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NON-ENZYMATIC DEGRADATION OF ACID-SOLUBLE CALF SKIN COLLAGEN BY SUPEROXIDE ION: PROTECTIVE EFFECT OF FLAVONOIDS

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Abstract—Calf skin acid-soluble collagen in microfibrillar form was incubated with free oxygen radicals produced by the system xanthine oxidase + hypoxanthine. This incubation liberated peptides of a size smaller than that of α-chains, as demonstrated by SDS–PAGE and by evaluation of the 4-hydroxyproline contained in small peptides. The amount of liberated peptides was found to increase with time. The process was inhibited by addition of superoxide dismutase to the medium but not by addition of catalase. Two flavonoids extracted from bilberries and a third one from grapes were demonstrated to protect collagen against this non-enzymatic proteolytic activity. This work confirms that collagen may be degraded during the process of inflammation and that some flavonoids are endowed with protective properties.

The superoxide ion and the derived oxygen free radicals are highly reactive and capable of destroying a variety of molecules. Their liberation during phagocytosis as potent bactericidal agents [1, 2] as well as their action in inflammatory events [3] have been clearly demonstrated. Their effect is probably not limited to foreign materials as the host tissues may be attacked. Several reports have shown that various types of molecules may be degraded, particularly in the connective tissues, where most of the inflammatory reactions take place. Hyaluronic acid is cleaved [4] and collagen gelation is prevented by treatment with superoxide ions, a fact indicative of collagen modifications [5–7].

In this paper, we reinvestigated the effect of free oxygen radicals on collagen microfibrils incubated *in vitro*. We found that even the triple helical region of collagen is cleaved by incubation with the system hypoxanthine + xanthine oxidase (XO). In addition, considering the well-known protective effect of flavonoids [8] which is due to their oxygen free-radical scavenging properties, we tried to demonstrate that the protective effect extended to non-enzymatic collagen degradation. Indeed, we demonstrated a protective effect of three different flavonoid preparations.

MATERIALS AND METHODS

Reagents. All the usual reagents were obtained from Prolabo (Paris, France), and were of analytical grade. XO was supplied by Sigma (St. Louis, MO), grade I, Ref. 1875, in the form of a suspension in an ammonium sulfate solution containing 0.02% (w/v) sodium salicylate. The purification and standardization of the enzymatic activity is described later. Superoxide dismutase (SOD) was purchased from Sigma, type I, Ref. S 8254. It was found to be devoid of proteolytic activity and was used without further purification. Catalase was supplied by Worthington (Freehold, NJ), Ref. 1872 C T R 30 J 987.

Acid-soluble collagen was prepared and purified from calf skin in our laboratory according to Piez *et al.* [9]. Its purity was found to be more than 98% by acrylamide gel electrophoresis (SDS–PAGE) and by the amino acid composition.

We used three different flavonoids. The first one is an anthocyanosidic extract prepared from the bilberry *Vaccinium myrtillus* sp. marketed by Merck-Sharp-Dohme-Chibret (Paris, France) as "Difrarel". The second one is an extract of the same anthocyanoside after a yeast fermentation step. The third is a grape anthocyanoside generously supplied by Dr. S. Brun (Faculty of Pharmacy, Montpellier, France). In all cases, the fraction of anthocyanoside that was soluble in 0.05 M Tris–HCl buffer (pH 7.4) was used and the insoluble fraction was discarded.

Determination of XO activity and control of its purity. Prior to use, the activity of every batch of XO is tested as follows: 20 μl of a solution of crude XO are dissolved in 3 ml of 0.05 M Tris–HCl buffer (pH 7.4) containing 10^{-7} M EDTA, 0.5×10^{-3} M hypoxanthine and 0.32×10^{-4} M nitroblue tetrazolium and incubated for 5 min in the cuvette of a Beckman UV 25 spectrophotometer. The absorbance is recorded at 560 nm and the slope of the initial linear portion of the curve calculated.

As XO preparations often contain a proteolytic activity, we checked every batch as follows: 1 ml of a 0.5% (w/v) azocasein (Sigma) solution in 0.05 M Tris–HCl buffer (pH 7.4) is incubated for 1 hr at 37°. After precipitation by addition of 1 vol. of 12% (w/v) trichloroacetic acid solution to 1 vol. of enzymatic solution, the supernatant is neutralized by adding an equal volume of 0.5 N NaOH solution. The absorbance of the supernatant is read at 520 nm. Most of the XO preparations contain a substantial proteolytic activity, making it necessary to purify the

Table 1. Addition of reagents to the suspension of collagen fibrils in order to detect the degradation induced by oxygen free radicals

	Volume (ml) of reagents added in each test-tube*					
	Collagen control	Hypoxanthine control	Xanthine oxidase control	Sample	Sample inhibited by superoxide dismutase	Sample inhibited by catalase
Collagen fibril suspension (1 mg/ml)	1	1	1	1	1	1
Hypoxanthine solution (1.0×10^{-3} M)	0	1.5	0	1.5	1.5	1.5
Xanthine oxidase solution (0.0075 U/ml)	0	0	0.1	0.1	0.1	0.1
Superoxide dismutase solution (1500 U/ml)	0	0	0	0	0.01	0
Catalase solution (25,000 U/ml)	0	0	0	0	0	0.01
0.05 M Tris–HCl buffer (pH 7.4) containing 0.1 M NaCl	2	0.5	1.9	0.4	0.39	0.39

* All the reagents are dissolved in 0.05 M Tris–HCl buffer (pH 7.4) containing 0.1 M NaCl, and heated to 37° prior to mixing with the collagen suspension. The pattern of reagent addition is the same when the fibrils have been pretreated by anthocyanosides prior to incubation.

enzyme by chromatography on Sephadex G-100 according to Greenwald and Moy [6]. A second chromatography of the enzyme is then routinely performed, in order to insure the purity of the product which is finally dialyzed against a 0.05 M Tris–HCl buffer (pH 7.4) containing 0.1 M NaCl.

Incubation of collagen fibrils. Prior to the incubation, the collagen preparation is dialyzed against a 0.05 M Tris–HCl buffer (pH 7.4) containing 0.1 M NaCl at 37° for 24 hr in order to obtain an homogeneous suspension of fibrils containing approximately 1 mg/ml, as evaluated through hydroxyproline measurements. Samples of 1 ml of this suspension are distributed into a series of test tubes equilibrated at the same temperature.

The reagents, all maintained at 37°, are added to the various tubes according to Table 1. All the tubes are incubated in quadruplicate for 3, 6 or 20 hr at 37° under gentle stirring. By the end of the incubation period, the suspension is centrifuged for 30 min at 20,000 *g* at 4° in order to sediment the remaining fibrils. The clear supernatant is separated for analysis of the contained peptides. On the first aliquot, 4-hydroxyproline is liberated by 6 N HCl hydrolysis and evaluated through a sensitive chromatographic and fluorimetric technique set up in the laboratory [10]. On another aliquot, the soluble peptides are analyzed by SDS–PAGE electrophoresis in a 10% acrylamide gel [11, 12]. The collagenic nature of these peptides is demonstrated by the fact that they all disappear when a bacterial collagenase digestion is performed prior to PAGE [13]. The sizes of these peptides are compared to those obtained by CNBr digestion of type I collagen. Small-size peptides are also sought by evaluation of 4-hydroxyproline in the dialyzates of the supernatants after collagen incubation.

The *N*-terminal amino acids are determined in the mixture of liberated peptides following published dansylation and TLC techniques [14–16].

The protective effects of the anthocyanosidic extracts are evaluated by preincubating the collagen fibrils in 0.05 M Tris–HCl buffer containing 0.1 M NaCl and known weights of the anthocyanosidic extracts to be tested. The mol. wts of the active principles are not known, so that it is impossible to express their concentrations on a molar basis. After a 1-hr preincubation at 37°, the fibrils are separated by centrifugation at room temperature for 10 min at 5000 *g* and washed twice in a 0.05 M Tris–HCl buffer (pH 7.4) containing 0.1 M NaCl at 37°, in order to separate the unbound anthocyanosidic extract. Then, these treated fibrils are reacted like the untreated ones.

RESULTS

Incubation of acid-soluble calf skin collagen with superoxide anions generated by the system XO + hypoxanthine results in the liberation of soluble peptides that contain 4-hydroxyproline. The amount of 4-hydroxyproline contained in these peptides increases with the period of incubation (Table 2). In contrast, incubation of collagen under the same conditions in controls containing buffer only, or hypoxanthine alone, or XO alone, furnishes negligible amounts of hydroxyproline-containing peptides. When SOD is added to the system, the degradation of collagen strongly diminishes. When catalase is added, the degradation is not diminished.

The soluble peptides released under the action of superoxide ions are analyzed by SDS–PAGE. A number of bands of a mobility higher than that of the α-chains of collagen are found (Fig. 1). As compared to the SDS–PAGE pattern of a CNBr lysate of type I collagen, the liberated peptide mol. wts are in the range 10,000–90,000.

The *N*-terminal amino acids of the liberated polypeptides are determined through dansylation and semi-quantitative TLC on silica gel. Approximately

Table 2. Superoxide-induced degradation of collagen, expressed in nmoles of 4-hydroxyproline (see text)

	Amount of 4-hydroxyproline-containing peptides liberated by incubation of 1 mg collagen containing 111.2 μg hydroxyproline for the following incubation periods		
	3 hr	6 hr	20 hr
Control hypoxanthine	<1.35	<1.35	<1.35
Control xanthine oxidase	2.12 ± 0.08 (0.25)	2.87 ± 0.19 (0.34)	8.08 ± 0.45 (0.95)
Incubated sample	16.16 ± 0.20* (1.90)	22.82 ± 0.41* (2.69)	44.30 ± 0.74* (5.22)
Incubated sample with superoxide dismutase	3.54 ± 0.19 (0.42)	5.20 ± 0.23 (0.61)	n.d.
Incubated sample with catalase	14.75 ± 0.38 (1.74)	24.69 ± 0.83* (2.91)	n.d.

Data are the means of quadruplicate results ± S.D. The figures in parentheses refer to the percentages of lysed collagen, the initial amount of collagen corresponding to 100%. The limit of detection of 4-hydroxyproline is 1.35 nmoles per test-tube.

n.d. = not determined.

* Difference from the controls was significant at the $P < 0.05$ level.

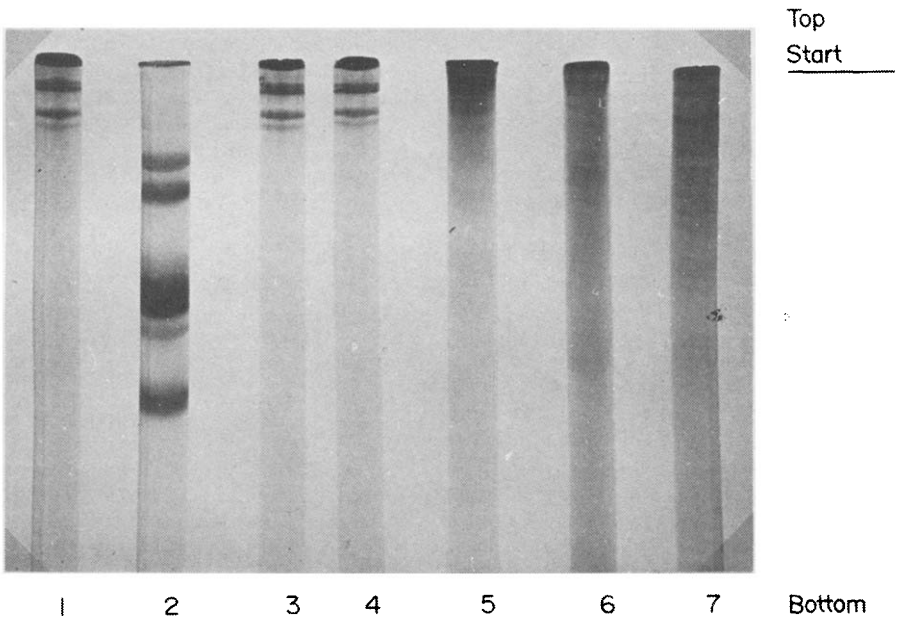

Fig. 1. SDS–PAGE electrophoresis in 10% acrylamide gels. All the samples are denatured by 2 min boiling prior to deposition. From left to right: lane 1, calf skin acid-soluble collagen; lane 2, mixture of CB-peptides obtained by CNBr digestion of calf skin acid-soluble collagen; lane 3, acid-soluble collagen after a 20-hr incubation in 0.05 M Tris–HCl buffer (pH 7.4); lane 4, acid-soluble collagen after treatment by xanthine oxidase in 0.05 M Tris–HCl buffer (pH 7.4); lane 5, acid-soluble collagen after treatment by hypoxanthine + xanthine oxidase for 20 hr in 0.05 M Tris–HCl buffer (pH 7.4); lane 6, acid-soluble collagen previously treated by anthocyanoside extract (the two fast migrating bands correspond to the anthocyanosides); lane 7, acid-soluble collagen previously treated by anthocyanoside extract and after washing the fibrils treated by hypoxanthine + xanthine oxidase, under the same conditions as lane 5. In the last two lanes, the fraction deposited corresponds to the soluble material but there remains an important insoluble residue.

90% of the *N*-terminal residues are glycine. In addition, traces of leucine, isoleucine and valine are found.

When collagen has been treated with the anthocyanosidic extract prior to electrophoresis, most of the collagen becomes unable to enter the gel. The amount of α_1-chains is greatly decreased with a correlative increase in the β-chains that appear more diffuse than usual.

When collagen has been preincubated with the anthocyanosidic extracts prior to incubation with superoxide ions, a protective effect appears even for low concentrations of these substances (Table 3) and this effect is dose-dependent. When the soluble fraction is analyzed by PAGE, the pattern is the same for anthocyanoside- and non-treated samples.

It is not worthwhile to incubate collagen directly with both the superoxide ion generating system and the anthocyanosidic preparations because the latter alone were found to directly inhibit the superoxide-forming system (Table 4).

It was found that the anthocyanoside-treated fibrils do not contain any more free anthocyanoside and that the bound anthocyanosides do not exert a scavenging effect because when these treated fibrils are incubated in the presence of XO and hypoxanthine, the formation of uric acid is not inhibited whereas nitroblue tetrazolium is reduced, provided that the treated fibrils have been washed from any free anthocyanoside prior to the incubation as described in Materials and Methods.

DISCUSSION

By the use of a system generating oxygen free radicals *in vitro*, we were able to cleave fibrillar preparations of acid-soluble collagen into peptides of a relatively small size. These peptides are smaller than α-chains of collagen, which demonstrates that, under our experimental conditions, the cleavage is not limited to the telopeptides, the non-helical extensions of collagen. Greenwald and Moy [6] and Venkatasubramanian and Joseph [7] have proposed that superoxide ions could cleave these telopeptides. We find here that the action of superoxide is even more complete and drastic than expected. The evaluation of the amount of hydroxyproline present in these peptides permits the calculation of the percentage of cleaved collagen. Glycine constitutes 90% of the *N*-terminal amino acids of the liberated peptides. As glycine is largely predominant in collagen (33% of the residues), this finding does not exclude a random effect of the superoxide ions. Nevertheless the figure of 90% seems greatly in excess of the expected value in the case of a random effect. Further research will be devoted to this point. It was demonstrated that the purified XO was devoid of proteolytic activity and that the amount of uric acid generated during the reaction had no effect on the collagen molecule. Additional evidence of the action of superoxide ion is found in the protective effect of SOD added to the incubation medium.

The electrophoretic patterns of the superoxide-

Table 3. Protective effect of anthocyanoside extracts from *Vaccinium myrtillus* and from grapes on collagen degradation by superoxide

	Amount of 4-hydroxyproline-containing peptides liberated by incubation of 1 mg collagen containing 72 μg hydroxyproline for the following concentrations of anthocyanoside extracts					
	0 μg/ml	40 μg/ml	80 μg/ml	160 μg/ml	320 μg/ml	640 μg/ml
Control hypoxanthine	<1.35	<1.35	<1.35	<1.35	<1.35	<1.35
Control xanthine oxidase	4.23	n.d.	n.d.	n.d.	n.d.	n.d.
Anthocyanoside extract ("Difrarel")	32.40	22.55*	17.34*	15.75*	14.31*	12.09*
Anthocyanoside extract (yeast-fermented extract)	32.40	20.52*	19.98*	17.28*	18.36*	16.68*
Grape anthocyanoside extract	32.40	31.44	24.29*	18.06*	12.46*	9.72*

Results expressed as amounts (nmoles/tube) of 4-hydroxyproline contained in the peptides liberated during the 6-hr incubation period.
The limit of detection of 4-hydroxyproline is 1.35 nmoles/tube, n.d. = not determined.
* Difference between the results of incubation in the presence of anthocyanoside and without anthocyanoside significant at the $P < 0.05$ level.

Table 4. Scavenging effect on superoxide ions by the anthocyanosidic extracts

Concentration of anthocyanosidic extracts (μg/ml)	Bilberry anthocyanosides (Difrarel) (mean ± S.D.)	Bilberry yeast-fermented anthocyanins (mean ± S.D.)	Grape anthocyanosides (mean ± S.D.)
0	100	100	100
3.2	117.0 ± 1.8	87.8 ± 0.5	55.0 ± 2.0
32.0	42.0 ± 0.9	50.1 ± 3.5	11.3 ± 0.4
320.0	15.0 ± 1.6	18.0 ± 3.1	6.8 ± 0.1

The system hypoxanthine–xanthine oxidase (see text) is incubated for 5 min with the amount of anthocyanosidic extract shown in the table and the remaining superoxide activity measured by reduction of nitroblue tetrazolium (see text).
Results expressed as per cent of the activity found without a scavenger.

treated fibrils demonstrates that fragments of a size smaller than the α-chain are cleaved by this treatment. On the other hand, the preincubation of fibrils with anthocyanosides induces the disappearance of α-chains with an increase in β-chains, demonstrating that these substances induce some kind of cross-linking of the α-chains. When the collagen fibrils have been pretreated by anthocyanosides prior to the exposure to superoxide ions, the amount of solubilized peptides decreases but some cleavage still remains and the solubilized peptides show the same pattern as without pretreatment.

It remains now to demonstrate to what extent such an effect is possible *in vivo*. The formation of superoxide ions during phagocytosis as well as in the course of the inflammatory syndrome [17–21] has been clearly demonstrated. Also, the protective effect of chemical complexes with SOD activity such as copper-penicillamine [22] has been shown. In addition, there are frequent pathological situations such as atherosclerosis, in which an inflammatory state proceeds at a low chronic rate. In these situations, the proteasic activity is found to be increased. It appears possible that this enzymatic degradation of proteins should be paralleled by a non-enzymatic proteolytic process induced by superoxide ions. If the action found *in vitro* on the non-denatured collagen molecules corresponds to the *in vivo* situation, it may generate peptidic fragments of collagen endowed with chemotactic activity which activate the fibrotic events necessary for repair.

The scavenger activity of flavonoids of anthocyanosidic extracts have been emphasized by several authors [8]. These flavonoids have an activating influence on the collagen biosynthesis and a stimulating effect on the reticulation of collagen fibrils [23, 24]. In this paper, we demonstrate not only a scavenger activity of the bilberry and grape extracts, but, in addition, a protective effect on the collagen fibrils. Further experiments will be necessary to confirm whether the same effect is operative *in vivo* and may constitute a way to the treatment of atherosclerosis.

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