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EVIDENCE BY *IN VIVO* AND *IN VITRO* STUDIES THAT BINDING OF PYCNOGENOLS TO ELASTIN AFFECTS ITS RATE OF DEGRADATION BY ELASTASES

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Abstract—Procyanidol oligomers and (+) catechin bound to insoluble elastin markedly affect its rate of degradation by elastases. Insoluble elastin pretreated with procyanidol oligomers (PCO) was resistant to the hydrolysis induced by both porcine pancreatic and human leukocyte elastases. The quantitative adsorption of pancreatic elastase was similar on either untreated or PCO-treated elastin suggesting that the binding of this compound to elastin increases the non-productive catalytic sites of elastase molecules. (+) Catechin–insoluble elastin complexes were partially resistant to the degradation induced by human leukocyte elastase but were hydrolysed at the same rate as untreated samples by a constant amount of pancreatic elastase. In addition, the coacervation profile of kappa-elastin peptides as a function of temperature is greatly modified in presence of these flavonoids.

We conclusively evidenced that PCOs bind to skin elastic fibres when injected intradermally into young rabbits. As a result, these elastic fibres were found more resistant to the hydrolytic action of porcine pancreatic elastase when injected to the same site.

These *in vivo* studies further emphasized the potential effect of these compounds in preventing elastin degradation by elastase(s) as occurred in inflammatory processes.

The fragmentation of elastic fibres, as observed histologically in several pathological states or during aging, could be attributed either to the deficiency of lysyl oxydase [1] and/or to the imbalance between elastase(s) and its natural inhibitors [2].

Many cell types including polymorphonuclear neutrophils, platelets, macrophages, aorta smooth muscle cells and fibroblasts do contain such elastase-type proteases which could be released from cells by various stimuli [3]. *In vivo*, the action of these endopeptidases is controlled by natural inhibitors present in physiological fluids and tissues [4]. However, in several conditions, these protease inhibitors were shown not to be able to exert their biological activity due to a functional or a genetic deficiency or to other causes [5].

Exogenous inhibitors purified from microorganisms or synthetized in order to fit the extended substrate binding site of the protease were used as tools to inhibit *in vivo* elastolysis [6]. The main drawback of this therapeutic approach relied in the multiplicity of cellular elastases which react differently to synthetic inhibitors [3]. In addition, in some conditions, specific elastase inhibitors may prove totally inefficient.

For instance, trypsin and α-chymotrypsin with no or little intrinsic elastolytic potential can solubilize elastin when acting synergistically [7].

We therefore adopted an alternative approach to this problem, based on molecular interactions between elastin and elastase and consisting in the protection of the substrate from proteolytic degradation.

Although compounds related to tannins are generally considered to be non-specific protein binding agents, it was recently demonstrated that pro-anthocyanidins may well precipitate one protein in the presence of a large excess of another protein depending on their respective isoelectric points and structures [8]. It was also shown that proteins and polypeptides rich in proline exhibited a high affinity for tannins [8]. Collagen and elastin are proline-rich macromolecules and this may partly explain the high affinity of several flavonoids for connective tissues [8, 9].

(+) Catechin affects collagen biosynthesis by inhibiting prolyl and lysyl hydroxylases and also influences the reticulation of collagen fibrils [10, 11]. It was also reported that the treatment of Guinea-pig skin derived soluble collagen with (+) catechin renders the collagen resistant to the action of bacterial collagenase [12].

In the present investigation, we studied by *in vitro* and *in vivo* experiments, the potentiality of flavonoids to modulate the rate of elastin degradation by elastases.

MATERIALS AND METHODS

Reagents. Two flavonoids were used in the present study: (+) catechin 3,3′,4′,5,7-flavanepentol was obtained from Sigma Chemicals (St. Louis, MO, U.S.A.), and procyanidolic (flavane-3,4-diols) oligomers from Labaz Laboratories (France). Flavane-3,4 diols differ from catechin by the presence of an additional OH group in the 4th position of the tetrahydropyrane ring. This explains the tendency of this compound to form dimers and oligomers in solution.

Insoluble elastin was prepared from calf ligamentum nuchae by the hot alkali technique and was found to be devoid of any contaminating protein by conventional analytical procedures [9].

Kappa-elastin peptides derived from calf insoluble ligamentum nuchae elastin were prepared by alkaline hydrolysis in presence of ethanol as described [9].

In addition, low molecular weight elastin peptides (~15,000) were further eliminated by molecular sieve chromatography on a Sephadex G-150 column.

The apparent molecular weight of the kappa-elastin preparation used in these experiments was between 20 and 60 KD.

Porcine pancreatic elastase (125 U/mg) was purchased from Sigma Chemicals (St. Louis, MO, U.S.A.).

Lysozomal elastase was purified from human spleen as recommended by Starkey and Barrett [13]. The enzyme was purified 1405-fold and the preparation was homogenous by SDS-page electrophoresis. We found that 1 mg of calf ligamentum nuchae elastin is dissolved after 1 hr incubation at pH 8.25 respectively by 0.15 mg of human leukocyte elastase or by 0.02 mg of porcine pancreatic elastase.

Elastase assays. Succinoyl-trialanine-paranitroanilide (Suc(Ala)$_3$ NA, Choay Laboratories, France), was used as synthetic substrate as recommended by Bieth [14].

Elastinolytic activities were quantitated with calf ligamentum nuchae insoluble elastin labelled with NaB^3H_4 as substrate according to a previously published procedure [15]. The specific activity of the ^{3}H-insoluble elastin was equal to 0.35 10^6 cpm/mg.

Coacervation of kappa-elastin peptides. The coacervation of kappa-elastin peptides as a function of pH and temperature was determined essentially as described by Partridge and Whiting [16]. 100 mM acetate buffers (pH 3.6 to 5.2) were used as solvents and the final concentration of elastin peptides was 5 mg/ml.

Intradermal injections of flavonoids and elastase in rabbits. Procyanidolic oligomers (2 g), basic fuschin (1 g) and ferric chloride (100 mg) were dissolved in 100 ml of 30% ethanol/water v/v. The solution was allowed to stand 2 hr at room temperature and was then filtered through Whatman No. 1 filter paper. 0.5 ml of this solution was injected intradermally in young rabbits as previously described [17]. After 3 hr, 0.2 ml of phosphate buffered saline (PBS) containing various amounts of porcine pancreatic elastase was injected at the same site. Biopsies were made at the site of injection and were exhaustively washed with PBS containing protease inhibitors [18]. The biopsies were then frozen at −30° in a cryostat.

Frozen sections (6 μm) were successively washed with PBS, with 70% alcohol (3 times) with pure grade ethanol (3 times) and finally with xylen (3 times).

Microscopic observations were made in a Polyvar Young Reichert microscope. Control experiments consisted in injecting similar quantities of basic fuchsin and ferric chloride not containing procyanidolic oligomers.

The capacity of porcine pancreatic elastase to hydrolyse the skin elastic fibres system was also controlled using classical stains for elastin. Independently of the staining technique it was found that, in our experimental conditions, 10 μg of porcine pancreatic elastase induced a nearly complete disappearance of the elastic fibres. We usually used one rabbit per set of experiments (including the controls) and the experiments were repeated 3 times using 3 different young rabbits.

Expression of the results. The degradation of skin elastic fibres directly visualized on microscopical sections (20–50 in each case) was quantitated by determing the length of such fibres (in μm) on a Reichert Jung video screen [17]. The statistical variations of the length of the elastic fibres were evaluated by the Student *t*-test. Alternatively, the density of elastin present on photographs was evaluated as follows: a transparent grid was placed at random onto skin thin sections photographs. In order to take into account photographic enlargements and corresponding elastic fibres' length in control experiments, the grid consisted of squares having a ratio equal to 5 mm [19].

The number of intercepts between elastic fibres and the x,y axis of the grid was determined and the density index of elastin ($El_{d,i}$) was defined as the total number of intercepts per unit area (a fixed number of squares).

RESULTS

In vitro *studies*

One mg of insoluble elastin purified from calf ligamentum nuchae could be saturated by respectively 125 μg of procyanidolic oligomers and 20 μg of catechin. The binding of these flavonoids to elastin was not appreciably modified by changes in pH. Preliminary data also indicate that proteins including gelatin, bovine serum albumin, high molecular weight soluble kappa-elastin peptides as well as several dipeptides (Ser-Leu, Pro-Pro, Val-Gly, Val-Pro) did not quantitatively affect the interactions between these two pycnogenols and insoluble elastin near neutral pH. Subsequently, 1 mg of ^{3}H-labeled insoluble elastin (0.35 10^6 cpm/mg) was pretreated with an excess of procyanidolic oligomer (250 μg) or (+) catechin (250 μg) and the rates of solubilization of these flavonoid treated samples by 100 ng of porcine pancreatic elastase were compared to those of untreated elastin (Fig. 1A): the kinetics of solubilization of elastin were linear up to 24 hr of incubation at 37° in 100 mM Tris–HCl buffer pH 8.25. The rate of solubilization of (+)catechin treated elastin by porcine pancreatic elastase was similar to that obtained with the control samples. Pretreatment of elastin with procyanidolic oligomers however greatly reduced (more than 10-fold) its degradation by the same amount of protease.

In order to exclude a possible inhibitory effect of these polyphenolic substances on the enzyme itself, succinoyl-trialanine-paranitroanilide (Suc(Ala)$_3$NA) was used as substrate. It could be demonstrated that none of the two flavonoids did modify (at a concentration up to 100 μg/ml) the rate of hydrolysis of this substrate by porcine pancreatic elastase. It

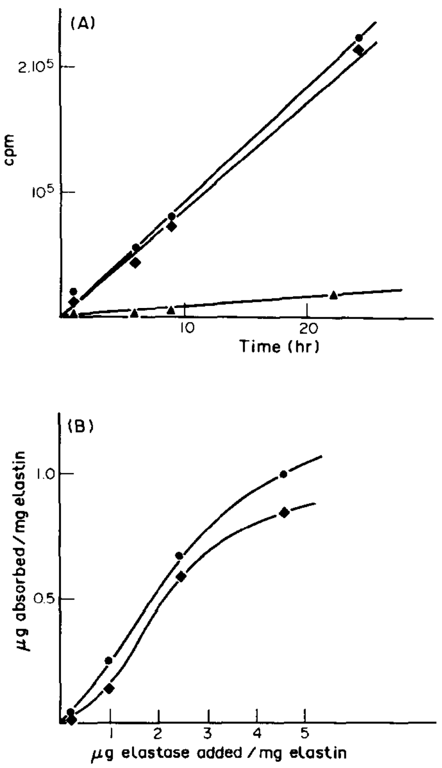


Fig. 1. A. Kinetics of solubilization of flavonoids treated and untreated ^{3}H-insoluble elastin (0.35 10^6 cpm/mg) by 100 μg of porcine pancreatic elastase (125 U/mg). 100 μl of a 10 mg/ml suspension of ^{3}H-insoluble elastin are pretreated with 250 μg of either (+) catechin or procyanidolic oligomers for 1 hr at 37° in 1 ml of 100 mM Tris HCl buffer pH 7.5. The tubes were then centrifuged at 10,000 g and washed 3 times with 1 ml of this buffer. The insoluble pellets were then dispersed in 1 ml of 100 mM Tris HCL pH 8.25 containing 0.02% sodium azide and 100 ng of porcine pancreatic elastase (125 U/mg). The tubes were then allowed to stand at 37° under mechanical shaking; the enzymatic reaction was stopped by adding to the tubes 10 μl of 100 mM solution of phenylmethanesulfonylfluoride (PmsF). They were then centrifuged at 10,000 g for 15 min and 50 μl aliquots were withdrawn for the determination of the radioactivity in an Intertechnique scintillation counter.

Abscissa: time in hours; ordinates: total cpm in the supernatant; ●—●, control sample; ♦—♦, (+) catechin treated elastin; ▲—▲, procyanidolic oligomer treated elastin (P.C.O.). The values represent the mean of triplicate and two separate experiments. S.E.M. $< 10^2$ cpm.

B. Adsorption of porcine pancreatic elastase to procyanidolic oligomers treated and untreated insoluble elastins. Unlabelled calf ligamentum nuchae insoluble elastin was pretreated with procyanidolic oligomers as indicated in A. The insoluble elastins were then dispersed in 1 ml of 100 mM Tris HCl buffer pH 7.5 containing increasing amounts of porcine pancreatic elastase. The tubes were allowed to stand at room temperature for 15 min with occasional shaking. They were then centrifuged at 10,000 g for 5 min and 25 μl aliquots of the supernatants were withdrawn to quantitate non adsorbed elastase on Suc(Ala)$_3$NA as substrate.

Abscissa: μg porcine pancreatic elastase added per mg elastin; ordinates: μg porcine pancreatic elastase adsorbed per mg elastin; ●—●, untreated elastin; ♦—♦, procyanidolic polymers treated elastin.

could therefore be stated that procyanidolic oligomers inhibited elastolysis by modifying elastin–elastase interactions.

Figure 1B indicates that the saturation binding curve of porcine pancreatic elastase onto elastin was not appreciably modified by the pre-treatment of the macromolecules with procyanidolic oligomers. One mg of untreated elastin binds maximally 1 μg of pancreatic elastase.

Procyanidolic oligomer-treated elastin binds in the same experimental conditions 0.75 μg of elastase. This low difference in enzyme adsorption could not explain the observed inhibition of elastin solubilization following pretreatment of the macromolecule with procyanidolic oligomers.

These results suggest that binding of procyanidolic oligomers to elastin might increase the non-productive binding sites for elastase molecules.

Leukocyte elastase purified from human spleen was also used as another source of elastolytic enzyme in order to investigate the potential of flavonoids as protective agents during inflammatory reactions (Table 1).

The inhibitory effect of procyanidolic oligomers is even more pronounced with human leukocyte elastase. Pretreatment of elastin with this flavonoid completely inhibits the action of 10 μg of purified enzyme. Interestingly and in keeping with previous comparative studies on the specificities of these elastases [20], pretreatment of elastin with (+) catechin considerably decreased its susceptibility to solubilization by human leukocyte elastase. In addition, treating (+) catechin in boiling HCl under oxygen (treatment producing oligomers), increased the inhibitory potential of this compound when added to elastin towards both pancreatic and leukocyte elastases.

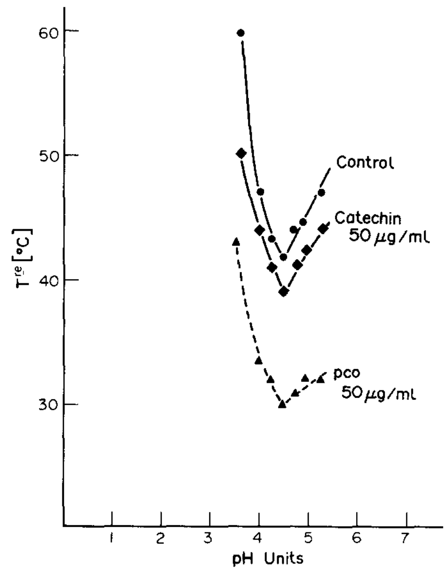


Fig. 2. Coacervation profile of high molecular weight kappa-elastin (5 mg/ml). Solvent: 100 mM acetate buffer; Abscissa: pH units; ordinates: temperature (°); ●—●, control kappa-elastin solution; ♦—♦, coacervation profile in presence of 50 μg/ml (+) catechin; ▲—▲, coacervation profile in presence of 50 μg/ml of procyanidolic oligomers (PCO).

Table 1. Effect of pycnogenols on the degradation of ^{3}H elastin by elastases

Effector	Porcine pancreatic elastase (cpm released)	Porcine pancreatic elastase (% of control)	Human leukocyte elastase (cpm released)	Human leukocyte elastase (% of control)
None	137,320	100	96,870	100
Procyanidolic oligomers	18,430	13.4	40	00
(+) Catechin	151,610	110.4	35,960	37.1
(+) Catechin* oligomers	101,860	74.2	10,140	10.5

100 μl of a 10 mg/ml ^{3}H insoluble elastin dispersion (0.35 10^6 cpm/mg) was pretreated with 250 μg of procyanidolic oligomers, 250 μg of (+) catechin or 250 μg of (+) catechin oligomers (previously treated with boiling HCl under O_2 during 15 min in 1 ml of 100 mM Tris HCl buffer pH 7.5 for 1 hr at 37°.

The tubes were then centrifuged and the elastin pellets resuspended in 100 mM Tris HCl, 0.02% NaN_3 pH 8.25 containing either 5 μg of porcine pancreatic elastase or 80 μg of human leukocyte elastase. The tubes were allowed to stand 2 hr at 37° with mechanical stirring.

They were then centrifuged at 10,000 *g* for 5 min and 100 μl of the supernatant were withdrawn for radioactivity determination. The results represented the mean of two separate experiments performed in triplicate.

The S.E.M. values never exceeded 1000 cpm.

The coacervate of elastin peptides has been considered as a model for the relaxed fibrous state of the polymer. Changes in the temperature at which coacervation first occurs is likely to be a sensitive indicator of conformational changes in different solvents [16]. The coacervation profile of kappa-elastin as a function of temperature is dramatically modified in presence of procyanidolic oligomers at low concentrations (Fig. 2).

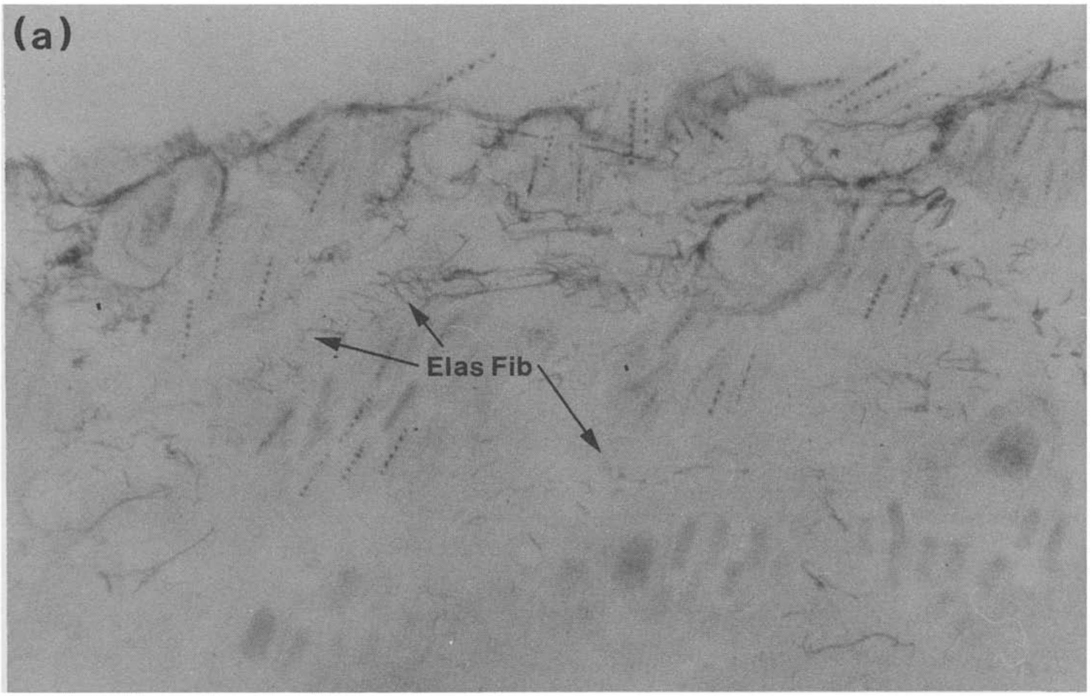
The coacervation temperature of elastin peptides is lowered to 10–15° near the isoelectric point of the peptide moieties in the presence of 50 μg/ml of procyanidolic oligomers. This may partly explain the beneficial effect of this compound in favouring fibrillogenesis during experimental lathyrism [21]. Such effect was also observed with (D +) catechin but to a much lower extent.

Hydrogen binding between phenolic hydroxyl residues and peptide bound proline carbonyl residues was proposed to be the major stabilizing flavonoid-protein complexes [8, 22]. In elastin, most of the proline residues are found in sequences which favour the formation of β turns [23].

Addition of flavonoids to solutions of kappa-elastin may well produce conformational changes towards a more ordered structure as indicated by the decrease in the coacervation temperature.

In vivo *experiments*

Previous published data emphasized that various types of polyphenolic structures including flavonoid type substances bound effectively and in a specific manner to elastic fibres as evidenced by histological observations [24, 25]. In preliminaries of the present



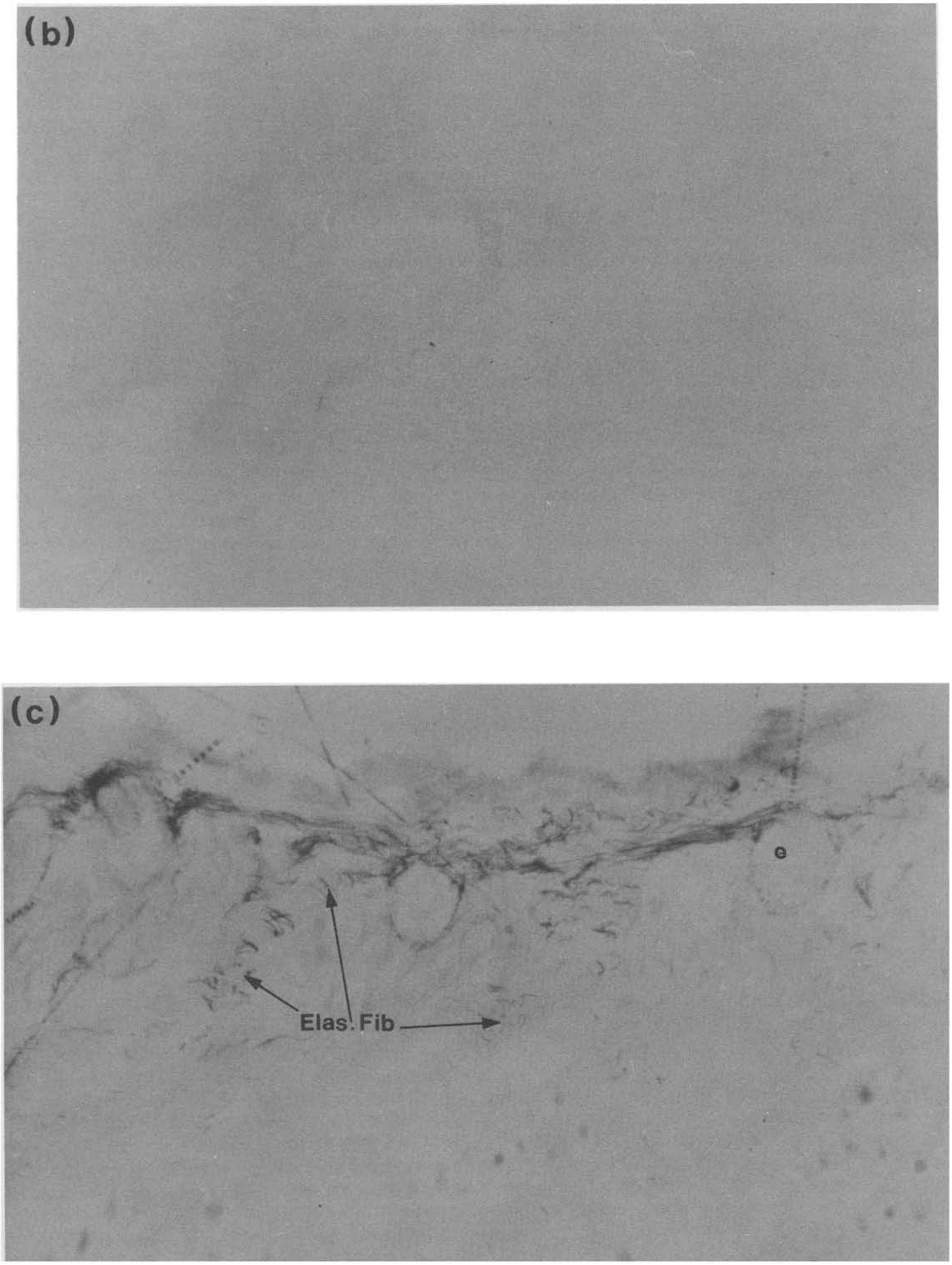


Fig. 3. (A) Control rabbit skin treated *in vivo* with procyanidolic oligomers stained according to the technique described in Material and Methods. Elastic fibres (El. Fib.) are well visualized and the background is colourless (×360).

(B) Rabbit skin treated with pancreatic elastase (10 μg). No elastic fibres are visualized after the enzymatic action of pancreatic elastase (10 μg) (×360).

(C) Rabbit skin treated with procyanidolic oligomers and then with pancreatic elastase (10 μg) (×360).

Table 2. Effect of preintradermal injection of PCO on to the degradation of rabbit skin elastic fibres by porcine pancreatic elastase

	(1) Control	(2) Elastase treated	(3) PCO and elastase treated
Mean length of skin elastic fibres (± S.E.M.)	13.95 ± 2.5	00	9.04 ± 1.3
Elastin density index. $El_{d,i}$ (± S.E.M.)	4.4 ± 0.2	00	3.08 ± 0.2

1. Rabbit(s) received 0.5 ml of phosphate buffer saline (PBS) or an ethanolic solution of basic fuschin and $FeCl_3$. They were then stained for the identification of elastic fibres.

2. Rabbit(s) received 0.5 ml of PBS containing 10 μg of porcine pancreatic elastase (0.75 Unit) and thin tissue sections were postfixed with PCO (2 g), basic fuschin (1 g) and $FeCl_3$ (100 mg) ethanolic solution 1/2 hr after the injection of elastase.

3. Rabbit(s) were first injected with the PCO solution (0.5 ml) then received 0.5 ml of PBS (× 2) and finally the elastase solution as in (2). (Enlargement ×360).

S.E.M.: standard error of the mean.

investigations, we demonstrated that a solution consisted of procyanidolic oligomers (2 g), basic fuschin (1 g) and ferric chloride (100 mg) in ethanol could be used as a stain for revealing elastic fibres on rabbit or/and human thin tissue sections. Basic fuschin by itself was unable to stain to any extent elastic fibres of several different elastic tissues.

In addition, this staining procedure was found selective for elastic fibres as evidenced by colourless background.

It was then proposed to use such a technique for evaluating elastin contents in a morphometric analytical basis [25].

When such a solution was injected intradermally to a young rabbit, the elastic fibre system could still be directly identified on tissue section from skin biopsies performed at the injected site (Fig. 3A). This *in vivo* staining was similar if not identical to those obtained *in vitro* from non-injected tissue, using the same reaction mixture to identify rabbit skin elastic fibre system. Elastase injections and further staining of tissue sections resulted in a gradual loss of the length and density index of elastin [25]. In our experimental conditions, injection of 10 μg of porcine pancreatic elastase (0.75 U) was sufficient to induce a complete disappearance of elastic fibres (Fig. 3B).

When a PCO solution was injected at the same site prior to elastase, a conspicuous protection of elastic fibres against proteolytic degradation could be demonstrated (Fig. 3C).

This significant protective index could be quantitatively determined as described in the method section and is equal to 70% independently of the type of determinations used (Table 2).

DISCUSSION

Inflammatory processes result in the recruitment of cells as polymorphonuclear neutrophils and macrophages to the site of injury. These cells contain proteolytic enzymes including elastase-type proteases [26], which are secreted from cells upon stimulation. Under this situation, the local concentration of elastase(s) would certainly exceed by several orders the magnitude the local concentrations of natural occuring inhibitors, e.g. alpha-1 antitrypsin and alpha-2 macroglobulin. As the respective elastases possess different specificities, exogenous synthetic elastase inhibitors designed for one particular protease could be inefficient in controlling elastolysis [27].

As a result, we explored the potential of several agents able to bind to the elastic fibres in a "quasi-specific" fashion and protect further degradation of the polymer by the elastases. In the present work, we evidenced that flavonoids could efficiently and selectively bind to elastic fibres. Our *in vitro* studies showed conclusively that such binding protected the elastin against degradation by either purified porcine pancreatic elastase or leukocyte elastase. H. Kagan *et al.* have conclusively shown that elastase(s) may bind to elastin in at least two competitive ways [28]. The active enzyme may be attracted to anionic sites on the elastin surface at which alanyl residues accessible to the active site of the protease afford the appropriate conditions for productive proteolytic scission of the polypeptide chain. Alternatively, a second competing mode of binding would involve similar ionic binding forces but at sites on elastic which do not present the appropriate aminoacid side chain. Our results indicated that procyanidolic compounds bound on to insoluble elastin increased the non-productive binding sites of elastase(s).

This contention is supported by our data which showed that PCO treated elastin bound similar quantities of protease as untreated elastin samples. Nevertheless, these PCO treated samples were completely refractory to the proteolytic action of either porcine pancreatic elastase or/and human leukocyte elastase. It was also suggested that elastin dyes bound to the crosslinked regions of insoluble elastin [29]. This will partly explain the observed differences of the degradation of catechin treated elastin by porcine pancreatic and human leukocyte elastases since it was demonstrated that human leukocyte elastase differed from porcine pancreatic elastase by the presence of an unusual hydrophobic binding site which can accomodate desmosine analogues [30].

The above described data indicated that pretreatment of elastin with flavonoid prevented further degradation of this connective tissue macromolecule by elastase(s). This property may be helpful to control elastolysis and indeed we recently observed that flavonoids could protect hamsters against emphysema induced by intratracheal instillation of elastase.

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