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Partial characterization of sulfohydrolase from *Gracilaria dura* and evaluation of its potential application in improvement of the agar quality

Mahendra K. Shukla, Manoj Kumar, Kamlesh Prasad, C.R.K. Reddy*, Bhavanath Jha

Discipline of Marine Biotechnology and Ecology, Central Salt and Marine Chemicals Research Institute, Council of Scientific and Industrial Research (CSIR), Bhavnagar 364021, India

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ABSTRACT

Sulfohydrolase extracted from *Gracilaria dura* was purified to homogeneity and investigated for improving the quality of commercial agar. The purified enzyme (50 kDa) showed optimum activity at pH 8.0 and temperature 35 °C. The agar treated with ~50 U of purified enzyme exhibited 1.66-fold increase in 3,6-AG content with 60% sulfate removal and also resulted an increase of ≥2-fold in viscosity and gel strength with a recovery of 90% agar. Further, the gelling and melting temperatures were markedly decreased to 31 °C and 82 °C respectively over the control values of 39 °C and 90 °C. The scanning electron microscopy revealed higher cross-linking and rigidity in the treated agar while FT-IR spectral analysis confirmed the increased 3,6-AG content with decreased sulfate. Therefore, the possibility for cloning of sulfohydrolase encoding gene(s) for its commercial production and exploitation in desulfation of agar could be an eco-friendly and alternative method to alkali treatment.

1. Introduction

Agars, the sulfated galactans are the most abundant structural polysaccharide of red algal cell walls and are generally considered as the mixture of agarose and agaropectin, and accounts for ca. 50% of algal dry weight (Yoon & Park, 1984). These sulfated galactans constitute a large family of hydrocolloids composed of linear chains of galactose (Gal) with alternating α-1,3 and β-1,4 linkages and differ in degree and position of sulfate esterification. The agar quality in terms of gel strength is solely attributed to the 3–6, AG content while the sulfate moiety and the contaminating floridean starch (storage sugars of red algae) undermine the quality of agar. In addition, presence of acidic groups such as sulfonic, pyruvic and uronic acid in agar deteriorate its gel strength by blocking the formation of double helices between the molecules. The proportion and distribution of the aforementioned substituents in the skeletal chain of the galantans modify the physico-chemical properties of the agar (Lahaye & Rochas, 1991; Yaphe & Duckworth, 1972). Among the others, viscosity and gelling capacity are the key features that confer diverse industrial applications for these hydrocolloids as stabilizers, thickeners and gelling agents (Usov, 1992).

The Gal-6-sulfurylases found in agarophytes and the analogous sulfohydrolase activities extracted from carrageenophytes represent a novel class of enzymes owing to the chemical reaction they catalyze and have only been found in the red algal lineage. The conversion of porphyran to agarose and μ-carrageenan to κ-carrageenan has been demonstrated previously using partially purified enzyme from *Porphyra* (Rees, 1961a) and *Chondrus crispus* (Wong & Craigie, 1978) respectively. Recently, Sulfurylase I and II were purified from *C. crispus* and evaluated their potentials for converting ν- into ι-carrageenan (Genicot-Joncour et al., 2010).

With increasing demand of agarose in biotech and pharmaceuticals industries, considerable emphasis has been made to remove the sulfate group from agar in order to enhance the gel strength. The widely used alkali pretreatment methods in the industries have proven their potential to improve the gel strength (Freile-Pelegrin & Murano, 2005; Marinho-Soriano & Bourret, 2005; Meena, Prasad, Ganeshan, & Siddhanta, 2008; Mehta et al., 2010) though they have drawbacks of diminished polysaccharide yield and generation of an effluent, which can have deleterious environmental effects if discharged untreated. Thus, there is a growing need to develop eco-friendly downstream processing technologies for recovery of products from bioresources. Recently, a method for obtaining high quality agarose with high yield has been established in *Gracilaria gracilis* circumventing the alkali pretreatment (Rodriguez, Matulewicz, Noseda, Ducatti, & Leonardi, 2009). A simple subjecting of temperate red seaweed *C. crispus* to dark for 10 days enhanced the gel quality of carrageenan (Villanueva, Hilliou, & Sousa-Pinto, 2009).

Nevertheless, there is no evidence for the sulfohydrolase assisted desulfation of agar in the literature. In view of this, we have for the first time attempted to purify and characterize the sulfohydrolase from the agarophytic red alga *Gracilaria dura* (J. Agardh) C.

* Corresponding author. Tel.: +91 278 256 5801/3805x614; fax: +91 278 256 6970/7562.
E-mail address: crk@csmcri.org (C.R.K. Reddy).

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Agardh. The purified enzyme was subsequently used for evaluating its abilities in improvement of agar quality based on 3–6 anhydrogalactose content, desulfation, gelling and melting temperature, gel strength and viscosity and these values were compared with that of untreated agar as control. The implications of our findings are discussed in the context of desulfation and the improvement of gel quality and yield for the commercial utilization of these sulfohydrolases in agar producing industries. We suggest that sulfohydrolase of *G. dura* allow agars to attain the conformation of agarose and thus could be used as an alternate to alkali treatment.

2. Experimental

2.1. Materials

G. dura was collected from Veraval coast (20°54′N, 70°22′E) of Gujarat, India. The healthy thalli were carried in a cool pack to the laboratory under cool conditions. In order to make unialgal material, the rhizoidal portions of the alga were removed to eliminate contaminants and were then cleaned manually with brush in filtered autoclaved seawater to remove the epiphytic foreign matters, freeze dried in liquid nitrogen and kept at −40 °C for further use. Agar sample was purchased from Hi-Media (Product Number RM666, Hi-Media Laboratories Pvt. Ltd, Mumbai, India), para-nitrophenol and para-nitrophenylsulfate (p-NPS) which are used as artificial substrates for sulfohydrolase were purchased from Sigma–Aldrich (St. Louis, Missouri, USA). Ultra pure water used for experiment was prepared in laboratory from a Milli-Q system (Millipore, USA).

2.2. Enzyme extraction and purification

Seaweed extract for determination of sulfohydrolase activity was prepared under ice-cold conditions in the extraction buffer [100 mM Tris–HCl (pH 9.5), 500 mM KCl and 10 mM β-mercaptoethanol] at a proportion of 1:3 (w/v) by stirring the suspension overnight at 4 °C. Extract was centrifuged at 12,000 × *g* for 30 min at 4 °C and proteins in the supernatant were precipitated between 30–70% ammonium sulfate saturation. Precipitate was collected after centrifugation at 15,500 × *g* for 60 min at 4 °C and dissolved in the buffer containing 100 mM Tris–HCl (pH 7.1) and 10 mM β-mercaptoethanol and dialysed against same buffer for 24 h with the change of buffer at an interval of 4 h. The dialysed enzyme solution was loaded on Sephadex G-50 gel filtration chromatographic column equilibrated and eluted with the buffer containing 100 mM Tris–HCl (pH 7.1) and 10 mM β-mercaptoethanol. The active enzyme fractions were pooled and again dialysed. The desalted enzyme solution was further purified with DEAE-sepharose column previously equilibrated with aforementioned buffer and washed with the same buffer at a flow rate of 1 ml min^{-1} untill effluent A_{280} was negligible. The enzyme solution was eluted with increasing gradient of NaCl in aforesaid buffer and the active fractions were pooled between 300 mM to 500 mM NaCl gradient and used for further experiment. At each step of purification, the active fractions were analyzed by SDS–PAGE (Laemmli, 1970) using 10% polyacrylamide gel. Molecular weight markers of 29–205 kDa (Genei, Bangalore) were also run simultaneously at l20 V and proteins were quantified by Bradford method using bovine serum albumin as a standard (Bradford, 1976).

2.3. Sulfohydrolase activity assay and determination of kinetic parameters

Sulfohydrolase activity was determined by measuring the amount of p-nitrophenol released from p-NPS. The assay mixture containing diluted enzyme, 100 mM Tris–HCl (pH 8.0) and 25 mM p-NPS was incubated for 30 min at 35 °C in water bath. The reaction was terminated by the addition of 0.2 ml of 0.2 N NaOH and the product of the reaction p-nitrophenol was quantified spectrophotometrically by recording at A_{410} nm. One unit of the sulfohydrolase activity is defined as the amount of enzyme causing transformation of 1 μmol of substrate per minute at optimal condition of temperature and pH. For the optimization of enzyme concentration with agar as a substrate sulfohydrolase activity was determined according to Kim et al. (2004).

To obtain kinetics parameters sulfohydrolase activity was measured at various concentrations ranging from 2 to 20 mM of p-NPS. The enzyme reaction was initiated by mixing aliquot of sulfohydrolase solution with the assay mixture containing 100 mM Tris–HCl (pH 8.0) and indicated amount of substrate. Apparent K_m and V_{max} were determined using a Lineweaver–Burk plot.

2.4. Effect of temperature, pH and additives on enzyme activity

Optimal concentration of substrate was observed as 16 mM and thus fixed the same for the determination of reaction rate under different temperatures or pH conditions. Buffer solutions for the determination of pH dependence of enzyme activity were prepared as follows: 0.1 M acetic acid/0.1 M sodium acetate (pH 3–6.0), 0.1 M Tris–HCl (pH 6.0–9.0), and 0.1 M glycine–NaOH (pH 9.0–12.0). To eliminate any possibility of an influence of buffer species, enzyme activity was measured in different buffers with overlapping pH points. The optimum temperature for the purified sulfohydrolase activity were measured at pH 8.0 over a temperature range of 25–65 °C and finally the enzyme activity was measured at pH 8.0 and temperature 35 °C at which activity was observed maximum. Different metal ion and organic solvents were studied to examine their effect on the enzymatic activity.

2.5. Desulfation of agar

Agar suspended in 100 mM Tris–HCl (pH 8.0) buffer at a proportion of 1:20 (w/v) was equilibrated for 30 min at 45 °C by stirring. Sulfohydrolase enzyme (∼50 U) was added in the agar mixture and kept at 35 °C for 12 h. The agar precipitate after centrifugation was washed with 50% ethanol solution and dehydrated with acetone. The dehydrated agar sample was hydrolyzed (Wolnik, 1988) and the amount of sulfate content in sample was determined by inductively coupled plasma atomic emission spectroscopy (ICP-AES, PerkinElmer, Optima 2000, USA). The 3,6-anhydrogalactose content were determined by the method of Yaphe and Arsenault (1965).

2.6. Determination of gel strength, gelling and melting temperature and viscosity

The desulfated agar was rinsed with five volume of 50% ethanol and dehydrated with two volume of acetone. The dehydrated agar was dried at 80 °C for 2 days and stored at room temperature for further analysis. For the determination of gel strength 1.5% solution of agar was prepared in milliQ water and kept at 10 °C for 12 h and gel strength (g/cm^2 at 20 °C) was measured using Nikkaksui-type gel tester (Kiya Seisakusho, Ltd, Tokyo, Japan). The measurement was performed on 1.5% (w/v) gel sample aged overnight at 4 °C, using a cylindrical plunger of 1 cm in diameter. Gel strength was measured as the required weight to break the gel. The gelling temperature was measured by cooling a 1.5% (w/v) hot agar solution (25 ml) placed in a glass test tube. Subsequently, temperature was allowed to gradually drop and this drop in temperature was monitored every 1 min time interval. The temperature at which a clear depression was formed after removing the digital thermometer was recorded as gelling temperature. The melting temperature was determined

Table 1
Purification yields of sulfohydrolase.

Purification step	Protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Yield (%)	Purity
Crude	116.31	282.03	2.43	100	1
$(NH_4)_2SO_4$	69.5	2651.1	38.14	59.76	15.73
Sephadex G-50	15.3	1184.53	77.41	13.16	31.92
DEAE-sepharose	4.72	987.11	209.2	4.1	86.28

by heating the 1.5% (w/v) agar gel with a temperature increase of about 0.5 °C min^{-1} recording the temperature at which a glass bead (15.78 mm diameter, 4.93 g) placed on top of the gel sank (Murano et al., 1992). Apparent viscosity was measured (in 1.5% agar solution) on a Brookfield DV-II + Pro viscometer with spindle number SC-18 (Synchrolectric Viscometer, Stoughton, MASS 02072), using Spindle No. 1 at the speed of 60 rpm at 80 °C.

2.7. Preparation of xerogels of agar and desulfated agar

Phase inversion of the respective agar hydro gel samples was done by immersing the respective samples in acetone for 48 h followed by de-solvation under vacuum for 24 h. The xerogels thus obtained were subjected for the SEM analyses. For recording SEM image, vacuum dried samples were mounted on a sample holder, coated with gold and the micrographs were recorded on a LEO scanning electron microscope-LEO 1430VP (Carl-Zeiss, Germany) at an accelerating voltage of 20 kV and 202× magnification.

2.8. FT-IR spectroscopy

The native agar and enzyme treated agar was characterized by FT-IR analysis using a PerkinElmer FT-IR (PerkinElmer Spectrum GX FT-IR System, USA) by using 10.0 mg of sample in 600 mg of KBr. All spectra were the average of two counts with 10 scans each.

3. Results

3.1. Purification of sulfohyrolase

The purification steps and their results are summarized in Table 1. The sulfohydrolase from *G. dura* was purified following a combination of ammonium sulfate precipitation, ion exchange and gel filtration chromatography. The purification steps resulted in obtaining an enzyme with a purification fold of 86 times and a yield of 4.1%. The specific activity of purified enzyme was 209.19 U/mg protein at pH 8.0 and 35 °C. The purified sulfohydrolase appeared as a single band in SDS–PAGE (Fig. 1) indicating that the enzyme was purified to apparent homogeneity. The molecular weight of the purified sulfohydrolase was estimated to be ~50 kDa, based on its mobility calculated with the help of standard protein marker. The results of SDS–PAGE revealed a monomeric protein to be the active conformation of the enzyme.

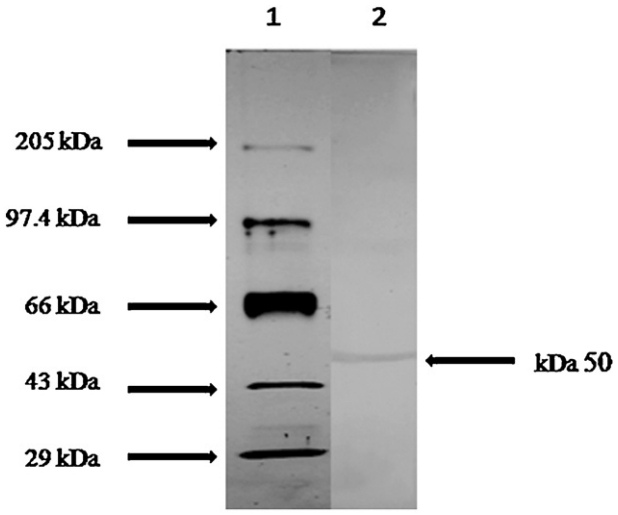

Fig. 1. SDS–poly acrylamide gel electrophoresis (SDS–PAGE) of purified sulfohyrolase. Lane 1: Protein marker with molecular weight ranging from 29 to 205 kDa (29 kDa, carbonic anhydrase; 43 kDa, ovalbumin; 66 kDa, bovin serum albumin; 97.4 kDa phosphorylase b and 205 kDa, myosin from rabit muscle). Lane 2: Sulfohydrolase after DEAE-sepharose chromatography.

The pH and temperature dependence of the purified enzyme is shown in Fig. 2. The enzyme activity for hydrolysis of sulfate ester bonds in p-NPS (16 mM) was determined by analyzing the content of sulfate release in the assay mixture. For hydrolysis of p-NPS the optimum pH was 8.0, though the enzyme showed higher activities from pH 6.0–8.0. The pH dependence varied significantly from acidic (pH 3.0–6.0) to alkaline (pH 9.0–12.0) ranges and were relatively higher in the acidic range. The average relative activity at acidic pH (<6.0) was 63.27% while at alkaline pH (>9.0) was 34.37% but the maximum being at pH 7.0–9.0 with 89.23%.

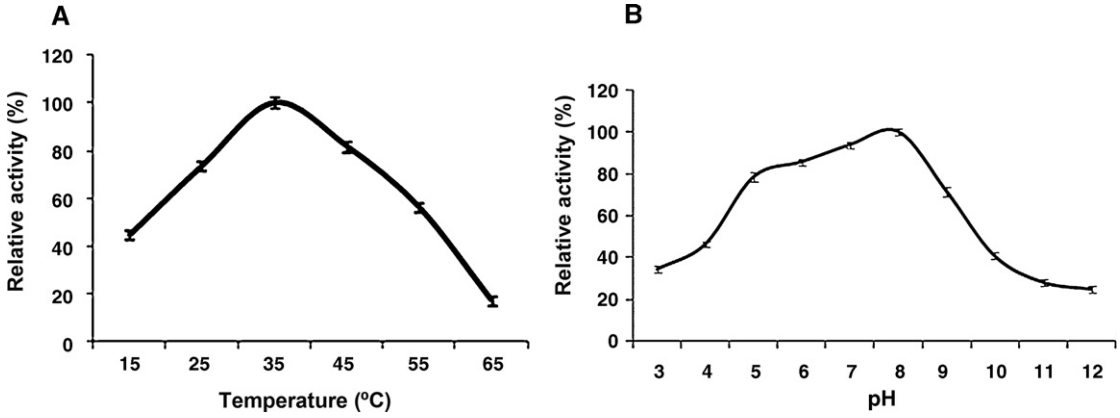

Fig. 2. Effect of (A) temperature and (B) pH on sulfohydrolase activity using p-NPS (16 mM) as a substrate.

Table 2
Effect of additives on the enzyme activity.

Additive	Residual activity
Control	100
Cu	78.71 ± 3.8
Ca	121.3 ± 3.8
Mg	139.8 ± 3.7
Na	106.86 ± 4.7
K	100.58 ± 3.15
Hg	ND
Pb	ND
PMSF	ND
$CHCl_3$	102 ± 3.58
n-Hexane	90.67 ± 2.72
Toluene	63.33 ± 2.7
Cyclohexane	44 ± 3.77
EDTA	59.7 ± 3.14

ND—not detected.

The enzyme activity against p-NPS was measured at different temperatures at pH 8.0 (Fig. 2). The enzyme when stored at 4 °C for 7–14 days did not show any loss of activity and thus, was quite stable. Activity gradually increased in the temperature range from 25 to 45 °C but optimum at 35 °C. The enzyme activity reduced to almost half of the optimum at 15 °C and 55 °C. The enzyme activity was completely absent at temperature ≥65 °C probably due to the thermal denaturation of enzyme. K_m and V_{max} of the enzyme was determined by linear regression, plotting the inverse enzyme activity against the inverse of substrate concentration. The apparent K_m and V_{max} were estimated to be 10 mM and 20 mM min^{-1} respectively at pH 8.0 and 35 °C.

The effect of additives on sulfohydrolase activity was examined in the presence of various metal ions, chelators (5 mM) and organic solvents (5%) and is shown in Table 2. Among the metals ions Ca^{2+} and Mg^{2+} ions enhanced the enzymatic activity by 21% and 40% respectively, while Cu^{2+} reduced the activity by 21% from the optimum values. The metal ions Hg^{2+} and Pb^{2+} completely inhibited the activity while no appreciable change in the activity was observed in the presence of Na^+ and K^+. The serine protease inhibitor (PMSF) and metal chelator EDTA significantly reduced the enzyme activity by 100% and 40% respectively. Most of the organic solvents used in this study inhibited the enzyme activity by 10–55%, however, the presence of $CHCl_3$ did not affect the enzyme activity.

3.2. Desulfation and physical properties of agar

The purified sulfohydrolase was allowed to react with agar sample under optimum experimental conditions. A series of enzyme concentrations (10, 25, 50 and 100 U) were used to optimize the enzyme concentration for agar desulfation. A gradual increase in sulfate release and 3, 6-AG together with the increase in viscosity of agar solution was observed when subjected to 10–50 U of enzyme while no appreciable change in these parameters observed when enzyme concentration was >50 U (Table 3). Therefore, the concentration of 50 U was chosen as optimum for subsequent experiment. A recovery of almost 90% was observed in the agar subjected to enzymatic treatment. The physical properties like gel strength, gelling and melting temperature and viscosity improved significantly in the enzyme treated agar (Table 4).

Table 3
Optimization of enzyme concentration for the removal of sulfate from agar.

Enzyme (U)	Sulfate (%)	3,6-AG (w/w) (%)
0	2.82 ± 0.19	18 ± .5
10	2.51 ± 0.096	19 ± 1.0
25	2.05 ± 0.05	23 ± 1
50	1.11 ± 0.046	30 ± 2
100	1.1 ± 0.076	30 ± 1.5

The ICP-AES findings further showed that sulfate content decreased remarkably from 2.81% (control) to 1.11% (enzyme treated agar) that corresponded to reduction of about 60% (Table 4). The release of sulfate was positively correlated with the amount of 3,6-AG units that increased from 18% (control) to 30% (enzyme treated agar). The capability of sulfohydrolase to increase the viscosity of agar solution was also measured and estimated to increase by two-fold with values 9.67 cp in (control) and 18 cp (enzyme treated agar). The sulfohydrolase treatment resulted in a noticeable decrease in gelling and melting temperature with 31 °C and 82 °C respectively and was quite low as compared with that of control agar with 38 °C and 90 °C. The enzymatic desulfation improved the gel strength as it enhanced its value from 190 g/cm^2 in control to 470 g/cm^2 in enzyme treated agar and contributed to almost 2.5-fold increase.

3.3. Scanning electron microscopy

The scanning electron micrographs (SEM) of the xerogels obtained for control agar and enzyme treated agar showed different types of network morphologies (Fig. 3). The xerogels of control agar showed fibrous structures of almost 215–650 nm width (Fig. 3A). The enzyme treated agar xerogel exhibited much blunt structure persisted with helixes of approximately 2500–3000 nm in width together with strong cross linking (Fig. 3B).

3.4. FT-IR spectra

The quantitative FT-IR spectra of both control agar and enzyme treated agar are shown in Fig. 4. The area of the band at 1250 cm^{-1} which represents the total sulfate content get reduced in the enzyme treated sample while the band in the range of 850–868 cm^{-1} was completely absent in the enzyme treated sample with a significant increase in the band at 930 cm^{-1}.

4. Discussion

The enzyme sulfohydrolase which catalyzes the formation of 3,6-AG ring from L-Gal-6-sulfate by removing sulfate ester moiety in porphyran has been first reported by Rees (1961a,b). This has opened new opportunities for researchers to improve the gel strength of sulfated galactans phenomenally. Subsequently, a few studies describing the conversion of μ-carrageenan into κ-carrageenan using protein fraction from *C. crispus* (Wong & Craigie, 1978) *Gigartina stellata* (Lawson & Rees, 1970) and ν-carrageenan to ι-carrageenan with *Calliblepharis jubata* extracts

Table 4
Comparison of native and enzyme treated agar.

Agar type	Gel strength (g cm^{-2})	Gelling temperature (°C)	Melting temperature (°C)	Sulfate (%)	Viscosity (cp)	ΔSulfate[a] (%)	3,6-AG content (%)
Commercial agar	190.0 ± 20.0	39 ± 1.5	90.67 ± 3.1	2.8 ± 0.2	9.7 ± 1.5	60.59	18 ± 0.9
Enzyme treated agar	486.7 ± 15.3	31 ± 1.0	82.67 ± 2.5	1.1 ± 0.1	18.0 ± 2.0		30 ± 0.98

[a] ΔSulfate (%): decrease in sulfate content.

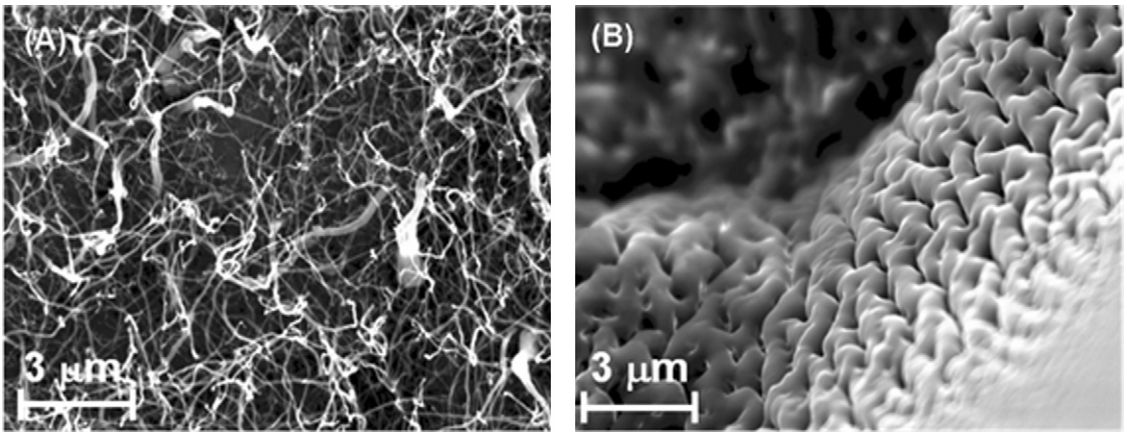

Fig. 3. Scanning electron microscopy (SEM) images of xerogel of (A) native agar (B) sulfohydrolase treated agar.

(Zinoun, Diouris, Potin, Floc'h, & Deslandes, 1997) have been reported. Later, Genicot-Joncour et al. (2010) demonstrated the potential conversion of ν-carrageen into ι-carrageen with the purified sulfurylase I and II from *C. crispus*. The present study for the first time reports the purification of sulfohydrolase from agarophyte *G. dura* and its potential application in improvement of commercial agar quality substantially. The purified enzyme had the ability to catalyze the desulfation and consequent formation of 3,6-AG. Our study clearly demonstrates that this desulfation event is reliant on the enzymatic reaction that is carried out by a single protein. Thus, compatible with the nucleophilic substitution reactions proposed for the formation of anhydro ring in alkaline solutions (Cianca, Matulewicz, & Cerezo, 1997; Viana, Noseda, Duarte, & Cerezo, 2004).

In the present study we achieved about 60% sulfate removal from the agar subjected to enzymatic treatment together with 1.66-fold increase in the content of 3,6-AG which was primarily responsible for the increased gel strength. Also, the improved gel strength and other physical properties such as gelling and melting temperature with values 31 °C and 82 °C respectively, signify the potentiality of the enzyme extracted from *G. dura*. The enzymatic treatment of agar meets the acceptable ranges of gelling and melting temperature for commercially available agaroses and thus could be an alternative for alkali treatment which has been reported to time dependent in *Gracilariopsis lemaneiformis* for obtaining the gelling and melting temperature with 81 °C and 32 °C respectively

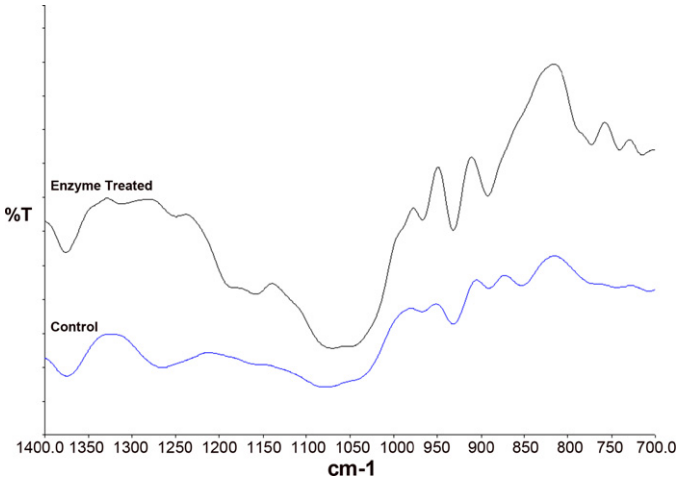

Fig. 4. FT-IR spectra of control agar and sulfohydrolase treated agar.

(Gonzalez-Leija et al., 2009). The findings observed in the present study are quite comparable with the results obtained for alkali treatment that results in 70% sulfate removal and 1.15-fold increase in 3, 6-AG content. In contrast, no effect of alkali treatment on desulfation of agar and consequently on the concentration of 3, 6-AG has been reported in *G. cervicornis* (Freile-Pelegrin & Murano, 2005). In another study, approximately 90% sulfate removal with a considerable increase in 3,6-AG content (1.5–2.8-fold) and gel strength (5–9-fold) have also been reported using either alkali treatment alone or in combination with temperature (Gonzalez-Leija et al., 2009; Meena, Prasad, Ganeshan, & Siddhanta, 2008). The alkali based methods have been questioned for the environmental concerns as it generates the toxic effluent if emancipate untreated. Also, such chemical treatments results in diminished polysaccharide yield. A practical alternative to these alkali or other chemical based methods have been proposed recently with the use of dark treatment (Villanueva, Hilliou, & Sousa-Pinto, 2009) with an advantage of higher polysaccharide yield. Further, the effect of irradiation with decreased sulfate content from 1.93 to 1.68% combined with increased agarose content from 12.13 to 19.72% on dry weight basis has been well documented (Hong, Hien, & Son, 2007).

The sulfohydrolase purified in the present study showed the higher activity at pH range 6.0–9.0 with an optimum being at pH 8 and 35 °C. Our results are in agreement with the earlier report (Rees, 1961a) from the *Porphyra umbilicalis* where the optimum activity of enzyme being at pH 7.6–7.8. Similarly, the two enzymes namely sulfurylase I (MW, 65 kDa) and II (MW, 32 kDa) from *C. crispus* with optimum pH 8.0–9.0 for the former and 7–8 for the latter has also been purified (Genicot-Joncour et al., 2010). On the contrary, the enzyme from *C. crispus* with ability to convert μ-carrageenan into the κ-carrageenan has exhibited the maximum activity at pH 6.5 and 40 °C (Wong & Craigie, 1978). Recently, sulfatase purified from *Sphingomonas* (Kim et al., 2004) has been employed for improving the agar quality substantially.

The effect of additives particularly EDTA, PMSF, Ca^{2+} and Mg^{2+} were of great interest as both EDTA and PMSF inhibit the enzyme activity while divalent cations such as Ca^{2+} and Mg^{2+} enhanced the activity of the enzyme. Inhibitory action of the PMSF suggests the existence of serine residue at the active site. In addition, EDTA induced enzyme inhibition indicates that divalent cations may play a crucial role during catalytic process. The divalent ions (Ca^{2+} and Mg^{2+}) presumably stabilize the negative charges developed in the sulfate during the nucleophilic attack by the hydroxyl group of serine.

Gelation is preceded by disordered (or less ordered)–ordered transition of the macromolecular conformation and many experimental data support the dimeric structure of agarose in the ordered

state which interpret a double helix structure of agarose (intertwined strand) (Anderson, Campbell, Harding, Rees, & Samuel, 1969; Rees, Morris, Thom, & Madden, 1982; Viebke, Piculell, & Nilsson, 1994) or a duplex of single helices (Bongaerts, Reynaers, Zanetti, & Paoletti, 1999; Cuppo, Reynaers, & Paoletti, 2002; Kim et al., 2004; Rochas & Rinaudo, 1984). In the present study, the SEM image signify the desulfation of agar by the enzyme treatment resulted in a morphologically distinct agar product with much stronger network of ordered helical structure when compared with the control agar confirming our observation of increased viscosity and gel strength of treated agar. After sulfohydrolase reaction the helical structure of agar crosslinked with each other to give the formation of thick helix. Both the control and enzyme treated samples showed characteristic bands of agar as reported earlier (Rosangela, Rosangela, & Marguerite, 2000). The presence of a band in the range of 850–868 cm^{-1} which is due to C-4 sulfate and C-6 sulfate in the control while the absence of this band in enzyme treated sample together with a significant increase for the band at 930 cm^{-1} indicate the complete formation of 3,6-AG. These results are in consistent with ICP results discussed above thus support the decreased sulfate and increased 3,6-AG content in the enzyme treated agar samples which in turn increased the gel strength.

So far, the methods for agarose preparation have focused mainly on the reduction of sulfate content using either fractionation or selective adsorption (Guiseley, Kirpatrick, Provonchee, Dumais, & Nochumson, 1993; Izumi, 1970). Although, both these methods successfully reduce the sulfate content but significantly lower the yield of agarose by 3–4-fold (Duckworth & Yaphe, 1971; Guiseley, Kirpatrick, Provonchee, Dumais, & Nochumson, 1993; Izumi, 1970). In this respect, sulfohydrolase could be healthy and ideal candidate for the removal of sulfate groups in agar together with high recovery of agarose.

5. Conclusions

In conclusion the present study demonstrates that the sulfohydrolase purified from the red alga *G. dura* has ability to improve the quality of agar significantly. The agar treated with enzyme exhibited improved gel strength, gelling and melting temperature, and viscosity. In addition, enzymatic treatment resulted in the conformational changes with the formation of strong cross linked helixes and removal of sulfate moiety from the control agar confirming the formation of 3,6-AG. Also, the obtained high recovery of agarose yield after the enzymatic treatment could be an alternative for the commonly used alkaline treatment for sulfate removal and improving the gelling properties. There is a considerable scope for cloning the gene(s) encoding sulfohydrolase for commercial production of this enzyme for preparing high quality agar from seaweed resources.

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