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Full Length Article

Comparison of Biochemical Components within White and Brown Cysts in the Cereal Cyst Nematode *Heterodera avenae*

A.S. Mo, Y.R. Long, Z.Q. Qiu, H.Y. Wu* and X.B. Zhou
Agricultural College of Guangxi University, Nanning 530004, China
*For correspondence: whyzxb@gmail.com

Abstract

Several biochemical components of white and brown cysts in the cereal cyst nematode were measured and compared in our study. These components included carbohydrate, glycerol, soluble protein, esterase and trehalase. Results showed that the total carbohydrate, glycogen and soluble protein contents were higher in the white cysts than in the brown cyst, whereas the glycerol content was lower in the white cysts than in the brown cysts. Trehalase activity was lower in the white cysts than in the brown cysts, whereas esterase activity was higher in the white cysts than in the brown cysts. Only one esterase band, EST0.20, in both types of cysts was observed in the esterase isoenzyme pattern; the esterase in the white cysts showed a darker band and exhibited more activity than the esterase in the brown cysts. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis indicated that the protein pattern in the white cysts had darker and more protein bands of high molecular weight (>44.3 kDa) than that in the brown cysts. For protein of lower molecular weight (≤44.3 kDa), the pattern bands in the brown cysts were remarkably darker than those in the white cysts. The amount of protein in the white cysts differed from that in the brown cysts. The biochemical components signals and response may provide useful information for exploring nematode physiology.

Keywords: *Heterodera avenae*; Carbohydrate; Esterase; Protein; Trehalose

Introduction

The cereal cyst nematode is an important plant pathogenic nematode, which harms oats, barley, wheat and other temperate grain crops. The disease, a soil-borne disease of temperate wheat-growing areas, develops quickly (Yan and Smiley, 2010). It has caused considerable economic losses over the last 40 years and seriously affected the production and quality of wheat (Holgado *et al*., 2006). *H. avenae* is an important economic species, and it has been reported in 16 provinces in China (Wu *et al*., 2014).

For the cereal cyst nematode, the second-stage juvenile (J2) enters the root and establishes a feeding site. As it feeds, develops and the female body swells, protrudes its posterior end, and bursts out of the root. The mature female dies, and her cuticle hardens and turns brown to form a cyst. Cysts are particularly leathery, play an important role as a stage of life protecting the eggs and juveniles from desiccation, cold, and heat between crop cycles (Smiley and Yan, 2010). They remain in the soil over summer until temperatures fall and the autumn rains begin, stimulating hatching to produce the next generation. Cereal cyst nematodes have only one life cycle per year in Shandong, China (Wu *et al*., 2014). Eggs inside a cyst can remain viable for several years. Once cereal cyst nematodes have been introduced into a field, eradication is nearly impossible. These nematodes are transmitted in any manner by which soil is moved from one location to another.

Cysts play an important role as a stage of life and protect themselves against adverse environmental conditions, such as freezing and desiccation. The chemical composition of the cyst wall and eggshells of the potato cyst nematode (*Globodera rostochiensis*) has been reported (Clarke *et al*., 1967, Clarke, 1968); however, the physiological and biochemical characteristics of *Heterodera* spp. are rarely reported. Information regarding the changes in the physiological and biochemical characteristics of *Heterodera* spp. from the white stage to the brown stage may elucidate the mechanism of hatching, diapause, resistance to desiccation, and the action of nematicides in this genus.

In the present study, we determined the changes in the total carbohydrate, glycogen, trehalose, glycerol and protein contents, as well as esterase and trehalase activities of *H. avenae* cysts from white to brown. This study aimed to (1) determine the biochemical differences between white and brown cysts and (2) examine the differential protein patterns of white and brown cysts to provide insights into the basis for cloning and functional analysis of the tanning and browning gene in *Heterodera* spp.

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Materials and Methods

J2 Collection

The study was conducted in a natural *H. avenae*-infested field in Hutun Town (36°12′N, 116°36′E) near Tai'an City in June 2011. When winter wheat was harvested, soil samples were collected from the plant rhizosphere (cv. Jimai 22), and brown cysts were separated using a 180 μm pore sieve by handpicking under a stereomicroscope (Liu, 2000). The cysts were stored at 4°C for eight weeks, placed on a 150 μm pore sieve, and broken by gentle rubbing. A rubber stopper was used to release the eggs, which were collected using a 36 μm pore sieve. J2s that were hatched from eggs were kept in water at 15°C. J2s (250±4.2 J2/mL) were then used for inoculation.

Culture of Nematode

Polyvinyl chloride tubes (25 cm height and 5 cm in diameter) were filled with an autoclaved soil mixture (50% sand, 0.5 mm diameter, 50% garden soil, autoclaved). The wheat seeds were placed on Petri dishes with filter paper after soaking in NaClO (3%) for 5 min. The seeds were then rinsed five times with sterile water and sprouted at 25°C. The seeds of germination were sowed when the shoot length was 0.5–1.0 cm. One pre-germinated seed was planted in each tube. Immediately after planting, freshly hatched J2s (250±4.2 J2) of *H. avenae* were dispensed into three 2 cm holes about 1 cm away from the root system, covered with soil again, and then inoculated every 3 days for a total of four times with about 1000 J2 per tube. The wheat seedlings were placed in an intelligent illumination incubator under the following controlled conditions: 19°C±1°C, 14 h of artificial light and 10 h of darkness every day, 60%–70% relative humidity, and Hoagland nutrient solution applied twice during the growth period.

Around 75 days after the first inoculation, the wheat plants were harvested, and the root system was washed gently in a basin with water to free the adhesive soil particles from the swollen white female and brown cysts attached to the roots. The white and brown cysts were collected in accordance with the method described by Jing *et al*. (2014). All the white females and brown cysts used in this experiment were cleaned using ultrasonic (SK1200H Shanghai Ultrasonic Instrument Co., Ltd.).

Carbohydrate Extraction

Forty-five white and brown cysts were extracted and placed into 1.5 mL centrifuge tubes each with 160 μL trichloroacetic acid (10%) respectively. Grinded 5 min on the ice with pestle, and centrifuged at 12 000 rpm for 10 min 4℃, the supernatant was used for the total carbohydrate assay.

Forty μL supernate was transferred into a 0.5 mL centrifuge tube, added into 160 μL absolute ethyl alcohol and stored at 4℃. Centrifuged at 12 000 rpm for 10 min after 16~18 h and the supernatant was discarded. Dissolved with 40 μL 10% trichloroacetic acid (TCA) after dried a few minutes, and the solution was stored. The total carbohydrate, trehalose, and glycogen contents were determined using the anthrone–sulfuric acid method (Feng, 1989; Yen *et al*., 1996; Li *et al*., 2009).

Total Carbohydrate and Glycogen Content Determination

Ten μL of 10% TCA, total carbohydrate and glycogen extract, respectively, were transferred in new centrifuge tubes each with 200 μL 0.2% anthrone reagent. Four treatments and repeated 4 times each. All the tubes were put into a 100℃ boiling water bath for 10 min, cooled in an ice bath, then 200 μL was transferred into 96-well culture plate, and OD value was measured under the 630 nm wavelength.

Trehalose Content Determination

Ten μL total carbohydrate extract and 10 μL sulphuric acid (1%) were transferred in 1.5 mL tube and mixed thoroughly, 100℃ boiling water bath for 10 min. Ten μL 30% KOH was added and 100℃ boiling water bath for 10 min. Two hundred μL anthrone reagent was added after cooled in the ice bath, 4 treatments each experiment and repeated 4 times, OD value was measured under the 630 nm wavelength. The trehalose content was expressed μg per cyst.

Glycerol Extract and Content Determination

Glycerol assay was based on the procedure described by Wu and Yuan (2004). Thirty white females and brown cysts were transferred into 1.5 mL tubes with 85 μL deionized water and grinded for 5 min in the ice bath. Centrifuged at 12000 rpm for 10 min, and the supernatant was stored for assay.

One hundred μL oxidizing and chromogenic agent were added into new 1.5 mL tube with 20 μL glycerol extraction and mixed thoroughly, 60℃ water bath for 15 min. Transferred 200 μL into 96-well culture plate, 4 treatments each experiment and repeated 4 times, OD value was measured under the 420 nm wavelength. The content was expressed μg per cyst.

Soluble Protein Extract and Content Determination

Soluble protein levels were measured using the Coomassie Brilliant Blue method with bovine serum albumin (BSA) as the standard protein used to construct a standard curve (Xiao *et al*., 2006). Thirty white and brown cysts each were transferred to a new tube, 120 μL extraction buffer (20% sucrose, 50 mmol/L pH 7.1Tris-HCI, 0.5% Tritonx-100) was added and grinded for 5 min in the ice bath. It was centrifuged at 12000 rpm for 10 min and supernatant was immediately stored at 4℃ for reserve.

One hundred and fifty μL Coomassie Brilliant Blue G-250 staining solution, 0.01% G-250，4.7% ethanol，8.5% (W/V) phosphoric acid, was added into tube with 10 μL soluble protein extraction, and OD value was measured under the 420 nm wavelength. The soluble protein content was calculated according to standard curve of bovine serum albumin (BSA). The content was expressed as μg per cyst.

Esterase and Trehalase Activity Assay

Esterase was extracted from 30 each white and brown cysts in accordance with the approach described by Li *et al*. (2009). The cysts were transferred into centrifuge tubes, 100 μL extract buffer was added and grinded for 5 min in the ice bath, centrifuged at 12000 rpm for 10 min and the supernatant was transferred into a 0.5 mL EP tube and immediately stored at 4℃.

Esterase activity was determined based on the method by Li *et al*. (2009). Twenty five μL crude extract of enzyme was transferred into a 1.5 mL Eppendorf, 100 μL α-naphthyl acetate was added and mixed thoroughly. The tube was placed at 37℃ for 15 min to allow the reaction to occur. After the reaction, 76 μL DBLS reagent (a mixed solution containing 10 g/L faster blue RR salt mixed with 50 g/L SDS in the ratio 2 to 5 by volume) was added and mixed thoroughly. 200 μL of the mixture was transferred onto a micro-plate for activity assay. The absorbance (optical density (OD) value) was recorded at a wave length of 600 nm. Esterase activity was indicated as one activity unit (U) per mg protein, and one unit was defined as the increase in 0.1 OD value per minute.

Trehalase was extracted from 60 each white and brown cysts according to method described by Lei *et al*. (2006). Sixty cleaned cysts were transferred into tubes, 150 μL extract buffer (0.02 mol/L PB, pH 5.8) was added and grinded for 5 min in the ice bath, centrifuged at 12000 rpm for 10 min and the supernatant was transferred into a new tube and immediately stored at 4℃.

Each new tubes with 40 μL enzyme extraction mixed with 50 μL standard trehalase solution (4 mM), were transferred into a boiling water bath for 3 min after incubated with 37℃ for 30 min, then cooled in the ice bath; 90 μL salicylic acid color-substrate solution (1% 3, 5- two nitro salicylic acid, 1% NaOH, 0.2% Phenol and 0.05% Na_2SO_3) was added and mixed, The tubes were incubated in 90℃ bath for 7 min, and then cooled in ice bath; 30 μL potassium sodium tartrate tetrahydrate (40%, W/V) was added and mixed fully. And an aliquot (200 μL) was transferred into an enzyme measuring microplate, The absorbance (OD value) was detected using Spectra Max M2 with a wave length of 575 nm trehalase activity was expressed as one activity unit (U) per mg protein, and one unit was defined as the increase in 0.1 OD value per minute. The protein content of samples was determined as above.

Esterase Isoenzyme Electrophoresis

Mixed enzyme was extracted using the method described by Li *et al*. (2009). A total of 80 each white and brown cysts were ground thoroughly with 100 μL of extraction buffer (0.03 M, pH 6.8, Tris-HCl, 2% (v/v) Triton X-100, 6% (v/v) glycerol, and 0.01 M NaCl) in a 1.5 mL Eppendorf tube in an ice bath. The homogenate was centrifuged at 15,000 g for 10 min at 4°C. The supernate was collected in a 0.5 mL Eppendorf tube and then immediately stored at −20°C.

Esterase isoenzymes were analyzed using vertical thin-layer native polyacrylamide gel electrophoresis (PAGE) equipment (DYG-28A electrophoresis cell, DYY-10C electrophoresis power supply; Beijing Liuyi Instrument Factory, Beijing, China), with a 7.5% slab gel and a 4.0% stacking gel. Protein samples (8 μg) were loaded in each sample hole using the microinjector, Electrophoresis was conducted at 120 V and 4°C. All protocols, including the electrophoresis parameter and stain procedure, were the same as reported in the study by Li *et al*. (2009). After electrophoresis, the esterase isoenzyme band was stained at 37°C until isoenzyme bands were visualized as cleared zones. The gels were subsequently placed into a fixative solution of 7% acetic acid and photographed.

Comparison of Total Protein Pattern

The difference in the total protein bands between the white and brown cysts was determined after electrophoresis. Modified sodium dodecyl sulfate–PAGE (SDS–PAGE) assay (Podzol and Noel, 1984) was conducted. The method used in protein extraction was the same as that in mixed enzyme extraction. Protein samples (10 μg) were mixed with a loading buffer (1% SDS containing 100 mM mercaptoethanol and tris-glycine buffer at pH 6.8), loaded in each sample hole using the microinjector, and 10% slab gel with 5% stacking gel was used. Electrophoresis was conducted at the same voltage as esterase isoenzyme electrophoresis, and the gel was stained using the method described in the study by Li *et al*. (2009). The protein standards used were myosin (200 kDa), β-galactosidase (116 kDa), phosphatase b (97.2 kDa), BSA (66.409 kDa), ovalbumin (44.287 kDa), carbonic anhydrase (29 kDa), trypsin inhibitor (20.1 kDa), lysozyme (14.3 kDa), and aprotinin (6.5 kDa).

Statistical Analysis

All data were subjected to analysis with SPSS 12.0; LSD tests were used and differences with $P < 0.05$ were considered significant.

Results

Total Carbohydrate, Glycogen and Trehalose Contents in White and Brown Cysts of *H. avenae*

The total carbohydrate content in white cysts (10.126 μg per

cyst) was significantly higher than that of in brown cysts (5.143 μg per cyst; $P < 0.01$). The glycogen content in white cysts (1.80 μg per cyst) was 106.9% higher than that in brown cysts (0.87 μg per cyst; $P < 0.01$). However, there was significantly higher trehalose content in the brown than that in the white cysts (Fig. 1).

Contents of Glycerol and Soluble Protein

The glycerol content in brown cysts (0.0286 μg per cyst) was significantly higher than that in white cysts (0.0183 μg per cyst; $P<0.05$). The results suggested that glycerol level of both cysts was low, and synthesize glycerol during the cyst change from white to brown as a protectant from dehydration of eggs in brown cyst. The soluble protein content was 3.18 μg in the white cysts, which was 1.1 times that of brown cysts (2.94 μg per cyst). The difference was considered significant ($P<0.05$) (Fig. 2).

Esterase and Trehalase Activities

The results showed that esterase activity in the white cysts (30.6 U/mg) was 1.2 times higher than that in the brown cysts (13.8 U/mg; $P < 0.05$). Trehalase activity in the white cysts (0.436 U/mg) was lower than that in the brown cysts (0.878 U/mg; $P < 0.05$) (Fig. 3).

Esterase Isoenzyme Patterns Total Protein SDS–Page Pattern Comparison of White and Brown Cysts

Esterase isoenzyme patterns indicated that the white and brown cysts had only one esterase band, EST0.20, which was found in the darker band and exhibited higher activity in the white cysts than in the brown cysts (Fig. 4-I). The composition of major protein bands in both white and brown cysts was revealed in the SDS–PAGE pattern (Fig. 4-II). For high molecular weight (>44.3 kDa), the protein pattern bands of the white cysts were darker than those in the brown cysts. For low molecular weight (≤44.3 kDa), the protein pattern bands in the brown cysts were remarkably darker than those in the white cysts. PAGE analysis indicated the amounts of protein differed in both cysts. Bands at 358.5, 93.3, 64.1, 59.9 and 56.8 kDa were present in the white cysts but not in the brown cysts (Fig. 4-II, red arrow). A band at 21.2 kDa was present in the brown cysts but not in the white cysts (Fig. 4-II, green arrow).

Discussion

For a cyst-forming nematode, the cyst stage is an important survival period over hot, dry summers. A previous study showed that under laboratory conditions, no J2s emerged from the white cysts that were incubated at different temperatures, even at incubation at 4°C for four weeks.

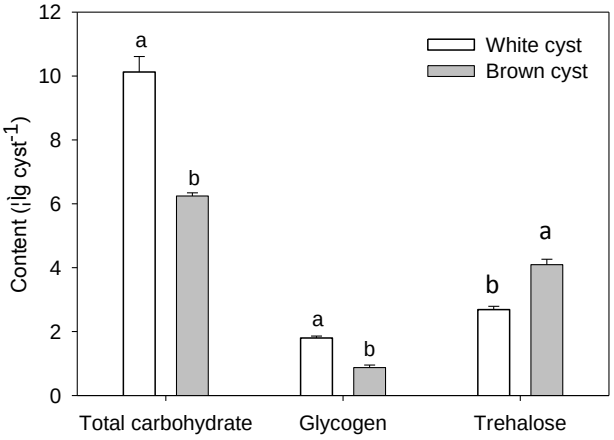

Fig. 1: Content of total carbohydrate, glycogen and trehalose in white and brown cysts. Bars are SE

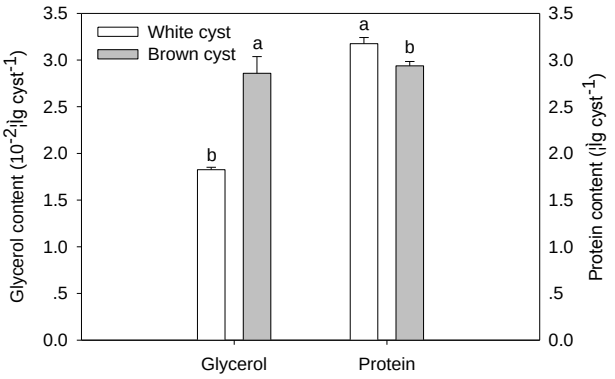

Fig. 2: Content of glycerol and protein in white and brown cyst. Bars are SE

Unlike the white cysts, J2s emerged from the brown cysts collected every month, except that no eggs were present in the cysts (Jing *et al.*, 2014). However, the physiological and biochemical characteristics of the white and brown cysts of the nematode are rarely reported.

Metabolic changes play a role in desiccation survival, previous studies have indicated that glycogen shift to trehalose during insect-killing nematode *Steinernema feltiae* dehydration (Gal *et al.*, 2001). In our present study, the total carbohydrate content and glycogen content in the white cysts was significantly higher than that in the brown cysts. There was lower trehalose in the white, which might be related to the maturity of eggs inside the white females. Trehalose accumulation needs to use the glycogen stored in the cyst, and the change in the glycogen content presented the opposite trend with the trehalose content.

Trehalose is considered important in the physiology of nematodes where it may function in sugar transport, energy storage, and protection against environmental stresses. There are strong correlations between *Aphelenchus auenae* survival in dry air and glycerol and trehalose contents were observed (Madin and Crowe, 1975).

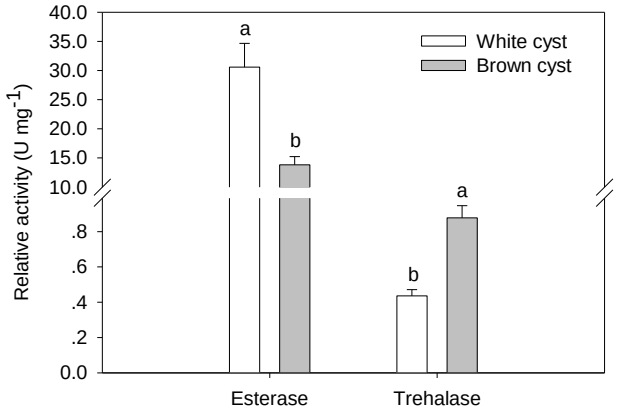

Fig. 3: Esterase and trehalase relative activity in white and brown cysts. Bars are SE

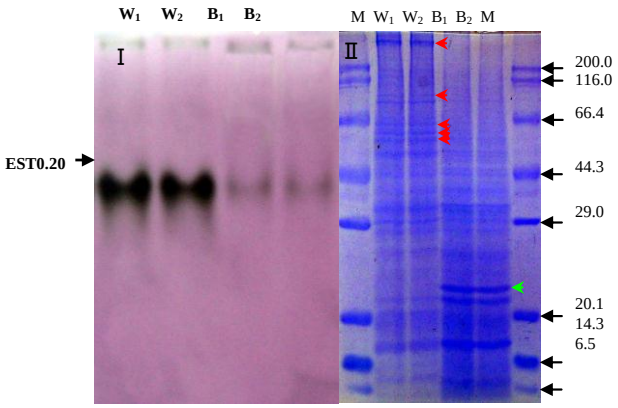

Fig. 4: Esterase isoenzyme (I) and protein (II) patterns of white. White (W) and brown (B) cyst extracts of *Heterodera avenae*

A large number of trehalose that accumulates in the hemolymph is derived from fat body glycogen in the diapausing pupae of *Philosamia cynthia pryeri* (Hayakawaa and Chino, 1981), which was studied by Saravanakumar *et al*. (2008). The trehalase gene was expressed in non-diapause eggs of the multivoltine silkworm *Bombyx mori* for 9 days, whereas that in the diapause eggs was not upregulated. The glycogen phosphorylase gene was expressed in the non-diapause eggs, whereas that in the diapause eggs was highly expressed in the early stage and then downregulated in the late stage. Trehalase activities enhanced by the diapause hormone originate mainly from the treh-2 protein regulated at the transcriptional level (Kamei *et al*., 2011). In previous studies, during dehydration in nematodes, metabolic changes show a link between glycogen and trehalose metabolism. For example, the entomopathogenic nematode *Steinernema feltiae* showed a twofold increase in trehalose and a reduction in glycogen accumulation after desiccation of the nematode (Solomon *et al*., 2000). In cells, the metabolism of glycogen and trehalose is linked by the common substrate, UDP-glucose. Trehalose plays an important role in the hatching sequence of some plant parasitic nematodes. The trehalose content of perivitelline fluid influences hatching and is important for the survival of dormant, unhatched juveniles (Perry, 1989). However, the trehalose content in the white cysts was significantly lower than that of brown cysts in the present study, and no J2 emerged from the white under favorable conditions in the laboratory (Jing *et al*., 2014). Pellerone *et al*. (2003) used *C. elegans* as a model and found that two putative trehalose-6-phosphate synthase (tps) genes are expressed as mRNA at all stages of the life cycle. They also determined that the trehalose content does not influence the viability or development of the nematodes. Further studies should be conducted to determine whether the genes are expressed in cyst nematodes.

Glycerol played an important role in rapidly balancing the osmotic pressure. Rapid and gradual increase of osmotic stress affect on survival of entomopathogenic nematodes (Chen and Glazer, 2004). A strong correlation between accumulation of glycerol and trehalose was present during dehydration and survival of a mycophagous nematode, *Aphelenchus avenae*, in dry air (Crowe and Madin, 1975). And a correlation between glycerol or trehalose accumulation and increased desiccation tolerance has also been observed in *Heterorhabditis megidis*, *Heterorhabditis indica* and *S. carpocapsae* (O'Leary *et al*., 2001), which could be the explanation of higher glycerol content in brown cyst than that of white cyst.

In the present study, the esterase activity of the white cysts was significantly higher than that of the brown cysts, suggesting that the metabolism in the white cysts was more exuberant than that in the brown cysts. Therefore, lipid metabolism may be faster in the white cysts than in the brown cysts. We deduced that the viscera (e.g., ovary) in the white females were degraded during this period, so those in the brown cysts disappeared.

The majority of the studies focus on the protein biosynthesis and transcription of *C. elegans* (Kazanietz *et al*., 1995). In the present study, the soluble protein content in the white cysts was higher than that in the brown cysts. Esterase is a hydrolase enzyme, esterase activity can helpful to understand metabolic pathway. However, more low-molecular-weight protein bands were found in the brown cysts than in the white cysts, the mechanism underlying this protein differences needs to be further investigated.

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