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Trypsin-Based Chemoenzymatic Assay for Detection of Pollutants and Safety Assessment of Food Additives

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Abstract: Chemoenzymatic assay systems are widely used to detect toxicants in various samples, including food and environment specimens. These methods are based on the ability of various types of toxicant to specifically inhibit/activate the functions of individual enzymes or enzyme systems. The present study examines the possibility of using the proteolytic enzyme trypsin as a specific marker to detect protease inhibitors in different samples. The study shows that trypsin activity is not affected by various heavy metals, pesticides, or quinones at levels considerably greater than their maximum allowable concentrations (MACs) in water bodies. At the same time, the IC_{50} value for the food preservative potassium sorbate (E202) is 15 mg/L, which is substantially lower than its acceptable daily intake (ADI). The quenching of trypsin fluorescence in the presence of potassium sorbate suggests that inhibition could occur due to the binding of the preservative to the enzyme in the region adjacent to the active center. The trypsin was immobilized in starch gel to ensure its stability in the enzyme inhibition based assay. Single-use reagents were prepared as dry starch disks that could be stored over long periods. Their sensitivity to copper (II) chloride, potassium sorbate, and chromium (III) chloride was similar to the sensitivity of the free trypsin.

Keywords: trypsin-inhibition-based assay; detection of pollutants; food additives; chemoenzymatic assay; trypsin immobilization; protein fluorescence

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1. Introduction

To maintain the health of the human population, it is critical to detect toxic substances in the environment, as there is a risk of the regular intake of various anthropogenic contaminants with food and drinks. These contaminants may include heavy metals, pesticides, phenols, and their oxidation products, quinones, which may be even more toxic than their respective polyphenols [1–5]. Another cause for concern is the use of numerous food additives, including preservatives, which are added to prevent food spoilage. The amounts of preservatives are controlled, and their concentrations are usually low: 0.01–0.2% of the weight of the product [6,7]. However, recent studies show that the consumption of increased amounts of food preservatives may produce deleterious effects through, for example, their complexing with blood proteins or the inhibition of the gene expression of the proteins involved in synthesis of coagulation factors [8,9].

Pollutants in the environment are commonly detected using biological testing systems, based on the assessment of changes in the vital functions of various test organisms [10], and chemoenzymatic methods, based on the ability of pollutants to inhibit/stimulate the activities of different enzymes or multi-enzyme systems [11]. Chemoenzymatic assay systems exhibit selective sensitivity to various classes of toxic substance and, thus, they can be used to study the effects of the mechanisms of action of toxicants on the functions

of separate components of metabolic pathways [11]. However, it should be noted that prior to using enzymes as test organisms, a considerable amount of preliminary work is required, including the stabilization of the enzymes or the pretreatment of the samples to be analyzed (establishing the optimum pH for enzyme activity, ensuring the solubility of the tested compounds, removing interfering impurities) [11]. Moreover, enzymes are generally expensive reagents.

The enzymes most frequently used in these inhibition assays are hydrolases and oxidoreductases. Of the chemoenzymatic assay systems, the most widely used are the systems based on cholinesterases, which are highly sensitive to organophosphorus and carbamate pesticides [12]. The assays most commonly applied for detecting heavy meals are the assay systems based on urease, peroxidase, and glucose oxidase [11].

Protease-based chemoenzymatic assay systems are used considerably less often. The main field of application of proteases is pharmaceutics, in which drugs inhibiting protease are screened [13]. Nevertheless, a number of studies demonstrate the potential of proteases such as trypsin—an enzyme produced by the pancreas—to serve as biomarkers in the bio-diagnostics of water or soil contamination [14–16]. The proteolytic activity of trypsin can be affected by a variety of substances, which can either stimulate or inhibit its action. Substances capable of decreasing trypsin activity include, for example, specific protease inhibitors from soybeans (such as Kunitz-type inhibitors (KSTIs) and Bowman–Birk-type inhibitors (BBIs)), cadmium, etc. [17,18]. The effect of trypsin stimulation was observed in the presence of monatomic alcohol isopropanol and biogenic amine putrescine [19,20]. The data on the effect of the food preservative sodium benzoate on trypsin activity are rather contradictory. A study by Mu et al. reported the stimulation of trypsin activity by sodium benzoate as a result of the formation of a new complex and a change in the trypsin structure [21]. On the other hand, there are data showing the inhibition of trypsin by sodium benzoate [22]. The contradictions between these data suggest the importance of collecting datasets on the effects of potentially hazardous substances on proteolytic enzymes.

In the current study, we tested trypsin as the basis for the chemoenzymatic assay to detect protease inhibitors in the samples we analyzed. The effects of different types of xenobiotic—a number of salts and ions of heavy metals, pesticides, quinones, and food preservatives—on the trypsin's activity were estimated. For trypsin to be used as the basis for chemoenzymatic assays, a stable form of the enzyme should be prepared, which should retain the sensitivity of trypsin to inhibitory effects. In the present study, we propose a method involving the immobilization of trypsin in starch gel, in which the enzyme molecules are incorporated into a three-dimensional network of closely interwoven polymer chains.

2. Materials and Methods

The reagents used in this study included lyophilized trypsin from porcine pancreas, 1300 BAEE U/mg (Sigma-Aldrich, St. Louis, MO, USA), specific substrate N_{α}-Benzoyl-L-arginine ethyl ester (BAEE) (Sigma-Aldrich, St. Louis, MO, USA), hydrochloric acid (SigmaTek, Khimki, Russia), and Clark-and-Lubs buffer pH 7.6. Solvents were nitric acid (Technological Laboratory Giredmet, Khimreaktivsnab, Ufa, Russia), acetonitrile (PanReac AppliChem, Barcelona, Spain), and distilled water.

Aqueous solutions of heavy-metal salts and nitric-acid solutions of metal ions were used to study the effects of heavy metals on trypsin activity. Salts of heavy metals—copper chloride ($CuCl_2$), zinc nitrate ($Zn(NO_3)_2$), manganese chloride ($MnCl_2$), chromium chloride ($CrCl_3$), and cobalt chloride ($CoCl_2$)—were analytical-grade chemicals (OOO "Khimkraft", Kaliningrad, Russia). State-standard reference samples of ions of cadmium (Cd^{2+}), nickel (Ni^{2+}), arsenic (As^{3+}), mercury (Hg^{+}), and aluminum (Al^{3+}) (Ural Plant of Chemical Reagents, Verkhnyaya Pyshma, Russia) were used. Other chemicals whose effects on trypsin activity were studied included the following: quinones—1,4-benzoquinone and toluquinone (Sigma-Aldrich, Taufkirchen, Germany); food preservatives—sodium benzoate (Sigma-Aldrich, Amsterdam, The Netherlands) and potassium sorbate (Supelco, Bellefonte,

PA, USA); high-purity active ingredients of pesticides—fenvalerate, deltamethrin, cypermethrin, imidacloprid, glyphosate (Sigma-Aldrich, St. Louis, MO, USA), metribuzin, tebuconazole, epoxiconazole, fenoxaprop-p-ethyl (Xi'anTai Cheng Chem Co., Ltd., Yancheng, China); and glyphosate-based commercial pesticide formulation Tornado Extra (JSC Avgust, Moscow, Russia). Pesticides were dissolved in acetonitrile, except glyphosate and Tornado Extra, which were dissolved in distilled water.

Trypsin activity was determined from hydrolysis rate of its specific substrate, BAEE, by recording temporal variations in the absorbance of the reaction mixture at 253 nm, using a Shimadzu UV-2600 spectrophotometer (Shimadzu Corporation, Kyoto, Japan). The reaction mixture contained 11 U of trypsin, 450 µL of the tested or control sample, 40 µL of 0.1 M Clark-and-Lubs buffer with pH 7.6, 460 µL of 0.5-mM BAEE solution, and 40 µL of 1-mM hydrochloric acid.

The effects produced by the solvents and tested substances on trypsin activity were estimated as relative activity determined from the formula $A = (A_{exp}/A_{contr})\cdot 100\%$, where A_{exp} and A_{contr} are enzyme activities in the presence of the tested substance and in control solution, respectively. The resulting data were used as the basis for determining the values of toxicological parameters IC_{20} and IC_{50}—concentrations of the tested substances that inhibit trypsin activity by 20 and 50%, respectively.

The absorption and fluorescence spectra of trypsin were measured using a Cary 5000 spectrophotometer (Agilent Technologies, Mulgrave, Australia) and a Fluorolog-3 spectrofluorometer (Horiba, Jobin Yvon, France), respectively. Fluorescence spectra under excitation at a wavelength of 295 nm were measured in the 300–450-nm range. The spectral-slit width was 2.5 nm. The spectra were corrected for the inner-filter effect and solvent background. The absorption spectra were measured in the 200–600-nm range.

The samples for measuring fluorescence spectra had a volume of 1 mL and contained 9 U of trypsin and various concentrations of the tested substance in the buffer. The trypsin-fluorescence spectra were also studied in the presence of the commercial pesticide formulation with glyphosate as the active ingredient, Tornado Extra, whose inhibitory action was shown in our previous paper, and IC_{50} = 2.4 g/L was obtained [23].

Visualization of spatial structure of trypsin (PDB ID: 1s0q) and assessment of the distances between tryptophan residues and catalytic triad were performed using VMD 1.9.2 [24]. The solvent accessible surface areas (SASAs) of the tryptophan residues were estimated by GROMACS GMX SASA plugin with solvent-probe radius of 1.4 Å [25]. Before calculation, hydrogens were added to the trypsin crystal structure (PDB ID: 1s0q) according to CHARMM36 force-field topology.

Immobilization of trypsin was performed as follows. First, potato-starch gel was prepared by boiling starch suspension for 2 min and cooling to 25 °C. Next, the trypsin solution was mixed with potato-starch gel, and 25-µL drops were placed onto fluoropolymer film and dried at 8 °C. The resultant preparations were 4–5-mm-diameter dry disks; each disk was intended for one assay. To measure the activity of immobilized trypsin, 1 disk with immobilized enzyme, 40 µL of 1-mM hydrochloric acid, 450 µL of the tested or control sample, 40 µL of 0.1-M Clark-and-Lubs buffer with pH of 7.6, and 460 µL of 0.5-mM BAEE were added to the spectrophotometer cuvette.

The data are presented as mean values (M) with standard deviation (s). All the measurements were repeated 5 times. The significance of differences was determined by Student's *t*-test. The results were considered statistically significant at $p < 0.05$.

3. Results

3.1. The Effects of Pollutants and Food Preservatives on the Activity of Trypsin-Assay System

Enzyme-based assays have a substantial advantage over assays based on living organisms: the conditions of enzymatic assays can be varied to adjust their sensitivity to toxicants. For example, assay sensitivity can be enhanced through the pre-incubation of the enzyme in the tested solution or by altering the proportions of the components in the reaction mixture. The sensitivity of chemoenzymatic assay systems to toxic substances is

largely determined by the amount of enzyme in the reaction mixture: the highest sensitivity is usually achieved at low enzyme concentrations. We varied the trypsin content and found the level that would allow its enzymatic activity to be detected while, at the same time, ensuring good reproducibility of the results, which ranged between 5 and 11 U (Figure 1). It would not have been justifiable to increase the trypsin content further, as this would have led to a decrease in the assay's sensitivity to toxicants and excessive consumption of the enzyme. A reduction in trypsin content to below 5 U resulted in a considerable decrease in the level of the detectable signal and, hence, a larger measurement error.

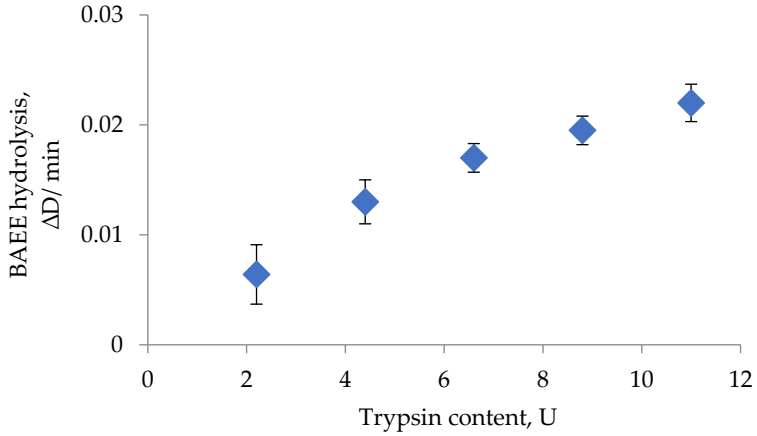

Figure 1. BAEE-hydrolysis rate versus trypsin content in the reaction mixture.

In the present study, we used trypsin amounts falling within the preliminarily determined range and analyzed the effects of a number of toxicants, such as metal salts, pesticides, quinones, and food additives on the trypsin activity. Initially, the solvents used to prepare solutions of toxicants—distilled water, nitric acid, and acetonitrile—were tested to determine their effects on the trypsin activity. The tests showed that the proportions of distilled water and acetonitrile in the reaction mixture should be no more than 45% and 3% of the total volume of the mixture, respectively. Higher percentages of these solvents inhibited the trypsin activity. The concentration of nitric acid in the reaction mixture should not exceed 0.1 mM.

The use of the optical method of signal detection imposes a number of limitations on the analysis of the effects of toxicants on enzyme activity [26]. This is particularly evident in the analysis of trypsin activity, as measurements are conducted at a wavelength of 253 nm, i.e., in the UV region, where various substances have pronounced absorption [26]. Moreover, in some of our experiments, the addition of the toxicant solutions to the reaction mixtures resulted in the production of suspended particles, turbidity, and color changes in the solution. For example, the addition of $Zn(NO_3)_2$ or more than 170 mg/L of $CuCl_2$, $MnCl_2$, $CrCl_3$, and $CoCl_2$ to the reaction mixture led to the formation of precipitate, probably due to interactions between these chemicals and the salts in the potassium-phosphate buffer. These interactions of the toxicants with the reaction-mixture components considerably limited the range of the concentrations of additives tested in the present study.

The effects of heavy metals on the activity of trypsin were studied using solutions of the heavy metals in water and in nitric acid. Concentrations of $Zn(NO_3)_2$ below 85 mg/L and $CoCl_2$ below 58.5 mg/L did not affect the trypsin's activity. The trypsin activity decreased in the presence of $CuCl_2$, with an IC_{20} value of 80 mg/L. The presence of manganese and chromium chlorides in the reaction mixture stimulated trypsin activity. The activity of trypsin exposed to 90 mg/L and 104 mg/L of $MnCl_2$ and $CrCl_3$, respectively, rose by 35%.

In the study of effects of the metals in the form of ion solutions on the trypsin activity, the range of metal concentrations was limited because of the considerable inhibitory effect of the solvent (nitric acid). Metal ions at concentrations below 0.2 mg/L did not affect the trypsin activity. An additional process—the 5-min incubation of trypsin in solutions of

metal salts or ions—did not lead to the emergence of an inhibitory effect within the tested range of concentrations.

Most of the tested pesticides were poorly soluble in water, and they were dissolved in acetonitrile. However, the addition of high concentrations of pesticides dissolved in acetonitrile to the reaction mixture often caused the turbidity of the solution and the production of suspended particles, which rendered the analysis impossible. Thus, we determined the highest possible concentrations of the tested pesticides: up to 9 mg/L for metribuzin, tebuconazole, fenoxaprop-P-ethyl, and epoxiconazole; and up to 10 mg/L for deltamethrin, fenvalerate, cypermethrin, and imidacloprid. At these concentrations of pesticides, no changes in trypsin activity were observed. The additional process—enzyme incubation in the tested pesticide solution—increased the trypsin sensitivity to metribuzin and fenoxaprop-P-ethyl only (Figure 2). In the presence of the water-soluble glyphosate, pesticide-concentration dependence was obtained without enzyme pre-incubation (Figure 3).

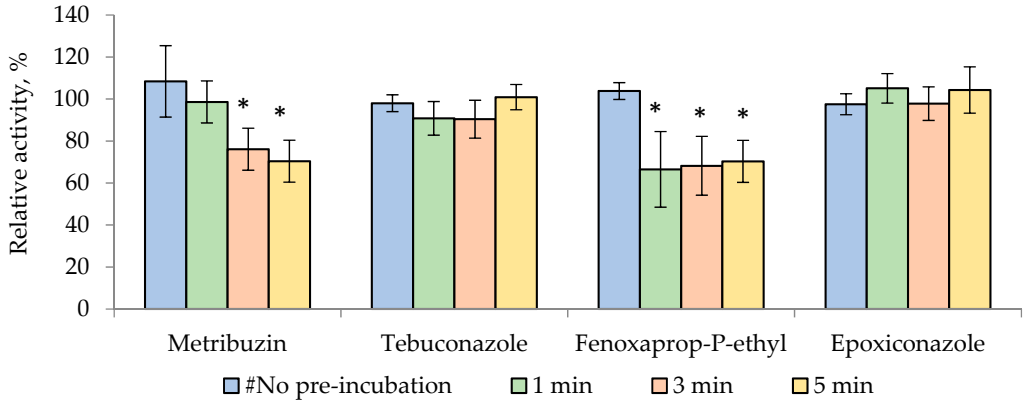

Figure 2. Dependence of relative activity of trypsin on the time of incubation of the enzyme in solutions with pesticide concentration of 9 mg/L. A 20% inhibition of trypsin activity was achieved by pre-incubation of trypsin in metribuzin and fenoxaprop-P-ethyl solutions for 3 and 1 min, respectively. #No pre-incubation denotes that the pesticide solution was added to the reaction mixture immediately before signal detection. * Values of $p < 0.05$ when comparing the relative activity values obtained without pre-incubation of trypsin in the pesticide solution.

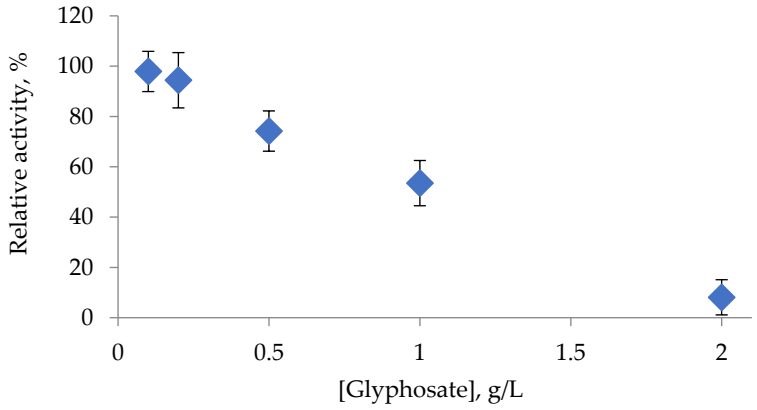

Figure 3. Relative activity of trypsin in the presence of glyphosate. The amount of trypsin in the reaction mixture was 11 U. The values of IC_{20} and IC_{50} for glyphosate were determined as 0.5 and 1 g/L, respectively.

The highest concentrations of the quinones—toluquinone and benzoquinone—in the reaction mixture that made it possible to detect changes in its absorbance were 6 and 16 mg/L, respectively. Benzoquinone at a concentration of 16 mg/L inhibited the trypsin activity by 20%. The decrease in the amount of trypsin to 5.5 U only insignificantly increased the sensitivity of the trypsin-assay system to benzoquinone and toluquinone (Figure 4).

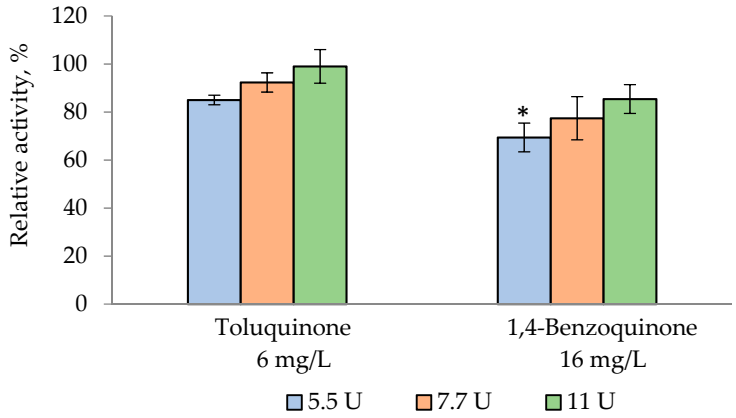

Figure 4. Dependence of the relative activity of trypsin in the presence of quinones on the amount of the enzyme. A decrease in the amount of trypsin in the reaction mixture led to an insubstantial increase in the sensitivity of the trypsin-assay system to benzoquinone and toluquinone. * Values of $p < 0.05$ when comparing the relative activity values obtained in the presence of 11 U of trypsin.

The highest tested concentration of the food preservative sodium benzoate, 144 mg/L, did not affect the trypsin activity. At the same time, another food preservative—potassium sorbate—considerably inhibited the trypsin activity; the IC_{50} value for the potassium sorbate was 15 mg/L, and it was substantially lower than its acceptable daily intake (ADI), which, for drinks, for example, is 300 mg/L [7].

Thus, the analysis of different types of xenobiotic revealed the selective sensitivity of trypsin to toxicants and food preservatives. Table 1 lists the IC_{20} and IC_{50} values for some toxicants and food preservatives.

Table 1. IC_{20} (mg/L) and IC_{50} (mg/L) values determined from the effects of toxicants and food preservatives on trypsin-assay system * #.

Inhibitors	Concentrations Studied (mg/L)	IC_{20} (mg/L)	IC_{50} (mg/L)	ADI RUS (mg/L)
Copper(II) chloride	<80	80	–	0.001–0.002
Glyphosate	100–2000	500	1000	0.02–5.0
Metribuzin &	<9	9	–	0.1–0.2
Fenoxaprop-P-ethyl &	<9	9	–	0.0003–0.04
1,4-Benzoquinone	<16	16	–	0.001–0.2
Potassium sorbate	<15	10	15	200–300

"–" No inhibitory effect of the toxicant was detected in the tested concentration range. * For the other additives (zinc nitrate, cobalt chloride; ions of cadmium, nickel, arsenic, mercury, and aluminum; toluquinone, sodium benzoate; pesticides: tebuconazole, fenoxaprop-P-ethyl, deltamethrin, fenvalerate, cypermethrin, and imidacloprid) either no effect was found or enzyme activation was observed (manganese chloride, chromium chloride) at studied concentrations. # Trypsin content in all cases was 11 U, except for the test with 1,4-benzoquinone, on which trypsin content was 7.7 U. & The pre-incubation for 3 min was performed for two toxicants marked by & and no pre-incubations—in the other assays in the table.

3.2. Interaction of Food Preservatives and Some Toxicants with Trypsin

One of the possible reasons for the variations in the enzyme activity is a change in the enzyme structure caused by its interactions with the toxicants. To reveal changes in the tertiary structure of the trypsin, we measured the fluorescence spectra in the presence of the toxicants that had a pronounced effect on the trypsin activity. These were the pesticide formulation Tornado Extra (the effect of which was studied in our previous work [23]), the salts $CuCl_2$, $MnCl_2$, and $CrCl_3$, and potassium sorbate. The toxicants were preliminarily dissolved in water. The protein spectra in the presence of a toxicant were compared with the spectra of the control sample, to which a corresponding amount of water was added. In each treatment, the trypsin-to-toxicant concentration ratio was close to the ratio obtained under the maximal inhibitory or stimulatory effect. The fluorescence spectra were measured at an

excitation wavelength of 295 nm, at which only the tryptophan residues of proteins were excited. Highly sensitive to microenvironment polarity, tryptophan fluorophores respond to variations in microenvironment parameters caused by disturbances in the protein structure or direct contact with bound additives via changes in fluorescence intensity and the position of the spectral maximum.

Figure 5 shows the fluorescence spectra of the trypsin in the presence of different concentrations of various additives. The trypsin exhibited fluorescence typical for this protein, with a maximum of about 330 nm, which did not change in the experiments. The addition of the pesticide formulations only slightly perturbed the intensity of the trypsin fluorescence, without producing concentration-dependent effects (an example is shown in Figure 5a). This can be attributed to the high level of background emissions of the complex mixtures, which were difficult to take into account and subtract completely. The salts $CrCl_3$, $MnCl_2$, and $CuCl_2$ produced no effect on the trypsin fluorescence (the example with $CuCl_2$ is shown in Figure 5b), implying no change in the protein structure after their addition. The only additive that caused a change in the fluorescence spectrum was potassium sorbate (Figure 5c). This food preservative is very soluble in water and has favorable spectral properties, which made it possible to detect clearly a gradual decrease in protein fluorescence with an increase in its concentration.

The Stern–Volmer plot for the quenching of the trypsin fluorescence by the potassium sorbate is shown in Figure 5d. The corresponding Stern–Volmer constant K_{SV} was calculated as $85.99 \pm 3.42\ M^{-1}$. Taking into account that the average fluorescence lifetime of trypsin in the buffer was measured as 2.51 ns [27], we estimated the bimolecular quenching constant k_q as $3.4 \times 10^{10}\ M^{-1}s^{-1}$. This value is greater than the diffusion-controlled limit of k_q, which is about $1 \times 10^{10}\ M^{-1}s^{-1}$ [28]. Thus, we concluded that both the dynamic mechanism of the quenching due to the collision with the quencher and the static mechanism of the quenching due to the binding of the potassium sorbate by the trypsin contributed to the total effect. To distinguish between the contributions of these two quenching types, a detailed study of the fluorescence lifetimes of trypsin in the presence of potassium sorbate and under varying experimental temperatures should be performed, which will be the subject of the future research.

To assess the possibility of a relationship between the inhibition of the trypsin activity and the quenching of its fluorescence by the potassium sorbate, we considered the relative positions of the catalytic triad and the tryptophan residues in the trypsin globe (Figure 6). Of the four tryptophans, Trp215 was the closest to the active site of the enzyme. It was located at 5.16–6.43 Å from the triad residues. Thus, the interaction of the potassium sorbate with the surface area adjacent to the site of the Trp215 localization could have influenced the trypsin activity. The tryptophan residues of trypsin have different solvent accessible surface areas (SASAs), which means that they are not equally available for quenching by additives. The following SASAs were estimated for the tryptophans ($Å^2$): Trp51—0, Trp141—3.3, Trp215—56.3, Trp237—37.9. This indicated that only the last two were able to make contact with the quenchers. Therefore, we suggest that Trp215 and Trp237 could have been responsible for the observed protein-fluorescence quenching with the potassium sorbate, and that one of the two was located close to the active site of the enzyme. Although the crystal structure of trypsin does not reflect protein dynamics in the solution, which could cause variation in the estimated distances, the relative positions of the amino acids and their accessibility to the solvent are unlikely to change considerably because of structural fluctuations. A previous study led to the conclusion that trypsin has nearly identical molecular architectures in solution and in three crystal forms [29].

Thus, the fluorescence spectroscopy revealed direct intermolecular contact between the trypsin and the additive, affecting its activity for potassium sorbate only. It can be concluded that the inhibitory effects of the $CuCl_2$ and the pesticides or the stimulatory effects of the $CrCl_3$ and the $MnCl_2$ resulted from other mechanisms, such as the effects on the ligands and enzyme substrates, or the disruption of the enzyme–substrate interaction.

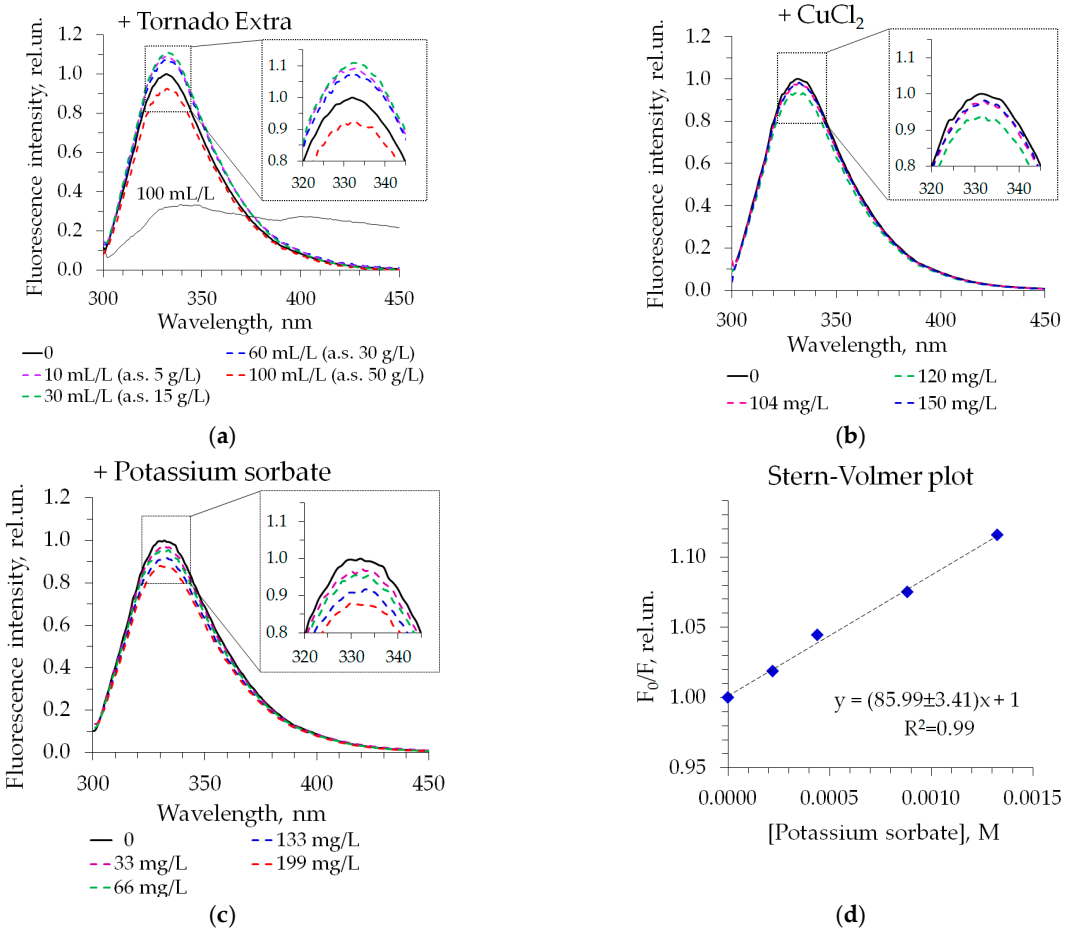

Figure 5. Fluorescence spectra of trypsin under 295-nm excitation in the presence (colored dashed lines) and in the absence (thick black lines) of additives: (**a**) Tornado Extra pesticide formulation, (**b**) $CuCl_2$, (**c**) potassium sorbate. Inserts show the intensity change near the spectral maximum. Protein content was 9 U. Thin black line in panel (**a**) refers to the fluorescence spectrum of the additive without trypsin at indicated level under 295-nm excitation. Panel (**d**) shows the Stern–Volmer plot for quenching of trypsin fluorescence by potassium sorbate. Equation for the linear approximation and an adjusted coefficient of determination R^2 are indicated.

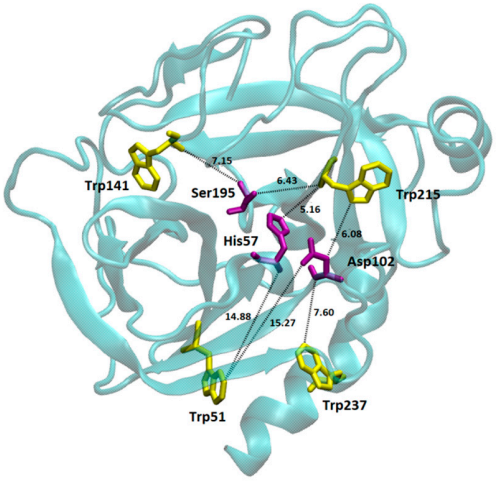

Figure 6. Spatial structure of trypsin (PDB ID: 1s0q) shown by ribbon representation with indicated position of catalytic triad Ser195-His57-Asp102 (violet sticks) and tryptophan residues (yellow sticks). Dotted lines connect the closest atoms of the triad and tryptophans; corresponding distances are indicated in Å.

3.3. Constructing the Biological Module for Chemosensor by Immobilizing Trypsin in Starch Gel

The trypsin-based assay can only be used as an indicator to detect protease inhibitors if the stable form of the enzyme is available. It is a challenging task to prepare a stable enzyme that retains the necessary sensitivity to toxicants. In the present study, trypsin was immobilized into starch-polymer gel for the first time. The resultant preparations were 4–5-mm-diameter dry starch disks; one disk was intended for one assay.

When developing a reagent based on immobilized trypsin, we studied the effects of the potato-starch concentration and drying temperature on the trypsin activity (Figure 7). The activities of the reagents immobilized into 2.5, 3, and 3.5% starch gels did not differ considerably. At the same time, the reagents prepared using the 2.5% starch gel were brittle, and the 3.5% gel was too viscous to measure accurately the sizes of the drops that were dried to make the starch disks. Therefore, the 3% starch gel was chosen as the matrix for the trypsin immobilization. No significant differences in the activities of trypsin immobilized in the starch gel were observed between the disks dried at 8 °C and 20 °C.

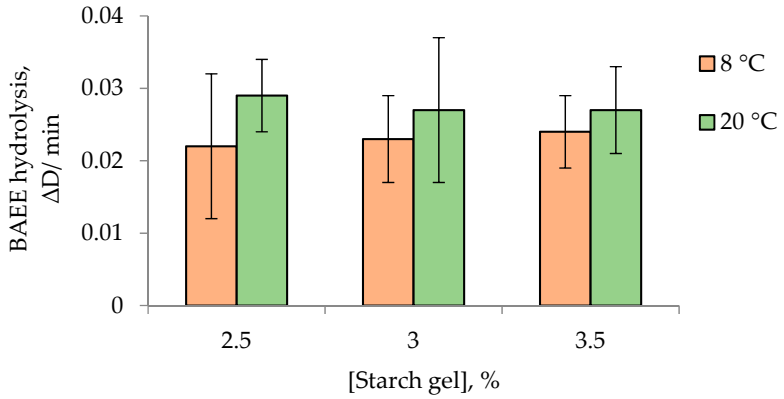

Figure 7. The rate of BAEE hydrolysis by trypsin versus concentration of potato-starch gel. Trypsin content was 11 U in the starch disks.

The single-use disks prepared in the present study contained 11 U of immobilized trypsin (the same amount as the solution), and disks containing increased amounts of trypsin—22 and 29 U—were also prepared. The activity of the disks with immobilized trypsin was about 100% compared to the activity of the free forms with the same amounts of trypsin. The immobilized trypsin reagents stored at 8 °C for 4 months retained 80% of their activity.

The sensitivity of the trypsin immobilized in starch gel to the toxicants was assessed in experiments with glyphosate as the active ingredient of the commercial pesticide Tornado Extra, $CuCl_2$, and potassium sorbate.

As the trypsin immobilized in starch gel had the form of dry starch disks, for the enzymatic reaction to occur, it was necessary to enable the diffusion of the substrate and inhibitor to the immobilized trypsin. This was achieved by causing the disk swell while it was pre-incubated in the tested solution, before the addition of the substrate. The disks with immobilized trypsin were incubated in the pesticide solution for 2 and 5 min. The results of the measurements of the trypsin activity in the disks with the lower trypsin contents (11 and 22 U) had poorer reproducibility (Figure 8). In addition, the incubation of the disks containing different amounts of trypsin in the glyphosate solution for 5 min did not result in any significant differences in the degree of inhibition of enzyme activity.

The sensitivity of the trypsin immobilized in starch gel to $CuCl_2$ and potassium sorbate was comparable to the sensitivity of the free enzyme. The IC_{20} and IC_{50} values for the potassium sorbate were 12 and 20 mg/L, respectively. Furthermore, the activity of the immobilized trypsin, as with that of the free enzyme, was stimulated in the presence of $CrCl_3$.

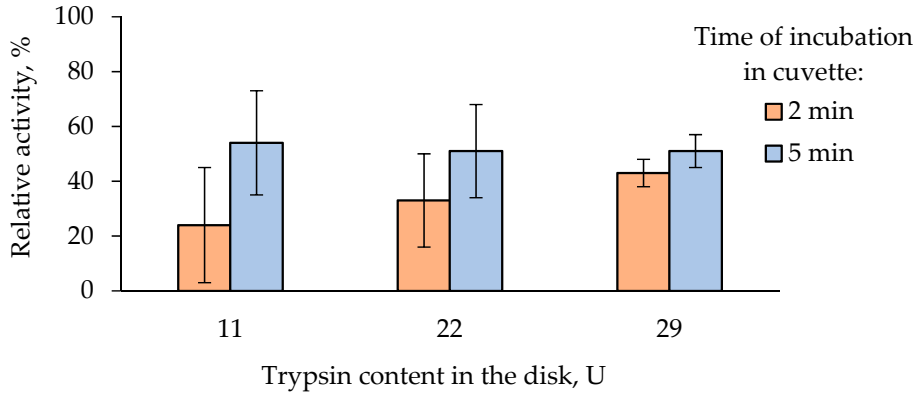

Figure 8. Relative activity of trypsin immobilized in starch gel in the presence of 5 g/L of glyphosate as the active ingredient of commercial pesticide Tornado Extra. Five g/L of glyphosate was determined as the IC_{50} value for trypsin immobilized in starch gel; this value was obtained through pre-incubation of the immobilized enzyme in the pesticide solution for 5 min.

4. Discussion

Chemoenzymatic assay systems are widely used to detect various chemical and biological components for clinical, food, and environmental monitoring [30]. Sensors based on enzyme activation or inhibition are effective tools because of their high sensitivity and simplicity. The methods used to detect analytes using chemoenzymatic assay systems can be either specific, i.e., sensitive to certain substances, or integrated, with the combination of substances contained in the tested sample affecting the assay system. The method chosen to detect analytes should correspond to the aims of the study, and the choice of the method determines the choice of the chemoenzymatic assay system. A good example of a specific detection method is provided by chemoenzymatic assays based on cholinesterases used to detect organophosphorus compounds and carbamate pesticides [11,30]. Integrated methods include assay systems based on enzyme systems conjugated with bacterial luciferase: the enzymes in these systems are sensitive to a wide range of toxicants, and the systems can be used to assess the overall toxicity of samples [11,31].

In the current study, we examined the possibility of using trypsin as the basis for a chemoenzymatic assay system for detecting toxic substances in aqueous media. The preparation of the solutions of the tested toxicants was a challenging task, which placed serious limitations on the investigations of the trypsin's sensitivity to the toxicants. For instance, most pesticides are soluble in acetonitrile, but the maximum volume of this solvent that could be added to the reaction mixture without affecting trypsin activity was only 3% of the reaction mixture volume. Moreover, in most of the experiments, when the toxicant solutions were prepared and when they interacted with the trypsin reaction mixture, the solution absorbance increased considerably, preventing the performance of accurate assays. However, certain adjustments to the procedure, such as decreasing the amount of the enzyme in the reaction mixture and/or increasing the time of enzyme incubation in the toxicant solution, enhanced the trypsin sensitivity to some of the toxicants.

Nevertheless, despite the measures taken to increase the sensitivity of the trypsin-based chemoenzymatic assay to the toxicants, the study showed that the trypsin activity was inhibited by some of the large number of tested substances only when their concentrations in the solutions were very high—considerably greater than their maximum allowable concentrations (MACs). Of all the tested heavy metals, the trypsin activity was slightly affected by $CuCl_2$, which inhibited it, and $MnCl_2$ and $CrCl_3$, which stimulated its activity. We assume that the distinctions between the effects caused by the four salts of the heavy metals originated from the properties of cations. This is for two reasons. Firstly, the content of chloride anion in the samples with the highest salt concentrations was in the narrow range of 0.9–1.2 mM, but the effects differed significantly. Secondly, a recent study of trypsin

inhibition by anions [32] demonstrated that the effect of chloride ions on this enzyme was rather weak (at a 1-M concentration, it suppressed trypsin activity by 25%).

Among the pesticides, the metribuzin and fenoxaprop-P-ethyl herbicides produced insignificant inhibitory effects on the trypsin activity, but their IC_{20} values (9 mg/L) were considerably greater than their MACs for water bodies—0.1 and 0.0003 mg/L, respectively. The glyphosate herbicide substantially inhibited the trypsin activity. However, again, the IC_{20} and IC_{50} values for the glyphosate—0.5 and 1 g/L—were much higher than its MAC in water bodies (0.02 mg/L). These results indicate that the trypsin-based chemoenzymatic assay is not sensitive to different types of pesticide. This method can only detect very high levels of pesticides in samples. Similar results were obtained in the experiments with the quinones. The trypsin activity was insignificantly inhibited by the benzoquinone at concentrations that were higher than its MAC by a factor of 160.

The study of the trypsin fluorescence in the presence of different concentrations of pesticide formulations indicated that protein tertiary structure did not change and the components of the formulations did not interact with the tryptophan residues of the trypsin. The same results were obtained for the salts $CrCl_3$, $MnCl_2$, and $CuCl_2$.

Of particular interest was the study of the effects of the food preservatives on the trypsin activity. Food preservatives are not toxic substances, and they are commonly used to prevent the spoilage of food during storage. Trypsin was found to be highly sensitive to potassium sorbate: the IC_{50} value was 15 mg/L, which was noticeably lower than the ADI established for the potassium sorbate—300 mg/L. In the presence of this additive, the quenching of the trypsin fluorescence was detected with a high bimolecular-quenching constant, which indicates the binding of the sorbate with the trypsin in the area near the tryptophan and the formation of a non-fluorescent complex. In a previous study, the strong interaction of potassium benzoate with trypsin was revealed by a number of spectroscopic techniques [20]; however, this food preservative did not substantially change trypsin's activity in either that study [20] or our current experiments.

Thus, the present study demonstrates that the trypsin-based chemoenzymatic assay is a promising tool to estimate the amounts of the food preservative potassium sorbate in food samples in order to prevent its excessive intake, which may cause health problems.

To use trypsin in chemoenzymatic assays, it is necessary to prepare its stable form. Trypsin immobilization has been carried out using a wide range of methods, such as covalent binding on magnetic nanoparticles [33] and surface adsorption using silicon dioxide [34], chitosan [35], alginate [36], and other carriers. The immobilization of enzymes usually results in a decrease in their activity [37–39]. For example, the activity of covalent immobilized trypsin onto poly[(methyl methacrylate)-co-(ethyl acrylate)-co-(acrylic acid)] latex particles was 51.2% of the free trypsin activity [39]. A number of studies showed, however, that immobilized proteolytic enzymes, such as trypsin, were even more effective than their free forms [40–43]. For instance, the catalytic activity of trypsin immobilized on cyclodextrin-modified gold nanospheres was 100–120% [40]. Other studies reported that compared with the free enzyme, immobilized trypsin exhibited greater resistance against thermal inactivation and denaturants and showed good durability for multiple recycling [32,33]. The potential of immobilized trypsin for use in large-scale proteomics studies and practical applications has been shown [33,34].

Starch gel is a promising carrier for trypsin immobilization. Indeed, natural starch, which is a renewable, biocompatible, biodegradable, and relatively inexpensive material, is a convenient biopolymer for biomedical and pharmaceutical applications [44,45]. Starch is composed of two main components, amylose and amylopectin, and their ratio is determined by the starch source and pretreatment [46]. Corn, rice, wheat, and potato starches contain about 70–80% of amylopectin and 20–30% of amylose. Starch granules are not soluble in cold water, but in large amounts of water at temperatures of >40 °C, they are gelatinized. Starches extracted from different sources have different gelatinization temperatures. Potato starch has the lowest gelatinization temperature (55–65 °C), making it convenient for immobilizing enzymes. The process of immobilizing enzymes into starch gel is very simple:

starch gel is mixed with the enzyme solution, pipetted, and dried. Starch gel was previously used as a carrier to prepare highly active reagents based on butyrylcholinesterase [47] and a bioluminescence enzyme system of luminescent bacteria [48], which exhibited a longer storage time and stability under varying physical and chemical environmental conditions (temperature, pH, and the ionic strengths of solutions) [44,49,50].

Despite the advantages of the integration of enzymes into polymeric matrices, trypsin has not been immobilized into starch gel until now. Based on our previous studies [50], here, we attempted to immobilize trypsin into starch gel and, at the same time, preserve its activity and sensitivity to toxicants. In the current study, we prepared single-use dry starch disks with trypsin to simplify the enzyme-inhibition assay. The trypsin was immobilized into the 3% starch gel, which was convenient to pipette and enabled the preparation of mechanically strong, dry, single-use reagents.

The sensitivity of trypsin immobilized in starch gel differs depending on the time it spends in contact with the toxicant solution. During its incubation in the solution, the starch disk with the immobilized trypsin swelled, and, probably due to the quickening of the diffusion processes, the enzyme became more accessible to the inhibitor and the substrate. Thus, by varying the incubation time, it is possible to control the sensitivity of chemosensors based on trypsin immobilized in starch gel to toxicants. The sensitivity of the immobilized trypsin to $CuCl_2$, potassium sorbate, and $CrCl_3$ was comparable to the sensitivity of the free trypsin.

To conclude, we must note that certain difficulties may be encountered when chemosensors are used to analyze real environmental/food samples rather than laboratory samples, as accurate interpretations of results cannot be achieved without taking into account the properties and effects of natural components of the tested media [31,51].

5. Conclusions

It is unlikely that chemoenzymatic assays based on the measurement of trypsin activity will be used to detect toxicants such as metals (copper, chromium, manganese, zinc, cobalt, cadmium, nickel, arsenic, mercury, and aluminum), pesticides (fenvalerate, deltamethrin, cypermethrin, imidacloprid, glyphosate, metribuzin, tebuconazole, epoxiconazole, and fenoxaprop-p-ethyl), or quinones (benzoquinone and toluquinone) in environmental or food samples. These toxicants can produce inhibitory/stimulatory effects on trypsin activity only if their concentrations in tested samples are considerably higher than their MACs. However, chemoenzymatic assays with trypsin may be used to assess the safety of food additives. The present study demonstrated a substantial inhibitory effect of the food preservative potassium sorbate on trypsin activity, which was probably associated with the binding of this additive to the enzyme at the active center. The trypsin immobilized in the single-use dry starch disks retained its high activity during storage and showed sensitivity to toxicants similar to that of the free enzyme. Thus, the present study demonstrates that trypsin immobilized in starch gel can potentially be used to design a chemosensor for assessing the safety of food additives.

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