Paper B55

Stabilization of collagen using aldehyde forming enzyme - an approach towards enzyme tanning

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Abstract

Type I collagen from rat tail tendon (RTT) fibres was crosslinked with Aldehyde forming enzyme to bring about stabilization of the matrix. The novel strategy for enzymatic tanning of fibrous protein collagen was investigated by crosslinking with variety of methods that include chemical, physical and UV irradiation with the aim of enhancing its physical characteristics such as mechanical strength, thermal stability, resistance to collagenolytic and proteolytic breakdown, thus increasing its overall biocompatibility. The formation of the crosslinked network between Aldehyde forming enzyme and the collagen fibres has brought about significant thermal and enzymatic stability to collagen. Aldehyde forming enzyme crosslinked collagen fibres exhibited an increase in hydrothermal stability by 18°C. Thermal stability and crosslinking efficiency was found to increase with concentration of enzyme. Aldehyde forming enzyme treated collagen exhibited 82% resistance to collagenolytic hydrolysis.

Keywords: Collagen; Interaction; Aldehyde forming enzyme; Denaturation

1. Introduction

Type I collagen, the structural protein of skin, bone and tendon, is an important biomaterial finding widespread applications in fields such as surgical sutures, cosmetics, wound healing and leather making [1]. This widespread use of collagen emphasizes the need to understand the mechanism of stabilization of collagen against biodegradation and heat, as these studies will have far reaching implications in both industrial and biological applications of collagen. Collagen being a protein is amenable to degradation by microbial attack. Therefore, the development of useful articles based on collagen depends on rendering them resistant to degradation. The thermal stability of collagen with the influence of various factors were studied widely [2]. It is well known that collagen crosslinked with various crosslinking agents such as plant polyphenols, metal ions and aldehydes is made resistant against the degradation by collagenase and the thermal stability of collagen was increased owing to crosslinking [3]. In recent times, ecological concerns demand alternative crosslinking chemicals. Hexavalent chromium, formaldehyde and glutaraldehyde are known to be carcinogenic]. Modified polysaccharides were proved to be biodegradable and toxologically acceptable.

In the present study the aldehyde forming enzyme is used as an attractive option for stabilization of collagen as it is abundant and one of the cheapest and eco-acceptable natural resource [5] . In the present work, an investigation is attempted to study the effect on crosslinking eficiency, thermal stability and collagenase resistance realted to type I collagen matrix of RTT using aldehyde froming enzyme as a crosslinker.

2. Materials and methods

2.1 Preparation of type I collagen

Acid soluble RTT type I collagen solution was also isolated according to the method described by Chandrakasan et al. [6]. 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 solution was determined from the hydroxyproline content according to the method of Woessner [7].

2.2 Extraction of aldehyde forming enzyme

After cultivation, the mycelia were harvested by filtering the broth, washed with deionised water, and dried by washing with acetone and ether cooled at -20ºC. After cultivation, the aldehyde forming enzyme was extracted from the acetone-dried mycelia of *Schizophyllum commune.* The enzyme was prepared and assayed according to the described protocol of Tachibana et al [5].

2.3 Characterization of modified collagen

The RTT fibres were washed extensively in double distilled water at 4°C and treated with 1% solution of enzyme at 25°C for 24 h at pH 8.0.

2.4 Crosslinking efficiency

Crosslinking efficiency of aldehyde forming enzyme in stabilization of collagen was determined by 2,4,6 – trinitrobenzenesulfonic acid (TNBS) assay that determines the ε-amino groups in collagen [8].

2.5 Thermal resistance

2.5.1 Hydrothermal shrinkage

The hydrothermal stability of enzyme treated collagen fibres were determined using a micro-shrinkage tester. The shrinkage temperature is also termed as hydrothermal stability as the measurement is carried out in the presence of water. The rate of heating was maintained at 2°C/min.

2.5.2 Enzymatic resistance

The enzymatic degradation of native and enzyme stabilized RTT by bacterial collagenase (Type IA) from Clostridium histolyticum was analyzed by estimating the amount of hydroxyproline released in the solution after hydrolysis. 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: enzyme ratio was maintained at 50:1 [9].

2.6 Determination of structural stabilization of collagen- scanning electron microscopic studies (SEM)

The control and 10 % modified preserved skin samples were cut into specimens of uniform thickness. The micrographs for the grain surface and cross section were obtained by operating the SEM at low vacuum with an accelerating voltage of 5 KV in different lower and higher magnification levels.

3. Results and discussion

3.1 Enzyme preparation and purification

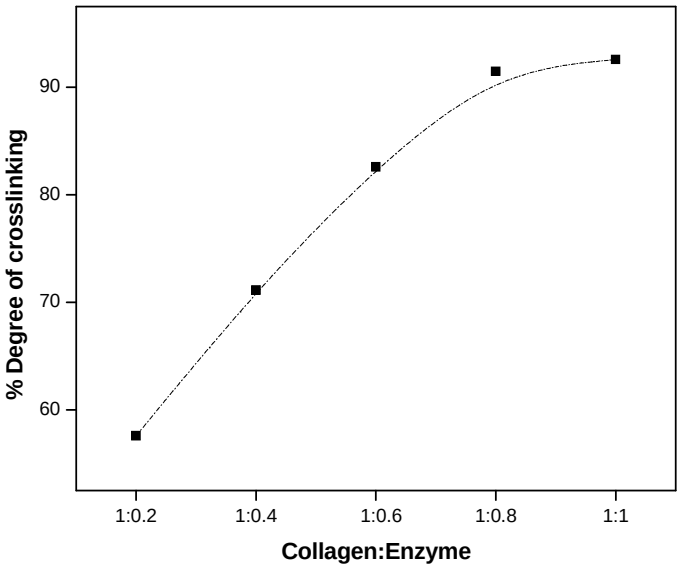
The aldehyde forming enzyme was prepared from the acetone-dried mycelia of *Schizophyllum commune.* The aldehyde forming enzyme was purified about 900-fold based on the activity of the crude cell-free extract. In order to confirm that enzymatic activity was associated with enzymatic activity in each piece was assayed.

3.2 Degree of enzymatic crosslinking with collagen

The cross linking degree increased with an increasing amount of enzyme as given in Figure 1. As the pH value of the collagen solution increased, a higher resultant cross linking degree was obtained until the optimum pH 7.5. However, with regard to the effect of incubation temperature, the cross linking degree of the matrices incubated at 25 ºC was higher than those for the other incubation

temperature. The maximum cross linking degree was observed for matrices treated with aldehyde forming enzyme at an optimum of 1% concentration with collagen in neutral pH incubated at 25ºC.

Figure 1. % Degree of cross linking collagen at varied enzyme concentration



3.3 Characterization of enzymatic crosslinked collagen

Denaturation temperature (T_d) values of matrices incubated at 25ºC are summarized in Figure 2. The T_d of the untreated collagen matrices recorded was around 59ºC, and the highest T_d was around 79ºC. In order to establish the effect of aldehyde forming enzyme in inhibiting collagenase, RTT collagen fibres treated with native collagenase and collagenase incubated with various concentrations of enzyme was studied. Enzyme treatment to collagenase exhibited dose independent inhibition on the collagenolytic activity against collagen. Aldehyde forming enzyme at 1:1 ratio with collagen exhibited varying % degradation of collagenase against RTT collagen fibres. From the Table 1, it is observed that the inhibition increased with increase in incubation time from 0 to 48 hours and found to be decreased gradually at 72, 96 and 120 hours respectively. Hence a treated collagen matrix exhibiting stability against the activity of bacterial collagenase can exhibit high resistance to degradation by any class of enzymes. % Collagen degradation (based on hydroxyproline released) for the native, enzyme treated RTT by collagenase at various times period was determined.

The cross-sectional morphology of matrices incubated at 25ºC was examined by SEM. According to the SEM images as given in Figure 3, untreated collagen matrices (Figure 3(a)) displayed orderly and bigger pore sizes as compared to enzyme-treated matrices (Figure 3(b)). There was no obvious difference among treated collagen matrices with respect to pore size and structure which showed irregular morphology and enlarged pore size.

4. Conclusions

The present study on the influence of aldehyde forming enzyme as a stabilizing agent establishes a possible mechanism for the stabilization of collagen. The study demonstrates that AFE brings about a significant increase in hydrothermal shrinkage temperature. The AFE treated collagen was found to be resistant to degradation by collagenase and the resistance to degradation was found to be high at higher pH conditions. The thermal stability and the enzyme stability of AFE treated collagen increased with increase in crosslinking of collagen.

Figure 2. Hydrothermal stability of modified collagen with aldehyde forming enzyme

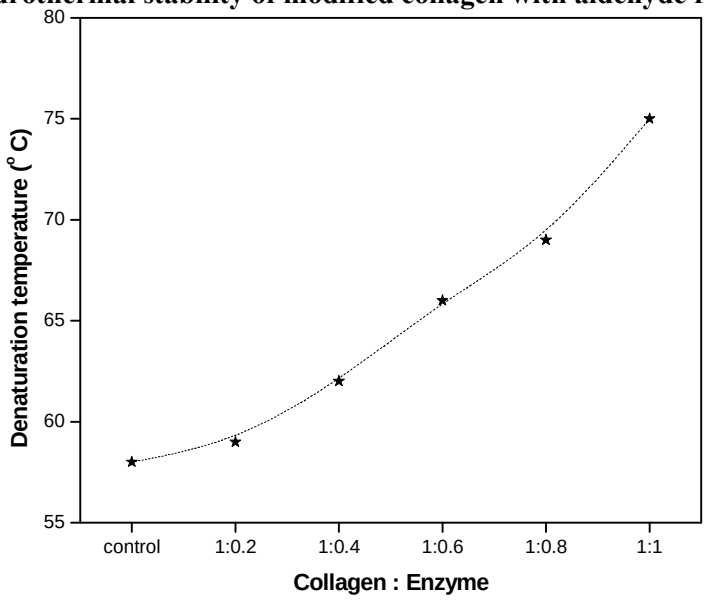
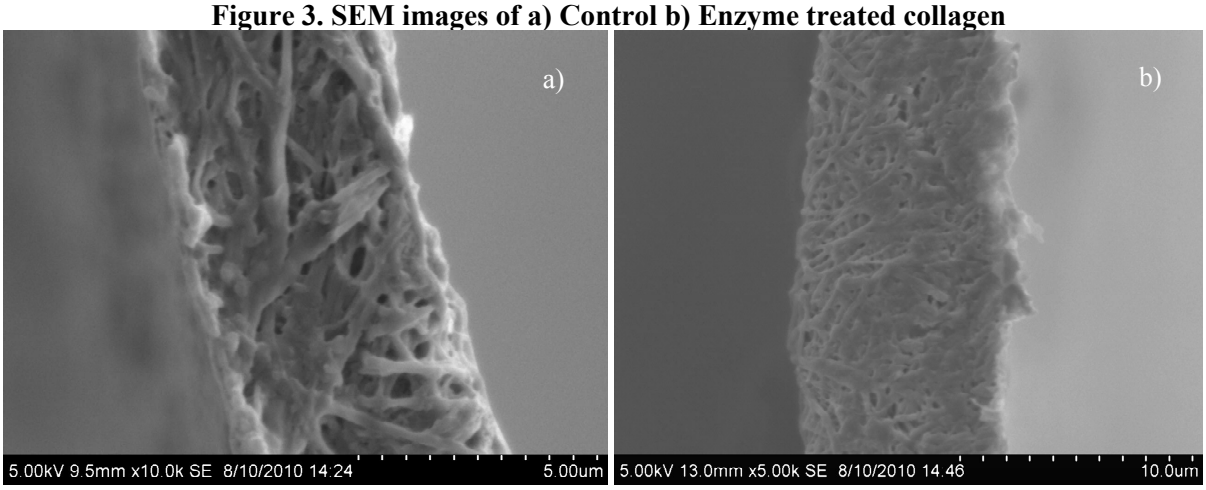


Table 1. % Degradation data for enzymatic crosslinked collagen

Incubation* (Hours)	X (O.D)	Y (µg of hp/mL)	Z (µg of hp/5mL)	A (µg/1500µL of supernatant)	B (A*7.4)	% Degradation
0	0.13	2	10	20	148	12.4
2	0.15	2.3	11.5	23	170.2	15.7
4	0.18	2.8	14	28	207.2	23.6
8	0.21	3.3	16.5	33	244.2	26.4
16	0.25	3.9	19.5	39	288.6	33.5
24	0.36	5.7	28.5	57	421.8	44
32	0.485	7.6	38	76	562.4	58.6
48	0.64	10	50	100	740	77.9
72	0.58	9.1	45.5	91	673.4	70.1
96	0.52	8.1	40.5	81	599.4	62.4
120	0.44	6.8	34	68	503.2	52.4

*The Aldehyde forming enzyme at 1:1 ratio with collagen incubated for different time interval

Figure 3. SEM images of a) Control b) Enzyme treated collagen



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