. no	Methods	Advantages	Disadvantages
1	Hot water extraction	Most widely used; easy-to-operate.	A little time-consuming; some polysaccharides are not dissolved in hot water.
2	Dilute alkali-water extraction	Some acidic polysaccharides can be extracted.	Extraction temperature should be kept below 10 °C.
3	Enzymolysis method	Reaction conditions are mild.	Seldom used alone; usually combined with other extraction methods.
4	Other methods (DMSO; 2-methoxyethanol-LiCl; acidic aqueous solution, etc.)	Seldom used.	High cost; low yield.

increasing the concentration of ethanol or acetone can precipitate different MW polysaccharides respectively [81]. It is often carried out as follows:

High concentration or anhydrous ethanol is slowly added to the solution of polysaccharide mixture when stirring to make the final concentration of ethanol reach 25% (v/v). After adding ethanol, the solution is placed for 2 h, and then centrifuged to get the supernatant and precipitate (can be designated as "1st precipitate"). The precipitate is high MW polysaccharide fractions. Ethanol is continued to slowly add to the supernatant when stirring to make the final concentration of ethanol reach 35% (v/v). The solution is placed for 2 h and then centrifuged to obtain the supernatant and precipitate (can be designated as "2nd precipitate"). The 2nd precipitate is also polysaccharide fractions, but its MW is lower than the 1st precipitate. The process of graded precipitation can proceed further, which depends on the practical case. The key of graded precipitation is to try to avoid co-precipitation to happen. In practical terms, the concentration of the ploysaccharide mixtures can not be too high, the speed of ethanol adding can not be too fast, and the pH of the solution should be around neutral. The result of purfication is better when the concentration of the polysaccharide solution is smaller and the co-precipitate effect is weaker. But if the polysaccharide concentration is too thin, the recovery of polysaccharide will decrease and the consumption of ethanol will greatly increase. In general, the concentration of polysaccharide in the mixture is adjusted to 0.25% (w/v)–3% (w/v) before using this method [79]. Graded precipitation method is often firstly used in the research and development of polysaccharide healthcare products as it is much easier than column chromatography method.

2.3.2. Salting-out method

The principle of this method is that different MW polysaccharide fractions have different solubility in salt solutions of a certain concentration. When a neutral salt (such as NaCl, KCl, $(NH_4)_2SO_4$ etc.) is added to the polysaccharide solution and reaches a certain concentration, the polysaccharide fraction will separate out as the form of precipitate. So this polysaccharide fraction and supernatant can be obtained respectively through centrifugation. Continue to add this salt to the obtained supernatant, thus another polysaccharide fraction will precipitate out. Salting-out method is widely used in protein purification. In early stage, the purification of PSK (Polysaccharide Krestin) used this method. The salting-out method is cost-effective, low-efficiency and easy to form co-precipitation [80]. The key factor of affecting the efficiency of salting-out method is the concentration of polysaccharide. The purfication result is better when the concentration of the polysaccharide solution is smaller. The secondary factors are pH value of the solution and salting-out temperature. In order to obtain a satisfactory experimental repeatability, the concentration and pH and temperature of the solution should be all controlled strictly. There are a lot of salting-out agents to be used in practice. Although neutral inorganic salts are all used as salting-out agents, $(NH_4)_2SO_4$ is most widely used in polysaccharide purification. The polysaccharide precipitate obtained by salting-out method contains many salts and these salts can be removed through dialysis.

2.3.3. Metal coordination method

Different polysaccharides can form the precipitate of coordination compounds with various metal ions (copper, barium, calcium, and lead etc.), respectively. This property also can be used to isolate and purify polysaccharides. Common coordination reagents are $CuCl_2$, $Ba(OH)_2$, $Pb(CH_3COO)_2$ and so on [81]. The coordination compound precipitate obtained is firstly fully washed using water, then is decomposed using acid to get free polysaccharide. In polysaccharide purification, the most commonly used is copper salt and $Ba(OH)_2$ coordination method. In the copper salt coordination method, the frequently used agents to precipitate polysaccharides are $CuCl_2$ solution, $CuSO_4$ solution, $Cu(CH_3COO)_2$ solution and Fehling's solution. Among which, the $CuCl_2$ solution, $CuSO_4$ solution, $Cu(CH_3COO)_2$ solution require excessive use. Fehling's solution can not be used excessively, otherwise the resulting precipitate will be redissolved. The obtained polysaccharide coordination compound precipitate is firstly washed using water, then is decomposed using 5% HCl (v/v) ethanol solution. Excessive copper salt is washed and removed by ethanol. When using $Ba(OH)_2$ coordination method, the saturated $Ba(OH)_2$ solution is often used to add in the polysaccharide solution. It is proved that when the concentration of $Ba(OH)_2$ is less than 0.03 mol/L, glucomannan and galactomannan can be completely precipitated while araban and galactan do not precipitate [79,81,86]. Therefore, we can use this property to separate the polysaccharides and then use acetic acid (2 mol/L) to decompose the precipitate. The resulting supernatant is precipitated by adding in ethanol and finally can get the free polysaccharide.

2.3.4. Quaternary ammonium salt precipitation method

Long chain quaternary ammonium salts can form coordination compounds with acidic polysaccharides or long chain high-MW polysaccharides. The coordination compounds can not dissolve in the aqueous solution of low ionic strength. According to this property, the coordination compound of quaternary ammonium salt-polysaccharide can be generated in the form of precipitate, then the coordination compound precipitate can be gradually dissociated and eventually dissolved by slowly increasing the ionic strength of the solution to a certain extent. This method is often applied in the separation of acidic polysaccharides and neutral high-MW polysaccharides. The quaternary ammonium salts commonly used are CTAB (cetyl trimethyl ammonium bromide) and CPC (cetylpyridinium chloride) [87,88]. In the experiment operation, besides controlling the ionic strength of solution, the pH value of solution also must be controlled. The pH value of solution should be less than 9.0 and no sodium tetraborate is present in the solution, otherwise neutral polysaccharides can also precipitate. The quaternary ammonium salt precipitation method has a very good effect. It can precipitate acidic polysaccharides or long chain macropolysaccharides from a very dilute solution

(e.g. 0.01% concentration) via selective precipitation. The coordination compounds formed have different solubility in salt solutions with different ionic strength, acid solutions and organic solvents, through which combined polysaccharides can be freed. The coordination compound formed is often dissolved in NaCl solution of 3–4 mol/L, then polysaccharides will be precipitated by adding 3–5 times ethanol and the quaternary ammonium salt still stays in the solution; or the coordination compound formed is dissolved in NaCl solution of 3–4 mol/L, then the quaternary ammonium salt will be precipitated by adding iodide or thiocyanate and the polysaccharides still stay in the solution. *n*-butanol, pentanol or chloroform and other organic solvents are also used to extract the quaternary ammonium salt-coordination compound solution. Finally, salts are removed by dialysis and the solutions are freeze-dried to obtain the pure polysaccharides. The quaternary ammonium salt precipitation method is a classical method and it is still used in the polysaccharide purification up to now, such as lentinan was once purified using this method in Japan.

2.3.5. Column chromatography method

The column chromatography is currently the most widely used method for polysaccharide purification due to its good purification effect and simple operation. Several methods of column chromatography are described respectively as below.

2.3.5.1. Cellulose column chromatography. Cellulose is the common stuff material in the column. Firstly, the cellulose in the column is equilibrated using ethanol solution, and then the polysaccharides are loaded on the cellulose column for purification. After that, the cellulose column is eluted using the eluents respectively, and thus the different polysaccharide fractions can be eluted successively. The low MW polysaccharides are eluted out first, then the high MW polysaccharides are eluted. So what is eluted out last is the highest MW polysaccharide fraction. During the elution, various polysaccharide fractions can be finally separated out each other after they undergo the process of dissolution-precipitation in the cellulose column for a great number of times. This method can be called "graded dissolution method", substantially contrary to the graded precipitation method. Since the number of theoretical plates is high in cellulose column chromatography, the purity of the eluate is high [89]. But the disadvantages of this method are low flow rate and long time-consuming. Especially for the acidic polysaccharides with high viscosity, it seems that the flow rate is too low.

2.3.5.2. Anion exchange column chromatography. This is the most commonly applied method in both polysaccharide purification and column chromatography at present. In particular, anion exchange column chromatography is usually used at first for bulky polysaccharide solution. Polysaccharide solution can be concentrated and preliminarily purified through this method, even some polysaccharides can be purified homogeneous fractions. The widely used anion exchanger so far are DEAE-cellulose, DEAE-Sephadex and DEAE-Sepharose, among which DEAE-cellulose is usually the first choice [81]. DEAE-cellulose possesses an open framework and polysaccharide molecules can freely enter this carrier and diffuse rapidly. DEAE-cellulose has a big surface area. Although its ion-exchange capacity is only 0.70–0.75 mmol/g, the absorption quantity of DEAE-cellulose to polysaccharides is much larger than ion exchange resin. In addition, since ion exchange groups on cellulose are less, loose in arrangement and alkalescent, the adsorption of DEAE-cellulose to polysaccharides is weak and polysaccharides can be eluted out using salt solution of a certain ion concentration.

Anion exchange column chromatography is fit for separating various acidic polysaccharide, neutral polysaccharide and mucopolysaccharide. The separation mechanism of anion exchange column chromatography is not only ion-exchange, but also adsorption-desorption. So anion exchange column chromatography can be used in the separation of neutral and acidic polysaccharides, and the separation of different neutral polysaccharides as well. In general, when the pH value is 6.0, acidic polysaccharide can be adsorbed to the exchanger whereas neutral polysaccharide can not be adsorbed [79–82]. Then the buffers which have same pH value and different ionic strength can be used to elute these acidic polysaccharides out respectively. The ability of polysaccharide to adsorb exchanger is related to polysaccharide structure. The adsorb ability usually increases with the increase of acidic groups in polysaccharide molecules. For linear molecules, larger MW neutral polysaccharide is easier to be adsorbed compared to smaller MW polysaccharide. The adsorb ability of straight chain polysaccharide is greater than that of branched chain polysaccharide. In most cases, 100 g of DEAE-cellulose can load 0.5–1.5 g of dry polysaccharide sample. The elution mode is usually to use buffers with different ionic strength to carry out gradient elution or stepwise elution. In addition, neutral polysaccharide can also form coordination compound with borax (sodium tetraborate) [86]. Based on this, DEAE-cellulose is sometimes processed into borax-type DEAE-cellulose. When the polysaccharide solution flows through the borax-type DEAE-cellulose column, polysaccharide will coordinate with borax and adsorb on the column. Then the column is eluted using borate solution of different concentrations. The eluate which flows out first is the polysaccharide fraction that does not coordinate with borax, and the eluate flowing out last is the polysaccharide fraction that coordinates with borax most strongly.

Besides DEAE-cellulose, the other two anion exchanger, i.e. DEAE-Sephadex and DEAE-Sepharose are also widely used. DEAE-Sephadex series has several products, such as DEAE-Sephadex A25 (often used for the MW < 30,000 polysaccharide purification) and DEAE-Sephadex A50. DEAE-Sepharose series also has several products, such as DEAE-Sepharose CL-6B (often used for the MW > 100,000 polysaccharide). Since these exchangers have three dimensional network structure, they possess not only ion exchange function but also molecular sieve effect. Compared with cellulose, they have higher charge density, and thus they have larger exchange capacity and better separation effect. However, when the pH value or ionic strength of the eluent changes, the two exchangers (DEAE-Sephadex and DEAE-Sepharose) will change much in volume and thus the flow rate will be affected. The process method of regeneration of DEAE-Sephadex and DEAE-Sepharose is same as DEAE-cellulose.

In summary, the above three anion exchangers (DEAE-cellulose, DEAE-Sephadex and DEAE-Sepharose) are widely used in polysaccharide purification. Meanwhile, there exists some disadvantages, especially when they are used for mucopolysaccharide purification. For example, the flow rate is low, the height of column bed may change with the change of buffer concentration and pH value and thus is not steady, the service life of exchanger is short, etc. The three exchangers are gradually replaced by another kind of anion exchanger which backbone is Sepharose FF with good chemical stability and fast flow rate after 1990's. The typical product model is DEAE-Sepharose FF. The usage method of DEAE-Sepharose FF is similar to DEAE-cellulose. The DEAE-Sepharose FF can not be stored in the form of dry powder and it must be suspended in water for preservation.

2.3.5.3. Gel column chromatography. The gel column chromatography is to separate polysaccharides according to the size and shape of polysaccharide molecules, i.e. the principle of molecular sieve. This chromatography method is widely used in the separation and purification of polysaccharides. In general practice, the anion exchange column chromatography is firstly used to do the preliminary purification on the obtained crude polysaccharide, and then the gel column chromatography is used to do further purification.

Table 2
Advantages and disadvantages of purification methods of polysaccharides.

Sr. no	Methods	Advantages	Disadvantages
1	Graded precipitation	Easy-to-operate; often used firstly.	Unable to obtain homogeneous polysaccharide fractions.
2	Salting-out method	Cost-effective.	Low efficiency; easy to form co-precipitation.
3	Metal coordination	Good specificity.	It is not easy to find a proper coordination reagent.
4	Quaternary ammonium salt precipitation	Often used to purify acidic polysaccharides and neutral high-MW polysaccharides; good selectivity.	Need to accurately adjust/control ionic strength and pH of the solution.
5	Column chromatography:		
	i. Cellulose column	Purity of the eluate is high.	Low flow rate; long time-consuming.
	ii. Anion exchange column	Most widely used; fit for purifying various acidic/neutral polysaccharides and mucopolysaccharides.	Sometimes the height of column bed may change when pH of buffer changes.
	iii. Gel column	Principle of molecular sieve; often used to further purify polysaccharides.	The ionic strength of eluent should not be less than 0.2 mol/L.
	iv. Affinity column	High efficiency; easy-to-operate.	Difficult to find a proper ligand for a given polysaccharide.
6	Ultracentrifugation	Good efficiency.	High requirement for equipment; mostly used for semi-micro preparation of polysaccharide.
7	Ultrafiltration	Principle of molecular sieve.	Low yield; long time-consuming.
8	Preparative zone electrophoresis	Good separation effect.	Long time-consuming; small purification capacity; only used for semi-micro preparation.

The commonly used gels are various types of Sephadex, Sepharose, Bio-gel, and later Sephacryl, Superdex, and Superose etc. The eluent is various concentrations of salt solutions and buffers. The ionic strength of eluent should not be less than 0.2 mol/L, otherwise serious tailing peaks will occur [79].

2.3.5.4. Affinity chromatography. Some specific polysaccharides can reversibly bind with certain specific molecules. For example, a kind of lectin (concanavalin) can specially bind with some branched polysaccharides. This binding ability between specific molecules can be called affinity. The two specific molecules can also dissociate after they bind. Using this property, polysaccharides can get purified through the process of binding-dissociation. The process is described simply as below: an affinity column should be prepared in advance. The affinity column is eluted using the polysaccharide solution as mobile phase. The polysaccharide solution is a mixture of polysaccharide fractions. During the elution process, only the polysaccharide fraction that can bind with the ligand would be combined and adsorbed to the column, and other polysaccharide fractions that can not bind with the ligand would flow out of the column. Then the ionic strength and pH value of the mobile phase can be properly changed to dissociate the polysaccharide fraction combined with the ligand. So finally the purified polysaccharide fraction can be obtained [81,89].

The advantage of affinity chromatography is high efficiency and easy-to-operate. Especially for separation of the low-content polysaccharide, the concentration rate can reach several hundred even several thousand using affinity chromatography only one time. However, the disadvantage is that it is difficult to find a proper ligand for a given polysaccharide molecule. Therefore, affinity chromatography is seldom applied in the polysaccharide purification.

2.4. Other methods for polysaccharide purification

2.4.1. Ultracentrifugation method

Different MW polysaccharides possess different sedimentation speed in the strong centrifugal force field. Based on this property, various polysaccharides can be separated and purified. The ultracentrifugation method includes two types: one is differential centrifugation method, the other is density gradient zonal centrifugation method [79,86].

The differential centrifugation method is to separate different MW polysaccharides in batch by gradually adding centrifugal speed. The high MW polysaccharides can be separated at a low speed, while the low MW polysaccharides can be separated at a high speed. This method is seldom used in polysaccharide separation. The density gradient zonal centrifugation method is often used in polysaccharide research, especially for the determination of polysaccharide homogeneity. The rationale of this method is that when polysaccharides are centrifuged in the inert gradient media and reach an equilibrium, different MW polysaccharides can be gathered and distributed to certain specific positions within gradient and form different zones, then these zones are to be separated so different polysaccharide fractions can be obtained. The commonly used inert media are water, NaCl solution and CsCl solution etc. The centrifugal speed is generally set up to 60,000 rpm or so. The density gradient zonal centrifugation method for polysaccharides is often used before 1980's and mostly performed for semi-micro preparation of polysaccharide.

2.4.2. Ultrafiltration method

Based on the different size and shape of polysaccharide molecules in a solution, these polysaccharide molecules can be separated when they pass through the ultrafiltration membrane under pressure because this membrane only can allow a certain MW range of polysaccharide to pass through. This method is called ultrafiltration method. In fact, the principle of ultrafiltration method is also molecular sieve. In theory, it is feasible to use this method to separate and purify polysaccharides. But in practical operation, there are some problems [31]. Most ultrafiltration membranes can adsorb polysaccharides, which leads to the biggish decrease of polysaccharide yield. For example, hollow fiber ultrafiltration membrane can greatly adsorb polysaccharides. In addition, the ultrafiltration speed is much low and the time-consuming is too long because many polysaccharide solutions are very viscous. Even the polysaccharides can go bad during the long ultrafiltration processing period. Furthermore, the shape of most polysaccharide molecules is not spherical. If the shape of polysaccharide is linear, polysaccharides also can pass through the membrane when their MW exceed cut-off of membrane.

2.4.3. Preparative zone electrophoresis method

Different polysaccharides can be separated under the action of electrical field according to their MW, shape and charges. The carrier is commonly glass powder. Operations are usually like this: use water to slurry glass powder, pack a column, and use electrophoresis buffer (e.g. 0.05 mol/L borax solution, pH 9.3) to equilibrate the column for 3 days. Then load the polysaccharide sample on the upper end of column and switch on electric current. The upper end is positive pole. Due to electro-osmosis, polysaccharide molecules generally shift to negative pole in electrophoresis. A great deal of heat is generated in the electrophoresis process, so this kind of column must be equipped with jacket cooling. The commonly used voltage is about 1.2–2.0 V/cm, current is 30–35 mA, and the electrophoresis time is 5–12 h. After electrophoresis, the glass powder carrier is pulled out from the column and segmented. The segmented parts can be eluted with water or dilute alkaline solution respectively. This method has a good separation effect. Meanwhile, the time-consuming is long and the purification capacity each time is small. So this method is suitable for semi-micro preparation in lab [79,81].

The advantages and disadvantages of all the above purification methods are listed in Table 2.

3. Summary

Separation and purification of polysaccharides is very complicated, so it is quite difficult to obtain homogeneous active polysaccharide fractions. This is one of the main factors to impede polysaccharide research development. There are many methods and approaches for isolation, separation and purification of polysaccharides; researchers must carefully choose proper methods for separation and purification based on specific properties/characteristics of the polysaccharide to be researched.

Acknowledgment

This study is financially supported by Translational and Innovation Fund of Ministry of Education, Singapore (No.: MOE2012-TIF-1-G-065), which is gratefully acknowledged.

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