Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

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

Do assorted approaches aid in estimation of uronic acids? Case studies on *Tinospora sinensis* polysaccharides

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ARTICLE INFO

Article history:

Received 18 May 2014

Received in revised form 25 June 2014

Accepted 8 July 2014

Available online xxx

Keywords:

Assorted approaches

Colorimetric method

Spectrophotometric methods

Tinospora sinensis

Uronic acid

ABSTRACT

Polysaccharides containing uronic acids are of immense biological significance. Colorimetric methods using different chromogens have been explored for uronic acid estimation in polysaccharides but these methods counter numerous difficulties, when neutral sugars are present in substantial amount. The uronic acid content of cold water-soluble polysaccharide (CWSP) and hot water-soluble polysaccharide (HWSP) isolated from *Tinospora sinensis*, widely known for marked therapeutic significance has been studied. The uronic acid was quantitatively estimated by spectrophotometric methods using carbazole, 3-phenylphenol and 3,5-dimethylphenol (DMP) vis-a-vis gas-liquid chromatography (GLC) analysis. Galacturonic acid in CWSP and HWSP was estimated as 1.41% and 1.20% respectively. The findings bring to light, the efficacy of chromogens and also reveal to what extent, they could be relied upon. The results also indicated higher specificity and selectivity of DMP over carbazole and 3-phenylphenol. However, GLC analysis despite time consuming remains finest method for precise estimation of uronic acids. Further, GLC analysis of alditol acetates indicated the monosugar composition of L-rhamnose, L-arabinose, D-mannose, D-galactose and D-glucose in molar ratio 0.178, 0.259, 0.140, 1.045 and 98.377 in CWSP and L-rhamnose, L-arabinose, D-xylose, D-mannose, D-galactose and D-glucose in molar ratio 0.608, 0.628, 0.978, 0.227, 0.111 and 97.448 in HWSP.

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1. Introduction

Polysaccharides having uronic acids are of immense biological significance and possess significant metabolic potential in living organisms. They have been reported to possess strong immunological, antitumor, antiadhesive, antiviral, anti-infective, antioxidant, antitumorigenic, and hematopoietic activities, etc. [1–3]. It has been established that uronic acid residues prominently influence the physiological, biological and pharmacological properties of polysaccharides and glycoconjugates. This is due to alteration in the basic structure of sugar moieties, thereby, modifying the physical and chemical properties, resulting in characteristic biological activities and solubility behavior [4,5]. Interestingly, the binding abilities of polysaccharides (key to polysaccharide-based drugs and vaccines) are strongly influenced by uronic acid content. Further, free-radical scavenging [6], anticoagulant [7], antiadhesive [8], antitussive [9], anticomplementary [10], antiulcer [11] activities etc. have been reported to enhance significantly with increasing uronic acid content. The uronic acid based polysaccharides are also involved in the metabolism of many drugs and endogenous

compounds. Glucuronic acid acts as a key agent in detoxication processes [12], while glucuronan polysaccharide is widely utilized by nutraceutical and pharmacological industries owing to its effective rheological and biological properties [13].

In view of vital role played by uronic acids in polysaccharides, it has been realized that an accurate and precise estimation of uronic acid is of key significance, thus affecting the commercial applications of the polysaccharide in pharmacological world. This understanding has shifted the interest of chemists toward developing a facile, quick, accurate and economical method for uronic acid estimation. Surprisingly, estimation of uronic acid constituents in polysaccharides is more difficult than quantifying their neutral (sugar) counterparts. It is due to the fact that under acidic hydrolytic conditions, release of uronic acids is comparatively difficult owing to relatively higher stability of their glycosyl linkages, resulting in incomplete hydrolysis. Further, once released, uronic acids under acidic conditions undergo more rapid degradation to non-carbohydrate moieties than neutral sugars [14].

Different colorimetric methods have been developed for the measurement of uronic acids in plant polysaccharides; wherein various chromogens (indole, phenol derivatives, carbazole, thiol compounds) react with the chromophores (obtained by treating hexuronic acids with mineral acids) to give a characteristic color and specific intensity contributing to qualitative and

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quantitative evaluation of acid content respectively. It has been reported that colorimetric methods are less reliable, particularly in case of polysaccharides, where a number of monosugars are present in addition to uronic acids [14]. Nevertheless, these methods are simple, economic, time effective and easy to perform. Therefore, it was considered worthwhile to evaluate the frequently used methods and find out the best method which could estimate uronic acid with higher degree of accuracy and least acceptable error.

In the present communication, the uronic acid content of cold water-soluble polysaccharide (CWSP) and hot water-soluble polysaccharide (HWSP) isolated from stems of *Tinospora sinensis* have been investigated as a case study. The species has been used in Ayurveda, Indian and Chinese folk systems attributing to its immense therapeutic significance [15,16]; however, no work has been carried out on the structure of polysaccharides till date which in turn may be responsible for its broad spectrum of activities. Therefore, uronic acid content in both the polysaccharides has been studied extensively using assorted approaches based on spectrophotometric methods versus gas-liquid chromatography (GLC) analysis. Further, the most widely used colorimetric methods have been experimented and each chromogen has been studied individually in our laboratory to recommend the most suitable method for analysis. In addition, monosugar composition of the two polysaccharides was also determined.

2. Experimental

2.1. Materials

The stems of *T. sinensis* were collected from Forest Research Institute, Dehradun. A voucher specimen was identified and accession number (164274A, 164274B, 164274C) was obtained from Herbarium, Botany Division, Forest Research Institute, Dehradun. All standard and test solutions were freshly prepared before use. Commercial carbazole, 3-phenylphenol, 3,5-dimethylphenol, D-galacturonic acid and monosugars were obtained from Sigma-Aldrich (St. Louis, MO, USA). Dialysis membrane-150 was purchased from Himedia Laboratories Pvt. Ltd., Mumbai. All other chemicals and solvents used were of analytical reagent grade. Optical rotation was determined on Autopol-II, automatic polarimeter (Rudolph Research, Flanders, New Jersey) at 589 nm, D-lines of sodium.

All the colorimetric tests were performed on Chemito UV-VIS Spectrophotometer UV-2500 at different wavelengths in three different methods. Gas-liquid chromatography of the sugar mixtures was carried out using Thermo Scientific Gas Chromatograph GC-600 fitted with a flame ionization detector (FID). The samples were analyzed in the form of their alditol acetates on a BPX70 capillary column under the following operating conditions: single ramp program with initial temperature 190 °C for 1 min, program rate of 3 °C/min and final temperature 260 °C for 10 min with N₂ flow rate of 1.5–2.0 mL/min.

2.2. Isolation of polysaccharide

The polysaccharide was isolated using standard procedure. The stems (2 kg) were pulverized and subjected to vigorous stirring in cold water (5 L) for 6 h at room temperature. The moisture content of the fresh stems was found to be 85.55%. The stirred mixture was filtered and centrifuged. The supernatant part was decanted off and concentrated under reduced pressure to a smaller volume (500 mL). To this concentrated extract (500 mL), ethanol (1.5 L) was added with constant stirring. The CWSP was precipitated out and then lyophilized to give dried light gray fluffy mass. It was again dissolved in water and re-precipitated with ethanol. The precipitate

was successively treated with acetone and dry solvent ether. It was lyophilized and kept in a desiccator. The residue left after filtering the stirred mixture obtained above, was subjected to hot water stirring at 100 °C for 6 h to isolate the HWSP using the same procedure as for CWSP to give a light gray fluffy polysaccharide.

The polysaccharide (19.25 g) thus obtained, was purified using ion exchange chromatography by employing Dowex-50 W-X8 and Seralite-SRA-400 exchange resins followed by dialysis for 72 h using dialysis membrane of molecular weight 1×10^4 – 1.5×10^4 . The dialyzed product was concentrated and re-precipitated with a large volume of ethanol to obtain finally the pure polysaccharide. It was kept overnight, and the solvent was decanted off. The precipitated polysaccharide was treated sequentially with solvent ether, acetone and absolute ethanol. It was filtered and lyophilized at -80 °C to obtain finally the pure polysaccharide in the form of a gray and light gray amorphous powder of CWSP (16.50 g) and HWSP (4.78 g) respectively [17,18].

2.3. Spectrophotometric analysis of uronic acid

The uronic acid present was confirmed by qualitative carbazole-sulphuric acid test [19]. The quantitative estimation of uronic acid was carried out via spectrophotometry using three colorimetric reagents viz. carbazole, 3-phenylphenol and 3,5-dimethylphenol. In order to ensure the accuracy of results, the polysaccharides samples of CWSP and HWSP were taken in two dilutions in replicates in all three methods.

In first method i.e. borate modified carbazole method [20,21], 0.1 mL and 0.2 mL volumes of CWSP and HWSP solutions ($100 \mu\text{g mL}^{-1}$) were added to 6 mL conc. sulphuric acid under ice-cold conditions. The mixture was agitated at vortex and cooled, followed by addition of 0.2 mL of 0.1% alcoholic solution of carbazole. The mixture was then heated at 55 °C for 30 min. The absorbance of the resultant color developed was measured at 530 nm. The content of galacturonic acid was calculated using the absorbance of polysaccharide sample, corresponding to the plotted graph of standard galacturonic acid solutions (0.1 mL^{-1} mL of $100 \mu\text{g mL}^{-1}$; absorbance versus concentration).

The estimation of uronic acid using 3-phenylphenol was done by Blumenkrantz and Asboe-Hansen method [22]. In the method, 0.1 mL and 0.2 mL volumes of CWSP and HWSP solutions ($100 \mu\text{g mL}^{-1}$) were added to 1.2 mL borate-sulphuric acid reagent (0.0125 M solution of sodium tetraborate in conc. sulphuric acid) under ice-cold conditions. The mixture was vortexed, cooled and heated on steam bath at 100 °C for 5 min. It was then cooled in ice bath, followed by addition of 20 μL of alkaline solution of 3-phenylphenol [0.15% 3-phenylphenol in 0.5% sodium hydroxide]. The absorbance was measured at 520 nm.

In 3,5-dimethylphenol method [23], 0.125 mL of CWSP and HWSP solutions ($100 \mu\text{g mL}^{-1}$) were treated with 2 mL conc. sulphuric acid in the presence of 0.125 mL of 2% NaCl followed by vigorous shaking. The mixture was heated at 70 °C for 10 min, cooled at room temperature, then 0.1 mL of 0.1% of acidic solution of 3,5-dimethylphenol (0.1 g 3,5-dimethylphenol in 100 mL acetic acid) was added and absorbance was measured instantly at 450 nm and 400 nm. In this method, the uronic acid concentration was calculated based upon difference of absorbance taken at two wavelengths.

2.4. Alditol acetates

The CWSP and HWSP of *T. sinensis* were subjected to complete hydrolysis with sulphuric acid (2 N, 5 mL), breaking the polymeric chain into its constituent sugar monomers. The resulting monosugars obtained after complete hydrolysis of CWSP and HWSP were estimated quantitatively by converting the hydrolyzates

to their corresponding alditol acetates and subjecting each to gas-liquid chromatography [24]. Alditol acetates were prepared by the method of Jansson et al. [25]. To hydrolyzate (0.050 g), sodium borohydride (0.020 g) was added and the mixture was kept for 18 h at room temperature. It was neutralized by addition of a mixture of acetic acid and methanol (1:49), till the pH reached upto 7. The mixture was concentrated to dryness in the vacuum rotator at 40 °C. Boric acid was removed by co-distillations, with methanol (3–5 mL) under reduced pressure. The alditols thus obtained were subjected to acetylation using redistilled acetic anhydride and pyridine, 1:1 (5 mL) and refluxed for 6 h. Toluene (6 mL) was added forming an azeotrope with acetic anhydride, and the mixture was distilled. Toluene (6 mL) was added further and the solution was concentrated to dryness. The final product was dissolved in water (10 mL) and the acetylated sugars fractionated with dichloromethane (4 × 25 mL). Traces of moisture in dichloromethane were removed by adding activated anhydrous sodium sulphate. The alditol acetates were analyzed by GLC [24].

2.5. Reduction of carboxyl groups of polysaccharide

Both CWSP and HWSP were reduced by the method of Taylor et al. [26]. The pH of polysaccharide (0.06 g) dissolved in distilled water was adjusted to 4.75 by addition of 1 M HCl solution and 1 mmol of solid 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) was added. The reaction proceeded for 45 min at pH 4.75 until evolution of hydrogen ion ceases. A 3 M solution of sodium borohydride was added dropwise at 25 °C in a period of 1 h. 4 M hydrochloric acid was also added simultaneously to maintain the pH at 7.0. Finally, the remaining sodium borohydride (if present any) was destroyed by addition of 1 M HCl and to make the reaction mixture slightly acidic. The solution was subjected to exhaustive dialysis against distilled water and lyophilized. The reduced dried polysaccharide (0.052 g) was dissolved in water (5 mL), precipitated with three volumes of ethanol (15 mL) and lyophilized.

The reduced CWSP and HWSP gave a negative carbazole-sulphuric acid test confirming the complete reduction of uronic acid. The alditol acetates of reduced polysaccharide were prepared by method of Jansson et al. [25] as discussed above and were subjected to gas-liquid chromatography.

3. Results and discussion

3.1. Spectrophotometric results of uronic acid

Colorimetric methods using widely known chromogens viz. carbazole [20,21], 3-phenylphenol [22], and 3,5-dimethylphenol [23] were employed as a facile approach to quantify the uronic acid content. Borate modified carbazole [20,21] and 3-phenylphenol methods furnished conflicting results giving overestimated and underestimated percentage of galacturonic acid respectively. Carbazole afforded 63.8% uronic acid content in CWSP and 44.5% in HWSP (Fig. 1a), whereas 3-phenylphenol revealed 0.81% and 0.78% in CWSP and HWSP respectively as shown in Fig. 1b. The third chromogen, 3,5-dimethylphenol, indicated 1.75% acid content in CWSP and 1.58% in HWSP (Fig. 1c).

3.2. GLC analysis of uronic acid

In GLC analysis, the carboxyl groups present in both CWSP and HWSP were reduced [26].

The reduced polysaccharides were hydrolysed to constituent monosugars. The monosugars were derivatized to their corresponding alditol acetates. GLC of the alditol acetates was carried out and the chromatograms were analyzed to give complete molar ratio

Fig. 1. (a) Determination of uronic acid in CWSP and HWSP with borate modified carbazole method. (b) Determination of uronic acid in CWSP and HWSP with Blumenthal and Asboe-Hansen method. (c) Determination of uronic acid in CWSP and HWSP with 3,5-dimethylphenol method.

of monosugars. The total molar ratio of monosugars obtained from reduced polysaccharide in comparison with the monosugar ratio of unreduced polysaccharide, revealed higher peak area of galactose, thereby, confirming galacturonic acid. The analysis showed 1.41% galacturonic acid in CWSP and 1.20% in HWSP. On comparing the GLC results with colorimetric assays, it was found that acid content value determined by DMP method was closest to the galacturonic acid content obtained from GLC analysis. Thus, unlike the other two chromogens, DMP gave reliable results, thereby corroborating the GLC studies.

3.3. Comparison of spectrophotometric techniques with GLC methods

The quantitative estimation of uronic acid was carried out using assorted approaches. While estimating uronic acid by a facile

approach, employing spectrophotometric methods using three chromogens, it was thought worthwhile to study the acid content through GLC analysis with a view to validate the uronic acid content present. On comparing the GLC results with colorimetric assays, it was found that acid content value determined by DMP method was nearest to the corresponding values derived from GLC analysis. Thus, unlike the other two chromogens, DMP gave reliable results thereby corroborating the GLC studies.

In order to understand discrepancies in results of carbazole and 3-phenylphenol, several allied experiments were performed to study the behavior of individual chromogens involved. The results showed that the chromogens (carbazole, 3-phenylphenol, 3,5-dimethylphenol) have different rate of action with respect to their sensitivity, specificity and selectivity. Moreover, individual sugars react differently in terms of speed of development of color and its absorbance [27]. Carbazole possessing greater heat stability has been known for decades, for uronic acid estimation but in the presence of substantial amount of neutral sugars, its specificity decreases [28]. Further, carbazole is not selective toward uronic acid in the presence of other monosugars and subsequent modifications in the method allow no possibility of differentiation between uronic acid and monosugars (hexoses and pentoses), thereby leading to overestimation of acid. Also there are no separate protocols for individual estimation of glucuronic acid and galacturonic acid. In the present study, both CWSP and HWSP contain excess of neutral sugars compared to uronic acid content and they strongly interfered with later thereby resulting in error.

3-Phenylphenol was found to be more sensitive and tenfold more specific than former but the assay was found to be affected by factors such as short sensitivity range (0.5–15 μg) [22], interference of neutral sugars when present in appreciable amount [28] and lack of specificity for individual uronic acid. Further, the assay showed very eccentric behavior i.e. absorbance value was found to decrease as the concentration of polysaccharide sample increased, leading to underestimation of uronic acid content. The reason behind such anomalous display was that the absorbance given by 3-phenylphenol with glucose after addition of sulphuric acid (5-hydroxymethyl-2-furancarboxaldehyde) decreases throughout the range of measurement (520 nm) of 5-formyl-2-furancarboxylic acid, prime chromogen formed by hexuronic acid with sulphuric acid and responsible for color [23]. In the present study, both the polysaccharides CWSP and HWSP contain glucose to the extent of 98.37% and 97.44% respectively, due to which, the absorbance of 3-phenylphenol decreases at much higher rate than its relative increase due to galacturonic acid content with increasing sample concentration. 3,5-Dimethylphenol, on the contrary, has proved to be an appropriate and more selective chromogen than the above two since glucuronic and galacturonic acid can be estimated individually. Moreover, glucose interference was largely eliminated, thereby leading to precise estimation of uronic acid with a far lesser extent of neutral sugars interference.

3.4. Monosugar analysis of *T. sinensis*

The monosugar composition of CWSP and HWSP was quantitatively studied by complete hydrolysis and derivatization of monosugars obtained, to corresponding alditol acetates [25] and their GLC analysis [24]. The retention time of six peaks, coincide with the retention time of standard α -rhamnose, L-arabinose, D-xylose, D-mannose, D-galactose and D-glucose. In CWSP, the molar ratio of α -rhamnose, L-arabinose, D-mannose, D-galactose and D-glucose was found to be 0.178, 0.259, 0.140, 1.045 and 98.377 while composition of HWSP was α -rhamnose, L-arabinose, D-xylose,

D-mannose, D-galactose and D-glucose in molar ratio 0.608, 0.628, 0.978, 0.227, 0.111 and 97.448.

4. Conclusion

The study for the first time conclusively proves that though colorimetric methods are facile and time effective, but the choice of chromogen profoundly affects the results. The findings disclose the efficacy of chromogens and also reveal to what extent, they could be relied upon. Based on the experimental results, the present analysis established the superiority of DMP over the carbazole and 3-phenylphenol due to its relatively higher specificity and selectivity and is recommended as the best colorimetric method for uronic acid estimation affording satisfactory results. However, GLC analysis, despite time consuming, remains the finest method for precise estimation of uronic acids. Further, monosugar analysis of the polysaccharide of *T. sinensis* has been carried out for the first time, which may prove to be useful from a prospective of therapeutic applications. The determination of primary structure of the polysaccharide is a critical prerequisite to the understanding of the plant activity leading to improved utilization of the plant.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

The authors are grateful to the Director, Forest Research Institute (FRI), Dehradun and the Head, Chemistry Division, FRI, Dehradun for providing laboratory facilities. One of the authors (SN) is grateful to Indian Council of Forestry Research and Education for financial assistance as Junior Research Fellow. Thanks are also due to Dr. H. B. Naithani, Systematic Botany Discipline, Botany Division, Forest Research Institute, Dehradun for authentication of the plant material.

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