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Biocatalytic Synthesis of Water-Soluble Oligo(catechins)

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Catechins are polyphenolic compounds found in green tea. These flavonoids possess anti-oxidant, anti-carcinogenic, and anti-inflammatory properties. The primary goal of this work is to synthesize water-soluble oligomers of catechins. Although, oxidative polymerization of catechins catalyzed by enzymes such as Horseradish Peroxidase (HRP) have been reported, the polymeric products have limited solubility. This restricts their compatibility with biological systems and their effectiveness as antioxidants or anti-carcinogenic agents. Here we report a unique enzymatic approach for the synthesis of water-soluble oligo(catechins). Polyelectrolytes such as Sulfonated Polystyrene (SPS) and surfactants such as sodium dodecylbenzenesulphonate (SDS) have been used as templates for the HRP catalyzed polymerization of (+)-, (−)-catechins and (−)-epicatechin. Oligocatechins synthesized exhibit enhanced anti-tumorigenic activity to human colon cancer cells as compared with the monomeric catechins.

Keywords enzymatic polymerization, oligo (catechins), cancer therapy, template-assisted polymerization, surfactants

Introduction

Natural products such as green tea have attracted a great deal of attention in the last decade because of its potent anti-oxidant properties and distinct anti-cancer activity (1) Catechins are important constituents of green tea. The green tea flavonols are rich in phenolic antioxidants, which are believed to be responsible for the chemoprotective nature of the tea extracts. The major polyphenolic constituents found in green tea are (−)-epicatechin, (+)-catechin, (−)-catechin, (−)-epicatechin gallate, (−)-epigallocatechin gallate (EGCG) and (−)-epigallocatechin. Commercially prepared green tea extracts are standardized to contain 50–60% polyphenols. Recent studies have revealed that the naturally occurring polyphenolic catechins inhibit some forms of

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breast cancer cell proliferation (2) and tumor growth and prevent recurrence of some forms of breast cancer in women (3). Green tea polyphenols have also been used as chemopreventive agents for skin cancer (4) and for the reduction in colon cancer (5) and are believed to reduce the risk of cancer of the pancreas, rectum (6)and lungs (7). These flavonoids provide tremendous opportunity as eco-friendly starting materials that can be enzymatically modified to yield a range of oligomeric flavonoids with potentially higher antioxidant/anticancer activity.

Here we report a unique and simple one-pot synthesis of water-soluble oligomeric catechins that exhibit enhanced stability than the naturally occurring monomers. This novel approach involves the use of different stereoisomers of catechin, isolated from natural sources (green tea) that are polymerized using oxidoreductases like Horseradish Peroxidase (HRP) in biocompatible polyelectrolytes such as Sulfonated PolyStyrene (SPS). The polymerization can also be carried out in aqueous micellar solutions of anionic and neutral surfactants such as sodium dodecylbenzenesulfonate, (SDS) and polyoxyethylene(10) isooctylphenyl ether (Triton X-100), respectively.

Experimental

Materials

Horseradish peroxidase (HRP, EC 1.11.1.7) type II, 150–200units/mg solid was purchased from Sigma chemicals Co. (St. Louis, MO). All catechin monomers were also purchased from Sigma Chemical Co. (St. Louis, MO). SPS, sodium dodecylbenzenesulfonate, (SDS) and hydrogen peroxide (30 wt%) were purchased from Aldrich Chemicals Inc., Milwaukee, WI and were used as received. All other chemicals were purchased from Aldrich and were of reagent grade or better. UV-Visible spectra were obtained using a Perkin-Elmer Lambda 9 UV-Vis-near-IR spectrophotometer. The fluorescence spectra were obtained on a Perkin-Elmer LS 55 Luminescence spectrometer.

Procedure for Synthesis of Oligo(catechins)

All oligomerization reactions were carried out enzymatically at room temperature, in 10 mM sodium phosphate buffer. 28 mg of SDS was dissolved in sodium phosphate buffer solution at pH 7. (−)-Catechin monomer (10 mg, 3.44 mM) was added to this solution. This was followed by the addition of 4 mg of HRP. The polymerization was initiated by the addition of 1021 μl of 0.3% hydrogen peroxide. The hydrogen peroxide was carried over in small aliquots (100 μl each) over a period of one hour to prevent inhibition of HRP activity. The formation of Oligo(catechins) was monitored using UV-Visible Spectroscopy. A similar procedure was adopted for the polymerization of the other stereoisomers of catechin. The anti-tumorigenic activity of the oligocatechins was studied several weeks after the synthesis of these oligomers.

Proliferation Assay

All samples were surface sterilized by dissolution/suspension in a very small volume of absolute ethanol, which was subsequently diluted with aqueous culture medium. Further serial dilutions in medium were then prepared over the concentration ranges indicated within parentheses Oligo(−)-Catechin/SPS (50 pg/ml–516 μg/ml), Oligo(±)catechin/SPS (25 pg/ml–250/μg/ml), Oligo(−)-Catechin/SDS (50 pg/ml–501 μg/ml). Cells

from a human colon cancer cell line were cultured in 96-well plates and exposed to the individual test compounds for either 22–24 or 46–48 h, after which the viability of the cells was assessed by uptake of a supra-vital dye, followed by cell lysis and measurement of optical densities using an automated plate reader. The technique allows comparison, within the same plate, of the number of surviving cells following incubation with or without the test compound, and permits a high number of replicates. It does not, however, measure changes in mitosis or apoptosis, but is very useful in the initial screening of potential modifiers of cell proliferation.

Results and Discussion

Oligocatechins Synthesized in the Presence of Polyelectrolytes

Previous work on the polymerization of catechins has only yielded water-insoluble polymers (8). Further, in these cases the reactions were carried out in toxic solvents such as methanol. For ease of deliverability into biological systems, it is imperative that the polymerization is carried out under benign conditions using biocompatible catalysts. In the method described here, a typical reaction to synthesize the oligo(catechins) occurs in aqueous media buffered at pH 7, with the monomeric catechin, the template/micellar environment and a catalytic amount of the enzyme and hydrogen peroxide. Figure 1 shows UV-Visible spectra of (−)catechin monomer and (−)-catechin/SPS complex as well as the fluorescence spectra of the oligomer synthesized at pH 7. As seen in the figure, the monomer shows significant absorption in the range of 250–300 nm and no absorption beyond 300 nm. The initiation of polymerization by the addition of H_2O_2 leads to the appearance of a broad absorption peak in the 325–550 nm range with a maxima around 390 nm. While the monomer solution was

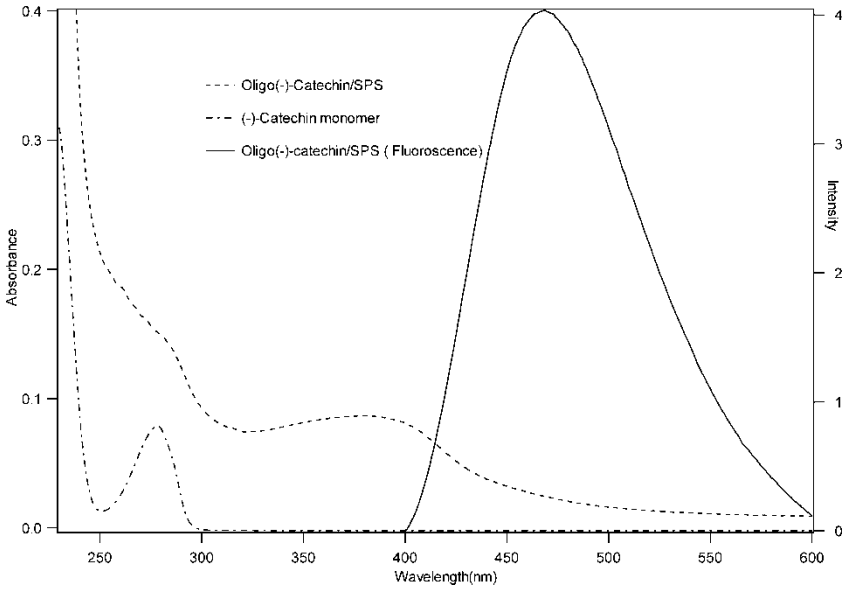

Figure 1. The UV-Visible and fluorescence spectra for oligomerization of (−)-catechin using SPS as a template.

colorless, the oligomerization resulted in the formation of dark-red-brown solution. The fluorescence spectrum indicates a broad emission with a maximum centered at 467 nm. To the best of our knowledge, the fluorescence spectra of oligomeric catechins have not been reported to date.

Oligomerization of (−)-Epicatechin in Micellar Solution

Oligomerization of (−)-epicatechin was carried out in anionic surfactants such as SDS (Figure 2), as well as non-ionic biocompatible surfactants like Triton X-100 (Figure 3). The UV-Visible and fluorescence spectra of the oligomerized (−)-Epicatechin/SDS complex are shown in Figure 2. In the case of (−)-epicatechin polymerized in a micellar solution of SDS, the reaction yielded oligocatechins with a broad UV-vis absorption in the range of 300 to 550 nm. (−)-epicatechin monomer is known to exhibit a broad fluorescence spectrum with maximum around 321 nm while the dimer of (−)-epicatechin has been known to exhibit a fluorescence in the region of 300–340 nm (9). However, the enzymatically synthesized oligomeric (−)-epicatechin shows a broad featureless fluorescence with emission maximum centered around 491 nm. The absorption in the 300–550 nm range combined with the fluorescence emission in 491 nm seems to suggest that these oligomeric catechin compounds could have conjugated structures.

In order to investigate if a macromolecular template or a micellar environment was absolutely essential to solubilize the oligomeric catechin, the oligomerization of (−)-epicatechin reaction was also carried out in the presence of small molecules such as *p*-toluenesulphonic acid.

It was possible to obtain water-soluble oligo(epicatechin) even in the absence of a macromolecular template. In fact a comparison of the UV-Vis spectra of the oligomers synthesized using identical concentrations of monomers in the presence of PTSA and

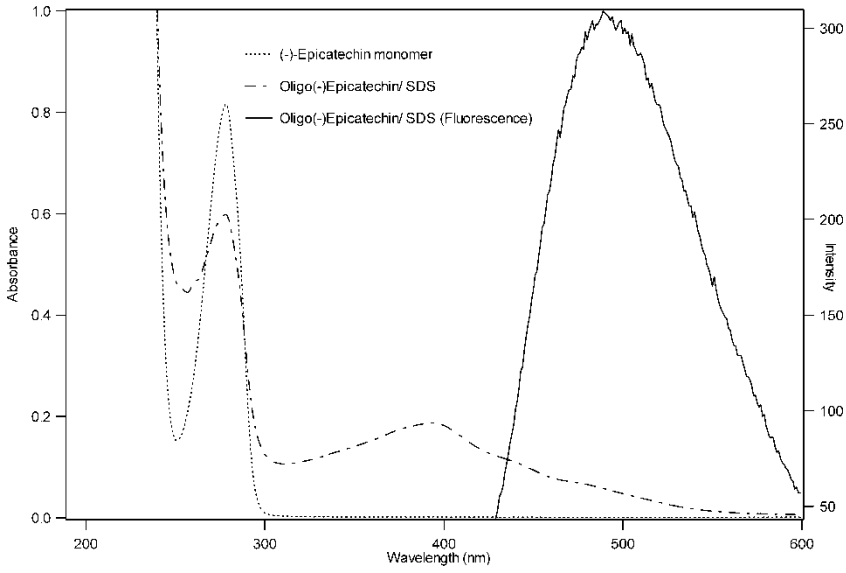

Figure 2. The UV-Visible and fluorescence spectra for oligomerization of (−)-epicatechin using SDS as a template.

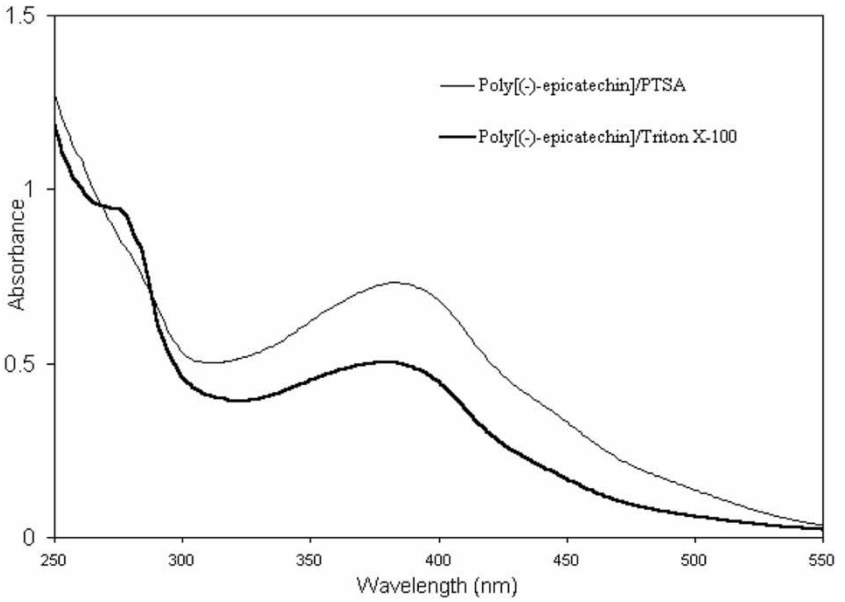

Figure 3. The UV-Visible spectrum of oligo(−)-epicatechin synthesized using PTSA and neutral surfactant Triton X-100 as templates.

Triton-X seems to indicate that the concentration of oligomers formed in the presence of PTSA is higher. While Triton-X is a non-ionic surfactant, PTSA has considerable ionic character in the pH conditions in which the reaction is carried out. The nature of interaction between the catechins with various templates (polyelectrolytes/surfactants/small molecules) and effect of ionic/micellar environment on the formation of oligomeric products are currently being investigated.

Anti-cancer Studies

The most promising and widely studied polyphenol among the green tea flavonoids is Epigallocatechin gallate (EGCG) for its potent anti-tumorigenic applications (10). However, there have been very few studies on the efficacy of the various stereoisomers of catechin [(+)-, (−)-, (±)-] on cancer cells. These monomeric catechins do not exhibit significant anticancer activity as compared to EGCG. We have tested the inhibitory effect of these oligocatechins on the proliferation of human colon cancer cells.

The thresholds for an effect on viability were 5 μg/ml for Oligo(−)-Catechin/SDS, 25 μg/ml for Oligo(±)-Catechin/SPS and 50 μg/ml for Oligo(−)-Catechin/SPS. The most effective compound was Oligo(−)-Catechin/SDS which reduced viability by 88 and 93% at 24 and 48 h, respectively (Figure 4) at the highest concentration tested.

In contrast, Oligo(−)- Catechin/SPS had no effect at 24 h, but reduced the viability of cells by 66% after 48 h exposure (Figure 5).

The Oligo (±)-Catechin/SPS was synthesized using the similar procedure outlined above and caused a small reduction (21%) in cell viability after 24 h incubation, which was enhanced to 48% after an additional 24 h (Figure 6). This paper reports the first step in the investigation of the enzymatic synthesis of oligo(catechins) and their

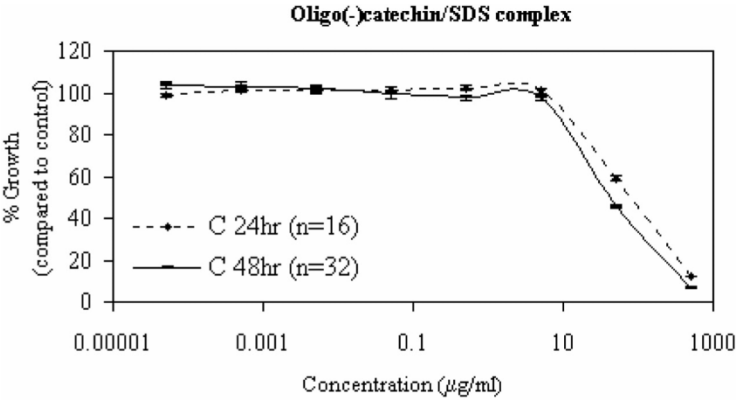

Figure 4. Efficacy of oligo(−)-catechin/SDS complex in suppressing cell viability.

effectiveness as anticancer agents. Further detailed investigations are now underway to study the growth inhibitory effects of the oligo(catechins) on different types of cancer cells (human breast cancer cells) as well as normal epithelial cells.

Conclusions

A novel method for the enzyme-catalyzed synthesis of water-soluble oligomers of catechins and (−)-epicatechin is presented. The reaction was carried out either in the presence of macromolecular templates such as SPS or in aqueous micellar solutions of anionic surfactants or neutral surfactants. (−)Epicatechin was also oligomerized in the presence of *p*-toluene sulfonic acid to yield water-soluble oligo(epicatechin). The macromolecular templates and micellar solutions impart water-solubility to the final oligomeric complex. These oligomeric catechin complexes show inhibitory effect for the proliferation of colon cancer cells. Oligo(−)-Catechin/SDS showed the highest activity with significant effect on cell viability at a dosage as low as 5 μg/ml. The inhibitory effects of

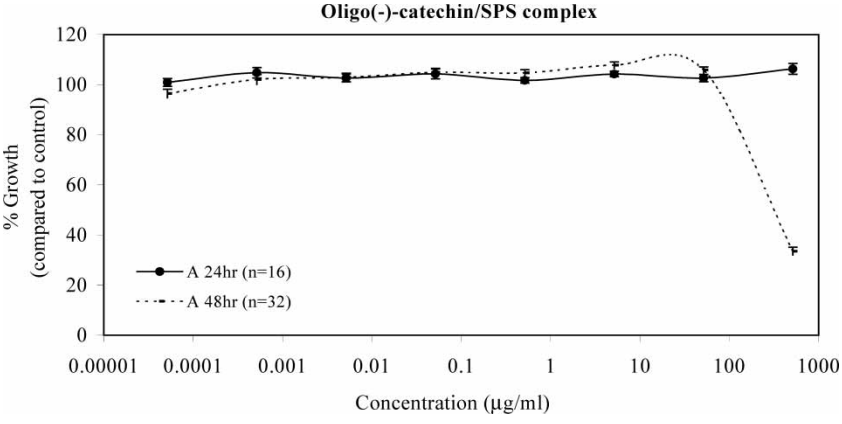

Figure 5. Efficacy of oligo (−)-catechin/SPS complex in suppressing cell viability.

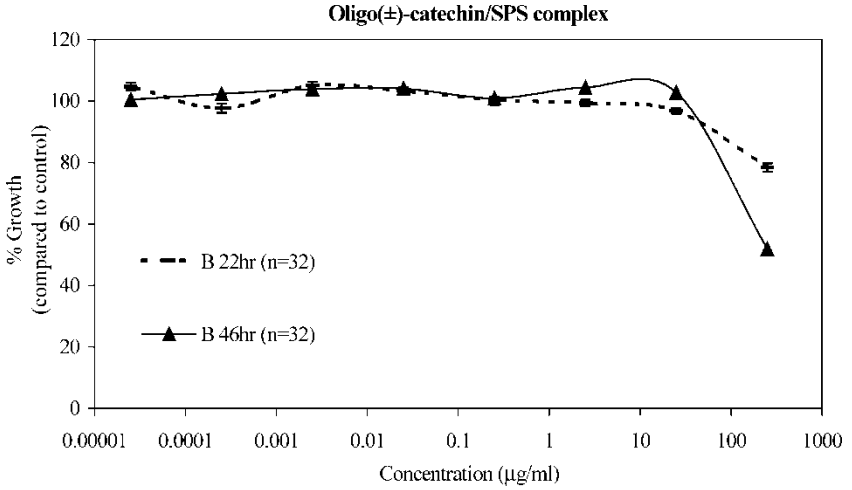

Figure 6. Efficacy of oligo (±)-catechin/SPS complex in suppressing cell viability.

oligo(epicatechins) are being investigated. Further investigations are underway to understand the role of the polyelectrolyte templates and micellar media in the formation of the oligomers and their effect on solubility of the final oligomeric products. This class of enzymatically oligomerized, naturally occurring green tea flavonoids show tremendous promise as anticancer agents.

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