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The effect of immobilized chitinase enzyme on the biocontrol of *Sitophilus zeamais*

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Abstract: Microbial enzymes produced using biological processes are of great importance due to their contribution to sustainable solutions for agricultural production. In our previous study, the chitinase enzyme from *Lactobacillus coryniformis* was purified, immobilized to zinc oxide (ZnO) nanoparticles, and its effectiveness as a bioinsecticide against the storage pest *Sitophilus zeamais* Motschulsky, 1855 (Coleoptera: Curculionidae) was determined by forming a nanoformulation. In this study, the effectiveness of immobilized chitinase (nanoformulation), free chitinase, malathion, ZnO, and *L. coryniformis* on intestinal enzymes such as alkaline phosphatase (ALP), adenosine triphosphatase (ATPase), and lactate dehydrogenase (LDH) were tested to elucidate some of the mechanism of insect death. The effects of the applications on the activity of ALP, ATPase, and LDH enzymes were found to be statistically significant ($p < 0.01$). The lowest activity in the ALP enzyme was measured in Malathion and 8 mg/L applications. While the lowest activity in the ATPase enzyme was measured in the 8 mg/L application, the lowest activity in the LDH enzyme was observed in the malathion and 6 mg/L applications. Applications caused a decrease in enzyme activities (LDH, ALP, and ATPase). This situation affected the intestinal activities negatively, disrupted the feeding physiology of the insect, and caused its death. Obtained results showed that the formulation created may have an important role in the protection of corn from this pest.

Key words: Alkaline phosphatase, adenosine triphosphatase, lactate dehydrogenase, *Sitophilus zeamais*

1. Introduction

Maize weevil, *Sitophilus zeamais* Motschulsky, 1855 (Coleoptera: Curculionidae), is one of the most destructive pests of stored grains (de Araújo Ribeiro et al., 2020; de Oliveira et al., 2020). The infestation of the pest begins in the field, but most of the damage occurs during storage (Danho et al., 2002). The insect causes weight losses of up to 80% in stored maize (Langsi et al., 2020). The pest not only causes great losses due to grain consumption but also causes increases in temperature and humidity, which facilitates the growth of molds that include toxigenic species (Bernal and Medina, 2018; Brito et al., 2019). The damages caused by the pest are of great concern for food safety (Danso et al., 2017). Chemical insecticides are widely used in the control of storage pests. This also applies to *S. zeamais*. The control strategies for *S. zeamais* mainly involve the use of traditional insecticides such as pyrethroids, malathion, and fenitrothion, which can be dangerous to humans, animals, plants, and other nontarget organisms (Vásquez-Castro et al., 2012). The use of synthetic chemicals in the management of pests has failed due to the selection of resistant individuals, the creation of resistant populations, and environmental pollution (Bravo et al., 2011; Zhang et al., 2015). As a result, an intense effort has been made to find alternative control methods. Botanical insecticides, microorganisms with insecticidal activity, and the hydrolytic enzymes they release (chitinases, proteases, lipases, and glucanases) can be accepted as highly effective, safe, and ecological solutions (Berini et al., 2019) Chitinase, one of the enzymes secreted by microorganisms, can degrade chitin found in the body walls, peritrophic matrices, salivary glands, trachea, eggshells and muscle attachment points of insects (Rathore and Gupta, 2015; Abulikemu et al., 2021). The degradation of chitin by certain microorganisms and chitinolytic enzymes cause serious harm and even death in pathogens and pests that contain the polymer on their exterior surfaces. In this context, chitinolytic microorganisms and enzymes play an important role in the control of postharvest pests and many other biotechnological applications as biocontrol agents and pathogen antagonists in agriculture (Hartl et al., 2012; Nagpure et al., 2014; Veliz et al., 2017).

The investigation of the action mechanism of an insecticide helps to determine where and how a chemical

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substance will affect, the situations where physical and chemical events deviate from the normal, in short, the real causes of death. Importance should be attached to entomological, physiological, and biochemical studies in this regard. The conscious and supplementary aspects of biological control will be better understood as the studies on insect physiology advance and especially the hidden aspects of biochemical events are clarified.

The insecticides used in the control of pests cause multiple sublethal effects on enzymatic activity (Singh and Marwaha, 2000). The activities of tissue-specific enzymes are used to diagnose damage to specific tissues and organs resulting from chemical toxicity. Due to their roles in various functions in the insect body, LDH, ATPase, and ALP are used in the determination of the effects of biological materials and chemicals that are used in scientific studies on the intestinal enzymes of insects (Khosravi and Sendi, 2013; Kalaivani et al., 2018; Hamadah, 2019; Demirci and Güngördü, 2020). ALP (E.C.3.1.3.1) are hydrolytic enzymes that hydrolyze phosphomonoesters under alkaline conditions. ALP is responsible for the cytolysis of tissues during insect growth (Mishra et al., 2020). Insect ALPs show tissue-specific distributions with membrane transport such as intestinal epithelial cells, hemolymph, and salivary glands (Han et al., 2021). The midgut has a higher ALP activity than other tissues (Zibaee and Bandani, 2010).

LDH (EC 1.1.1.27) is a tetrameric protein that catalyzes the reversible conversion of pyruvate to lactate (Farhana and Lappin, 2021). Dehydrogenases are important tools in the investigation of the metabolic activities of insects during growth. The relative activities of insect dehydrogenases may be related to the functions and energy-giving demands of tissues (Sh, 2010). LDH is a glycolytic enzyme that can be found in almost all tissues. It plays a role in carbohydrate metabolism and is an indicator of exposure to chemical stress (Farhana and Lappin, 2021). LDH is also a widely used parameter to diagnose cell, tissue, and organ damage in toxicology and clinical chemistry. ATPases are a group of enzymes that catalyze the hydrolysis of a phosphate bond in adenosine triphosphate (ATP) to form adenosine diphosphate (ADP). ATPases are essential enzymes in all known life forms and have essential roles in energy conservation, active transport, and pH homeostasis (Rule et al., 2016).

In this study we conducted to elucidate a part of the mechanism responsible for the death of the corn storage pest *S. zeamais*; it was aimed to evaluate the effects of different doses of immobilized chitinase nanoformulation (2, 4, 6, 8, 10 mg/L), chitinase, malathion, ZnO Nps, and *L. coryniformis* on the metabolic enzymes (ALP, LDH, and ATPase) of *S. zeamais*, which were determined to cause the death of the insect in the previous study.

2. Materials and methods

This study was carried out in DAYTAM (Eastern Anatolia High Technology Application and Research Center) laboratories at Atatürk University in 2021.

2.1. Purification and immobilization of ZnO nanoparticles of chitinase and determination of the effects of chitinase on *S. zeamais*

The purification of chitinase from *L. coryniformis*, immobilization of chitinase on ZnO nanoparticles, characterization of chitinase, determination of the application doses to insects, and application to *S. zeamais* were carried out in a previous study. In practice, 5 different nanoformulation doses (2, 4, 6, 8, and 10 mg/mL) were created and applied to *S. zeamais* with 3 replications by spraying method (Dikbaş et al., 2021).

2.2. Extraction of insect intestinal tissues

The digestive systems of the treated insects (three repetitions of each application) were dissected under the microscope (Leica EZ4) with the help of a fine-tipped needle and the activity of three enzymes was determined from intestinal samples (Nathan et al., 2008).

2.3. Determination of the activity of alkaline phosphatase

The intestinal extract in a sodium phosphate buffer (50 µL; 300 µg protein) was mixed with 450 µL 0.05 M Tris-HCl pH 8.0 (for ALP activity). Then, 500 uL 12.5 mM ρ-nitrophenyl phosphate that was prepared in either acetate or a Tris buffer was added to the mixtures. After a 15-min incubation in a water bath at 37 °C, 100 uL 0.5 M sodium hydroxide was added. Then, the mixtures were centrifuged (4000 g; 5 min) and the enzymatic reaction was stopped. Finally, the absorbances of the supernatants at 410 nm were recorded (Nathan et al., 2004).

2.4. Determination of the activity of lactate dehydrogenase

The intestines were homogenized in a citrate-phosphate buffer (pH 6.8) that was cooled to 4 °C with ice for three min using a tissue grinder. The homogenized intestinal sections were suspended in a cold buffer and diluted to 1 mL. The homogenate was centrifuged at 15 min at 500 rpm and the supernatant was used as an enzyme source.

To standardize the volumes, a 0.2-mL NAD + solution was added to the test tubes, and 0.2 mL water and 0.01 mL solution were added to the control test tubes that contained 1 mL of the buffered substrate. The samples were incubated at 37 °C for exactly 15 min. Then, 1 mL color reactive (2,4-dinitrophenyl hydrazine reactive) was added to each tube and captured in the tubes and incubation continued for another 15 min. The contents were cooled to room temperature and 10 mL 0.4 N NaOH was added to each tube to make a strongly alkaline solution to maximize hydrazine formation. Color density was measured at 440 nm exactly 60 s after the addition of alkali to each tube (King, 1965; Nathan et al., 2006). After the determination

of enzymatic activity, the presence of inhibition was controlled by comparison to the control sample (untreated insect).

2.5. Determination of the activity of adenosine triphosphatase

The insect intestines were homogenized using a homogenizer and 1.0 mL 0.1 M Tris-HCl buffer (pH 7.5). Then, the contents were centrifuged at 4 °C for 15 min at 1000 rpm, and the clear supernatant was used to determine the activity of sodium-potassium-ATPase. A total of 0.3 mL Tri-HCl buffed (pH 7.5), 0.1 mL 0.02 M ATP, 0.1 mL 100 mM NaCl, and 0.1 mL KCl were transferred to the test tubes and 0.1 mL distilled water and 0.1 mL tissue extract were added to the blind sample. The reaction mixture was incubated in a water bath at 37 °C for 15 min and the reaction was stopped using 2.00 mL 5% TCA. The tubes were kept at 4 °C for 30 min and centrifuged for 5 min at 500 rpm. A total of 1 mL ammonium molybdate and 0.4 mL ANSA were added to the supernatant and the mixture was kept at room temperature for 10 min. The intensity of the blue color was measured against the reactive at 680 nm (Fiske and Subbarow, 1925; Nathan, 2005a)

2.6. Statistical analysis

The treatments were examined with one-way variance (ANOVA) analysis and the differences between the mean values were determined using Duncan's multiple comparison tests at the 5% probability level.

3. Result

In a previous study (Dikbaş et al., 2021), chitinase with a molecular mass of ~33 kDa was purified from *L. coryniformis* using the HiTrap Capto DEAE column and its optimum pH and temperature values were determined to be 6.0 and 65–75 °C, respectively. The immobilization of the enzyme on ZnO nanoparticles was achieved and the enzyme was characterized using the FTIR, XRD, SEM, and AFM methods. Five different nanoformulation (ZnONps+ chitinase) doses were obtained (2, 4, 6, 8, and 10 mg/L) and applied to maize grains to allow the intake of the formulation by the insect (*S. zeamais*) during feeding. Considering the mortality rate (100%) and mean death time (2.4 days), the best dose was determined to be 6 mg/L.

To determine the effect of the applications on the intestinal enzymes of the insect, the intestines of the insects were dissected under the microscope and the changes in the activity of three enzymes were determined from the intestinal samples (Figure 1).

The effects of the applications on ALP, ATPase, and LDH activities were highly significant ($p < 0.01$) (Table 1, Figure 2). ALP, ATPase, and LDH activity in this study varied ranging from 5.05–10.59 U/L; 30.83–92.33 U/L; and 19.06–49.21 U/L, respectively. The lowest ALP activity was obtained from the 8 mg/mL administration and the activity was reduced by 49% compared to the control. Compared to the control, the lowest ATPase activity was obtained from 8 mg/mL administration and the activity decreased by 33%. The lowest LDH activity was obtained from the administration of 6 mg/mL and the activity decreased by 39% compared to the control.

Table 2 shows whether there are significant relationships between mortality rate, mean death time, ATPase, LDH, and ALP and the direction of the relationships. The correlation test was used to determine whether there was a relationship between two continuous variables. There was a strong negative (–0.98) and significant correlation between time of death and mean death rate ($p < 0.05$). As the death rate increases, the time of death decreases. When the effects of time of death and death rate on enzymatic activity were examined, there was a positive (0.71) and significant relationship between ATPase and

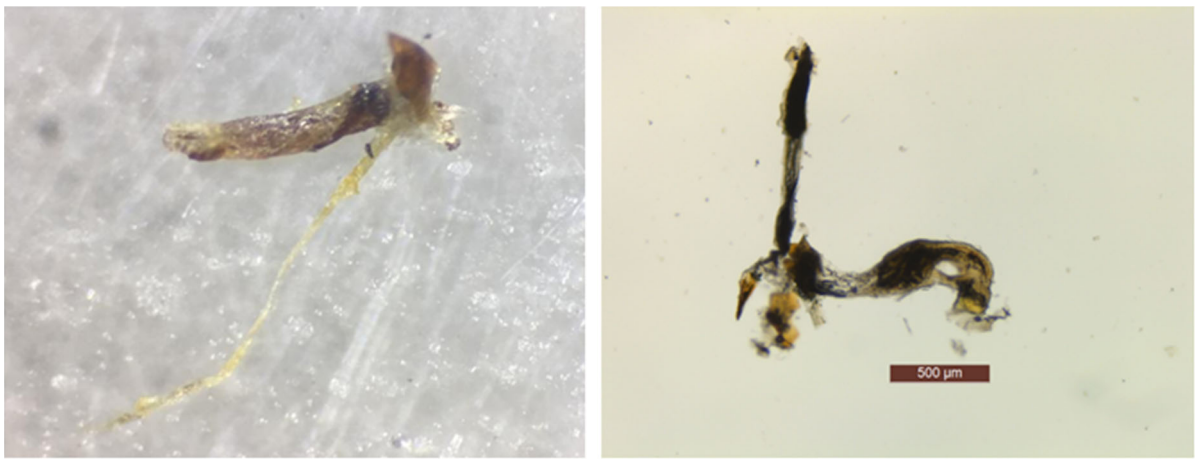

Figure 1. Microscopic images of the insect gut.

Table 1. Enzymatic activities concerning the applications[1].

Application	ALP (U/L)	ATPase (U/L)	LDH (U/L)
2 mg/L	7.71^{b} (±0.1)	50.28^{gh} (±0.5)	38.01^{b} (±2.06)
4 mg/L	6.27^{c} (±0.1)	51.51^{fg} (±0.6)	25.71^{cde} (±2.7)
6 mg/L	6.08^{c} (±0.1)	46.59^{h} (±0.5)	19.06^{e} (±1.2)
8 mg/L	5.18^{ef} (±0.1)	30.83^{i} (±0.5)	29.26^{cd} (±3.8)
10 mg/L	6.19^{c} (±0)	55.33^{f} (±1.9)	26.16^{cde} (±5.6)
Enzyme	5.59^{de} (±0.1)	70.96^{c} (±1.5)	20.84^{de} (±1.6)
Bacteria	6.00^{cd} (±0.1)	65.70^{d} (±1.5)	25.71^{cde} (±1.9)
Water	7.87^{b} (±0)	84.10^{b} (±2.6)	32.36^{bc} (±1.3)
ZnO	5.51^{ef} (±0.1)	60.44^{e} (±1.5)	26.60^{cde} (±1.5)
Malathion	5.05^{f} (±0.1)	45.55^{h} (±2)	18.62^{e} (±1.5)
Control	10.59^{a} (±0.5)	92.33^{a}(±1.6)	49.21^{a} (±1.5)
Total	6.55	59.42	28.32
F value (application)	103.78**	129.45**	11.77**
Variation coefficient (%)	4.21	4.58	15.98

[1]: The differences between the mean values with the same letter in the same column are not significant at the 5% probability level. **: Highly significant. Mean ± standard deviation.

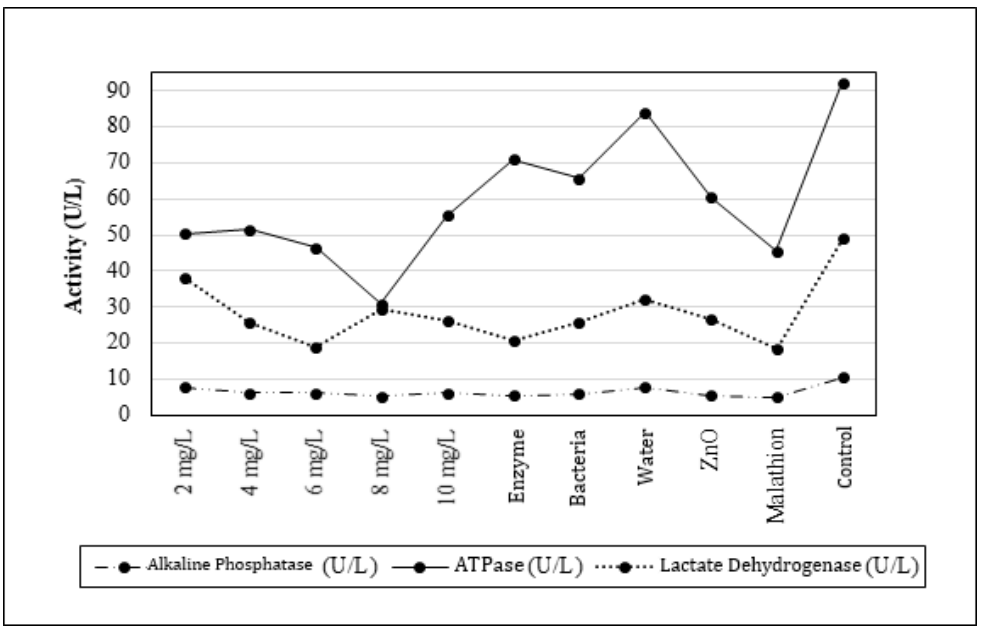

Figure 2. Plot showing the relationships between the enzymes and applications.

time of death ($p < 0.05$). There was a strong negative (–0.78) and significant relationship between ATPase and mortality ($p < 0.05$), and ATPase activity decreased with increasing mortality. There was a positive (0.726) significant correlation between ALP and LDH ($p < 0.05$), and LDH activity increased with increasing ALP activity.

4. Discussion

The use and promotion of biopesticides in agriculture will greatly help reduce the use of toxic pesticides. The use of such nontoxic and biodegradable biopesticides in pest management will prevent harmful chemicals and protect the crop.

Table 2. Results of the correlation test.

	ALP	ATPase	LDH	Mortality rate
ATPase	0.48			
LDH	0.73*	0.12		
Mortality rate	–0.60	–0.078**	–0.33	
Mean death time	0.63	0.71*	0.36	–0.98**

* $p < 0.05$ (2-tailed) ** $p < 0.01$ (2-tailed).

Chitinase production has been a criterion in the selection of biocontrol agents for use against insects. Various studies on the applicability of chitinase and ZnO nanoparticles as bioinsecticides have been carried out and yielded positive results (Suganthi et al., 2017; Athanassiou et al., 2018; Silva et al., 2018; Rafiq et al., 2018; Mansour et al., 2019; Jameel et al., 2020; Laribi-Habchi et al., 2020; Mahmood et al., 2020).

The changes in metabolism are very important in understanding insect deaths. Therefore, in our study; it has been demonstrated that exogenous applications of ZnONps-immobilized chitinase to maize reduce intestinal enzyme activities (ALP, LDH, and ATPase) of *S. zeamais* compared to control, impair pest health, and reduce survival time.

ALP plays a role in the transphosphorylation reaction (Millán, 2006). ATPases are necessary for the transport of glucose, amino acids, and organic molecules (Sengottayan, 2013). LDH is an important glycolytic enzyme found in almost all animal tissues (Kaplan and Pesce, 1996). It also plays a role in carbohydrate metabolism and is an indicator of chemical stress. LDH is a common parameter in toxicology and clinical chemistry for identifying damage to cells, tissues, and organs (Sengottayan, 2013). Any disruption to the activities of these enzymes can affect the physiology of insect intestines.

When compared with the control, the lowest ALP activity was obtained with the malathion, 8 mg/L, and ZnO applications and the differences between the applications were not statistically significant ($p > 0.05$). However, the difference between the malathion application and other applications was significant ($p < 0.05$). Nathan et al. (2004) reported that botanical insecticides and bacterial toxins cause weight loss in the larvae of *Cnaphalocrocis medinalis* (Guenée) (Lepidoptera: Crambidae), a rice pest, and significantly reduced the activities of ACP, ALP, and ATPase. The decrease in activity seen in the related study supports our findings.

The lowest ATPase activity was obtained in the 8 mg/L application. The difference between the application and other applications was significant ($p < 0.05$). Following the 8 mg/L application, the lowest activities were obtained with the malathion, 6 mg/L, and 2 mg/L applications in ascending order, but the differences between the applications were not statistically significant. Kalaivani et al. (2018) reported that methyl salicylate (MeSA) affected the growth and development of *C. medinalis* and reduced the activities of intestinal enzymes (LDH, ACP, ALP, and ATPase). Nathan et al. (2004) observed the changes in the activities of acid phosphatases (ACP), ALP, and ATPases in *C. medinalis* after treatment with neem extracts. The researchers concluded that changing the physiological balance of the midgut can affect enzymatic activity. In their study, the decrease in the activity of these enzymes after treatment with neem extract indicates that these materials affect intestinal physiological events (i.e. ion transport) that may affect these enzymes.

ATPases are necessary for the transport of glucose, amino acids, etc. Researchers have attributed the differences in ATPase activity levels after treatment with neem extract against *C. medinalis* to the role of membrane lipids and the microenvironmental changes in the lipids at the physical and chemical levels. Membrane ATPase helps the transport of metabolites and nutrients, especially in the intestinal epithelium, and secondary transport of ions and nonelectrolytes (Lechleitner and Phillips, 1988; Fogg et al., 1991). Babu et al. (1996) showed that the ATPase activity in the intestines of *Helicoverpa armigera* significantly decreased as a result of the toxic effects of azadirachtin. In another study, the researchers have shown that ATPase inhibition can cause changes in the regulation of electrolytes by affecting active ion transport and a decrease in enzymatic activity after treatment with neem extract indicated that the insect metabolism was disturbed (Nathan et al., 2005a). The findings obtained in previous studies show parallelism with our study.

When compared with the control, the lowest LDH activity was obtained in the malathion and 6 mg/L applications, followed by the enzyme, 4 mg/L, bacteria, 10 mg/L, and ZnO applications in ascending order. The differences between these applications were not statistically significant ($p > 0.05$). On the other hand, the difference

between the malathion and 6 mg/L applications and other applications was significant ($p < 0.05$). Following the applications of the enzyme, 4 mg/L, bacteria, 10 mg/L, and ZnO, the lowest enzymatic activity was observed in the 8mg/L application and the differences between the applications were not statistically significant, while there was a significant difference between the enzyme and 8mg/L application. In their study, Khosravi and Sendi (2013) reported that LDH activity of *Glyphodes pyloalis* larvae decreased with the treatment of neem extract. Nathan et al. (2006) examined the effects of bacterial toxins (*Bacillus thuringiensis*) and insecticides of plant origin (*Azadirachta indica* and *Vitex negundo*) on the LDH activity of *C. medinalis*. The combined effects of the three biopesticides significantly reduced the enzymatic activity and showed strong enzyme inhibition. Nathan et al. (2005b) tested the effects of neem (*Azadirachta indica*) limonoids, azadirachtin, salannin, deacetylgedunin, gedunin, 17-hidroksyazadiradion, and deacetylnimbin, on the LDH activity of the larvae of *C. medinalis*, and observed a decrease in enzymatic activity at all concentrations when compared with the control. The decrease in the LDH activity after the treatment with the neem limonoids indicates that the insect metabolism was disturbed, which can be attributed to the toxic effects of neem derivatives, especially on the membrane permeability of the intestinal epithelium (Zibaee et al., 2008). Considering the biopesticide studies, the decrease in intestinal enzyme activities is consistent with the findings obtained in our applications. The decrease in enzyme activities is thought to be a partial cause of insect deaths by causing deterioration in the intestinal structure.

There was a strongly negative and significant relationship between death time and mean mortality rate. In other words, mean death time decreased with an increasing mortality rate ($r > 0.5$ and $p < 0.01$). The effects of death time and mortality rate on enzymatic activity showed that there was a positive relationship between LDH and death time (LDH activity decreased as the mortality rate increased) and a negative relationship between LDH and mortality rate (the activity of LDH decreased wiincreaseding mortality rate). However, the relationships were not significantly correlated. There was a positive and significant relationship between ATPase and death time ($p < 0.05$) and the activity of ATPase increased with increasing death time. There was a strongly negative and significant relationship between ATPase and mortality rate ($r > 0.5$ and $p < 0.01$) and the activity of ATPase decreased with increased mortality rate. There was a positive relationship between ATPase and LDH, but the relationship was not significantly correlated (the activity of LDH increased in the same direction as the increase in the activity of ATPase). There was a positive relationship between ALP and death time (the activity of ALP increased with increasing death time) and there was a negative relationship between ALP activity and mortality rate (the activity of ALP decreased with increased mortality rate). However, the relationships were not significantly correlated. There was a significant relationship between ALP and LDH ($p < 0.05$) and the activity of LDH increased with increasing ALP activity. There was a positive relationship between ALP and ATPase, but the relationship was not significantly correlated.

The effectiveness of the biological enzyme chitinase immobilized on ZnO nanoparticles on intestinal enzymes of *S. zeamais* was evaluated. The activities of enzymes varied depending on the dose of the application, and as a result of the previous study, it was determined that the dose with the highest efficiency (6 mg/L) had a reducing effect on intestinal enzyme activities.

The results of this study show that there is a possible interaction between a biological enzyme chitinase immobilized on ZnO nanoparticles and intestinal enzymes and that the immobilized enzyme plays an important role in biocontrol against *S. zeamais*. The results obtained will provide natural options that can work within biological pesticide programs in insect control and integrated pest management as a biopesticide. The use of a biological enzyme immobilized on ZnO nanoparticles as a bioinsecticide may make it an important tool in pest management.

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Author contributions
ND and SU conceived the research. ND, SU, and GT conducted experiments. ND, TÖÖ, GT, and RK analyzed data. ND and SU wrote the manuscript. All authors read and approved the manuscript.

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Declaration of competing interest
The authors declare that they have no conflict of interest.

Ethical approval
This research did not involve any studies with human participants or vertebrate research subjects.

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