BIOACTIVE COMPOUNDS IN THE FLORA OF BELARUS. 2. ASTRAGALIN, AN EFFECTIVE PROTECTOR OF CATALASE FROM ULTRASONIC INACTIVATION IN AQUEOUS SOLUTIONS

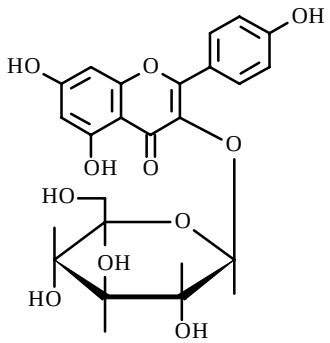
N. V. Kovganko, Zh. N. Kashkan, S. N. Krivenok, M. V. Potapovich, A. N. Eremin, and D. I. Metelitsa

UDC 547.814.5+541.118+577.152.1

*The kinetics of catalase decomposition of H_2O_2 (50 mM) inhibited by the flavonolic glycoside astragalin (**1**) from the aerial part of* Gymnocarpium dryopteris *were studied. **1** (0.3-30 M) is a strong inhibitor of the catalase process and was tested as a catalase (CAT) protector from ultrasonic activation at 45 °C by treatment of enzyme solutions (1.2 nM) in phosphate buffer (7.5 mM) at pH 5.5 with low-frequency ultrasound (US, 27 kHz) at initial specific power 60 W/cm^2. **1** inhibited US-inactivation of CAT according to the rate constants for US-inactivation of CAT in the presence of **1** with and without inhibitor. **1** at concentrations of 1.0 and 20.0 μM decreases US-inactivation up to 22.3 and 19.5% of the initial level. The useful properties and effectiveness of **1** are better than the synthetic inhibitors—antioxidants propylgallate and 4-t-butylpyrocatechin.*

Key words: astragalin, glycosides, catalase, ultrasound, enzyme protectors, antioxidants.

The flavonol glycoside astragalin (**1**) was isolated from the aerial part of *Gymnocarpium dryopteris*. Its structure was solved by spectral methods [1]. The oak fern *G. dryopteris*, or Linneus fern, is widely distributed in the forests of Belarus and represents a practically unlimited source of astragalin, which possesses anti-inflammatory [2], anti-allergenic [3], and antimicrobial activities [4]. It together with other flavonoids is used to treat rheumatoid arthritis and osteoarthritis [5].



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Considering the potential anti-radical activity of **1** [6], it seemed expedient to test it as a protector of enzymes from inactivation and destruction during ultrasonic treatment of their solutions. This aspect is timely because low-frequency ultrasound (LFUS, 20-27 kHz) is used for recanalization of vessels after myocardial infarct and for acute thrombosis of peripheral arteries and deep-vein thrombosis in combination with the mediated action of various plasminogen activators [7-9]. High-frequency US (HFUS, 880 kHz and 2.64 MHz) is used in therapy of diseases of the nervous system, the ambulatory-support system, and other pathologies.

Institute of Bioorganic Chemistry, National Academy of Sciences of Belarus, 220141, Minsk, ul. Kuprevicha, 5/2, fax (375172) 63 72 74, e-mail: kovganko@iboch.bas-net.by. Translated from Khimiya Prirodnykh Soedinenii, No. 1, pp. 62-65, January-February, 2004. Original article submitted July 9, 2003.

0009-3130/04/4001-0071

TABLE 1. Extent of CAT Inhibition and Protective Action of **1** at Various Concentrations

Concentration		Extent of inhibition $(k-k_i)/k$, %	$k_{in}^{astr}(US)/k_{in}(US)$, %
[**1**], μM	[DMF], mM		
1.0	0.64	2.5	22.3
20.0	12.92	-	19.5
30.0	19.38	25.0	-

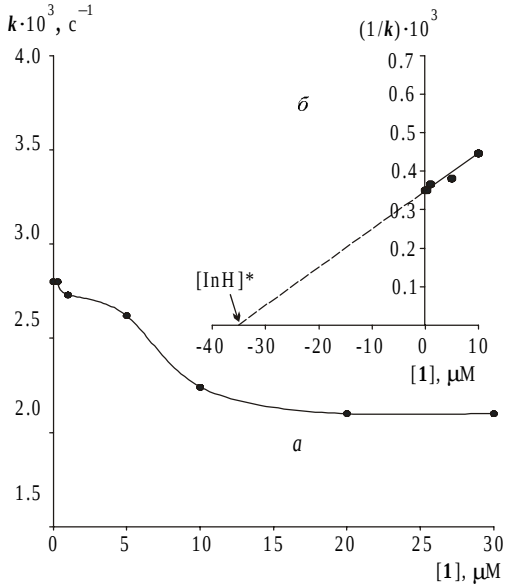

Fig.1

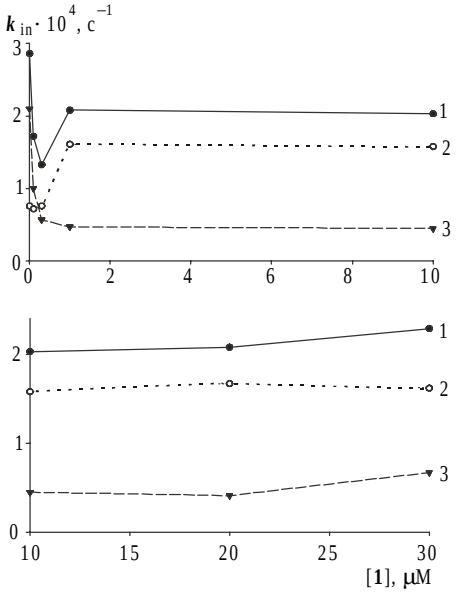

Fig.2

Fig. 1. Rate constant of H_2O_2 (0.05 M) decomposition as a function of concentration of **1** (a) and its transformation in Dixon coordinates (b): 30°C, PB (10 mM), pH 5.5, CAT (0.5 nM).
Fig. 2. Rate constants of CAT (1.2 nM) inactivation as functions of concentration of **1** at 45°C and effect of LFUS (27 kHz, 60 W/cm^2) on enzyme solution in PB (7.5 mM), pH 5.5. k_{in} (1), k^*_{in} (2), k_{in}(US) (3).

We used effective first-order rate constants k_{in} and k_{in}(US), which characterize the overall (total) inactivation and its US-component, as quantitative criteria for inactivation of the aforementioned enzymes. It has been found [10-17] that human serum albumin and HO˙ radical scavengers (DMF, mannitol, ethanol, butanol, etc.) strongly inhibit US-inactivation of enzymes, reducing k_{in} and k_{in}(US).

We studied the effect of astragalin on the kinetics of the catalytic activity of CAT and its ultrasonic inactivation upon LFUS treatment of buffered enzyme solutions (27 kHz). We determined the quantitative properties of **1** as a protector of the enzyme from US-cavitation.

Potential protectors of CAT from US-inactivation of the enzyme in solution are characterized by their effect on the catalytic activity and degree of interaction with the protein. An ideal protector should not interact with the protein and lower the catalytic activity of the enzyme. Such a situation is unrealistic because aromatic compounds will interact with CAT, which is a rather hydrophobic protein. The effect of **1** on CAT at 30°C was evaluated by adding it in concentrations of 0.3-30 μM to a reaction medium containing H_2O_2 (50 mM) in phosphate buffer (PB) (10 mM) at pH 5.5. Figure 1a shows the rate constant of H_2O_2 decomposition as a function of the concentration of **1** ([**1**]). It can be seen that **1** decreases the rate of the CAT reaction. Up to an inhibitor concentration of 10 μM, the dependence of k_i on $[\mathbf{1}]_0$ is nearly straight in Dixon coordinates [18] (Fig. 1b). The quantity $[InH]^* = 35$ μM. Knowing the H_2O_2 concentration ([S]), equal to 0.05 M, and the Michaelis constant without **1** ($K_m = 0.33$ M) that was previously determined [19], K_i can be calculated using the equation $[InH]^* = K_i([S]/K_m + 1)$ [18], which was 30.43 μM. It should be noted that the degree of CAT inhibition is practically unchanged at [**1**] > 10 μM. Table 1 lists the extent of CAT inhibition at two values of [**1**].

It can be seen that **1** at concentrations 6000 times greater than the CAT content (0.5 nM) inhibits the enzyme by only 25%. The organic cosolvent DMF plays a certain role in CAT inhibition. At 0.15% DMF in PB (10 mM) and pH 7.4, CAT retains about 67% of the initial catalytic activity.

It was found earlier that DMF inhibits CAT dissociation into subunits. At 52% (by vol.) DMF and CAT (15.4 μM) in citrate—acetate buffer (0.1 M) at pH 6.0, the enzyme completely decomposes into subunits [20]. CAT monomers exhibit a relatively high peroxidase activity for oxidation of tetramethylbenzidine [20], *o*-phenylenediamine [21], and *o*-dianisidine [21-23]. This means that partial CAT dissociation into subunits is possible in the presence of DMF in our experiments. These can catalyze the oxidation of **1** by the peroxidase mechanism at 0.05 M H_2O_2.

The ability of **1** to protect against US-inactivation of CAT (1.2 nM) was studied at 45°C in PB (7.5 mM) at pH 5.5. Figure 2 shows rate constants for CAT inactivation k_{in} (curve 1), k_{in}^* (2), and k_{in}(US) (3) at [**1**] = 0.1-30.0 μM in a solution treated with LFUS (27 kHz) at high specific power 60 W/cm^2. For [**1**] = 1 μM, k_{in} and k_{in}(US) decrease sharply. Thermal inactivation and total inactivation of CAT increase with increasing [**1**]. However, up to [**1**] = 20 μM, they remain at a constant relatively low level and again increase only for [**1**] > 20 μM.

Table 1 shows that the residual level of US-inactivation of CAT is low at [**1**] = 1.0-20.0 μM. The sonication solution and the solution for determining CAT activity contained DMF, which plays a dual role. On one hand, it can partially inhibit CAT; on the other, protect it from the action of active HO$^{\cdot}$ radicals, for which DMF is an acceptor [12-14, 16, 17]. The quantitative characteristics for the protective action of **1** are much greater than those of propylgallate (residual US-inactivation 59% at a concentration of 30 μM) and 4-*t*-butylpyrocatechin (US-inactivation 32% at a concentration of 30 μM), which we tested earlier under comparable conditions.

The overall properties (extent of CAT inhibition and effectiveness of inhibiting US-inactivation of CAT upon LFUS irradiation) of **1** make it promising for protecting enzymes from inactivation in an US-cavitation field. Upon administering **1** through an US probe into arteries and veins with thrombolysis, its thermal inactivation at 36°C decreases greatly whereas the effectiveness of its protective action increases.

We used **1** to achieve complete inhibition of US-inactivation of CAT. This can be explained firstly by the fact that **1** interacts with the enzyme whereas active radicals are generated in the cavitation field and partially migrate into the solution volume, i.e., part of the radicals may not be accepted by the protector. Secondly, US-inactivation of CAT and other enzyme subunits can occur not only through the action of radicals but also by other mechanisms connected with mechano-acoustical and sono-capillary effects, which should be the subject of a special study for oligomeric enzymes.

EXPERIMENTAL

Reagents. We used catalase from bovine liver (EC 1.11.1.6) with optical purity index RZ of 0.61 and concentration 21.3 μM (Fluka, Switzerland). CAT concentration was determined spectrophotometrically using a molar absorption coefficient at the maximum of the Cope band of 324,000 $M^{-1}cm^{-1}$ [24]. We used diluted perhydrol (Reakhim, Russia). The H_2O_2 concentration was determined spectrophotometrically using a molar absorption coefficient ε_{230} = 72.4 $M^{-1}cm^{-1}$ [25]. Other reagents were from Reakhim (Russia). DMF was distilled before use.

Astragalin (1) was isolated from the aerial part of *G. dryopteris* and characterized as before [1]. DMF was added to a certain concentration (the DMF concentration is given in mM) depending on the concentration of **1** owing to the limited solubility of **1** in water and PB (10 or 7.5 mM) at pH 5.5.

Ultrasonic treatment of CAT solutions in PB (7.5 mM) at pH 5.5 with and without **1** was performed in a glass cylinder 2.6 cm in diameter and 12 cm high. The volume of irradiated CAT solutions was 40 mL. We used a LFUS generator (Tekhnosonik, N. E. Bauman MHTU, Moscow) equipped with a piezoceramic transducer and titanium waveguide at working frequency 27 kHz. The starting specific power could be varied in the range 5-60 W/cm^2. This corresponded to a vibrational amplitude of the waveguide end from 19 to 57 μm. The waveguide was immersed in the irradiated solution so that the distance of its end from the cylinder bottom was 4 cm. CAT solutions with and without **1** were irradiated at 45°C continuously for 1-2 h. Aliquots were taken and placed into a thermostatted spectrometer cuvette containing H_2O_2 (50 mM) to determine the residual CAT activity at the US treatment temperature.

The thermal stability of CAT at 45°C in PB (7.5 mM) at pH 5.5 with and without **1** was measured under the same conditions as for enzyme sonication. Aliquots were taken during the thermal inactivation to determine the residual CAT

activity. Initial CAT concentrations in the cuvette were 0.5 nM; H_2O_2, 50 mM.

The initial (A_0) and residual (A) CAT activities were found spectrometrically at 45°C on a SF-46 instrument (LOMO, Russia) from the absorption decrease of consumed H_2O_2 at 230 nm. A first-order rate constant k (sec^{-1}) for H_2O_2 consumption was used to characterize quantitatively A_0 and A and was determined from semi-logarithmic analysis of kinetic curves for the H_2O_2 absorption decrease at 230 nm.

The influence of **1** on CAT catalytic activity was studied at 30°C in PB (10 mM) at pH 5.5 in the presence of DMF, the content of which depended on [**1**]. The CAT activity in the presence of **1** was found as described above. The first-order rate constant k_i (sec^{-1}) was characterized. The extent of inhibition $(k - k_i)/k$ was calculated for each [**1**] and expressed in percent where k_i is the H_2O_2 consumption rate constant in the presence of **1** at a certain concentration and K_i is the inhibition constant from the functions in Dixon coordinates [20] $1/k_i$—$[\mathbf{1}]_0$ at the intercept $[InH]^*$ intersected on the abscissa by a straight line.

Quantative Characteristics of CAT Inactivation in Solution. Total (ultrasonic and thermal) inactivation of CAT was characterized by effective first-order rate constants k_{in} (sec^{-1}) that were determined from semi-logarithmic analysis of rate constants k as functions of time for each aliquot of CAT solution collected during sonication under certain conditions at 45°C.

Thermal inactivation of CAT was characterized by effective first-order rate constants k_{in}^* (sec^{-1}) that were determined graphically from semi-logarithmic analysis of kinetic curves for the change of k for aliquots collected during CAT thermal inactivation.

Effective rate constants for US inactivation of CAT $k_{in}(US)$ (sec^{-1}) were calculated as the difference between k_{in} and k_{in}^*: $k_{in}(US) = k_{in} - k_{in}^*$. The influence of **1** on US-inactivation of CAT was expressed as the ratio $k_{in}^{astr}(US)/k_{in}(US)$, %, where $k_{in}^{astr}(US)$ is the rate constant for US-inactivation of CAT in the presence of a certain [**1**] in the sonicated enzyme solution.

It should be emphasized that US-treatment of CAT solutions with and without **1** was performed in solutions saturated with air. Continuous irradiation did not raise the temperature of thermostatted solutions by more than 1 K.

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