Kinetics of Inhibition of Type I Collagenase by Dialdehyde Cellulose in Stabilization of Type I Collagen

by

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Abstract

Collagen is one of the widely studied biomaterial for various industrial applications. However, search of eco-friendly and biocompatible stabilizing agent is a thrust research domain. In this research work, application of dialdehyde cellulose (DAC) was studied to understand the effect on the enzymatic and conformational stability in collagen. The secondary structure of collagen is not significantly altered on interaction with DAC. But, it was found that DAC lead to changes in the amplitude of the circular dichroic (CD) spectrum but did not alter the triple helical conformation of collagen. DAC treated collagen exhibited 93% resistance to collagenolytic hydrolysis. Conversely, DAC treated collagenase exhibited 89% inhibition against collagen degradation and the inhibition was found to be concentration dependent. The kinetics of inhibition of collagenase by DAC was derived from the extent of hydrolysis of (2-furanacryloyl-L-leucyl-glycyl-L-prolyl-L-alanine), FALGPA. DAC exhibited non-competitive mode of inhibition against collagenase. CD data on DAC-modified collagenase substantiate the hypothesis that the inhibition of collagenase by DAC arises from secondary and quaternary structural changes in the enzyme. Gaining new insights in understanding the mechanism of stabilization of collagen by DAC through kinetics of inhibition of collagenase was presented.

Introduction

Native type I collagen, found abundantly in mammalian tissues is susceptible to attack only by Type I collagenase at physiological pH, temperature and ionic strength.[1,2] Collagen as a protein has a distinguishing feature, each molecule has a coiled structure with three polypeptide chains, wound together to form a triple helix.[3,4] It provides structural integrity as a scaffold, a matrix, upon which other cells can proliferate. It is also known to have function of many proteins in the control of cell shape and differentiation, migration and the synthesis.[5-7] Mammalian collagenase is highly specific, cleaving collagen at the Gly-Ile (772-773) bond in the α-chain[5] Bacterial collagenase, on the other hand, exhibits wider specificity and cleaves collagen predominantly at the Y-Gly bond in sequences of the type -Pro-Y-Gly-Pro-, where Y is most frequently a neutral amino acid.[6-8] Cleavage of the triple helix by collagenase is known to render the collagen molecule susceptible to attack by other proteases.[9]

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Collagen is found to inhibit collagenase after crosslinking by aldehydes from natural sources during stabilization.[10] Although aldehyde tanning is easily described, definitions and mechanistic insight into the molecular basis were difficult. One of the probable causes postulated for the aldehyde-induced stability of collagen against collagenase is the inhibition of collagenase activity by mono- and dialdehydes. Aldehydes mediated activation or inhibition of enzymes in general have been described in literature. Reports on aldehyde stabilization postulate that irreversible binding of aldehydes by the protein occurs due to covalent interactions between amino sites of collagen and aldehydes yielding a crosslinked collagenous matrix.[11] This cross-linking is the main objective of stabilization/tanning, a process that converts raw hide/skin into leather. Traditionally, aldehydes were used in the leather industry to stabilize the hide/skin against degradation. They are also being used in fields such as medicine as cross-linking agents for surgical sutures, cosmetics, wound healing etc. Aldehydes are also excellent cross-linking agents for the electron microscopy study of proteins, cells, etc.[12] Ecological concerns are demanding alternative crosslinking agents and hence, DAC, a modified biopolymer of cellulose, is an eco-option to conventional formaldehyde and glutaraldehyde used in several applications.[13]

The stabilized collagen's resistance to chemical and enzymatic treatments and wet heat involves a number of factors. In the present work, an investigation was made to study the effect of DAC on the enzymatic stability of type I collagen matrix of RTT using DAC as a crosslinker. Chiro-optical properties give more subtle information on the conformational behavior of biopolymers in solution. In order to elucidate the conformation changes occurring in collagen and collagenase owing to cross-linking with DAC, CD analysis is presented.

Materials and Methods

Materials

Type I collagenase and 2-furanacryloyl-l-leucylglycyl-l-prolyl-l-alanine (FALGPA) were sourced from Sigma chemicals Co., USA. The type I collagenase was used after purification by gel filtration on Sephadex G-200 according to the method of Keller and Mandl.[14] All other reagents and chemicals used for the study were sourced from SRL Ltd., India.

Methods

Preparation of Type I Collagen

Tails were excised from 6-month-old male albino rats (Wistar strain) and frozen at -20°C. On removal from the freezer, tails were thawed and tendons were teased out. Teased collagen fibers were washed with 0.9% NaCl at 4°C, to remove the adhering soluble proteins. Acid soluble rat tail tendon type I collagen was isolated according to the method described by Chandrakasan *et al*.[15] The procedure included acetic acid extraction and salting out with NaCl. The purity of collagen preparation was confirmed by SDS-Polyacrlyamide gel electrophoresis (PAGE). The collagen concentration in the solutions was determined from the hydroxyproline content according to the method of Woessner.[16]

Preparation of DAC

DAC was prepared according to the methods reported earlier with modifications as described.[11,17] Cellulose (~100 g) was hydrolyzed in 5 N hydrochloric acid (10 h, 85°C). Hydrolyzed cellulose was suspended in demineralized water and subsequently cooled in an ice bath. Sodium metaperiodate (120 g) was added to the sample while stirring with a magnetic stirrer. The pH of the solution was maintained around 4 during the reaction. The reaction was performed in the dark at 35°C and stopped after 48 h to obtain DAC of 99% oxidation. The product was extracted with centrifugation in t-butyl alcohol (1:,3 ratio sample:, solvent). The supernatant was tested for presence of iodine in the solvent using iodimetry. The product was resuspended in the same volume of in t-butyl alcohol and the centrifugation cycle was repeated several times to remove iodic compounds. The product was dried at 35°C. The degree of oxidation was determined by measuring the concentrations of unconsumed periodate by iodometry whereas the dialdehyde content was determined using procedures reported earlier.[18] DAC exhibited low solubility, thus autoclaved DAC was used for all the experiments. Autoclaving was carried out by suspending DAC as 10% solution in distilled water at 120°C under 15 lbs of pressure for two hours.

Preparation of Crosslinked Collagen

The RTT were washed extensively in double-distilled water at 4°C and treated with 1% solution of DAC at 25°C for 24 h at pH 8.0. The crosslinked collagen samples were thoroughly washed and aged for 24 h and were stored in water at 4°C. Collagenase resistance experiments were carried out on crosslinked samples.

Resistance to Collagenase

The native and DAC treated RTT fibers were treated with Type IA collagenase from *Clostridium histolyticum*. Native and DAC treated RTT collagen fibers were treated with collagenase (collagen:collagenase ratio 50:1) in 0.04 M $CaCl_2$ solution buffered at pH 7.2 with 0.05M Tris-HCl, for different periods of incubation viz., 12, 24, 36, 48, 60 72, 84, 96, 108 and 120 h and incubated at a temperature of 37°C. The collagenolytic hydrolysis of native and DAC treated RTT was monitored by the release of soluble form of hydroxyproline from insoluble collagen.[19] Supernatant of the incubated samples were analyzed for collagen degradation. Aliquots of 750 µL of supernatant were withdrawn after centrifugation (10,000 rpm/ 10 min). The collagenase hydrolysate was hydrolyzed in sealed hydrolysis tubes with 6 N of 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.[16] 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. Soluble collagen, solubilized due to collagenase hydrolysis, is quantified as hydroxyproline (%) ´ 7.4. Based on the soluble collagen content in the supernatant solution of the collagenase treated RTT fibers the % degradation of collagen for native and DAC treated fibers can be calculated as:

$$\%\ collagen\ degradation = 100 - \left(\left(\frac{Initial\ collagen - Soluble\ collagen}{Initial\ collagen}\right)\times 100\right)$$

Inhibition of Collagenase Activity

Collagenase (0.04 mg/ml) was dissolved in 0.05 M tricine buffer (with 0.4M NaCl and 0.01M $CaCl_2$, pH 7.5), and incubated with DAC (0, 10, 20, 40, 80, 120, 160 and 200 μM) at 25°C for 24 h. The ratio of collagen, collagenase was maintained at 50:1. RTT collagen fibers (2 mg) were added to the mixture and further incubated for 96 h at 37°C and the mixture was centrifuged for 15 min at 10000 rpm. Collagen degradation (%) was estimated from the supernatant by analyzing hydroxyproline. Inhibition (%) by DAC was calculated as difference in the degradation of RTT collagen treated by native and DAC treated collagenase.[20]

$$\%\ Inhibition = \left(\frac{\%\ collagen\ degradation_{native\ collagenase} - \%\ collagen\ degradation_{DAC\ treated\ collagenase}}{\%\ collagen\ degradation_{native\ collagenase}}\right)\times 100$$

Kinetic Investigation

Assay of Native Collagenase

Type I collagenase assay using FALGPA as substrate was performed according to the method reported earlier.[20,21] Assays were carried out spectrophotometrically by continuously monitoring the decrease in absorbance of FALGPA after the addition of collagenase. The FALGPA (at concentrations of 0.2-1.6 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 collagenase (100 mL 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–visible spectrophotometer by following the decrease in absorbance at 324 nm when [FALGPA] = 0.2 mM. At higher concentrations of FALGPA viz. (0.4 mM) and (0.8-1.6 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.[22]

Inhibition of Collagenase by DAC

The reaction of DAC treated collagenase with FALGPA was performed under the same conditions mentioned for native collagenase above. The collagenase was treated with varying concentrations viz., 0, 20, 40, 80 and 160 μM of aqueous solution of DAC for 24 h at 25°C. The final concentrations of the collagenase in all treatments were maintained constant (0.4 mg/mL). The FALGPA substrates at concentrations of 0.2–1.6 mM) were taken in appropriate amount of Tricine buffer and DAC incubated collagenase (100 ml) was added and the final volume adjusted to 1 mL. The hydrolysis of substrate was monitored at the corresponding wavelengths (immediately after the addition of DAC incubated collagenase) similar to native collagenase. Rates of hydrolysis were calculated employing initial rate methods. The rate data were analyzed in terms of Michaelis–Menten method. From the Linewaver-burk plots of v^{-1} vs $[S]^{-1}$ 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 total hydrolysis and multiplying by the substrate concentration.

Conformational Changes of DAC Stabilized Collagen

Circular dichroism spectrum was recorded using Jasco-J715 spectropolarimeter. Type I collagen at0.06 μM in pH 4.0 acetate buffer (10^{-6} M) was prepared at 25°C. The rate of nitrogen purging was maintained at 5 L/min up to 200 nm and increased to 10 L/min below 200 nm wavelengths. A 2-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 to remove suspended particles. Cuvette of 0.1 cm path was used throughout the experiments. A slit width of 1 nm was used. The scan speed of 20 nm/min was used with an average of 5 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 interacting with DAC were investigated after incubating the enzyme with varying concentrations (0.6 μM – 120 μM) of DAC. The spectra obtained were deconvoluted using G and F and K2D programs and the mean values of secondary structure components were tabulated.

RESULTS AND DISCUSSION

Inhibition of Collagenase

Native Collagenase Resistance of DAC Stabilized Collagen

The stability of the DAC-treated Type I collagen fibers against Type I collagenase degradation was studied by analyzing the rate of hydrolysis of collagen on treatment with collagenase. The extent of solubilization of collagen by collagenase was examined over a range of time intervals as given in Figure 1.

The figure clearly displays the stabilization effect on the collagen fibers treated with DAC where a marked decrease in the release of hydroxyproline is observed, compared to the untreated collagen fibers. Native RTT collagen fibers have undergone extensive hydrolysis with the treatment of collagenase releasing about 131 mg of hydroxyproline per gram of collagen. Collagen samples treated with DAC exhibit a higher degree of stability with a release of only 0.9 mg of hydroxyproline per gram of collagen. DAC treated RTT exhibited 7% degradation of collagen as against 99% degradation in the case of native collagen at 120 h period of incubation. DAC interacts with collagen through covalent and hydrogen bonding. The enzymatic stability of DAC treated collagen is due to inter- and intra-molecular crosslinking arising from such interactions. Hence, the stability of DAC treated collagen fibers against collagenase could be achieved by protecting active sites in collagen recognized by collagenase. Thus, DAC brings about maximum cross-linking with collagen as it carried one dialdehyde group in each glycoside of cellulose and its own molecule contains more than 1000 such glycoside units in its structure. Inter- and intra-molecular crosslinking is possible by interaction of amino groups of the collagen structure with the larger number of accessible aldehyde groups in DAC.

DAC Treated Collagenase Resistance of Native Collagen

RTT collagen fibers treated with native collagenase and collagenase incubated with various concentrations of DAC was studied to establish the effect of DAC in inhibiting collagenase. Inhibition on the collagenolytic activity of DAC treated collagenase against collagen degradation was dependent on the quantity of DAC used (Figure 2). DAC at 40 mM exhibited 64% inhibition to collagenase against the degradation of RTT collagen fibers. The inhibition increased with increasing concentration of DAC. At 200 mM, 89% inhibition was observed at 96 h of incubation of DAC and collagenase. From these observations it can be concluded that DAC is effective in direct inhibition of collagenase compared to the inhibition exhibited through the binding with collagen.

Conformational Changes in DAC Stabilized Collagen

The triple helical conformation of collagen gives rise to a CD spectrum characterized by a positive band at 220 nm and a negative band at 197 nm. In order to investigate the effect of DAC on the secondary structure of collagen, CD studies of collagen in the presence of DAC were carried out and shown in Figure 3. The ratio of the positive peak intensity and the negative peak intensity (Rpn) that is used in establishing triple helical conformation in collagen solution in the presence of different concentrations of DAC is listed in Table I. From the Rpn values, it can be observed that treated samples do not show significant changes in Rpn values compared to native triple helical conformation of collagen. The Rpn ratio for collagen solution of concentration 0.6 μM is found to be 0.118 and in the presence of DAC at 120 μM is 0.131. The CD spectral changes in collagen with only small deviations in the

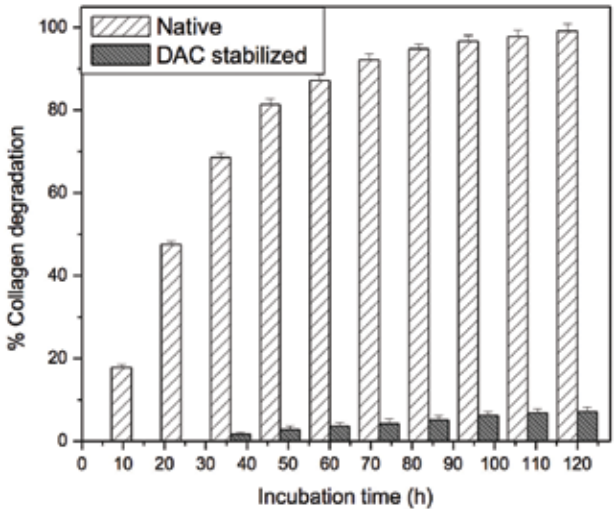

Figure 1. Plot of Native and DAC treated RTT collagen degradation (%) by collagenase at different intervals of incubation.

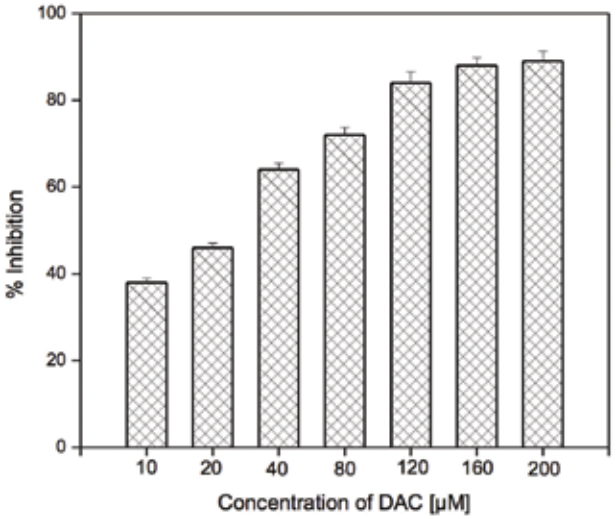

Figure 2. Plot of Inhibition (%) of collagenase activity at diferent concentrations of DAC.

TABLE I
Rpn ratio of DAC treated collagen.

Concentration of DAC (μM)	Rpn (Characteristic ratio)
0.6	0.119
6	0.121
30	0.124
90	0.127
120	0.131

Note: Rpn ratio of native collagen solution (0.06 μM) – 0.118.

Rpn ratio in the presence of DAC is indicative of the effect of the dialdehyde (DAC) on the conformation of collagen. Collagen on denaturation leads to red shift of the negative band and disappearance of the positive band,[24,25] but no such effect is observed in the DAC-treated collagen. Thus, the change in the CD spectra of collagen in the presence of DAC is not due to the loss of triple helicity or denaturation and this marginal increase in dichroic intensity could be attributed to the crosslinking between DAC and collagen.

Conformational Changes in DAC Treated Collagenase

In order to investigate whether inhibition of collagenase by DAC is due to alterations in the secondary structure, CD spectral studies on the DAC-collagenase systems were carried out. 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 [26] Collagenase has a helix, b sheet, b turn and random coil structure, with both a helix and b sheet almost equally distributed as its secondary structure, which are predicted using deconvolution techniques.[27-29] Significant changes in the CD spectra are observed in the presence of DAC. As given in Table II, the secondary structure contents of native collagenase contain almost equal distribution of a helix and b sheet with 37 and 34%, respectively. Considerable loss of a helical structure of collagenase occurs in the presence DAC. At 30 mM of DAC, the structure of changes to b sheet. With increasing concentration of DAC, the collagenase structure is shifted more towards random coil conformation. The secondary structure contents analyzed as given in Table II at a concentration of 30 mM influence the conformation of collagenase to shift towards 29% b sheet and 50% random coil and at higher concentration of DAC (90 mM), the collagenase conformation changes to 86% random coil. It can be observed from the numeric values and figure that DAC induces substantial conformational changes in collagenase.

Kinetic Analysis of Inhibition of Collagenase by DAC

In order to establish the mechanism of inhibition of collagenase by DAC, hydrolysis of the synthetic substrate FALGPA at different concentrations of DAC treated collagenase was investigated. The Michaelis-Menten constant obtained for collagenase was K_m = 0.55 mM for the substrate FALGPA at 25°C, pH 7.5 in 50 mM Tricine buffer. This value substantiates the value reported earlier for bacterial collagenase.[30] A detailed analysis of the kinetics of inhibition and mechanism of action of DAC was performed by analyzing the kinetic type from the nature of inhibition observed by varying concentrations of FALGPA.. The mode of inhibition by DAC was determined by plotting double reciprocal Lineweaver-Burk plots (v^{-1} against $[S]^{-1}$) to study the effect of DAC on collagenase activity. The kinetic parameters K_m, V_{max} and K_i are listed in Table III. The Lineweaver-Burk plots (Figure 3) obtained for FALGPA hydrolysis by collagenase incubated in the presence of different concentrations of DAC reveal that there was no appreciable change in K_m values. The K_m values were found to range from 0.94 to 0.96 mM. Addition of DAC at concentrations of 20, 40, 80 and 160 µM to collagenase caused a decrease in the velocity to 0.62, 0.44, 0.35, 0.28 $mmols^{-1}$ respectively, without appreciable change in the K_m value as shown in Figure 4, which suggests that DAC apparently acts as a non-competitive inhibitor of collagenase during inhibition against FALGPA. A Dixon plot of v^{-1} vs [I] at different substrate concentrations yielded an inhibition constant (K_i) of 34 µM. This study on the influence of DAC on the activity of collagenase illustrates the function of DAC in the stabilization of collagen against collagenase-promoted hydrolysis.

Mechanism of Inhibition of Collagenase by DAC

The present study provides convincing evidence that DAC can be used as a good crosslinking agent for the stabilization of

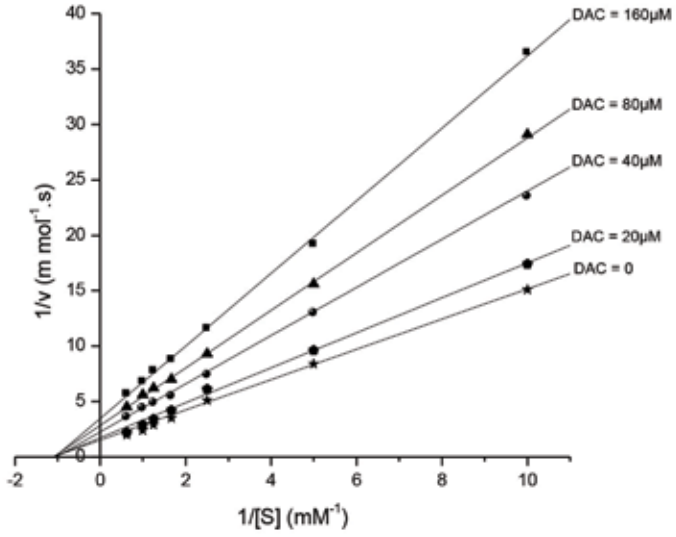

Figure 3. Lineweaver–Burk plots of FALGPA hydrolysis by collagenase in the presence of DAC. Assays were performed in Tricine buffer pH 7.5, with varying concentrations of FALGPA (0.1–1.6 mM) and DAC at concentrations of 0, 20, 40, 80 and 160 µM. Each data point is an average of values from three different equations.

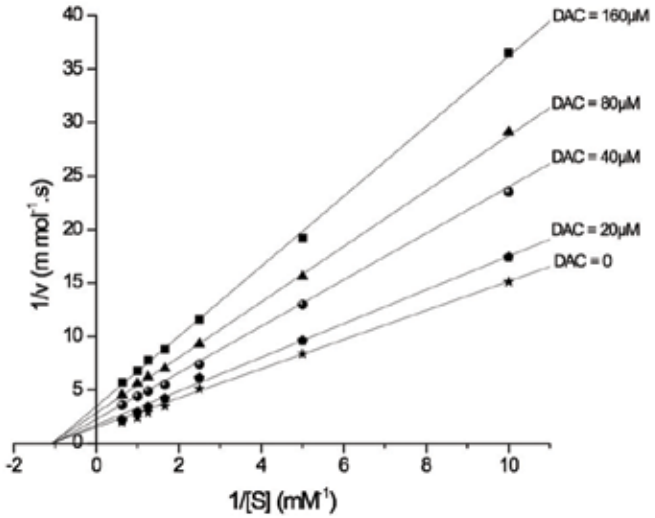

Figure 4. Mehcanism of collagen – DAC – collagenase interaction.

collagen (Figure 5). The study also displays that DAC inhibits the activity of collagenase and the ability of DAC to function as an inhibitor is deduced from the values of V_{max} and K_m obtained for the hydrolysis of FALGPA by collagenase. In the presence of DAC, the kinetics of inhibition of collagenase was found to be non-competitive. In a classical model for non-competitive inhibition, the inhibitor would have no effect on substrate binding and vice-versa. The inhibitor and substrate bind reversibly at diverse sites. The substrate binds to E (enzyme) and EI (enzyme-inhibitor) complex and I (inhibitor) binds to E and ES (enzyme-substrate). There appears to be a reduced amount of active enzyme at any given concentration of the DAC possibly because of the formation of a non-productive EIS (enzyme-substrate-inhibitor) complex. As long as DAC is present, some of the enzyme will always be in the non-productive EIS form even at saturating substrate concentrations. Based on the results of this study, it can be concluded that the deactivation of collagenase by DAC contained in stabilized collagen is entirely possible. The presence of free DAC to the extent of 2670 ppm (166 mM) was found in the stabilized collagen to deacctivate the collagenase activity. Amino acid composition of bacterial collagenases shows the side chain amino acids viz. lysine and arginine.[31,32] A similar composition is observed in the case of mammalian collagenases also.[33] Therefore, a probable covalent binding of inhibitor with these side chain amino groups present around the active site of collagenase could be, an explanation to these findings.

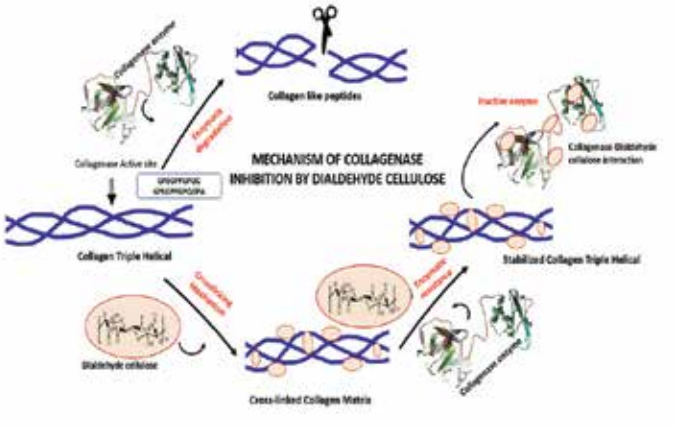

Figure 5. Mehcanism of collagenase inhibition by dialdehyde cellulose.

TABLE II
Secondary structure contents of collagenase at various concentrations of DAC.

Compound	Concentration (μM)	α-Helix (%)	β-Sheet (%)	β-Turn (%)	Random (%)
Native	–	37	34	12	32
DAC	0.6	28	32	5	34
	6	22	31	5	42
	30	16	29	5	50
	90	4	8	2	86

TABLE III
Michaelis-Menten parameters for collagenase hydrolysis of FALGPA at 25°C, pH 7.5 in the presence of varying concentrations of DAC.

Compound	DAC [μM]	K_m (mM)	V_{max} (m mol s^{-1})	K_i [μM]	Type of Inhibition
Control (only collagenase)	–	0.55±0.06	0.244±0.02	–	–
Dialdehyde Cellulose	20	0.955±0.005	0.623±0.01	34	Non-Competitive
	40	0.943±0.005	0.442±0.06		
	80	0.962±0.005	0.354±0.02		
	160	0.949±0.005	0.287±0.04		

CONCLUSIONS

The present study on the influence of DAC as an inhibitor for collagenase induced hydrolysis establishes a possible mechanism for the stabilization of collagen. The study demonstrates that DAC induces a significant increase in resistance to collagenase. From this study, it is clear that the change in the conformation of collagenase by DAC is a major factor in the inhibition of collagenolytic activity by collagenase. The aldehyde groups of DAC interacts with the side chain functional groups of amino acids of collagenase, such aslysine and arginine. The hydroxyl groups of the polymeric aldehyde can involve as hydrogen bond acceptors or donors with the backbone amide and other side chain functional groups, such as hydroxyl and carboxyl groups of collagenase. Hence, aldehyde and hydroxyl groups of DAC can exhibit covalent and hydrogen bonding and also hydrophobic interactions with collagenase that can induce significant changes in the conformation of collagenase resulting in considerable inhibition in the activity of collagenase in the stabilization of collagen.

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