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Inhibition of angiogenesis by chitooligosaccharides with specific degrees of acetylation and polymerization

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ABSTRACT

Chitooligosaccharides (CHOS) inhibit angiogenesis and may be used in the treatment of cancer tumors. We have studied the effect of the fraction of acetylation (F_A) and the degree of polymerization (DP) on CHOS anti-angiogenic activity. We tested enzymatically produced CHOS-mixtures with F_A0.15, F_A0.3 and F_A0.6, and DP ≤ 12 in initial experiments with chorioallantoic membranes. All of the samples reduced the formation of new blood vessels, CHOS with F_A0.3 giving the best effect. Single-DP fractions from the F_A0.3 sample purified by size-exclusion chromatography (DP3-DP12) were then tested for inhibition of migration of human endothelial cells, which is an important element of the angiogenesis process. All of the fractions inhibited migration, meaning that, within the DP area tested in this study, F_A is more important than DP for the effect. Generally, the results reveal that DP3–DP12 CHOS have considerable potential as anti-angiogenic compounds.

1. Introduction

Chitooligosaccharides (CHOS) or chitosan oligomers are produced enzymatically or chemically from chitosans. Chitosans are linear heteropolymers of $\beta(1\rightarrow4)$ linked *N*-acetyl-D-glucosamine (GlcNAc) and its deacetylated counterpart D-glucosamine (GlcN). Chitosan can be made from chitin by partial or complete *N*-deacetylation, either by homogeneous (Sannan, Kurita & Iwakura, 1975) or by heterogeneous deacetylation (Rigby, 1934). Chitin is a linear, insoluble homopolymer of GlcNAc and is found in large quantities in nature as a structural component of the cell wall of fungi and yeasts and in the exoskeletons of insects and arthropods (*e.g.*, crabs, lobsters and shrimps).

Chitinases and chitosanases are chitin- and chitosan-degrading enzymes. Chitinases are found in glycoside hydrolase (GH) families 18 and 19, while chitosanases are found in GH families 46, 75 and 80, and not so often in GH families 5, 7 & 8 (according to the CAZy database; (Cantarel, Coutinho, Rancurel, Bernard, Lombard & Henrissat, 2009). As long as the fraction of acetylation does not become too low, most chitinases and chitosanases can cleave most chitosans. Cleavage specificities (*i.e.* preferences for certain acetylation patterns at the cleavage point) vary and, consequently, product mixtures will vary with respect to the length and sequences of the CHOS they contain. For example, CHOS produced with family 18 chitinases will have a GlcNAc on their reducing ends, whereas the CHOS produced with most chitosanases will predominantly have GlcN on their reducing ends (Aam, Heggset, Norberg, Sørlie, Vårum & Eijsink, 2010; Fukamizo, Honda, Goto, Boucher & Brzezinski, 1995; Heggset, Hoell, Kristoffersen, Eijsink & Vårum, 2009; Heggset et al., 2010; Horn et al., 2006; Mitsutomi, Hata & Kuwahara, 1995; Sasaki, Vårum, Itoh, Tamoi & Fukamizo, 2006; Sikorski, Stokke, Sørbotten, Vårum, Horn & Eijsink, 2005). The extent of chitosan degradation is given as degree of scission, α (= 1/number – average degree of polymerization, DP_n) (Sørbotten, Horn, Eijsink & Vårum, 2005), which represents the fraction of glycoside bonds that has been cleaved. Complete conversion of the chitosan polymers to dimers (DP = 2) would yield an α value of 0.50.

CHOS are defined by their fraction of acetylated sugar residues (F_A), their length or degree of polymerization (DP) and their pattern of *N*-acetylated sugar residues (P_A; also referred to as "sequence"). It is possible to make reasonably well defined mixtures of CHOS

Abbreviations: CHOS, chitooligosaccharides; CAM, chorioallantoic membrane; DP, degree of polymerization; F_A, fraction of acetylation; HBC cells, human breast cancer cells; ECV304 cells, human umbilical vein endothelial cells; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2,-diphenyl tetrazolium bromide.

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by tailoring the conditions of enzymatic turnover; the F_A of the chitosans, the type of enzyme used, and the reaction time (Aam, Heggset, Norberg, Sørlie, Vårum & Eijsink, 2010). Various CHOS in mixtures can be separated according to their length by size exclusion chromatography (SEC) (Sørbotten, Horn, Eijsink & Vårum, 2005), or by their charge, which reflects the number of deacetylated residues, by cation exchange chromatography (Haebel, Bahrke & Peter, 2007).

CHOS possess a number of different bioactivities including antifungal and immunostimulatory effects (Gorzelanny, Poppelmann, Pappelbaum, Moerschbacher & Schneider, 2010; Nishimura, Ishihara, Ukei, Tokura & Azuma, 1986). CHOS may be used as vectors in gene therapy, and are known to accelerate wound healing processes (Muzzarelli, 2009, 2010). CHOS have the potential to prevent bacterial adhesion to intestinal epithelial cells and thereby prevent bacterial invasion and disease, and CHOS increase the differentiation of mesenchymal stem cells to osteoblasts and increase Ca^{2+} bioavailability in bone tissue, leading to increased bone strength. Finally, CHOS of specific DP and P_A are inhibitors of chitinases, which might be exploited in treatment of asthma by inhibition of the acidic mammalian chitinase (AMCase) and for preventing malaria by inhibition of *Plasmodium* parasite chitinases [see Aam et al., 2010; Muzzarelli, 2009, 2010 for recent reviews (Muzzarelli, 2009, 2010; Aam, Heggset, Norberg, Sørlie, Vårum & Eijsink, 2010)].

The anti-tumor effects of CHOS have been known for several decades (Muzarelli, 1977). In 1986, Suzuki et al. reported that both fully acetylated and fully deacetylated chitohexaose had antitumor activity (Suzuki, Mikami, Okawa, Tokoro, Suzuki & Suzuki, 1986). Since then, different aspects of the anti-tumor activity of CHOS have been studied, such as the effects of Mw (Qin, Du, Xiao, Li & Gao, 2002) and charge/F_A (Huang, Mendis, Rajapakse & Kim, 2006). Several mechanisms for anti-tumor activity have been proposed. CHOS could induce apoptosis of tumor cells (Harish Prashanth & Tharanathan, 2005; Xu et al., 2008) or CHOS may indirectly inhibit tumors by activating host immune functions (Maeda & Kimura, 2004). Several studies suggest that CHOS could inhibit tumor metastasis by inhibiting the expression of matrix metalloproteinase-9 in human fibrosarcoma cells (HT1080) (Van Ta, Kim & Kim, 2006), or by inhibiting TNF-alpha-induced E-selectin expression in endothelial cells *via* the JNK/NF-κB pathway (Lin, Chen, Lee, Lee, Lin & Chiu, 2007). It has also been reported that CHOS inhibit proinflammatory cytokine-induced invasiveness of HT-29 cells *via* reduced production of nitric oxide (Nam, Kim & Shon, 2007). Most of these studies have been done with rather undefined CHOS fractions and little is known about exactly which CHOS properties are the most dominant factors in determining anti-tumor potential.

Angiogenesis is necessary for tumor growth. Early in 1971 the critical role of tumor angiogenesis in cancer progression was proposed by Folkman (1971). He hypothesized that tumors undergo three distinct phases of growth: an initial avascular phase of slow limited growth, an angiogenesis phase where the tumor cultivates its own blood supply, and finally a phase of rapid vascular growth. In the second phase, cancer cells can generate various pro-angiogenic factors such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF) and epidermal growth factor (EGF). These factors can promote the migration, proliferation and tube formation of endothelial cells and these activities are essential steps of angiogenesis (Ferrara, 2000).

While (polymeric) chitosans are known to have beneficial effects on angiogenesis in the context of wound healing (Muzzarelli, 2009), several studies have indicated that (oligomeric) CHOS possess anti-angiogenic activities in the context of tumor growth (Wang, Zheng, Yang, Niu, Chu & Lin, 2007; Wu, Yao, Bai, Du & Lin, 2008; Xiong et al., 2009). Earlier work done by our group has shown that fully N-acetylated CHOS are more effective in preventing angiogenesis than fully deacetylated CHOS (Wang, Zheng, Yang, Niu, Chu & Lin, 2007). In a follow-up study, we showed that fully deacetylated hexameric CHOS were more effective inhibitors of angiogenesis compared to shorter deacetylated oligomers (Xiong et al., 2009). These results indicate that both lengths and fraction of acetylation are important factors. Clearly, further studies are needed to identify the most crucial CHOS parameters that determine anti-angiogenic activity. CHOS with DPs larger than those of commercially available fully acetylated or fully deacetylated CHOS need to be tested. Here, we describe a series of studies with enzyme-generated home-made CHOS mixtures, including relatively pure fractions. Using these CHOS preparations, we were able to test the effect of DP and F_A on the anti-angiogenic potential of CHOS. This enabled us to identify combinations of F_A and DP that are optimal for inhibition of angiogenesis.

2. Materials and methods

2.1. Preparation of CHOS from chitosans/chitin

Chitosans with three different F_A were hydrolyzed with a chitinase or a chitosanase to produce CHOS.

KitoNor chitosan, F_A0.15 (Norwegian Chitosan, Gardermoen, Norway) and a chitosan with F_A0.3 (Heppe Medical Chitosan GmbH, Halle, Germany) were hydrolyzed with purified recombinant chitosanase ScCsn46A from *Streptomyces coelicolor* A3(2) (Heggset et al., 2010).

A highly acetylated chitosan was prepared in our lab by homogenous deacetylation of chitin from shrimp shell (Chitinor, Senjahopen, Norway) to F_A0.6 (Sannan, Kurita & Iwakura, 1975; Vårum, Anthonsen, Grasdalen, & Smidsrød, 1991a,b). 100 g 40% (w/v) NaOH was mixed with 4 g chitin and incubated at 4 °C for 16 h. 300 g ice was added and the solution was stirred thoroughly until all the ice was melted. After centrifugation at 4 °C to remove undissolved chitin, the supernatant was sparged with N_2 gas for 10 min to remove oxygen prior to incubation at 25 °C for 46.5 h. The deacetylation was stopped by cooling the sample with 280 g ice and adding HCl until the pH reached 4.5. After filtration, to remove chitin that was not sufficiently deacetylated to be water soluble, the solution was dialyzed against dH_2O. After several changes of water to remove the salt, the pH in the solution was adjusted to 4.5 with HCl, followed by sterile filtration and lyophilization. The F_A0.6 chitosan was hydrolyzed with purified recombinant ChiB from *Serratia marcescens* (Sørbotten, Horn, Eijsink & Vårum, 2005).

The F_A of the chitosans before enzymatic hydrolysis, and the α after degradation were found by performing ^{1}H NMR spectroscopy analysis on a Varian Gemini instrument at 300 MHz (Sørbotten, Horn, Eijsink & Vårum, 2005; Vårum et al., 1991b).

The F_A0.15 and F_A0.3 chitosans are soluble in 0.5% acetic acid, whereas the F_A0.6 chitosan are soluble in water. To set-up the enzymatic reactions, all three chitosans were dissolved in buffer (40 mM NaAc, 100 mM NaCl, pH 5.5) to a concentration of 10 mg/mL. In case of the F_A0.15 and F_A0.3 chitosans 0.5% HCl was added, and after all of the chitosan was dissolved the pH was adjusted back to 5.5 by using NaOH. Enzymes were added to prewarmed chitosan solutions to a final concentration of 0.5 μg/mg chitosan and the reactions were incubated with shaking (225 rpm) at 37 °C. Reactions were stopped by decreasing the pH to 2.5 with HCl.

2.2. Separation of CHOS

The CHOS were separated by size exclusion chromatography (SEC) on three XK 26 columns packed with Superdex™ 30 prep grade (GE Healthcare) coupled in series with an overall dimension

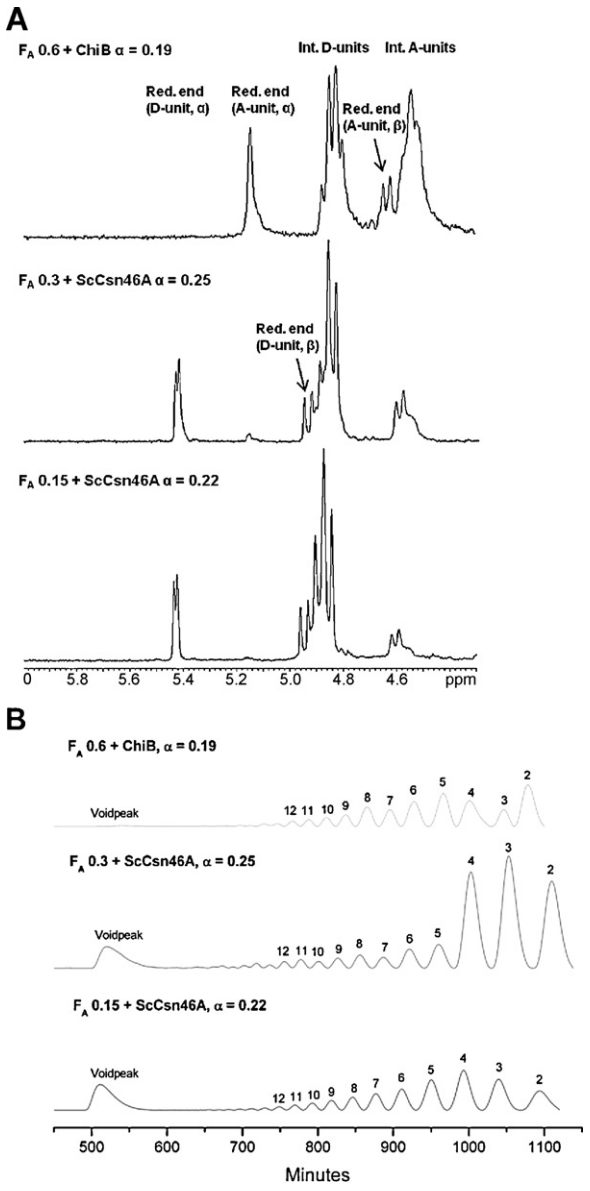

Fig. 1. ^{1}H NMR analysis of CHOS preparations (A) and size exclusion chromatograms (SEC) of the three different CHOS hydrolysates (B). The degree of scission (α) is calculated from the ^{1}H NMR spectra (A) by $\alpha = 1/DP_n$ (average degree of polymerization) where $DP_n = (A\alpha + A\beta + D\alpha + D\beta + A + D)/(A\alpha + A\beta + D\alpha + D\beta)$. A = GlcNAc and D = GlcN. Aα/Aβ/Dα/Dβ equals the integral of the reducing end signals (α and β anomers), and A and D equals the integral of the peaks representing the internal and nonreducing ends (Int. D/A-units) (Sørbotten et al., 2005). Since the reducing end signals of the β anomers are overlapping with the internal signals, we have used the α/β ratio 60:40 in the calculations. The spectra show that all reducing ends are GlcNAc for the reaction with ChiB, whereas in the reactions with ScCsn46A mainly CHOS with GlcN on the reducing ends are produced. In the SEC chromatograms of the three different CHOS hydrolysates (B), peaks are labeled by numbers indicating the DP (see also Sørbotten et al., 2005). Raw-data chromatographic spectra were baseline corrected using the statistical software R, version 2.6.1 (The R Foundation for Statistical Computing)

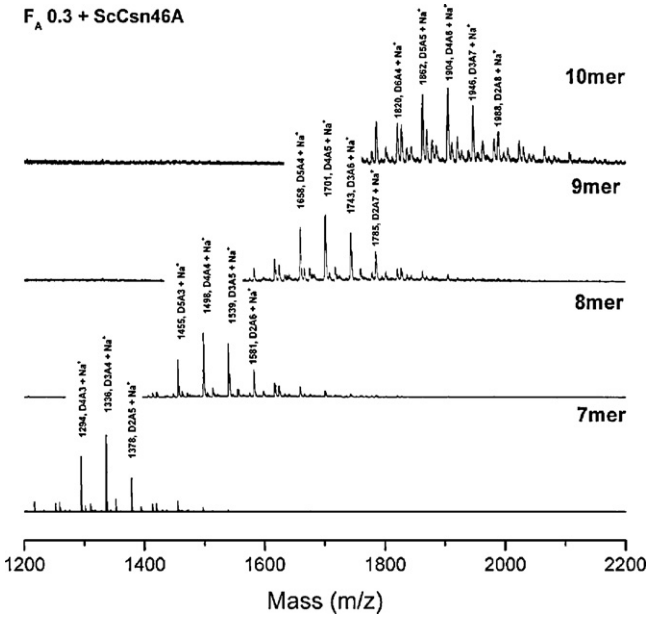

Fig. 2. MALDI-TOF-MS analysis of CHOS fractions obtained by SEC of hydrolyzed F_A0.3 chitosan. The 7–10mer fractions are shown. Major signals are labeled by mass, sugar composition (A, GlcNAc; D, GlcN). All of the labeled peaks represents Na^+ adduct.

of 2.6 cm × 180 cm. The mobile phase (150 mM NH_4AC pH 4.6) was run at a constant flow of 0.8 mL/min (Sørbotten, Horn, Eijsink & Vårum, 2005). The signals were read on a RI detector (Gilson model 133). In each run 100 mg of chitosan hydrolysate was applied (*i.e.* 5 mL) and the 3mer–12mer fractions were collected. Identification of the fractions was performed with MALDI-TOF-MS. The fractions were dialyzed with Float-A-Lyzers (MWCO 100–500 Da, Spectrum-Labs) to remove salts, sterile filtrated through Filtropur S 0.2 μm sterile filters (Sarstedt, Germany) and lyophilized. Prior to use, the CHOS were dissolved in phosphate buffered saline (PBS).

To limit the number of assays, initial experiments were done with mixtures of CHOS (*i.e.* pooled fractions): F_A0.15/3–7mers, F_A0.15/8–12mers, F_A0.3/8–12mers, F_A0.6/4–7mers and F_A0.6/8–12mers. We further collected CHOS from the F_A0.3 chitosan into single fractions (meaning that each sample contained CHOS with one specific DP), since the F_A0.3 sample gave the best effects against angiogenesis in the CAM experiments in the initial studies.

2.3. Preparation of cell lines and eggs

Human umbilical vein endothelial cells (ECV304) and MCF-7 human breast cancer cells (HBCs) were purchased from the Cell Bank of the Committee of Type Culture Collection, Chinese Academy of Sciences (Wuhan, China). Cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 U/mL penicillin and 100 μg/mL streptomycin (Pen Strep, Invitrogen, USA) at 37 °C in a 5% CO_2 atmosphere. The HBC medium was collected when the HBC cells were in the logarithmic phase, filtrated through 0.45 μm microporous membranes (Shanghai XingYa Cleaning Stuff Factory, China) and stored at 4 °C for further use. In the cell proliferation assay and in the cell migration assay 5% (v/v) HBC medium was used as an angiogenesis inducing factor and equal volumes of ECV304 medium prepared in the same way were used as a control.

Fertilized chicken eggs were purchased from a local supplier (Dalian Fengxiyuan Farm, China).

2.4. Chicken chorioallantoic membrane (CAM) assay

The CAM assay was carried out as previously described (Zhao, Miao, Zhao, Zhang & Yin, 2005). In brief, fertilized eggs were incubated in a humidified atmosphere at 37 °C. On the seventh day, a window (~2 cm in diameter) was opened on the air space end of the egg shell to expose a part of the CAM. Different concentrations of CHOS samples in 50 μl PBS were added to sterilized gelatin sponges (4 mm^2) that were placed on the CAM. PBS and

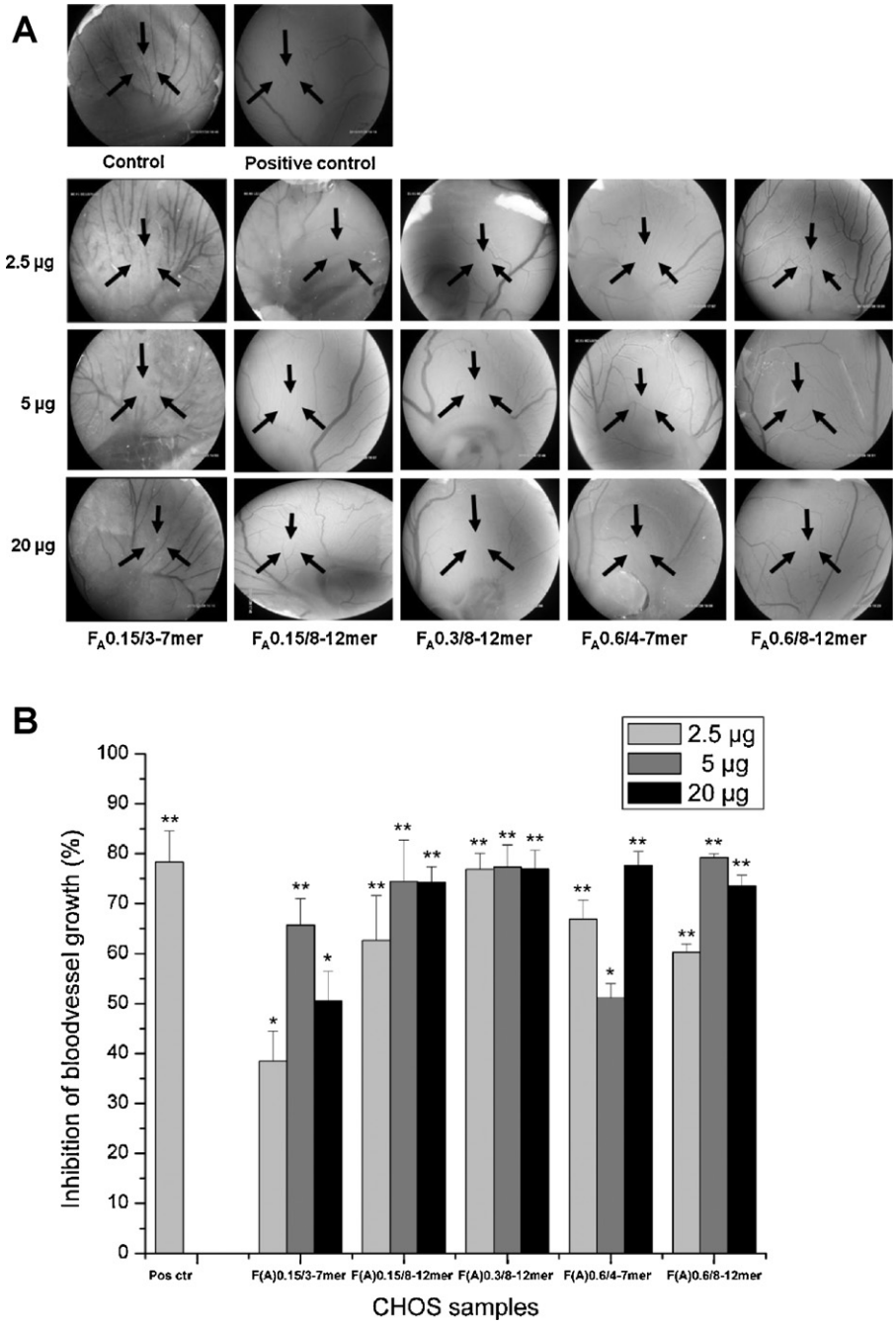

Fig. 3. Effect of CHOS on CAM angiogenesis. CAM were treated with different amounts of CHOS for 48 h and photographed. The total amount of CHOS added to each egg was 2.5, 5 or 20 μg. (A) Show examples of the CAMs after various treatments. The arrows show the position of the gelatin sponge. Control is CAM treated with PBS; the positive control is CAM treated with 500 μg hydrocortisone. (B) Shows the inhibition of vessel growth. The neovascular area of the CAM under the sponges was analyzed by Microsoft AutoCAD. The area of the blood vessels under the sponges in the negative control CAM was regarded as 100%. Statistical analysis was performed by Student's *t*-test. $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. control.

hydrocortisone (5 mg/mL, Jinyao Co. Ltd., Tianjin, China) were used as negative and positive controls respectively. The windows were covered with parafilm, and the eggs were incubated for additional 48 h at 37 °C. 50 μl of the same CHOS samples were added again onto the gelatin sponge after 24 h of incubation. The final doses of CHOS were 2.5, 5 and 20 μg/egg. Minimum 8 eggs were used for each CHOS concentration. The neovascular zones in the CAM under the sponges were photographed under an inverted microscope (Leica, Germany) and the pictures were analyzed by the Microsoft Auto CAD (Autodesk, US). This allowed quantification of vessel growth by computing the area of new vessels under the sponges. The vessel growth inhibiting of the positive control and the different CHOS groups were calculated. The area of blood vessels in the negative control was set to 100% vessel growth/0% inhibition of angiogenesis.

2.5. Cell proliferation assay

The effect of CHOS on the proliferation of normal and HBC medium-induced ECV304 cells was determined with the 3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) colorimetric assay (ATCC, VA, USA).

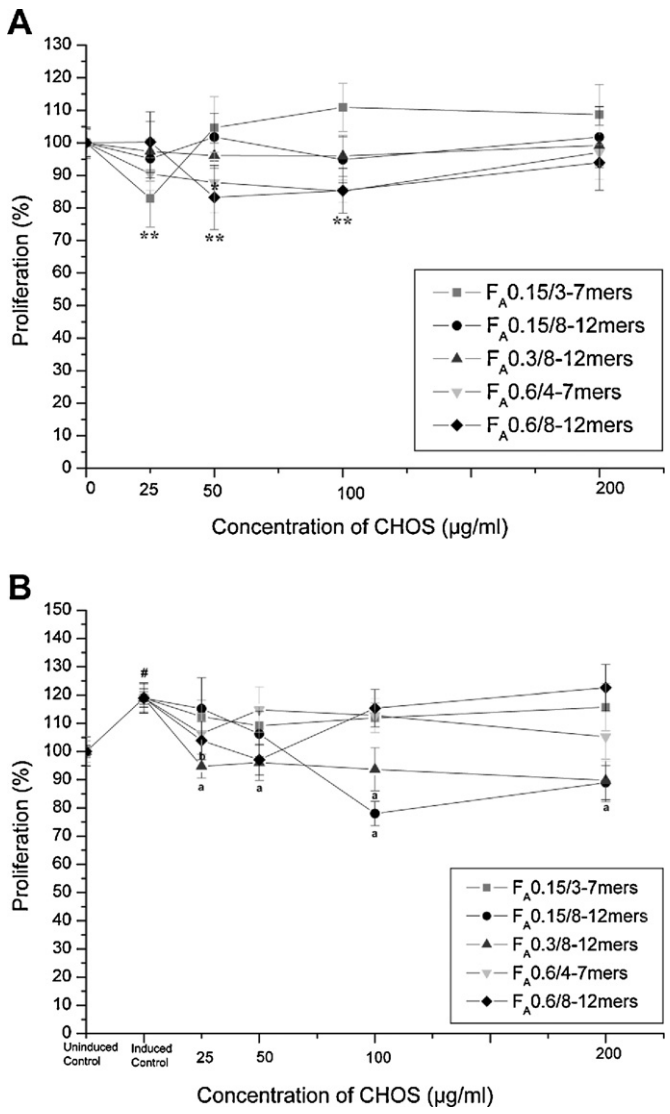

Fig. 4. CHOS effects on cell viability of normal ECV304 cells (A) and HBC medium-induced ECV304 cells (B). Different concentrations of CHOS were applied to the cells for 24 h and cell viability was assayed by the MTT assay. Statistical analysis was performed by Student's *t*-test. In figure (A), $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. non-induced ECV304 cells with no added CHOS. In (B), $^{\#}p < 0.01$ vs. non-induced ECV304 cells with no added CHOS; $^{a}p < 0.01$ and $^{b}p < 0.05$ vs. induced ECV304 cells with no added CHOS.

First, the effect of the CHOS samples on normal ECV304 cell was determined. In brief, ECV304 cells were seeded in 96-well flat-bottomed culture plates (6×10^3 cells per well) and cultured for 24 h. The medium was then replaced with 200 μl new medium containing CHOS. The final concentrations of CHOS were 25 μg/mL, 50 μg/mL, 100 μg/mL and 200 μg/mL. After incubation for another 24 h, 20 μl 5 mg/mL MTT solution was added to each well, and plates were incubated for another 4 h. After removal of the medium, 100 μl dimethyl sulfoxide (DMSO) was added to each well to dissolve the purple crystals of MTT. The absorbance was measured using an ELISA micro plate reader (Rayto instrument, US) at 570 nm, measuring the intracellular purple formazan that was formed. Six replicates were used for each concentration of CHOS tested.

Second, the effect of the same CHOS samples on the proliferation of HBC medium-induced ECV304 cells was tested. The procedure was as described above, but in addition 5% (v/v) HBC medium was added to each well. Six replicates were used for each concentration of CHOS tested.

2.6. Cell migration assay

To assess the activity of single fractions of CHOS derived from the $F_A0.3$ chitosan on migration of HBC medium-induced ECV304 cells, the "scratch wound" assay was performed (Sato & Rifkin, 1988). ECV304 cells were seeded (2×10^5 cells per well) in a 24-well culture plate. Once the cell had grown to confluence, the cell monolayer was interrupted with a 0.5 mm cell scraper, washed twice with PBS, and then further incubated for 24 h in RPMI-1640 medium with or without 5% HBC culture fluid (induced and non-induced controls respectively), or in RPMI-1640 medium with 5% HBC culture fluid containing single oligomeric fractions derived from the $F_A0.3$ chitosan (3mer–12mer; 25 μg/mL, 50 μg/mL, 100 μg/mL and 200 μg/mL). The migration of the cells was photographed under an inverted microscope (Leica, Germany). The number of cells migrating to the clear area in the induced control sample was regarded as 100%, *i.e.* no inhibition of migration. The inhibition of migration in other samples was calculated by expressing the number of migrated cells as a percentage of the maximum number. Six replicates were used for each concentration tested.

3. Results

3.1. Production, separation and characterization of CHOS

The chitosans with $F_A0.15$ and $F_A0.3$ were hydrolyzed with the chitosanase ScCsn46A from *Streptomyces coelicolor* A3(2) to $\alpha = 0.22$ and 0.25 respectively. The $F_A0.6$ chitosan was enzymatically hydrolyzed by ChiB from *Serratia marcescens* to $\alpha = 0.19$. ^{1}H NMR spectra of the product mixtures at the point of termination of the reaction are shown in Fig. 1A. The SEC chromatograms showing the distribution of CHOS in each of the three samples are shown in Fig. 1B.

$F_A0.3$ DP3–12 fractions were analyzed with matrix-assisted laser desorption ionization–time of flight mass spectroscopy (MALDI-TOF-MS) on a Bruker instrument to confirm the peak annotations in Fig. 1B. Fig. 2 shows the DP7–DP10 fractions as an example. Fig. 2 clearly shows that the fractions tested here indeed mainly contain oligomers of the expected length. The spectra also show that, as expected, there is considerable heterogeneity among the oligomers in terms of composition (clearly visible in Fig. 2) and sequence (not analyzed). Considering the results of a previous in-depth characterization of the cleavage specificity of ScCsn46A (Heggset et al., 2010), the large majority of the reducing end sugar residues in the oligomeric fractions are GlcN. This is confirmed by the ^{1}H NMR analysis (Fig. 1A) where D-unit α is the major reducing end signal for both of the chitosans hydrolyzed by ScCsn46A.

3.2. Effect of CHOS on CAM angiogenesis

To determine whether the different fractions of CHOS could suppress blood vessel formation *in vivo*, we employed an *in vivo* angiogenesis model, the chicken CAM assay. The ability of different concentrations of different fractions of CHOS to inhibit CAM angiogenesis is shown in Fig. 3. The blood vessels under the gelatin sponge grew productively in the negative control group (no inhibitor present), whereas almost no blood vessels were observed under the gelatin sponge in the positive control group (addition of hydrocortisone). The growth of blood vessels, in the neovascular area under the sponges, of the negative control was regarded as 100% growth (*i.e.* 0% inhibition of blood vessel growth/angiogenesis), and the degree of inhibition was calculated by expressing observed neovascular areas relative to this

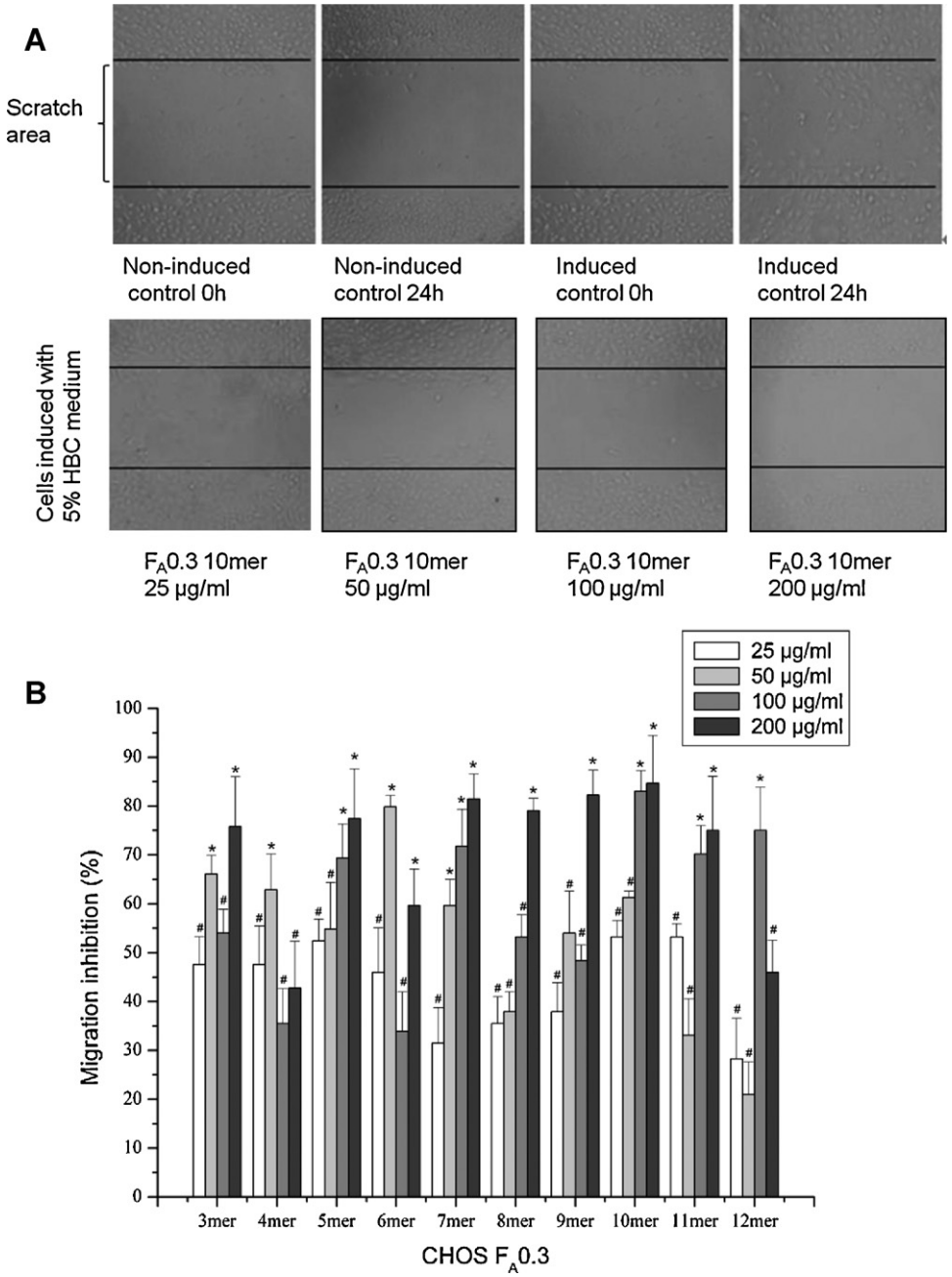

Fig. 5. Effect of individual CHOS fractions from hydrolyzed F_A0.3 chitosan on migration of HBC medium-induced ECV304. An ECV304 cell monolayer was interrupted by a cell scraper and subsequently incubated with RPMI-1640 medium (non-induced control), with RPMI-1640 medium containing 5% HBC culture medium (induced control), or with RPMI-1640 medium containing 5% HBC medium added different concentrations of single CHOS fractions, for 24 h. (A) Shows micrographs for the controls (upper row) and induced cells treated with various dosages of the 10mer fraction (lower row). (B) Show the inhibition of migration for all tested CHOS fractions. The migration of the cells in the induced control after 24 h, was regarded as 100%. Migration inhibiting of the different CHOS single fractions on induced ECV304 cells were calculated. Statistical analysis was performed by Student's *t*-test. $^{*}p < 0.05$ and $^{\#}p < 0.01$ vs. induced control.

100% value. Growth inhibition in the positive control was 79(±6)%. Almost all of the CHOS fractions tested had inhibitory activity on the blood vessel formation in CAM. The weakest inhibitory activity was seen for the F_A0.15/3–7mers fraction, where vessel growth inhibition was 39(±6)%, 64(±5)% and 51(±6)% for the 2.5 μg, 5 μg and 20 μg dosages, respectively. The highest inhibitory activity was seen for the F_A0.3/8–12mers fraction, where vessel growth inhibition was similar to that in the positive control even at the lowest tested concentration (77(±3)%, 78(±4)% and 77(±4)% for the 2.5 μg, 5 μg and 20 μg dosages, respectively). In this case, almost no blood vessels are visible under the gelatine sponge (Fig. 3).

3.3. Influence of different fractions of CHOS on the proliferation of ECV304 cells and HBC medium-induced ECV304 cells

The MTT method was used to evaluate the influence of the CHOS samples on the proliferation of both ECV304 cells and HBC medium-induced ECV304 cells. The proliferation of normal ECV304 cells was set to 100%. As shown in Fig. 4A, the effects of CHOS on growth of non-induced ECV304 cells were generally small. F_A0.15/8–12mers and F_A0.3/8–12mers showed no effects and the effect of F_A0.15/3–7mers was irregular. The F_A0.6/4–7mers and F_A0.6/8–12mers seem to inhibit the proliferation of ECV304 cells

to some extent but the effects are small and only significant at the lower two of the three tested dosages. Fig. 4B shows the effect of the CHOS samples on growth of HBC medium-induced ECV304 cells. As expected, addition of the HBC medium promoted proliferation of ECV304 cells (119%). All of the CHOS samples inhibited proliferation at least at some of the tested dosages, except for the F_A0.15/3–7mers which had no effect (as in the CAM assay). Several of the CHOS did not show convincing dose–response effects, and from the data in Fig. 4 we conclude that only two CHOS samples, F_A0.3/8–12mers and F_A0.15/8–12mers, show convincing inhibition of proliferation, with levels of about 90% as compared to 119% for the non-inhibited cells.

3.4. Effects of individual CHOS fractions on migration of HBC medium-induced ECV304 cells

Since the F_A0.3 sample gave the best effects against angiogenesis in the CAM experiments, we investigated the effects of individual CHOS fractions derived from the F_A0.3 chitosan (Fig. 1B) on HBC medium-induced ECV304 cell migration, using the "scratch wound" assay. The results are shown in Fig. 5. The upper row of panels in Fig. 5A shows that induction of the ECV304 cells with HBC medium leads to massive migration of cells to the scratch area, whereas the lower row shows an example of how a CHOS fraction was able to inhibit such migration. The migration observed without any CHOS present was set to 100% migration (or 0% inhibition migration inhibition) and the inhibitory effect of each CHOS fraction tested (F_A0.3 DP 3–12) was expressed by calculating the relative numbers of migrated cells. All CHOS fractions suppressed migration of the induced cells, albeit to different extents (Fig. 5B). The decamer was among the most effective inhibitors and panel A shows the microscope pictures for this CHOS fraction. Migration of the cells was inhibited 53(±3)%, 61(±1)%, 83(±4)% and 85(±10)% by 25 μg/mL, 50 μg/mL 100 μg/mL and 200 μg/mL of the F_A0.3–10mer fraction, respectively.

4. Discussion and conclusions

The anti-angiogenic activity of CHOS depends on F_A and DP, but it has so far been difficult to map this dependency in detail. One challenge is to produce well-defined and contaminant-free CHOS; another challenge lays in the fact that the bio-assays that are needed to assess anti-angiogenic activity are not straightforward and endowed with relatively large error margins. In the present study, we have used purified relatively well-defined CHOS fractions and tested in several bio-assays.

CAM is perhaps the most widely used *in vivo* vessel development model. The result of the CAM experiments with multiple DP CHOS mixtures in this study reduced vessel formation for all tested CHOS fractions. The data indicated that the F_A0.15/3–7mers sample was among the least powerful, whereas F_A0.3/8–12mers fraction showed the most powerful inhibiting effect on the angiogenesis *in vivo*. The inhibiting power of the F_A0.3/8–12mers fraction was equal to the activity of the positive control for inhibition (hydrocortisone).

According to the tumor angiogenesis theory, the activation of vascular endothelial cells is an early key step in angiogenesis. The activation of endothelial cells is initiated by the binding of pro-angiogenic factors (Bouis, Kusumanto, Meijer, Mulder & Hospers, 2006), and cancer cells often secrete such factors. Therefore culture fluid of MCF-7 human breast cancer cells (HBC) was used to induce angiogenic behavior in ECV304 cells. Indeed, Figs. 4 and 5 show that HBC medium did induce such behavior (proliferation and migration, respectively), in accordance with the results of previous studies (Wu, Yao, Bai, Du & Lin, 2008).

Although the effects of the MTT proliferation assay generally were small, the results of this assay (Fig. 4) did confirm that the F_A0.3/8–12mer fraction was among the most interesting CHOS mixtures tested in this study. This fraction, as well as the F_A0.15/8–12mer fraction was able to offset the proliferation of endothelial cells that is induced by HBC-medium, in a concentration dependent manner.

Combining the results of the CAM and MTT experiments, we focused on the F_A0.3 sample and the effects of single CHOS fractions (*i.e.* one defined DP) derived from the F_A0.3 chitosan on induced ECV304 cell migration were studied. All of the samples (DP3-DP12) tested had an inhibitory effect on the migration, but did not reveal a relationship between the magnitude of the effect and the DP.

In conclusion, our studies clearly show the anti-angiogenic potential of CHOS and they provide a rare example of CHOS bioactivities being demonstrated with highly pure samples. Overall, the effect of CHOS is very clear, and the data also clearly indicate that the inhibition of angiogenesis seen in Fig. 3 is primarily due to inhibition of migration (Fig. 5), whereas inhibition of cell proliferation (Fig. 4) seems to play a secondary role. This conclusion is in accordance with conclusions drawn in previous studies (Wang, Zheng, Yang, Niu, Chu & Lin, 2007; Wu, Yao, Bai, Du & Lin, 2008; Xiong et al., 2009). Somewhat surprisingly, our results show relatively small variations in the effects caused by the DP and the F_A of CHOS, although the CAM assay, and to some extent also the proliferation assay, do indicate that an F_A0.3 is favorable for obtaining the best effects. The effects of DP (Fig. 5) seem less clear than the effects of F_A, although the decamer does seem to be the most active in the experiment that was done. It would probably be useful to address the DP issue also in future studies; many additional F_A/DP combinations could be tested. From a practical point of view, it is perhaps encouraging that the DP does not seem to be that important in the DP-interval tested in this study (DP3-DP12). If most DPs have a reasonable effect, the need to carry out CHOS purifications becomes less pressing and mixtures instead of pure CHOS may be applied. This will of course reduce the production costs of these highly interesting bioactive compounds.

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