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Functional modification of agarose: A facile synthesis of an agarose-saccharate derivative

Mahesh U. Chhatbar, Chirag B. Godiya, A.K. Siddhanta *

Marine Biotechnology and Ecology Discipline, CSIR-Central Salt & Marine Chemicals Research Institute, Council of Scientific & Industrial Research, G.B. Marg, Bhavnagar 364002, Gujarat, India

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ABSTRACT

A water-soluble agarose-saccharate (AGS) was synthesized *in situ*. AGS was characterized by FT-IR, ^{13}C NMR, UV–vis and CD-ORD spectra, X-ray diffraction (crystallinity index = 0.45) as well as by studies of physicochemical properties. The product in 150 ppm solution was sweeter than sucrose although significantly less sweet than pure sodium saccharate (SA). In 675 ppm solutions or in the solid state AGS did not have bitter aftertaste whereas pure SA did taste bitter under the same conditions. This water-soluble sweet agarose derivative would be of potential use as a food additive.

1. Introduction

Agarose (AG) is a seaweed polysaccharide, chemically consists of alternating 3-O-linked D-galactopyranose and 4-O-linked 3,6-anhydro-L-galactopyranose (Fig. 1a). It is mainly derived from red seaweed *Gracilaria dura* (Meena et al., 2007), and is used in the food industry as a texturizing and thickening agent (Phillips & Williams, 2000, chap. 2). It is an industrially important high value material and is also used extensively in biotechnology and molecular biology applications (Meer, 1980; Renn, 1984). Sodium saccharate (1,2-benzisothiazolin-3-one, 1,1-dioxide, sodium salt; Fig. 1b) is an artificial sweetener and is about 300 times as sweet as sucrose, but has an unpleasant bitter aftertaste, especially at high concentrations (Mitchell & Pearson, 1991).

Modification of natural polymers is a promising method for the preparation of new materials with potential applications. There exist numerous reports in the literature on the modification of polysaccharides employing various strategies, *e.g.* grafting, cross-linking, *etc*. We reported modifications of agarose as part of an ongoing program of our lab on modification of seaweed polysaccharides for preparing new materials with improved functional properties (Meena, Prasad, & Siddhanta, 2006; Meena et al., 2007; Meena, Chhatbar, Prasad, & Siddhanta, 2008; Oza, Meena, Prasad, Paul, & Siddhanta, 2010; Prasad, Trivedi, Meena, & Siddhanta, 2005; Prasad, Mehta, Meena & Siddhanta, 2006).

We report herein for the first time a facile synthesis of a sweet water-soluble agarose-saccharate (AGS) using sodium saccharate.

2. Materials and methods

Agarose used in this study was extracted from the seaweed *G. dura* as described in our previous work (Meena et al., 2007). Other chemicals used in this study [*e.g.* Iodine (I_2), dimethyl formamide (DMF) and pyridine] were purchased from M/s S.D. Fine Chemicals Ltd., Mumbai (India). Triphenyl phosphine (Ph_3P) was purchased from M/s Spectrochem Pvt. Ltd., Mumbai (India) and sodium saccharate (SA) was purchased from M/s Central Drug House (CDH) Pvt. Ltd., New Delhi (India). When dry solvents are referred to, they were dried by the following procedures: (i) DMF was dried over CaH_2 and filtered and (ii) pyridine (C_5H_5N) was dried over CaH_2 and distilled. To prepare physical mixture, agarose and sodium saccharate (1:1 mole) were dissolved in distilled water and then the solution was lyophilized.

2.1. Synthesis of agarose-saccharate (AGS)

The synthesis is a two-step *in situ* procedure. The first step involved substitution of hydroxyl group by iodine at the C-6 of agarose, and then iodine was substituted by sodium saccharate sodium salt yielding agarose-saccharate (AGS) and sodium iodide (Scheme 1).

* Corresponding author. Tel.: +91 278 2567760; fax: +91 278 2567562.
E-mail address: aks@csmcri.org (A.K. Siddhanta).

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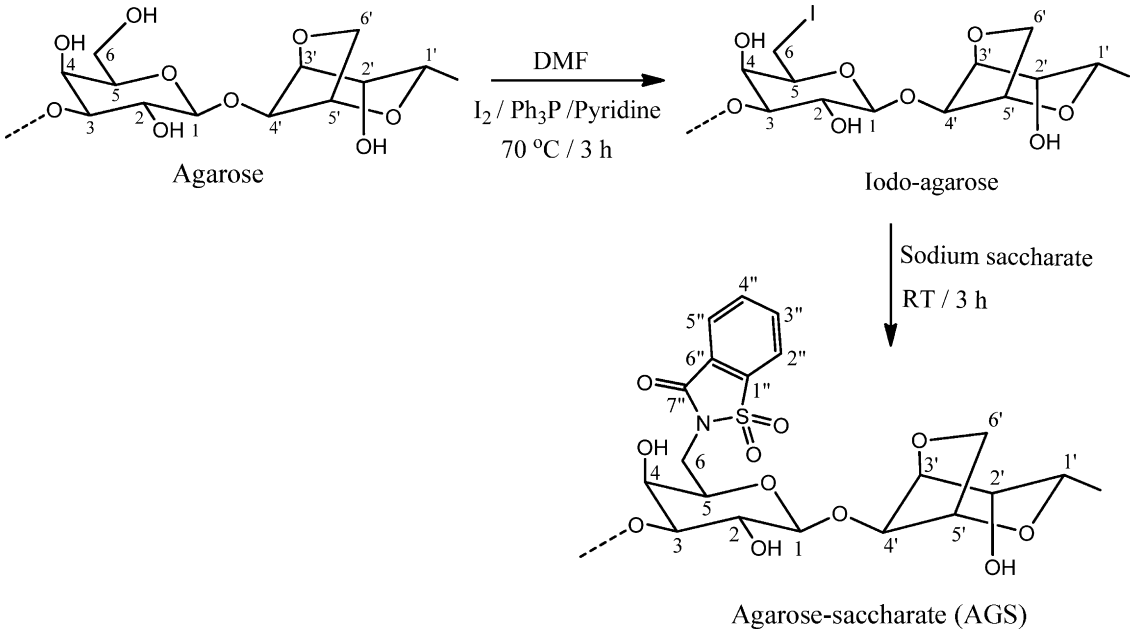

Scheme 1. Synthetic routes of agarose-saccharate (AGS).

(a) Preparation of 6-iodo-agarose was done adapting the method described by Ashton, Königer, and Stoddart (1996). Agarose (3 mmol, 918 mg) was dissolved in 30 ml dry DMF with constant stirring over 10 min with the evolution of heat raising the temperature to *ca.* 60 °C. Dry pyridine (5 ml) was added to the latter at room temperature (30 °C). A brown solution (25 ml) of triphenyl phosphine (Ph_3P; 6 mmol, 1572 mg) and iodine (I_2; 6 mmol, 1512 mg) in dry DMF was added dropwise into the agarose solution. The reaction mixture was heated in an oil bath to 70 °C for 3 h under an atmosphere of N_2 under stirring.

(b) The reaction mixture was allowed to cool to room temperature (30 °C), to this was added sodium saccharate (2,3-dihydro-1,2-benz-isothiazole-3-on-1,1-dioxide sodium; 3 mmol, 723 mg) and was stirred for 3 h at room temperature in N_2 atmosphere. Then the reaction mixture was poured into isopropyl alcohol (IPA; 200 ml) to form a precipitate. The precipitate was dissolved in distilled water (50 ml), centrifuged and dialyzed against distilled water using a dialysis tube (MW cut off 1200). The water was replaced three times a day for three days, and finally the modified agarose-saccharate (AGS) was obtained by lyophilization of the dialyzate.

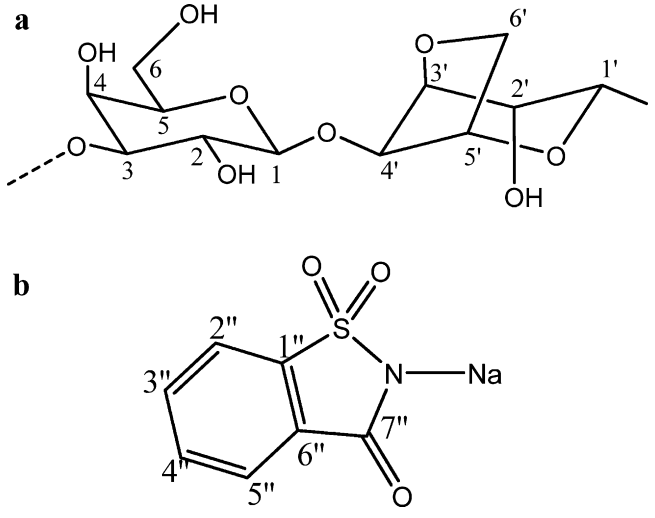

Fig. 1. (a) Repeating unit of agarose (AG) and (b) sodium saccharate (SA).

2.2. Spectral characterization

The FT-IR spectra were recorded in KBr pellets (2.0 mg sample in 600 mg KBr) on a PerkinElmer FT-IR, model Spectrum GX. All spectra were average of two counts with 10 scans each and resolution of 4 cm^{-1}.

^{13}C NMR spectra were recorded on a Bruker Avance-II 500 (Ultra shield) Spectrometer, Switzerland, 125 MHz spectrometer. Samples were dissolved in D_2O (50 mg/ml), and the spectra were recorded with 5000 accumulations, pulse duration 5.9 μs, acquisition time 1.2059 s and relaxation delay 6 μs using DMSO-d_6 (*ca.* δ 39.5) as internal standard.

2.3. Quantitative assay of the extent of modification of the agarose

The extent of modification of agarose by sodium saccharate was evaluated by UV absorbance measurements at 260 nm, according to method reported in the literature on alginate derivative (Abu-Rabeah, Polyak, Ionescu, Cosnier, & Marks, 2005). AGS sample was dissolved in water to produce 0.01% (w/v) solution and the absorbance was measured on a Varian CARY 500 UV–vis-NIR spectrophotometer at 260 nm. The degree of modification was calculated from the calibration curve obtained by measuring the absorbances of different concentrations of sodium saccharate in 0.01% (w/v) agarose solution. A solution of agarose at a concentration of 0.01% (w/v) was used as a blank.

2.4. Optical rotation and circular dichroism

Optical rotations were measured with Digipol 781 automatic polarimeter Rudolph Instrument (*ca.* 0.25%, H_2O) at 30 °C. Circular dichroism (CD) spectra were recorded on JASCO model J-815 CD Spectrometer, in the range 190–250 nm using sample concentration of *ca.* 0.8 mg/ml (800 ppm). Molar ellipticity values, [θ] are reported in units of deg cm^2 $dmol^{-1}$. All measurements were performed at room temperature using 1.0 cm quartz cells. The ratio

of peak height to trough depth was calculated using Eq. (1) as described by Morris, Rees, and Thom (1980) and Chhatbar, Meena, Prasad, and Siddhanta (2009):

$$\frac{\text{Peak}}{\text{trough ratio}} = \frac{\theta_{\text{trough}} - \theta_{\text{peak}}}{\theta_{\text{trough}}} \quad (1)$$

2.5. Other characterizations

Powder X-ray diffractions were recorded on a Philips X'pert MPD system in the 2θ range 10–60° for vacuum dried samples of the powder of agarose, AGS, SA as well as the physical mixture of agarose and sodium saccharate. The crystallinity index (C.I.) of the materials was determined using the following equation described by Herman and Weidinger (1948):

$$\text{C.I.} = \frac{\text{Area of crystalline Peak}}{\text{Area of crystalline peak} + \text{Area of amorphous peak}} \quad (2)$$

Total nitrogen was estimated by Kjeldahl method on a KEL PLUS-KES 201 Digestion unit attached to a KEL PLUS-CLASSIC DX Distillation unit (M/s PELICAN equipments, Chennai, India). The sulphur content was estimated by inductively coupled plasma (ICP) spectrophotometry on a Perkin-Elmer ICP-OES Optima 2000DV machine. The apparent viscosity was measured on a Brookfield viscometer (Synchrolectric Viscometer, Stoughton, MA 02072, USA) using Spindle No. 1 at 60 rpm at 80 °C. Measurements were done in triplicate in each experiment. The molar mass (weight average molecular mass, M_w; number average molecular mass, M_n and polydispersity index, PDI) of the agarose (AG) and agarose-saccharate (AGS; 500 ppm solution in 0.1 M Na_2NO_3 at 45 °C) were determined by Gel permeation chromatography (GPC, Waters alliance HPLC using Ultra Hydrogel columns 120 and 500 (length 300 mm, dia. 7.8 mm) on a Waters 2695 separation modules equipped with a 2414 refractive index detector, USA).

2.6. Sweetness index

The relative sweetness of SA and AGS was measured following the method described by Cardello, Da Silva, and Damasio (1999) in relation to sucrose concentration. The taste panel consisted of 10 trained panelists, comprising of 5 males and 5 females of age group 25–35 years, who were instructed to keep the sample to be tasted in oral cavity for about 30 s. Each panelist tasted the solutions three times in an hour to get a mean value. After every expectoration the members were asked to rinse oral cavity with water. They were asked to rate the sample for sweetness on a scale of 1–10 and state whether a bitter aftertaste could be detected (yes/no). Points were allotted for sweetness (scale 1–10), while comments for bitter aftertaste (yes/no). The points which were obtained for sweetness were made to add to get the total of each parameter to decide if the solution of AGS was sweeter and/or had no bitter aftertaste as compared to SA, was noted by the panel members. A 5 ml sample of the oral solution were taken for the study.

3. Results and discussion

3.1. Physical properties

Optimization studies revealed that a 3 h duration of the reaction in both steps (iodination and saccharation) was sufficient to produce the sodium saccharate derivative of agarose in good yield and purity. Optimized yield of the product was 75% (with 1:1 mole of agarose and sodium saccharate), which was calculated on the basis of the nitrogen content of the product (Kjeldahl's estimation) with respect to the total quantities of agarose and sodium saccharate that were used in the synthesis. Total nitrogen contents in agarose (AG), sodium saccharate (SA) and agarose-saccharate (AGS) were $0.02 \pm 0.01\%$, $7.5 \pm 0.11\%$, $2.45 \pm 0.10\%$, respectively (Table 1). The apparent viscosity of the AGS (*ca.* 1% in distilled water, 80 °C) was lower (5.5 ± 1.5 cP) than that of parent agarose (AG) (*ca.* 1% in distilled water, 80 °C; 12.5 ± 2.0 cP) (Table 1). The sulphur contents of AG, SA and AGS were found 0.01%, 17.2% and 2.4%, respectively (Table 1), which indicated insertion of SA on to the AG backbone. The weight average molecular mass (M_w), number average molecular mass (M_n) and polydispersity indices (PDI) of AG (M_w 145 kD; PDI 2.9) and AGS (M_w 129 kD; PDI 3.3) are given in Table 1. The results indicated that the agarose polymer backbone remained largely undegraded during the reaction. However, it was observed that when duration of the iodination step increased, the M_w decreased, which may be attributed to the increased degradation of agarose polymer (Supplementary data Table S1).

3.2. Quantification of the sodium saccharate modified agarose

A $\pi \rightarrow \pi^*$ transition occurs in the sodium saccharate (SA) ring present in the AGS, allowing quantification of the amount of SA by UV–Vis absorption spectroscopy at 260 nm. UV absorptions were measured in several samples within the concentration range 0.5–2 mM of SA dissolved in agarose solution (0.01%, w/v) at 260 nm. The data collected from UV absorbance measurements had a very good linearity ($R^2 = 0.99$). The extinction coefficient (ε) = 145.12 M^{-1} cm^{-1} for SA was calculated from the slope of the calibration curve (Supplementary data Fig. S3). The average value of the degree of substitution (DS) in the AGS was 0.28 ± 0.05. Further we calculated DS on the bases of %*N* (DS = 0.35 ± 0.04) and %*S* (DS = 0.30 ± 0.05) which are in good agreement.

3.3. Spectral characterization

The FT-IR spectra of agarose AG, SA, AGS and the physical mixture of agarose and SA are depicted in Fig. 2. Strong bands at 1643 cm^{-1} for agarose and 1645 and 1586 cm^{-1} for SA were observed in the FT-IR spectra (Fig. 2). The spectrum of AGS exhibited new bands at 1261 (—C—N bending), 804 and 540 cm^{-1} (—SO_2 bending and scissoring vibrations) indicating the presence of SA moiety in the product. Furthermore, characteristic bands at 931 (3,6-anhydro moiety of agarose), 878 and 667 cm $^{-1}$ (β-skeletal bending of basic carbohydrate moieties) in the IR spectrum of the copolymer indicated that during grafting reaction the agarose polymer did not get decomposed (*cf.* Prasad et al., 2006).

^{13}C NMR spectra of AG, SA, AGS are given in Fig. 3a–c, having indicated the chemical shift values and the probable assignments. Five carbons of SA appeared at 121.69, 125.06, 134.75, 135.25 and 173.48 ppm, which were assigned to C-2″/C-5″, C-6″, C-4″, C-3″ and C-7″ (Fig. 1), respectively (Supplementary data Table S2). The assignments of SA were done by comparison with the corresponding data reported by Romañuk et al. (2009). The carbon chemical shifts of agarose were assigned by comparison with the data reported by Meena et al. (2007). All the 19 carbon resonances were discernible in the ^{13}C NMR spectrum of AGS (Fig. 3), having a new signal at δ 49.14 for the new C—N bond (Scheme 1) associated with an upfield shift of C-7″ of SA in AGS. The assignments were done by comparison with the values obtained for SA and agarose in this study.

3.4. Optical rotation and circular dichroism

The optical rotation values of parent agarose (AG) $[\alpha]_{589}{}^{30}$ (*ca.* 0.25%, H_2O) and SA $[\alpha]_{589}{}^{30}$ (*ca.* 0.25%, H_2O) were −34.51° and

Table 1
Physical properties of agarose (AG) and agarose-saccharate (AGS).

Samples	% Yield[a] (±SD)	Total nitrogen[a] % (±SD)	Sulphur[a] % (±SD)	Apparent viscosities at 80 °C (cP)	M_w (kDa)	M_n (kDa)	PDI
Agarose (AG)	NA	0.02 ± 0.01%	0.01 ± 0.01%	12.5 ± 2.0	145.1	49.9	2.9
Sodium saccharate (SA)	NA	7.2 ± 0.1%	17.0 ± 0.1%	NA	NA	NA	NA
AGS	75.0 ± 0.5	2.45 ± 0.10%	2.4 ± 0.12%	5.5 ± 1.5	129.0	38.5	3.3

[a] Data presented are mean of triplicate measurements (±SD); NA = not applicable.

−72.84°, respectively, while of AGS $[\alpha]_{589}{}^{30}$ (*ca.* 0.25%, H_2O) was −86.53°, indicating a significant makeover of the symmetry elements of agarose.

The CD spectrum of non-modified agarose was fully in the positive region and observed peak value of [θ] 69.34 at 189 nm. The CD spectrum of SA also showed positive trends and had a peak value of [θ] 109.04 at 198 nm. The spectrum of AGS had a small peak [θ] −3.03 at 202 nm, along with another [θ] 146.77 at 192 nm indicating the insertion of SA onto the agarose polymer (Supplementary data Fig. S4). The peak-to-trough ratio of SA 0.48 (<1), while those of agarose and AGS were >1 (69.7 and 7.5, respectively), suggesting significant chiroptical modification of agarose in AGS (*cf.* Chhatbar et al., 2009; Morris, Rees, Sanderson, & Thom, 1975; Morris et al., 1980).

3.5. X-ray diffraction analysis

The X-ray diffraction patterns of parent agarose (AG), SA, AGS and the physical mixture of AG and SA are depicted in Fig. 4. The X-ray diffraction pattern of agarose showed that it was amorphous in nature, while that of AGS showed two distinctly sharp peaks at 5.81° and 23.03° indicating induced crystallinity (C.I. 0.45) on the parent polymer, as a result of addition of crystalline SA (Fig. 4) bringing about order in the polymeric molecular architecture. Similar observations have been reported for hydrogels based on grafting in our previous work (Meena et al., 2006, 2008; Oza et al., 2010). The XRD pattern of the physical mixture of agarose and sodium saccharate resembled that of SA which indicated that chemical modification of agarose has happened (Fig. 4).

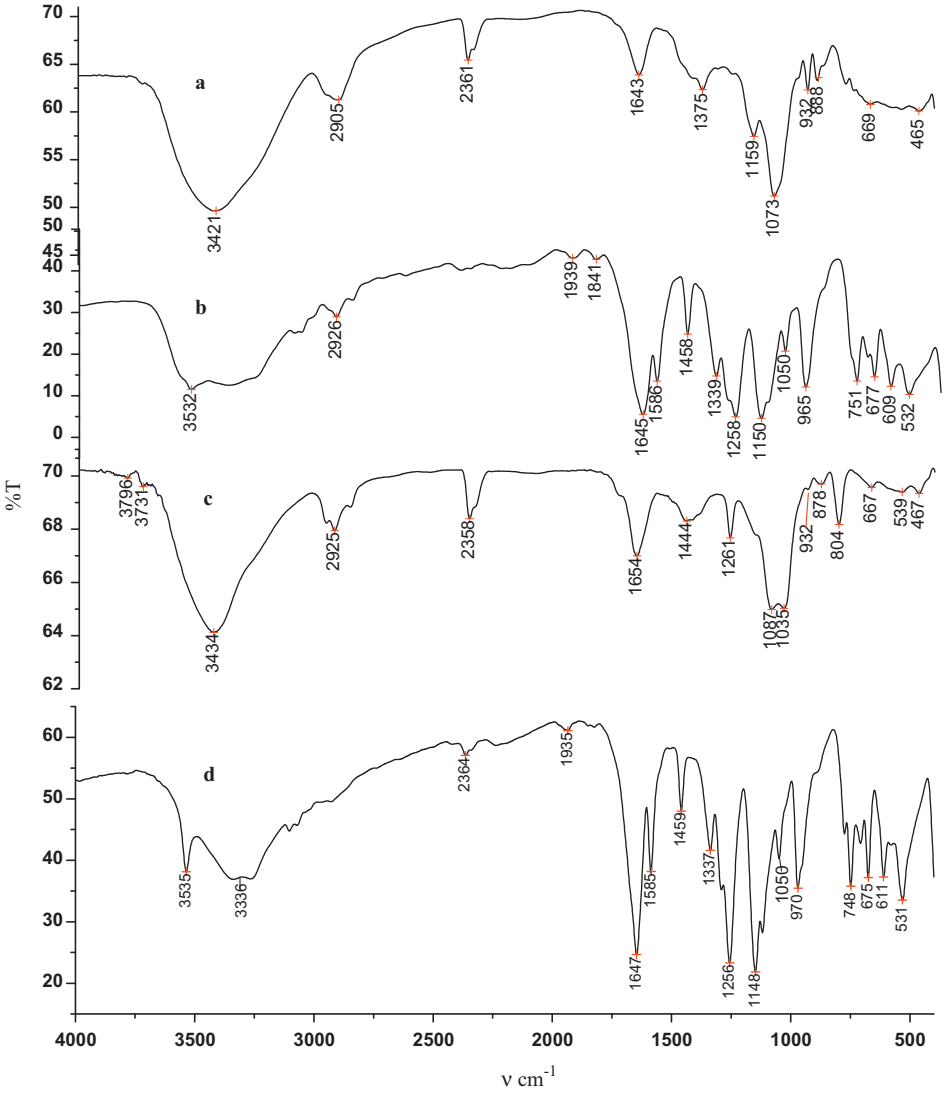

Fig. 2. FT-IR spectra of (a) agarose (AG), (b) sodium saccharate (SA), (c) AGS and (d) physical mixture of AG and SA.

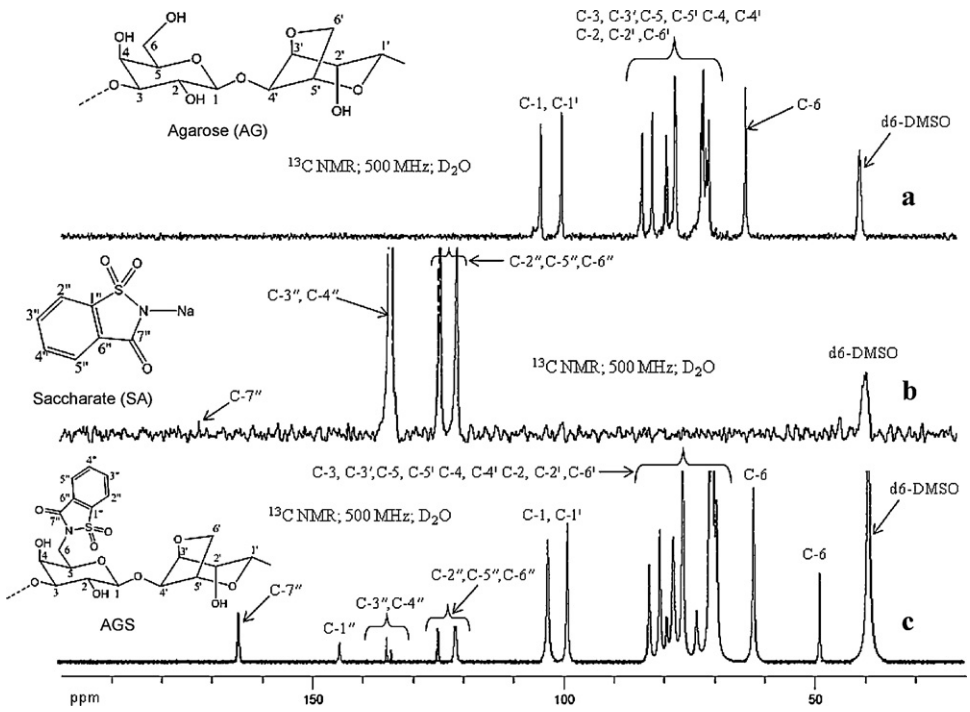

Fig. 3. ^{13}C NMR of (a) agarose (AG), (b) sodium saccharate (SA) and (c) AGS.

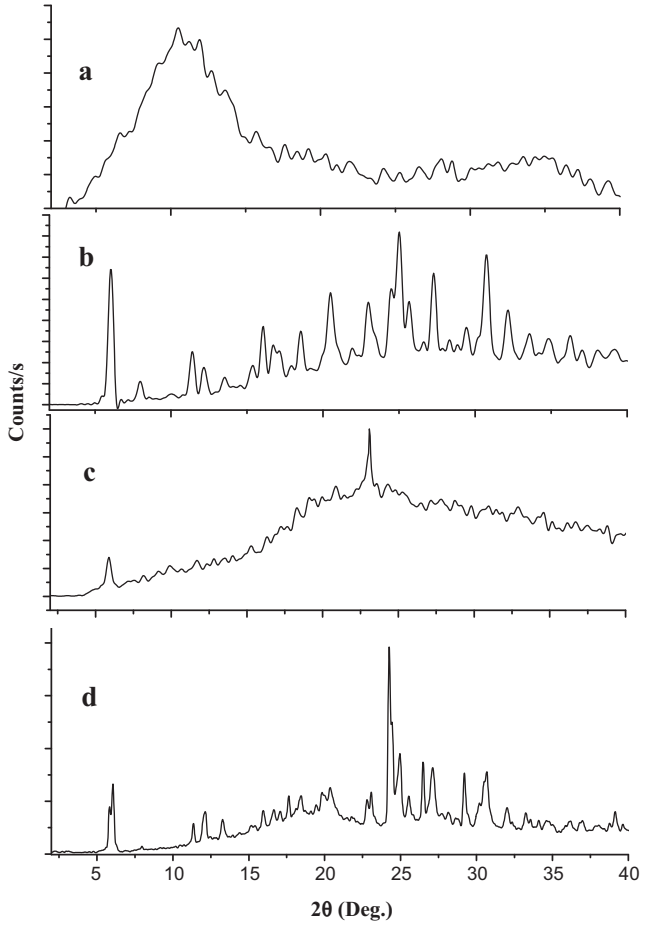

Fig. 4. XRD patterns of (a) agarose (AG), (b) sodium saccharate (SA), (c) AGS and (d) physical mixture of AG and SA.

3.6. Sweetness index

Sodium saccharate (SA) is 300–450 times sweeter than sugar (La Via & Hill, 1977) but it has a bitter aftertaste at high concentrations while AGS is not bitter at higher concentrations. In this study we prepared 150 ppm solution of SA in distilled water and its sweetness (10 in sweetness scale) was higher than that of sucrose (1 in scale) at the same concentration (Table 2). The relative sweetness index of AGS in 150 ppm solution in distilled water was significantly lower (4 in scale) than that of SA and that of the AGS solution was higher than that of sucrose (1 in scale) at the same concentration. However, the sweetness potency of the AGS in 675 ppm solution, containing 150 ppm equivalent of pure sodium saccharate was comparable (9 in scale) with that of a 150 ppm pure SA solution (10 in scale). In 675 ppm solution AGS or in solid state it did not have any bitter aftertaste whereas pure SA did taste bitter under the same conditions (Table 2). The sweetness index of agarose-sodium saccharate physical mixture (1:1, w/w) was lower (7 in sweetness scale) than that of pure SA in 150 ppm solution, as one would expect (Table 2). Further, in the solid state the physical mixture had a bitter aftertaste. It appears that the N either in saccharate (—N—Na) or in saccharine (—NH) contributes to the bitter

Table 2
Sweetness potency—taste panels' scores/comments.

Samples	Panel 1[a]	Panel 2[a]	Bitter aftertaste
Sucrose[b]	1	1	No
SA[b]	10 ± 0.5	10 ± 0.6	Yes
AGS[b]	4 ± 0.4	4 ± 0.5	No
AGS[c]	9 ± 0.5	9 ± 0.4	No
Physical mixture of SA and agarose[b] (1:1, w/w)	7 ± 1.0	7 ± 0.6	Yes

[a] The taste panel consisting of 10 trained panelists – 5 males (Panel 1) and 5 females (Panel 2) of age group 25–35 years.
[b] Prepared 150 ppm solution in distilled water.
[c] Prepared 675 ppm solution, containing 150 ppm equivalent of pure sodium saccharate.

taste, but when it is substituted with an alkyl group, which is 6-linked agarose herein, the bitter aftertaste seems to have been lost. This is presumably because the N atom of the saccharate moiety was not available for binding with the taste buds for steric reasons. Further, this leads one to conclude that the N atom makes little contribution to the sweetness potential. However, more studies are in order to confirm these aspects.

3.7. Proposed mechanism of the formation of (AGS)

The proposed mechanism for preparation of agarose-saccharate (AGS) has been given in Scheme 1. This procedure is a two-step one. First step constituted substitution of hydroxyl at the C-6 of agarose with iodine yielding 6-iodo-agarose, followed by the second step wherein iodine is substituted by sodium saccharate molecule *in situ* affording agarose-6-saccharate.

4. Conclusions

Agarose polymer has been modified with a sweetener saccharine. A sweet water-soluble agarose-saccharate (AGS) with no bitter aftertaste, unlike sodium saccharate (SA), was synthesized *in situ*, for the first time. The agarose-saccharate derivative exhibited crystallinity (XRD; C.I. of the AGS = 0.45), unlike agarose polymer. The modified product AGS had a sweetness index in a certain concentration (675 ppm wherein the saccharate moiety was present in 150 ppm equivalent), which was comparable (9 in sweetness scale) to that of pure SA in 150 ppm solution (10 in scale). Pure AGS was sweet with no bitter aftertaste whereas SA was sweet with a bitter aftertaste. This water-soluble and sweet agarose derivative would be of potential use as a food additive.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carbpol.2012.01.086.

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