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Relationship Between the Structure of Some Humic Compounds and Their Inhibitory Effects on Carp Catalase

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Phenolcarboxylic acids, aldehydes and flavonoids are common components of the plant organism; among others, they are precursors and degradation products of the plant cell wall (Haslam 1988; König and Scholz 1994). As breakdown products of plants, phenolics pass into the soil, where they are converted into components of humic compounds under microbial mediation (Wetzel 1992). The humic compounds (phenolcarboxylic acids) may be washed into natural waters, where they form a major component of the dissolved organic acids and can constitute about four-fifths of the total organic matter (Wetzel 1993); concentrations of 4-8 mg or more polyphenolic acids per liter are common (Wetzel 1983; Perdue and Gjessing 1990).

Increasing attention has recently been paid to these substances, since they have proved to provide chemoprotection against carcinogenesis and the probability of progression of many common chronic diseases (Gali et al. 1992; Frankel et al. 1993), due to their antioxidant activity, i.e. they scavenge free radicals or induce protective enzyme activities (Majid et al. 1991; Terao et al. 1994; Barth et al. 1995; Salah et al. 199.5; Ueda et al. 1996).

The polyphenolic compounds can form complexes reversibly with protein molecules, serving as polydentate ligands which provide hydrogen-bonding sites via the phenolic groups and aryl rings on the periphery of the phenolic molecule (Ladd 1985; Haslam 1988; Haslam and Lilley 1988; Spencer et al. 1988). Through their ability to combine with proteins, polyphenolic compounds and humic substances can partially or entirely inactivate various enzymes (Stewart and Wetzel 1982; Ladd 1985); thus, these substances might play a regulatory role in freshwater ecosystems (Wetzel 1991, 1993; Kim and Wetzel 1993).
Ever more studies have been published concerning the enzyme-inhibitory effects of plant phenols in algae, bacteria, plants and mammals (Goldstein and Swain 1965; Wetzel 1992, 1993; Erdei et al. 1994; Reed 1995). While kinetic studies have shown that the inhibition of several enzymes by phenolics (humic substances) is non-competitive (Wetzel 1983; 1991; Murakami et al. 1991, 1992; Kim and Wetzel 1993; Zhang and Das 1994), the inhibitory effect of plant polyphenols have been less well examined. *In vitro* studies can contribute to a clarification of the relation of the structure to the inhibitory properties of phenolics, and such results might be of clinical significance or might help to solve certain ecological problems in the near future. *In vitro* studies can also help to reveal the possible toxicity of phenolic compounds (as we report in this study on tannic acid), which may be essential for further studies.

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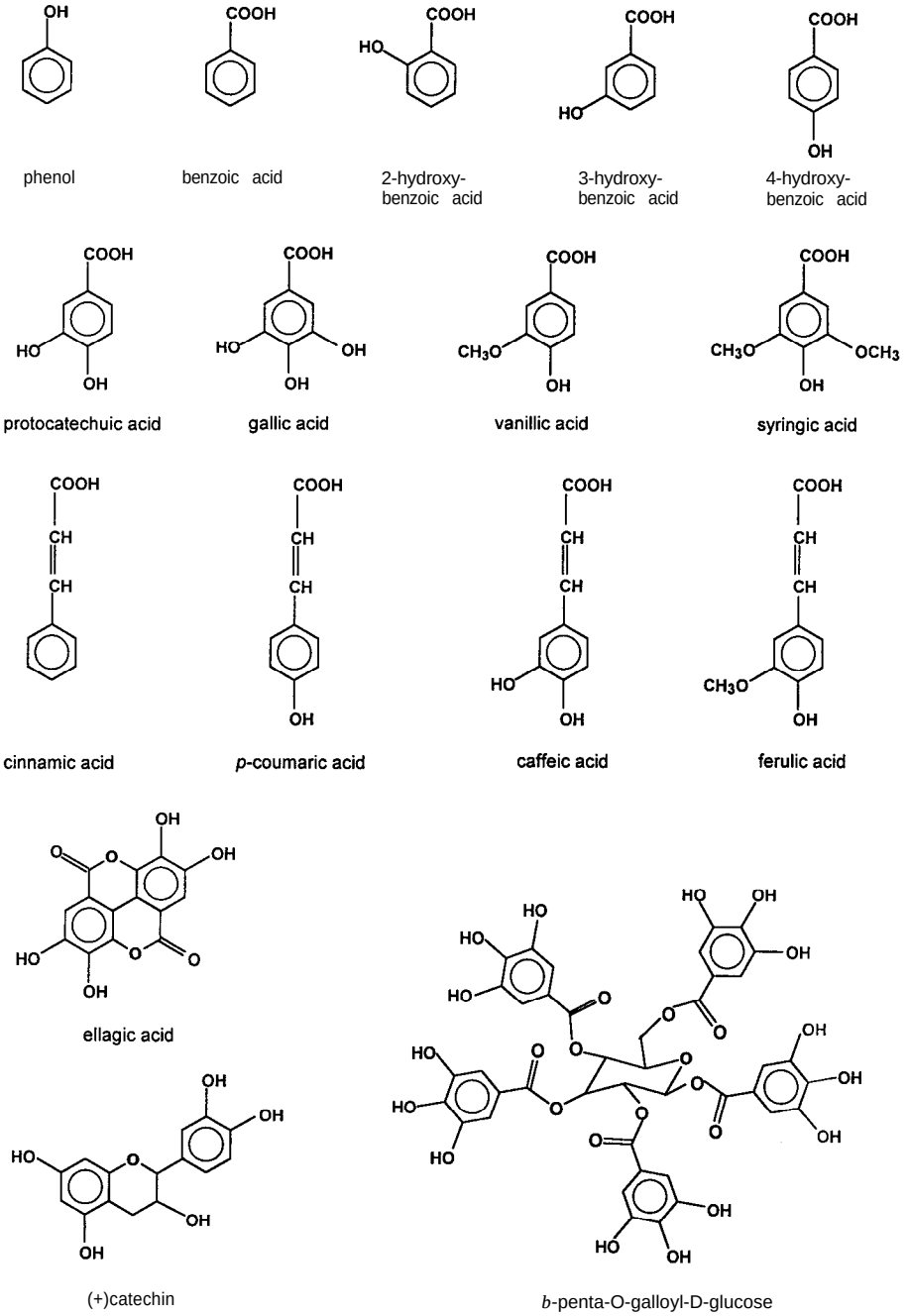

Figure 1. Structures of the examined phenolics. These compounds are precursors and/or degradation products of plant cell wall substances. *b*-Penta-O-galloyl-D-glucose is a model compound of hydrolyzable tannins.

Freshwaters may be comparatively rich in phenolics (Wetzel 1983; Perdue and Gjessing 1990), and fish are at the top of the food chain, so they can accumulate larger quantities

of contaminants than do other organisms. Accumulated humics can act not only as antioxidants in the fish organism, but also as (e.g. antioxidative) enzyme inhibitors. Antioxidative enzyme inhibition may lead to a higher free radical concentration, which may induce enzyme inactivation (Bartkowiak et al. 1981) and therefore metabolic deficiencies. Since phenolics have antioxidative and enzyme-inhibitory effects, and since catalase is a very active antioxidant enzyme of aerobic organisms, *in vitro* analyses on the effects of plant-derived phenolics on catalase could furnish theoretical information of value in further studies. On the above basis, the main aim of the present study was to investigate the possible relations between the number and locations of the substituents and the inhibitory properties of phenolics on catalase as a model enzyme. For this purpose, *in vitro* experiments were carried out with 16 different phenolic compounds. Three structural groups (hydroxybenzoic and hydroxy-phenylpropenoic acids and phenolics with complex structures) were investigated. IC_{50} values were calculated via concentration and time-dependent assays in order to evaluate the toxicity of the phenolics studied in terms of enzyme inhibition.

MATERIALS AND METHODS

All chemicals were of analytical grade. Phenolics: phenol (crystallized) was purchased from BDH, benzoic acid, 2-hydroxybenzoic acid and cinnamic acid (3-phenylpropenoic acid) were purchased from Reanal, 3-hydroxybenzoic acid and 4-hydroxybenzoic acid were purchased from Aldrich, and protocatechuic acid (3,4-dihydroxybenzoic acid), gallic acid (3,4,5-trihydroxybenzoic acid), vanillic acid (4-hydroxy-3-methoxybenzoic acid), syringic acid (4-hydroxy-3,5-dimethoxybenzoic acid), *p*-coumaric acid (4-hydroxycinnamic acid), caffeic acid (3,4-dihydroxycinnamic acid), ferulic acid (4-hydroxy-3-methoxycinnamic acid), (+)catechin ([2R,3S]-2-[3,4-dihydroxyphenyl]-3,4-dihydro-1[2H]-benzopyran-3,5,7-triol), ellagic acid (4,4',5,5',6,6'-hexahydroxydiphenic acid 2,6,2',6'-dilactone) and tannic acid ([1401-55-4] M_r 1700.2, structure not given) were purchased from Sigma. The structures of the studied phenolics are shown in Figure 1. All other reagents (H_2O_2, EDTA, Triton X-100, $Na_2HPO_4x2H_2O$ and $KH_2PO_4x2H_2O$) were obtained from Reanal, and bovine serum albumin (BSA) was purchased from Sigma.

Carp *(Cyprinus carpio* L.) were used in the experiments: among fish for breeding, the carp is of major economic importance in Central Europe, and it is easy to obtain and keep for experiments. Fish of both sexes, weighing 800-1000 g, were obtained from the Tisza Fish Farm, Szeged, Hungary. They were kept in air-saturated water (pH 7.0-7.1) at 20±1 °C in 100-liter aquaria and acclimatized for 7 days before the experiment. After the acclimatization, the animals were killed and drained of blood, and the livers were taken from them for the preparation of enzyme solutions. The tissue was washed with fish saline solution (0.62% NaCl) and cleaned with paper towels. The liver was then homogenized (l/5, wt/v) with a Braun 853302/4 homogenizer in 12.5 m*M* sodium phosphate buffer (pH 7.0) containing 0.4 M NaCl, 0.1344% EDTA and 0.5% Triton X-100. The suspension was centrifuged at 10,000g for 70 min. The supernatants were stored at -80 °C.

Catalase activity was measured by Cohen's method (1970). The reaction was conducted for 1 min at 25 °C. The activities of peroxidase and glutathione-peroxidase proved to be rather low in the supernatant of the homogenate of carp liver (less than 0.1% of the catalase activity), so their H_2O_2-decomposing activities were negligible.
Time-dependent assays were carried out to determine the infinite incubation time for phenolics, needed for IC_{50} determinations. In these assays, the liver homogenate

supernatant (diluted 750-fold in reaction mixture) was incubated with a phenolic (each was dissolved in 50 m*M* pH 7.0 phosphate buffer, final concentration: l-1000 µ*M)* for 1, 3, 5, 10, 20, 40, 60 or 100 min, H_2O_2 was then added and the catalase activity was measured.

Concentration-dependent assays were carried out as described above, but with incubation only for 30 min (on the basis of the results of time-dependent assays). The final concentration of a phenolic compound in the reaction mixture varied in the range 0.1-4000 µ*M* (each was dissolved in 50 m*M* pH 7.0 phosphate buffer). In control assays, the mixture did not contain phenolic.

The IC_{50} value for each phenolic was determined via the Hill plot (Cornish-Bowden 1979) from the data of concentration-dependent assays. Protein concentration was measured according to Bradford (1976). Three parallel measurements were carried out and the reported data represent mean values ± SD. Statistical significance was assessed with the Student t-test. The level of statistical significance was taken as $P < 0.05$.

RESULTS AND DISCUSSION

The stability of the untreated fish catalase at 25 °C is shown in Figure 2. It lost about 5-7% of the original activity during 30 min, and a further 7-9% during storage for 60-100 min (Figure 2). Time-dependent assays demonstrated that the phenolics exerted 90-100% of their inhibitory effect in 30 min, with the exception of tannic acid, which needed about 50-60 min for its total inhibitory effect (Figure 2). The incubation time for concentration-dependent assays was therefore chosen to be 30 min.

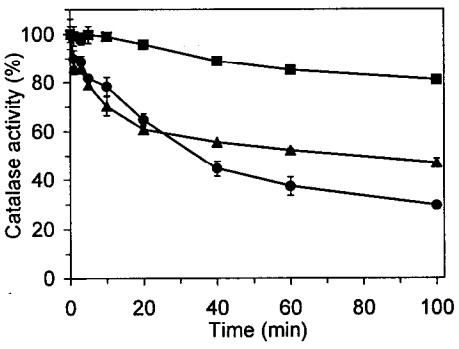

Figure 2. The two types of time-dependent curves and the spontaneous activity loss of catalase. The untreated carp catalase (■) lost about 5-7% of the original activity during 30 min, and a further 7-9% during 60-100 min. Caffeic acid [10^{-5} *M]* (A), representing the tested phenolics, exerted 90-100% of its inhibitory effect in 30 min, whereas tannic acid [10^{-6} *M]* (●) needed about 50-60 min for its total inhibitory effect.

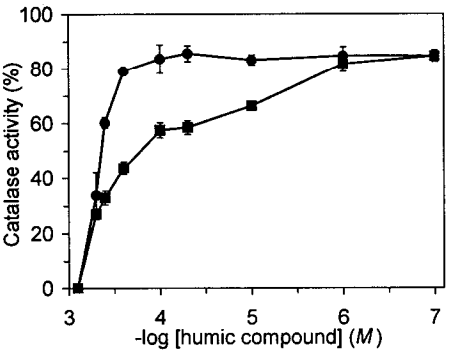

Figure 3. The two types of concentration dependence of the inhibition of catalase. Cinnamic acid (●) and (+)catechin (■), as representative compounds for which the concentration intervals from the concentration affording 100% catalase inhibition down to the smallest inhibitory concentration are approximately one and two or more orders, respectively.

Several authors (e.g. Wetzel 1993) have indicated that various enzymes can be inhibited by phenolics. Our results demonstrated that this finding is true for phenolic concentrations as low as 0.1-10 µ*M* the case of catalase (Figure 3).

We found two types of dose-dependent effects of phenolics (Figure 3) on catalase activity: a) phenolics acting in an interval of two or more orders of concentration (from

the concentration affording 100% inhibition to the smallest inhibitory concentration), i.e. phenol, ferulic acid, gallic acid, syringic acid, (+)catechin, caffeic acid and tannic acid, and b) phenolics acting in an interval of approximately one order of concentration (from the concentration affording 100% inhibition to the smallest inhibitory concentration), i.e. benzoic acid, 2-hydroxybenzoic acid, 3-hydroxybenzoic acid, 4-hydroxybenzoic acid, *p*-coumaric acid, protocatechuic acid, vanillic acid, cinnamic acid and ellagic acid (Figure 3).

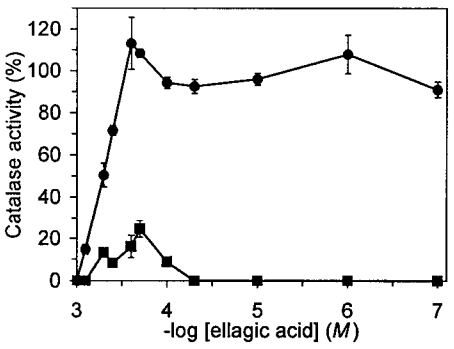

Figure 4. Comparison of H_2O_2 decomposition in the presence of native (●) and denatured (■) catalase. The activity of the native catalase in the supernatant not containing ellagic acid is taken as 100%.

In the case of ellagic acid, an interesting phenomenon was observed in the range 0.1-0.4 m*M* an apparent activation of catalase activity (about 115%) (Figure 4), which can be explained in that the ellagic acid formed a complex with at least one of the components of the reaction mixture. In order to examine this assumption, measurements were performed with catalase denatured by heat: apparent catalase activities of 0-26% were measured (Figure 4). In the assay performed without catalase (without supernatant), no significant change in H_2O_2 absorbance was detected. Consequently, the complex formed between ellagic acid and one or more of the components of the supernatant (e.g. metal ions) may catalyze the H_2O_2 decomposition, confirming the H_2O_2-scavenger role of ellagic acid in biological systems.

The IC_{50} values of the phenolic compounds are shown in Figure 5. The IC_{50} values of the homologous hydroxybenzoic acids and hydroxyphenylpropenoic acids were compared statistically in order to establish whether there is any relation between the inhibitory effect and the positions of the substituents. The significantly different IC_{50} values (the *P* values in Table 1) were used for analysis. Benzoic acid, 4-hydroxybenzoic acid and cinnamic acid were used as references, since they are the simplest (basic) hydroxybenzoic acids and hydroxyphenylpropenoic acids, respectively. The IC_{50} values were assessed on the basis of the structures of the references and of their substituted derivatives (with hydroxy groups or methoxy groups). It was concluded that a) as concerns the value of IC_{50}, the presence of the carboxyl group is more important than the number of hydroxy groups, (it is presumed that the small molecules may easily reach the active site, where the carboxyl group undergoes electrostatic interaction with the ionic amino acids or with the Fe^{3+} of the prosthetic group); b) the inhibitory effect is stronger if the phenolic molecule contains both carboxyl and hydroxy groups, c) the positions of the substituents on the phenyl ring influence the inhibitory effect more than does the number of hydroxy groups (or other substituents, e.g. methoxy groups), d) the presence of a hydroxy group *para* to the carboxyl group in the hydroxybenzoic acids leads to a stronger inhibitory effect, while in the hydroxyphenylpropenoic acids the effect is the opposite, e) the IC_{50} values of the *p*-hydroxybenzoic acids containing a *meta* methoxy group are lower than those of the hydroxybenzoic acids not containing such a methoxy group, while this relation is the opposite for the hydroxyphenylpropenoic acids, f) in good agreement with

the results of Wetzel (1993), the hydroxyphenylpropenoic acids are stronger inhibitors than the corresponding hydroxybenzoic acids, excluding *p*-coumaric acid, g) the very low IC_{50} of tannic acid makes this substance a rather toxic compound. The possible explanation might be that the tannic acid molecule is rather bulky, with many hydroxy groups; these may interact with several enzyme molecules, resulting in a stable precipitate (Ladd 1985; Haslam 1988; Wetzel 1991, 1993).

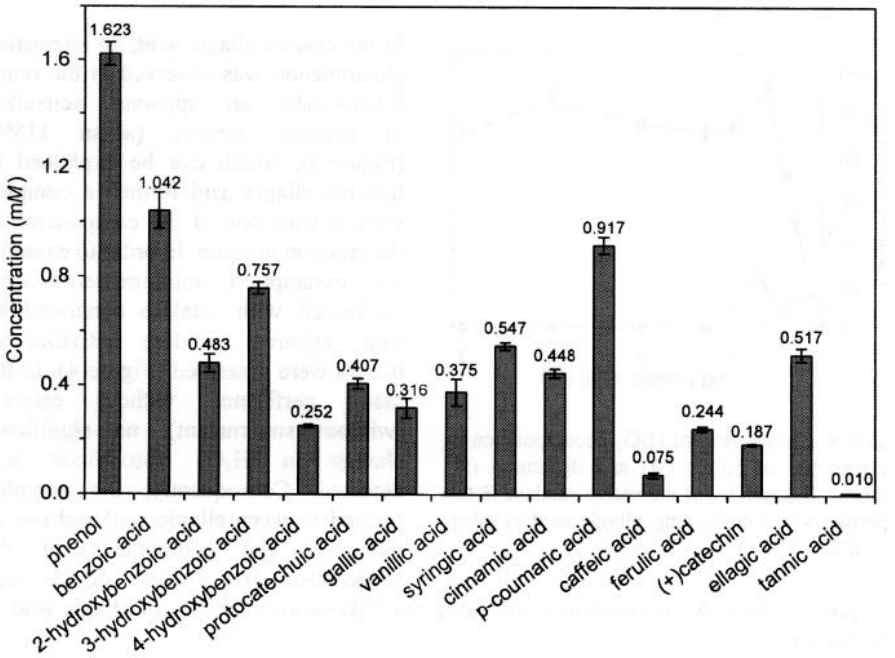

Figure 5. IC_{50} values of phenolics. Values were calculated via the Hill plot (Cornish-Bowden 1979).

Table 1. Comparison of IC_{50} values with Student t-test[1].

Compared pairs of compounds	*P*	Compared pairs of compounds	*P*
benzoic acid – 4-hydroxybenzoic acid	0.0001*	4-hydroxybenzoic acid – *p*-coumaric acid	0.0016*
benzoic acid – protocatechuic acid	0.0042*	protocatechuic acid – caffeic acid	0.0007*
benzoic acid – gallic acid	0.0007*	protocatechuic acid – vanillic acid	0.4701
benzoic acid – vanillic acid	0.0037*	protocatechuic acid – gallic acid	0.0719
benzoic acid – syringic acid	0.0022*	vanillic acid – syringic acid	0.0198*
benzoic acid – cinnamic acid	0.0058*	vanillic acid – ferulic acid	0.0517
4-hydroxybenzoic acid – protocatechuic acid	0.0457*	vanillic acid – caffeic acid	0.0109*
4-hydroxybenzoic acid – gallic acid	0.0263*	cinnamic acid – *p*-coumaric acid	0.0079*
4-hydroxybenzoic acid – vanillic acid	0.0616	cinnamic acid – caffeic acid	0.0058*
4-hydroxybenzoic acid – syringic acid	0.0052*	cinnamic acid – ferulic acid	0.0213*
		caffeic acid – ferulic acid	0.0032*

[1]The IC_{50} values were calculated via the Hill plot (Cornish-Bowden 1979). The P values denoted by asterisks indicate that the two IC_{50} values in question differ significantly ($P < 0.05$) from each other.

The time-dependent and concentration-dependent inhibition of catalase demonstrates that the relatively small phenolic molecules can inactivate catalase in a shorter time than does the large tannic acid molecule (M_r 1700.2), which needs an approximately two-fold incubation time for full inhibition. This may be explained by the difference in the diffusion coefficients. Moreover, the phenolics can bind to the active site via hydrogen-bonds, preventing H_2O_2 from binding to the active site (Murakami et al. 1991, 1992; Erdei et al. 1994). Tannic acid can form much more aspecific hydrogen-bonds with the external surface of the enzyme than do the small phenolics, but it cannot approach so closely to the internal structure of the enzyme (Ladd 1985; Haslam 1988; Kim and Wetzel 1993; Erdei et al. 1994).

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