Effect of pea flour and pea flour extracts on *Sitophilus oryzae*

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Abstract—Protein-rich pea flour is an antifeedant and a repellent and is toxic to the rice weevil, *Sitophilus oryzae* (L.), but its mode of action is not known. Results showed that protein-rich pea flour had no fumigant effect on adult survival or offspring production of *S. oryzae*. In a contact experiment, immobilized weevils were fed every other day and had their abdomens brushed with protein-rich pea flour or wheat flour on the alternate days. Insects treated with protein-rich pea flour had an average longevity of 9.6 days, which was significantly shorter than that for insects treated with wheat flour (11.3 days) or brushed controls (17.6 days). These results suggest that toxins from the protein-rich pea flour may be able to penetrate the insect cuticle. Midguts from weevils fed protein-rich pea flour, a pea flour extract, or a mixture of pea peptides contained numerous bubbles. Midgut tissues in these treated adults were injured, as shown by dual staining with the fluorescent dyes calcein AM and propidium iodide. The volume of the bubbles increased rapidly when insects were fed protein-rich pea flour or pea flour extract. There were no bubbles found in the midguts of *S. oryzae* that fed on wheat kernels or wheat flour.

Résumé—La farine de pois riche en protéine est un antiappétant et est toxique à la charançon du riz, *Sitophilus oryzae* (L.), mais on ne connaît pas le mode d'action. Les résultats ont montré que la farine de pois riche en protéine n'agissait pas comme fumigant ni sur les adultes, ni sur la production de la progéniture avec *S. oryzae*. Dans une expérience où la farine de pois riche en protéine était mise en contact avec les insectes, ils étaient immobilisés, et nourris aux 2 jours. Les jours alternés, les abdomens des insectes étaient brossés, soit avec de la farine de pois riche en protéine ou avec de la farine de blé. Les insectes traités avec la farine de pois riche en protéine avaient une durée de vie moyenne de 9,6 jours; moins que les insectes traités avec la farine de blé (11,3 jours), ou les témoins (17,6 jours). Ces données suggèrent que les toxines de la farine de pois riche en protéine peuvent franchir la cuticule de l'insecte. L'intestin moyen des charançons avait plusieurs bulles de gaz quand les insectes étaient nourris avec de la farine de pois riche en protéine, un extrait de farine de pois, ou un mélange de peptides en provenance des pois. Les tissus d'intestin moyen dans ces adultes étaient endommagés, démontré par les colorants fluorescents à double action, calcéine AM et iodure propidium. Le volume des bulles augmentait rapidement quand les insectes étaient nourris à la farine de pois riche en protéine ou à l'extrait de pois. Il n'y avait pas de bulles dans les intestins moyens de *S. oryzae* nourri aux graines de blé ou à la farine de blé.

Introduction

Highly evolved plants are an important source of novel insecticides (Prakash and Rao 1997). Numerous plant species or extracts from plants have insecticidal properties (Jacobson 1989; Golob *et al.* 1999; Weaver and Subramanyam 2000). They affect insects through repellency, contact toxicity, or fumigation (Dev and Koul 1997; Shaaya *et al.* 1997; Golob *et al.* 1999). Legume seeds contain a wide range of allelochemicals with toxic and deterrent effects against insect pests (Harborne *et al.* 1971; Bell 1977).

Yellow split pea (*Pisum sativum* L.) (Fabaceae) mixed with wheat (*Triticum aestivum* L.) (Poaceae) reduces the survival and reproduction of *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae), one of the major pests of stored cereals (Coombs *et al.* 1977; Holloway 1986). Protein-rich pea flour made

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Received 15 February 2005. Accepted 25 November 2005.

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from yellow split pea has negative effects on stored-grain insect pests (Bodnaryk *et al.* 1999; Delobel *et al.* 1999; Fields *et al.* 2001; Hou and Fields 2003*a*, 2003*b*; Hou *et al.* 2004*a*, 2004*b*, 2004*c*). It is repellent (Fields *et al.* 2001) and toxic (Hou and Fields 2003*b*), reduces the reproduction of many stored-product insect pests (Bodnaryk *et al.* 1999), and is compatible with parasitoids (Hou *et al.* 2004*a*) used as biological control agents. Since pea flour is used as an animal feed and human food, it is less likely to have negative effects on mammals. Bodnaryk *et al.* (1999) produced an extract with 80% methanol from protein-rich pea flour that is 20–100 times more toxic than protein-rich pea flour itself. Taylor *et al.* (2004*a*) isolated an insecticidal mixture of pea peptides from this methanol extract. However, the mode of action of the protein-rich pea flour and the pea flour extracts has not been determined. Like synthetic insecticides, botanical insecticides can enter an insect in three ways: through ingestion, through absorption across the cuticle, and through the spirucular openings and respiratory system. The purpose of this study was to determine whether the gut of *S. oryzae* is affected by protein-rich pea flour and the pea flour extracts and to test whether the toxins in peas enter insects as a gas or through the cuticle of adult weevils.

Materials and methods

Sitophilus oryzae, from a culture maintained in the laboratory for over 5 years, were reared on whole kernels of wheat at 30 °C, 70% RH. Protein-rich pea flour (Progress Protein; 54% protein, 30% starch, and 7% moisture; Parrheim Foods, Saskatoon, Saskatchewan) was used in this study. It is produced commercially by grinding peas and isolating a protein-rich fraction by air classification. Pea flour extract was obtained from protein-rich pea flour by the method described in Hou *et al.* (2004*b*). A mixture of pea peptides was isolated by anion exchange chromatography as described by Taylor *et al.* (2004*a*).

Fumigant toxicity

Wheat (20 g; moisture content 14%) was placed in a small cloth bag (10 cm × 10 cm) with thirty 1- to 2-week-old adults of *S. oryzae*. A petri dish containing 2 g of protein-rich pea flour was placed at the bottom of a 1 L jar. The cloth bag with wheat and insects was hung 5 cm above the petri dish and the jar lid was sealed with tape. After 1 week, the adults were removed and the numbers of live and dead insects were determined. The fumigated wheat was held separately in 10 cm × 3.3 cm vials at 30 °C, 70% RH, for 5 weeks without additional exposure to protein-rich pea flour, and the number of emerged adults was noted. Survivors of the fumigation were placed on fresh wheat and allowed to lay eggs for 1 week. The wheat was cultured for 5 weeks before counting the number of emerged adults. Controls included wheat with no protein-rich pea flour. There were five replicates per treatment.

Contact toxicity

Seven-day-old adults of *S. oryzae* were chilled at 4 °C for 10 min to reduce their activity and then glued (LePage® 5-Minute Epoxy, Henkel Canada Corp., Brampton, Ontario) with their ventral surface up in a petri dish. The insects' legs were glued to their thorax to prevent the legs from moving material placed on the abdomen to the mouth. Weevils were fed or treated on alternate days to reduce the stress due to starvation. On treatment days, the insect abdomen was brushed three times with a small, soft brush dipped in one of the powders, so that the abdomen was covered with a thin layer of dry protein-rich pea flour or wheat flour. To feed the insects every other day, wheat flour was added to the petri dish to a depth that covered the mouth parts. All insects were thoroughly cleaned with a micro-vacuum and blower between each feeding or treatment. Insects were kept at 25 °C and 70% RH. The numbers of dead and live insects were noted every 24 h for 15 days. There were four treatments: (1) brushing with protein-rich pea flour and feeding on alternate days, (2) brushing with wheat flour and feeding on alternate days, (3) brushing with no flour and feeding on alternate days (brushed controls), and (4) no brushing, with feeding every day (unbrushed controls). Each treatment had three replicates with 20 insects per replicate.

Midgut and vital staining

Twenty 7-day-old *S. oryzae* adults were held at 30 °C and 70% RH on one of seven treatment diets: no food, 2 g of wheat kernels, 2 g of wheat flour, 2 g of protein-rich pea flour, five disks made from 0.2 g of wheat flour (Xie *et al.* 1996), five disks made from 0.2 g of wheat flour mixed with 0.8 mg of pea flour extract, or five disks made from 0.2 g of wheat flour mixed with 0.8 mg of pea peptides. The numbers of dead and live insects were noted every 24 h for 10 days. There were three replicates for each treatment. Other groups of insects treated as above were dissected in physiological saline at 4 °C (Maddrell 1969) every day for 3 days. For the dissection, two pairs of fine forceps were used; the head of the insect was snapped off, the first two segments of the thorax were broken away, and the abdomen was opened up dorsally. After this, the intact midgut was pulled from the insect. A pretest showed that the isolated guts of *S. oryzae* still moved after 19 h in the saline solution at room temperature. The dissections of guts of three insects per treatment were videotaped through a dissecting microscope, and the length and width of each gas bubble produced in the midgut were measured on the television screen with a calibrated micro-ruler.

Viability of insect tissue was determined by dual fluorescent staining, a method commonly used to detect the viability of cells in insects (Collins and Donoghue 1999), bacteria (Bunthof *et al.* 2001), and plants (Haugland 2002). Three guts per treatment were stained immediately after dissection. Guts were placed on glass slides for 5 min and stained with 25 μL of calcein AM (20 μmol/L) and 25 μL of propidium iodide (15 μmol/L) (Molecular Probes Inc., Eugene, Oregon) diluted from stock solutions with physiological saline just before use. The calcein AM stock solution was prepared by dissolving 20 μg of calcein AM in 500 μL of anhydrous dimethylsulfoxide on the day of use. The propidium iodide stock solution was prepared by dissolving 1 mg of propidium iodide in 1 mL of deionized water. Stock solutions were stored at 4 °C and protected from light.

Stained guts in saline were covered with cover slips raised by four fine copper wires. A fluorescence microscope (Leica DMRB, St. Gallen, Switzerland) with a red–blue filter was used to observe and photograph the guts at a magnification of 100×.

Data analysis

To normalize the data and to stabilize the variance, the number of insects produced and the midgut bubble volumes were transformed with $\log(x + 1)$ and the mortality of insects (percentage) was transformed with the arcsine of the square root of $x + 1$. Means are presented with standard errors. SAS/STAT® PROC GLM (general linear models procedure) with the GT2 option (SAS Institute Inc. 2000) was used to compare the effects of treatments ($\alpha = 0.05$). LT_{50} values (time to the death of 50% of the insects) were calculated using Kaplan–Meier survival analysis and the Gehan–Breslow test, and pairwise comparisons were made using the Holm–Sidak method (SigmaStat®, SPSS Inc. 2003; Daly 2000).

Most of the bubbles formed in the midguts were spherical; therefore, the volume of each bubble was calculated using the formula $\pi d^3/6$, where d is the average of the width and the length of the bubble. The total gas volume in each midgut was then derived from the sum of the volumes of all bubbles in the midgut. The differences in gas volume among treatments were compared with Tukey's multiple range test in SAS/STAT® PROC GLM.

Results

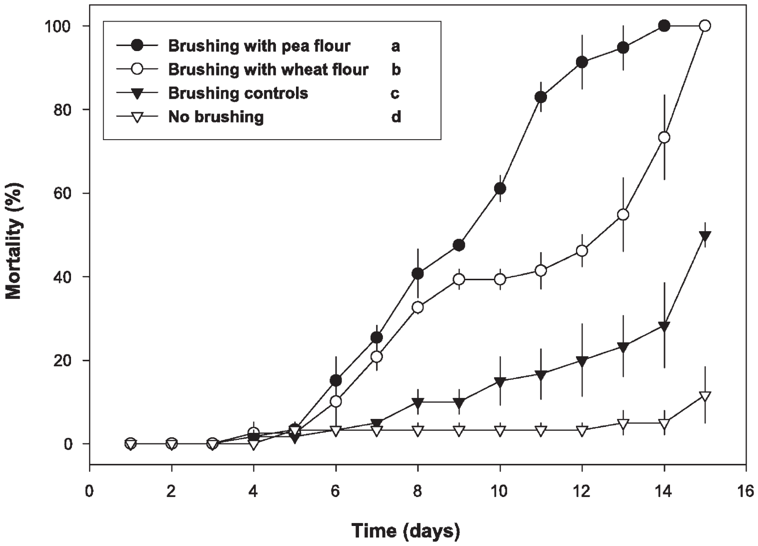
Fumigant toxicity

A 7-day fumigation with protein-rich pea flour caused no mortality in *S. oryzae* adults. No difference in percent mortality ($F_{1,8} = 2.69$; $P = 0.14$) or number of emerged adults ($F_{1,8} = 0.50$; $P = 0.50$) was detected between protein-rich pea flour (mortality, 10% ± 0.5%; emerged adults, 257 ± 18) and the control (12% ± 1%; 241 ± 13). There was no significant difference in percent mortality ($F_{1,8} = 2.28$; $P = 0.17$) or number of emerged adults ($F_{1,8} = 0.18$; $P = 0.68$) produced in fresh wheat by fumigated *S. oryzae* adults between protein-rich pea flour (mortality, 15% ± 2%; emerged adults, 204 ± 28) and the control (10% ± 2%; 189 ± 31) ($P > 0.05$).

Contact toxicity

The mortality of glued, unbrushed controls, which were provided with food daily, was less than 15% after 15 days (Fig. 1). The LT_{50} of *S. oryzae* treated with protein-rich pea flour (10.0 days; 95% confidence interval (CI), 9.0–10.0 days) was significantly less than that of *S. oryzae* treated with wheat flour (13.0 days; 95% CI, 10.9–15.1 days) ($P < 0.05$), which was less than that of the brushed controls (15.0 days; 95% CI not available) ($\chi^2_3 = 168.27$, $P < 0.01$). Percent mortality was significantly different among all treatments (Fig. 1; Kaplan–Meier

Fig. 1. Cumulative percent mortality (mean ± SE) of *Sitophilus oryzae* glued on petri dishes and brushed with protein-rich pea flour, brushed with wheat flour, brushed with no flour (controls), or not brushed. Wheat flour was provided as food for 24 h every second day in the brushing treatments and continuously in the no-brushing treatment. There were three replicates, with 20 insects per replicate. Treatments followed by different letters are significantly different (Kaplan–Meier survival analysis, Gehan–Breslow test, Holm–Sidak multiple comparison method, df = 3, $P = 0.05$).



survival analysis, Gehan–Breslow test, Holm–Sidak multiple comparison method, df = 3, $P = 0.05$.)

Midgut and vital staining

No bubbles were observed in the midguts of *S. oryzae* fed wheat kernels (Fig. 2A), wheat flour, or wheat flour disks. However, bubbles were produced in the midguts of *S. oryzae* fed protein-rich pea flour (Fig. 2B) or wheat flour disks treated with pea flour extract or pea peptides, as well as midguts of starved insects. Less fat tissue was observed on the anterior midguts of *S. oryzae* that fed on protein-rich pea flour than on the anterior midguts of insects that fed on wheat kernels (Figs. 2A, 2B). The midguts of insects fed with the various pea treatments became transparent because of the distention caused by large bubbles (Fig. 2B). Over the three sample days, the volume of gas bubbles in *S. oryzae* fed pea peptides (5.4 mm^3 ± 0.7 mm^3), pea flour extract (8.9 mm^3 ± 3.1 mm^3), or protein-rich pea flour (8.1 mm^3 ± 3.7 mm^3) was larger than the gas volume in starved insects (0.8 mm^3 ± 0.4 mm^3) ($F_{3,24} = 10.68$; $P = 0.001$). The volume of the gas increased rapidly after 2 days of treatment with pea flour extract or protein-rich pea flour (Fig. 3). On the third day, the gas volume in the pea flour treatment was significantly higher than that in the pea peptide or starvation treatment ($F_{3,8} = 10.92$; $P = 0.003$). Dissections to measure the size of the bubble in the midgut became impossible after 3 days because the guts were too fragile to handle without breaking.

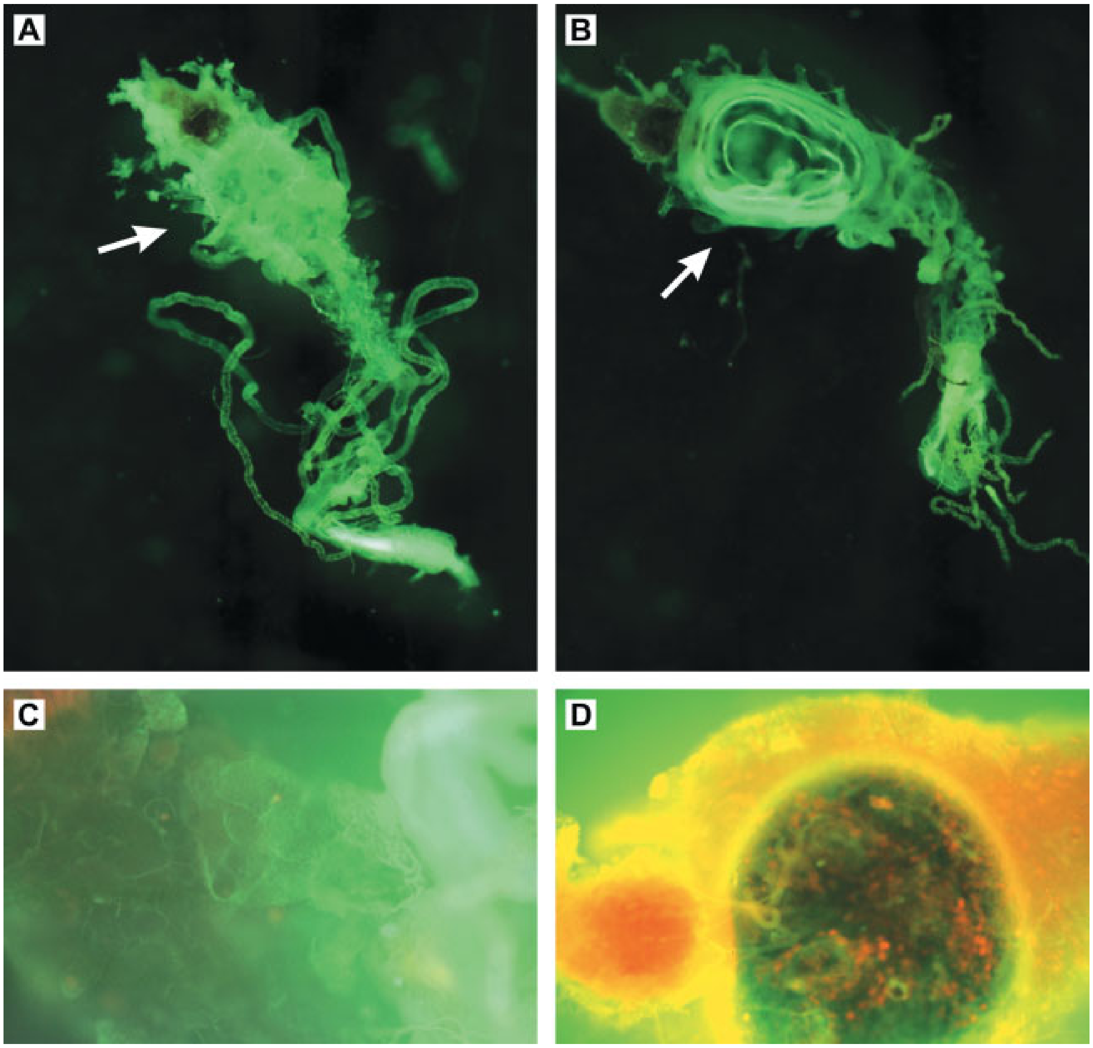
The LT$_{50}$ of *S. oryzae* held on only protein-rich pea flour (3.0 days; 95% CI, 2.8–3.2 days) was significantly less than that of starved *S. oryzae* (4.0 days; 95% CI, 3.7–4.3 days) and insects that fed on wheat flour disks treated with pea flour extract (6.0 days; 95% CI, 5.7–6.3 days) or pea peptides (6.0 days; 95% CI, 5.6–6.4 days) ($\chi^2_3 = 166.73$; $P < 0.001$) (Fig. 4). There was no mortality of insects held on wheat kernels within 10 days.

There were more red spots, indicating dead tissue (Haugland 2002), in the midguts of *S. oryzae* that fed on protein-rich pea flour (Fig. 2D), pea flour extract, or pea peptides than in the midguts of those that fed on wheat kernels (Fig. 2C).

Discussion

Chemicals enter an insect's body by three means: through the respiratory system, through

Fig. 2. Isolated midguts of *Sitophilus oryzae* adults. Insects were fed wheat kernels (A, C) or protein-rich pea flour (B, D). Gas bubbles formed in the midgut (indicated by arrows) only in the protein-rich pea flour treatment (A, B). The midgut was stained with calcein AM and propidium iodide and viewed under a fluorescence microscope (C, D); dead tissue fluoresces red and live tissue fluoresces green.



the cuticle, or through the digestive system. We examined the response of *S. oryzae* to pea flour and its extract as a fumigant, a contact insecticide, or a stomach poison.

Many plants and their extracts produce volatile chemicals that act through insects' respiratory systems as fumigants (Shaaya *et al.* 1997). There are many compounds in protein-rich pea flour, and some of them are volatile, as protein-rich pea flour has a distinct odor. However, protein-rich pea flour had no fumigant toxicity to *S. oryzae*.

Wheat flour and protein-rich pea flour brushed on the abdomens of *S. oryzae* increased the mortality of the weevils, although the significant mortality in the brushed control indicates that brushing and starvation (*i.e.*, feeding every other day) may have also contributed to the mortality. The higher mortality of *S. oryzae* brushed with protein-rich pea flour than of those brushed with wheat flour suggests that toxins from protein-rich pea flour may have penetrated the cuticle and reached the site of action within the insect body. We suspect that

Fig. 3. Changes over time in the volume (mean ± SE, mm^3) of bubbles present in the midgut of *Sitophilus oryzae* given no food or fed 100% protein-rich pea flour, wheat flour disks with 0.4% pea flour extract, or wheat flour disks with 0.4% pea peptides. Treatments followed by different letters are significantly different (Tukey's multiple range test, $F_{3,24} = 10.68$; $P = 0.0001$). No bubbles were found in insects feeding on wheat flour.

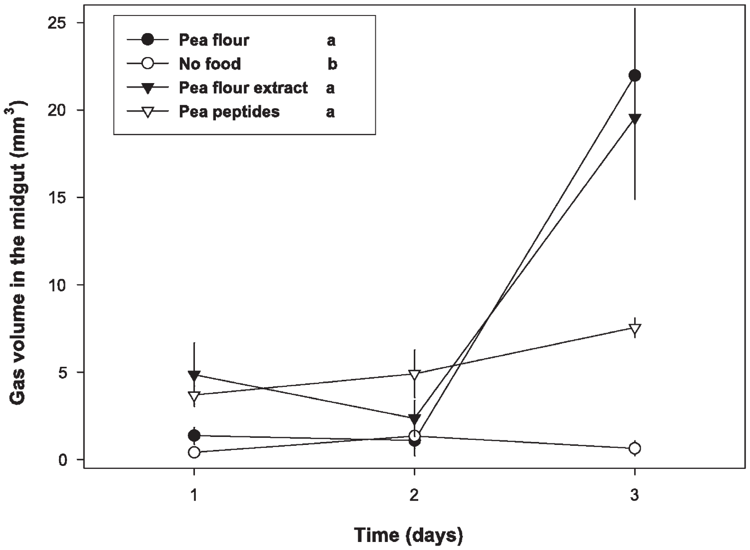
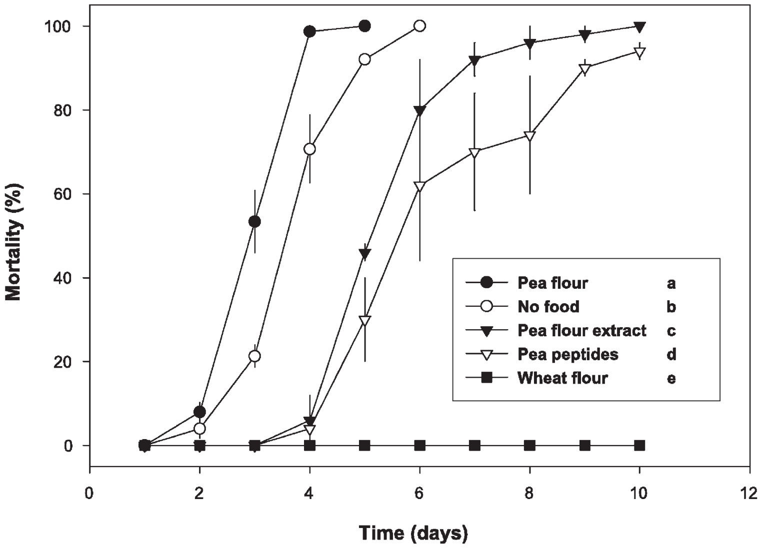


Fig. 4. Percent mortality (mean ± SE) of *Sitophilus oryzae* over time when given no food or fed 100% protein-rich pea flour, wheat flour disks with 0.4% pea flour extract, wheat flour disks with 0.4% pea peptides, or wheat flour. Treatments followed by different letters are significantly different (Kaplan–Meier survival analysis, Gehan–Breslow test, Holm–Sidak multiple comparison method, df = 3, $P = 0.05$).



the increased mortality in the wheat flour and protein-rich pea flour treatments was due to one or more of the following three possibilities. First, flour on the abdomen may be an irritant that causes increased movement, thus causing the insects to starve more quickly. This may explain the higher mortality observed among insects brushed with wheat flour than among the brushed controls. Second, when the flours were brushed onto the abdomens every other day, there could have been damage to the insect cuticle. Finally, the toxins from the protein-rich pea flour may have entered the insects across the cuticle, through sites of damage, or via the respiratory system.

Feeding on protein-rich pea flour, pea flour extract, or pea peptides increased the gas volume in *S. oryzae* midguts. One way in which feeding is regulated is through the stretch receptors on the gut wall, which measure the distention of the gut and inhibit feeding when the gut is full (Bernays and Simpson 1982). It is possible that the bubble produced in the *S. oryzae* midgut triggered these receptors and thus caused the inhibition of feeding. Also, symbiotic bacteria in the midgut of young *S. oryzae* adults (Nardon and Grenier 1989) may act on the gut contents to produce gas after insects feed on pea flour.

Pea flour extract, pea peptides, and protein-rich pea flour caused tissue death in the midgut of *S. oryzae*. This tissue death might have been caused by the bubble stretching the midgut and causing mechanical damage to the cells. We observed that the midgut was too fragile for dissection after 4 days of treatment, approximately when the first insects died. Another possibility is that the pea peptides were directly toxic to the peritrophic membrane or the microvilli of the midgut epithelium cells, which is the case with the extracts of neem (*Azadirachta indica* Adr. Juss., Meliaceae) (Nogueira *et al.* 1997) and *Celastrus angulatus* Maxim. (Celastraceae) (Liu *et al.* 1998). Protein-rich pea flour has been shown to contain sulfur-rich, 37-amino-acid peptides of the pea albumin (PA1b) type (Higgins *et al.* 1986; Delobel *et al.* 1999; Taylor *et al.* 2004*b*). These insecticidal peptides, with a molecular mass of about 3800 Da, are much smaller than toxic proteins of *Bacillus thuringiensis* (Slaney *et al.* 1992) and could penetrate the midgut peritrophic matrix. It has been found that the PA1b peptides bind to target sites in the cell membrane (Gressent *et al.* 2003) and might affect ion channels (Jouvensal *et al.* 2003) in the membrane. Therefore, the dead tissue might also be the result of the interaction of pea peptides and the membrane of the midgut cells.

Pea flour extracts also contain various soyasaponins and lysolecithins (Taylor *et al.* 2004*c*). Because of their surface active properties, these natural products might enhance the absorption of toxic peptides into cell membranes, including those of the insect midgut. This may explain why the pea extracts caused larger gas bubbles in the midgut and were more toxic than the pea peptides without soyasaponins. Soyasaponins have previously been suggested as possible anti-insect factors of legume seeds (Applebaum *et al.* 1969; Su *et al.* 1972; Applebaum and Birk 1979).

Acknowledgements

We thank T. Mayert for technical assistance, S. Woods for statistical advice, T. Aung and M. Jordan for providing microscopes, E. Huebner for his advice, Parrheim Foods for providing the protein-rich pea flour, and the Agri-Food Research and Development Initiative and the University of Manitoba for providing financial support.

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