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Effect of Seed Proteinaceous Extracts from Two Wheat Cultivars against *Phthorimaea operculella* (Lepidoptera: Gelechiidae) Digestive α-amylase and Protease Activities

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ABSTRACT

The potato tuberworm, *Phthorimaea operculella* (Zeller) is a worldwide pest of solanaceous crops. Larvae feed inside the galleries in foliage, stems and tubers which makes chemical control unsuccessful, and other control methods should be applied. In recent years many plants have received genes that encoding toxic proteins as a strategy to resist against insect pests. *P. operculella* is a solanaceous-specific pest and it has no evolutionary background with cereals. Therefore the goal of current study was to investigate the effect of wheat (*Triticum aestivum* L. cv. MV17 and cv. Azar) seed proteinaceous extract against digestive α-amylase and protease activities of potato tuberworm and was also to determinate the optimal pH of these enzymes using 1% starch and 2% azocasein as substrate, respectively. The optimum pH of α-amylase and protease activities was found to be highly alkaline. Enzyme inhibition assays showed that amylase activity was significantly affected by extracts from MV17 and Azar at different pH values and maximum effect was observed at pH 9. The inhibitory effect of MV17 and Azar extracts on protease activity did not markedly vary between pH 8 and 11 and among pH 8, 9, and 11, respectively. Inhibition manner of various concentrations (1.5, 0.75, 0.375, 0.187, and 0.093 (mg protein/ml) of extracts were dose-dependent. Maximum inhibitory effect occurred at the highest concentration and the minimum was at the lowest concentration. In polyacrylamide gel assay, both enzymes, without inhibitors showed two isozymes. At highest concentration of extracts, both bands disappeared or their intensity decreased. So, these proteins can be tested in producing resistant potato crops against potato tuberworm.

Key words: α-amylase, protease, wheat, potato tuberworm.

INTRODUCTION

The potato tuberworm, is a universal pest of solanaceous crops and especially damages to potatoes. Although it is mainly a pest of potato, it can also be found in other solanaceous plants. It is one of the most important pests of potato in many temperate and tropical regions of the world. The larvae mine leaves, stems, and petioles causing irregular galleries and cave tunnels through tubers. Foliar damage to the potato crop usually does not result in considerable yield losses but infested tubers especially in

non-refrigerated systems may have reduced marketability. Several approaches are available for the development of an integrated pest management system for potato tuberworm. Since the larvae feed inside the tubers and they are not exposed to the insecticides, pesticide application in order to control this insect was not successful (Rondon, 2010). Wide use of pesticides has caused malefic effects to human health and environment and the occurrence of resistance in the phytophagous insect-pests against the pesticides has led to focus on enzymes inhibitors encoded in transgenic plants as an alternative strategy to control the insect pests, because insect pests rely on their digestive enzymes such as α-amylases and proteases to keep on feeding on host plants (Gatehouse *et al*., 1999; Franco *et al*., 2002). Cereals and legumes seeds are the rich sources of digestive enzyme inhibitors (Franco *et al.*, 2002). So, it is recommendable to characterize digestive enzymes as well as to do *in vitro* and *in vivo* bioassay with plant proteinaceous inhibitors in order to achieve a control strategy based on digestive enzyme inhibitors (Harrison and Bonning, 2010). So, the goal of current study was to investigate the effect of seed proteinaceous extracts from wheat cv. MV17 and cv. Azar on the amylolytic and proteolytic activities of the potato tuberworm.

MATERIAL AND METHODS

Rearing of insect

A population of potato tuberworm was taken from the insect physiology laboratory (University of Mohaghegh Ardabili) and maintained on potato tubers (*Solanum tuberosum* L. cv. Agria) located in plastic containers in the incubator set at 30±1˚C and 55% RH.

Preparation of enzyme extract

Insect enzyme extraction was done based on procedures described by Mehrabadi *et al.* (2012). Fifth instar larvae of potato tuberworm was used for enzyme extraction, because the most feeding occurs in this instar. The larvae were randomly selected, cold-immobilized on ice for 10 minutes and carefully dissected in distilled water under stereomicroscope (Nikon WD®). Guts were separated and grounded in 1.5 ml of phosphate buffer at pH 7 and homogenized with homogenizer (Ultra Turrax T8®). The 1.5 ml homogenates from preparations were centrifuged at 13,000 rpm for 30 min at 4˚C. The supernatants were transferred to a new tube and stored at -20˚C for further use as an enzyme source.

Determination of optimum pH of α-amylase and protease activitiy

The optimal pH of α-amylase and protease activities were determined using different pH values; 8, 9, 10, 11, and 12 of universal buffer (Hosseinkhani and Nemat-Gorgani, 2003) containing Glycin (0.02 M), 2-morpholinoethansulphonic acid (0.02 M) and succinate (0.02 M). To determine the optimal pH of α-amylase activity, 10 µl of enzyme extract was dissolved in 65 µl universal buffer at distinct pH, then 25 µl of 1% starch solution as substrate was added to the reaction and the mixture was

incubated at 40˚C for about 30 min. Then the reaction was stopped by adding 100 µl DNS (dinitrosalicilic acid) according to (Bernfeld, 1955) and heated in boiling water for 10 min. Then absorbance of reaction mixture was read at 540 nm by using ELISA reader. To determine the optimal pH of protease activity, 10 µl enzyme extract was incubated with 50µl of 2% azo-casein as substrate in 40 µl of distinct pH at 45˚C for about 60 min. According to Saadati *et al*. (2011), the reaction was stopped by adding 100 µl TCA (trichloroacetic acid) and kept in refrigerator at 4˚C for about 30 min, followed by centrifugation at 13,000 rpm for about 20 min to precipitate non-hydrolysed substrate. Finally the absorbance of reaction mixtures was measured at 405 nm.

Seed protein extraction procedure

Seeds of MV17 cultivar were supplied by Seed and Plant Improvement Institute, Karaj, Iran and seeds of Azar were obtained from Agricultural Jahad Organization of Saghez, Kurdistan, Iran. According to Baker (1987) and Melo *et al*. (1999), seeds were milled completely and then 30 grams of grinded seeds from each plant separately was mixed with 100 ml of 0.1M NaCl and stirred for 90 min, then the mixture was centrifuged at 8,000 rpm for about 30 min at 4˚C. The pellet was discarded and proteins were concentrated using a saturation of 70% ammonium sulfate followed by centrifugation the mixture at the same condition. The pellet was dissolved in ice-cold Tris-HCl buffer (0.02 M and pH 7.0) and dialyzed against the same buffer for about 20 h. Then this dialyzed solution was transferred to 1.5 ml tubes and placed at 70˚C for about 15 min in order to inactivate the enzymes within the seeds. Finally, the 1.5 ml tubes were centrifuged at the same condition and the supernatants were transferred to other tubes and they were frozen at -20˚C as inhibitor source for enzyme inhibition assays.

The effect of pH on inhibitory activity of seed extracts

In-vitro assay of the effect of different pH on inhibitory activity of seed extracts from MV17 and Azar on α-amylase and protease activities was assayed. At 55 µl of given pH value of universal buffer (8, 9, 10, 11, and 12), 10 µl enzyme extract was pre-incubated with 10 µl of each seed extracts solution at 40˚C for 15 min for amylase inhibition assay and for 60 min at 45˚C for inhibition assay of protease. Then 1% starch solution as α-amylase substrate and 2% azocasein as protease substrate were added to each enzyme mixture. Appropriate blanks were included in the experiments, too. The inhibition percentage of α-amylase and protease (%I) was calculated according to Mehrabadi *et al*. (2011):

$$\%I_{\alpha\text{-amylase}} = 100 * ((\Delta A540 \text{ Control} - \Delta A540 \text{ Experiment})/\Delta A540 \text{ Control})$$

$$\%I_{\text{protease}} = 100 * ((\Delta A405 \text{ Control} - \Delta A405 \text{ Experiment})/\Delta A405 \text{ Control})$$

The effect of different concentrations of seed extracts on enzymes activity

The effect of seed proteinaceous extracts on α-amylase and protease activities was determined as described by Mehrabadi *et al*. (2010). Various concentrations including 1.5, 0.75, 0.375, 0.187, and 0.093 mg/ml protein of seed extracts were prepared by diluting the most dens extract (1.5 mg/ml protein). Then 10 µl of enzyme extract at

difined pH (pH 9 for α-amylase and pH 11 for protease) was pre-incubated with each of above-mentioned concentrations. Thereafter, specific substrate of both enzymes was added to the mixtures and the continuation of assay was done as described in the previous section.

All assays were performed with three replicates using 20 samples per replicates.

Semi-denaturing native-page

Electrophoretic detection of amylolytic and proteolytic activity was done basically according to the procedures described by Laemmli (1970) and Walker *et al.* (1998). Amylolytic activity were detected using 10% (w/v) polyacrylamide gel co-polymerized with 0.5% starch according to Mehrabadi and Bandani (2010) and 4% for stacking gel with 10% SDS (Gel electrophoresis apparatus includes a gel cassette filled with polyacrylamide gel and placed in a tank filled with electrode buffer and an electrical field is applied via the power supply to the rear. The negative terminal is at the top end (cathode), so proteins migrate downword to the anode. Electrophoresis was conducted at a voltage of 70V at 4°C until the blue dye reached the bottom of the gel. Then, the gel was rinsed with distilled water and washed by 1% (v/v) Triton X-100 buffer for about 30 min followed by incubation in Tris-base buffer (pH 9.0) containing 2 mM $CaCl_2$ and 10 mM NaCl for about 2 h. Finally, the gel was treated with a solution of 1.3% I_2 and 3% KI to stop the reaction and stain the un-reacted starch background. Proteolytic activity was detected using 10% (w/v) polyacrylamide gel co-polymerized with 1% gelatin. Electrophoresis was conducted at a voltage of 70V at 4°C until the blue dye reached the bottom of the gel. Then, the gel was rinsed with distilled water and washed by 2.5% (v/v) Triton X-100 buffer for about 60 min followed by incubation in Tris-base buffer (pH 11) for about 24 h. Finally, the gel was treated with staining buffer as described by Hosseininaveh *et al.* (2007) containing 50% (v/v) methanol, 10% (v/v) acetic acid, and 0.25% (w/v) Coomassie blue R-250 to stain the un-reacted gelatin background for about 24h and finally was treated with destain buffer containing 10% (v/v) methanol and 5% (v/v) acetic acid for about 4 h.

Zones of α-amylase and protease activities appeared at light bands against dark background.

Protein determination

Protein concentration of enzyme extracted from insect gut and proteinaceous extract of seeds was measured according to the method of Bradford (1976), using bovine serum albumin (Bio-Rad, Munchen, Germany) as a standard.

Material supply

Azocasein, bovine serum albumin (BSA), succinic acid disodium salt, and ammonium persulfate for electrophoresis (APS) were supplied by Sigma (St Louis, MO, USA). Tris, phosphate buffer solution (pH 7.0), 2-hydroxy-3,5-dinitrosalicylic acid (DNS), potassium sodium tartrate tetrahydrate, starch soluble, trichloroacetic acid (TCA), sodium hydroxide, ammonium sulfate, acrylamide, N,N′-methylene

diacrylamide, dodecyl sulfate sodium salt (SDS), 2-morpholinoethanesulfonic acid (MES), sodium chloride, calcium chloride, phosphoric acid, glycerol, potassium iodide, iodine, coomassie brillant blue G 250, bromophenol blue, and N,N,N′,N′-tetramethyl ethylenediamine (Temed) were purchased from Merck (Darmstadt, Germany). Methanol was from Arman Sina (Tehran, I.R.I); glycin from Scharlau (Barcelona, Spain) and Triton X-100 from Applichem (GmbH in Darmstadt, Germany). Spectrophotometric measurements were made using ELISA reader, BioTek® (Winooski, VT), ELx800.

Statistical analysis

Data was analyzed using MSTAT-C software by Single factor ANOVA followed by mean comparison with Tukey's Honestly Significant Difference Test (HSD). The error bars in the figures represent SE (Standard Error).

RESULTS

Optimum pH of α-amylase and protease activity

Some features of α-amylase and protease enzymes in the potato tuberworm gut were determined in introductory experiments. We found that the optimal pH of α-amylase (Fig. 1a) and protease (Fig. 1b) was in the alkaline range with a peak at about pH 9 and 11, respectively. The α-amylase and protease activity level in optimal pH was 0.024319 and 0.130000 U/min/mg protein, respectively.

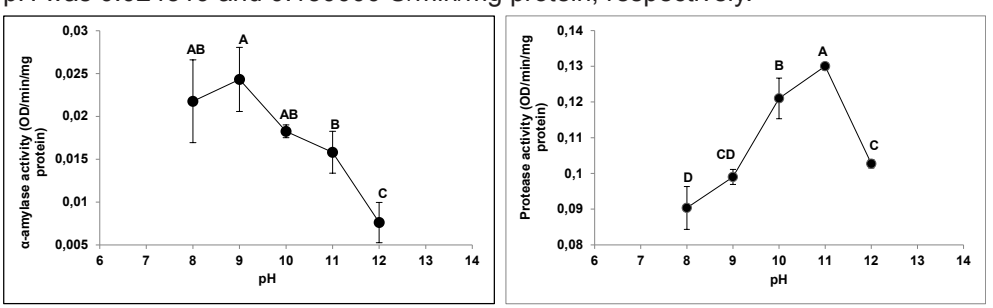

Fig. 1. The effect of pH on α-amylase (a) and protease activities (b) of potato tuberworm. Means followed by the same letters are not significantly different by Tukey's test ($P<0.05$).

The effect of pH on the inhibitory activity of seed proteinaceous extracts

Inhibitory activity of seed extracts on α-amylase and protease activities was evaluated in different pH values to study the importance of pH factor in insect midgut. The effect of extracts from MV17 and Azar on α-amylase differed at several pH values and the highest inhibition percentage of α-amylase by both extracts was observed at pH 9 (Fig. 2a). Whereas the inhibition of protease activity by extract from MV17 did not significantly differ between pH 8 and 11 and by extract from Azar did not markedly vary among 8, 9, and 11 pH values (Fig. 2b).

The effect of different concentrations of seed extracts on the α-amylase and protease activity

Preliminary assay to estimate the protein concentration of seed extracts was done to start the enzyme inhibition assays. Both extracts showed dose dependent style of inhibition. Various concentrations; 1.5, 0.75, 0.375, 0.187, and 0.093 (mg protein/ml) of extract from MV17 inhibited α-amylase activity by 59.72, 45.44, 34.88, 23.21, and 12.89% and of extract from Azar decreased the enzyme activity by 50.63, 38.78, 27.16, 25.53, and 17.62% (Fig. 3a). Protease activity was also reduced by extract from MV17 by 42.46, 33.73, 23.77, 20.46, and 16.56% and by extract from Azar by 49.34, 35.22, 20.29, 12.82, and 4.88%, (Fig. 3b).

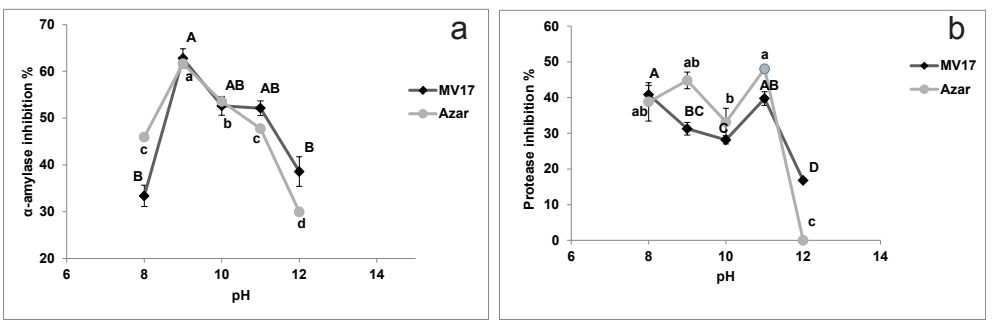

Fig. 2. The effect of pH on inhibition of potato tuberworm α-amylase (a) and protease (b) by extracts from MV17 and Azar. Means followed by the same letters are not significantly different by Tukey's test (P<0.05).

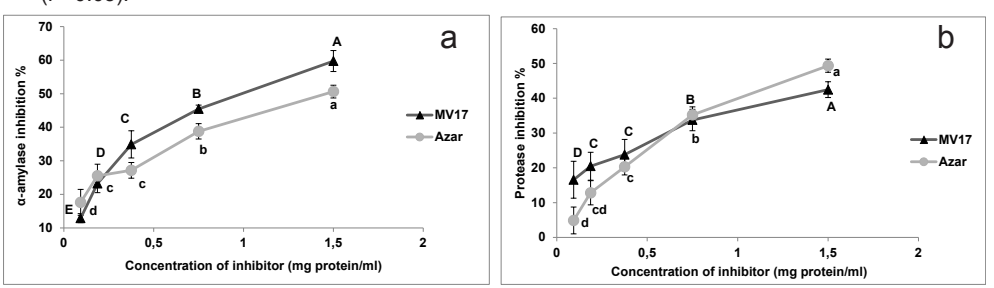

Fig. 3. Inhibition of potato tuberworm α-amylase (a) and protease (b) by different concentrations of MV17 and Azar. Means followed by the same letters are not significantly different by Tukey's test (P<0.05).

Gel assays of the effect of seed proteinaceous extracts on the α-amylase and protease activity

Gel assays showed that there were two major isozymes of α-amylase and protease enzymes in the larval gut. When different concentrations of seed extracts were used, the intensity of bands was differed in both cases. At lowest concentration of seed extracts (0.093 mg/ml protein) both bands were faint. At the highest concentration of extract from MV17 both bands of α-amylase (Fig. 4a) and protease (Fig. 5a) disappeared. Whereas the highest concentration of extract from Azar only decreased

the intensity of bands of both enzymes (Figs. 4b and 5b).

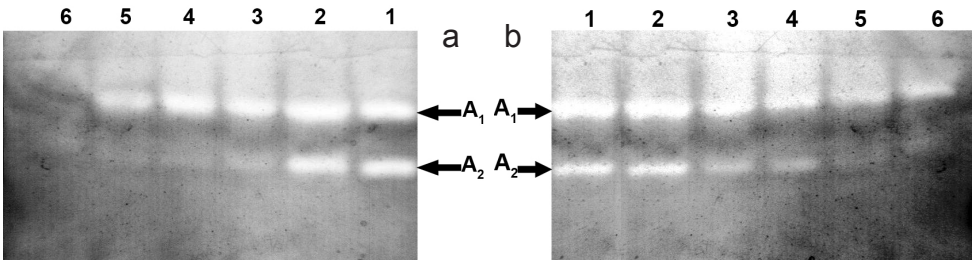

Fig. 4. In gel inhibition assay of the effect of different concentrations of plant extracts on the α-amylase of potato tuberworm using 0.5% starch as substrate. Lane numbers are as follow: (1) enzyme extract with no inhibitor, (2) 0.093 mg/ml, (3) 0.187 mg/ml, (4) 0.375 mg/ml, (5) 0.75 mg/ml, (6) 1.5 mg/ml protein of extract from MV17 (a) and Azar (b). A1: α-amylase first isozyme, A2: α-amylase second isozyme.

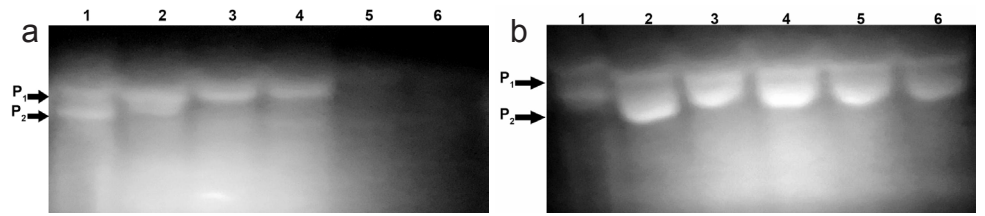

Fig. 5. In gel inhibition assay of the effect of different concentrations of plant extracts on the protease of potato tuberworm using 1% gelatin. Lane numbers are as follow: (1) enzyme extract with no inhibitor, (2) 0.093 mg/ml, (3) 0.187 mg/ml, (4) 0.375 mg/ml, (5) 0.75 mg/ml, (6) 1.5 mg/ml protein of extract from MV17 (a) and Azar (b). P1: Protease first isozyme, P2: Protease second isozyme.

DISCUSSION

The potato is one of the most important food crops along with rice, wheat, and maize all over the world (Ross, 1986; Douches *et al.*, 2004). Unfortunately, severe losses may occur in storage, especially in developing countries where low income farmers cannot afford refrigerated storages (Rondon, 2010).

In this study, the digestive α-amylase and protease enzymes of potato tuberworm were characterized for the first time and also the effect of extracts from two wheat varieties was tested on enzymes activities. Many of Lepidopteran insects live on a polysaccharide-rich diet and require digestive α-amylase to break down and utilize the starch in their food sources. These amylases play a very important role in starch digestion and in insect survival (Borzoui *et al*., 2013).

Plant seeds are known to contain a diversity of enzyme inhibitors that are thought to be involved in defense mechanisms against herbivores. Among these proteins, the α-amylase and proteinase inhibitors are found in several legume and cereal species. There are many examples where amylase inhibitors, especially those isolated from cereals, inhibit amylases from insect guts (Valencia-Jiménez *et al*., 2008). The role and mechanism of action for most of these inhibitors are being studied in detail and their respective genes isolated. These genes have been used for the construction

of transgenic plants to be incorporated in integrated pest management programs (Lawrence and Koundal, 2002).

The acidity of the contents of digestive tract is the main factor affecting the digestive enzymes (Terra and Ferreira, 1994). It has been reported that high levels of acidity in the phytophagous lepidopteran gut is to deal with high levels of tannins in their foods. These materials at low acidity join the insect enzymes and resulting in reduced digestion performance. The digestive tract of Lepidopteran insects such as mediterranean flour-moth *Anagasta kuehniella* Zeller, *Plodia interpunctella* Zeller, and many other Lepidoptera is extremely alkaline (Baker, 1989; Sivakumar *et al*., 2006; Amorim *et al*., 2008; Pytelkova et al., 2009). In the present study, the results of pH assays were in consistent with previous experiments. As we observed, potato tuberworm α-amylase and protease exhibited elevated activity at alkaline pH 9 and 11, respectively. Also, it was found that pH factor affected the inhibition of α-amylase and protease activity of potato tuberworm by seed extracts. An inhibitor which reduces enzyme activity at the optimum pH of enzyme activity, can be used in producing transgenic plants against insect pests. The highest inhibition of α-amylase by extract from MV17 and Azar was observed at pH 9 (the optimum pH of α-amylase activity). Whereas the inhibition of protease by the extracts was not significantly affected by pH factor. There are many other reports confirming that inhibition manner of digestive α-amylase and protease by seed proteinaceous extracts is pH dependent e.g., the inhibitory effect of wheat seed extract on digestive α-amylase of *Plutella xylustella* L. (Borzoei *et al.*, 2013), and inhibition of proteolytic and amylolytic activity of *Tenebrio molitor* L. by plant proteinaceous seed extracts (Dastranj *et al.*, 2013).

The effect of seed proteinaceous extracts also was dose dependent. A gradual increase in the amount of the enzyme inhibition was observed along with a gradual increase in the amount of the seed extracts concentrations. In line with these data, Mehrabadi *et al.* (2010) found that the effect of triticale seed extract on the α-amylase activity of the Sunn pest (*Eurygaster integriceps* Puton) was also dose dependent. They found that lowest concentration of triticale seed extract (0.25 mg) inhibited about 10% of enzyme activity, while the highest dose of seed extract (1.5 mg) caused 80% inhibition of enzyme activity.

CONCLUSIONS

Since the insects such as potato tuberworm are substantially dependent on α-amylases to their survival and digestion the starch as the main and the most necessary material in their food, amylase could be a good target for insect control through α-amylase inhibitors (Franco *et al*., 2002; Svensson *et al.*, 2004; Sivakumar *et al*., 2006). The current results showed that potato tuberworm α-amylases are more sensitive than protease in terms of the inhibition by tested proteinaceous seed extracts. The plants possess several mechanisms against herbivore insects. One of these mechanisms are proteinaceous inhibitors which block the active site of digestive enzymes and consequently prevent their usual function. In plant-pest co-evolution pathway, the insect pests have overcomed to defense mechanism of host plants

such as proteinaceous enzyme inhibitors and have been abled to keep on feeding on host plants. Hence in the strategy of transgenic plant production against insect pests, proteins of non-host plants should be encoded in transgenic plants which the insect pests have no evolutionary background with these proteins. Finally, since MV17 and Azar could affect the insect digestive enzymes specially α-amylase, these seed extracts can be tested to produce resistant potato crops against potato tuberworm.

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