Acceleration of Fibril Formation and Thermal Stabilization of Collagen Fibrils in the Presence of Taxifolin (Dihydroquercetin)

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 144, No. 12, pp. 640-643, December, 2007
Original article submitted July 14, 2006

We studied the effect of flavonoid taxifolin (dihydriquercetin) on the structure and thermal stability of collagen I fibrils. Taxifolin accelerated fibril formation with reconstruction of periodical cross-striation characteristic of these fibrils. Differential scanning calorimetry showed elevation of melting temperature of collagen fibrils formed in neutral or weakly alkaline media, but not of individual tropocollagen molecules in acid medium. Taxifolin capacity to stimulate fibril formation and promote stabilization of fibrillar forms of collagen can be used in medicine.

Key Words: *collagen; taxifolin; dihydroquercetin; flavonoids; polyphenols*

Flavonoids (polyphenols of plant origin) are widely used in medicine, due to their positive effects on human health [6]. Moreover, they are important components of vegetable food [8]. Due to their antioxidant activity, capacity to bind heavy metals, and modulate cell signaling systems, flavonoids protect human organism from various environmental stress factors [14]. The mechanisms of their effects on human organism are studied insufficiently [11]. Metabolites of some flavonoids can be toxic, exhibit mutagenic and, presumably, carcinogenic effects, detected for quercetin, the best studied and widely used flavonoid [10]. Dihydroquercetin (taxifolin) is significantly less toxic, presumably due to the absence of C-2,3 double bond [10]. Therefore, study of taxifolin characteristics seems to be a prospective task.

We studied interactions of taxifolin with collagen and the effects of this agent on collagen fibril formation and fibril stability.

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MATERIALS AND METHODS

Dihydroquercetin (taxifolin; Flavokon) obtained from *Larix sibirica* was provided by Bioflavon Company. A similar preparation from Sigma not differing from taxifolin by the studied parameters served as the reference drug.

Stock solution of collagen I was prepared from rat tendons by extraction with 0.5 M acetic acid with subsequent double re-precipitation with ethanol and dissolving in 0.1 M acetic acid. Collagen concentration in the solution was evaluated by dry protein weight and was 6 mg/ml.

For fibril formation, stock solution of collagen was diluted with 0.01 M phosphate buffer (pH 7.4) at 5°C to a final collagen concentration of 0.1 mg/ml and ethanol solution of taxifolin was added so that its final concentrations in the samples were 10^{-5}-10^{-1} weight percent. Collagen solutions containing ethanol (50 μl/10 ml) served as the control. Hence, the final concentration of ethanol in the solution did not exceed 0.5%, pH was brought to 7.4 by adding 0.1 M NaOH with constant stirring on cold, after which one half of the volume was left at 20°C

and the other was placed in a thermostat at 37°C. The samples were analyzed after 24 h.

Thermodynamic characteristics of collagen were evaluated by scanning calorimetry on a DASM-4 microcalorimeter (Biopribor Company). Warming rate was 1 K/min, accuracy of temperature recording ±0.1°C. The data were registered automatically using SCAL software.

Right angle light scatter in collagen suspension (0.1 mg/ml) was measured on a Perkin Elmer MPF-44B spectrofluorometer at λ=510 nm. Phosphate buffer (10 mM; pH 7.4; 25°C) served as the incubation medium.

Collagen preparation in 10 mM phosphate buffer (pH 7.4; 20°C) was used for electron microscopy. The fibril formation process coursed during 24 h. Collagen was applied on microscopic grids coated with Formvar film. The preparations were stained with 1% uranyl acetate and poststained with 1% sodium phosphotungstate (pH 7.4), 2-3 min each procedure. The preparations were examined under a JEM-100B electron microscope (JEOL) at ×30,000.

RESULTS

Collagen molecules in phosphate buffer spontaneously formed fibrils. The appearance of collagen preparation in neutral medium (pH 7.4) in the absence and presence of 0.1% taxifolin differed significantly. After 24 h the transparency of control preparation was significantly higher in comparison with the preparation with taxifolin. The formation of large collagen threads clearly seen with plain eye was observed in the preparation with taxifolin. The dynamics of fibril formation was registered by changes in light scatter [12]. According to the light scatter curves reflecting the dynamics of this process, the parameter slowly increased in the control sample without taxifolin and significantly more rapidly in the presence of taxifolin, starting earlier than in the control. Increasing taxifolin concentration led to the corresponding increase in light scatter, i.e. taxifolin stimulated the formation of collagen fibrils (Fig. 1).

Electron microscopy also showed the formation of collagen fibril. Figure 2 shows collagen fibrils formed in the presence of taxifolin in different concentrations. Microphotographs show cross-striation with a period of 64-67 nm characteristic of collagen fibrils. Typical structural features of fibrils were well retained in the presence of taxifolin, indicating that it did not impair fibril structure. Moreover, periodicity was more pronounced at high concentrations of taxifolin.

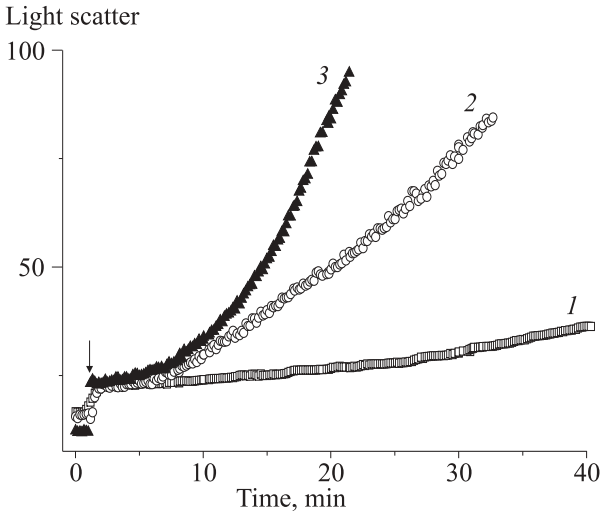

Fig. 1. Changes in the light scatter intensity. *1*) control collagen preparation; *2*) with 0.001% taxifolin; *3*) with 0.01% taxifolin. Arrow shows the moment of taxifolin addition.

Thermograms of collagen preparations obtained by differential scanning calorimetry showed that protein melted at 41.3±0.3°C in acid medium and at 50±2°C in neutral medium (Fig. 3). It is known that collagen molecules in acid medium are monomeric and their melting temperature is lower; in neutral medium this protein spontaneously forms fibrils with a higher melting temperature [12]. Melting curves of our preparations always had one peak; hence, in acid medium the protein was presented by the monomeric form, while in neutral medium it was involved in the fibril formation process.

Melting temperature of monomeric collagen in acid medium did not change in the presence of taxifolin in concentrations of 10^{-5}-10^{-1}%, while collagen fibrils formed in neutral medium acquired greater thermostability (Fig. 2; Table 1). The in-

TABLE 1. Thermodynamic Characteristics of Collagen Fibrils Formed in 0.01 M Phosphate Buffer, pH 7.4 (°C)

Taxifolin %	Incubation at 20°C*		Incubation at 37°C*	
	T_m	$T_{1/2}$	T_m	$T_{1/2}$
—	52.0	3.2	48.3	2.8
10^{-5}	52.0	3.2	49.3	1.8
10^{-4}	52.5	2.1	52.6	2.3
10^{-3}	53.5	2.2	55.5	2
10^{-2}	55.8	2.3	60.3	1.9
10^{-1}	56.7	1.0	61.9	1

Note. T_m is the temperature of transition maximum, $T_{1/2}$ is the transition half-width. *Temperature at which the preparation was stored for 24 h after making before being charged into calorimeter.

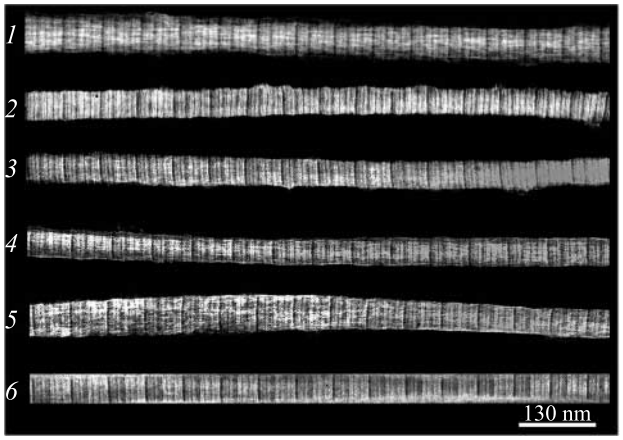

Fig. 2. Electron microphotographs of collagen fibrils, formed at 20°C over 24 h without taxifolin (*1*), with taxifolin in a concentration of 0.00001% (*2*), 0.0001% (*3*), 0.001% (*4*), 0.01% (*5*), and 0.1% (*6*).

crease in taxifolin concentrations was paralleled by exponential growth of thermostability of collagen fibrils, which was seen from almost linear relationship between collagen fibril thermostability and taxifolin concentration logarithm (Fig. 2, *b*). Incubation of the protein at 37°C at low or zero (control sample) concentrations of taxifolin led to reduction of its thermostability (Fig. 2, *b*). These differences disappeared completely in the presence of taxifolin in a concentration of 10^{-4}%, and at higher concentrations thermostability of collagen fibril at 37°C significantly increased. These results suggest that the stabilizing effect of taxifolin was most pronounced in neutral media at its concentrations of 10^{-4}% and higher under conditions promoting the formation of collagen fibrils, while individual molecules of collagen in acid medium were insensitive to taxifolin.

It is known that thermal denaturation of collagen is paralleled by destruction of hydrogen bonds and hydrophobic interactions between the protein subunits and helix-coil transformation. Hydrophobic interactions determined predominantly by glycine residues and hydrogen bonds formed between hydroxyproline residues [9] play an important role in stabilization of collagen molecules. Molecules of polyphenol (one of which is taxifolin) have many hydroxyl groups, but are poorly soluble in water because of the presence of hydrophobic aromatic rings. Presumably, the combination of high hydrophobicity and capacity to form hydrogen bonds permits these molecules incorporate in certain areas of collagen fibrils and promote stabilization of their structure. In addition, it was shown that flavonoids can form intermolecular sutures, stabilizing the protein [3].

Collagen gels stabilized with flavonoids are suggested to be used for wound healing, creation of biocompatible materials, cosmetic preparations, and microshells for drug delivery [4,5,7]. The detected capacity of taxifolin (dihydroquercetin) to promote fibril formation and stabilize collagen fibrils clears out some aspects of biological activity of this substance [1,2] and opens new prospects for its use in various spheres of medicine, cosmetology, and food industry.

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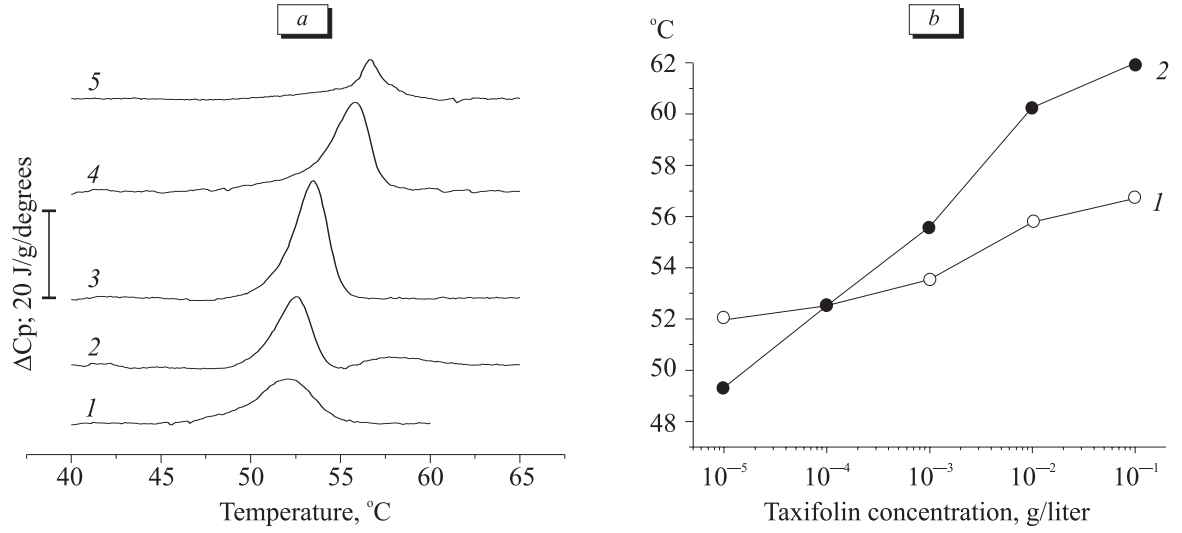

Fig. 3. Differential scanning calorimetry of collagen preparation in 0.01 M phosphate buffer (pH 7.4). *a*) collagen melting curves (0.1 mg/ml) without taxifolin (*1*) and with taxifolin in a concentration of 0.0001% (*2*), 0.001% (*3*), 0.01% (*4*), and 0.1% (*5*). The preparation was stored at 20°C for 12 h; *b*) relationship between the phase transition temperature of collagen fibrils and taxifolin concentration: *1*) preparation stored at 20°C for 12 h; *2*) preparation stored at 37°C for 12 h.

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