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Role of green tea polyphenols in the inhibition of collagenolytic activity by collagenase

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Abstract

Inhibitory effect of green tea polyphenols viz., catechin and epigallocatechin gallate (EGCG) on the action of collagenase against collagen has been probed in this study. Catechin and EGCG treated collagen exhibited 56 and 95% resistance, respectively, against collagenolytic hydrolysis by collagenase. Whereas direct interaction of catechin and EGCG with collagenase exhibited 70 and 88% inhibition, respectively, to collagenolytic activity of collagenase against collagen and the inhibition was found to be concentration dependent. The kinetics of inhibition of collagenase by catechin and EGCG has been deduced from the extent of hydrolysis of (2-furanacryloyl-L-leucyl-glycyl-L-prolyl-L-alanine), FALGPA. Both catechin and EGCG exhibited competitive mode of inhibition against collagenase. The change in the secondary structure of collagenase on treatment with catechin and EGCG has been monitored using circular dichroism spectropolarimeter. CD spectral studies showed significant changes in the secondary structure of collagenase on treatment with higher concentration of catechin and EGCG. Higher inhibition of EGCG compared to catechin has been attributed to the ability of EGCG to exhibit better hydrogen bonding and hydrophobic interaction with collagenase.

Keywords: Collagenase; *N*-(3-[2-Furyl]acryloyl)-Leu-Gly-Pro-Ala (FALGPA); Collagen; Polyphenols

1. Introduction

The plant polyphenolic compounds are one of the secondary metabolites present in various plant parts and they are important components of both human and animal diets [1–3]. The structures of natural polyphenols vary from simple molecules, such as phenolic acids, to highly polymerized compounds, such as condensed tannins [4]. These compounds contain sufficient hydroxyls and other suitable groups such as carboxyls to form strong complexes with proteins and other macromolecules [5]. They have an ideal structure for free radical-scavenging activities, and have been shown to be more effective antioxidants in vitro than vitamins E and C on a molar basis [6].

Catechins are a group of bioflavonoids that exhibit powerful antioxidant property in a number of biochemical systems [7]. Chemically, catechins are polyhydroxylated with water soluble characteristics that differ in the number and position of the hydroxyl groups in the molecule [8]. It has long been postulated that due to such polyhydroxylated structure, catechins could act as antioxidant either through the chelation of metals with redox properties or by acting as scavengers of free radicals [9,6]. Catechins interfere with several stages of the inflammatory process involved in atherosclerosis [10,11] and may influence hemostatic indexes and reduce thrombosis [12]. EGCG is a major component of the polyphenolic fraction of green tea and they exhibit antioxidant, antitumor and antimutagenic activities [13–15]. It is shown that green tea polyphenols are non-toxic to human chondrocytes and inhibits the expression of inflammatory mediators in arthritic joints [16,17]. EGCG have been demonstrated to inhibit matrix metalloproteinase-2 (MMP-2) (also known as gelatinase A) and matrix metalloproteinase-9 (MMP-9) (also known as gelatinase B) and some of these enzymes has been known to play an important role in tumor invasion and metastases [18–21]. The crude tea catechins were tested for their ability to inhibit the prokaryotic and eukaryotic cell derived collagenase activities. Preincubation of collagenase with tea catechins reduced the collagenase activity as well. The collagenase activity in the gingival cervicular fluid from highly progressive adult periodontitis was inhibited by the addition of tea catechins [22].

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The most abundant protein found in mammalian tissues is type I collagen. It is the main structural protein of skin, bone and tendon [23]. Collagen provides structural integrity acting as a scaffold, a matrix, upon which other cells can proliferate. Collagen is also known to have a role in the control of cell shape and differentiation, migration, and the synthesis of a number of proteins. Collagen as a protein has a distinguishing feature, each molecule has a coiled coil structure with three polypeptide chains, wound together to form a triple helix [24,25]. Collagen is also an important biomaterial finding widespread applications in fields such as surgical sutures, cosmetics, wound healing and leather making. It constitutes more than 90% of the total solid matrix of the skin and this protein is stabilized into leather during tanning processes. Native collagen is susceptible to attack only by collagenase at physiological pH, temperature and ionic strength [26,27]. Collagen molecule is susceptible to attack by other proteases only after initiation of cleavage of the triple helix by collagenase [28]. Mammalian collagenase is highly specific, cleaving collagen at the Gly-Ile (772–773) bond in the α-chain [29]. Bacterial collagenase, on the other hand, exhibits less specificity and cleaves collagen predominantly at the Y-Gly bond in the sequences of type -Pro-Y-Gly-Pro-, where Y is most frequently a neutral amino acid [30–32].

The use of plant polyphenols for the stabilization of collagen matrix dates back to the history of mankind itself in the form of vegetable tanning. In the process of stabilization of collagen, rendering stability against collagenase is an important aspect. In order to understand the role of polyphenols in the mode and degree of inhibition of collagenase, a study on the interaction of collagenase with green tea polyphenols viz., catechin and EGCG has now been attempted.

2. Materials and methods

2.1. Materials

All reagents and chemicals used were of analytical grade. Catechin, Epigallocatechin gallate (EGCG), collagenase (Type IA) and *N*-[3-(2-furyl)acryloyl]-Leu-Gly-Pro-Ala (FALGPA) were sourced from Sigma Chemicals Co., USA. All other reagents and chemicals used for the study were sourced from SRL Ltd., India.

2.2. Collagenase hydrolysis of polyphnenols treated RTT

Tendons from tails of 6-month-old male albino rats (Wistar strain) were teased and washed with 0.9% NaCl at 4 °C to remove the adhering muscles and other soluble proteins. The rat-tail tendon (RTT) was subsequently washed extensively in double distilled water and treated with 0.02 M catechin and EGCG for 24 h at room temperature (27 °C) without any agitation. After 24 h, the catechin and EGCG treated RTT were stored in water at 4 °C before testing for resistance to collagenase.

The native, catechin and EGCG treated RTT fibres were further treated with collagenase (Type IA) from Clostridium histolyticum. Collagenase treatment was carried out in 0.04 M $CaCl_2$ solution buffered at pH 7.2 with 0.05 M Tris–HCl. The collagen:collagenase ratio was maintained at 50:1. The samples were incubated at a temperature of 37 °C. Samples were collected at various time intervals ranging from 6 to 96 h and stored in freezer. The cleavage of native and treated RTT was monitored by the release of soluble form of hydroxyproline from insoluble collagen [33]. Aliquots of 750 μl of supernatant were withdrawn after centrifuging at 10,000 rpm for 10 min. The collagenase hydrolysate was hydrolyzed in sealed hydrolysis tubes with 6N HCl for 16 h. The hydrolysates were evaporated to dryness in a porcelain dish over a water bath to remove excess acid. The residue free of acid was made up to a known volume and the percentage (%) of hydroxyproline was determined using the method of Woessner [34]. Hydroxyproline is a unique amino acid for collagen and it offers itself as a useful marker for identifying collagen in the presence of non-collagenous proteins. The method of determining hydroxyproline involves the oxidation of hydroxyproline to pyrrole-2-carboxylic acid, which complexes with *p*-dimethylaminobenzaldehyde exhibiting maximum absorbance at 557 nm:

$$\%\ \text{soluble collagen} = \%\ \text{hydroxyproline} \times 7.4$$

Based on the soluble (solubilized due to enzymatic hydrolysis) collagen content in the supernatant solution of the collagenase treated RTT fibres the % degradation of collagen for native, catechin and EGCG treated fibres are calculated as

$$\%\ \text{collagen degradation} = 100 - \left(\frac{\text{initial collagen} - \text{soluble collagen}}{\text{initial collagen}} \times 100\right)$$

2.3. Monitoring of collagen degradation by inhibition of collagenase activity

A known amount of collagenase was incubated with 0, 20, 40, 80 and 160 μM of catechin and EGCG solution for 18 h in 1 ml of 0.1 M Tris–HCl (pH 7.4) containing 0.05 M $CaCl_2$ at 25 °C. Subsequently the RTT collagen fibres were treated with the incubated samples of native, catechin and EGCG treated collagenase. The ratio of collagen:collagenase was maintained at 50:1 and the reaction buffered at pH 7.4 using 0.1 M Tris–HCl and 0.05 M $CaCl_2$. The treated samples were incubated at 37 °C and after 72 h the reaction was stopped and the mixture was centrifuged for 15 min at 10,000 rpm. The supernatant was analyzed for hydroxyproline and % collagen degradation was determined. % Inhibition by polyphenols viz., catechin and EGCG was calculated as differences in the % degradation of RTT collagen treated by native and polyphenol treated collagenase:

$$\%\ \text{inhibition} = \frac{\%\ \text{collagen degradation}_{\text{native collagenase}} - \%\ \text{collagen degradation}_{\text{polyphenol treated collagenase}}}{\%\ \text{collagen degradation}_{\text{native collagenase}}} \times 100$$

2.4. Kinetic investigations on the assay of native collagenase

Clostridium histolyticum collagenase I (ChC) assay using FALGPA as substrate was performed according to the method reported earlier [35]. Assays were carried out spectrophotometrically by continuously monitoring the decrease in absorbance of FALGPA after the addition of ChC. The FALGPA (at concentrations of 0.1–1.6 mM) was taken in appropriate amount of tricine buffer (0.05 M tricine, 0.4 M NaCl and 10 mM $CaCl_2$, pH 7.5) and ChC (100 μl of 0.4 mg/ml) was added and the final volume was adjusted to 1 ml. The course of hydrolysis of FALGPA was monitored using Varian Cary 100 UV–vis spectrophotometer by following the decrease in absorbance at 324 nm when [FALGPA] = 0.1 mM. At higher concentrations of FALGPA viz. (0.2 mM) and (0.4–1.6 mM), the decrease in absorbance were measured at 338 and 345 nm, respectively. An initial rate treatment was adopted by treating the first 10% of hydrolysis according to standard methods [36].

2.5. Kinetic investigations on inhibition of collagenase by polyphenols

The reaction of catechin and EGCG treated ChC with FALGPA was performed under the same conditions mentioned as those employed for the assay of native collagenase in this study. The ChC is treated with varying concentrations viz., 10, 20, 40, 80 and 160 μM of aqueous solution of catechin and EGCG for 18 h at 25 °C. The final concentrations of the ChC in all the treatments are maintained constant (0.4 mg/ml). The FALGPA at concentrations of 0.1–1.6 mM was taken in appropriate amount of Tricine buffer (0.05 M tricine, 0.4 M NaCl and 10 mM $CaCl_2$, pH 7.5) and polyphenol incubated ChC (100 μl) was added and the final volume is adjusted to 1 ml. The hydrolysis of substrate was monitored at the corresponding wavelengths (immediately after the addition of polyphenol incubated ChC) as done in the case of native collagenase. The concentrations of substrate (FALGPA) used were in the range of 0.1–1.6 mM. Rates of hydrolysis were calculated employing initial rate methods. The rate data were analyzed in terms of Michaelis–Menten treatment. From the Linewaver–Burk plots of v^{-1} versus $[S]^{-1}$ the kinetic parameters such as V_{max}, maximum velocity and K_m, the Michaelis–Menten constant of the enzyme were calculated. Initial velocities were calculated from the slope of the absorbance changes during the first 10% of hydrolysis and converted into units of microkatals (μmol/s) by dividing to full hydrolysis and multiplying by the substrate concentration.

2.6. Structural investigations on collagenase–polyphenol binary system using circular dichroism

Circular dichroism spectrum of Type IA collagenase of concentration 0.18 mg/ml in 1 mM acetate buffer (pH 4.0) was acquired at 25 °C using Jasco-J715 spectropolarimeter. The rate of nitrogen purging was maintained at 5 l/min up to 200 nm and increased to 10 l/min below 200 nm. A two-point calibration was done with (+)10 camphorsulfonic acid. The samples were prepared in double distilled water. All the solutions were filtered through 0.25 μm filters to remove suspended particles. A 0.1 cm path length cell was used for the experiments. A slit width of 1 nm was used. Scan speed of 20 nm/min was used with an average of five scans per sample. Each spectrum was corrected by a baseline measured with the same solvent used in the sample. A reference spectrum was recorded with acetate buffer. The conformational changes in collagenase on interaction with catechin and EGCG were investigated after incubating the enzyme with varying concentrations (0.6–90 μM) of catechin and EGCG. The spectra obtained were deconvoluted using G and F and K2D programs and the mean values of secondary structure components were tabulated [37–39].

2.7. Computational details

Catechin and EGCG molecule have been built using builder tools outfitted with Silicon Graphics O2 workstation. Consistent Valence Force Field (CVFF) has been assigned to all atoms of the polyphenolic molecules. The geometry of catechin and EGCG has been minimized using steepest decent method followed by conjugate gradient algorithm. Energy minimized coordinates of catechin and EGCG have been used in determining the contact surface area using Connolly method implemented in Insight II software package.

3. Results and discussion

Plant polyphenolics are known to inhibit collagenase activity [16,19]. It is of profound interest to establish how the green tea polyphenols viz., catechin and EGCG exhibit inhibition to the activity of ChC against collagen. The energy-minimized structures of the polyphenolic molecules catechin and EGCG along with their contact surface areas are shown in Fig. 1a and b, respectively. Both catechin and EGCG can involve in hydrogen bonding interactions with various functional groups of proteins, as they have several hydroxyl groups, which can acts as hydrogen acceptor and donor. EGCG can involve itself with higher non-covalent interactions than catechin as they have greater contact surface and more number of hydroxyl groups.

3.1. Stability of polyphenols treated collagen against collagenase

% Collagen degradation (based on hydroxyproline released) for the native, catechin and EGCG treated RTT by collagenase at various time periods has been determined (Fig. 2). Significant reduction in the degradation of collagen is observed for the fibres treated with the green tea polyphenols viz., catechin and EGCG compared to native RTT. Catechin treated RTT exhibited 44% degradation of collagen as against 93% degradation in the case of native collagen at 96 h period of incubation; whereas EGCG treated RTT collagen exhibited only 5% collagen degradation for the same time period of incubation. Both catechin and EGCG can interact with collagen through hydrogen bonding and hydrophobic interactions. The stability of catechin and EGCG treated collagen fibres against collagenase would have

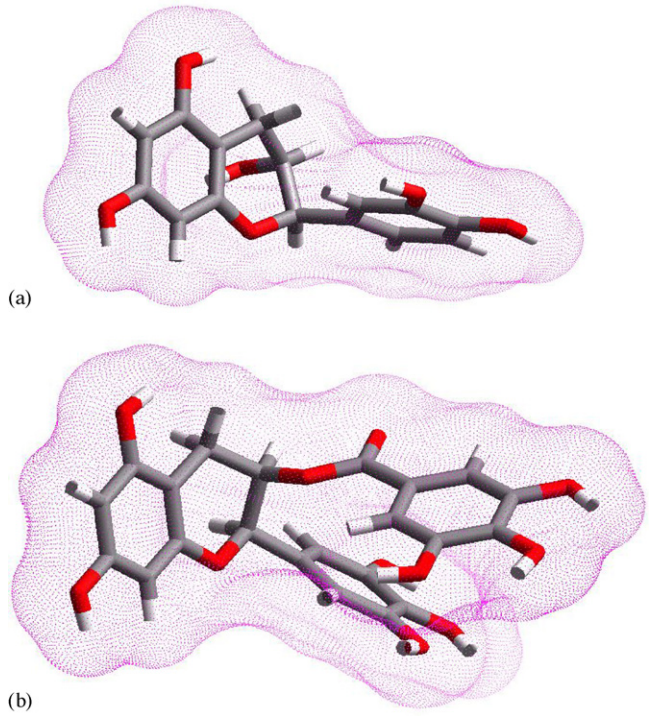

Fig. 1. Canonical surface of energy-minimized structure of green tea polyphenols: (a) catechin and (b) epigallocatechin gallate.

been brought about by protecting the active sites in collagen (through interaction with polyphenols) recognized by collagenase. The significant differences in the enzymatic stability offered by catechin and EGCG could be due to the effectiveness of the later in exhibiting better interaction with collagen

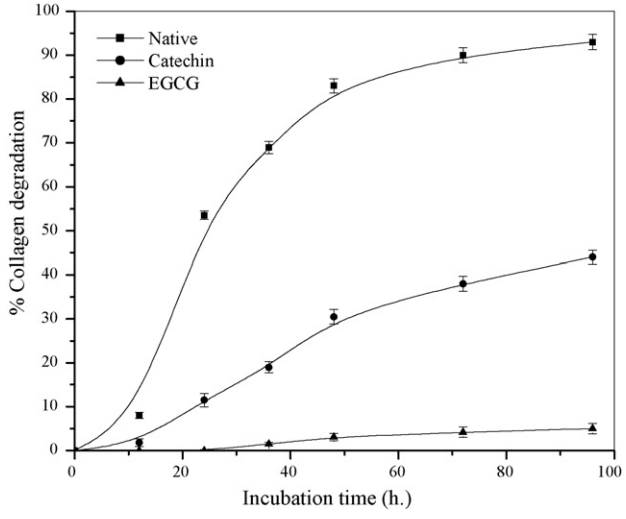

Fig. 2. Plot of % collagen degradation vs. incubation time of collagenase hydrolysis of native, catechin and EGCG treated RTT collagen fibres. Catechin and EGCG treated RTT collagen fibres along with native RTT collagen fibres were further treated with collagenase in 0.04 M $CaCl_2$ solution buffered at pH 7.2 with 0.05 M Tris–HCl. The collagen:collagenase ratio was maintained at 50:1. The samples were incubated at a temperature of 37 °C. The treatment of collagenase to RTT collagen fibres was carried out for different periods of incubation viz., 12, 24, 36, 48, 72 and 96 h. Supernatant of the incubated samples were analyzed for collagen degradation.

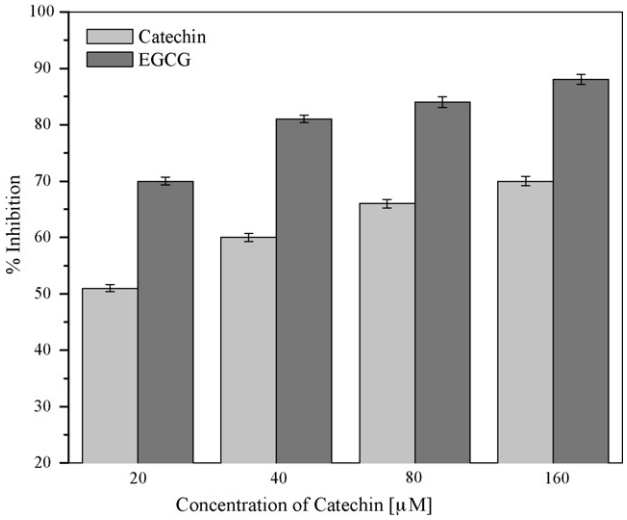

Fig. 3. The percentages of ChC inhibition activities of catechin at concentrations of 10, 20, 40, 80, and 160 μM. The ChC (0.04 mg/ml) were dissolved in 0.05 M tricine buffer (with 0.4 M NaCl and 0.01 M $CaCl_2$, pH 7.5), and preincubated with and without catechin compounds at 25 °C for 24 h. Then, 2 mg RTT collagen fibres was added to the mixture and incubated for 72 h and the collected supernatant analyzed for hydroxyproline and collagen degradation was calculated. % Inhibition calculated as difference in collagen degradation by collagenase samples incubated with and without various concentrations of catechin.

through multiple hydrogen bonded crosslinks. It is important to probe further the ability of these two polyphenols in the direct inhibition of collagenase.

3.2. Inhibition of collagenase by polyphenols against collagen degradation

In order to establish the effect of catechin and EGCG in inhibiting collagenase, RTT collagen fibres treated with native collagenase and collagenase incubated with various concentrations of catechin and EGCG has been studied. EGCG and catechin treatment to collagenase exhibited dose dependent inhibition on the collagenolytic activity against collagen (Fig. 3). EGCG at a concentration of 20 μM exhibited 70% inhibition to collagenase against the degradation of RTT collagen fibres. The inhibition increased with increase in concentration of EGCG and at 160 μM concentration 88% inhibition has been observed, whereas EGCG treatment of RTT collagen exhibited 95.8% inhibition against collagenase hydrolysis (at 72 h). In the case of catechin (at 160 μM) treated collagenase the inhibition to collagen degradation has been observed to be 70% whereas the inhibition has been found to be only 62% when catechin treated collagen fibres had been subjected to collagenase hydrolysis (at 72 h), which is indicative of the effectiveness of the catechin in direct inhibition of collagenase compared to the inhibition exhibited through the binding with collagen. In arthritic condition it is known cartilage degrdation is mainly mediated by MMPs. The green tea catechins especially EGCG had exhibited almost complete inhibition to collagenase at 160 μM. Eventhough the inhibiting concentration of green teas may not be achieved physiologically through oral consumption but may readily be achieved through local administration.

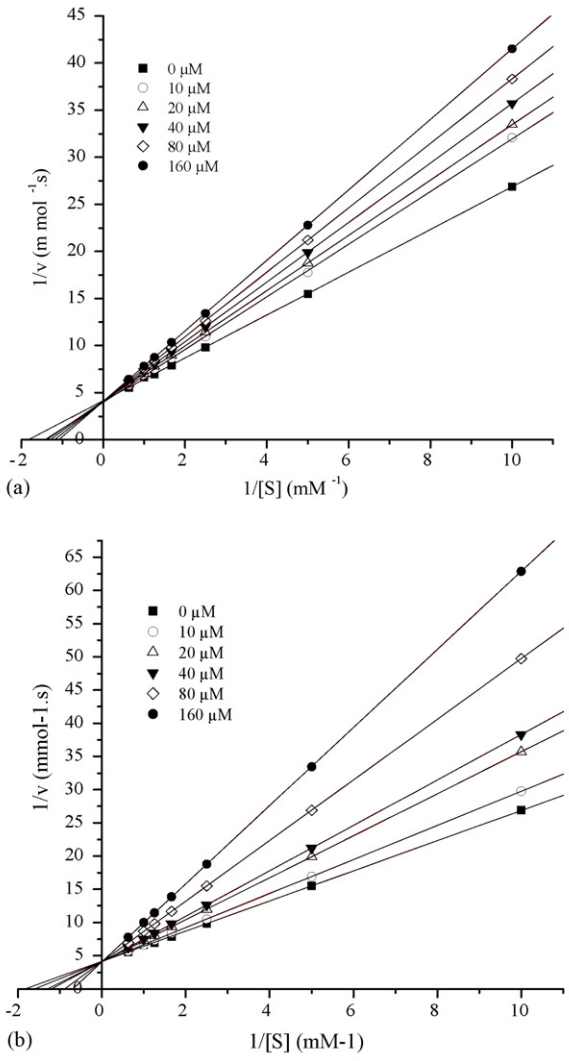

Fig. 4. Lineweaver–Burk plots of FALGPA hydrolysis by ChC in the presence of green tea polyphenols. Assays were performed in tricine buffer pH 7.5, with varying concentrations FALGPA (0.1–1.6 mM) and green tea polyphenols: (a) catechin and (b) EGCG.

3.3. Kinetic analysis of the inhibition of collagenase by polyphenols

In order to establish the mechanism of inhibition of enzyme activity by catechin and EGCG; collagenase treated with varying concentrations of green tea polyphenols viz., catechin and EGCG have been studied for their ability to hydrolyze the synthetic substrate FALGPA at different concentrations. To analyze the inhibition of ChC by catechin and EGCG, Lineweaver–Burk plots, i.e. double-reciprocal plot for the hydrolysis of FALGPA by catechin and EGCG treated collagenase have been determined (Fig. 4 a and b). The kinetic parameters K_m and V_{max} are calculated (Table 1). The Michaelis–Menten constant obtained for native collagenase was $K_m = 0.55$ mM for the substrate FALGPA at 25 °C pH 7.5 in 50 mM Tricine buffer containing 0.4 M NaCl and 10 mM $CaCl_2$. The K_m value of the FALGPA hydrolysis by collagenase is found to be similar to that obtained earlier [33]. The V_{max} was found to be 0.2439 ± 0.05 mmol s^{-1}. The Lineweaver–Burk plots obtained for FALGPA hydrolysis by collagenase incubated in the presence of different concentrations of catechin and EGCG revealed that there was no change in the V_{max} compared to control. Both catechin and EGCG exhibited dose-dependent inhibition on the activity of ChC, and Lineweaver–Burk plots clearly showed a competitive inhibition (Fig. 4a and b). From the Michaelis–Menten constants (Table 1), it is clear that EGCG exhibits significantly higher inhibition on the activity of ChC compared to catechin. The addition of 160 μM of catechin and EGCG to collagenase resulted in K_m values of 0.91 ± 0.07 and 1.43 ± 0.12 mM, respectively. The competitive inhibition exhibited by both catechin and EGCG may work through direct competition with the substrate by binding to the active site, or binding to a remote site and causing a conformational change in the enzyme. Both mechanisms give identical kinetic results. It is important to study if there are any changes in the conformation of collagenase on the binding of the green tea polyphenols.

3.4. Polyphenol induced conformational changes in collagenase

The effect of the catechin on the conformation of collagenase has been investigated by monitoring circular dichroic spectral

Table 1
Michaelis–Menten parameters for collagenase hydrolysis of FALGPA at 25 °C, pH 7.5 in the presence of varying concentrations of catechin and EGCG

Compound	Concentration (μM)	K_m (mM)	V_{max} (mmol s^{-1})	Type of inhibition
Control (only collagenase)	–	0.55 ± 0.06	0.244 ± 0.02	
Catechin	10	0.67 ± 0.05	0.244 ± 0.02	Competitive
	20	0.71 ± 0.11		
	40	0.77 ± 0.06		
	80	0.83 ± 0.08		
	160	0.91 ± 0.07		
EGCG	10	0.68 ± 0.06	0.244 ± 0.02	Competitive
	20	0.79 ± 0.08		
	40	0.88 ± 0.09		
	80	1.11 ± 0.05		
	160	1.43 ± 0.12		

Table 2
Secondary structure contents of collagenase at various concentrations of catechin and EGCG

Compound	Concentration (μM)	α-Helix (%)	β-Sheet (%)	β-Turn (%)	Random (%)
Native	–	37	36	12	15
Catechin	0.6	32	36	11	21
	6	29	39	9	23
	30	25	42	8	25
	90	11	48	5	36
EGCG	0.6	28	35	5	32
	6	23	33	4	40
	30	15	31	3	51
	90	5	8	2	85

changes. In the far UV region, collagenase exhibits double minima at about 210 and 220 nm and a maximum at 195 nm with a crossover point at about 200 nm [40]. Collagenase has α helix, β sheet, β turn and random coil structure. CD spectrum of the native collagenase (Fig. 5) exhibits double negative bands at 208 and 222 nm and a positive band around 195 nm. These results are characteristics of α helix, whereas a peptide/protein having β sheet conformation alone will exhibit a single negative band between 215 and 225 nm. Collagenase has both α helix and β sheet almost equally distributed as its secondary structure, which may not be clearly distinguishable through the CD spectrum. However, the secondary structural details can be predicted using deconvolution techniques [37–39].

The structure of collagenase changes significantly to β sheet after interaction with catechin (90 μM), which is observed in the spectra where a negative band at 208 nm shifts to 215 nm, which is characteristic of β sheet (Fig. 5a). Similar observation has also been observed earlier when collagenase was treated with catechin-aldehyde polycondensate [41]. Treatment of low concentration (30 μM) of EGCG changes the structure of collagenase to β sheet and with further increase in concentration of EGCG; the structure is shifted more towards random coil conformation (Fig. 5b). Analysis of the CD spectra using CONTLL package has been carried out and from the analysis it is observed that the native collagenase contains almost equal distribution of α helix and β sheet with 37 and 36%, respectively (Table 2). The secondary structure content analyzed is also indicative that the conformation of collagenase treated with 90 μM catechin changes towards 48% β sheet and 36% random coil (Table 2). In the case of EGCG at 30 μM the conformation of collagenase shift towards 31% β sheet and 51% random coil and with further increase in the concentration of EGCG to 90 μM the collagenase conformation changes to 85% random coil (Table 2).

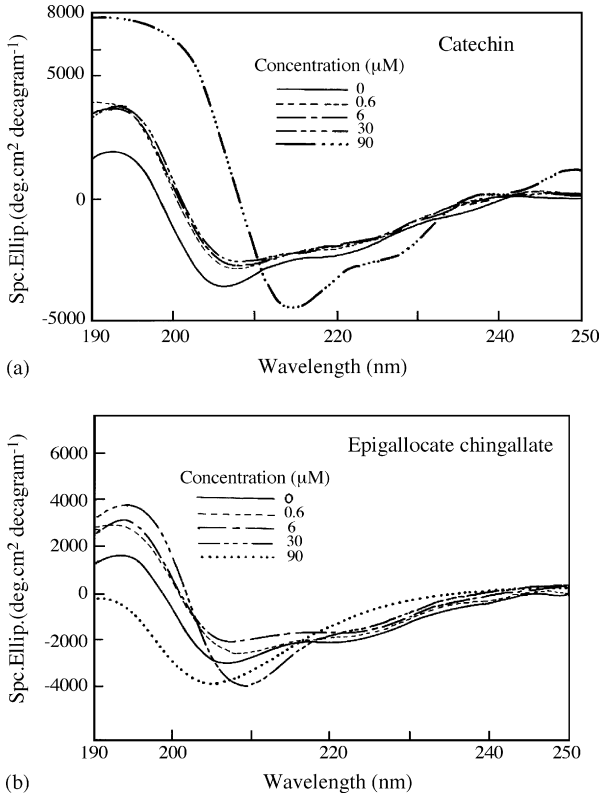

Fig. 5. CD spectra of ChC treated with different concentrations of (a) catechin and (b) EGCG.

4. Conclusions

From this study it is clear that the changes in the conformation of collagenase by the green tea polyphenols is a major factor in the inhibition of collagenolytic activity by ChC. The hydroxyl groups of polyphenols can act as hydrogen bond acceptor/donors with the backbone amide and other side chain functional groups viz., hdroxyl, amino and carboxyl groups of collagenase. The benzene ring of the polyphenols can also involve in hydrophobic and π–π interactions with collagenase. EGCG having additional galloyl unit and hydroxyl groups compared to catechin, can exhibit better hydrogen bonding and hydrophobic interactions with collagenase and hence can influence significant changes in the conformation of collagenase resulting in high inhibition in the activity of collagenase.

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