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Depolymerization of fucosylated chondroitin sulfate from sea cucumber, *Pearsonothuria graeffei*, via ^{60}Co irradiation

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ABSTRACT

A method for depolymerization of a novel fucosylated chondroitin sulfate from *Pearsonothuria graeffei* (fCS-*Pg*) using ^{60}Co irradiation in water solution was developed in the current study. Fragments with varying molecular weights were obtained by ^{60}Co irradiation at different dosages and sample concentrations. The chemical compositions and structures of these fragments were further investigated using high-performance liquid chromatography (HPLC), infrared spectroscopy (IR) and nuclear magnetic resonance spectroscopy (NMR). Our results indicated that ^{60}Co irradiation induced depolymerization via selective breakage of glucuronic acid units in the fCS-*Pg* backbone, with no obvious influence on sulfated fucose branches under mild conditions. The recommended conditions for fCS-*Pg* degradation were 2–10% solution concentration and irradiation dosages of 10–50 kGy. The anticoagulant activities of the low molecular weight fragments were additionally evaluated. Notably, anticoagulant activities were reduced with decreasing molecular weights. Compared to the native fCS-*Pg*, low molecular weight fragments displayed significantly decreased anticoagulant activities. Based on the collective findings, we propose that these fragments are potentially applicable as antithrombotic agents with reduced bleeding risk relative to native fCS-*Pg*.

1. Introduction

Fucosylated chondroitin sulfate (fCS) is a glycosaminoglycan from sea cucumber, composed of alternating β-D-glucuronic acid and N-acetyl-β-D-galactosamine units (Mourão et al., 1996). The β-D-glucuronic acid residues contain sulfated fucose branches, which have distinguishable patterns and proportions of sulfate substitution in different sea cucumber types. Recently, we isolated a novel fCS from sea cucumber, *Pearsonothuria graeffei*, with a distinct chemical composition and molecular weight from previously reported fCSs, including those from *Stichopus japonicas* (Yoshida, Minami, Nemoto, Numata, & Yamanaka, 1992) and *Ludwigothurea grisea* (Mourão et al., 1996). ^{1}H NMR experiments revealed a unique sulfation pattern of its fucose branches, mainly comprising 4-sulfated-Fuc (Chen et al., 2011). However, our preliminary structural analysis was based on a comparison of proton signals on ^{1}H NMR spectra with reference data, and required further confirmation. Data from anticoagulant and thrombotic assays supported the potential of fCS-*Pg* as a novel anticoagulant drug with prolonged activated partial thromboplastin time (APTT) and thrombin time (TT), as well as evident antithrombotic activity (Chen et al., 2011). However, a previous study on fCS from sea cucumber, *S. japonicus*, indicated that fCS induced an undesirable effect of platelet aggregation (Li & Lian, 1988). Moreover, polysaccharides with large molecular weights have been shown to cause a number of problems, such as high viscosity and low permeability into cells (Hasegawa, Isogai, & Onabe, 1993; Ilyina, Tikhonov, Albulov, & Varlamov, 2000). A low molecular weight derivative, depolymerized holothurian glycosaminoglycan (DHG), was prepared by Suzuki, Kitazato, Takamatsu, & Saito (1991), with a view to minimizing these limitations. While the pharmacological functions of fCS have been widely investigated, the development of effective methods for preparation of its low molecular weight derivatives has attracted comparatively little attention. To generate research interest in this area, we recently extended our investigations to obtain low molecular weight fragments of fCS isolated from the sea cucumber, *P. graeffei* (Chen et al., 2011).

Abbreviations: fCS-*Pg*, fucosylated chondroitin sulfate from *Pearsonothuria graeffei*; HPLC, high-performance liquid chromatography; IR, infrared spectroscopy; NMR, nuclear magnetic resonance spectroscopy; fCS, fucosylated chondroitin sulfate; APTT, activated partial thromboplastin time; TT, thrombin time; DHG, depolymerized holothurian glycosaminoglycan; PMP, 1-phenyl-3-methyl-5-pyrazolone; Fuc3,4S, 3,4-*O*-disulfated fucose; Fuc2,4S, 2,4-*O*-disulfated fucose; Fuc4S, 4-*O*-sulfated fucose; Fuc, fucose; GlcA, glucuronic acid; GalNAc, *N*-acetylgalactosamine; CSE, standard chondroitin sulfate E.

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Several methods for degradation of polysaccharides have been reported to date, including irradiation (Bertolini, Mestres, Colonna, & Raffi, 2001), ultrasonic degradation (Portenlanger & Heusinger, 1997), free radical depolymerization with or without metallic catalysts (Yang, Li, & Guan, 2004; Petit et al., 2006), acid hydrolysis (Hjerde, Stokke, Smidsrod, & Christensen, 1998; Karlsson & Singh, 1999) and enzymatic depolymerization (Cheng & Prud'homme, 2000). Among these procedures, mild acid hydrolysis and free radical depolymerization for fCS degradation have been extensively researched (Mourão et al., 1996; Wu, Xu, Zhao, Kang, & Ding, 2010; Wu et al., 2012). Common mild acid hydrolysis could easily induce partial desulfation and loss of sulfated fucose branches (Mourão et al., 1996; Wu et al., 2012), reported to play key roles in the anticoagulant, antithrombotic, anti-atherosclerosis and antitumor metabolism activities of fCS (Mourão et al., 1996; Mourão, Giumarães, Mulloy, Thomas, & Gray, 1998; Mourão et al., 2001; Borsig et al., 2007). In contrast, free radical depolymerization selectively breaks glucuronic acid units in the fCS backbone, with no impact on sulfated fucose branches. Earlier, Wu et al. (2010) reported a method for degrading fCS from sea cucumber, *Thelenata ananas*, using hydrogen peroxide with copper ions, which produced fCS fragments with no obvious loss of sulfate and fucose branches. However, the preparation procedures were complicated and inconvenient, including controlling the titration rate of hydrogen peroxide, reaction time, pH shift, and removing the chemical regents used, such as hydrogen peroxide and copper ions, from the reaction mixture, and resulted in reduced yield of low molecular weight fCSs. Furthermore, owing to the poor reproducibility of these methods, it was difficult to control the degree of degradation efficiently, leading to possible problems with quality control during the production process. Therefore, there remains an urgent need to develop efficient and simple methods for preparing low molecular weight fCSs economically and safely.

Compared with other degradation procedures, irradiation technology overcomes the limitations in obtaining large quantities of depolymerized macromolecules by controlled degradation, and promotes reproducibility and yield without the requirement for chemical reagents or special equipment (Charlesby, 1977). Furthermore, irradiation is simpler and more eco-friendly, compared to other conventional methods (Choi et al., 2009). This procedure has therefore been approved by the FAO/IEAE/WHO joint committee on the wholesomeness of irradiated food, and is practiced commercially in several countries (Lacroix & Ouattara, 2000). Irradiation, reported to promote polysaccharide depolymerization via cleavage of glycosidic bonds (Sokhey & Hanna, 1993), is currently applied for the depolymerization of simple, linear non-sulfated polysaccharides, including hyaluronic acid, sodium alginate, pectin, starch and chitosan (Kim & Srinivasan, 2008; Zaied, Youssef, Desouky, & Dien, 2007; Xu, Sun, Yang, Ding, & Pang, 2007; Dogan, Kayacier, & Ic, 2007; Liu, Ma, Xue, & Shi, 2012; Zainol, Akil, & Mastor, 2009). However, application of irradiation to marine sulfated polysaccharides has rarely been documented. In 2009, Choi et al., reported that irradiation not only decreased the molecular weight of fucoidan, but also enhanced the antioxidant potency of polysaccharides.

The present study reports the development of an effective method for depolymerization of highly sulfated fCSs from sea cucumber using irradiation. The chemical compositions and molecular sizes of the fCS-*Pg* fragments generated from irradiation were analyzed, and their structures investigated with ^{1}H NMR, with a view to establishing the mechanisms underlying irradiation-induced degradation. Furthermore, the fCS-*Pg* structure was corrected using 2D NMR analysis of the oligosaccharide mixtures generated by irradiation. Anticoagulant activities of the degraded fCS-*Pg* fragments were additionally investigated using APTT and TT assays.

2. Materials and methods

2.1. Materials

Dry sea cucumber, *P. graeffei* (Indo-Pacific), was purchased from a local market in Qingdao (China). TSK-G4000 and -G3000 PWXL columns were acquired from TOSOH Biosep (Tokyo, Japan) and DEAE-cellulose anion-exchange resin from Whatman (Brentford, England). Papain and cysteine were purchased from Fluka (Seelze, Germany). The carbohydrate standards (e.g., D-mannose, L-fucose, L-arabinose, D-galacturonic acid, lactose, and chondroitin sulfate A from bovine trachea) were from Sigma (St. Louis, MO, USA). The derivatization reagent, 1-phenyl-3-methyl-5-pyrazolone (PMP), was obtained from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China).

2.2. Isolation and purification of fCS-Pg

Crude sea cucumber polysaccharide was prepared based on a previously described method (Chen et al., 2011, 2012). Briefly, the dry sea cucumber body wall (ca. 100 g) was minced and homogenized. The homogenate was treated with chloroform/methanol (4:1, v/v) to remove lipids, and autoclaved at 50 °C. The resulting residue was digested with papain at 60 °C for 10 h in solution containing 5 mM EDTA and 5 mM cysteine, and subjected to centrifugation (2000 × *g* for 15 min at 10 °C). Polysaccharides in the clear supernatant fractions were precipitated with 160 mL of 10% cetylpyridinium chloride solution. After incubation at room temperature for 24 h, the mixture was centrifuged (2000 × *g* for 15 min). The precipitated sulfated polysaccharide was dissolved with 1000 mL of 2 M NaC1: ethanol (100:15, v/v) solution, and precipitated with 600 mL of 95% ethanol to isolate fCS-*Pg*. After incubation for 24 h at 4 °C, the precipitate formed was collected by centrifugation (2000 × *g* for 15 min), dissolved in water, and dialyzed against water for 24 h. The final lyophilized solution was used as crude fCS-*Pg*.

The crude polysaccharide (~1 g) was fractionated using anion exchange chromatography on a DEAE-cellulose column (2.6 cm × 40 cm), with elution via a linear gradient of 0–1.5 M NaCl (in 0.1 M sodium acetate, pH 5.0) in 1000 min at a flow rate of 1.0 mL/min. Carbohydrate fragments were detected with the phenol/sulfuric assay (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956).

Polysaccharide purities and molecular weights were determined using high-performance liquid chromatography (HPLC) (Waters, MA, USA) on a TSK-G4000 PWXL column by elution with 0.2 M NaCl and detection via refractive index. The molecular weight of fCS-*Pg* was determined as 73.2 kDa, consistent with previous findings by our group (Chen et al., 2011).

2.3. Radiation degradation of fCS

fCS-*Pg* (50 mg) was dissolved in 5 mL of water, and fCS-*Pg* solutions (1%, w/w) attained. The fCS-*Pg* solutions were irradiated in the atmosphere with γ-rays from a ^{60}Co source at dosages of 10, 20, 50 and 100 kGy. Irradiation was carried out at ambient temperature with a dosage rate of 10 kGy/h. The experiment was performed to examine the effect of the irradiation dosage on fCS-*Pg* degradation.

Various concentrations (50, 100, 250 and 500 mg) of fCS-*Pg* were dissolved in 5 mL of water, respectively, and fCS-*Pg* solutions with different mass concentrations (1%, 2%, 5% and 10%) attained, respectively. The fCS-*Pg* solutions were irradiated in the atmosphere with γ-rays from a ^{60}Co source at a dosage of 50 kGy, with a view to establishing the effects of the sample concentrations on fCS-*Pg* degradation. Irradiation was additionally performed at ambient temperature with a dosage rate of 10 kGy/h.

After irradiation, polysaccharide solutions were centrifuged (7000 × *g* for 5 min) and clear supernatants lyophilized, prior to analysis. Changes in the molecular weights of degraded fCS-*Pg* samples were monitored via HPLC (Waters, MA, USA) using a TSK-G3000 PWXL column, and changes in chemical composition determined.

2.4. Estimation of average molecular weights

HPLC was performed using TSK-G4000 and -G3000 PWXL columns at a sample injection volume of 20 μL (1 mg/mL) and flow rate of 0.5 mL/min on a Waters 2870 system (MA, USA), with a 2414 refractive index detector. The mobile phase consisted of 0.2 M NaCl. The column was maintained at 40 °C. GPC chromatograms were recorded on a computer with LC solution version 1.25 software, and analyzed with a "GPC Postrun" function.

2.5. Determination of chemical compositions

Monosaccharide compositions were determined using the PMP-HPLC method, as described previously (Strydom, 1994). In brief, native fCS-*Pg* and degraded oligosaccharide samples (typically, 2 mg) were hydrolyzed with 2 M TFA at 110 °C for 8 h. Hydrolysate was carried out with 450 μL PMP solution (0.5 M in methanol) and 450 μL of 0.3 M NaOH at 70 °C for 30 min. The reaction was terminated by neutralization with 450 μL HCl, followed by extraction with chloroform (1 mL, 3×). HPLC analyses were performed on an Agilent ZORBAX Eclipse XDB-C18 column (Agilent, USA, 5 μm, 4.6 mm × 150 mm) at 25 °C with UV detection at 250 nm. The mobile phase was aqueous 0.05 M KH_2PO_4 (pH 6.9) with 15% (solvent A) and 40% (solvent B) acetonitrile, respectively. A gradient of B (8–19%) within a time-period of 25 min was applied.

Sulfate content was determined with ion chromatography, as described earlier (Ohira & Toda, 2006). Briefly, ~1 mg of fCS-*Pg* or low molecular weight fCS fragments were hydrolyzed with 2 M TFA at 110 °C under nitrogen for 8 h. The hydrolysate was dried under vacuum before dissolving in water, prior to ion chromatography.

2.6. IR and NMR spectroscopy

IR-measurements were performed with a Perkin-Elmer instrument (Thermo Nicolet Corporation, Madison, USA). IR spectra are usually registered in the middle infrared (4000–400 cm^{-1}) with a resolution of 4 cm^{-1} in the absorbance mode for 8–128 scans at room temperature. The samples for IR analysis are prepared by grinding the dry blended powders with powdered KBr, often in the ratio of 1:5 (Sample: KBr) and then compressed to form discs (Leal, Matsuhiro, Rossi, & Caruso, 2008).

For NMR analysis, fCS-*Pg* fragments (30 mg) were co-evaporated with 500 μL D_2O (99.8%) twice via lyophilization before final dissolution in 500 μL high-quality D_2O (99.96%) containing 0.1 μL acetone. ^1H NMR experiments were performed at room temperature on a Bruker AVANCE III 500 spectrometer. The observed ^1H chemical shifts were reported relative to internal acetone standard (2.03 ppm). 2D NMR experiments were conducted on a Bruker AVANCE III 600 spectrometer. All two-dimensional spectra were acquired using a pulse-field gradient incorporated into NMR pulse sequences. ^1H, ^{13}C, homonuclear $^1H/^1H$ correlation experiments (COSY, TOCSY), nuclear overhauser effect spectroscopy (NOESY), and heteronuclear single quantum coherence (HSQC) experiments were conducted at room temperature. The number of scans (ns) in each experiment was dependent on the sample concentration.

2.7. Anticoagulant assay

Human blood was donated by a local young, healthy male aged 24 years, and the investigation performed according to the "Guidance for the Use of Human Blood" published by the Shandong Provincial Government. Human blood was added into a solution of 3.8% sodium citrate. Plasma was separated by centrifugation at 3000 rpm for 10 min. Anticoagulant assays, including APTT (assay kit from Organon-Tecknica, Fresnes, France) and TT (5 NIH U/mL human thrombin, from Diagnostica Stago, AsnieAres, France), were performed according to the procedures recommended by the manufacturer. Results were expressed in international units/mg using a parallel standard curve based on the International Heparin Standard (150 units/mg).

2.8. Statistical analysis

All the data were statistically evaluated with SPSS/13.00 software. $p < 0.05$ and $p < 0.01$ were considered to indicate statistical significance. All the results were expressed as mean ± SE.

3. Results and discussion

3.1. Effect of ^{60}Co irradiation on the fCS-Pg chain size

In order to develop a more efficient and controllable method for preparation of low molecular weight fCSs, we examined the effects of irradiation dosage and solution concentration during irradiation on the molecular weight of fCS-*Pg*. Data are presented in Table 1 and Fig. 1.

Compared to the native fCS-*Pg* (73.2 kDa), the average molecular weight of ^{60}Co irradiated fCS-*Pg* samples was considerably decreased. Specifically, the average molecular weights of samples (1%, w/w) irradiated with 10 kGy (DfCS-1), 20 kGy (DfCS-2), 50 kGy (DfCS-3) and 100 kGy (DfCS-4) were 5.6, 4.4, 3.7 and 2.5 kDa, respectively (Fig. 1A). The extent of degradation was enhanced with increasing irradiation dosages, but this relationship was not linearly proportional. All fragments generated at irradiation dosages of below 50 kGy showed good homogeneity, with a molecular weight distribution index between 1.1 and 1.4. However, at the highest irradiation dosage (100 kGy), fragment homogeneity was significantly decreased, possibly owing to destruction of the polysaccharide backbone. Previous reports on sulfated

Table 1
Composition analysis of the native fCS-*Pg* and its low molecular weight fragments prepared by ^{60}Co irradiation.

Sample	MW(kDa)	Molar ratio*			
		GlcA	GalNAc	Fuc	Sulfate
fCS-*Pg*	73.2 ± 7.9[a]	1.3	1.0	1.7	3.3
DfCS-1**	5.6 ± 0.7[b]	0.5	1.0	1.5	3.4
DfCS-2	4.4 ± 0.5[c]	0.5	1.0	1.4	3.6
DfCS-3	3.7 ± 0.4[d]	0.2	1.0	1.3	3.9
DfCS-4	2.5 ± 0.2[e]	0.1	1.0	0.9	4.2
DfCS-1′	3.7 ± 0.4[f]	0.2	1.0	1.3	3.9
DfCS-2′	4.0 ± 0.4[g]	0.3	1.0	1.5	3.8
DfCS-3′	6.2 ± 0.8[h]	0.6	1.0	1.7	3.6
DfCS-4′	7.6 ± 0.9[i]	0.9	1.0	1.7	3.4

Means with different superscripts (a–e) in columns differ significantly ($p < 0.01$).
Means with different superscripts (a, f–i) in columns differ significantly ($p < 0.01$).

* The molar ratio of sugar and sulfate were compared by defining the GalNAc content as 1 molar.

** DfCS-1, DfCS-2, DfCS-3 and DfCS-4 were prepared by ^{60}Co irradiation degradation of the fCS-*Pg* solution (1%) at dosages of 10, 20, 50 and 100 kGy; whereas DfCS-1′, DfCS-2′, DfCS-3′ and DfCS-4′ were prepared by ^{60}Co irradiation degradation of the fCS-*Pg* solution with concentrations of 1%, 2%, 5% and 10% at the degradation dosage of 50 kGy.

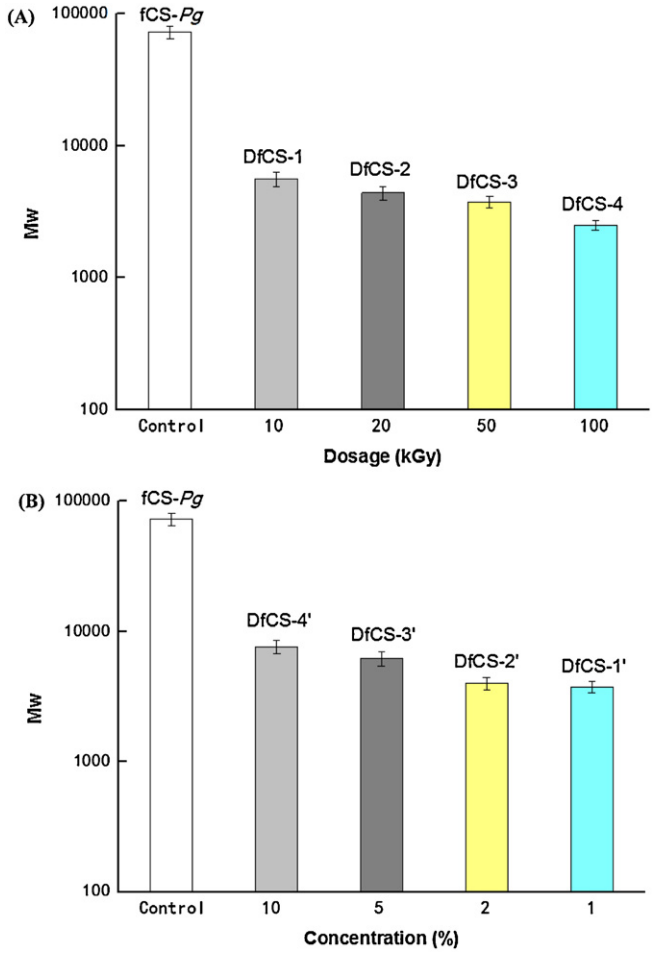

Fig. 1. Effect of ^{60}Co irradiation on the average molecular weight changes of fCS-*Pg*. (A) DfCS-1, DfCS-2, DfCS-3 and DfCS-4 were prepared by ^{60}Co irradiation degradation of the fCS-*Pg* solution (1%) at dosages of 10, 20, 50 and 100 kGy; (B) DfCS-1′, DfCS-2′, DfCS-3′ and DfCS-4′ were prepared by ^{60}Co irradiation degradation of the fCS-*Pg* solution with concentrations of 1%, 2%, 5% and 10% at the dosage of 50 kGy.

polysaccharides, such as carrageenans, have indicated that high irradiation dosages lead to a release of sulfates (Karlsson & Singh, 1999; Abad et al., 2009). Wu et al. (2012) additionally reported that with reducing molecular weights, the molecular weight distribution of polysaccharides was slightly increased, inducing poor homogeneity. Therefore, in the current study, we selected 50 kGy as the optimal dosage, at which degraded fCS-*Pg* fragments showed good homogeneity, to further establish the effects of sample concentrations on irradiation-induced breakage of fCS-*Pg*.

The effects of fCS-*Pg* concentration on the molecular weight distribution of irradiated fCS-*Pg* fragments are presented in Fig. 1B. The fCS-*Pg* fragments prepared displayed an evident decrease in molecular weight with decreasing solution concentrations. Specifically, average molecular weights of samples (irradiated at a 50 kGy dosage) at concentrations of 1% (DfCS-1′), 2% (DfCS-2′), 5% (DfCS-3′) and 10% (DfCS-4′) were approximately 3.7, 4.0, 6.2 and 7.6 kDa, respectively. The results indicate that the extent of degradation is enhanced with decreasing polysaccharide concentrations, but the relationship is not linearly proportional.

In relation to DfCS-1′ with the poorest homogeneity, other fragments (DfCS-2′, DfCS-3′ and DfCS-4′) with molecular weight distribution indices between 1.1 and 1.3 displayed good homogeneity. Accordingly, we propose that irradiation of fCS-*Pg* should be performed at a dosage of 50 kGy and sample concentration of above 2%.

3.2. Effect of ^{60}Co irradiation and sample concentration on chemical composition

In addition to the impact of the molecular size of polysaccharides on activity, monosaccharide composition, sulfate content and pattern should be taken into consideration (Shanmugam & Mody, 2000). Monosaccharide composition analysis of fCS-*Pg* and its low molecular weight fragments was performed using HPLC following acid hydrolysis and derivatization with PMP (Strydom, 1994), and the sulfate content determined with ion chromatography (Ohira & Toda, 2006).

The effects of irradiation dosages on the chemical composition of fCS-*Pg* fragments are shown in Table 1. Native fCS-*Pg* contained glucuronic acid (GlcA), galactosamine (GalNAc), fucose (Fuc) and sulfate at a molar ratio of 1.3:1.0:1.7:3.3 (Table 1). With increasing irradiation dosages, the GlcA content was gradually reduced, compared to native fCS-*Pg*, while the relative molar ratio of sulfate increased. Moreover, at a dosage below 50 kGy, we observed no significant changes in the molar ratio of GalNAc and Fuc. In contrast, when the radiation dosage was increased to 100 kGy (DfCS-4), almost no GlcA was detected and the Fuc content was obviously decreased. These results indicate that irradiation initially targets GlcA in the backbone and subsequently destroys Fuc. Thus, control of the irradiation dosage is important to maintain fucose branches in the structure.

The effects of fCS-*Pg* solution concentrations on the chemical compositions of irradiated fCS-*Pg* fragments were additionally investigated (see Table 1 for details). At fCS-*Pg* concentrations above 2%, the compositions of irradiated fCS-*Pg* fragments altered slightly, with only a small decrease in the GlcA content observed at decreasing solution concentrations. However, at fCS-*Pg* concentrations below 2%, the GlcA content in irradiated fCSs fragments decreased sharply, indicating severe destruction of glucuronic acid units. In contrast, only the slight loss of Fuc, considered the key factor for prolonging APTT and TT, as well as antithrombotic activity (Mourão et al., 1998, 2001), was observed.

Thus, to obtain fCS fragments with high bioactivity, it is essential to determine the optimal range of irradiation dosages and fCS-*Pg* concentrations. The recommended irradiation conditions were established as dosages below 50 kGy and concentrations of above 2%, whereby no obvious destruction of the main fCS-*Pg* structure was recorded in our experiments.

3.3. IR spectroscopy

Based on analysis of the changes in molecular and chemical compositions of fCS-*Pg* fragments, we further investigated the precise alterations in glucosidic linkage and sulfation patterns. Both IR and NMR spectra were examined to clarify the detailed mechanisms underlying irradiation-induced degradation.

The IR spectra of native fCS-*Pg* and its low molecular weight fragments (DfCS-1, DfCS-2, DfCS-3, DfCS-4 and DfCS-1′, DfCS-2′, DfCS-3′, DfCS-4′) are shown in Fig. 2. Both native and low molecular weight fCS-*Pg* fragments displayed similar spectral bands. (1) In the 4000–1800 cm^{-1} region, we observed characteristic O—H and C—H stretching vibrations at 3471 and 2960 cm^{-1}, respectively (Matsuhiro, Osorio-Román, & Torres, 2012). (2) The 1800–400 cm^{-1} region contained characteristic bands of glycosaminoglycans, including the amide I band at 1646 cm^{-1}, an amide group II vibration at 1545 cm^{-1} and C—N vibration of the N-acetyl group at 1423 cm^{-1} (Conley, 1970; Foot & Mulholland, 2005; Matsuhiro et al., 2012). (3) The band at 1372 cm^{-1} was assigned to symmetric deformation of CH_3 (Chandía & Matsuhiro, 2008). (4) Three characteristic signals were assigned to sulfate groups, including S=O asymmetric stretching vibration at 1240 cm^{-1}, symmetric C—O—S stretching vibration at 850 cm^{-1}, and S—O stretching

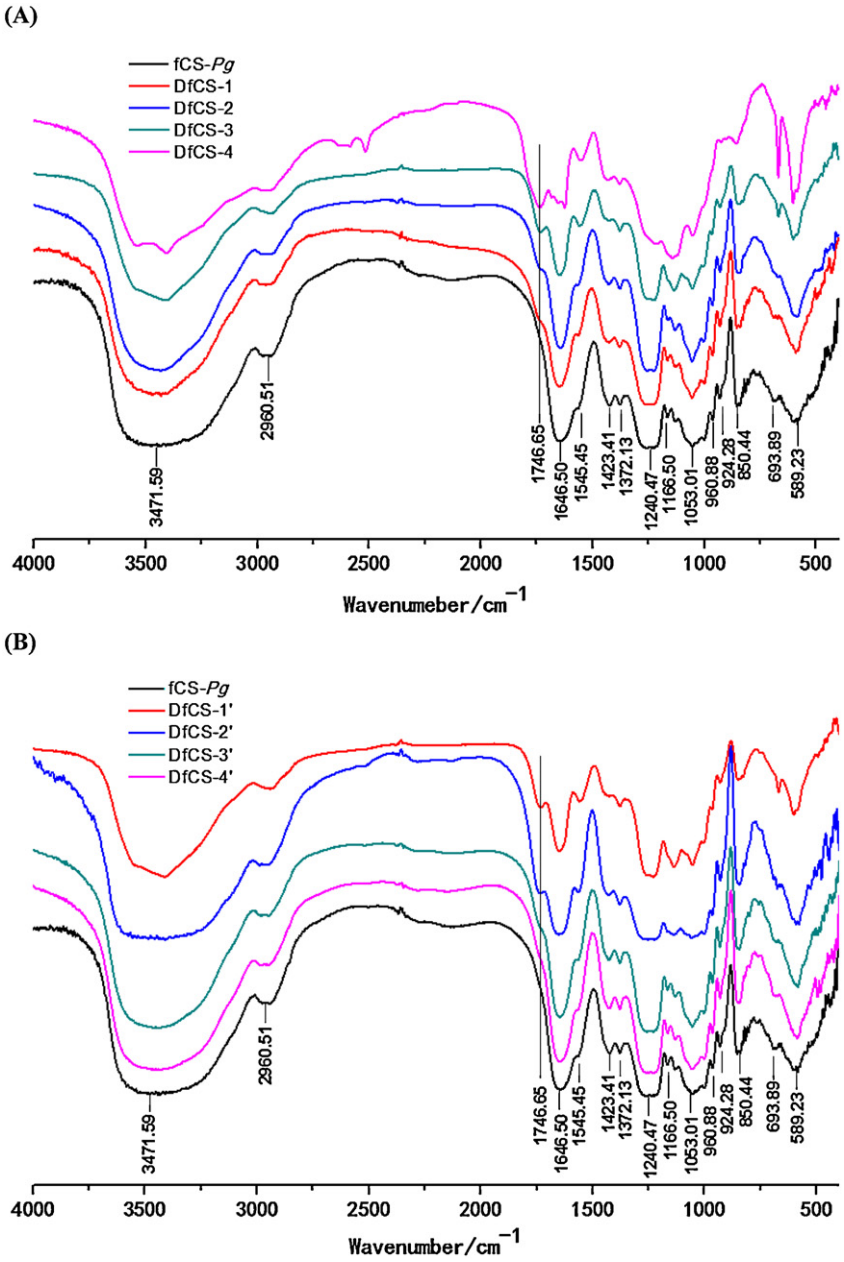

Fig. 2. IR spectra of native fCS-*Pg* and low molecular weight fCS-*Pg* fragments prepared by ^{60}Co irradiation degradation. (A) DfCS-1, DfCS-2, DfCS-3 and DfCS-4 were prepared by ^{60}Co irradiation degradation of the fCS-*Pg* solution (1%) in dosages of 10, 20, 50 and 100 kGy; (B) DfCS-1′, DfCS-2′, DfCS-3′ and DfCS-4′ were prepared by ^{60}Co irradiation degradation of the fCS-*Pg* solution with concentrations of 1%, 2%, 5% and 10% at the dosage of 50 kGy.

vibration at 589 cm^{-1} (Matsuhiro, 1996; Matsuhiro et al., 2012). Moreover, the absorption bands of native fCS-*Pg* and its fragments in IR spectra were similar to fCSs from other sea cucumber types reported in the literature (Hoshino & Heiwamachi, 1990; Matsuhiro et al., 2012).

However, compared to native fCS-*Pg*, the spectra of irradiated fCS-*Pg* fragments (DfCS-1, DfCS-2, DfCS-3, DfCS-4 and DfCS-1′, DfCS-2′, DfCS-3′, DfCS-4′) showed some differences. A new absorption band at 1746 cm^{-1} was observed in irradiated fCS-*Pg* fragments, which gradually increased in intensity with the irradiation dosage or decreasing solution concentration (Fig. 2A and B). This band may be attributed to cleavage of glycosidic bonds induced by radiation or oxidation of carbohydrate radicals generated within the fCS-*Pg* residue (Nagasawa, Mitomo, Yoshii, & Kume, 2000; Abad et al., 2009). Moreover, an increased signal at 693 cm^{-1} with increasing radiation dosage or reduced solution concentration (Fig. 2A and B) was observed, due to deformation of the O—H group (Cardenas-Jiron, Leal, Matsuhiro, & Osorio Roman, 2011). In the spectra of fCS-*Pg* fragments prepared using a radiation dosage of above 50 kGy or below a sample concentration of 2%, characteristic bands at 1240, 850 and 589 cm^{-1} were decreased, indicating loss of sulfate groups. At a dosage of 100 kGy, a number of bands present in the native fCS-*Pg* spectrum were significantly reduced, including those at 1646 cm^{-1} representing the amide I band, 1166 cm^{-1} assigned to C—O symmetric stretching vibration of glycosidic linkage, 1053 cm^{-1} attributed to C—O—C stretching vibration, 960 cm^{-1} corresponding to C—C stretching vibration with contributions of C—C—H deformation, and 924 cm^{-1} due to asymmetrical ring vibration (Matsuhiro et al., 2012). These findings collectively indicate severe destruction of the entire fCS-*Pg* structure at the high radiation dosage, and support the importance of performing irradiation under limited conditions.

3.4. ^1H NMR analysis

As IR spectra only provide a primary comparison of the structural changes of degraded fragments, ^1H NMR spectra of native and low molecular weight fCS-*Pg* fragments were further investigated to obtain precise information, such as changes in sulfation patterns or linkage of sugar units, for clarification of the degradation mechanism. Compared to native fCS-*Pg*, degraded fragments showed similar spectra containing characteristic signals, and the main peaks were assigned, as reported previously (Chen et al., 2011). Specifically, signals at 1.21 and 1.89 ppm were readily assigned to the methyl protons of Fuc (CH_3) and GalNAc (CH_3CO), respectively, those between 5.1 and 5.6 ppm to the anomeric protons of various sulfated fucose branches (Mourão et al., 1996; Yoshida et al., 1992), whereby the major signal at 5.17 ppm was assigned to 4-*O*-sulfated fucose (Fuc4S), minor peak at 5.23 ppm to 4-*O*-sulfated fucose (Fuc4S) and another minor peak at 5.56 ppm to 2,4-*O*-disulfated fucose (Fuc2,4S), based on previous results (Chen et al., 2011). However, these preliminary assignments were based on comparison of chemical shifts with reference data (Mourão et al.,

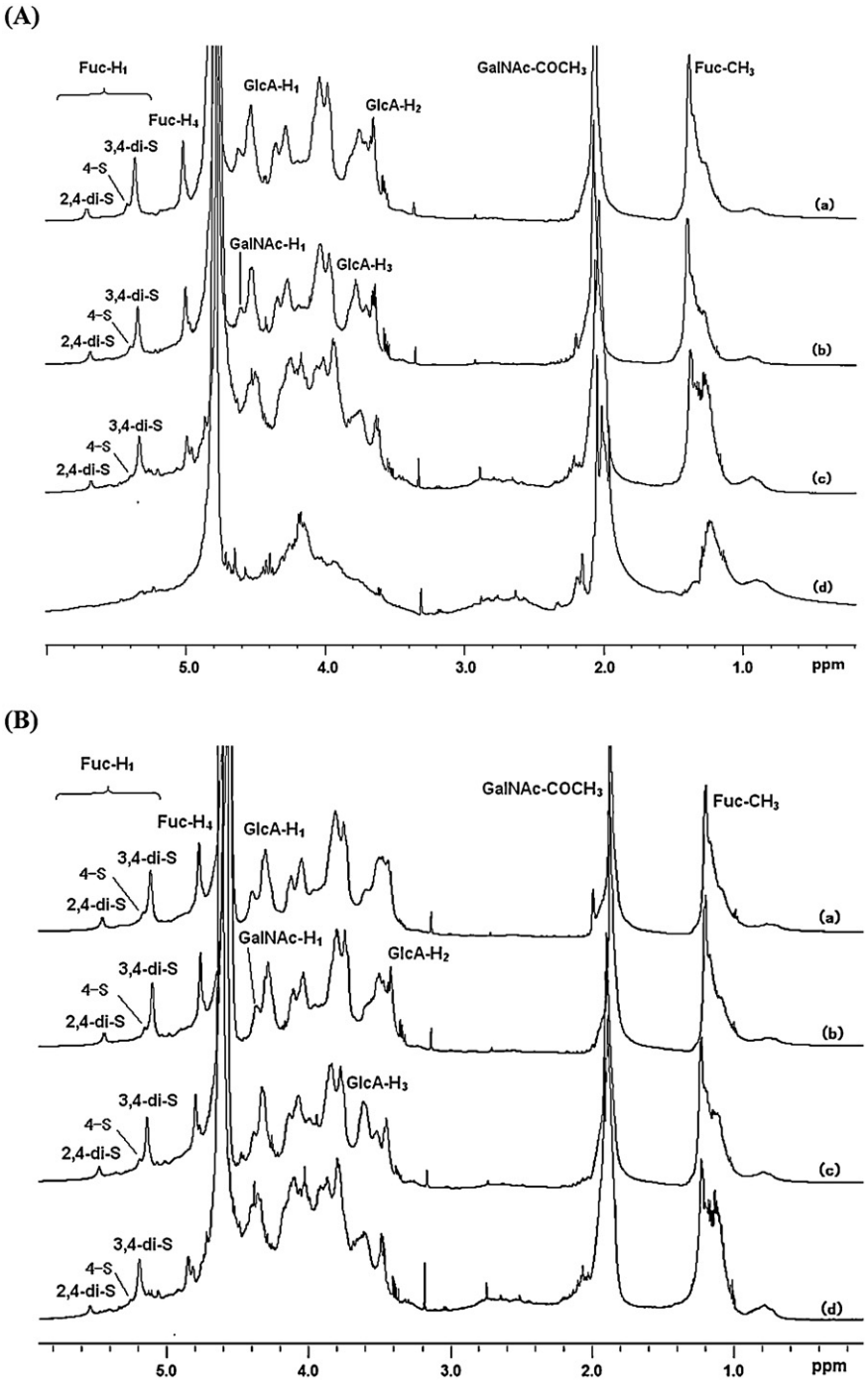

Fig. 3. ^1H NMR spectra of low molecular weight fCS-*Pg* prepared by ^{60}Co irradiation degradation. (A) DfCS-1, DfCS-2, DfCS-3 and DfCS-4 were prepared by ^{60}Co irradiation degradation of the fCS-*Pg* solution (1%) at dosages of 10, 20, 50 and 100 kGy: (a) DfCS-1, (b) DfCS-2, (c) DfCS-3 and (d) DfCS-4 (all the peaks of DfCS-4 suffered a great decrease and some signals were even not detected); (B) DfCS-1′, DfCS-2′, DfCS-3′ and DfCS-4′ were prepared by ^{60}Co irradiation degradation of the fCS-*Pg* solution with concentrations of 1%, 2%, 5% and 10% at the dosage of 50 kGy: (a) DfCS-4′, (b) DfCS-3′, (c) DfCS-2′ and (d) DfCS-1′.

1996; Yoshida et al., 1992), and required further confirmation with 2D NMR.

Fig. 3A presents a comparative analysis of ^{1}H NMR spectra of the degraded fCS-*Pg* fragments (DfCS-1, DfCS-2, DfCS-3 and DfCS-4) subjected to different dosages of irradiation and native fCS-*Pg*. DfCS-1 and DfCS-2 (irradiated at 10 and 20 kGy, respectively) showed similar spectra to native fCS-*Pg*, and the proportion of anomeric protons of their fucose branches at 5.17, 5.23 and 5.56 ppm remained almost identical, indicating that irradiation had no obvious effects on the sulfation pattern of fucose branches. Other signals at around 3.4–4.5 ppm, assigned to the cross ring proton, also displayed no significant changes. However, at increasing irradiation dosages above 20 kGy, signals from both the anomeric proton of fucose branches and cross-ring proton became weaker. In particular, signals around 3.50–3.65 ppm, assigned to the H2 and H3 positions of glucuronic acid units, were markedly decreased with increasing dosages, consistent with the finding that GlcAs undergo severe destruction upon irradiation in the monosaccharide composition assay. In the spectrum of DfCS-4 subjected to 100 kGy irradiation, both the anomeric signals of fucose proton and cross-ring proton were decreased to a significant extent, compared to native fCS-*Pg*, indicating that apart from the glucuronic acid unit, other sugar units were destroyed. The disappearance of the anomeric proton signal of fucose branches at 5.17, 5.23 and 5.56 ppm and sharp decrease in the methyl proton at 1.21 ppm signified destruction of sulfated fucose branches under these conditions. These results are in agreement with those from the monosaccharide composition assay.

The ^{1}H NMR spectra of fCS-*Pg* fragments (DfCS-1′, DfCS-2′, DfCS-3′ and DfCS-4′) irradiated with 50 kGy ^{60}Co at different solution concentrations are presented in Fig. 3B. The spectra of DfCS-2′, DfCS-3′ and DfCS-4′ (irradiated above a concentration of 2%) were similar to those of native fCS-*Pg*, including signals at 3.4–4.5, 5.17, 5.23, and 5.56 ppm, suggesting that the sulfation pattern of fucose branches and backbone structure of fCS fragments were maintained in preparations at solution concentrations higher than 2%. In contrast, in the spectrum of DfCS-1′ (irradiated at a solution concentration of 1%), signals around 3.5–3.65 ppm assigned to H-2 and H-3 of glucuronic acid units decreased sharply, indicative of obvious destruction of glucuronic acid units. Similarly, the signals attributed to the anomeric proton of fucose branches at 5.17, 5.23, and 5.56 ppm were slightly weaker than those of native fCS-*Pg*, suggesting fucose branch destruction. Thus, based on the ^{1}H NMR data, we propose that irradiation conditions should be limited to dosages below 50 kGy and sample concentrations of above 2% to maintain the main structure and bioactivities of native fCS-*Pg*.

Previous reports on linear repeated polysaccharides (sulfated or non-sulfated), such as carrageenans, glucan, chitosan and hyaluronic acid, have suggested that free radicals from irradiated water are the indirect effect of irradiation of diluted aqueous solutions (Relleve et al., 2005), which could lead to depolymerization of basic units mainly via breakage of glycosidic bonds (Andrzej, 2010; Alijani, Balaghi, & Mohammadifar, 2011) and yield smaller polysaccharide units of the radiolytic product (Aliste, Vieira, & Mastro, 2000; Alijani et al., 2011). Thus, the significant decrease in molecular weight of fCS-*Pg* in the present study may additionally be attributed to the breakage of glycosidic bonds. In view of the collective results from chemical composition assays, IR and ^{1}H NMR spectra, we conclude that fCS-*Pg*s release their sulfates during irradiation, consistent with the cleavage of sulfates from sulfated galactans, such as carrageenans, during irradiation (Abad et al., 2009). Additionally, our results indicate that the GlcA content gradually decreases with increasing irradiation dosages or decreasing concentrations, which has not been reported previously. This novel discovery should provide a basis for further research on irradiation-induced degradation.

3.5. 2D NMR of a low molecular weight fCS-Pg fragment (DfCS-2)

Based on a comparison of chemical shifts with reference data (Mourão et al., 1996; Yoshida et al., 1992), our ^{1}H NMR data provided information on the possible structure of fCS-*Pg*. However, this could not be confirmed with 2D NMR, owing to poor resolution of fCS-*Pg* with high molecular weight. Nevertheless, after degradation, fCS-*Pg* fragments showed better resolution with 2D NMR. Here, we corrected the structure of fCS-*Pg* via 2D NMR analysis of DfCS-2 as a representative fragment prepared via irradiation at a concentration of 1% and 20 kGy dosage.

Assignments of ^{1}H and ^{13}C chemical shifts (Table 2) of fucose branches as well as the CSE backbone in DfCS-2 were made from

Table 2
^{1}H and ^{13}C chemical shifts from NMR spectra of DfCS-2.

Samples	DfCS-2					Fuc0S** (standard)	CSE***	
	GlcA(β)	GalNAc4,6S(β)	Fuc3,4S*(α)	Fuc4S(α)	Fuc2,4S(α)		GlcA(β)	GalNAc4,6S(β)
H1****	4.51	4.58	5.17	5.23	5.56	5.03	4.49	4.65
(C1)	(104.9)	(101.5)	(100.5)	(99.9)	(96.5)	–	(104.2)	(102.1)
	J = 7.4	*J* = 6.5	*J* = 3.6		*J* = 3.7			
H2	3.56	3.98	3.96	3.71	**4.43**	3.96	3.41	4.07
(C2)	(75.0)	(54.4)	(73.2)	(73.2)	(**76.7**)	–	(73.0)	(52.2)
H3	3.64	3.98	**4.51**	3.90	3.97	4.01	3.60	4.07
(C3)	(77.4)	(74.3)	(**77.3**)	(74.3)	(68.3)	–	(74.7)	(76.1)
H4	3.96	**4.67**	**4.92**	**4.56**	**4.64**	3.96	3.80	4.80
(C4)	(76.4)	(**82.4**)	(**81.4**)	(**81.3**)	(**81.4**)	-	(82.6)	(76.6)
H5	3.76	3.92	4.53	4.12	4.60	4.35	3.68	4.10
(C5)	(71.9)	(69.4)	(67.6)	(67.6)	(68.6)	–	(77.5)	(73.3)
H6	3.63	**4.25/4.15**	1.10	1.12	1.11	1.21	–	4.30
(C6)	(73.0)	(**67.8**)	(16.7)	(16.8)	(16.6)	–		(68.5)
CH_3	–	1.92	–	–	–	–	–	2.04
		(24.0)						(22.4)
C=O	–	–	–	–	–	–	–	–
	(175.2)	(176.3)						(174.6)

* Fuc3,4S represents more than 75% of the fucose branches and the rest were Fuc2,4S and Fuc4S.
** The chemical shifts of Fuc0S were from a standard non-sulfated monosaccharide fucose, cited from literature (Ribeiro, Vieira, Mourão, & Mulloy, 1994).
*** Chemical shifts of CSE were cited from literature (Yoshida et al., 1992), and chemical shifts of the sulfation sites of DfCS-2 were highlighted in bold.
**** Chemical shifts in ppm and coupling constants (*J*) in Hz.

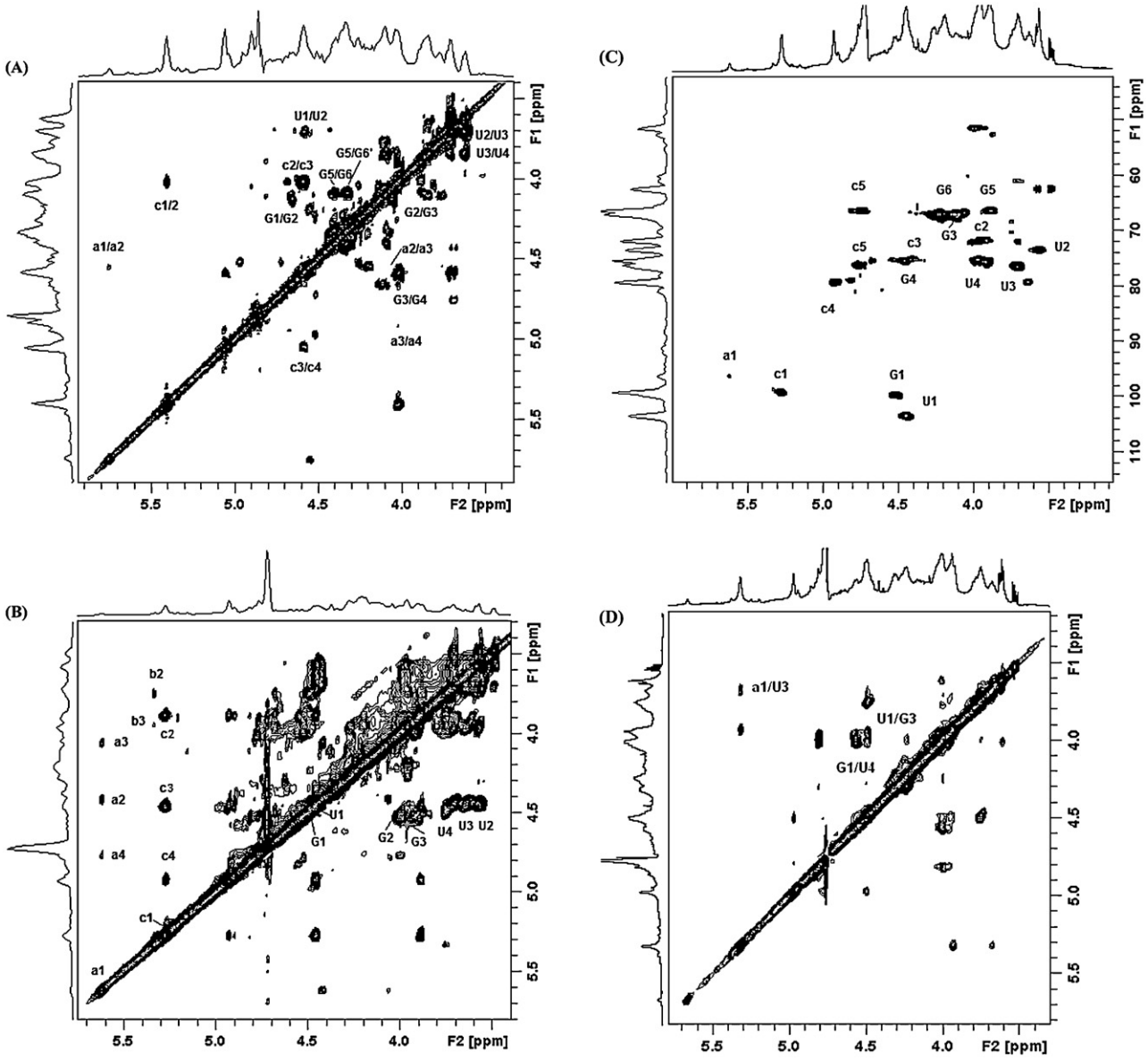

Fig. 4. The 2D NMR spectra of DfCS-2 (the low molecular weight fCS-*Pg* fragment) prepared by irradiation at the concentration of 1% and at the dosage of 20 kGy: (A) COSY; (B) TOCSY; (C) HSQC; (D) NEOSY. Signals designated with a, b and c refer to those produced by Fuc2,4S, Fuc4S and Fuc3,4S; signals designated with G and U refer to GalNAc and GlcA, respectively.

COSY (Fig. 4A), TOCSY (Fig. 4B), HSQC (Fig. 4C) and NOESY (Fig. 4D) spectra. In terms of sulfation patterns in fucose branches of the oligosaccharide mixture, we observed two clearly anomeric signals (a1 and c1) in the COSY spectrum (Fig. 4A), whereas the signal of b1 (Fig. 4B) in DfCS-2 was significantly decreased after degradation. The COSY spectrum (Fig. 4A) showed cross peaks between anomeric protons H1 and H2 of fucose branches, e.g., a1/a2 and c1/c2 (Fig. 4A). The TOCSY spectrum (Fig. 4B and Table 2) displayed proton signals in the same residue, which aided in extending assignment to the remaining proton signals, e.g., H3, H4 and H6 (Table 2). For instance, c1 at 5.17 ppm cross was from residue c (Fig. 4B), and H2–H5 identified as 3.96, 4.51, 4.92 and 4.53 ppm, respectively. Sulfate substitutions were assigned by careful comparison of proton chemical shifts of fucose residues in the polysaccharide chains with that of standard monosaccharide fucose (Table 2). The sulfate position was deduced from its downfield shift (Table 2). For instance, downfield shifts of H3 and H4 signals of residue c, compared to those of the monosaccharide, Fuc, indicated sulfation at both 3- and 4-positions. Similarly, we identified that residue a (5.56 ppm) was at 2,4-*O*-disulfated.

Assignment of a CSE backbone, –[4GlcAβ1-3GalNAc(4,6S)β1]–, of DfCS-2 was additionally made from COSY (Fig. 4A), TOCSY (Fig. 4B), HSQC (Fig. 4C), and NOESY spectra (Fig. 4D). The β anomeric configuration of both GlcA and GalNAc could be deduced from the H1/H2 coupling constants, 7.4 and 6.5 Hz, respectively (Table 2). The downfield shifts of H4/C4 (4.67/82.5) and H6/C6 (4.25/67.8) of GalNAc (Table 2) suggested sulfation at the 4-*O*- and 6-*O*-positions. Careful comparison with spectral data of standard CSE (Yoshida et al., 1992) provided further evidence of a CSE backbone (Table 2). The correlation signals, G1/U4 and U1/G3, in the NOESY spectrum (Fig. 4D) led to the unambiguous identification of GalNAc linked to the C4 position of the GlcA, while the latter linked to the C3 position of GalNAc.

In view of the collective data, DfCS-2 was identified as mainly containing 3,4-*O*-disulfated fucose (Fuc3,4S) branches (>75%), 2,4-*O*-disulfated fucose (Fuc2,4S) branches to a minor extent, and a

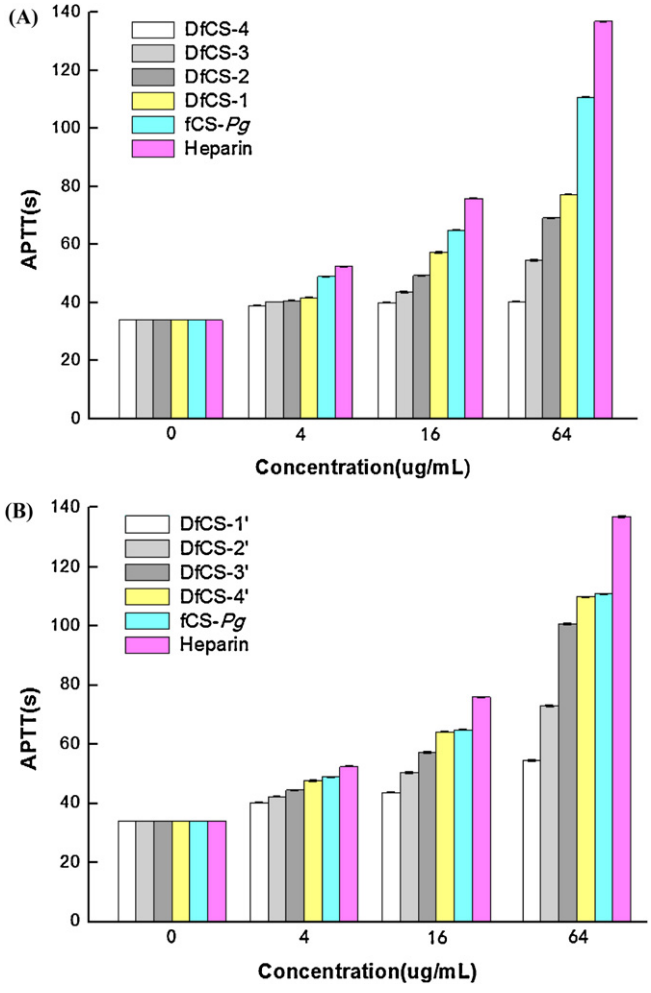

Fig. 5. Effect of native fCS-*Pg*, low molecular weight fCS-*Pg* fragments prepared by ^{60}Co irradiation degradation and unfractionated heparin (14 kDa) on prolong of the APTT. (A) DfCS-1, DfCS-2, DfCS-3 and DfCS-4 were prepared by ^{60}Co irradiation degradation of the fCS-*Pg* solution (1%) at dosages of 10, 20, 50 and 100 kGy; (B) DfCS-1′, DfCS-2′, DfCS-3′ and DfCS-4′ were prepared by ^{60}Co irradiation degradation of the fCS-*Pg* solution with concentrations of 1%, 2%, 5% and 10% at the dosage of 50 kGy.

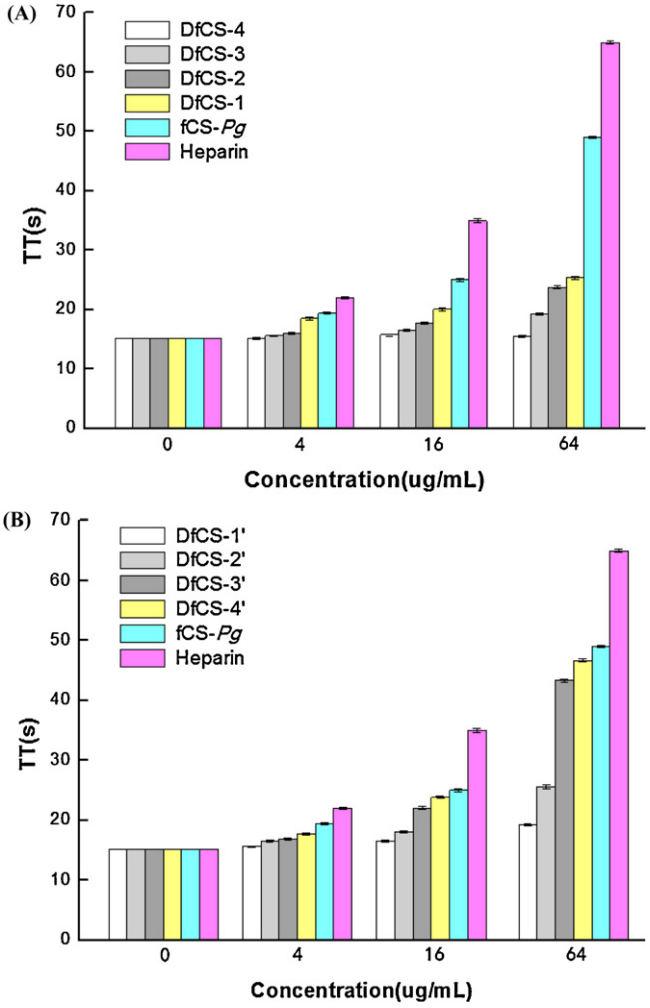

Fig. 6. Effect of native fCS-*Pg*, low molecular weight fCS-*Pg* prepared by ^{60}Co irradiation degradation and unfractionated heparin (14 kDa) on prolong of the TT. (A) DfCS-1, DfCS-2, DfCS-3 and DfCS-4 were prepared by ^{60}Co irradiation degradation of the fCS-*Pg* solution (1%) at dosages of 10, 20, 50 and 100 kGy; (B) DfCS-1′, DfCS-2′, DfCS-3′ and DfCS-4′ were prepared by ^{60}Co irradiation degradation of the fCS-*Pg* solution with concentrations of 1%, 2%, 5% and 10% at the dosage of 50 kGy.

chondroitin E backbone. This finding is distinct from our previous ^{1}H NMR analysis of fCS-*Pg*, where the anomeric signal at 5.17 ppm was assigned to Fuc4S branches by comparison with reference data (Chen et al., 2011).

3.6. Anticoagulant activities

The APTT assay determines interference with the intrinsic coagulation process and TT represents the last coagulation step of thrombin-mediated fibrin formation (Lin et al., 2007). The anticoagulant activities of a range of low molecular weight fragments (DfCS-1, DfCS-2, DfCS-3, DfCS-4 and DfCS-1′, DfCS-2′, DfCS-3′, DfCS-4′) and native fCS-*Pg* were therefore assessed by measuring APTT and TT. All polysaccharides displayed anticoagulant activity, as evident from the significant prolongation of APTT and TT (Figs. 5 and 6). However, compared with native fCS-*Pg*, degraded fCS fragments had shorter APTT and TT (Figs. 5 and 6), suggesting significantly lower anticoagulant activity.

The low molecular weight fCS fragments (DfCS-1, DfCS-2, DfCS-3 and DfCS-4) prepared using different irradiation dosages showed distinct anticoagulant activities (Figs. 5A and 6A). DfCS-1 with the largest molecular mass exhibited higher anticoagulant activity than other lower molecular weight fragments. Conversely, DfCS-4 with the smallest molecular weight showed the lowest anticoagulant activity, possibly owing to the removal of sulfated fucose during irradiation. Unlike other fCS fragments whose anticoagulant activities showed a positive dosage-dependent relationship, the anticoagulant activity of DfCS-4 remained relatively unchanged with increasing concentrations.

The low molecular weight fCS fragments (DfCS-1′, DfCS-2′, DfCS-3′ and DfCS-4′) prepared at different solution concentrations additionally displayed distinct anticoagulant activities (Figs. 5B and 6B). DfCS-3′ and DfCS-4′ with larger molecular masses showed higher activities than DfCS-1′ and DfCS-2′ with smaller molecular masses.

Since the structural features of native fCS-*Pg* and its low molecular weight fragments were similar, their variable anticoagulation activities were primarily considered to be the influence of different molecular masses (Zhou, Sheng, Yao, & Wang (2006); Zhou et al., 2004). DfCS-4 displayed the lowest anticoagulation activity among all the fragments, mainly due to loss of sulfated fucose branches that represents the key factor for the maintenance of fCS activity (Mourão et al., 1996, 1998, 2001; Borsig et al., 2007). Thus, relative to native fCS-*Pg* with higher anticoagulant activity, degraded fCS fragments may have significantly less bleeding risk at

equivalent therapeutic dosages, and therefore present a safer option as anticoagulant medicine (Nagase et al., 1995, 1996).

4. Conclusions

In summary, we have developed a novel method for the controlled degradation of fCS from sea cucumber, *P. graeffei*, using ^{60}Co irradiation in water solution. This procedure does not involve the addition of chemical additives at ambient temperature, and presents a more convenient, environmentally friendly and efficient approach than previously reported procedures (Wu et al., 2010). The effects of radiation dosages and sample concentrations on molecular weight, chemical composition and structural change were investigated. Our results indicated that with increasing irradiation dosage or decreasing sample concentration, the molecular weight of fCS-*Pg* was decreased, accompanied by a decrease in the GlcA content. Different molecular weight fCS fragments with primary structures similar to native fCS-*Pg* could be obtained under controlled conditions, specifically, at a concentration range of 2–10%, and irradiation dosages of 10–50 kGy.

As a result of improved resolution, a degraded fragment (DfCS-2) was used to confirm the structure of native fCS-*Pg* in 2D NMR analysis. We identified fCS-*Pg* as mainly containing 3,4-*O*-disulfated fucose (Fuc3,4S) branches (>75%) with a chondroitin E backbone. This finding was distinct from our previous ^{1}H NMR results, where the sulfation pattern was assigned to 4-*O*-sulfated fucose (Fuc4S) branches by comparing with reference data (Chen et al., 2011).

Our experiments disclosed that the anticoagulant activities of fCS fragments are significantly related to molecular mass, and enhanced with increasing molecular mass. Compared with the native fCS-*Pg*, irradiated fCS fragments had lower anticoagulant activities, supporting their utility as a safer anticoagulant medicine. However, further studies are required to improve our knowledge on fCS fragments, their anticoagulant mechanisms in vitro, and the critical structural features required for keeping this activity. It is additionally necessary to assess the anticoagulant and antithrombotic effects of these novel low molecular weight fCS fragments i*n vivo* in animal models.

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