The effects of humic compounds alone and in combination with agricultural chemicals in carp (*Cyprinus carpio* L.)

PhD thesis

Zsolt Varanka

University of Szeged Department of Biochemistry
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INTRODUCTION AND AIMS OF THE STUDY

Phenolcarboxylic acids and other phenolics are released through the autooxidative and microbe-mediated enzymatic degradation of the tannin and lignin components of plants. After polymerization reactions they turn into the part of the humus. Humic substances are biological origin, naturally occurring heterogeneous organic acids characterized by yellow to black colour with medium to large molecular weight and considerable biological stability. Humic acids comprise the main part (up to 80%) of the soluble organic matter of the freshwater. The average concentration of the organic acids is considered to be 5 to 25 mg L^{-1} in the freshwater, however, it might be as high as 50 to 70 mg L^{-1} in the "blackwaters". The molecule of humics contains several functional groups (e.g. –COOH, –OH, $–OCH_3$, =O) with complex-formation capability. The physico-chemical state, toxicity, degradation and transport of the organic and inorganic compounds might be modified in consequence of complexation by phenolics. Due to the complexing ability, the considerable enzyme inhibitory nature, the significant light-absorption (especially in the UV-range) and the pH-buffering capacity of humics, these substances potentially alter the physical and chemical characteristics of the aquatic environment, thereby play regulatory role in the aquatic ecosystems.

Plant-derived phenolics have long been used in the folk medicine as inflammation reducing and blood-clotting agent, or even more, they got into the centre of interest of the modern pharmacology due to their noteworthy antioxidant behaviour. Scientific studies demonstrated that regular consumption of antioxidant phenolics decrease significantly the human mortality, which is caused by coronary- and heart disease. Further, phenolics inhibit the initiation and development of thrombosis, arteriosclerosis, joint inflammation, carcinogenesis and mutagenesis. Catergen, a hepato-protecting medicine with the active ingredient of the flavonoid (+)-catechin is an example of the practical application of phenolics in pharmacology.

The relationship between the molecular structure and the antioxidant capacity of phenolics is well studied, but the relation between the structure and the enzyme inhibitory feature is not properly defined. Based on earlier studies, which were performed with low number of phenolics the following hypothesis was come up: the enzyme inhibitory power of phenolics depends only on the number of the hydroxy-groups. In order to clarify the dependency of the inhibitory feature of phenolics we performed comparative concentration- and exposure time-dependent *in vitro* experiments on a test enzyme. The test enzyme was a very active, commonly occurring antioxidative enzyme, the catalase. In these experiments we examined 11 hydroxybenzoic and hydroxy-phenylpropenoic acids with similar structure and with additional 3 phenolic compounds (ellagic acid, (+)-catechin and tannic acid) with complex structure. Phenol and benzoic acid was used as reference, as their molecules have only one functional (hydroxy- or carboxyl-) group. The concentration, which causes 50% enzyme inhibition (IC_{50}) was calculated for each phenolic. Then the IC_{50} values of the homologous hydroxybenzoic acids and hydroxy-phenylpropenoic acids were compared statistically in order to establish whether there is any relation between the inhibitory effect and the positions of the substituents.

Although, plant degradation products, as humic components, are present both in the soil and in the aquatic environment, most of the *in vivo* studies examine the biological effects of phenolics from pharmacological aspect. That's why the experiments are carried out on mammals, mainly. Notwithstanding, based on the above-mentioned complex-forming capacity of humics, the ecological approach has came to the front, recently.

The stability of the aquatic ecosystems might be disturbed even by 2 mg L^{-1} Cu^{2+}, as first the bacterium flora, then the number of crustaceae is decreased drastically. Copper is used in the agriculture as fungicide and it can reach the surface waters with the precipitate. Animals living in the water accumulate heavy metals; thus even low aquatic copper concentration may evoke toxic effects.

Modern agriculture applies synthetic chemicals in order to increase the yield, and such a chemical agent is the pyrethroid insecticide deltamethrin (DM), which is used also by helicopter spraying against mosquitoes. Although, the degradation of DM is rapid in the water, its enhanced toxic effects in fish were also proven. For example, DM was one of the factors, which triggered extended fish devastations in Lake Balaton in the last decade.

So, besides humics, numerous artificial (water polluting) agents persistent in the aquatic environment, which together with the naturally occurring components (e.g. humic acids) may exert complex effects in the aquatic animals, like fish. As we mentioned, the most of the *in vivo* experiments with phenolics have pharmacological approach, but the metal-complexing ability and the antioxidant behaviour of these compounds is studied by *in vitro* experiments, mainly. Therefore, little information is available about the influence of phenolic–toxicant combinations on the defense enzyme

systems of fish. However, these pieces of information are important in terms of the stability of the aquatic ecosystems.

Based on the above open points, our aim was to reveal the effects of the humic ellagic acid (EA) and tannic acid (TA), known as plant antioxidants, on the antioxidant defense system and on the cytochrome P450 monooxygenase CYP 1A izoenzymes of fish. Further, our scope was to estimate the influence of the two phenolics on the effects of the environmental pollutant fungicide $CuSO_4$ and the insecticide DM on the above enzyme systems. The chemical stress and tissue necrosis inducing action of the two toxicants are well known. Therefore, the study was extended over the examination of the influence of the chemicals on the blood glucose level (general stress parameter) and on the activity of the blood aminotransferases (tissue necrosis indicator). The alterations in the ultrastructure of hepatocytes were studied by electronmicroscopy. The common carp was chosen to be used as test animal, as it is the best-known and most important fish species economically and gastronomically in central Europe.

METHODS

IN VIVO METHODS

Treatments of the experimental animals

Both sexes of common carp (*Cyprinus carpio* L.) weighing 600-800 g were used for the treatments. Two fish were kept in air-saturated water (pH~7.0) at 13-14°C in each 100-litre aquarium and acclimatized for 7 days before the treatment. The control fish were injected intraperitoneally (*i.p.*) with distilled water. The applied dose of TA was 5.9 μmol kg^{-1} (=10 mg kg^{-1}) body weight (b.w.), 33.1 μmol kg^{-1} (=10 mg kg^{-1}) b.w. for EA, 157.4 μmol kg^{-1} for Cu^{2+} (=10 mg kg^{-1}) b.w., and 0.4 nmol kg^{-1} (=0.2 μg kg^{-1}) b.w. for DM. Each group of 6-8 fish got an injection containing TA, EA, $CuSO_4$ or DM alone, or the group got a combined injection containing TA+$CuSO_4$, EA+$CuSO_4$, TA+DM or EA+DM. The effects of the above substances were studied after an exposure time of 24 or 48 h.

Sample preparation

After an exposure time of 24 or 48 h venous blood was taken with use of heparin as anticoagulant and centrifuged to remove the blood cells. Glucose concentration and the aminotransferase activities were determined from the plasma. Animals were then killed and the liver was removed. Measurements were prepared with homogenization of liver in phosphate-buffer. The homogenate was centrifuged at 10,000 *g* for 20 min at 4°C. The supernatants were used for the redox-parameter determinations (excluding thiobarbituric acid (TBA) test, which was assessed from the whole homogenate). Microsomes, for cytochrome P450-dependent enzyme assays, were prepared by differential centrifugation of liver homogenate as described by Förlin.

Measurement of the activities of P450 izoenzymes and the redox-parameters from the liver, and the stress-parameters from the plasma

In order to estimate the single and combined effects of phenolics and toxicants in fish we determined the following parameters after the *in vivo* treatment. The activity of ethoxyresorufin-O-deethylase (EROD) was followed by the method of Burke et al. (1985) and the ethoxycoumarin-O-deethylase (ECOD) by Kamataki et al. (1980). Superoxide dismutase (SOD) and manganese-superoxide dismutase (Mn-SOD) activities were determined by the method of Misra and Fridovich (1972); catalase (CAT) activity by the method of Beers and Sizer (1952); the amount of thiobarbituric acid-reactive substances (TBARS), as products of lipid peroxidation (LP), was assessed by the method of Placer et al. (1966); reduced tissue glutathione (GSH) by the method of Sedlak and Lindsay (1968); cytosolic glutathione-peroxidase (GPx) activity by the method of Chiu et al. (1976); protein by the method of Lowry et al. (1951); plasma glucose (GLU) by the method of Trinder (1969); the aspartate aminotransferase (AspAT) and alanine aminotransferase (AlaAT) activities by the method of Reitman and Frankel (1957).

Determination of the copper content of the liver

The liver samples were suspended in HNO_3 – H_2O_2 mixture, heated at 160°C for 24 h, and after dilution to the same aliquot the copper content was measured by polarized atomic absorption method.

Determination of the deltamethrin content of the liver

The liver samples were homogenized in acetone, purified via extraction with organic solvents (Akhtar, 1982), the lipid fraction was separated via solid-phase extraction (Chapman and Harris, 1978), then DM was measured by GC-MS.

Electronmicroscopy

Small cuboidal sections were cut from the carp liver and were prefixed in Karnovsky's (1965) fixative, postfixed in OsO_4 solution, dehydrated in graded ethanol, then embedded in Durcupan ACM. The ultrathin (100 nm) sections were stained with uranyl acetate, contrasted with lead citrate, then examined in a Tesla BS 500 electronmicroscope.

IN VITRO METHODS

Examination of the relationship between the molecular structure and the enzyme inhibitory property of phenolics

These experiments are divided into exposure time- and concentration-dependent assays. Time-dependent assays were carried out to determine the infinite incubation time for phenolics, needed for IC_{50} determinations. The concentration-dependent inhibition of catalase activity by 16 phenolics was examined with application of 30-min preincubation time, as a result of the above time-dependent assays. Then, the phenolic concentrations, which cause 50% activity decrease (IC_{50}) were calculated via the Hill-plot (Cornish-Bowden, 1979). The IC_{50} values of the homologous hydroxybenzoic acids and hydroxy-phenylpropenoic acids were compared statistically in order to establish whether there is any relation between the inhibitory effect and the positions of the substituents.

Examination of the *in vitro* effects of phenolics and/or toxicants on the redox-parameters

The method of measurements of some redox-parameters (e.g. SOD, GSH, and GPx) involves the possibility of artefact formation, which could be taken into account when evaluating the *in vivo* results. Therefore we performed concentration-dependent *in vitro* assays with the chemicals examining single and combined effect, also. The results of these studies were regarded as reference for the *in vivo* experiments.

RESULTS AND DISCUSSION

1. We found two types of dose-dependent effects of the 16 studied phenolics on CAT 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.

2. Generally, we found the IC_{50} values (on CAT) to be around some hundred μM, except caffeic and tannic acid with its low value under 100 μM and 10 μM, respectively. Only the references (phenol and benzoic acid), which molecules hold only hydroxy- or carboxyl-group, have an IC_{50} value higher than 1 mM. We concluded that as concerns the inhibitory power, the presence of the carboxyl-group in the phenolic molecule is more important than the number of hydroxy-groups. Therefore, the influence of additional ligands on the IC_{50} value is limited.

3. The positions of the substituents on the phenyl ring (*meta*, *orto*, *para* configuration) influence the inhibitory effect more than does the number of hydroxy groups (or other substituents, e.g. methoxy groups).

4. When comparing the dependency of the inhibitory power of hydroxybenzoic and hydroxy-phenylpropenoic acids with homologous structure, we found that an additional substituent (hydroxy- or methoxy-group) in the same position evoke opposite effect on the inhibitory power. For example, the presence of a hydroxy-group *para* to the carboxyl-group in the hydroxybenzoic acids leads to a stronger inhibitory effect, while in the hydroxy-phenylpropenoic acids the effect is the opposite.

5. The time- and concentration-dependent inhibition of CAT demonstrates that the relatively small phenolic molecules can inactivate CAT in a shorter time than does the large TA molecule (M_r 1700.2), which needs an approximately two-fold incubation time for full inhibition. However, TA exerts the strongest inhibitory effect. This may be explained by the difference in the diffusion coefficients. Moreover, the small phenolic molecules can bind to the active site via hydrogen-bonds, preventing H_2O_2 from binding to the active site, or may alter the internal structure of the enzyme. TA 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. Further, the TA molecule, which is rather bulky, with many hydroxy-groups may interact with several enzyme molecules, resulting in a stable precipitate.

6. The *i.p.* administered TA caused partial inhibition of the hepatic P450 1A izoenzyme and the antioxidant enzyme activities, and according to the non-enzymatic redox-parameters TA behaved as prooxidant in carp. TA was found not to be metabolized by ECOD and EROD. Some non-deleterious alterations developed in the ultrastructure of the hepatocytes: the electron-density of the nucleus and mitochondria were enhanced, the rough endoplasmic reticulum (rEr) and the perinuclear cisterns were enlarged, and some glycogen loss was observed. *In vitro* studies revealed the enhanced CAT and GPx inhibitory, but weak apparent GSH concentration decreasing capacity of TA.

7. Opposed to TA action EA acted as antioxidant in carp as proved by the decreased LP level and the increased GSH concentration as compared to the control. The low P450 1A activities with the enhanced CAT and SOD activities show diminished oxyradical production, but augmented free radical degradation rate in the tissues. We found EA not to be metabolized by ECOD and EROD, as well. EA induced a relatively stronger *in vivo* inhibition of some enzymes (e.g. aminotransferases) than TA. EA triggered similar ultrastructural alterations in the liver compared to those of TA with an exception: the glycogen loss was much enhanced after the EA treatment. Opposed to TA action, apparent GSH concentration-decreasing activity was not detected *in vitro*, but a weaker enzyme inhibitory capacity and a considerable apparent CAT activity of EA was proved clearly. These multiple actions of EA refer to complex behaviour *in vivo*.

8. Both $CuSO_4$, and the phenolic+copper combinations acted as prooxidant and evoked enzyme inhibition in fish. The parallel expression of the exposure time-dependent effect of the two chemicals was observed after the phenolic+copper combined injections. Accordingly, the strong prooxidant, therefore antioxidant enzyme inductor and tissue necrotizing effects after 24 h exposure are due to the action of $CuSO_4$, but the decreased enzyme activities detected after 48 h might be the consequence of enzyme inhibition triggered by EA or TA. The phenolic+copper combinations behaved as prooxidant against GSH not only *in vivo*, but *in vitro*, also. In other words, these mixtures exerted notable apparent GPx activity. This way the GPx inhibition by $CuSO_4$ or the phenolic+copper combinations could not be evaluated. Besides their enhanced enzyme inhibitory capacity the EA+Cu(II) and TA+Cu(II) combinations show more enhanced apparent CAT activity than EA or TA alone. This H_2O_2-degrading activity might be additive to the 'enzymatic' CAT activity *in vivo*. Macroscopically the liver was not deleteriously necrotized. Electronmicroscopy revealed moderate alterations in the hepatocytes after the TA+$CuSO_4$ or EA+$CuSO_4$ treatments, comparing to the consequence of $CuSO_4$ injection alone. Although, moderate damage developed in the nucleus and in the rEr after the combined treatments, the total glycogen loss refers to high energy demand of the detoxification/excretion of the phenolic-toxicant combinations. The biochemical parameters several times indicated increased toxicity of a phenolic+copper combination compared to that of the alone-added copper. However, electronmicrographs demonstrated the opposite. Therefore, it is concluded, that the copper toxicity decreasing action of the two phenolics is expressed on ultrastructural, rather than on biochemical parameter level

9. Identical changes developed in the biochemical parameters after the DM and the TA+DM treatment: the activity of a DM metabolizing enzyme, the ECOD, increased in the fish. The metabolization of DM was relatively fast in carp, as following 24 or 48 h the treatment DM was not detected in the tissues by GC-MS. The improvement of the oxidative reactions was accompanied by increased free radical production. The consequence of the prooxidant effect was GSH loss and enhanced LP with rose antioxidant enzyme activities. The parallel expression of the exposure time-dependent effect of the two chemicals was observed after the TA+DM treatment, similarly, like after the TA+Cu(II) injection. Hence, the changes in the enzyme activities of fish after 24 h is due to the elevated free radical production induced by the synthetic toxicant, but the loss in enzyme activities after 48 h exposure is the consequence of the characteristic enzyme inhibitory action of TA. In contrast, a significantly moderate oxidative stress was induced after the EA+DM treatment, than after the DM or the TA+DM injection, as demonstrated by the non-enzymatic redox-parameters. The reduced toxicity of DM in the presence of EA is due to the considerable free radical scavenging (therefore antioxidant) activity of the phenolic. The fact, that the values of the blood parameters were increased only after longer exposure (2 days) to EA+DM also refers to weaker and slow developing oxidative stress in fish. Although, *in vitro* studies proved, that DM is a rather strong enzyme inhibitor as it can inhibit CAT and GPx even in nanomolar concentration, this feature could not been manifested well *in vivo*, because of the very low applied DM dose. Electronmicroscopy revealed that both TA and EA was able to decrease the cell destroying

activity of DM in the liver: the swelling of the nucleus and the rEr cisterns, and the increase in the electrondensity of the mitochondria was not so enhanced, and the marginal heterochromatin loss was moderate comparing to those of induced by DM treatment alone. Generally, we can say that the habitus of the hepatocytes after the phenolic+DM treatments was similar to that after the single phenolic treatment, and the appearance of "dark" and "light" cells was not observed in contradiction to the consequence of DM injection alone. Based on the comparison of the *in vivo* and *in vitro* results, collated with the electronmicrographs we concluded that EA significantly increased the DM tolerance of fish in contrast to TA action.

10. Collation of electronmicrographs with transaminase activities led to a conclusion: one should be cautious when using of transferases as indicator of tissue damage when strong enzyme inhibitors (such as TA) are applied.

11. The examined compounds both alone and in combination decreased the glycogen content of the hepatocytes and affected the plasma glucose concentration. Consequently, each substance triggered chemical/metabolic stress in fish.

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