

Detection of *Rhyzopertha dominica* larvae in stored wheat using ELISA: The impact of myosin degradation following fumigation [☆]

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Abstract

Hard red winter wheat kernels were infested with eggs of *Rhyzopertha dominica*. After 20 d, when the larvae reached the fourth instar, they were killed by exposing the infested kernels to phosphine gas for 24 h. The infested kernels were then divided into four portions and treated as follows: one portion was immediately frozen at -80°C to avoid myosin degradation; the other three portions were kept at 32°C and 65% relative humidity, and then frozen at -80°C after 14, 28, and 56 d post-fumigation, respectively. Each treatment was replicated five times. Myosin was measured using a commercial enzyme linked immunosorbent assay (ELISA) method that specifically detects this protein (Biotect[®], Austin, TX). Myosin degradation was most rapid in the first 2 weeks after the larvae were killed, decreasing from 1.672 to 0.695 ng/well during this period (a 58.4% reduction). There were no significant differences in myosin degradation between samples that were 14, 28, and 56 d post-fumigation. Grain is often fumigated to control insects. Frequently, this occurs many weeks before the grain is milled and may be repeated during the storage period. Therefore, estimates using the ELISA test may underestimate internal insect infestation because of myosin degradation. Insect fragment estimates for previously fumigated grain could be underestimated by as much as 58%.

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1. Introduction

The presence of insects or insect fragments in grain is a major problem for grain millers. Because several of the insect species that attack stored grain develop inside the kernels, it is difficult to determine at the time of purchase if the grain is infested. The immature stages of internal-feeding insect species are essentially invisible to grain inspectors. The grain industry has tried to develop methods to detect internal infestations in raw grain, such as X-ray (Schatzki and Fine, 1988); however, X-ray is not routinely used because of the expense and large amount of time

needed to process and develop X-ray film. Recently, near-infrared spectroscopy (NIRS) has been investigated (Maghirang et al., 2003; Perez-Mendoza et al., 2004). However, NIRS currently fails to achieve the very low level of insect fragment detection required by industry.

In the USA, the Food and Drug Administration (FDA) has set a defect action level of insect contamination of 75 insect fragments per 50 g of wheat flour (FDA, 1988). The insect fragment count has been used as the standard procedure for food analysis for a long time; however, it requires technical training, it is time consuming, and relatively expensive (Kitto et al., 1992). This test evaluates the number of insect fragments without regard to their size, and therefore is not a good measure of the mass of insect material present in the food product. Insect fragment count is highly variable and is dependent on the species, life-stage, and whether the insect is alive or dead at the time the grain is milled (Harris et al., 1952). Insect fragments found

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in food come from the exoskeleton of live or dead insects, particularly sclerotized body parts from larvae or adults living inside the grain kernels. Dead insects yield many more fragments than do live ones, and dead adult insects can contribute up to 50 times as many fragments as dead larvae (Sachdeva, 1978).

Tests to verify insect contamination in stored products should be able to detect minute quantities of insect material within the milled food product. The high selectivity of immunological assays makes them well suited to this purpose (Kitto et al., 1992). The insect muscle protein myosin is uniformly distributed in insects, and is present in large quantities in both adult and immature insect muscle (Quinn et al., 1992). Schatzki et al. (1993) found that the enzyme linked immunosorbent assay (ELISA) test for myosin was a sensitive method for detecting hidden insects in wheat. Myosin has been found in many immature insects and changes in its concentration are related to the developmental stage of the insects. An increase in myosin was noted during the larval stage, followed by a drop in the pupal stage, and a subsequent increase in adult insects was reported by Dorsey and Lockshin (1983) and Schatzki et al. (1993). In naturally occurring populations of insects in stored grain there is normally a mixture of all life stages present. Schatzki et al. (1993) originated the idea of using an “average insect unit” for the immunoassay procedure, taking into account the proportions of each stage of *Sitophilus granarius* (L.) likely to be found in stored grain and the myosin concentration per stage.

A biochemical assay was developed based on the ELISA test to detect and quantify the presence of myosin in insect muscle fragments attached to the exoskeleton (Quinn et al., 1992; Schatzki et al., 1993; Kitto et al., 1996). The method is applicable to a wide variety of stored products at a relatively low cost. The ELISA test employs polyclonal antisera that cross-react extensively among species of grain insects so that total insect infestation of grain or grain products can be quantitatively determined. The assay is based on a colorimetric reaction that provides a consistent response in evaluating the presence of insects. It is highly specific, rapid, and costs less than the insect fragment count test (Quinn et al., 1992; Schatzki et al., 1993; Belay et al., 1997).

Some studies have indicated that myosin may be unstable under certain conditions (Tanikawa et al., 1987). Dorsey and Lockshin (1983) reported that the intersegmental muscles of *Manduca sexta* (L.) (Lepidoptera: Sphingidae), degenerate 48 h after the emergence of the adult. They suggested that one or more proteases initiate the myosin degradation. Because the purpose of the ELISA myosin test is to use myosin as an indicator of the amount of insect contamination in the flour, it is important to know if myosin degrades after insect death, and if so, the rate of degradation. If myosin does degrade over time, then insect density and the expected number of insect fragments in the flour could be underestimated by the ELISA test. The objective of this study was to determine the effects of the duration of post-fumigation period on the ability of a

commercial ELISA test for myosin to quantify the number of wheat kernels infested with larvae of *Rhyzopertha dominica* (F.).

2. Materials and methods

2.1. Infestation of wheat kernels

Rhyzopertha dominica were obtained from cultures maintained at the USDA-ARS Grain Marketing and Production Research Center in Manhattan, Kansas. *Rhyzopertha dominica* eggs were obtained from the culture by placing 400 adults on 200 g of wheat, which was sieved 16 h later to obtain freshly laid eggs. A dissecting needle was used to make a hole in individual wheat kernels and a single egg was placed inside the hole. We cultured enough infested kernels so that we would have at least 40 infested kernels by the time the larvae reached the fourth instar (2 kernels per sample \times 4 storage durations \times 5 replications). The infested wheat kernels were placed into wells of a micro-well plate (one kernel per well), which was kept at 32 °C and 65% relative humidity (r.h.) until the larvae reached the fourth instar. The infested kernels were evaluated daily to look for the presence of frass, which indicated viable larvae inside the grain kernels. To verify the presence of fourth instars, wheat kernels were X-rayed 17 and 19 d post-oviposition. The micro-well plate, containing the infested kernels, was placed on a sheet of film (Kodak Industrex M X-ray film, Eastman Kodak, Rochester, NY) 56 cm below an X-ray source (Model 43855A Faxitron, Hewlett-Packard, McMinnville, OR) and exposed for 3 min at 18 kV and 3 mA. The processed X-ray films were then examined under a stereomicroscope at 12 \times magnification to verify the presence of a fourth-instar larva inside each kernel (Dowell et al., 1998).

After 20 d, when the larvae reached the fourth-instar, they were killed by exposing the wheat kernels to 79 ppm of phosphine gas for 24 h. This phosphine concentration was selected because preliminary experiments showed that this concentration caused 100% mortality to the larvae. The infested kernels were then divided into four portions and treated as follows: one portion was immediately frozen at -80 °C (to prevent myosin degradation), the other three portions of infested kernels were kept at 32 °C and 65% r.h. and aged for 14, 28, and 56 d after the fumigation event. No adults emerged from any of the kernels, so it was assumed that the fumigation killed the larvae inside the kernels. After ageing, the infested kernels were held at -80 °C until they were analysed to prevent myosin degradation.

2.2. Sample preparation

Myosin was measured using a commercial ELISA method that detects the insect muscle protein myosin (Biotect[®], Austin, TX). Uninfested hard red winter wheat kept at -4 °C was cleaned using a Hart-Carter Dockage

Tester (Model XT2; Simon-Carter Co., Minneapolis, MN). For each sample preparation, a 12.5 g portion of the cleaned grain was placed into a Waring blender, using a mc-2 mini container (Waring, Torrington, CT). Two infested wheat kernels that were aged 1, 14, 28, or 56 d post-fumigation were added to each sample. We used two infested kernels per 12.5 g of uninfested wheat to ensure that the test would detect fourth-instar larvae, taking into consideration the possibility of myosin degradation over time. Each wheat sample with the two infested kernels was ground for 15 s at low speed and then another 45 s at high speed. Twenty-five ml of cold buffer was then added to the sample and blended for 2 min at high speed. A 1.5 ml sample was pipetted into a microcentrifuge tube, and cooled on ice for 5 min. The sample was then centrifuged at 14,000 rpm for 10 min or until a clear supernatant was obtained. Six hundred μ l of the supernatant was retained and stored at 4 °C until analysis. This procedure was replicated five times for each storage duration.

Standards were prepared using the Biotect[®] kit directions. Serial dilutions were prepared using the buffer solution and the insect myosin standard included in the kit. The standards were prepared so that the standard insect equivalents ranged from 0.5 to 2.0 insects per 50 g of wheat.

2.3. ELISA

The standards, blanks, and infested grain samples were run simultaneously and in duplicate. We followed the Biotect[®] kit directions, which consisted of washing the plate four times, adding 50 μ l of the samples or standards, and incubating for 18 min at room temperature. The plate was then washed four times with the buffer solution after which 50 μ l of the buffer plus AB complex solution was added to each of the wells. The plate was incubated for 12 min at room temperature and then washed four times with the buffer solution. We added 50 μ l of buffer plus enzyme complex solution to each of the wells and the plate was incubated for 8 min. The plate was then washed four times and 100 μ l of the substrate solution was added to each well. The plate was covered and incubated for 5 min at room temperature after which 100 μ l of the stop solution was added to each well. The absorbance at 405 nm was measured for each well using a plate reader (EL 340, BIO-TEK Instruments Inc., Winooski, VT).

Myosin concentration data were subjected to one-way ANOVA (SAS Institute, 1988) to determine the effects of post-fumigation kernel ageing on insect myosin degradation. Means were compared using Fisher's Protected LSD test (SAS Institute, 1988). All treatment comparisons were considered significant at the $\alpha = 0.05$ level.

3. Results and discussion

Myosin concentration decreased from 1.672 to 0.695 ng/well (58.4% reduction) at 14 d post-fumigation (Fig. 1).

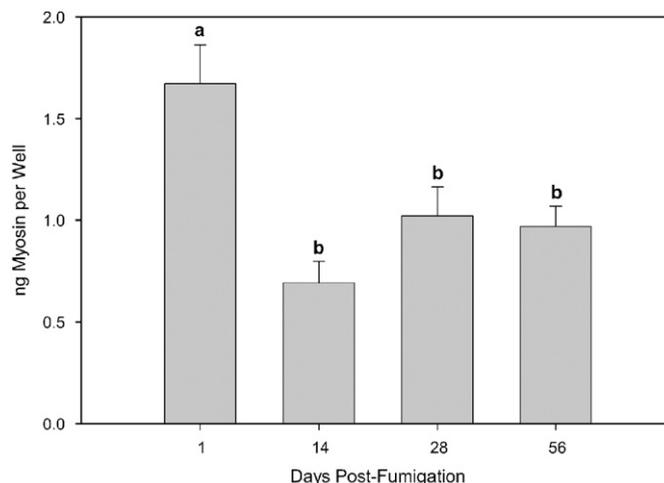


Fig. 1. Myosin degradation in wheat infested with *Rhyzopertha dominica* larvae, after 1, 14, 28, and 56 d post-fumigation. Vertical bars indicate standard error of the mean. Bars designated by the same letter were not significantly different ($P > 0.05$, least significant difference Test).

There were no significant differences in myosin concentration for kernels that were aged for 14, 28, and 56 d post-fumigation ($P > 0.05$). Thus, most of the myosin degradation occurred within 2 weeks when the larva died inside the wheat kernel. Comparative studies of myosin from various species of insects showed rapid inactivation of ATPase activity of insect myosin when stored in 0.6 M KCl at 0 °C, showing the instability of this protein when stored in a buffered solution (Tanikawa et al., 1987).

Myosin is a good indicator of insect infestation because it is less variable than insect fragment count in grains and byproducts, which depends on how many fragments the insect breaks up into during milling, the level of training of the analyst, and other factors (Brader et al., 2002). However, our data showed that myosin degraded during storage after the larvae were killed by fumigation. Thus, the ELISA myosin test may underestimate the number of insects that were present in the grain prior to fumigation.

Brader et al. (2002) compared four methods of analysing grain infestation: ELISA, insect fragment count, the flotation method and X-ray. The results showed that ELISA was the most efficient, followed by X-ray. Insect fragment count was shown to be the least reliable of the methods. However, Brader et al. (2002) did not investigate the effects of myosin degradation following death of the larvae inside the kernels, which could affect the reliability of the ELISA method. Schatzki et al. (1993) also investigated the ELISA method for stored grain infestation. They reported “no substantial difference” between kernels maintained at –25 °C and those kept at room temperature for 8–22 d before testing. Results from our study indicate that after 14 d post-fumigation, myosin had degraded by 58.4% for samples kept at 32 °C compared to samples kept at –80 °C. Differences between the Schatzki et al. (1993) results and ours may be due to the fact that their insects were killed by freezing and ours by phosphine

fumigation. In addition, their samples were kept at room temperature, while ours were aged at 32 °C, which is closer to grain storage temperatures during the summer. Phosphine fumigation is normally used to control insect infestations in stored grain, so the degradation detected in our study probably represents a more realistic measurement of myosin degradation that occurs after grain fumigation.

Although we do not know the rate of myosin degradation between 1 and 14 d, the fact that there was 58% degradation after only 2 weeks is important regarding the efficacy of this test under field conditions. In the field, grain that has been fumigated at different times will often be mixed and therefore will contribute to the variability of the ELISA method. Even though, under field conditions, the ELISA test for insect myosin may not be as accurate as originally proposed by Brader et al. (2002), it is still probably less variable in quantifying insect contamination than the standard insect fragment count method, because of the highly variable number of fragments each insect will contribute to the flour.

In the USA, wheat flour samples are labelled as sample grade when the number of insect fragments exceeds 75 in 50 g of flour (US Food and Drug Administration, 1988). Flourmills tend to reject wheat when radiographs indicate more than 5–6 insects per 50 g of kernels. Immunoassays are able to detect the majority of stored-product pests and the results are proportional to the weight of the insect (Quinn et al., 1992; Brader et al., 2002). Previous studies Schatzki et al. (1993) and Brader et al. (2002) have shown that ELISA can detect as few as 2 *S. granarius* larvae in 50 g of wheat flour, which is well under the previously mentioned FDA thresholds. Based on the results of our study, it appears that the amount of degradation is greatly reduced after 2 weeks. Therefore, even if the grain was fumigated 1–2 months before the test, the ELISA method should still detect at least 3–4 larvae per 50 g of wheat flour. Insect density estimates for previously fumigated grain could be underestimated by as much as 58% of the original insect density. Based on the results of this study, it is advisable to immediately test, or to refrigerate flour samples, as soon as possible after milling to increase the reliability of the ELISA method.

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