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Flavonoids as Effective Protectors of Urease from Ultrasonic Inactivation in Solutions

E. I. Tarun[a], V. P. Kurchenko[b], and D. I. Metelitza[a, 1]

[a] *Institute of Bioorganic Chemistry, National Academy of Sciences of Belarus, ul. akademika Kuprevicha, 5/2, Minsk, 220141 Belarus*

[b] *Faculty of Biology, Belarussian State University, ul. Kurchatova 10, Minsk, 220064 Belarus*

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Abstract—Inactivation of soybean urease in aqueous solution at pH 5.4, 36°C, and high-frequency sonication (2.64 MHz, 1.0 W/cm^2) is substantially reduced in the presence of seven structurally different flavonoids. A comparative kinetic study of the effect of these flavonoids on the effective first-order rate constants that characterize the total (thermal and ultrasonic) inactivation k_i, thermal inactivation k_i^*, and ultrasonic inactivation k_i(US) of 25 nM enzyme solution was carried out. The dependences of the three inactivation rate constants of the urease on the concentrations of flavonoids within the range from 10^{-11} to 10^{-4} M were obtained. The following order of the efficiency of the flavonoids used in respect of the urease protection from ultrasonic inactivation was found: astragalin > silybin > naringin > hesperidin > quercetin > kaempferol > morin. The results confirm a significant role in the inactivation of the urease of $HO^{\bullet}$ and $HO_2^{\bullet}$ free radicals, which are formed in the ultrasonic cavitation field.

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Key words: flavonoids, free radicals, ultrasound, urease, urease stabilization from inactivation

INTRODUCTION

A wide use of ultrasound in biotechnology, medical diagnostics, and therapy causes the necessity of studying its effect on biologically important subjects at molecular level [1–3].[2] Soybean urease (EC 3.5.1.5), a homohexameric enzyme containing two nickel atoms in each subunit, plays an important role among the biopolymers, affected by sonication in solution. It catalyses the urea hydrolysis yielding ammonia cation and carbonate anion [4–7]. Practical aspects of US effect on the urease are obvious: they are related to the use of this enzyme in immunobiotechnology (enzyme immunoassay, biosensors), where a part of processes are connected with sonication of media and embedded subjects. A fundamental interest in US inactivation of urease is due to the oligomeric nature of this enzyme, and, as a consequence, to the manifestation of the loss of its catalytic activity via the dissociative mechanism, all the features of which are detected in the process of thermal inactivation of urease [7–12].

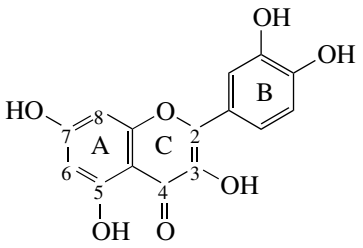
In our works [9–12], the kinetics of US inactivation of urease in aqueous solutions, subjected to the action of either LFUS (27 kHz, specific power of 10–60 W/cm^2), or HFUS (2.64 MHz, specific power of 1 W/cm^2), was studied in detail. The choice of the frequencies is determined by the fact that LFUS is most often used in the laboratory practice and for the destruction of thrombi in blood vessels during myocardial infarction and acute thrombosis of peripheral arteries [13]. In its turn, HFUS (2.64–3.5 MHz) is widely used for therapy of a wide range of pathologies.

The complex inactivation process of urease and other enzymes was quantitatively characterized by the effective first-order rate constants (min^{-1}): k_i, total (thermal and US) rate constant; k_i^*, thermal inactivation constant; and k_i(US), US inactivation constant. The latter is calculated as the difference between k_i and k_i^* at the presumption that k_i^* and k_i(US) are independent of each other [9–12]. We showed for urease and other enzymes that the constants k_i and k_i(US) depend not only on frequency and US power, but also on the initial enzyme concentration, solution pH, and temperature, at which they are sonicated [9–12].

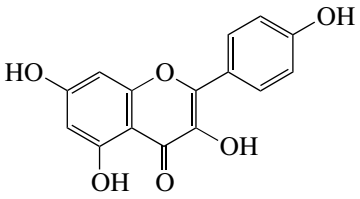
The US treatment of aqueous solutions of urease and other enzymes leads to the generation in the cavitation field of active oxygen-containing radicals, such as $O_2^{-\bullet}$, $HO_2^{\bullet}$, and $HO^{\bullet}$, whose formation was many times proven by direct and indirect methods (see, e.g.,

[1] Corresponding author: fax: 375-172-63-7274; e-mail: metelitza@iboch.bas-net.by

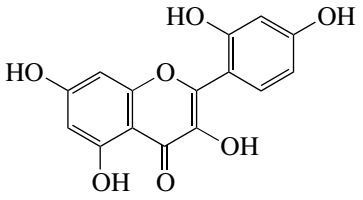
[2] Abbreviations: HFUS, high frequency ultrasound; LFUS, low frequency ultrasound; and US, ultrasound.



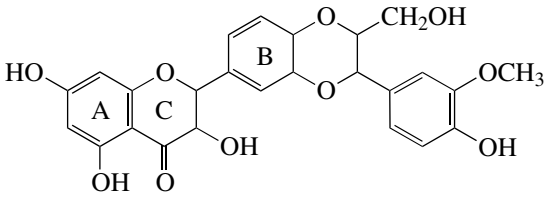
Quercetin



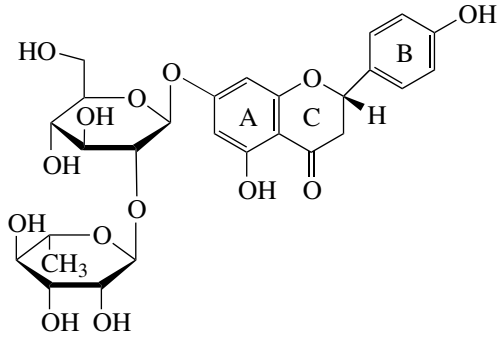
Kaempferol



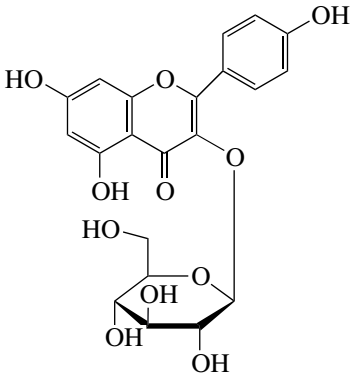
Morin



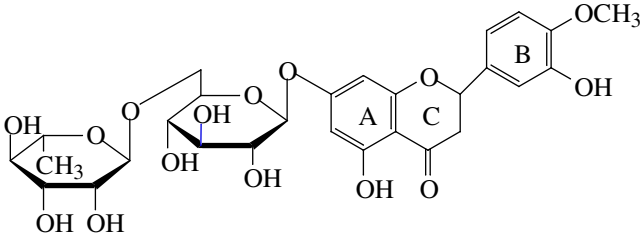
Silybin



Naringin



Astragalin



Hesperidin

review [2]). Radicals play an important role in aqueous solutions in US inactivation of urease, and the rate of total and US inactivation is substantially reduced in the presence of acceptors of hydroxyl radicals: ethanol, butanol, DMSO, DMF [12], synthetic antioxidants, such as propyl gallate, gallic acid polydisulfide, and its complexes with albumins [10, 11].

Many flavonoids of plant origin are well known to be potent inhibitors of the chemical and biological processes involving free radicals [14]. The antioxidant activity of plant flavonoids significantly determines their antiallergic, anticancer, antimutagenic, antiphlogistic, and antiviral properties [15]. It has recently been shown in our laboratory that the flavonoic glycoside astragalin, isolated from oak fern *Gymnocarpium dryopteris*, reduces at a high effeciency the rate of US inactivation of catalase from bovine liver (EC 1.11.1.6) in a buffer solution subjected to the action of LFUS with the frequency of 27 kHz and a high initial specific power (60 W/cm^2) [16].

The goal of this work is a comparative kinetic study of the effect of seven flavonoids of different structure, quercetin, kaempferol, morin, silybin, hesperidin, naringin, and astragalin, on the HFUS-induced (2.64 MHz, 1.0 W/cm^2) inactivation of soybean urease in aqueous

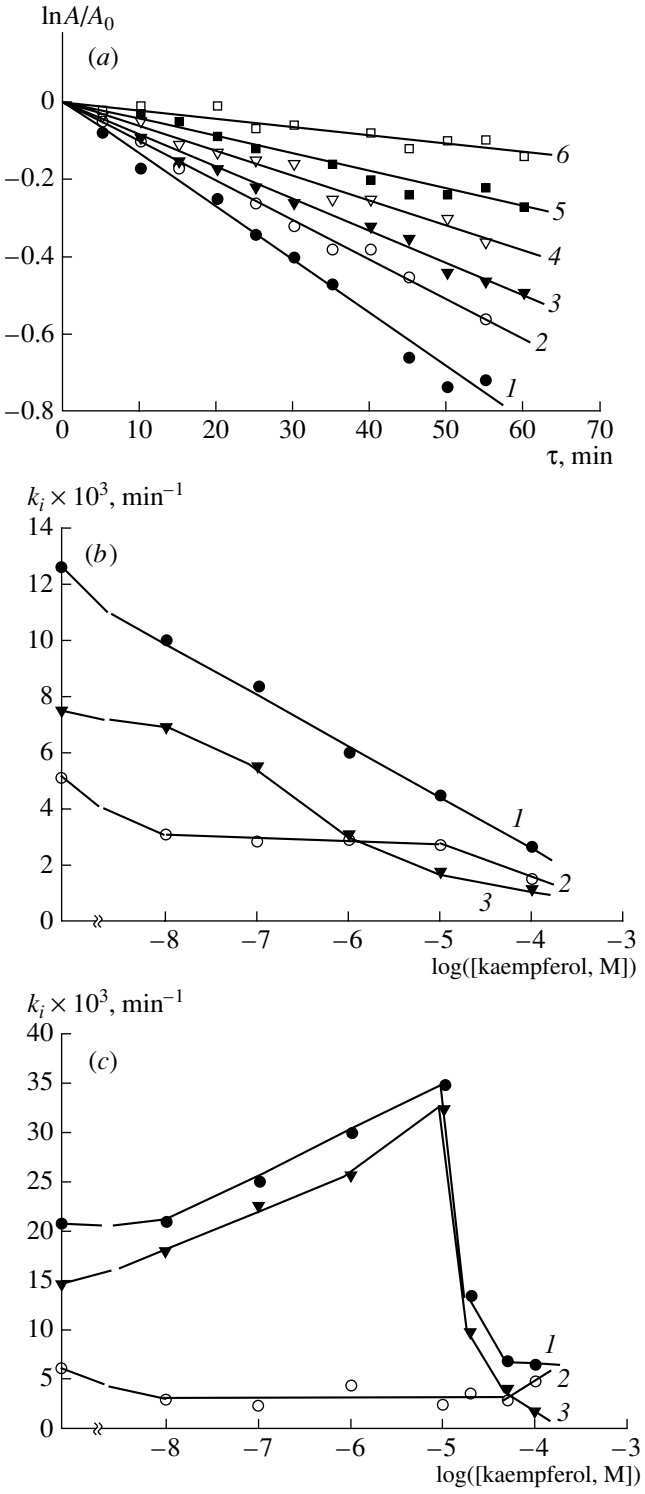


Fig. 1. Urease inactivation in solution (25 nM) by HFUS (2.64 MHz, 1.0 W/cm^2): (*a*) half-logarithmic plots of the kinetic curves at 36°C (*1*) in the absence and in the presence of kaempferol at the concentrations of (*2*) 0.01, (*3*) 0.1, (*4*) 1, (*5*) 10, and (*6*) 100 μM; (*b*) dependences of the rate (*1*) k_i, (*2*) k_i^*, and (*3*) k_i(US) of urease inactivation on the kaempferol concentration at 36°C; (*c*) the same at 46°C, where A_0 and A are the urease activity prior and after sonication, respectively.

solutions and the choice of optimal protector of urease from the US inactivation.

RESULTS AND DISCUSSION

Effect of Nonglycosidic Flavonoids on HFUS-induced Inactivation of Urease in Aqueous Solutions

The study of the effect of kaempferol on the kinetics of urease inactivation induced by HFUS showed that, in the whole range of concentrations (0.01–100 μM) and up to high levels of the enzyme inactivation, the process is described by the kinetics of the first-order reaction (Fig. 1*a*). Kaempferol reduces the rates of not only the US (Fig. 1*b*, curves *1*, *3*), but also the thermal inactivation of the enzyme (Fig. 1*b*, curves *2*), exhibiting an appreciable stabilizing effect on urease under the experimental conditions. The total inactivation of the urease (k_i) is 4.7-fold reduced, and its US component, k_i(US), by a factor of 6.4. The increase in the temperature of solution up to 46°C noticeably changes the character of the dependences of rate constants on the kaempferol concentration (Fig. 1*c*): k_i and k_i(US) constants grow up with increase in the flavonoid concentration up to 10 μM, and then sharply fall, so that the US inactivation disappears.

The growth in the inactivation rate, k_i and k_i(US), at 46°C is explained by the oxidation of kaempferol by either participation of molecular oxygen in the thermal inactivation or by the oxygen containing radicals during the US inactivation. The flavonoid ability to self oxidize in solutions at pH > 7 is well known [17].

The effect on the urease inactivation of the remaining six flavonoids was similarly studied under the strictly comparable conditions (25 nM urease, aqueous solution, pH ~ 5.4, 36°C, HFUS of 2.64 MHz, 1.0 W/cm^2).

Quercetin reduces US inactivation down to zero at the concentration of 10 μM. However, at the concentration above of 1 μM, thermal and total inactivation of the enzyme (Fig. 2*a*) grows up due to the flavonoid oxidation.

One can conclude from the results in Fig. 2*b* that, at the morin concentrations of 0.01–1 μM, all of the three rate inactivation constants decrease, while, at the concentration above of 1 μM, their substantial growth takes place.

The urease inactivation rate constants decrease at the silybin concentration of 0.001–1 μM, and the US component decreases up to zero at the flavonoid concentration of 1 μM (Fig. 2*c*). A further increase in the silybin concentration leads to increase in the urease inactivation rate, but the rate remains below its initial values in the absence of flavonoid.

Effect of Glycosidic Flavonoids on the HFUS-induced Inactivation of Urease in Aqueous Solutions

Naringin. It may be concluded from the results in Fig. 3*a* that an increase in naringin concentration from 0.01 nM to 100 μM leads to decrease in the k_i and k_i(US) rate constants, and, at the protector concentration of 100 μM, the US inactivation is reduced to zero. It is important to note that, at high naringin concentrations, a thermal stabilization of urease is also observed.

Hesperidin. It provides a thermal stabilization of urease, and, at the concentration of 1 μM, the US inactivation rate is 12.5-fold reduced (Fig. 3*b*).

Astragalin. The results described in Fig. 4*a* allow the conclusion that, at 36°C and within the concentration range of 0.01 nM–10 μM, astragalin reduces the thermal and US inactivation of urease. The US component becomes a zero at a concentration of 10 μM. When the temperature of the solutions increases up to 46°C, a growth in the k_i, k_i^*, and k_i(US) rate constants is observed. This is likely to be due to an increase in the oxidation rate of astragalin and an inactivating effect of the oxidation products on urease at this temperature (Fig. 4*b*).

The experimental results presented in Figs. 1–4 suggest that all the seven flavonoids are protectors of urease from inactivating effect of HFUS at 36°C. Their efficiency is different; it is determined not only by their structure, but also by their effect on thermal stability of urease under the experimental conditions. A quantitative comparison of this protecting efficiency of the flavonoids is impeded by their effect on thermal inactivation of the enzyme. However, this can be arbitrarily determined through a comparison for a single flavonoid concentration (e.g., for 1 μM) of the k_i^{fl}/k_i ratio of the rate effective constants, where the k_i^{fl} are the urease inactivation rate constants in the presence of 1 μM flavonoid in solution. These ratios, calculated from the experimental data, allow to arrange flavonoids according to the reduction of efficacy of their protecting effect on urease as follows: astragalin (0.23) > silybin (0.26) > naringin (0.29) > hesperidin (0.31) > quercetin (0.38) > kaempferol (0.47) > morin (0.61).

From the k_i^{fl} (US)/k_i(US) ratios of the inactivation rate constants for 1-μM concentration of the flavonoids, another order of efficiencies of their protecting effect in the decreasing order is obtained: silybin (0.01) > naringin (0.04) > astragalin (0.06) > hesperidin (0.08) > quercetin (0.26) > kaempferol (0.41) > morin (0.66).

Although the character of comparison is fully conventional, it should be noted that both of the orders are similar to each other. In both cases, the glycosidic flavonoids are most effective. They are at the beginning of the series, while the nonglycosidic flavonoids are at the end.

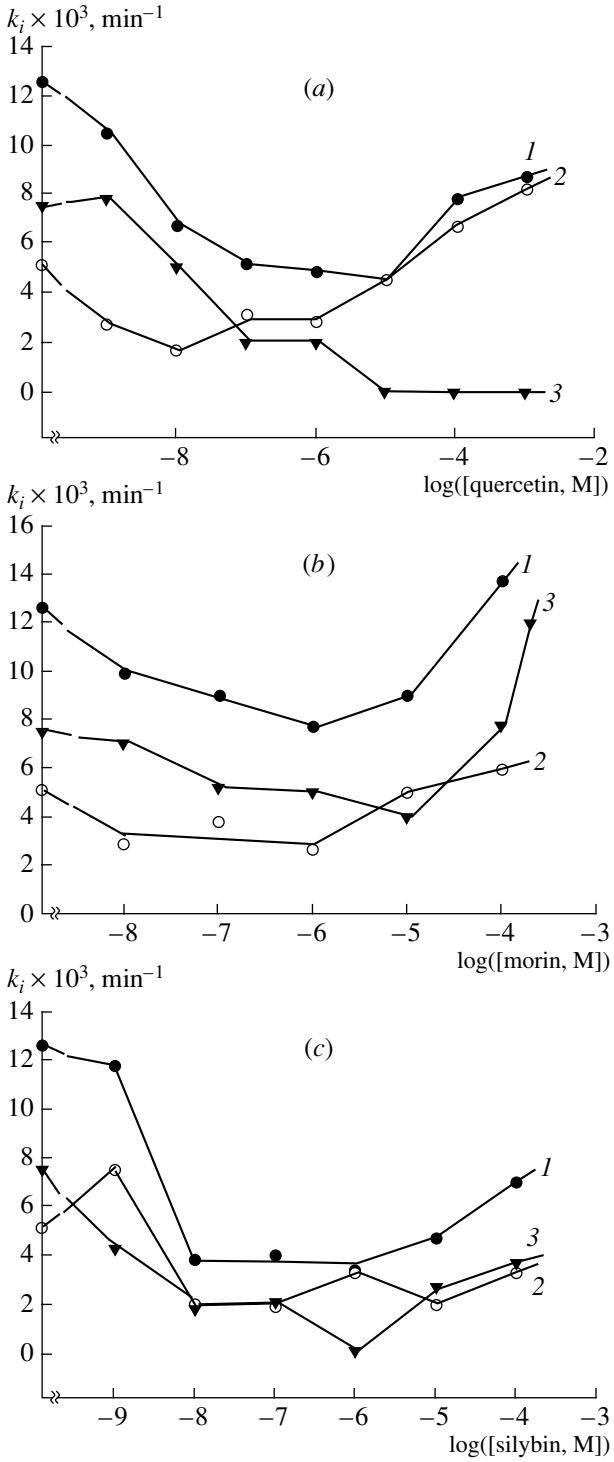


Fig. 2. Dependences of the inactivation rate constants, (*1*) k_i, (*2*) k_i^*, and (*3*) k_i(US), in solution at 36°C on the concentration of (*a*) quercetin, (*b*) morin, and (*c*) silybin. For the experimental details, see Fig. 1.

Our results on the thermal inactivation of urease in the presence of flavonoids permit the conclusion that, below the concentration of 10 μM, which is 400 times higher than the urease concentration (25 nM), the fla-

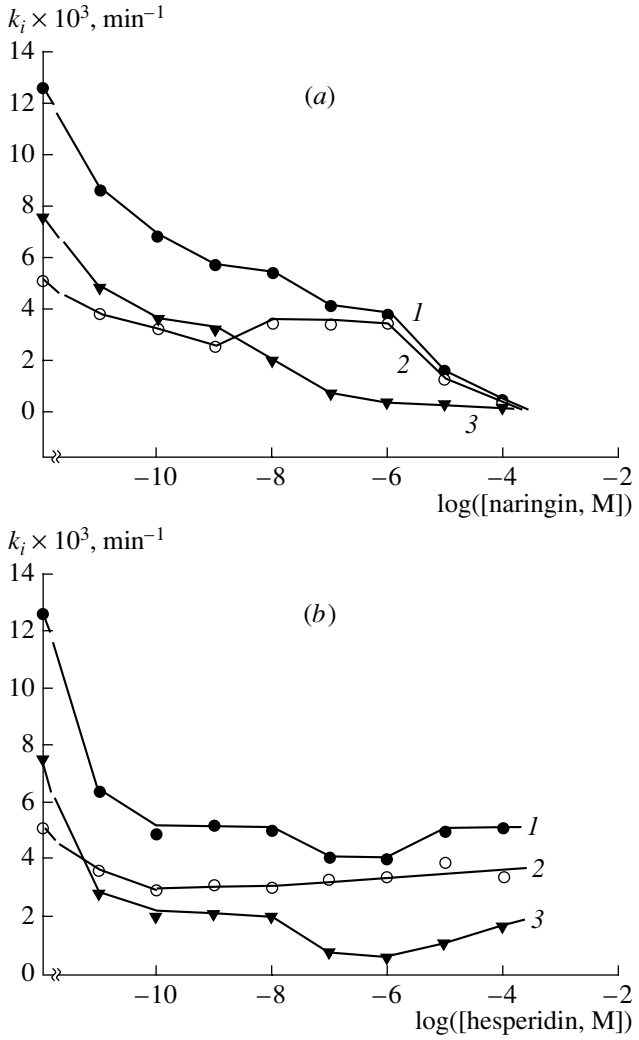


Fig. 3. Dependences of the urease inactivation rate constants (*1*) k_i, (*2*) k_i^*, and (*3*) k_i(US) in solution at 36°C on the concentration of (*a*) naringin, and (*b*) hesperidin. For the experimental details, see Fig. 1.

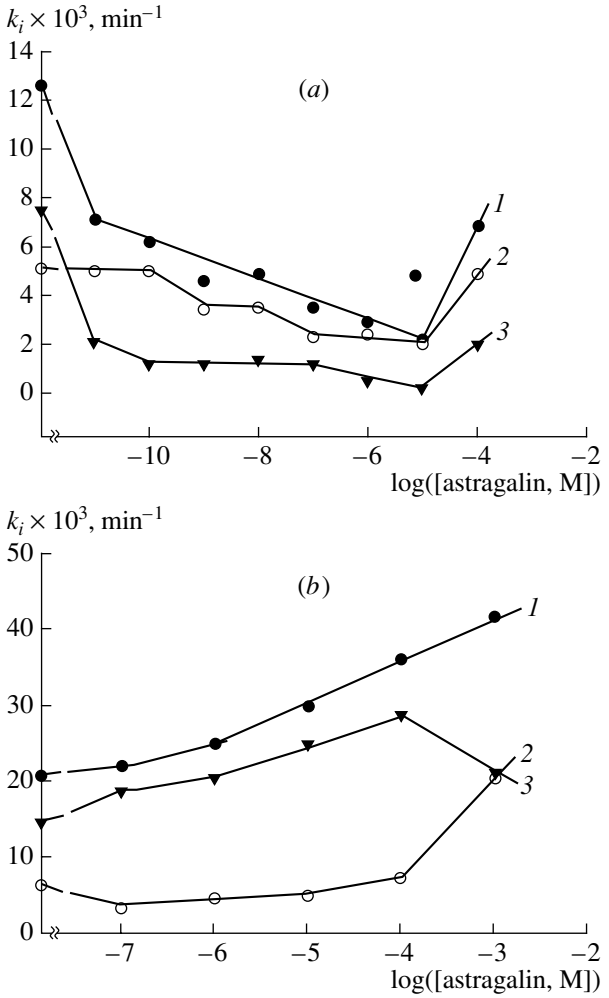


Fig. 4. Dependences of the urease inactivation rate constants (*1*) k_i, (*2*) k_i^*, and (*3*) k_i(US), in solution on astragalin concentration at (*a*) 36°C and (b) 46°C. For experimental details, see Fig. 1.

vonoids differently affected the process. Kaempferol (Fig. 1*b*) plainly reduced k_i^*. Quercetin and morin (Fig. 2) initially reduced the rate of the enzyme inactivation, while, above of 1 μM concentration, decreased it. Naringin and astragalin reduced the k_i^* values within the concentration range of 10^{-11} M to 10 μM, as follows from the information in Figs. 3, 4. Hesperidin insignificantly affected the thermal inactivation of urease (Fig. 3*b*). Various effects of the flavonoids on the thermal inactivation of urease at 36°C can be explained by the difference in their self-oxidation under the conditions of experiment. The flavonoids are known [17] to be oxidized at their hydroxy groups located in different positions of A and B rings. At high concentrations of the flavonoids, the radical products of their oxidation interact with urease and reduce its catalytic activity. Obviously, the radical products of the flavonoid oxidation possess different reactivities toward the enzyme, which depend on the flavonoid structure. The fenoxyl radicals, formed by the oxidation of OH groups located in *m*-positions relative to each other, are known to be most reactive [18]. Such groups are present in ring A of all the flavonoids we used, except for naringin and hesperidin. Morin also has these groups in the ring B. It is not surprising that, at an increase in morin concentration above of 1 μM, it induces a growth in the thermal inactivation of urease (Fig. 2*b*).

The sonication of urease solutions at 46°C in the presence of kaempferol (Fig. 1*c*) and astragalin (Fig. 4*b*) results in an increase of the total and US inactivation rate of the enzyme at the concentrations of 10 and 100 μM, respectively, because the oxidation products of these flavonoids with free radicals formed in the

field of US cavitation intensively interact with the enzyme and "poison" the biocatalyst.

All the flavonoids we used stabilize the urease subjected to sonication with HFUS (2.64 MHz, 1.0 W/cm^2) to a different extent; quercetin, naringin, hesperidin, and astragalin practically nullify the US component of the enzyme inactivation (see Figs. 1–4). The experimental results obtained in this and earlier works [9–12] support that oxygen-containing free radicals play an important role in the urease inactivation under the effect of LFUS (27 kHz, 60 W/cm^2) and HFUS (2.64 MHz, 1.0 W/cm^2). The cause of this phenomenon is that the traps of $HO^{\bullet}$ radicals, such as DMSO, DMF, ethanol, butanol [12], propyl gallate, polydisulfide of gallic acid and its complexes with albumin [10,11], and seven flavonoids, studied in this work, appreciably decrease the total and US inactivation rates, and, under specific conditions, nullify the US inactivation.

The direct correlation between the flavonoid structure and their antiradical efficacy, as well as the identification of specific groups that accept radicals during the sonication of the urease solutions is currently impossible, because there are no experimental data on the products of the flavonoid conversion in the field of US cavitation in the presence and in the absence of the enzyme. Nevertheless, in fact, the studied plant flavonoids are the effective protectors of urease from US inactivation in aqueous solutions and their efficiencies exceeds those of the synthetic antioxidants we used previously, such as propyl gallate, polydisulfides of substituted phenols, etc [10, 12].

The features of the associative–dissociative mechanism [8, 19, 20] are manifested as the bends in the half-logarithmic plots of the inactivation kinetic curves at the thermal inactivation of urease, which we have studied previously [5, 7, 9] under the specific concentrations of the enzyme and 50–60°C. A preliminary dissociation of urease into subunits or its absence is the most important aspect of US inactivation during US treatment of aqueous solutions of the enzyme. Previously, we have shown [11] that, at the US treatment of aqueous solutions of urease (25 nM) at 50°C, a clear bend in the half-logarithmic plot of the kinetic curve of the fall of the enzyme activity is observed.

In this work, the HFUS irradiation of urease solutions (25 nM) at 36 and 46°C in the presence of the flavonoids or their absence did not lead to any bends on the kinetic curves. This fact does not mean that the dissociation of the homohexamer of the urease, which can occur extremely rapidly and could not become kinetically apparent, is completely absent. Therefore, the stages of the dissociation of the oligomeric enzyme into trimers (dimers) and, then, into monomers are included in the common scheme of the US inactivation of urease given below.

$$\begin{array}{ccccccc} E_6 & \underset{k_{-1}}{\overset{k_1}{\Leftrightarrow}} & E_n & \underset{k_{-2}}{\overset{k_2}{\Leftrightarrow}} & E_1 & \xrightarrow{k_{den}} & E_{den} \\ \downarrow k_{6den} & & \downarrow k_{nden} & & & & \\ E_{6den} & & E_{nden} & & & & \end{array}$$

where E_6 is the native hexameric enzyme; E_n is trimer (and/or dimer); E_1 is urease monomer (subunit); and E_{6den}, E_{nden}, and E_{den} are the irreversibly denatured forms of hexamer, trimer (dimer) and monomer of urease, respectively. In the field of US cavitation, the simultaneous dissociation of urease can occur (rapidly) and the US inactivation of the initial enzyme and its dissociated forms according to the corresponding rate constants. The scheme of US inactivation of urease is represented by a set of sequential and parallel, reversible and nonreversible stages; it cannot be described by a system of equations, which can be analytically solved [8, 19, 20].

Free radicals $HO^{\bullet}$ and $HO_2^{\bullet}$ attack the urease in the cavitation field and destroy hydrogen bonds and hydrophobic interactions between the enzyme subunits, initiating their dissociation leading to the loss of the catalytic activity of enzyme. The absence of lag-periods on the kinetic curves of the urease US inactivation indicate the rapid stage of homohexamer dissociation. This clearly distinguishes it from another enzyme, glucose-6-phosphate dehydrogenase, which is also built up of subunits, the sonication of which under similar conditions is accompanied by clear lag-periods [9, 21, 22]. The Asp and Glu residues are the main targets of free radicals in urease. These are abundant in the protein (12.76 and 10.09%, respectively [23]) and can be the ligands of nickel ions in its active site and can participate in the control of its tertiary structure [10, 11].

EXPERIMENTAL

Reagents. Soybean urease from Biolar (Olaine, Latvia) with an activity of 1032 Samner's units per g and containing six subunits of *M* 90.7 kDa each was used. The urease concentration was determined by the known absorption coefficient $A^{1\%}_{1\ cm}$ of 6.2 at 280 nm [7]. Urea was used as a substrate, and a Bromocresol Purple dye (Reakhim, Russia), as a pH indicator. Ethanol and DMF were distilled prior to use. All solutions were prepared using only bidistilled water.

Flavonoids. Astragalin was isolated from the aeral part of oak fern (*G. dryopteris*) and was characterized as described previously in [24]. Astragalin (*M* 416) was kindly supplied by Dr. N.V. Kovganko (IBCh, NAS of Belarus). *Kaempferol* (*M* 286) was obtained from astragalin by its acidic hydrolysis. *Quercetin* (*M* 302), *morin* as a dihydrate (*M* 338), *silybin* (*M* 482), and *hesperidin* (*M* 610.57) were from Sigma (United States). *Naringin* (*M* 580.55) was from Fluka (Switzerland). All the flavonoids were used without additional purification. Stock flavonoid solutions were prepared in DMF,

except for hesperidin (4 mg) dissolved in a mixture of DMF (0.8 ml) and 50% EtOH (0.2 ml).

Sonication of aqueous solutions of urease (25 nM) at pH 5.4 was carried out at 36 and 46°C (in the absence and presence of various flavonoid concentrations) in a polystyrene vial with an inner diameter of 3.3 cm and a height of 8 cm containing a total volume of 30 ml of the enzyme solution. The temperature of the sonicated solution was maintained with an accuracy of ±0.5°C. The sonicated aqueous solutions of urease at pH 5.4 were saturated with air, which provided practically time-independent concentration of the dissolved oxygen, which was approximately ~10^{-4} M, according to [25].

An UZT-1.01F Apparatus for Ultrasonic Therapy (EMA, Moscow, Russia) was used for the US treatment. An US source with a piezoelectric converter working at a frequency of 2.64 MHz was used. The effective source area was 3.14 cm^2, and the specific power was 1 W/cm^2. The waveguide was immersed into the solution in a such way that the distance between its butt-end and the bottom of the flask was 2.0 cm. The sonication of solutions was carried out continuously for 1–2 h.

From the total solution volume of 30 ml, which contained urease and flavonoids at various concentrations, aliquots of the volume of 2.5–4.0 ml were taken to study the thermal inactivation of urease, which was studied at the same temperature as the sonication of the solutions. The aliquots of 0.176 ml were taken every 5 min to determine the residual activity of urease (A).

Catalytic activity of the urease prior to its sonication (A_0) and during the inactivation (A) were determined using pH-indicator according to the technique earlier described in [6].

The preparation of substrate mixtures was achieved using a 0.03 M urea solution. EDTA was added to 0.03 M urea aqueous solution (100 ml) to a final concentration of 0.05 mM, to bind impurities of heavy metal ions. Bromocresol Purple was dissolved in a mixture containing 0.2 ml of 0.05 M NaOH and 0.3 ml of water. The final concentrations of urea, Bromocresol Purple, and urease in the reaction mixture were, respectively, 23 μM, 38.2 μM, and 5.5 nM.

Changes in the absorption of pH indicator at 591 nm were monitored in the course of the urea hydrolysis, and the kinetic curves in the coordinates absorption–time were plotted. All the measurements were carried out on a Specol-221 (Carl-Zeiss, Germany) spectrophotometer. The initial rate of the reaction was expressed in arbitrary units (absorption change per s). It was assumed to be 100% before the thermal inactivation or sonication of urease. The relative activity of the partially inactivated urease was expressed as the ratio A/A_0, percent.

Characterization of urease inactivation in aqueous solutions. The effective rate constants k_i (min^{-1}) of total (sum) inactivation of urease were determined at 36 and 46°C from the half-logarithmic plots of the kinetic curves of the enzyme activity changes plotted versus time, A/A_0–time. Similarly, for the process of urease thermal inactivation, the effective rate constants, k_i^*, were calculated from the half-logarithmic plots of the curves in the A/A_0–time coordinates. The rate constants of the US inactivation of urease, k_iUS in the presence of flavonoids and in their absence, were calculated as the difference $k_i - k_i^* = k_i(\mathrm{US})$. The error in the determination of the rate constants was about 13%.

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